THE RELATIONSHIP BETWEEN CANCER PREDISPOSITION AND PRIMARY IMMUNODEFICIENCY

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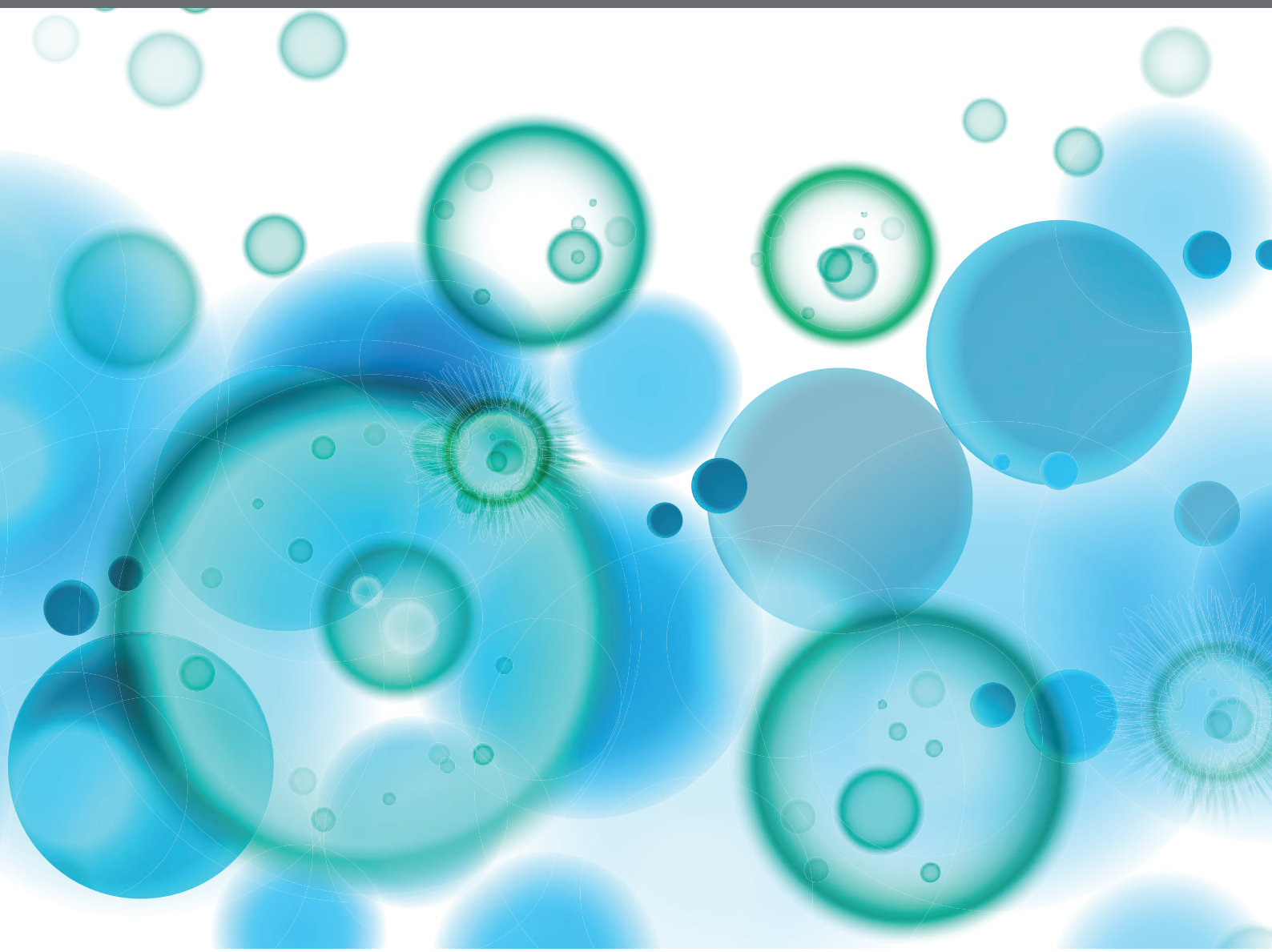
PUBLISHED IN: Frontiers in Immunology, Frontiers in Oncology and Frontiers in Pediatrics



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88963-172-8
DOI 10.3389/978-2-88963-172-8

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THE RELATIONSHIP BETWEEN CANCER PREDISPOSITION AND PRIMARY IMMUNODEFICIENCY

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Citation: Hauck, F., Gennery, A. R., Seidel, M. G., eds. (2019). The Relationship Between Cancer Predisposition and Primary Immunodeficiency. Lausanne: Frontiers Media. doi: 10.3389/978-2-88963-172-8

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frontiers in Immunology

EDITORIAL
published: 30 July 2019
doi: 10.3389/fimmu.2019.01781

Editorial: The Relationship Between Cancer Predisposition and Primary Immunodeficiency

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Keywords: inborn error of immunity, IEI, primary immunodeficiency, PID, cancer predisposition syndrome, CPS

Editorial on the Research Topic

The Relationship Between Cancer Predisposition and Primary Immunodeficiency

INTRODUCTION

The risk of malignancies is higher in patients with genetically determined inborn errors of immunity (IEI) than in the general population (1, 2). However, the degree of tumor predisposition and the underlying cellular and molecular mechanisms vary through the categories of IEI (3, 4). In addition to perturbed tumor immune surveillance in IEI and chronic inflammation or infections, the molecular defect *per se* that causes IEI may predispose to tumorigenesis (5). This suggests that malignancy in IEI may not merely be a consequence of immune deficiency, but occur in parallel to or even precede immune deficiency. Furthermore, in genetically determined DNA repair deficiencies the particular IEI may be perceived as an add-on to tumor predisposition (6–8). Additionally, deregulation of epigenetic factors related to IEI or its treatment such as alterations of the microbiome can contribute to tumor predisposition (9, 10).

The Research Topic covers many aspects of this increasingly appreciated clinical and basic scientific field. Furthermore, based on presentations and discussions directly or indirectly related to the process of the present collection, additional initiatives were launched and are ongoing. The 2019 focused meeting of the European Society of Immunodeficiencies in Brussels—"Malignancy and PID" (https://esidmeeting.org)—exemplifies this bringing together of specialists.

COMPOSITION

Large Cohort Studies

Two nationwide studies of common variable immunodeficiency (CVID) and one large international study of patients with haploinsufficiency of CTLA4 were conducted and provide insight into the cancer risk in these relatively frequent and highly relevant entities (Egg et al.; Kralickova et al.; Pulvirenti et al.). The need for awareness and appropriate screening measures is highlighted.

Systematic Review

A comprehensive meta-review on lymphoid malignancies IEI structured according to the classification of the international union of immunological societies (IUIS), gives a clear, detailed,

OPEN ACCESS

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Specialty section:
This article was submitted to Primary Immunodeficiencies, a section of the journal Frontiers in Immunology

Received: *28 May 2019*
Accepted: *15 July 2019*
Published: *30 July 2019*

Citation:
Hauck F, Gennery AR and Seidel MG (2019) Editorial: The Relationship Between Cancer Predisposition and Primary Immunodeficiency. Front. Immunol. 10:1781. doi: 10.3389/fimmu.2019.01781

and helpful overview of the current knowledge, types, and distribution of B and T cell lymphoid malignancies associated with IEI (Riaz et al.).

Perspective

The "*Current understanding and research priorities…*" in the challenging field of malignancies in IEI were defined, discussed, prioritized, and summarized by an interdisciplinary working group consisting of hematologists, oncologists, immunologists, tumor biologists, and geneticists and are presented (Bomken et al.).

Small Cohort Studies, Single Entity or Patient Reports, or Reviews

A diverse collection of relevant clinical observations was reported, ranging from a single center long-term experience of malignancies in IEI (Maffeis et al. *in press*), over lymphomagenesis in STK4 deficiency or ataxia telangiectasia, variable phenotypes of Cernunnos/XLF deficiency, as well as the study of clinical and biological signs of immune deficiency in patients with the cancer predisposition syndrome *constitutional mismatch repair deficiency* (Recio et al.; Schipp et al.; Tatfi et al.; Tesch et al.). Additionally, the risk of malignancies in patients with secondary immunodeficiency due to immunosuppressive drugs in the framework of solid organ transplantation is reported, aiming at identifying specific drug-dependent mechanisms and risk factors (Cangemi et al.).

Conceptual Review and Mini Reviews

One large conceptual review embedded the "*Closely related concepts*" of IEI and cancer predisposition syndromes into an integrative framework (Haas), while smaller (mini) reviews focused on tumor profiles in IEI, in Down Syndrome, or on common genetic bases of cancer and IEI (Derpoorter et al.; Satge; Satge and Seidel).

Basic and Methodological Research

A mouse study on the effects of the loss of JAK1 on innate immunity, with a potential consequence of reduced tumor surveillance (Witalisz-Siepracka et al.), and a methodological study on improved early detection of the transformation risk in severe congenital neutropenia (Klimiankou et al.) complete the spectrum of articles.

CONCLUSIONS AND PERSPECTIVES

While it is evident that the concept of cancer predisposition and immune deficiency as opposite sites of the same genetic coin is still in its infancy, we at this point in time can state that it is born and rapidly growing.

We envision that in the era of systems biology and "omics" technologies there will be major advances not only in basic science, but as well in the ways geneticists, tumor biologists, immunologists, and oncologists will work together to finally improve diagnosis, treatment and patient outcome both in the sense of overall survival and in terms of quality adjusted life years and reproductivity.

AUTHOR CONTRIBUTIONS

FH and MS drafted the article. AG approved it.

FUNDING

FH was funded by the Care-for-Rare Foundation (C4R, project number 160073), the German Center for Infection Research (DZIF, project number TTU 07.909), the Else Kröner-Fresenius Stiftung (EKFS, project number 2017_A110), and the German Federal Ministry of Education and Research (BMBF, project number 01GM1910C). MS was in part sponsored by the Styrian Children's Cancer Aid (*Steirische Kinderkrebshilfe*).

ACKNOWLEDGMENTS

We acknowledge the contribution of all authors, reviewers, and editors that have contributed to the realization of the Research Topic.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pediatrics

ORIGINAL RESEARCH
published: 19 June 2019
doi: 10.3389/fped.2019.00232

Primary Immunodeficiencies and Oncological Risk: The Experience of the Children's Hospital of Brescia

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Background and aims: Primary immunodeficiencies (PID) are characterized by recurrent infections and increased risk of malignancies because of the reduced immunological surveillance against cancer cells and oncogenic viruses.

Methods: We report the incidence of tumors among 690 patients with PID, diagnosed from 1990 until 2017 in Brescia.

Results: Out of 690 patients , 25 patients (3.6%) developed 33 tumors. Of the 25 affected patients, 8 patients suffered from common variable immunodeficiency (CVID), 5 from combined immunodeficiency (CID), 3 from Ataxia-telangectasia (AT), 2 from Hermanksy-Pudlak type 2 (HSP2), 2 from gammaglobulinemia X-linked (XLA), 2 from Wiskott-Aldrich syndrome (WAS), 2 from Hyper IgE syndrome (HIES), 1 from severe combined immunodeficiency (SCID). The age at diagnosis ranged from 1 to 52 years, with a median age of 19.6 years. The time between the diagnosis of PID and onset of tumor was short, often <1 year between diagnosis and the appearance of cancer in the case of CID. Moreover, in two cases of CID, the diagnosis of cancer was made before the diagnosis of PID, so cancer was the onset clinical manifestation. Hematological malignancies were prevalent (22/33, 66.7%) with a minority of solid tumors (11/33, 33.33%). In particular Non-Hodgkin lymphomas were the most frequent (16/33, 48.48%). In total 13 patients survived (52%) and tumor was the main cause of death (7 cases). Two patients underwent BMT once the disease was in remission.

Conclusions: Therefore, the correct management of tumors that arise in patients with primitive immunodeficiency still represents a challenge in the pediatric field. For this reason now it is mandatory to collect in a unique international registry the cases of malignancies in PID that could lead to a better understanding of the etiopathogenesis and of the biological and clinical characteristics of these tumors, with the aim of defining adequate preventive measures and guaranteeing an early diagnosis which also creating a shared and specific therapeutic strategy, with the prospect of obtaining a better prognosis for these patients.

Keywords: primary immumunodeficiencies, tumors, pediatric, bone marrow transplant (BMT), rare diseases

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Pediatrics

Received: *21 February 2019*
Accepted: *22 May 2019*
Published: *19 June 2019*

Citation:
Maffeis M, Notarangelo LD, Schumacher RF, Soncini E, Soresina A, Lanfranchi A and Porta F (2019) Primary Immunodeficiencies and Oncological Risk: The Experience of the Children's Hospital of Brescia. Front. Pediatr. 7:232. doi: 10.3389/fped.2019.00232

BACKGROUND AND AIMS

Primary immunodeficiencies (PID) represent a heterogeneous group of congenital diseases characterized by an alteration of the functionality of the immune system. These diseases are considered rare, in fact in Italy the incidence of these forms is variable, ranging from 1:500 for the most frequent forms to 1:500 000 for other rare forms. The overall incidence of PID can be calculated around 1:2000[1]. The classification of these diseases is very complex and constantly evolving in relation to genetic mutations and the etiopathogenetic mechanisms that are identified progressively over the years. Depending on the component of the immune system that is altered, they can be divided into two main groups: the deficiencies of innate immunity and the deficiencies of adaptive immunity. In particular, the latter group includes the deficits of humoral immunity, the combined immunity deficits, the immune deficiencies associated with syndromes, and the immune dysregulation diseases.

These diseases are characterized by an increased susceptibility to infections, by the frequent development of autoimmune diseases and by a predisposition toward the onset of neoplasia. This particular case became even more evident in recent years since the development of drugs for the prophylaxis and the treatment of opportunistic infections made it possible to have an increase of the life expectancy of these patients, providing the necessary time for neoplastic development. Indeed, tumors are currently the second leading cause of death for patients with PID after infections.

The high incidence of tumors is primarily evident in the combined immunodeficiencies in which there is a defect of cell-mediated immunity that plays a fundamental role in the control of birth and neoplastic growth, a mechanism that it is called Tumor Immunosurveillance (1–3). In fact, cytotoxic T lymphocytes are able to specifically recognize tumor cells and induce them to death. Moreover, the development of tumors also occurs as a consequence of the numerous infections affecting these patients: first of all the oncogenic viruses (Epstein-Barr virus, Human Papilloma Virus), which can directly cause a degeneration of the cells toward neoplastic forms; we also need to consider the chronicization of infections: by establishing a persistent inflammatory state, they create tissue damage, which can be precursor of a subsequent malignant transformation (such as for Helycobacter pilori) (4–6). Moreover, these mechanisms are joined by the tendency to acquire frequent genetic mutations, the result of genomic instability that characterizes some PID. This condition occurs in the following cases:

- presence of defects in DNA damage repair (Ataxia-telangectasia) (7)
- presence of defects of apoptosis that, causing cellular immortalization, allow cells to survive even in the presence of irreversible damage to the genome (Autoimmune Lymphoproliferative Syndrome) (6)
- presence of deficiencies in the cell cycle check-points for which the cell cycle, fundamental to allow a correct repair of the damage, is missing (Cartilage-hair hypoplasia) (8)
- defects in the cytokinesis which, by hindering cell division, lead to the formation of genetically unstable tetraploid cells (Neutropenia X linked and Wiskott-Aldrich syndrome) (9).

Since the PID are numerous, the mechanisms that explain the increased susceptibility of these patients to the development of tumors are multiple and often different in the various pathologies. Certainly, the most important process, which is common to many immune defects with a predisposition to carcinogenesis, is represented by the reduction of cell-mediated immunosurveillance (as occurs in combined immunodeficiencies). This can be explained by the fact that this component of the immune system plays a fundamental role in protecting against tumors. In support of this thesis, there is then the evidence that the most frequent tumors found in patients with PID are represented by lymphomas, neoplasms that afflict the cells of the specific immunity. Finally, in many cases [for example in Omenn Syndrome or in WAS (6)] the presence of a predominance of type 2 cytokines and a reduction of type 1 cytokines, such as INF-α, was found to be essential for the control of the development of tumoral pathologies, in particular those of lymphoproliferative type. Instead, infections, whether *de novo*, reactivated or chronic, can play a decisive role in the genesis of both blood tumors and solid tumors [>20% of carcinomas in patients with PID are induced by infections (4)], through two mechanisms main: activation of oncogenic viruses and chronic antigenic stimulation. As regards the first case, an example is represented by the Epstein Barr virus (EBV), which is the most frequently found infectious agent in these tumors, especially in type B lymphomas, but also in those of type T. In particular, according to the ICR data, it is found in 30–60% of patients with lymphoproliferative disorders and PID. This condition is the result of a reduction, caused by the underlying immunodeficiency, of EBV-specific CD8 T cells. Therefore, PIDs with T cell defects are those in which the greatest susceptibility to the development of EBV+ (10) lymphomas is expected to be found. Furthermore, another effect favoring the development of tumors depends on the EBV's ability to express genes that inhibit cell-mediated immunity. In this way the virus becomes immortal within B cells, which proliferate uncontrollably under the viral stimulus, acquiring mutations due to loss of heterozygosity and/or cytogenetic rearrangements (6).

Instead, as regards the mechanism of chronic antigenic stimulation, an example is represented by Helicobacter pylori (HP) infections, which is implicated in the genesis of gastric carcinoma and gastric MALT lymphoma. The increased risk of infections from this microorganism in patients with PID [in particular, it is frequently found in common variable immunodeficiency (CVID)] suggests a genesis due to a reduction in the production of gastric IgA and hydrochloric acid, which facilitates the colonization of HP. This microorganism promotes carcinogenesis by establishing chronic inflammation by stimulating the local production of cytokines, which alter the adhesive properties of the surface of the gastric mucosa and

[1]Document drawn up by AIEOP (Italian association of pediatric oncohematology) https://www.aieop.org/web/famiglie/schede-malattia/immunodeficienze/

promote ectopic proliferation of lymphoid tissue (6). Finally, in some cases the tumor arises as a result of cellular genomic instability, which favors the acquisition of numerous genetic mutations. In fact, some immunodeficiencies are characterized by the presence of defects in DNA repair mechanisms (as in the AT, where the ATM gene mutation reduces the functionality of the homonymous protein, which normally acts as a DNA double helix damage sensor and activates different repair mechanisms of these ones). In these cases, the DNA is continually exposed to potentially harmful insults, both extrinsic (for example from radiation of the environment), and intrinsic (for example from products of metabolism), which, not being able to be repaired, necessarily cause the appearance of mutations which favor cell degeneration toward neoplastic forms. In other cases, the acquisition of mutations is favored by the presence of apoptosis deficiency, which prevent the cell from meeting a programmed natural death following the appearance of DNA damage (as occurs in ALPS, for FAS, FAS mutations ligand or caspase 8) (6).

Furthermore, in some immunodeficiencies the acquisition of mutations is favored by the presence of defects in the cell cycle check-points (as occurs in cartilage-hair hypoplasia, which is caused by mutations in the RMRP gene, which determine an alteration of the cleavage of the Ciclin B2 mRNA, fundamental for the cell cycle control function). These mechanisms are normally able to detect possible DNA damage and stop the cell cycle for the time necessary to guarantee a correct repair (6), which, instead, will be absent in these pathologies. Finally, alterations in cytokinesis, as occurs in the XLN (Neutropenia X-linked) and probably also in the WAS, determine the formation of genetically unstable tetraploid cells, which facilitate the development of aneuplode tumor cells. Thus, as has been pointed out above, the mechanisms that explain the occurrence of tumors in patients with PID are numerous. Since many steps are needed to determine the appearance of a tumor, it is likely that several processes are simultaneously present in the same patient (7). For example, data from the ICR show that often brothers with the same immunodeficiency develop tumors of the same histotype. This could indeed be explained by the coexistence of multiple common favoring factors, such as exposure to the same carcinogens and having inherited greater susceptibility to the malignant transformation of specific clones and/or selective inability to destroy certain neoplastic cells (11).

METHODS

We decided to carry out a retrospective analysis at the Brescia Children's Hospital (Italy) reporting the incidence of tumors among 690 patients with a diagnosis of PID, diagnosed between 1990 and 2017 in Brescia. The study was approved by the Ethical Committee of the Spedali Civili of Brescia, president Aldo Maria Roccaro.

The study has the main purpose of reporting the experience of an Italian Pediatric Children's Hospital with patients suffering from PID who developed an oncological pathology during their lifetime, trying to report their characterizing elements.

RESULTS AND DISCUSSION

Out of 690 patients, 25 patients (3.6%) developed 33 tumors. They were treated differently depending on the tumor and the immunodeficiency: in particular, for solid tumors surgery was performed, with or without chemotherapy, while for hematological tumors, the treatment was based on National or International therapeutic protocols (**Figures 1**, **2**).

In the literature it is reported that 4–25% of patients with PID develop a neoplasm (12), but we must consider that it is difficult to establish the exact incidence of tumors that arise in these patients. First of all, this is due to the fact that PID are rare diseases that make it difficult to collect an amount of data big enough to allow for results to be truly representative of the phenomenon. Secondly, it is possible that in many cancer patients no underlying PID is diagnosed, especially when this presents a mild clinical manifestation and/or a late onset, leading to an inevitable underestimation of the cases (13). Furthermore, the incidence of cancer often presents regional divergences, which are probably the consequence of a different distribution of the various PID subtypes in the population, of the variability of the exposure to pathogens that promote carcinogenesis and of the high frequency of genetic variants that influence the individual's susceptibility to the development of tumors (14), making the comparison of data obtained from studies on different populations even more complex.

We discovered that tumors appeared only in some of the primitive immunodeficiencies present in our Center (**Figure 3**). Indeed of the 25 affected patients, 8 patients suffered from CVID, 5 from combined immunodeficiency (CID), 3 from Ataxia-telangectasia (AT), 2 from Hermanksy-Pudlak type 2 (HSP2), 2 from agammaglobulinemia X-linked (XLA), 2 from Wiskott-Aldrich syndrome (WAS), 2 from Hyper IgE syndrome (HIES), 1 from Severe combined immunodeficiency (SCID) suggesting that not all these immunodeficiencies present an equal susceptibility to the development of cancer (15). In fact, no case has been identified among patients with Chronic granulomatous disease (CGD) or IgA deficiency (IgAD), confirming the data present in the literature, in which only a few cases of cancer are reported

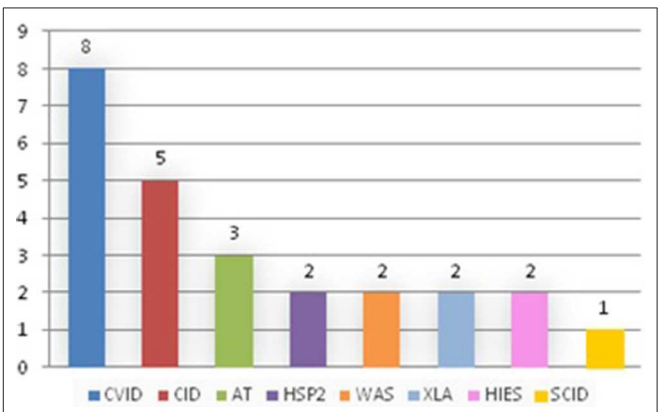

FIGURE 1 | Patients with PID.

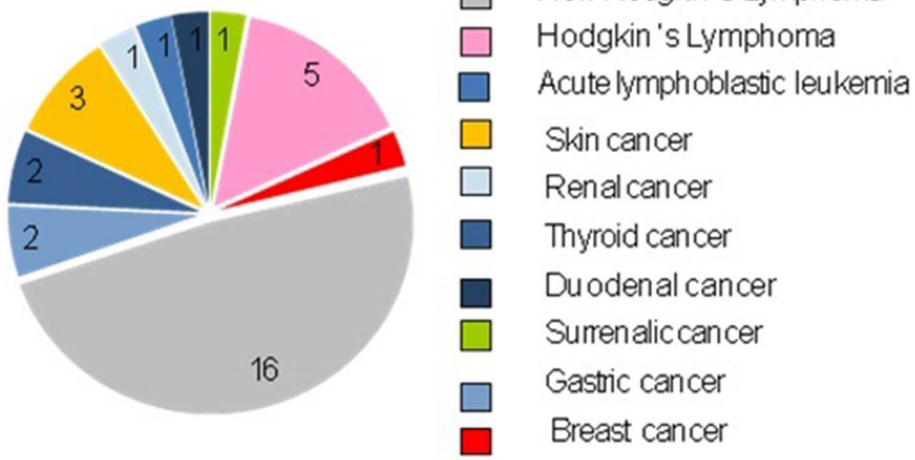

FIGURE 2 | Types of tumors present in our records.

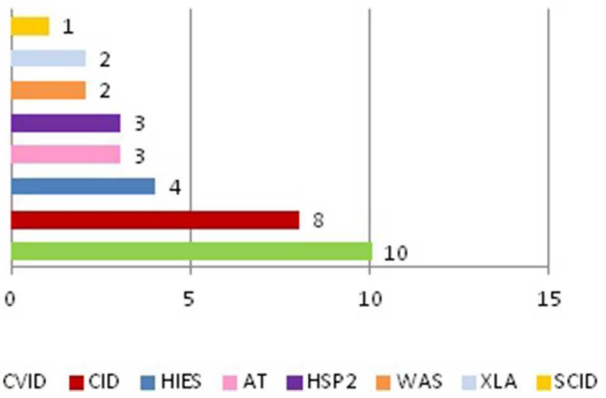

FIGURE 3 | Cases of tumors in patients with PID.

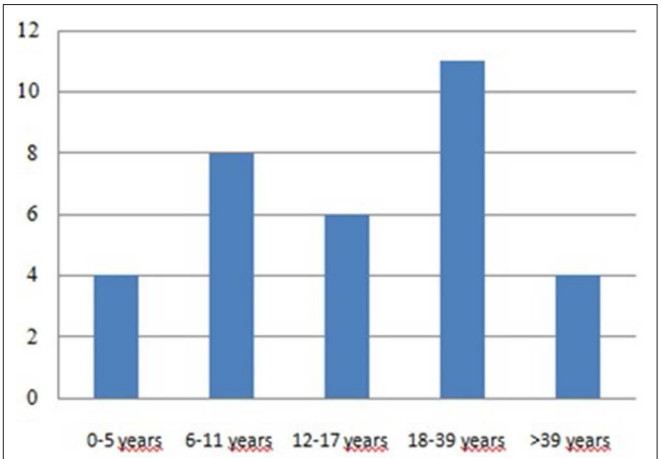

FIGURE 4 | Age of tumor development.

in these pathologies (10, 16, 17). It is also important to say that SCID and WAS are currently treated early with bone marrow transplantation, which has the advantage of ensuring a complete reconstitution of the functionality of the immune system and which could justify the small number of cases of cancer found in our records (1 out of 123 patients with SCID and 2 out of 52 patients with WAS). Moreover, in recent years the tendency of our Center is to rapidly perform a bone marrow transplant even in patients with CID, since experience has shown that executing of this procedure only after the development of a hematological tumor may be insufficient in the control of these neoplasms (two patients presented with tumor relapse despite bone marrow transplant). Therefore, in the next few years we expect a reduction in cancer cases in these patients too.

Another interesting finding is about the two patients with HSP2, who are two siblings, who both developed Hodgkin's lymphoma, a neoplasm that had never previously been identified in this immunodeficiency. In fact, before now, this syndrome was known only to determine a predisposition to the development of skin tumors (in our case a Dermatofibroma was detected) (18).

Among the cases we analyzed, patients who developed cancer were mostly male: 17 males (68%) and 8 females (32%). These data appear to be discordant with what emerges from the major studies in the literature. In fact, in two of these a distribution of cases of similar cancer was found in males and females (16, 17), while in one of them the females appeared more affected than males (17). In the latter case, if the individual tumors are analyzed, however, it is clear that the most common (non-Hodgkin's lymphoma, leukemia, and stomach tumors) have been presented with equal frequency in males and females and that the excess of cases present in the female subjects is attributable to the high number of cases of breast cancer (20% of all cancers).

Instead, the most frequent occurrence of cases in males, which was highlighted in the present study, is probably the consequence of the fact that the cohort of patients with PID in our center is characterized by a clear male prevalence (435 male patients out of 690 total, 63%), which can at least partially be explained by the fact that many of these diseases (XLA, WAS, XSCID, and 60% of CGD cases) have an X-linked transmission and, therefore, only affect males. These divergences explain the heterogeneity of the distribution of immunodeficiencies and tumors in the different populations and the consequent difficulty in studying the general characteristics of this phenomenon.

The age at diagnosis ranged from 1 to 52 years, with a median age of 19.6 years. These data are similar to those derived from the ICR (Immunodeficiency Cancer Registry), where the average age was 20 years (19). Considering all 25 patients with tumors, 18 tumors occurred in 14 patients aged below 18 years, while 15 cancers occurred in 11 patients aged over 18 years old (**Figure 4**; **Tables 1**, **2**). We have to consider that the age of onset of the various neoplasms appeared different related to the underlying immunodeficiency: in the CVID the diagnosis occurred at a mean age of 32.75 years and this is in line with what is found in the literature, which shows how CVID is an immunodeficiency that in itself is usually manifested in adulthood, as well as also a possible subsequent cancer (11). In the patients with SCID and CID the tumor arose at a mean age of 9 years which seems to conform to what is evident from the literature, in which the peak of onset is described between 0 and 10 years (11). In other PID the age of onset of the first neoplasia was very variable: in XLA at 1 and 37 years, in WAS at 4 and 27 years, in AT at 5, 14, and 29 years and in HSP2 at 8 and 10 years.

We also evaluated the latency time between the diagnosis of PID and the appearance of the first tumor, which was very different from case to case (from 1 month to 29 years). For example, there was a short latency (in 5 patients <1 year) between the diagnosis of SCID/CID and the subsequent appearance of cancer. Indeed, in two cases of CID the tumor was diagnosed even before the underlying immunodeficiency, constituting the clinical manifestation of onset.

This finding has already been reported in the literature, in particular in those PIDs that present a delayed onset and/or a mild clinical manifestation (4). Therefore, the wide variability that was found did not allow to identify either an age or a time of homogeneous latency of presentation of the neoplasms. For this reason, screening investigations for the most frequent tumors in these patients should be performed early, immediately after the diagnosis of PID.

The presence of a marked predisposition to the development of neoplasms is suggested in our study by the number of tumors: 25 patients showed 33 tumors, of which 26 were early tumors, of which two were synchronous and one bilateral, 6 were second tumors, and 1 a third tumor.

Most of the tumors were malignant (30 cases, 90.9%), only 3 cases (9.1%) were non-invasive tumors (non-infiltrative intraepithelial neoplasia of the stomach, dermatofibroma, and 1 squamous cell carcinoma *in situ*).

The neoplasms were mostly hematological (22 cases, 66.67%): 21 cases of lymphomas (5 cases of Hodgkin's lymphomas and 16 cases of Non-Hodking's lymphomas), 1 case of Acute Linfoblastic Leukemia.

Non-Hodgkin's lymphomas were the most frequent tumors (16/33, 48.48%). We identified the different subtypes: 8 diffuse large cell lymphomas, 2 Burkitt lymphomas, 1 gastric MALT lymphoma, 1 lymphoblastic T cell lymphoma, 1 plasmablastic B cell lymphoma, 1 folliculotropic mycosis fungoides, 1 anaplastic large cells lymphoma, and 1 non-determined subtype.

These lymphomas presented peculiar characteristics: (1) frequent extranodal onset (12/15 cases); (2) Prevalence of B phenotype (12/15); (3) Majority of diffuse large cell Lymphoma B (8/15). These results also appeared to be aligned with those

TABLE 1 | Tumors in pediatric age (<18 years).

Patients ($n = 14$)	Age	Tumors ($n = 18$)
1 G.Z.	10	Linfoblastic NHL T
2 D.C.	10	Scleronodular Classic LH
3 N.C	4	NHL (nd)
5 F.R.	1	Acute Linfoblastic Leukemia
6 R.A.S.	7	Adrenal Leiomyosarcoma
	12	Diffuse large B-cell lymphoma
7 M.P.	11	Diffuse large B-cell lymphoma
10 O.B.A.	5	Diffuse large B-cell lymphoma
11 F.D.	14	Papillary thyroid cancer
13 N.M.	14	Diffuse large B-cell lymphoma
14 M.V.*	10	Nodular lymphocyte predominant Hodgkin lymphoma
15 G.V.*	8	Nodular lymphocyte predominant Hodgkin lymphoma
	11	Dermatofibroma
16 L.M.	5	Burkitt NHL
17 A.C.	10	Nodular sclerosis classical Hodgkin lymphoma
	17	Mycosis Fungoide
18 S.G.	12	Mixed cellularity classical Hodgkin lymphoma
	17	Diffuse large B-cell lymphoma

**Brothers.*

TABLE 2 | Tumors in adult age (>18 years).

Patients ($n = 11$)	Age	Tumors ($n = 15$)
4 G.B.	20	Papillary thyroid cancer
8 M.B.	37	Gastric carcinoma
9 G.C.	29	Diffuse large cells lymphoma
12 A.M.	43	Burkitt's lymphoma
19 F.C.	27	Plasmablastic lymphoma
20 L.C.	35	Diffuse large cells lymphoma
	37	Squamous cell carcinoma *in situ*
21 E.A.	22	MALT NHL
22 I.V.	35	Gastric NIN
23 E.V.	45	Clear cell renal cell carcinoma
	45	Duodenal GIST
24 E.L.	24	Anaplastic large cell lymphoma
	28	Basal cell carcinoma
	32	Diffuse large cells lymphoma
25 R.M.	52	Lobular carcinoma of the breast

present in the literature and this is fundamental, since it is thanks to the knowledge of the common characteristics of these tumors that it is possible to make a diagnostic plan aimed at their early identification (15).

There was a minority of solid tumors (11 cases, 33.33%), which appeared to be very heterogeneous both by histotype and by localization: 3 cases of skin tumors (1 dermatofibroma, 1 squamous cell carcinoma *in situ* and 1 basalioma), 2 cases of thyroid tumors (papillary thyroid carcinoma), 2 gastric tumors (1 diffuse gastric carcinoma, and 1 non-infiltrative intraepithelial neoplasia of the stomach), 1 case of surrenalic tumor (leiomyosarcoma), 1 case of duodenal tumor (gastrointestinal stromal tumor, GIST), 1 case of breast cancer (lobular breast cancer), and 1 case of renal tumor (clear cell renal cell carcinoma).

Regarding specifically the tumors that appeared at a pediatric age, it was interesting to note that many were atypical for this age: Dermatofibroma, Fungal Mycosis, Papillary thyroid carcinoma, and Leiomyosarcoma. In contrast, the most common types of solid tumors of infancy were not found, such as Neuroblastoma, Wilms Tumor, and Rhabdomyosarcoma, as previously reported in the literature (11). This condition suggests that the immune system has a decisive role in controlling the development of only some types of cancer and to date the cause of this phenomenon has not yet been understood **Table 3**.

Moreover, in our study in various situations, the anatomo-pathological evaluation allowed to identify microbial agents in the tumor microenvironment: EBV, HP, and HPV, confirming their role in the etiopathogenesis of these neoplasms. Therefore, it is important to identify and treat these infections early, with the aim of preventing related cancers **Figure 5**.

In the period considered, 12 patients died (48%) in total and the tumor was the leading cause of death (7 cases). In particular, in our experience among patients who developed cancer at pediatric age, 7/11 patients (63.6%) survived 5 years after diagnosis, a number that appears lower than the general population of patients with cancer of the same age, in which the 5-year survival is 82% (20). This discrepancy could be due to the appearance in these patients of rapidly progressive tumors, as confirmed by our study in which, in pediatric patients who died for the spreading of the tumor, death occurred in all cases a short time after the diagnosis (from 3 to 13 months).

TABLE 3 | Tumor in our patients.

PID	Number of patients with PID	Number of patients with PID and tumor	Number of tumors	Type of tumor		
				1° tumor	2° tumor	3° tumor
CVID	74	8	10	1 HL	1 Squamous cell carcinoma *in situ*	
				1 Papillary thyroid cancer		
				3 NHL: (1 MALT, 1 Burkitt e 1 diffuse large B cell lymphoma)		
				1 Lobular carcinoma of the breast		
				1 Gastric NIN		
				1 Clear cell renal cell carcinoma		
				1 Duodena GIST		
CID	29	5	8	2 HL	3 NHL:	
				2 NHL: (1 linfoblastic T; 1 diffuse large B cell lymphoma)	(2 Diffuse large B cell lymphoma; 1 Mycosis fungoide)	
				1 Adrenal leiomyosarcoma		
SCID	123	1	1	1 Diffuse large cells lymphoma		
AT	19	3	3	3 NHL: (2 diffuse large B cell lymphomas; 1 Burkitt)		
XLA	46	2	2	1 Acute linfobastic leukemia		
				1 Gastric carcinoma		
HSP2	2	2	3	2 HL	1Dermatofibroma	
HIES	13	2	4	Papillary thyroid cancer	1 Basalioma	1 Diffuse large cells lymphoma
				1 Anaplastic large cells lymphoma		
WAS	52	2	2	2 NHL: (1 Plasmablastic lymphoma 1 nd)		

Patients	Tumor	Infection	Type PID
3	NH lymphoma	EBV	WAS
6	Adrenal leiomyosarcoma	EBV	CID
7	Diffuse large B cell lymphoma	EBV	SCID
8	Gastric carcinoma	HP	XLA
10	Diffuse large B cell lymphoma	EBV	CID
13	Diffuse large B cell lymphoma	EBV	AT
18	Diffuse large B cell lymphoma	EBV	CID
20	Squamous cell carcinoma in situ	HPV	CVID
21	MALT Lymphoma	EBV	CVID
22	Gastric NIN	HP	CVID

FIGURE 5 | Tumors and infection.

This condition does not seem to be due to the presence of tumors that are more resistant to treatment, but it is more likely the consequence of inadequate therapeutic management. In fact, in the absence of randomized clinical trials performed on these patients, the currently available chemotherapy treatment does not differ from that of immunocompetent patients, except for an individual modulation of the chemotherapeutic dosage and for the execution of a tight anti-infective prophylaxis. Despite the application of these measures, our experience has often shown a non-optimal response, as shown by the reduced survival, as well as the appearance of disease progression during the treatment of chemotherapy in 3 cases and relapses in 6 patients, all affected by lymphoma. As far as solid tumors are concerned, they have all been successfully treated and relapse has not occurred. The only exception was the case of gastric adenocarcinoma, which however appeared already metastatic at onset, to the point of contraindicating any treatment.

CONCLUSIONS

Our descriptive study certainly has the advantage of a very large number of cases (690) for a single Center considering the rarity of these diseases, a very long observation period (27 years) and good expertise in PID. The limits are instead represented by the difficulty of finding all the blood tests of older patients, above all because in many cases some immunological tests were not yet carried out. Furthermore, such a long observation time helps to highlight the importance of monitoring patients with PID in order to recognize and treat tumors early.

Therefore, the correct management of tumors that arise in patients with PID still represents a challenge in the pediatric field. For this reason now it is mandatory to collect in a unique international registry the cases of malignancies in PID that could lead to a better understanding of the etiopathogenesis and of the biological and clinical characteristics of these tumors, with the aim of defining adequate preventive measures and guaranteeing an early diagnosis which also creating a shared and specific therapeutic strategy, with the prospect of obtaining a better prognosis for these patients.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

FP, RS, ES, and LN contributed conception and design of the study. AS organized the database. AL was involved in the manipulation of stem cells of transplant patients and has coordinated the activities of the stem cell laboratory. MM performed the statistical analysis, wrote the first draft of the manuscript and sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

SYSTEMATIC REVIEW
published: 16 April 2019
doi: 10.3389/fimmu.2019.00777

A Systematic Review on Predisposition to Lymphoid (B and T cell) Neoplasias in Patients With Primary Immunodeficiencies and Immune Dysregulatory Disorders (Inborn Errors of Immunity)

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OPEN ACCESS

Edited by:
Andrew R. Gennery, Newcastle University, United Kingdom

Reviewed by:
Fabian Hauck, LMU Munich, Germany
Qiang Pan-Hammarström, Karolinska Institute (KI), Sweden
Eleonora Gambineri, University of Florence, Italy

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Specialty section:
This article was submitted to Primary Immunodeficiencies, a section of the journal Frontiers in Immunology

Received: *07 November 2018*
Accepted: *25 March 2019*
Published: *16 April 2019*

Citation:
Riaz IB, Faridi W, Patnaik MM and Abraham RS (2019) A Systematic Review on Predisposition to Lymphoid (B and T cell) Neoplasias in Patients With Primary Immunodeficiencies and Immune Dysregulatory Disorders (Inborn Errors of Immunity). Front. Immunol. 10:777. doi: 10.3389/fimmu.2019.00777

Primary immunodeficiencies and immune dysregulatory disorders (PIDDs; now referred to as inborn errors in immunity) are rare disorders with a prevalence of 41. 4 or 50.5 per 100,000 persons (1). The incidence of malignancy in PIDD patents is the second-highest cause of death in children as well as adults, after infection, and is higher in certain PIDDs compared to others. We performed a systematic review of the literature to identify reports of B cell and T cell neoplasias in PIDDs and clustered them based on their classification in the IUIS schema. As would be expected, higher susceptibility to malignancies are typically reported in patients with Common Variable Immunodeficiency (CVID), combined immunodeficiencies affecting cellular immunity, in particular, DNA repair defects, or in the context of impaired immune regulatory control. There is not much evidence of increased risk for cancer in patients with innate immune defects, indicating that not all types of infection or genetic susceptibility predispose equally to cancer risk. Viral infections, in particular EBV, HHV and HPV, have been shown to increase susceptibility to developing cancer, but also patients with defects in immune regulation, such as Autoimmune Lymphoproliferative Syndrome (ALPS), activated p110delta syndrome (APDS type 1) and IL-10 receptor deficiency among others have a higher incidence of neoplastic disease, particularly lymphomas. In fact, lymphomas account for two-thirds of all malignancies reported in PIDD patients (2), with either a combined immunodeficiency or DNA repair defect predominating as the underlying immune defect in one registry, or antibody deficiencies in another (3). The vast majority of lymphomas reported in the context of PIDDs are B cell lymphomas, though T cell lymphomas have been reported in a few studies, and tend to largely be associated with chromosomal breakage disorders (4) or

Cartilage Hair Hypoplasia (5). There appears to be a much higher prevalence of T cell lymphomas in patients with secondary immunodeficiencies (6), though this could reflect treatment bias. We reviewed the literature and summarized the reports of B and T cell lymphoma in PIDD patients to survey the current state of knowledge in this area.

Keywords: primary immunodeficiencies, B cell lymphoma, T cell lymphoma, systematic (literature) reviews, immunodeficiency

INTRODUCTION

Monogenic and other genetic defects of the immune system, now collectively grouped as primary immunodeficiencies and immune dysregulatory disorders (PIDDs) affect various components of the immune system with susceptibility to infections, but also to autoimmunity, malignancies, and other manifestations of immune dysregulation (7–9). The number of genetically defined PIDDs is increasing with the current tally at well over 300 genes (10), and several of these are associated with an increased predisposition to developing neoplastic disease (11). Early studies have suggested a variable prevalence of malignancies in PIDDs with approximately 25% affected with cancer at the higher end of the spectrum (2). A recent large study spanning 12 years for patients enrolled in a national registry (USIDNET) revealed an age-adjusted cancer risk, as well as a gender-associated cancer risk with male patients predominating in this cohort (12). The largest proportion of risk was conferred by susceptibility to hematopoietic malignancies rather than solid tumors, in particular lymphoma. Herein, we describe a targeted literature review on the associations of B and T cell lymphomas with PIDDs, particularly with regard to specific immune defects. We will also reflect on current state of knowledge as regards to pathogenesis and management of lymphoid neoplasias in PIDDs.

METHODS AND RESULTS

In these sections, we describe the strategy to identify relevant citations gathered for this review and a summary of the results. A comprehensive search of the MEDLINE database was initially conducted from its inception to October 17th, 2018. The search was updated on February 1st, 2019. Controlled vocabulary supplemented with keywords (which included lymphoma) was used to search for individual primary immunodeficiencies as per the IUIS 2017 classification. Search results and study selection are outlined in **Figure 1**. Number of cases and type (B, T or unspecified) are shown in **Figure 2**. The cases of B cell lymphomas, T cell lymphomas and unspecified lymphomas in PIDDs are summarized in **Tables 1**–**3** respectively.

B CELL LYMPHOMAS IN PIDDS

A thorough search of the literature using Medline database (via PubMed; **Figure 1**) identified 86 studies reporting B cell lymphoma in PIDD patients. **Table 1** gives details of the 456 patients identified from literature plus two unpublished cases from our center (Mayo Clinic, Rochester). The types of B cell lymphoma were unspecified non-Hodgkin Lymphoma (NHL) (37%, $n = 171$), diffuse large B cell lymphoma (DLBCL) (15%, $n = 68$), Hodgkin lymphoma (HL) (13%, $n = 59$), marginal zone lymphoma (MZL) including extranodal and intranodal MZL (5%, $n = 23$), Burkitt lymphoma (BL) (4%, $n = 17$) and diffuse histiocytic lymphoma (DHL) (0.4%, $n = 2$). Unlike T cell lymphomas where most of cases were reported in males, gender distribution was similar in males (29%, $n = 130$) and females (34%, $n = 157$), it was not specified (NS) in 37% ($n = 169$). The age of onset/diagnosis of lymphoma ranged from 7 months to 76 years (median age: 12 years). EBV association was seen in 25% ($n = 113$) of the patients. The majority of patients received combination chemotherapy as a standard treatment. While allogeneic hematopoietic cell transplantation (HCT) was not performed in many of these cases, it appeared to be successful in achieving a complete response (CR) in some cases. Serious infectious complications and death were frequently associated with chemotherapy treatment. Individual groups of PIDDs are examined in further detail based on the IUIS classification.

IUIS: Immunodeficiencies Affecting Cellular and Humoral Immunity

There were 12 studies which included 13 patients with B cell lymphoma. Adenosine deaminase 1 (ADA1) deficiency was most common immunodeficiency disease in this category (19–24). The other 7 cases include patients with an underlying diagnosis of Coronin 1A and DNA Ligase IV deficiencies, Artemis-SCID, RAG1, and ZAP70 defects (13–18, 24, 107). Of these 13 cases, 69% ($n = 9$) patients were females, 15% ($n = 2$) were males and 15% ($n = 2$) were NS. DLBCL (62%, $n = 8$) was the most common type of B cell neoplasm identified followed by unspecified B cell lymphoma (15%, $n = 2$). The median age at presentation was 1.5 years (range 0.9–14 years) and all patients were EBV-positive. The most common clinical presentation was lymphadenopathy and high fevers. The underlying etiology for the development of lymphoma appeared to be DNA repair defects and EBV association. All patients were treated with some form of combination chemotherapy, most commonly, rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone (R-CHOP). Of these 7 cases, only 2 patients survived and were in complete remission at follow-up. One patient who developed DLBCL in the setting of RAG1 deficiency had a partial response with rituximab, which was consolidated by HCT from an HLA-matched unrelated donor. Though there is no long-term follow-up data in this report, the patient had no evidence of disease at 3 years follow-up (16).

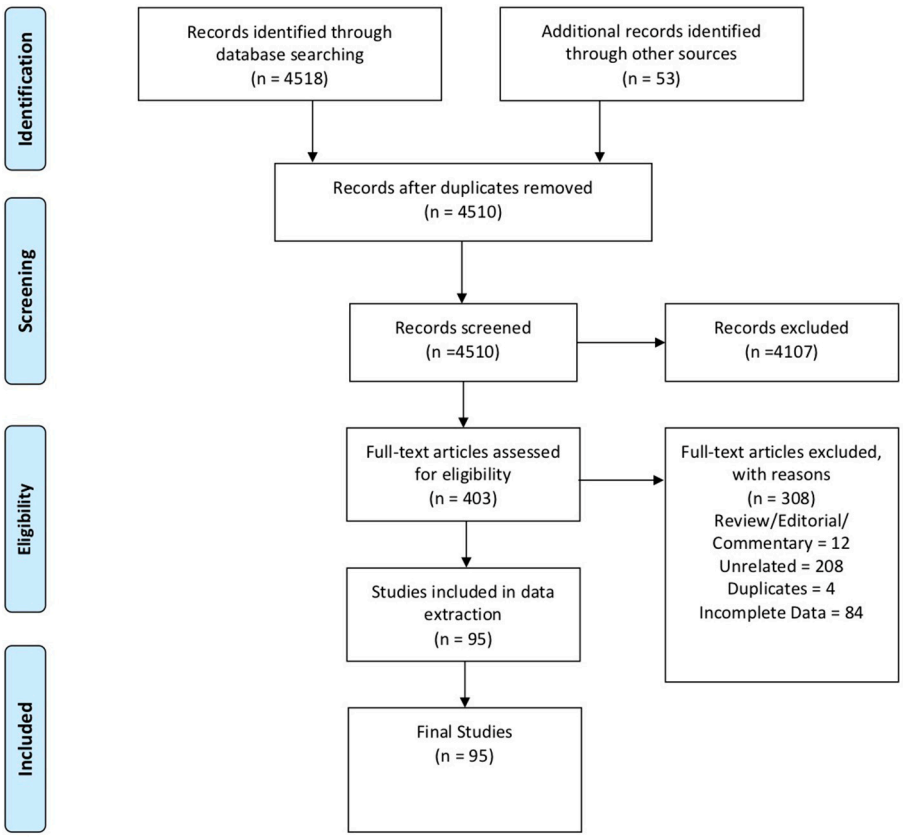

FIGURE 1 | PRISMA flow diagram: The PRIMSA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) diagram details our search and selection process applied during the overview.

Adenosine Deaminase 1 (ADA1) Deficiency

B cell lymphoma with ADA1 deficiency was seen in six patients, three of whom had EBV association (19–24). Only one patient was reported to be in complete remission 20 months after diagnosis, others died despite treatment.

IUIS: Combined Immunodeficiency Disorders With Associated or Syndromic Features

In this category, 27 studies plus two unpublished cases reported B cell lymphomas in 191 patients with combined immunodeficiency disorders with associated or syndromic features. Of these, 41% ($n = 78$) cases were associated with EBV infection, and the lymphoma was diagnosed at median age of 10 years. In this cohort there appeared to be a preponderance of males at 38% ($n = 73$) with females accounting for 32% ($n = 62$) and 30% ($n = 56$). Some of the more common genetic disorders are discussed further.

Ataxia Telangiectasia (AT)

Over one-third (35%, $n = 67$) of the reported cases of lymphoma in this IUIS category were in the setting of ataxia telangiectasia, which is an autosomal recessive disorder with immunodeficiency, DNA repair defects and neurological complications(3, 25–28, 38, 41). Lymphomas were diagnosed across all ages of patients typically manifesting before 10 years of age with the oldest cases being reported at 16 years of age. Original reports reported the histology as DLBCL or unspecified NHL in 75% ($n = 50$), HL in 21% ($n = 14$), BL in 3% ($n = 2$), and MZL in 1% ($n = 1$) of cases. The most common presenting symptoms included extensive lymphadenopathy. A French study reported a crude incidence of cancer of 24.7% in AT with B cell lymphoid malignancies representing the majority of cases. The pathological diagnosis of lymphoma in these patients can be challenging, and it is relevant to note that three of the four T-cell NHLs, based on pathology, were reclassified as B-cell NHLs after centralized pathology review. The median overall survival (OS) decreased from 24 to 15 years in AT patients with malignant disease. In fact, the common causes of mortality in this group were either cancer (47%) or infectious complications (34%). There was a trend toward increased survival if there was an excellent response to chemotherapy. Though the overall prognosis in AT remains relatively poor, a subset of these of patients might benefit from treatment of the malignancy with an improved survival (25).

Wiskott -Aldrich Syndrome (WAS)

There were 6 studies identified, which reported B cell lymphoma in 6 patients (median age 14 years) with WAS, which is an

TABLE 1 | Summary of B cell lymphomas in PIDDs.

PID	References	*N*	Proposed mechanism	Cancer	Specific mutations	Age/sex	Manifestation/course	Treatment	Outcome
IMMUNODEFICIENCIES AFFECTING CELLULAR AND HUMORAL IMMUNITY									
Coronin 1a deficiency*	(13)	2	EBV associated	Pt1: DLBCL	Coronin-1a mutation: V134M/V134M	1/M	Left orbit mass	Chemotherapy	F/U at 11.5 y = CR
				Pt2: DLBCL	Coronin-1a mutation: V134M/V134M	0.6/F	Cervicothoracoabdominal LN and cerebral lesion	Chemotherapy	Died during induction therapy at 8.5 m of age
DNA Ligase IV mutation*	(14)	1	EBV associated	DLBCL	LigIV gene: M249V substitution and a 5 nucleotide del from 1,270–1,274	14/F	Progressive gingival swelling and high fever	Vincristine, cyclophosphamide, and prednisolone	Died of respiratory aspergillosis
Artemis-SCID*	(15)	1	DNA repair defect	BCL	Hypomorphic mutations of the Artemis gene	5/F	Liver, lung, LN, skeletal muscle involvement	Rituximab	Died of lymphoma
RAG 1 mutation*	(16)	1	NR	DLBCL	RAG1 gene: Allele 1 R314W* Allele 2 R507W/R737H	2/F	Tumor of right tonsil	Rituximab, HSCT	CR
DNA Ligase IV mutation*	(17)	1	DNA repair defect EBV associated	DLBCL	Compound heterozygous for a null allele and a hypomorphic mutation in DNA LigIV	2/F	High fever, cervical LN, hepatomegaly, necrotizing mucositis.	Chemotherapy	Pt. died of aspergillosis
ZAP70 deficiency*	(18)	1	EBV associated	DLBCL	Zap70 Gene: c.836_837 delAT	1/F	Generalized lymphadenopathy	Vincristine, cyclophosphamide methylprednisolone	Died of DIC, multiorgan failure
ADA deficiency*	(19)	1	EBV associated	BCL	NR	NR	NR	NR	Dead 1 week later
ADA deficiency	(20)	1	EBV associated (suspected)	Immunoblastic plasmacytoid	NR	NR	NR	NR	Died at 4 y
ADA deficiency	(21)	1	NS	DLBCL	A83D; Exon5 splice donor site c.573 + 1G>A	10/M	6-week history of head- ache, eye deviation, and weakness.	COP + APO + 6-MP; MTX	Died after 5 m
ADA deficiency	(22)	1	Immune dysregulation	BL	Homozygous Q3X	14/F	Right hip pain and limping	Chemotherapy	CR 20 m later
ADA deficiency	(23)	1	Immune dysregulation	DLBCL	Homozygous W272X (c.815G > A)	3/F	Respiratory complaints and symptoms	BFM 2004 protocol	Died of septic shock and intracranial hemorrhage after starting treatment
ADA deficiency*	(24)	1	EBV associated	Plasmablastic	Homozygous 462delG	18/F	Persistent fever, multiple lymphadenopathies and bilateral periorbital edema	Rituximab; APO; and cyclophosphamide + dexamethasone	Patient died of a hemorrhagic alveolitis, 12 days after starting chemotherapy
COMBINED IMMUNODEFICIENCIES WITH ASSOCIATED OR SYNDROMIC FEATURES									
Ataxia Telangiectasia*	(25)	12	NS	HL	NS	10/8M, 4F	NR	NS	10 pts died
		38	NS	NHL	NS	9/22M, 16F	NR	NS	28 pts died
Ataxia Telangiectasia*	(26)	1	DNA repair defect	MZL	NS	16/M	Chronic lymphadenitis, LN at left jaw, Cervical, celiac, para-aortic	Rituximab	CR
Ataxia Telangiectasia*	(27)	1	DNA repair defect	HL	NR	14/M	Cervical, axillary and mediastinal LN	COPP	CR
Ataxia Telangiectasia*	(28)	1	EBV associated	NHL	ATM gene (exon 39 and c.6095G4A)	13/M	Waldeyer's ring, intrapulmonary, abd., tonsillar, cervical LN	Rituximab, doxorubicin, dexamethasone, cytarabine, cyclophosphamide, etoposide, vincristine, HSCT	No recurrence on 3 y FU
Wiskott Aldrich*	(29)	1	EBV associated DNA repair defect	DLBCL	NR	14/M	Cervical adenitis, neurological symptoms	Rituximab	Died
Wiskott Aldrich*	(30)	1	NS	BL	WASP gene missense mutation (105 C > T) in exon 1	12/M	Recurrent colicky abd. pain and bloody stools	Rituximab, CHOP	CR

(Continued)

TABLE 1 | Continued

PID	References	*N*	Proposed mechanism	Cancer	Specific mutations	Age/sex	Manifestation/course	Treatment	Outcome
Wiskott Aldrich*	(31)	1	EBV associated	DLBCL	NS	15/M	Progressive respiratory distress, night sweats	Cyclophosphamide, prednisone, radiotherapy, laser surgery	Died of lung infection
Wiskott Aldrich*	(32)	1	EBV associated	HL	NS	1	Pulmonary hilar LN	ABVD	CR for more than 4 y
Wiskott Aldrich*	(33)	1	EBV associated	NHL	NR	20/M	Lymphadenopathy, CNS, gastric wall, pulmonary lesions	Acyclovir, adenosine arabinoside	Died of sepsis
Wiskott Aldrich*	(34)	1	EBV associated	IL	NS	13/M	Rectosigmoid tumor	Chemotherapy, excision, L-asparaginase therapy	Died of colon perforation
DiGeorge Syndrome*	(35)	1	EBV associated	INHL	NS	10m/F	Hemiparesis due to cerebral mass, mediastinal LN	Untreated	Died
Cartilage hair dysplasia*	Unpublished	1	EBV associated	DLBCL	RMRP gene	51/M	Incidental lung nodule	R-CHOP	CR
Cartilage hair dysplasia*	Unpublished	1	EBV associated	MZL	RMRP gene	47/M	Fever, night sweats, lymphadenopathy	Rituximab	Under treatment with Rituximab for recurrence
Cartilage hair dysplasia	(36)	10	NS	Pt1: HL	70A > G/70A > G	20/M	NR	NR	Died in 1 m
				Pt2: NHL	70A > G/262G > T	40/M	NR	NR	Died same m.
				Pt3: NHL	dupTACTCTGTGA at 13/70A > G	45/F	NR	NR	Died in 3 m
				Pt4: NHL	70A > G/70A > G	46/F	NR	NR	Died in 6 m
				Pt5: NHL	Not tested	22/F	NR	NR	Died in 2 m
				Pt6: NHL	70A > G/70A > G	6/F	NR	NR	Died in 9 m
				Pt7: NHL	Not tested	21/M	NR	NR	Died in 1 m
				Pt8: NHL	Not tested	26/M	NR	NR	Alive 4.5 y
				Pt9: NHL	70A > G/70A > G	32/M	NR	NR	Alive 11 y
				Pt10: NHL	70A > G/70A > G	33/F	NR	NR	Alive 4.5 y
NBS	(37)	8	NS	Pt1: DLBCL	Homozygous 657del5	10/M	DLBCL at 10, 18, 23 and 26y	1st event unknown, 2nd LMB-89, 3rd R-CHOP, 4th DHAP	Alive, CR at 27 y
				Pt2: DLBCL	Homozygous 657del5	14/F	DLBCL at 14, 20y	1st event unknown, 2nd LMB-89 without MTX	Died of liver failure after CYM protocol at 20 y
				Pt3: DLBCL	Homozygous 657del5	4/F	NR	LMB-89, without CTX, followed by individualized chemotherapy	Died of disease after second individual protocol at 5 y
				Pt4: DLBCL	Homozygous 657del5	6/F	NR	LMB-89, without CTX	Died of disease at 7 y
				Pt5: DLBCL	Homozygous 657del5	11/F	NR	LMB-89 without CTX, followed by individualized chemotherapy	Died of disease at 12 y
				Pt6: DLBCL	Homozygous 657del5	23/M	Switch from DLBCL to AILT at 26y	LMB-89; for AILT: BFM-90 followed by individualized chemotherapy	Died of disease after individual protocol at 27 y
				Pt7: BL	Homozygous 657del5	9/M	Burkit like lymphoma at 9, followed by DLBCL at 10	1st LMB-89, 2nd LMB-89 followed by individualized chemotherapy and splenectomy	Alive at 16 y

(Continued)

TABLE 1 | Continued

PID	References	*N*	Proposed mechanism	Cancer	Specific mutations	Age/sex	Manifestation/course	Treatment	Outcome
				Pt8: BL	Homozygous 657del5	5/F	NR	LMB-89	PR, alive at 12
NBS	(38)	11	NS	Pt1: DLBCL	NR	9/M	NR	NHL-BFM86	CR 11 y after diagnosis
				Pt2: DLBCL	NR	15/F	B-NHL at 31 y, third malignancy Bi-linage leukemia at 34 y	NHL-BFM86 modified	Died at 34y from 3rd malignancy
				Pt3: BL	NR	4/M	2nd BL at 10 y	NHL-BFM90	CR at 18y
				Pt4: IL	NR	10/F	NR	NHL-BFM90 switched to CHOP because of progressive disease	Died 4.5 m after diagnosis
				Pt5: FL	NR	7/F	2nd DLBCL 13 m after diagnosis, third NHL (DLBCL) 8 y after diagnosis	NHL-BFM95	Current under treatment (NHL-BFM modified)
				Pt6; BL	NR	5/M	NR	NHL-BFM95	CR 6 y after diagnosis
				Pt7: DLBCL	NR	5/F	2nd malignancy: ALCL-T 3.5 y after diagnosis	NHL-BFM95 modified	Death of lung infection 8 y after diagnosis
				Pt8: DLBCL	NR	15/M	NR	NHL-BFM95 + Rituximab	CR 4 y after diagnosis
				Pt9: NHL	NR	10/F	NR	NHL-BFM95 modified	Death of lung infection 1 m after end of treatment
				Pt10: DLBCL	NR	9/F	2nd malignancy: T-ALL 3.5 y after diagnosis	B-NHL BFM04 modified + Rituximab	Death during treatment, 8 m after diagnosis of T-ALL
				Pt11: ALCL	NR	15/M	NR	NHL-BFM90	CR 6 y after diagnosis
Ataxia Telangiectasia	(38)	11	NS	Pt1: BL	NR	16/M	NR	B-NHL-BFM04 modified	Chronic lung disease, death of pulmonal infection 1.9 y after diagnosis
				Pt2: BL	NR	7/M	NR	B-NHL BFM04 modified + Rituximab-Window	CR 2.7 y after diagnosis
				Pt3: DLBCL	NR	6/M	NR	B-NHL BFM04 modified	CR 3.8 y after diagnosis
				Pt4: DLBCL	NR	9/F	NR	NHL-BFM90 modified	Death of early relapse 1 y after diagnosis
				Pt5: DLBCL	NR	5/M	2nd malignancy: DLBCL 10 y after diagnosis	NHL-BFM95 modified	CR
				Pt6: DLBCL	NR	11/M	2nd malignancy: DLBCL 3 y after diagnosis	NHL-BFM95 modified	Death with pulmonary failure
				Pt7: DLBCL	NR	7/F	NR	NHL BFM95 modified	CR 9.6 y after diagnosis
				Pt8: DLBCL	NR	10/F	NR	NHL-BFM95 modified	Death of therapy toxicity 4 m after diagnosis
				Pt9: DLBCL	NR	9/M	NR	NHL-BFM95 modified	CR 7.1 y after diagnosis
				Pt10: DLBCL	NR	9/F	NR	NHL-BFM95 modified	CR 7 y after diagnosis
				Pt11: HL	NR	12/M	NR	No chemotherapy	Death of progressive disease 1 m after diagnosis
NBS	(39)	10/26	Defective antitumor immunosurveillance	Pt1: NHL	NR	5/F	NR	NR	NR
				Pt2: NHL	NR	8/F	NR	NR	Death at 10 y
				Pt3: NHL	NR	7/F	NR	NR	Death at 7 y
				Pt4: NHL	NR	12/F	NR	NR	Death at 12 y

(Continued)

TABLE 1 | Continued

PID	References	*N*	Proposed mechanism	Cancer	Specific mutations	Age/sex	Manifestation/course	Treatment	Outcome
				Pt5: NHL	NR	7/F	NR	NR	Death at 10 y
				Pt6: NHL	NR	7/F	NR	NR	Death at 19 y
				Pt7: NHL	NR	7/F	NR	NR	Death at 7 y
				Pt8: NHL	NR	11/M	NR	NR	NR
				Pt9: NHL	NR	29/F	NR	NR	Death at 29 y
				Pt10: HL	NR	12/M	NR	NR	Death at 14 y
NBS*	(40)	11	Two cases possibly EBV associated	Pt1: BL	NR	9/M	Abdominal, splenomegaly (cervical tumor)	NR	Alive at 15 y
				Pt2: DLBCL	NR	24/M	Abdominal (inguinal LN)	NR	Died at 27 y
				Pt3: DLBCL	NR	11/F	Generalized (axillary LN)	NR	Died at 12 y
				Pt4: DLBCL	NR	6/F	Cervical LN	NR	Died at 7 y
				Pt5: DLBCL	NR	4/F	Cervical LN	NR	Died at 6 y
				Pt6: DLBCL	NR	15/F	Generalized (axillary LN)	NR	Died at 20 y
				Pt7: DLBCL	NR	11/M	Cervical LN	NR	Alive at 27 y
				Pt8: DLBCL	NR	4/M	Generalized cervical LN	NR	Died at 5 y
				Pt9: DLBCL	NR	8/F	Generalized (axillary LN)	NR	Died at 10 y
				Pt10: HL	NR	12/M	Cervical LN	NR	Died at 14 y
				Pt11: AILT-like	NR	8/F	Generalized cervical LN	NR	Died at 8 y
NBS	(41)	4	NS	Pt1: DLBCL	NR	15/F		NHL-BFM	CR, LFU +6 y
				Pt2: ALCL (B)	NR	6/F		NHL-BFM	Death of fungal sepsis after first course of therapy
				Pt3: DLBCL	NR	9/M		NHL-BFM	CR, LFU +2.5 y
				Pt4: IL	NR	10/F		NHL-BFM + RT	Initial nonresponse died after 5 m
NBS	(42)	1	Defective DNA repair	DLBCL	Homozygous 657del5	17/M	Bilateral cervical LN, malaise, headache, epistaxis, symptoms of URI, fever, night sweats, and loss of weight, followed by the development of protruding tissue mass in the epigastrium	Modified CHOP + Rituximab	CR, 3 y in CR on LFU
NBS*	(43)	1	EBV associated	HL	Homozygous 657del5	5/F	Fever lasting for 2 m and mediastinal adenopathy	COPP/ABV	CR, 2 y in CR on LFU
NBS*	(44)	12/57	Chronic antigenic stimulation	NHL	NR	NR	NR	NR	15 out of total 22 patients died, but 7 were clinically stable after early diagnosis and successful treatment
		2/57	Chronic antigenic stimulation	HL	NR	NR	NR	NR	
NBS	(45)	30/149	NS	NHL	NR	NR	NR	NR	44% of all patients in CR, 54% dead due to disease progression
		7/149		HL	NR	NR	NR	NR	
Dyskeratosis Congenita	(46)	1	Genetic insatiability	HL	NR	30/M	NR	Radiation-chemotherapy	Died after 25 y (gastric adenocarcinoma)
Ataxia Telangiectasia	(3)	1/10	NS	1 NHL	NR	NR	NR	NR	NR

(Continued)

TABLE 1 | Continued

PID	References	*N*	Proposed mechanism	Cancer	Specific mutations	Age/sex	Manifestation/course	Treatment	Outcome
Ataxia Telangiectasia	(41)	2	NS	Pt1: DLBCL	NR	9/F		NHL-BFM	Relapse after 10 m, died after 1 y of diagnosis
			NS	Pt2: DLBCL	NR	12/M		NHL-BFM	CR, LFU +1 y
PNP deficiency	(41)	1	NS	ALCL (B)	NR	2/F		dosages reduced	BMT (haploident.) in CR, BMT-related death
Schimke Immuno-Osseous dysplasia	(47)	1	NR	NHL	SMARCAL1 missense mutation (R561H)	8/M	Colicky abdominal pain and vomiting. Palpation of the abdomen revealed a hard mass in right upper abdomen. Intussusception secondary to NHL	Vincristine, cyclophosphamide, adriamycin and intrathecal methotrexate using half of their usual doses	Died due to septicemia following chemotherapy
Schimke Immuno-Osseous dysplasia	(48)	3/71	NS	NHL	SMARCAL mutation	NR	NR	NR	NR
Schimke Immuno-Osseous dysplasia*	(49)	1	EBV associated	BCL	Homozygous mutation of the SMARCAL1 gene (1146–1147delAA þ IVS6 þ 2delGT)	5/M	Fever, mild cough	Chemotherapy	Died 1 m later due to multiorgan failure
CHARGE association with Hyper-IgM	(50)	1	Chronic antigenic stimulation	MZL	NR	5/F	Suspected purulent bilateral conjunctivitis: Unresponsive On microscopy: salmon-colored, nodular lesions	Topical IFN-[alpha] 3 times a day 300,000 U/drop.	Resolved. No lesions at 1 y FU
PREDOMINANTLY ANTIBODY DEFICIENCIES									
Severe Ig deficiency	(41)	1	NS	DLBCL	NR	14/F		VBL for palliation only	Died after 3 m
IgG1, −3 and −4 deficiency	(41)	1	NS	BL	NR	6/F		NHL-BFM	CR, LFU +5 y
Selective IgA deficiency	(41)	1	NS	Burkitt-like	NR	2/M		NHL-BFM	CR, LFU +0.5 y
IgG-4 and IgM deficiency	(41)	1	NS	BL	NR	5/F		B-NHL therapy per protocol full dosage	CR, LFU +4.5 y
IgA, IgG2, and −4 deficiency	(41)	1	NS	BL	NR	11/M		NHL-BFM	Reached CR, died of sepsis after second therapy course
APDS	(51)	2/8	NS	Pt1: DLBCL	GOF mutation in the PIK3CD gene: E1021K (c.3061G > A)	8/M	2nd DLBCL at 19 y	Initially UKCCSG 9002 protocol, 2nd malignancy: CHOP + Rituximab	Died from large bowel perforation and bleeding 12 days after the third course of chemotherapy.
				Pt2: HL	Heterozygous GOF mutation in the PIK3CD gene: E1021K	11/M	Cervical LN enlargement	Chemotherapy and RT	CR, alive FU of more than 10 y
APDS	(52)	6/53	NS	2 DLBCL, 1 HL, 1 NMZL, 1 Hodgkin-like	E1021K mutation	1.5–27/NR	NR	NR	3 died
APDS	(53)	1/17	NS	MZL	E1021K mutation	NR	NR	NR	NR
Selective IgA deficiency	(54)	1/386	NS	NHL	NR	38/M	NR	NR	NR
CVID	(55)	1	NS	DHL	NR	47/M	Abd pain, numbness, and weakness in legs.	RT, cyclophosphamide, vincristine, procarbazine, prednisone	FU 3.5 y: CR
CVID	(56)	1	NS	EZMZL		16/F	Cough, wheezing	Prednisolone	Alive
CVID	(57)	1	NS	EZMZL		44/F		Chlorambucil and prednisone	Recurrence after 6m

(Continued)

TABLE 1 | Continued

PID	References	*N*	Proposed mechanism	Cancer	Specific mutations	Age/sex	Manifestation/course	Treatment	Outcome
CVID	(58)	54	EBV possibly	BCL	NR	NR	NR	NR	NR
CVID*	(59)	3/220	EBV associated	BCL	NR	NR	NR	NR	NR
CVID	(60)	7	NS	BCL	NR	46**/F	NR	NR	NR
		9	NS	BCL	NR	42**/M	NR	NR	NR
CVID*	(61)	1	EBV or ITK mutation	HL	NR	25/F	NR	Rituximab and brentuximab vedotin.	CR
CVID	(62)	3	NS	Pt1: BCL	NR	2/M	Generalized LN	NR	Died at 23y
				Pt2: BCL	NR	61/F	NR	NR	NR
				Pt3: BCL	NR	66/F	NR	NR	NR
CVID	(63)	22/248	NS	Pt1: NHL	NR	13/F	NR	Chemotherapy (CHOP)	Died, age 13
				Pt2: NHL	NR	31/F	NR	Surgery	Alive 1 y later
				Pt3: NHL	NR	41/M	NR	Chemotherapy, HSCT	Died, age 41
				Pt4: NHL	NR	44/F	NR	Surgery	Unknown, alive 8 y later
				Pt5: NHL	NR	45/M	NR	None	Alive, 1 y later
				Pt6: NHL	NR	46/F	NR	Chemotherapy (Type?)	Died
				Pt7: NHL	NR	48/M	NR	None	Died, age 48
				Pt8: NHL	NR	52/F	NR	Chemotherapy (CHOP)	Died, age 56
				Pt9: NHL	NR	52/F	NR	Radiation	Alive, 2 y later
				Pt10: NHL	NR	54/F	NR	Surgery, surgery, chemotherapy	Alive at age 68
				Pt11: NHL	NR	54/F	NR	Surgery	Died other causes, 15 y later, age 69
				Pt12: NHL	NR	56/F	NR	M-BACOD, CHOP, RT, CP	Alive, 12 y later
				Pt13: NHL	NR	63/M	NR	CHOP, M-BACOD, M2	Died, age 65
				Pt14: NHL	NR	67/F	NR	Chemotherapy (C-MOPP)	Died, age 68
				Pt15: NHL	NR	67/F	NR	Chemotherapy (CHOP)	Alive, stable 8 y later
				Pt16: NHL	NR	71/F	NR	Chemotherapy (C-MOPP)	Died, age 72
				Pt17: NHL	NR	72/F	NR	Alpha interferon	Died, age 73
				Pt18: NHL	NR	75/F	NR	Radiation	Alive, stable, 2 y later
				Pt19: NHL	NR	77/M	NR	Chemotherapy (Type?)	Died, age 77
				Pt20, Pt 21, Pt 22: HL	NR	8/M Rest NR	NR	Splenectomy and chemotherapy in Pt20	Pt20 relapsed after 12 years
CVID	(64)	5/5	NS	Pt1: EZMZL	NR	56/F	Parotid, 2nd Breast, 3rd DLBCL in sternum after 11 y	Radiation, excision, CHOP, rituximab	Recurrence
				Pt2: EZMZL	NR	35/F	Lung	None	Well
				Pt3: EZMZL	NR	31/F	Parotid	Excision, radiation	Recurrence
				Pt4: EZMZL	NR	42/M	Lung involvement	CVP and Rituximab 2, CHOP	Recurrence
				Pt5: EZMZL	NR	35/F	Lung	R-CHOP	CR

(Continued)

TABLE 1 | Continued

PID	References	*N*	Proposed mechanism	Cancer	Specific mutations	Age/sex	Manifestation/course	Treatment	Outcome
CVID	(65, 66)	9/117	Unknown	Pt1: DLBCL	NR	12/F	Liver, Spleen	CHOP	Died at 13 y
				Pt2: DMSL	NR	54/F	Right Inguinal Node	CHOP	Died at 69 due to other causes
				Pt3: DMSL	NR	50/F	Pelvis,	CHOP	Died at 56 y
				Pt4: DLBCL	NR	48/F	Proximal jejunum	Surgery	Alive, 8 y later
				Pt5: Follicular	NR	54/F	Parotid, 1 y later FL in axilla	Surgery for both	Alive, after 3 y
				Pt6: diffuse small cleaved cell	NR	56/F	Pelvic nodes	M-BACOD, CHOP, RT, CP	Alive
				Pt7: DLBCL	NR	57/F	Supraclavicular area, abdomen	C-MOPP	Died at age 68
				Pt8: DLBCL	NR	65/F	Lymph nodes, lungs	C-MOPP	Died at age 72
				Pt9: DLBCL	NR	70/F	Cervical lymph nodes	Alpha-Interferon	Alive
CVID	(54)	5/176	NS	Pt1: NHL	NR	52/F	NR	NR	NR
				Pt2: NHL	NR	59/F	NR	NR	NR
				Pt3: NHL	NR	76/F	NR	NR	NR
				Pt4: HL	NR	49/F	NR	NR	NR
CVID	(67)	1	Defective antitumor immunosurveillance	DHL	NR	44/F	Abd. pain, slight diarrhea, weight loss. Palpable mass in right abdomen	Resection and irradiation	4-y F/U: CR
CVID	(68)	1	Chronic antigenic stimulation	IL	NR	75/W	Weight loss, numbness and tingling in extremities	Surgical resection	Death post-op due to septicemia
CVID	(69)	1	Defective antitumor immunosurveillance	MZL	NR	39/M	Periumbilical pain, severe diarrhea, weight loss	NR	NR
CVID	(70)	4/224	NS	NHL	NR	NR	NR	Chemotherapy + Rituximab + transplant	NR
CVID	(71)	5/247	NS	Jejunal, 1 histiocytic, 1 HL, 2 BCL	NR	NR	NR	NR	4 died, HL responded to RT
CVID*	(72)	1	NS	EMZL	NR	41/F	Malaise, dyspnea and productive cough	IVIG, chlorambucil and prednisone	
CVID	(73)	10/334	NS	NHL	NR	NR	NR	NR	5 died.
CVID	(3)	10/416	NS	1 DLBCL, 2 FL, 1 BL, 1 SLL, 1 EMZL, 3 NHL, 1 WM	NR	NR	NR	NR	NR
CVID	(74)	23	NR	NHL	NR	NR/28F, 11M	NR		11 died of lymphoma, 12 alive
		3	NR	DLBCL	NR				2 died of lymphoma, 1 also had severe lung disease, 1 alive
		4	NR	HL	NR				2 died of lymphoma, 2 alive
		5	NR	EMZL	NR			3 no treatment, 2 chemotherapy	All alive
		1	NR	MZL	NR				No treatment given, alive
		1	NR	Diffuse poorly differentiated	NR				Died of lymphoma

(Continued)

TABLE 1 | Continued

PID	References	*N*	Proposed mechanism	Cancer	Specific mutations	Age/sex	Manifestation/course	Treatment	Outcome
		1	EBV associated	T -cell rich BCL	NR				Died of lymphoma
Selective IgA deficiency	(75)	1	Chronic antigenic stimulation	PCMZL, MZL	MZL: monoclonal amplification of 98 base pairs in FR3A region of the IGH gene	43/F	Asymptomatic subcutaneous nodules in upper extremity. Enlarged axillary, mediastinal LN	Rituximab Radiotherapy and local excision	CR followed by repetitive relapses
DISEASES OF IMMUNE DYSREGULATION									
ALPS*	(76)	1	EBV associated	FL, DLBCL, HL	c.784A>T mutation in TNFRSF6,	33/M	Lymphadenopathy	R-CHOP	CR
XLP1	(77)	3	Defective antitumor immunosurveillance	Pt1: BL	SH2D1A p.E17D c.51 G > C	6/M	Acute abd. obstruction on presentation, night sweats, weight loss.	NHL-BFM95, full dosage, + Rituximab	CR
				Pt2: DLBCL	SH2D1A p.W64C c.192G > T	14/M	Painless mass of about 2.0 × 2.5 cm in size on the front right-side chest wall	B-NHL-M2004, full dosage + Rituximab	Died at the age of 19
				Pt3: DLBCL	SH2D1A c.53insA > T	6/M	Testicular DLBCL	NHL-BFM90 + Rituximab	Died at 8.3 y
XLP1*	(78)	1	NS	BL	exon 2 of the SH2D1A gene at position 146 (c146insG	4/M	Lymphadenopathy, HS, cerebellar tumor	ETO, DXM, cyclophosphamide	PR died of ICH
XLP*	(79)	2	EBV associated	Pt1: BL	NR	1.6/M	Palpable abd. mass Malaise, poor appetite, failure to thrive.	Protocol LMB-84	Died of hemorrhage 6 m after stopping therapy.
				Pt2: BL	t(8;14) (q24;q32)	2/M	Abd. Mass, malaise and failure to thrive.	Protocol LMB-84	Relapse after 17 m. Died.
ITK deficiency*	(80)	1	EBV associated	HL	48, X,X,+2,der(10)add(10)(p13) add(10)(q34),del(11)(q22q23), + 12,-13, + 1B2mar	5/F	Cervical and inguinal lymphadenopathy	IFS, vinorelbine, etoposide, cytarabine, gemcitabine) RT, HSCT	CR
ITK deficiency*	(81)	2	EBV associated	Pt1: NHL	NS	6/F	Cervical and occipital Lymphadenopathy	CHOP and anti CD20 Ab	Died of infection
				P2: HL	NS	2.5/M	NR	Chemotherapy	NR
ITK deficiency*	(82)	1	EBV associated	HL	NS		Lung and Kidney	HSCT, Rituximab, Fludarabine, Melphalan, ATG	CR
ITK deficiency*	(83)	4	EBV associated	Pt1: HL	IKT gene: 1764C>G: YS886	4/F	LN	NR	Died at age 6
				Pt2: HL	IKT gene: 1764C>G: YS886	5/M	LN	Induction Therapy	PreHSCT, age 10
				Pt3: HL	IKT gene: 1764C>G: YS886	3/M	LN	HSCT	Alive
				Pt4: DLBCL	IKT gene: 1497delT: D500T, F501L, MS03X	6/F	Lungs	HSCT	Died from GVHD in 6 m
ITK deficiency*	(84)	3	EBV associated	Pt1: HL	ITK gene: single homozygous mutation c. 1764 C->G which causes a premature stop-codon Y588X	5/F	Lymphadenopathy and Hepatosplenomegaly	Rituximab, VP-16, vinorelbine, GMC	Died of respiratory failure
				Pt2: HL		5/M	Lymphadenopathy	Chemotherapy	CR
				Pt3: HL		3/M	Lymphadenopathy and HS	HSCT with fludarabine, melphalan, ATG, rituximab	Alive
ITK deficiency*	(85)	2	EBV associated	Pt1: HL	Homozygous mutation in ITK on ch 5q31–5q32.	6/F	Generalized LN, HS, nasal, concha tumor	Prednisone, procarbazine, vincristine, cyclophosphamide, ADM + rituximab	Died of respiratory failure at 10

(Continued)

TABLE 1 | Continued

PID	References	N	Proposed mechanism	Cancer	Specific mutations	Age/sex	Manifestation/course	Treatment	Outcome
				Pt2: HL	homozygous mutation in ITK 5q31–5q32.	1/F	Retroperitoneal and abd. LN, HS	GNC, steroid, rituximab, HSCT	Died of ischemic brain injury
CD70 deficiency*	(86)	1	EBV associated	HL		3/M	Recurrent fever, lymphadenopathy, HS	Initially chemotherapy and radiotherapy Rituximab at 4 HSCT at 10	CR after chemo/radiotherapy No relapse of HL, however recurrent lymphoproliferative episodes. Well since HSCT.
CD70 deficiency*	(87)	3	EBV associated	Pt1: HL	Homozygous c.250delT resulting in p.S84Pfs27X	17/M	Peptic ulcer, gastritis, splenomegaly and lymphadenopathy	doxorubicin, bleomycin, vinblastine, and dacarbazine	CR, F/U at 29 y: Stable
				Pt2: HL	Homozygous c.555_557delCTT resulting in p.F186del	2/M	Diffuse cervical lymphadenopathy	Initially OPPA and COPP ABVD + radiotherapy after relapse	CR after ABVD/radiotherapy F/U at 16: CR
				Pt3: HL	Homozygous c.555_557delCTT resulting in p.F186del	3/M	Chronic cervical lymphadenopathy	ABVD gemcitabine, vinorelbine, and brentuximab were followed by auto-HSCT	Relapse after ABVD, CR after HSCT
CD27 deficiency*	(88)	5	EBV associated	Pt1: DLBCL	Homozygous c.G158A, p.C53Y	2/F	NR	None	Died at 2 y
				Pt2: DLBCL	Homozygous c.G158A, p.C53Y	22/F	NR	CHOP	Died at 22
				Pt3: HL	Compound c.G24A, p.W8X; c.C319T p.R107C	15/F	Persistent cervical lymphadenopathy	EuroNet-PHL-C1	Alive
				Pt4: HL	Homozygous c.G287A, p.C96Y	?/F	NR	None	Died at 20 y
				Pt5: HL	Homozygous c.G287A, p.C96Y	8/M	NR	ABVD, radiotherapy	CR
XMEN* (MAGT1 deficiency)	(89, 90)	2	EBV associated	1. BL	g.43183delC	7/M	2nd malignancy at 14	NR	Alive at 16 y
				2. HL	g.46604G>T	17/NR	2nd malignancy at 22	HSCT	Died at 23 y of HSCT complications
XMEN	(91)	1	EBV associated (suspected)	DLBCL	c.712C > T, p.R238X	57/M	Lymphadenopathy, HS and B-symptoms	RCHOP, RGCVP	Died at 58 y from DLBCL
XMEN*	(92)	1	EBV associated	HL	c.555dup, p. Tyr186Ilefs*2	15/M	Influenza-like symptoms	COPP and ABVD	Alive at 17 y
CTPS1 deficiency*	(93)	2	EBV associated	Pt1: NHL	NR	6/F	NR		Died at 6 y
				Pt2: NHL	NR	1/M	NR	HSCT	Alive at 2 y
Hypergammaglobulinemia	(41)	1	NS	DLBCL	NR	1/F		No therapy received	Died of disease progression after 6 m
IL-10R deficiency	(94)	5	Defective antitumor immunosurveillance	Pt1: DLBCL	IL-10RB gene: homozygous missense in exon 2 (p.Y59C)	5/M	NR	COP, COPADM 3 2, CYM 3 2	Died of disease progression
				Pt2: DLBCL	IL-10RB gene: heterozygous composite frameshift mutation in exon 7 (F269fsX275) + missense mutation in exon 5 (p.W204C)	5/M	NR	COP, R-COPADM	Died during treatment
				Pt3: DLBCL	homozygous nonsense in exon 3 of IL-10RB (p. E141X)	6/M	NR	COP, R-COPADM, R-CYM, HSCT	Remission, alive (+12 m)
				Pt4: DLBCL	homozygous del (g.11930-17413 del) in IL-10RB.	5/M	NR	COP, COPADM, R-CYM, R-ICE	Remission, alive (+18 m)
				Pt5: DLBCL	Homozygous c.368-10 C.G in intron 3 of IL-10RA.	6/M	NR	Up-front R prephase	Remission, alive

(Continued)

TABLE 1 | Continued

PID	References	*N*	Proposed mechanism	Cancer	Specific mutations	Age/sex	Manifestation/course	Treatment	Outcome
DEFECTS IN INTRINSIC AND INNATE IMMUNITY									
Whim Syndrome*	(95)	1	EBV associated	BCL	NR	30/M	Red dermal facial nodules Axillary mass Axillary, hilar, Inguinal LN	6 cycles CHOP + G-CSF before each cycle	Resolved after 6 cycles.
Whim Syndrome*	(96)	1	EBV associated	BCL	NR	26/F	Fatal EBV+ B cell Lymphoma in the intestine and other organs.	CHOP	No response. Died of perforation and hemorrhage

Abd, (abdominal); ABVD, (doxorubicin [Adriamycin], bleomycin, vinblastine, and dacarbazine); ALT, (angioimmunoblastic T cell lymphoma); APO, (doxorubicin, vincristine, 6-MP, and prednisone); APDS, (activated phosphoinositide 3-kinase d syndrome); BCL, (B cell lymphoma); BL, (Burkitt lymphoma); ALPS, (Autoimmune lymphoproliferative syndrome); COP, (cyclophosphamide, vincristine, prednisone); COPADM, (cyclophosphamide, vincristine, prednisone, doxorubicin, methotrexate); CHOP, (cyclophosphamide, adriamycin, vincristine, prednisolone); COPP, (Cyclophosphamide, Oncovin, Procarbazine, Prednisone); CP, (cis-platinum); C-MOPP, (cyclophosphamide, oncovin, procarbazine, prednisone); CYM, (cytarabine, methotrexate); DHL, (diffuse histiocytic lymphoma); EMZL, (extra nodal marginal zone lymphoma); F, (female); G-CSF, (Granulocyte colony-stimulating factor); HLH, (hemophagocytic lymphohisticocytosis); ICE, (ifosfamide, carboplatin, etoposide); IL, (immunoblastic lymphoma); INHL, (immunoblastic NHL); LFU, (last follow up); LN, (lymph node); LPD, (lymphoproliferative Disease); M, (male); m, (months); M-BACOD, (methotrexate, doxorubicin, cyclophosphamide, vincristine, dexamethasone); ML, (Malignant Lymphoma); M2, (vincristine, cyclophosphamide); NHL-BFM, (Non-Hodgkin Lymphoma-Berlin-Frankfurt-Münster); NHL, (Non-Hodgkin Lymphoma); NMZL, (nodal marginal zone lymphoma); LL, (lymphoplasmacytic lymphoma); n, (number of patients); OPPA, (Oncovin, procarbazine, prednisone, and doxorubicin); PDC, (plasmacytoid dendritic cells); pt, (patient); PCMZL, (primary cutaneous marginal zone lymphoma); PE, (Physical Examination); PTCL, (peripheral T cell lymphoma); RT, (radiation therapy); R, (Rituximab); WM, waldenstrom macroglobulinemia; XLP1, (X-linked lymphoproliferative disease type I); y, (years).

**EBV positive, **median age.*

X-linked PIDD (29–34). DLBCL (33%, $n = 2$) was the most common type of B cell lymphoma in this group, followed by BL, HL, unspecified NHL and immunoblastic B cell lymphoma. As with many of the other PIDDs, EBV association was present in all these types. It is relevant to note that only 33% patients ($n = 2$, HL and Burkitt lymphoma) had complete remission on follow-up, while the others died of disease progression or its complications. In a multi-center cohort of X-linked thrombocytopenia (XLT) patients, certain *WAS* genetic variants associated with XLT, a milder form of WAS, predisposed to a higher incidence of developing lymphoma suggesting that these patients would benefit from treatment with HCT (108).

DiGeorge Syndrome (DGS)

In this literature review, DGS did not appear to be associated with a high incidence of lymphomas, with only one reported case in a 10-month female who developed immunoblastic B cell NHL(35). Similar to other PIDDs this case was also associated with EBV infection. The patient manifested multi-system complications with hemiparesis due to a cerebral mass, mediastinal lymphadenopathy, liver and kidney involvement, and succumbed to the disease prior to initiation of therapy.

Cartilage Hair Hypoplasia (CHH)

CHH is a syndromic PIDD due to genetic defects in the *RMRP* gene and manifests with variable degree of immunodeficiency. While many patients who manifest with severe cellular immunodeficiency early in life may receive an HCT, patients with initially milder immunodeficiency may develop complications later in life, including malignancy. We identified 10 reported cases, with a median age of 29 years, equally divided between males and females. Majority of these cases were NHL (90%, $n = 9$) while the remaining (10%, $n = 1$) were HL. Interestingly, none of these were associated with EBV (36). This is in contrast to the 2 cases of adult CHH (male patients) in our center (unpublished, Mayo Clinic, Rochester), who were both EBV-positive and diagnosed in their 3rd to 4th decade of life. One of these patients was diagnosed with DLBCL after an incidental work-up of a lung nodule, while the other patient had MZL with recurrent fevers and night sweats. The patient with DLBCL was treated with R-CHOP and had CR, while the patients with MZL had recurrent disease, and was treated with rituximab. Therefore, like other PIDDs, CHH may also present with EBV-associated lymphomas, and it remains unclear if the EBV diagnosis could have been missed in other cases reported in the literature.

Nijmegen Breakage Syndrome (NBS)

In this review strategy, 9 studies with 97 cases of lymphomas in patients with NBS were identified (37–45). NBS is a syndromic DNA repair defect, and in these published reports, 76% ($n = 74$) of patients presented with unclassified NHL and DLBCL. Most cases (82%, $n = 80$) were EBV-negative. The underlying DNA repair defect puts these patients at high risk, unless they receive HCT early after diagnosis.

Dyskeratosis Congenita (DC)

Among the short telomere syndromes, while one would postulate high-risk of malignancy due to premature cellular senescence,

TABLE 2 | Summary of T cell lymphomas in PIDDs.

PID	References	Proposed mechanism	*N*	Cancer	Age/sex	Manifestation/course	Treatment	Outcome
PREDOMINANTLY ANTIBODY DEFICIENCIES								
CVID	(97)	Immune dysregulation	1	DLL	52/M	RUQ tenderness, early satiety. HS. erythematous skin papules.	Chlorambucil and Prednisone	Poor response, died in 2 years.
CVID	(98)	Immune dysregulation	1	PTCL	57/M	Fever, night sweats, progressive refractory anemia, tender inguinal lymphadenopathy	ProMACE CytaBOM	CR after 6 cycles
CVID	(99)	NS	1	PTCL	32/F	Persistent cytopenia, progressive neurologic disease presenting as a polyradiculopathy with aseptic lymphocytic meningitis	cranial radiation, systemic and intra-thecal chemotherapy	Symptoms progressed. Died in few months.
IgA deficiency	(100)	Immune dysregulation	1	PTCL	24/M	4 years after PID diagnosis: liver failure, abdominal lymphadenopathy, pancytopenia, and recurrent bacterial infections and increasing pulmonary infiltrates	None. Diagnosis on autopsy.	Died of pulmonary failure before diagnosis
XLA	(101)	Chronic antigenic stimulation Immune dysregulation	1	PTCL	21/M	Sustained fever, unresponsive to antibiotics	NR	NR
XLA	(102)	Immune dysregulation	1	GCTCL	33/M	Generalized asymptomatic papulonodular eruption No lymphadenopathy/organomegaly	NR	NR
IgA deficiency	(102)	Immune dysregulation	1	GCTCL	68/M	Progressive generalized papules, plaques, and tumors. Occult IgA def diagnosed after presentation.	cyclophosphamide, methotrexate, etoposide, and dexamethasone	CR followed by recurrence and eventual death in 5 years
CVID	(102)	Immune dysregulation	1	GCTCL	44/M	progressive, asymptomatic red papules and nodules on his trunk and extremities.	Bexarotene Gemcitabine	Relapse and disease progression
IgA deficiency	(41)	NS	2	Pt1: ALCL	11/M		NHL-BFM	CR, LFU +3.5 years
				Pt2: TNHL	2/M		Non-B therapy, induction only	Sepsis during chemotherapy, death after 3 weeks of therapy
CVID	(71)	NS	4/247	4 T-cell	NR	NR	NR	2 died, 2 responded to RT
CVID	(3)		1/416	1 PTCL	NR	NR	NR	NR
APDS	(52)	NS	1/53	ALCL	NR	NR	NR	NR
Hypogammaglobinemia	(41)	NS	1	ALCL	15/M		NHL-BFM	CR, LFU +2.75 years

(Continued)

TABLE 2 | Continued

PID	References	Proposed mechanism	*N*	Cancer	Age/sex	Manifestation/course	Treatment	Outcome
COMBINED IMMUNODEFICIENCIES WITH ASSOCIATED OR SYNDROMIC FEATURES								
NBS	(44)	Chronic antigenic stimulation	5/57	TNHL	NR	NR	NR	15 out of 22 patients (B and T lymphomas) died, most at the beginning of the study, but 7 were clinically stable after early diagnosis and successful treatment
		Chronic antigenic stimulation	2/57	TLBL	NR	NR	NR	
NBS	(45)	NS	21/149	TNHL	NR	NR	NR	44% of all patients were in remission, 54% died due to disease progression
NBS	(103)	EBV associated	1	1 TLBL	10/F	Fever, generalized lymph node enlargement, hepatosplenomegaly and mediastinal mass	BFM90 protocol	Alive 7 years later
NBS	(37)	NS	9	Pt1: TLBL	10/F	NR	BFM90	Died of disease after protocol
				Pt2: TLBL	8/F	NR	BFM90	Died of relapse during maintenance
				Pt3: TLBL	16/M	NR	BFM90	Died of relapse during maintenance
				Pt4: TLBL	12/M	NR	BMF-90 followed by chemotherapy (ICE)	Died of organ failure caused by sepsis
				Pt5: AILT	8/F	NR	BMF-90 followed by individualized chemotherapy	Died of disease
				Pt6: TLBL	7/F	NR	EURO-LB 02 protocol	Alive disease-free
				Pt7: TLBL	3/M	NR	EURO-LB 02 protocol	Alive disease-free
				Pt8: TLBL	9/M	NR	EURO-LB 02 protocol	Alive with disease
				Pt9: TLBL	19/F	NR	EURO-LB 02 protocol	Died of relapse during maintenance
NBS	(38)	NS	6	Pt1: TLBL	18/M	2nd malignancy: AML	EURO-LB 02 protocol	Death because of 2nd malignancy (AML), 3.5 years after diagnosis
				Pt2: TLBL	16/M	NR	EURO-LB 02 protocol	CR 1 year after diagnosis
				Pt3: TLBL	4/M	2nd malignancy: TLBL 14 years after diagnosis	NHL-BFM90	CR after treatment in accordance to GMALL protocol for 2nd malignancy
				Pt4: TLBL	6/F	NR	EURO-LB 02 protocol	CR 2.4 years after diagnosis
				Pt5: TLBL	8/M	NR	ALL-BFM-MR protocol	CR 1.7 years after diagnosis and 1 year after SCT

(Continued)

TABLE 2 | Continued

PID	References	Proposed mechanism	*N*	Cancer	Age/sex	Manifestation/course	Treatment	Outcome
				Pt6: TLBL	16/M	NR	NHL-BFM95	Death 1 year after diagnosis
NBS	(39)	Defective antitumor immunosurveillance	2/26	Pt1: TLBL	16/M	NR	NR	Death at 18 years
				Pt2: TNHL	24/M	NR	NR	Death at 27 years from sepsis
NBS	(40)	NS	3	Pt1: TLBL	8/F	Cervical LN	NR	Died at 11 years
				Pt2: TLBL	16/M	Cervical LN	NR	Died at 18 years
				Pt3: TLBL	12/M	Cervical LN	NR	Died at 13 years
Ataxia Telangiectasia	(38)	NS	1	Pt1: TLBL	0.5/M	2nd malignancy: B-NHL after 3.5y	NHL-BFM86 modified	Death of second malignancy
Ataxia Telangiectasia	(41)	NS	1	TNHL	0.5/M	2nd malignancy: low-grade B-NHL	Non-B therapy, induction with 50% dosage, then reduced maintenance; omission of therapy after 7 months from severe toxicity	Reached CR initially; died of 2nd malignancy after 3.75 years
NBS	(4)	Defective DNA repair	3	Pt 1: TLBL	5/M	NR	NHL-BFM95, phase I protocol, nonmyeloablative conditioning, HSCT	CR 1, alive disease-free for 1.4 years
				Pt 2: PTCL	16/M	NR	CHOP–CHOP–ICE–DHAP–DHAP–DexaBEAM	Died of disease at 5 months after the diagnosis
				Pt 3: TLBL	14/M	NR	NHL-BFM95, protocol I, full dosage	Death of sepsis in PR after 7 weeks of therapy
IMMUNODEFICIENCIES AFFECTING CELLULAR AND HUMORAL IMMUNITY								
CID	(4)	Impaired Immune function	1	TLBL	8/M	9	NHL-BFM95, full dosage	Death of sepsis in CR 2 at 2 years after the diagnosis
DISEASES OF IMMUNE DYSREGULATION								
CD27 deficiency	(88, 104)	EBV associated	1	Pt1: TCL	16/M	Oral ulcers, uveitis, chronic EBV viremia, and EBV-related LPD progressing to T-cell lymphoma	Rituximab, R-CHOP, cord HSCT	Alive
Hypergammaglobinemia	(41)	NS	1	PTCL	2/F		Non-B therapy, induction, followed by maintenance	Still on maintenance therapy in PR for 2 years

AILT, (angioimmunoblastic T cell lymphoma); AML, (acute myloid leukemia); CID, (combined immunodeficiency); CVID, (Common variable immune deficiency); DLL, (diffuse lymphocytic lymphoma); CR, (complete remission); F, (female); GCTCL, (granulomatous cutaneous T-cell lymphoma); HS, (hepatosplenomegaly); LN, (lymph nodes); M, (male); NR, not reported; NBS, Nijmegan breakage syndrome; NHL-BFM, Non-Hodgkin Lymphoma-Berlin-Frankfurt-Münster; NS, not specified, PR, partial remission; ProMACE, prednisone, doxorubicin, cyclophosphamide, etoposide, methotrexate, leucovorin; Pt, patient; PTCL, peripheral T cell lymphoma; R-CHOP, Rituximab + cyclophosphamide, doxorubicin, vincristine, and prednisone; RUQ, right upper quadrant; TLBL, T lymphoblastic lymphoma; XLA, X-linked agammaglobulinemia; TCL, T cell lymphoma.

TABLE 3 | Summary of unspecified lymphoma in PIDDs.

PID	Reference	*N*	Proposed mechanism	Cancer	Age/sex	Manifestation/course	Treatment	Outcome
XMEN*	(89)	1/3	EBV associated	Lymphoma	45/M	NR	NR	Died at the age of 45
ADA deficiency	(105)	1	Not specified	Lymphoma	NR/M	NR	HSCT	Died
CVID	(54)	1	Not specified	Malignant lymphoma	30/M	NR	NR	NR
CVID	(65, 66)	1/117	Not specified	Undifferentiated lymphoma	63/M	Bone marrow	Plasmapheresis, CHOP, M-BACOD	Died 2 years later
CVID	(106)	71/2212	Not specified	Lymphoma	NR	NR	NR	NR

*CHOP, (cyclophosphamide, doxorubicin, vincristine, and prednisone); CVID, common variable immune deficiency; EBV, Epstein-Barr virus; F, female; HCT, hematopoietic cell transplantation; M, male; M-BACOD, (methotrexate, doxorubicin, cyclophosphamide, vincristine, dexamethasone); NR, not reported. *EBV positive.*

this literature search only yielded a single case of an adult DC patient with HL not associated with EBV (46). The treatment strategy included combination chemotherapy and radiation, which likely was not appropriate in the context of the patient's underlying genetic defect, and presumed radiosensitivity, as the patient subsequently developed a gastric carcinoma and died of the disease. Short telomere syndromes are known to be associated with radiosensitivity, and therefore, radiation therapy is probably not a recommended treatment modality in this group, as well as in the patients with DNA repair defects.

Schimke Immuno-Osseous dysplasia

We identified 3 studies, with 5 cases of B cell lymphoma (47–49). EBV association was seen in a single case (20%, $n = 1$). Outcome data was given for two patients, both of whom died with complications soon after starting chemotherapy.

CHARGE Association With Hyper IgM Syndrome

We identified one report of a 5-year-old girl, with nonclassical CHARGE association and elevated IgM levels who developed bilateral extranodal (ocular) MZL. She was treated with topical interferon alpha with subsequent complete resolution of disease (50).

IUIS: Predominantly Antibody Deficiencies

In this literature review, we identified 27 studies, which reported B cell lymphomas in 208 patients with predominantly antibody deficiencies. The median age at presentation was 46 years (range: 2–76 years) with a gender distribution of 34% ($n = 71$) female, 13% ($n = 27$) male, and 53% ($n = 110$) NS.

Common Variable Immune Deficiency (CVID)

Twenty-two studies of 192 CVID patients with B-cell lymphoma are presented in **Table 1** (3, 54–74). Common lymphomas included unclassified NHL (32%, $n = 62$), MALT lymphoma (EMZL) (7%, $n = 14$), DLBCL (5%, $n = 9$), and HL (4%, $n = 8$). Of these, 31% ($n = 60$) cases appeared to be associated with EBV infection. Possible mechanisms of lymphomagenesis in these different cohorts included chronic antigenic stimulation and defective immune surveillance. The treatments of choice in these patients included surgical resection and/or radiotherapy with chemotherapy. Common Variable Immunodeficiency (CVID) is the most common adult humoral immunodeficiency, both in US and European studies. There are several reports documenting an increased risk of lymphoma in these patients. In a large cohort of 176 patients with CVID, an increased incidence of lymphoma (obs = 4; SIR = 12.1; 95% CI = 3.3–31.0) was noted (54). In an ESID (European Society for Immunodeficiencies) registry study of 2,212 CVID patients, a subset ($n = 902$) analysis identified 3% of patients with lymphoma (106). However, in another study from a single center, which followed 473 CVID patients over four decades, the incidence of lymphoma was higher at 8.2% (74).

Selective IgA Deficiency

Three studies reported three cases of selective IgA deficiency with B cell lymphoma; an adult patient with apparent selective IgA deficiency who presented with a primary cutaneous MZL, a 38-year-old male with unclassified NHL and a 2-year-old boy

with Burkitt-like lymphoma (41, 54, 75). It is relevant to note that a combined Danish and Swedish study of 386 patients with IgA deficiency did not show an increased risk for cancer (standardized incidence ratio = 1.0) (54).

Activated Phosphoinositide 3-Kinase D Syndrome (APDS)

Nine patients with gain of function (GOF) mutation in PIK3CD gene (APDS) developed B cell lymphoma in the three studies identified in this review (51–53). DLBCL was the most common type (33%, $n = 3$). An EBV association was not seen in these particular cases.

Other Ig Deficiencies

Seidemann et al. reported four cases of B cell lymphoma in different Ig subclass deficiencies (excluding selective IgA deficiency) (41). Seventy-Five Percent ($n = 3$) had Burkitt lymphoma (BL). Two died during treatment with chemotherapy, two were alive and in complete remission at last follow up.

IUS: Diseases of Immune Dysregulation

In this category, 42 cases from 20 studies were identified, and of these, 76% ($n = 32$) had an association with EBV (41, 76–94). The median age of the reported cases was 5 years (range: 1–33 years), 62% ($n = 26$) were male, 31% ($n = 13$) female and 7% ($n = 3$) were NS. HL (48%, $n = 20$) was the most common subtype in these disorders of immune dysregulation, followed by DLBCL (29%, $n = 12$), BL (12%, $n = 5$), unspecified NHL (7%, $n = 3$) and composite lymphoma—FL, DLBCL, HL (2%). Specific examples are discussed further.

X-linked Lymphoproliferative Syndrome Type 1 (XLP-1)

In patients with X-linked lymphoproliferative syndrome type 1 due to genetic defects in *SH2D1A*, there were 3 studies with six patients who developed B cell lymphoma [4 BL, 2 DLBCL (77–79)]. The median age at diagnosis of lymphoma was 5 years (range: 1–14 years), and of these cases, 50% ($n = 3$) were EBV-associated and 50% ($n = 3$) had no apparent EBV association. Since XLP1 is associated with a defective immune response to EBV related to impaired cellular cytotoxicity it is not unexpected that there would be impaired immune surveillance in these patients (109). The two patients who had a DLBCL not related to EBV presented with a testicular mass and right chest mass, respectively. In the cases with the BL, clinical presentation involved lymphadenopathy and palpable abdominal mass/obstruction or cerebellar tumor. With the exception of a single case, all patients died within 5 years of diagnosis regardless of treatment modality used.

Interleukin-2-Inducible T-Cell Kinase (ITK) deficiency

Patients with ITK deficiency have an intrinsic susceptibility to EBV. In six studies, 13 patients with ITK deficiency (**Table 1**) who developed B cell lymphoma were identified, and all were associated with EBV (80–85). The most common was HL (84%, $n = 11$) followed by NHL (7%, $n = 1$) and DLBCL (7%, $n = 1$). The median age at presentation was 5 years (range: 1–6) with 54% ($n = 7$) female and 46% ($n = 6$) male, presenting clinically with lymphadenopathy and hepatomegaly. The treatment of choice was chemotherapy and HCT.

Autoimmune Lymphoproliferative Syndrome (ALPS)

While there are several genetic defects associated with ALPS or ALPS-like disease, the most frequent genetic defect associated with a classic ALPS-like phenotype is heterozygous germline pathogenic variants in the *FAS* gene. In **Table 1**, there was a single report an EBV-associated composite lymphoma (FL, DLBCL, HL) in an adult male with ALPS, which was treated with R-CHOP resulting in CR (76).

IL10-R Deficiency

Monogenic inflammatory bowel disease (IBD) can be associated with complex presentations, and patients with genetic defects in the IL-10 receptor (*IL10RA* and *IL10RB*) have particularly severe disease with additional complications. A single case report (110) and a case series has reported DLBCL in 5 patients with IL10R deficiency (94). There was no EBV association noted in any of these cases, and the median age of developing lymphoma was 5 years (range: 5–6 years). Interestingly, all patients in these series were male. Chemotherapy with COP (cyclophosphamide, vincristine, prednisone), COPADM (cyclophosphamide, vincristine, prednisone, doxorubicin, methotrexate), CYM (cytarabine, methotrexate), and ICE (ifosfamide, carboplatin, etoposide) was used with variable success. Among the five patients, two died due to disease progression during treatment, while three who received HCT remained alive and in remission on follow-up. It is evident that in this context, HCT is not only curative but may very well prevent the occurrence or recurrence of lymphoma (111).

CD70 Deficiency

B cell lymphoma was reported in four male patients under the age of 20 with CD70 deficiency across two studies (86, 87). All patients presented with HL, associated with EBV infection and were managed with chemotherapy and radiotherapy. Two patients underwent HCT and complete remission was achieved in all cases.

CD27 Deficiency

We identified one study reporting five cases (mostly females) with CD27 deficiency who developed B cell lymphoma (88). Three patients were diagnosed with HL, and two with DLBCL. All were related to EBV infection. Only two patients with HL treated with the EuroNet-PHL-C1 (EuroNet-Pediatric Hodgkin's Lymphoma Group-C1) protocol and ABVD (doxorubicin, bleomycin, vinblastine, dacarbazine) plus radiotherapy respectively were alive and in complete remission.

XMEN (MAGT1) Deficiency

Four studies with four patients were reported to develop B cell lymphoma in the context of MAGT1 deficiency (89–92). All were males with EBV-associated lymphomas. The ages ranged from 7 to 57 years, and the distribution of B cell lymphomas included 2 HL, 1 BL, and 1 DLBCL.

CTP Synthase 1 (CTPS1) deficiency

One study reported two cases with EBV- associated unspecified NHL in patients with CTPS1 deficiency (93).

IUIS: Defects in Intrinsic and Innate Immunity

WHIM (Warts, Hypogammaglobulinemia, Immunodeficiency, and Myelokathexis) Syndrome

WHIM syndrome is caused by gain-of-function defects in the *CXCR4* gene frequently associated with severe neutropenia and variable degree of immunodeficiency. There are two reports (**Table 1**) of B cell lymphoma (type not specified) in two adult patients with WHIM syndrome (95, 96). Both were associated with EBV infection leading to lymphoproliferation and ultimately lymphoma. Treatment in both cases was with CHOP chemotherapy, and one patient had a CR, while the other patient who presented with intestinal lymphoma had no response and died of intestinal perforation.

T CELL LYMPHOMAS IN PIDS

The incidence of T cell lymphomas is infrequent in PIDDs (**Table 2**). In this particular literature review, 74 patients were identified through 20 different reports. The types of T cell lymphoma identified included T cell non-Hodgkin lymphoma (TNHL) 36%, T cell lymphoblastic lymphoma (T-LL) 32%, peripheral T cell lymphomas (PTCL) 9%, granulomatous cutaneous T-cell lymphoma (G-CTCL) 4%, anaplastic large cell lymphoma (ALCL) 4%, and T cell-diffuse lymphocytic lymphoma (T-DLL) 1%. Of these cases, 41% ($n = 30$) were male, 10% ($n = 10$) were females and 46% ($n = 34$) did not report gender. The median age at the diagnosis of lymphoma was 13 years (range 0.5–68 years). It is interesting and relevant to note that the cases of T cell lymphomas were mostly observed in patients with predominantly antibody deficiencies or combined immunodeficiencies with associated or syndromic features. Only a single report is available of a patient with a combined immunodeficiency (CID) (**Table 2**), who developed T-LL and was treated with full dose NHL-BFM95 (Non-Hodgkin Lymphoma-Berlin-Frankfurt-Münster 95) on a phase1 clinical trial (4), and was in CR but subsequently died of sepsis.

IUIS: Diseases of Immune Dysregulation

CD27 Deficiency

A 16 year old male presented with oral ulcers, uveitis, chronic EBV viremia, and EBV-related lymphoproliferative disorder (LPD) progressing to T-cell lymphoma was treated with rituximab, R-CHOP, and subsequently a cord blood HCT, and the patient was alive at last follow-up (104).

IUIS: Immunodeficiencies Affecting Cellular and Humoral Immunity

CID

Only a single report of a patient with a combined immunodeficiency (CID) is reported (**Table 2**) who developed T-LL and was treated with full dose NHL-BFM95 on a phase1 clinical trial, and was in CR but subsequently died of sepsis (4).

IUIS: Predominantly Antibody Deficiencies

In patients with humoral immune defects, there were 10 studies with 17 patients reported (**Table 2**). The types of T cell lymphomas observed were PTCL (29% $n = 5$), G-CTCL (18%, $n = 3$), ALCL (18%, $n = 3$), T-DLL (5% $n = 1$), and TNHL (5%, $n = 1$). The median age at diagnosis was 32 years (range: 2–68 years) with a distribution of males (59%, $n = 10$), females (6%, $n = 1$) and NS (35%, $n = 6$).

Common Variable Immunodeficiency (CVID)

Of the patients with humoral defects, 53% ($n = 9$) had CVID across six studies, and the main types of T cell lymphomas included were PTCL, T-DLL, G-CTCL, and 4 unspecified T-cell lymphomas (3, 71, 97–99, 102). The treatment modalities used include Prom ACE (prednisone, doxorubicin, cyclophosphamide, etoposide, methotrexate, leucovorin), CytaBOM (cytarabine bleomycin, vincristine, methotrexate), radiation, systemic and intra-thecal chemotherapy. A single CVID patient presented with G-CTCL, which relapsed and progressed after treatment with bexarotene and gemcitabine. Another CVID patient developed T-DLL and had a poor response to chlorambucil and prednisone and succumbed to disease within 2 years.

Selective IgA Deficiency (sIgAD)

As with B cell lymphoma, there were three reports of four patients with sIgAD who developed T cell lymphoma (G-CTCL, PTCL, ALCL, TNHL) (41, 100, 102). All were males, and the diagnosis was made by autopsy in one patient, while the diagnosis of occult IgA deficiency was made in the second patient after the development of malignant disease. Though this patient was treated with chemotherapy with apparent CR, there was subsequent relapse of disease and mortality within 5 years. In patients classified as having sIgAD and malignant disease, it remains an open question as to whether there was a more severe underlying immunological defect that was not identified at the time.

Hypogammaglobinemia

Seidemann et al. reported a case of 15-year-old male with hypogammaglobulinemia who developed ALCL (41). Complete remission was achieved on treatment with chemotherapy. Last follow up was almost three years after diagnosis. A molecular etiology for the hypogammaglobulinemia was not available in this patient.

XLA

In the 2 patients reported with XLA, the types of T cell lymphoma observed were G-CTCL and PTCL (101, 102).

IUIS: Combined Immunodeficiencies With Associated or Syndromic Features

In this category, we identified nine studies with 54 patients. Median age was 10 years (0.5–24). Similar to other categories with T cell lymphomas, males had a higher percentage (33%, $n = 18$)

compared to females (15%, $n = 8$) and 52% ($n = 28$) were NS. TNHL (52%, $n = 28$) was the most common type of TCL followed by TLBL (44%, $n = 24$).

Nijmegen Breakage Syndrome (NBS)

Similar to B cell lymphomas (BCL) in NBS, there are reports of T cell lymphoma (TCL) in this group of patients. In **Table 2**, there are eight studies with 52 patients, all under the age of 20 (4, 37–40, 44, 45, 103). The underlying mechanism for development of malignant disease is the same as for BCL and related to defects in DNA repair. The commonly used chemotherapy protocols in these patients included NHL-BFM-90, NHL-BFM-95, and EURO-LB 02.

AT

We identified two studies reporting two cases of TCL in AT patients (38, 41)**.** A 6-month-old boy was diagnosed with TLBL and treated with a modified NHL-BFM 86 regimen. He developed B-NHL at the age of four leading to death. The second patient was also a 6-month-old boy who developed T-NHL and achieved complete remission initially, but like the first patient died at the age of four due to a second malignancy (B-NHL).

DISCUSSION

In this section, we discuss the mechanisms responsible for lymphomagenesis in the various inborn errors of immunity and provide an overview of the treatment.

DEFECTS IN IMMUNE RESPONSES THAT PREDISPOSE TO LYMPHOMAGENESIS IN PIDDS

The complex immune mechanisms and their interplay that predisposes to neoplastic transformation of B or T cells and development of lymphomas in PIDD patients has not been fully elucidated. However, it is expected that the etiology in most cases is multifactorial and related to a dynamic regulation of immune response and environmental triggers (**Figure 3**). An underlying intrinsic susceptibility to DNA damage in some of these PIDDs, may provide a substrate for uncontrolled cell growth, impaired apoptosis of damaged cells, premature cellular senescence, all of which may be compounded by increased antigenic stimulation of adaptive lymphocytes by viruses, such as EBV (112). This uncontrolled stimulation in the setting of altered immune checkpoints and immune dysregulation characterized by T cell exhaustion, defective anti-tumor immune surveillance and overproduction of inflammatory cytokines provides a fertile setting for neoplastic transformation and lymphoproliferation (**Figure 3**).

Defective DNA Repair

DNA integrity is constantly challenged at various levels as part of physiological processes. In lymphocytes, there is particular susceptibility specifically related to intrinsic mechanisms that are part of the immune diversity generation apparatus, such as V(D)J recombination, class switch recombination (CSR), and somatic hypermutation (SMH) (113). These intrinsic "stress points" coupled with other factors, including exposure to internal mutagens (e.g., endogenous metabolites) and external factors such as ultraviolet rays, ionizing radiation and chemicals (107) sets the stage for development of dysregulated cellular proliferation in B or T cells. Therefore, it is not surprising that normally there is a highly conserved DNA repair system orchestrated by a network of enzymes, which constantly assesses and detects DNA damaging lesions, modifies or removes damaged DNA, and reconstitutes the integrity of DNA through nucleotide resynthesis and ligation (114). When these checks and balances fail in the context of monogenic defects, it promotes the development of lymphoid tumors (113). Patients with premature cellular senescence related to shortened telomeres not only have an intrinsic susceptibility to DNA damage-inducing stimuli but also may have impaired apoptosis of damaged cells, which in turn may promote lymphoproliferation.

Role of Viral Infections and Defective Immune Surveillance, and Immune Dysregulation

Several of the documented PIDDs increase susceptibility to viral infections such as EBV, human papilloma virus (HPV), human herpes virus-6 (HHV-6), HHV-8, human T-cell lymphotropic virus (HTLV), Kaposi sarcoma–associated virus (KSV), and other viruses due to defective immune surveillance and immune dysregulation (115). Among these viruses, EBV represents the biggest threat because of its high prevalence (95%) and ability to transform epithelial cells, B cells, T cells and NK cells (116).

In the majority of cases, EBV virions entering through the oro-pharynx infect epithelial cells and B cells via the CD21 receptor. From a host immune system standpoint, NK cells and antigen-specific CD8+ T-cells are the main defense against primary EBV infection. Therefore, if EBV-infected B cells escape the cellular immune response, it can provoke an inflammatory outburst, resulting in cellular hyperproliferation and abnormal cell survival eventually leading to EBV-associated lymphoproliferative disorders (EBV-LPDs). EBV-LPDs consist of virus-associated hemophagocytic syndrome, non-malignant and malignant B-cell LPDs including non-Hodgkin and Hodgkin's types of B lymphomas and rarely EBV-positive T/NK cell lymphoma (117). The critical role of NK and T cells is demonstrated by the fact that combined B and T cell deficiency PIDDs account for approximately 2/3rd of EBV-associated LPDs in PIDDs, whereas defects in innate immunity do not significantly increase the risk of EBV-associated LPDs (116). Thus, underlying genetic defects in *SH2D1A* (SAP–XLP-1), *ITK*, *MAGT1, CD27, CD70, CTPS1*, *RASGRP1*, *CORO1A* and others account for the majority of EBV-LPDs.

The lack of adequate immune surveillance in the context of immune dysregulation is not uncommon in PIDDs, and though these cannot be exhaustively discussed here, failure of both innate (NK cells) and adaptive (T cells) immune cellular mechanisms facilitate viral escape and subsequent viral transformation of lymphocytes with poor cytotoxic clearance of virally-infected cells (89, 117). On the other

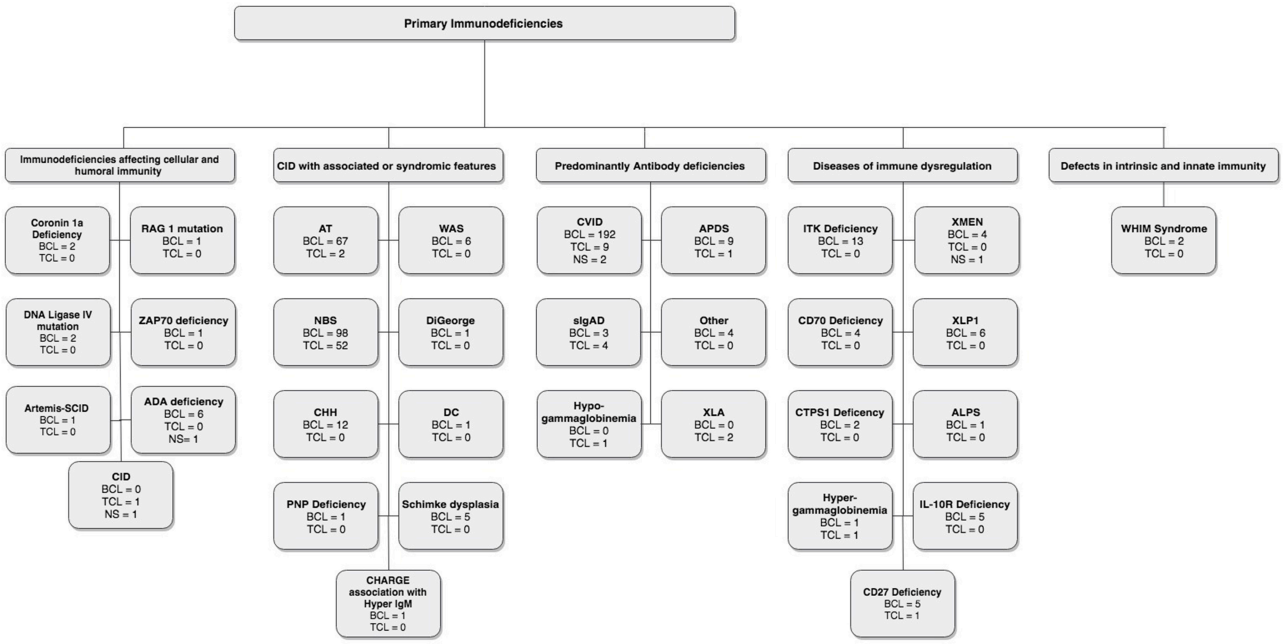

FIGURE 2 | Lymphoma distribution according to IUIS classification.

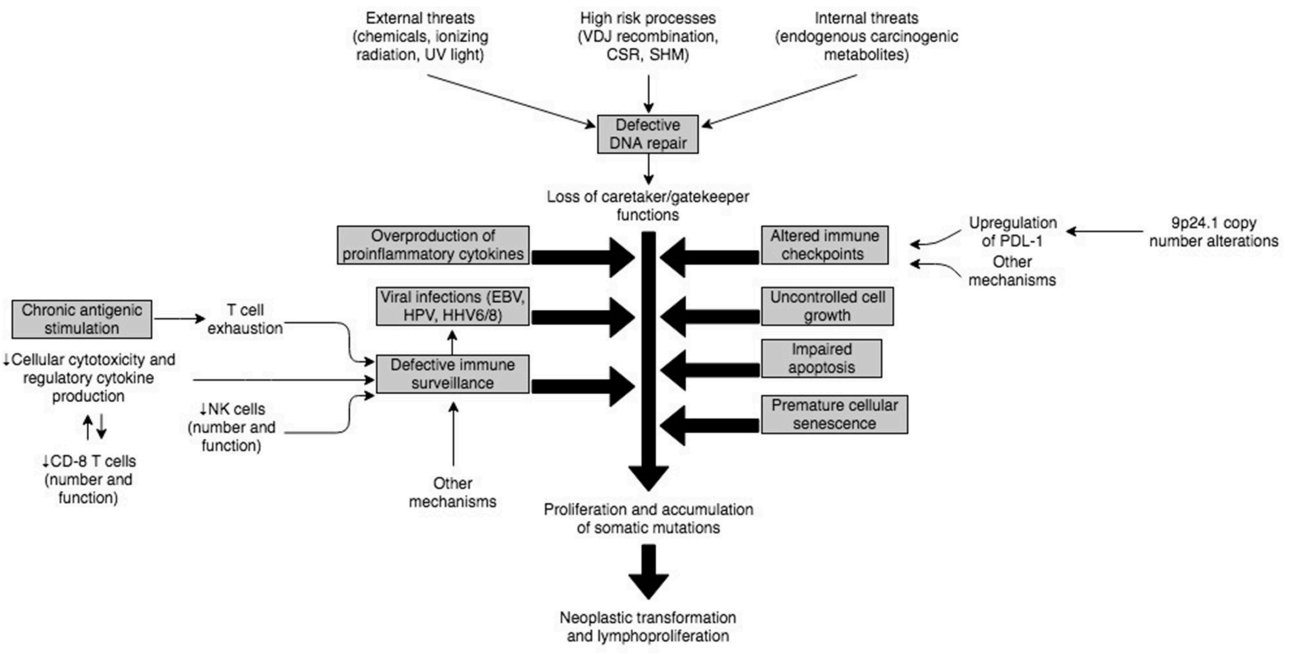

FIGURE 3 | Illustration of potential interplay of mechanisms implicated in pathogenesis of malignancies in PIDs.

hand, in certain other PIDDs, for example, the IL-10 receptor defects, there is production of pro-inflammatory cytokines due to the lack of IL-10-based regulation, and NFκB activation, with defective intra-tumoral CD8+ T cell immune surveillance and impaired cytotoxicity. This results in B cell proliferation and accumulation of somatic mutations (94, 111). Therefore, this is associated with a non-EBV-associated lymphoproliferation.

Chronic Antigenic Stimulation

Patients with PIDDs are more likely to develop persistence of antigens, and therefore ongoing stimulation of effector immune

cells. T and B cells respond to external stimuli (antigens) and subsequently eliminate non-self-antigens (pathogen-infected and/or transformed cells). Sustained antigenic stimulation, in the context of cellular and systemic immune compromise, is likely to increase T cell exhaustion with progressive loss of cytokine secretion (IL-2, TNF-<), impairment of IFN-γ production, and in extreme cases premature apoptosis of CD8+ T cells (118). This quantitative and qualitative loss of the CD8+ T cell immune response against tumor cells eventually results in the development of lymphomas, and another plausible mechanism for tumor development in PIDDs.

Altered Immune Checkpoints

One of the primary mechanisms by which tumors escape the immune system is by engaging immune checkpoints (119), which is why immune checkpoint inhibitors (ICIs) were approved for the treatment of cancers and are now used in 14 different types of cancer (120). This issue is relevant to the lymphomas, which develop in PIDDs as it has been shown that PDL-1 protein is overexpressed in most EBV-associated tumors (121). Further, copy number variations (CNVs) at 9p24.1 have also been reported, and this is a locus, which harbors PD-1 (122). However, only one of two studies found amplifications of chromosome 9p via array CGH (comparative genomic hybridization) in EBV+ DLBCL (123). These findings raise the possibility that EBV+ DLBCL evades immune surveillance by selectively targeting the PD1/PDL pathway. Therefore, there may be some value to using immune checkpoint inhibitors in PIDDs for the treatment of lymphoma, and this raises the need for clinical trials in this area.

TREATMENT OF B AND T CELL LYMPHOMAS IN PIDDS

Clinical Presentation, Diagnosis and Staging

PIDD patients often present with advanced disease, and extranodal sites of disease, including bone marrow, liver, and spleen, and the presence of B-symptoms is quite common. There is no difference in how the diagnosis of lymphoma is established, whether in in PIDDs or non-intrinsic lymphoid malignancies, and it usually involves tissue biopsy, most frequently of lymph nodes.

Clinical evaluation should include standard workflow of history and physical, complete blood count with differential, liver and kidney function and baseline pulmonary function test for adult patients, if they will undergo bleomycin-containing regimens in the context of HL. Additional work-up to assess the degree of immune impairment, based on underlying genetic defect is essential for this patient population, and should include evaluation of radiosensitivity (especially if the genetic defect is known to predispose to this) so that appropriate treatment regimens can be formulated. Principles of diagnostic imaging may be extrapolated from imaging recommendations for Hodgkin's lymphoma in HIV patients, which suggests 18FDG-PET with integrated or concurrent CT (computerized tomography) of the neck, chest, abdomen, and pelvis. Since 18FDG-PET imaging has not been validated in this setting, it is unclear whether a bone marrow biopsy can be omitted. The interpretation of 18FDG-PET scan be challenging in presence of concurrent infections, which is not uncommon in these patients (124).

Treatment Options

While there are no specific treatment options for lymphoma in PIDD patients, radiation-based therapies should be avoided in patients with known genetic defects that predispose to radiosensitivity. Most frequently standard treatment options are based on type of lymphoma, such as B or T cell, indolent, aggressive or very aggressive and patient-related characteristics such as age, comorbidities, immune status, and degree of immune compromise. While there are no large or randomized studies to confirm this, small series and case reports show that response to the treatment and prognosis is inferior in PIDD patients when compared to non-PIDD patients, largely related to their inability to tolerate standard chemotherapy, and susceptibility to life-threatening infections (25). HCT remains the only viable curative option for many of these diseases, though its efficacy of HCT is variable across different PIDDs. For example, the role of HCT in severe combined immune deficiencies (SCID) is well established and it has been consistently replicated (125). HCT is not a standard treatment of choice in CVID, especially those without a molecular defect, though it may be warranted in certain cases depending on the presentation and associated co-morbidities. In circumstances, of a specific molecular diagnosis replacing or clarifying the underling CVID, HCT may be the most optimal long-term treatment of choice, even though more targeted therapies may be available. HCT for CVID was reported in a multicenter experience for 25 patients transplanted at 14 centers in Europe, the US, and Japan, and though it may be considered for lymphoma in this context, the risk of transplant-related mortality (TRM) should be clearly weighed against the potential benefits (126). Several retrospective studies have reported outcomes of HCT as across a range of PIDDs, including SCID (127), and Wiskott Aldrich Syndrome (128). Only case reports or small case series are available for successful treatment in patients with other PIDDs such as GATA2 haploinsufficiency, IL-10 receptor or XIAP deficiencies. Despite the limited experience, data strongly favors the early use of HCT in the setting of IL-10 receptor deficiency (111). Recently, a retrospective case series of 29 adult patients with a variety of PIDDs (mean age: 24 years, range: 17–50 years) treated with reduced intensity conditioning (RIC)-HCT was published. This study reported an overall survival of 85.2% at 3 years (129). These data provide support for moving forward with HCT in adult PIDD patients. As such, in addition to the treatment of lymphomas, HCT can be lifesaving in patients with PIDDs who develop very severe complications, including susceptibility to life-threatening infections, bone marrow failure, autoimmune and autoinflammatory diseases, other malignancies, and hemophagocytic syndrome. Despite all these potential benefits, it remains to be ascertained as to whether early HCT can reduce the risk of developing lymphoma in these PIDD patients in the long term.

There is no consistent evidence to support the use of newer immunotherapies for treatment of lymphoma in PIDDs but these therapies hold significant mechanistic promise for personalized, chemotherapy-free treatments. Potential immunotherapy options include monoclonal antibody-based immunotherapy (e.g., Rituximab, Obinutuzumab, Epratuzumab), conjugated antibodies (Brentuximab Vedotin), Bi-Specific T cell engaging (BiTE) antibodies (Blinatumumab), Anti-PD1(Pembrolizumab, Nivolumab), Anti PDL-1 (Atezolizumab and Darvalumab), and anti-CTLA4 checkpoint inhibitors (e.g., Ipilimumab). The role of adoptive T cell therapies such as chimeric antigen receptor (CAR) T-cells remains uncertain at this point (130). While these targeted therapies may have value, they may also pose an increased risk as a result of immune manipulation in the context of an underlying immune deficiency or immune dysregulation.

While there are no reports for successful use of immune checkpoint inhibitors or BiTE antibodies, complete remission with Rituximab and Brentuximab Vedotin was reported in an adult female patient with CVID -associated classic HL, while two other cases of pediatric CVID-associated HL succumbed to severe infection related to chemotherapy (61).

CONCLUSIONS

Though this is not a comprehensive summary of malignancies in PIDDs, or even lymphoproliferative disease in this area, this review summarizes the Medline-indexed published reports of B and T lymphomas in patients with PIDDs. This report highlights the diversity of malignant lymphoproliferative disorders in setting of PIDDs, and its associated challenges of diagnosis and treatment. The pathological classification and nomenclature for the lymphoid malignancies with variably reported and postulated underlying mechanisms were inconsistent and inadequate for many of these published reports. A wide range of treatment options were utilized, and response rate was highly variable suggesting an empirical approach rather than a systematic and tailored treatment regimen, based on underlying genetic defect, and degree of immunological impairment. HCT and gene therapy (where available) remains the best treatment option for many, but not all of these patients, and should be promptly initiated after diagnosis, particularly in some conditions, such as SCID, WAS, IL10 receptor deficiency among others. HCT, as a therapeutic option, remains significantly under-utilized in adult patients, likely related to inadequate awareness among adult hematologists, and these patients may benefit from increased utilization of HCT in appropriate settings. We highlight the significant need of unifying nomenclature, pathological analysis, and assessment of mechanisms of lymphomagenesis in these patients to develop better and more personalized treatment regimens. Also, there is a considerable urgency to conduct clinical trials to develop evidence-based treatment plans that considers the underlying immunodeficiency rather than using approaches extrapolated from non-PIDD settings (131). It is recognized that there has been a recent effort to standardize classification and nomenclature in immunodeficiency-associated malignant LPDs, but this is largely focused on secondary immunodeficiency disorders, in contrast to primary, though presumably some of the same standards could be applied (132), but this should be undertaken specifically for PIDDs, to fully understand the similarities and differences in pathology.

AUTHOR CONTRIBUTIONS

All authors designed the study. IR and WF performed the literature search, conducted data extraction of relevant studies, and wrote the first draft of the manuscript. MP and RA critically reviewed the literature search and made revisions in the manuscript. All authors read and approved the final version of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 27 March 2019
doi: 10.3389/fonc.2019.00160

Dissecting the Multiplicity of Immune Effects of Immunosuppressive Drugs to Better Predict the Risk of *de novo* Malignancies in Solid Organ Transplant Patients

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De novo malignancies constitute an emerging cause of morbidity after solid organ transplant (SOT), significantly affecting the long-term survival of transplant recipients. Pharmacologic immunosuppression may functionally impair the immunosurveillance in these patients, thereby increasing the risk of cancer development. Nevertheless, the multiplicity and heterogeneity of the immune effects induced by immunosuppressive drugs limit the current possibilities to reliably predict the risk of *de novo* malignancy in SOT patients. Therefore, there is the pressing need to better characterize the immune dysfunctions induced by the different immunosuppressive regimens administered to prevent allograft rejection to tailor more precisely the therapeutic schedule and decrease the risk of *de novo* malignancies. We herein highlight the impact exerted by different classes of immunosuppressants on the most relevant immune cells, with a particular focus on the effects on dendritic cells (DCs), the main regulators of the balance between immunosurveillance and tolerance.

Keywords: immunosuppressive drugs, solid organ transplant, dendritic cells, cancer, immune cells

OPEN ACCESS

Edited by:
Andrew R. Gennery, Newcastle University, United Kingdom

Reviewed by:
Arya Biragyn, National Institute on Aging (NIA), United States
Shawn Demehri, Massachusetts General Hospital Cancer Center, United States

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Oncology

Received: *26 September 2018*
Accepted: *25 February 2019*
Published: *27 March 2019*

Citation:
Cangemi M, Montico B, Faè DA, Steffan A and Dolcetti R (2019) Dissecting the Multiplicity of Immune Effects of Immunosuppressive Drugs to Better Predict the Risk of de novo Malignancies in Solid Organ Transplant Patients. Front. Oncol. 9:160. doi: 10.3389/fonc.2019.00160

INTRODUCTION

Solid organ transplant (SOT) is an established procedure for patients with end-stage disease and the availability of potent anti-rejection drugs (1) significantly reduced the occurrence of acute and chronic allograft rejections, even though long-term survival is still unsatisfactory. Indeed, viral infections/reactivations, cardiovascular complications and tumor onset are among the major causes of morbidity and mortality in SOT patients (2, 3). In particular, SOT recipients have a 2 to 5-fold higher risk to develop a *de novo* neoplasm than the general population (4, 5). The tumor types with the highest risk relative to the general population are Kaposi sarcoma, lip carcinoma, non-melanoma skin cancers, non-Hodgkin lymphoma, liver, vulvar, and anal carcinoma (4, 5). Notably, the majority of these cancers are pathogenically related to oncogenic viruses, including Human Herpesvirus 8 (HHV8), Epstein-Barr Virus (EBV), Human Papillomaviruses (HPV), and Hepatitis B and C (3), whose control by host immune system is impaired in the transplant setting. Skin

cancers are the most frequent malignancy observed in SOT recipients, being observed in 8% of patients. The high incidence of skin cancers has been related to the high mutation burden due to UV exposure. These tumors, which have enhanced immunogenicity due to UV-induced mutations, are poorly controlled in immunosuppressed SOT recipients, thus explaining their increased incidence in this setting as compared to the general population. Other virus-unrelated malignancies such as carcinomas of the breast and prostate are not increased in transplant recipients. Post-transplant malignancies are often characterized by high aggressive clinical features and poor prognosis, thus representing an important medical need (6). Although iatrogenic immunosuppression has the power to inhibit the rejection of the transplanted organ, this treatment may limit the ability of patients' immune system to control nascent and overt tumors. Immune-evasion plays a pivotal role in tumorigenesis in the transplant setting, being directly promoted by the immunosuppressive effects of the drugs used and indirectly favored by the increased rate of oncogenic virus infections and reactivations, which may further contribute to impair host immune functions. The main mechanisms that drive the onset of *de novo* tumors in SOTs can be grouped into three major categories: (1) direct pro-oncogenic properties of select immunosuppressive drugs; (2) increased risk of oncogenic virus reactivation; (3) impaired immunosurveillance of tumor cells (7).

The most frequent *de novo* tumors arising after transplantation include Non-melanoma skin cancers (NMSC) (8, 9), often associated with Human papilloma virus (HPV) infection (10), Merkel cell carcinomas (MCC) (11, 12), related to Merkel cell polyomavirus (MCV) (13), post-transplant lymphoproliferative disease (PTLD), associated with Epstein-Barr Virus (EBV) (14), and Kaposi's sarcoma (KS), driven by Human Herpesvirus-8/KS herpesvirus infection (15).

If on one side SOT is the only treatment available for some end-stage diseases, on the other hand, the duration and type of immunosuppression can increase the risk of *de novo* malignancies in these patients. This may be at least in part due to the defective immune control of infections and/or reactivation by oncogenic viruses. Nevertheless, emerging evidence indicates that the various immunosuppressive drugs and regimes administered to SOT patients may have heterogeneous and still poorly defined effects on immune cell populations that may variably affect the cancer immunosurveillance (16) in these patients. On these grounds, the immune effects of immunosuppressive drugs may ultimately dictate the extent of risk to develop a *de novo* malignancy in SOT recipients.

On these grounds, there is the pressing need to better characterize the immune dysfunctions related to the immunosuppressive treatment of these patients to better understand the impact of the various immunosuppressive drugs on the immune system and how the chronic use of these drugs may favor the tumor onset in SOT patients. This may ultimately lead to a more precise and safe tailoring of the immunosuppressive schedule and limit as much as possible the risk of cancer development in these patients. The purpose of this review is to highlight the impact exerted by different classes of immunosuppressants on the immune system, with a particular focus on the effects on dendritic cells (DCs) and their central role in orchestrating both tolerance and anti-tumor immunity.

IMMUNOSUPPRESSIVE DRUGS IN SOLID ORGAN TRANSPLANTATION AND THEIR RELATIVE RISK OF CANCER DEVELOPMENT

Corticosteroids

Corticosteroids are a class of steroid hormones used primarily to reduce inflammatory and immune responses in various clinical conditions, and constitute an important component of the immunosuppressive regimens administered to SOT recipients. These drugs exert their effects by binding to an intracellular receptor, which then act to modulate gene transcription in target tissues, also including genes regulating immune responses. After binding to glucocorticoid receptors (GR) in the cytoplasm, corticosteroids inhibit the nuclear translocation and function of transcription factors, such as Activator Protein 1 (AP1) and Nuclear Factor-κB (NF-κB), resulting in a decreased inflammatory response through inhibition of pro-inflammatory cytokines such as interleukin (IL)-1, IL-2, IL-6, interferon (IFN)-γ and tumor necrosis factor (TNF)-α (17, 18). These drugs may also induce the production of anti-inflammatory proteins, including lipocortin and the inhibitor of NF-κB (IκB). Evidence accumulated so far clearly indicates that most of the immune effects triggered by corticosteroids are due to their ability to induce apoptosis of immune cells, particularly T lymphocytes and monocytes/macrophages. The possible contribution of corticosteroids to the risk of cancer development in transplanted patients is still unclear. It has been suggested that the ability of these drugs to promote anti-apoptotic and proliferative effects in various cell types (19, 20) could increase the risk of a *de novo* malignancy in SOT patients, although the specific contribution of corticosteroids is difficult to assess considering that these drugs are often administered in combination with other immunosuppressive agents.

A systematic review of the effects related to steroid avoidance and withdrawal after kidney transplantation did not disclose significant differences in the occurrence of malignancies in these patients up to 5 year after transplantation (21). Nevertheless, long-term consequences of steroid avoidance and withdrawal remain still unclear and prospective long-term studies are needed to draw definite conclusions.

Antimetabolites

Azathioprine

Azathioprine (AZA) was the first anti-proliferative agent available for clinical use and is commonly used in SOT patients in combination with other drugs, mainly Cyclosporine and Prednisone. AZA is converted to 6-mercaptopurine (6-MP) *in vivo*, which in turn is converted into 6-thiouric acid, 6-methyl-MP, and 6-thioguanine. These metabolites are incorporated into the DNA, block the *de novo* pathway of purine synthesis and inhibit cell proliferation. The main toxic effects induced by AZA are leukopenia, thrombocytopenia, anemia, and hepatotoxicity

(22). Immunosuppressive function of AZA is due to its ability to negatively interfere with the function and proliferation of T and B lymphocytes, with a relatively higher selectivity for T cells. In addition, AZA was shown to photosensitize the skin (23), promoting the accumulation of 6-thioguanine in DNA, which results in enhanced production of mutagenic reactive oxygen species after exposure to ultraviolet A (UVA) (24). These effects were suggested to have an impact on the development of squamous cell carcinomas (SCC) and other skin cancers in transplanted patients. A meta-analysis showed that treatment with AZA was associated with a significantly increased risk of SCC in SOT patients, whereas no significant association was observed between exposure to AZA and basal cell carcinoma (BCC) (25). A more recent study reported a significantly elevated risk of both BCC and SCC in SOT patients treated with AZA (26). Notably, multivariate analysis disclosed that AZA was associated with significantly higher risk than mycophenolate mofetil, sirolimus, cyclosporine, or tacrolimus (26). A recent whole exome sequencing study (WES) on cutaneous SCC from immunosuppressed patients has revealed a high mutation rate with an average of 50 mutations per megabase pair DNA. In particular, mutational signature analysis reveals the presence of a novel signature, whose presence correlates with chronic exposure to AZA (27). This signature is probably the result of combined action of UV exposure and incorporation of AZA metabolites into DNA, ultimately promoting tumor progression. These findings have clinical relevance for patients under treatment with AZA, who should be counseled about their skin cancer risk and UV photosensitivity.

Mycophenolate Mofetil

Mycophenolic acid (MPA), isolated in 1896 from *Penicillium brevicompactum* by Bartolomeo Gosio, is the active metabolite of mycophenolate mofetil (MMF) (28). MMF is a potent immunosuppressive drug used especially in combination with Calcineurin inhibitors (CNIs). MPA is the reversible uncompetitive inhibitor of inosine monophosphate dehydrogenase (IMPDH), the enzyme that synthesizes *de novo* guanosine nucleotides, thereby inhibiting DNA synthesis. In 2000, MMF was approved by FDA for use in liver transplantation, and in 2005 it has been shown (29) that treatment with MMF improved graft and survival of adult liver transplanted patients. Common side effects are nausea, vomiting, diarrhea (30) and high risk of opportunistic infection, in particular due to Cytomegalovirus (CMV) (31, 32). As compared to the use of AZA, MPA was associated with significantly lower risk of cutaneous SCC in cohorts of kidney, kidney–pancreas, heart and lung transplant patients (33, 34), an effect probably related to a decreased ability of MMF to induce ultraviolet light-related transforming effects (35).

Calcineurin Inhibitors

Calcineurin is a calcium/calmodulin-activated serine/threonine phosphatase that, once stimulated, de-phosphorylates and thereby activates members of the nuclear factor of activated T cells (NFAT) transcription factor family (36). Upon activation, NFAT family members migrate into the nucleus and activate transcription. Calcineurin is also responsible for the transcription of the genes encoding for IL-2 and several other cytokines, including TNF-α and IFN-γ. Calcineurin inhibitors (CNI), such as Cyclosporine A (CsA) and Tacrolimus (TAC), exert their immunosuppressive action through the inhibition of the Calcineurin pathway, inducing NFAT inhibition, which down-regulates IL-2 and INF-γ expression, and inhibits T-cell activation and proliferation in response to foreign antigens (37). Cyclosporine is a cyclic endecapeptide (38) commonly used to prevent rejection of liver, heart and kidney transplants. TAC, also called FK-506, is a macrolide antibiotic isolated from *Streptomyces tsukubaensi*. The use of this drug was initially restricted to patients with liver transplantation, but, more recently, it was also extended to patients with heart, pancreas and kidney transplantation (39, 40). Several studies reported increased risks of cancer related to the use of CNI (41, 42). Besides their ability to inhibit immune responses, CNI may also directly promote the aggressiveness and invasiveness of cancer cells by hampering antiviral immunity, supporting DNA-damage or up-regulating growth-promoting or pro-angiogenetic cytokines such as TGF-β, IL-10, or VEGF-2 (43–45). Indeed, adenocarcinoma cells treated with CsA *in vitro* were shown to undergo marked morphological and functional alterations, including increased cell motility and invasive growth (46). CNI also mediate activation of the Ras oncogene and promote renal cancer cell proliferation. Notably, VEGF overexpression induced by CSA in patients with renal carcinoma was dependent on Ras activation (47, 48). The pro-angiogenic effects of CNI-induced VEGF may probably constitute one of the major factors contributing to the increased rate of malignancies observed in transplanted patients treated with these drugs. Consistently, conversion from CNIs to Sirolimus (SRL) was shown to reduce the vascularization of cutaneous SCC in SOT patients (49). With regard to the risk of NMSC, CNIs were shown to cooperate with UVA and UVB in increasing the levels of TGF-β and suppressing p53 expression through the induction of ATF3 (50). Treatment with CSA was consistently associated with an increased incidence of EBV-driven post-transplant lymphoproliferative disorders (PTLDs), an effect probably dependent on the ability of CSA to induce EBV lytic replication in B lymphocytes and promote the release of the B-cell growth promoting cytokine IL-6 (51). Both CSA and TAC were shown to inhibit DNA repair (52), another possible contributory factor to the increased risk of cancer promoted by these drugs. The risk of *de novo* malignancies was increased in TAC-treated patients compared to that observed in patients treated with CsA (41).

mTOR Inhibitors

Mammalian target of rapamycin (mTOR) inhibitors are a large class of drugs that inhibit mTOR, a serine-threonine kinase involved in cell growth, proliferation, protein synthesis, and apoptosis (53, 54). The PI3K/Akt/mTOR pathway is often up-regulated in various malignancies and mTOR is a catalytic subunit of two functionally distinct molecular complexes called mTORC1 and mTORC2. mTORC1 is composed of five proteins, mTOR, RAPTOR, mLST8, PRAS40, and FKBP38, and the complex performs its function by phosphorylating the p70S6 and

4E-BP1 kinases, thereby regulating the expression of proteins that promote cell proliferation and survival, such as c-Myc, cyclin D1, and STAT3. The mTORC2 complex includes RICTOR, MAPKAP1, PRR5/PRR5L, Mlst8, and Deptor. mTORC2 directly phosphorylates Akt and regulates the organization of actin cytoskeleton by phosphorylating PKC-α. Unlike mTORC1, which is sensitive to acute treatment with rapamycin (RAPA), mTORC2 is less sensitive to the drug, although chronic treatments were shown to disrupt the integrity and function of this complex (55). The mTOR-inhibitor *Everolimus* (EVR) was derived from *Sirolimus* (SRL) and both compounds bind FK506-binding protein 12 (FKBP12) in the cytoplasm. mTOR inhibitors (mTORi) were initially designed as anti-cancer drugs, as well as immunosuppressive agent, because of their ability to suppress the growth and proliferation of tumor cells in mice (56). Dysregulation of cell cycle characterizes several types of tumors, and consequently, mTOR became an important therapeutic target for cancer patients. mTOR inhibition leads to an arrest of cells in the G1 phase of the cell cycle and a severe reduction of protein synthesis (57), demonstrating that the mTOR pathway is crucial for cell survival and protein synthesis. Notably, hyperactivation of the mTOR/PI3K/AKT pathway is present in virtually all types of tumors as a main consequence of somatic loss of the PTEN phosphatase, which is mutated or epigenetically inactivated in an large number of cancers (58). Inhibition of angiogenesis and decreased VEGF synthesis (59) constitute additional relevant effects characterizing mTORi. In HPV-positive transplanted patients, lifelong immunosuppressive therapy with mTORi has been associated with a significant reduction in the incidence of *de novo* neoplasms (60). Of particular interest was the case of a young liver transplant patient in whom the conversion to SRL therapy was followed by a rapid regression of skin warts suggesting that mTORi may be beneficial in immunosuppressed patients with HPV-induced relapsing warts (61). In addition, post-transplantation treatment with Rapamycin was shown to reduce the ability of B cells to undergo EBV lytic cycle replication (62), a well-known factor predisposing to EBV-PTLD. In the same setting, it has been demonstrated that the combination of mTOR and inhibitors of HSP90 (a dysregulated protein in EBV-related PTLDs) had a synergistic effect in inducing apoptosis and *in vitro* cytotoxicity of EBV-positive cells (63), suggesting the possible therapeutic efficacy of this combination in the control of PTLD.

Table 1 summarizes the main effects exerted by the different classes of immunosuppressive drugs on immune cells.

INVOLVEMENT OF IMMUNE CELLS IN THE MAINTENANCE OF IMMUNE COMPETENCE AND IN THE CONTROL OF CANCER DEVELOPMENT IN LIFE-LONG IMMUNOSUPPRESSED SOT PATIENTS

B Cells

Despite currently used immunosuppressive regimens are mainly investigated for their effects on T lymphocytes, the impact of these treatments on B-cell function may also be of pathogenic relevance. In fact, in addition to their activity as the producers of antibodies, B cells also function as potent antigen presenting cells and therefore their impairment may negatively impact on the immune control of nascent tumors. MPA and rapamycin were shown to strongly inhibit the proliferation of purified human B lymphocytes stimulated by CD40 ± Toll-like receptor triggering, whereas TAC and CSA had only marginal effects (73). Moreover, MPA and rapamycin also inhibited immunoglobulin production, which was independent of the degree of B-cell stimulation, and induced apoptosis of B lymphocytes (73). These findings clearly indicate that MPA and Rapamycin are able to profoundly inhibit B cells responses. On the other hand, it has been shown that CNIs inhibit humoral immune responses by interfering with T helper signals and not by eliciting direct effects on B lymphocytes (81). Nevertheless, SRL and TAC have different effects on the proliferation, activation and differentiation of B lymphocytes. In particular, clinically relevant doses of SRL, but not of TAC, inhibited the $CD19^{+}CD27^{+}$ B cell memory compartment. Moreover, SRL effectively blocked B cell differentiation into plasma cells and decreased absolute B cell counts. Despite inhibition, the residual B cells that do respond to stimulation in the presence of SRL result in a population shift toward more activated phenotypes. These activated B cells are able to induce a robust allogeneic T-cell activation and proliferation and a shift toward a Th1 phenotype (74).

An emerging body of evidence indicates that B cells may have regulatory properties that contribute to induction and maintenance of tolerance. Regulatory B cells (Bregs) is a relatively newly recognized subset of B lymphocytes showing potent regulatory activities in different inflammatory and autoimmune settings and playing a critical role in preventing transplant rejection. The characteristic phenotype identifying Bregs is not yet clear, and this subset of B cells is mainly identified by IL-10 production (82). Although IL-10 release constitutes the main mechanism by which Bregs perform their immunosuppressive activity, these cells may also impair immune responses through other mechanisms and cytokines, including IL-35, Fas-Ligand, PD-L1, and TGF-β (83). These activities allow Bregs to inhibit both innate and adaptive immune response and promote the expansion of immunosuppressive regulatory T cells (Tregs). In the transplant setting, it has been shown that increased Breg cell frequency correlated with reduced rejection episodes and long-term allograft survival (83). Higher numbers of Bregs were detected in operatively tolerant patients (in the absence of immunosuppressive drugs) compared to pharmacologically immunosuppressed patients, suggesting a protective function of the Breg cell subset (84, 85). Many commonly used immunosuppressive drugs, such as CSA, TAC, Prednisolone, AZA, and MMF, were shown to reduce Breg numbers (83), having thus a negative impact on the ability of transplanted patients to control the tolerance to the allograft.

Globally, immunosuppressive drugs used in the transplant setting may affect B lymphocytes resulting in both anti-tumor and pro-tumorigenic activities (86), although it remains unclear whether the net effect has any contributory role to the increased risk of cancer in the transplant setting.

TABLE 1 | Main effects exerted by immunosuppressive drugs on immune cells.

Mechanism of action	Drugs	Impact on immune cells	References
Regulation of gene expression	Glucorticoides	• Impair monocyte and macrophage function • Decrease circulating levels of CD4+ T cells.	(22)
Inhibition of *de novo* purine synthesis	Azathioprine	• Interfere with T-cells stimulation and proliferation	(22)
	Mycophenolate Mofetil	• Significant reduction of CD107 expression in NK cells • Significant reduction of INF-γ production by NK cells	(64)
		• Down-regulation of co-stimulatory and adhesion molecules human monocyte-derived DC	(65)
Kinase and phosphatase inhibitors	Calcineurin inhibitors	• Inhibit of NF-kB phosphorylation in $CD3^+$ T-cells, $CD4^+$ T-cells and $CD8^+$ T-cells	(66)
		• Prevent naïve T-cells differentiation	(67)
		• Preserve stable numbers of NK cells	(68)
		• Reduce IL-2 and TNF-α production by macrophages	(69)
		• Affect DC maturation *in vitro*	(70, 71)
		• Impair IL-12 production by DCs	(72)
	mTOR inhibitors	• Impair B-cells proliferation	(73)
		• Impair CD19+CD27+ memory B-cells	(74)
		• Promote $CD4^+CD25^{high}FOXP3^+$ Tregs expansion	(75)
		• Reduce the numbers of NK cells	(68)
		• Decrease M-MDSCs differentiation	(76)
		• Induce macrophages apoptosis	(77)
		• Impair DCs maturation and function	(78–80)

T Cells

Immunosuppressive drugs are mainly known for their ability to inhibit the function and survival of T lymphocytes and the NFAT and NF-kB are the main signaling pathways targeted by these drugs. These effects are best exemplified by TAC, which, in addition to its ability to inhibit the calcineurin/NFAT pathway, was also shown to inhibit NF-kB activity and TNFα production in $CD3^+$ T cells, $CD4^+$ T cells and $CD8^+$ cytotoxic T cells isolated from healthy donors (66). CNIs such as CsA and TAC may also prevent naive T cell differentiation into Th1, Th2, and Th17 subsets and inhibit the production of IFN-γ, IL-4, and IL-17 by memory $CD4^+$ T cells (67). These effects could at least in part account for the increased risk of *de novo* cancer in SOT patients treated with CNIs (60). A recent study investigated the contribution of residual T-cell immune function in mediating the decreased incidence of SCC showed by renal transplant recipients switching CNI-based therapies to mTORi. While both RAPA and TAC enhanced the survival of OVA-expressing skin grafts in mice, and inhibited short-term antigen-specific $CD8^+$ T cell responses, RAPA but not TAC induced a significant infiltration of $CD8^+$ effector memory T cells into UV-induced SCC lesions. Moreover, only RAPA was able to increase the number and enhance the function of $CD8^+$ effector and central memory T cells in a model of long-term contact hypersensitivity. In fact, RAPA was shown to promote the generation of long-lived memory precursors by altering the process of differentiation of short-lived precursors (87, 88). These findings are consistent with the possibility that the lower risk of *de novo* SCC showed by patients switched to mTORi regimens is probably due to enhanced $CD8^+$ memory T-cell responses to new antigenic stimulations occurring in their skin (89).

Due to the critical role of Treg cells in maintaining tolerance against self-antigens and controlling excessive immune responses, the effects exerted by immunosuppressive drugs on these cells were extensively investigated. Nevertheless, the possible promotion of cancer development associated with abnormal expansion or activation of Treg cells induced by these drugs remains an underexplored area. Treg cells are mainly characterized by the expression of CD25 and FoxP3 and represent 5–10% of all peripheral $CD4^+$ T cells (90). Tregs can be divided into resting Tregs ($CD45RA^+FoxP3^{low}$), effector Tregs ($CD45RA^-FoxP3^{high}$) and cytokine-producing Tregs, ($CD45RA^-FoxP3^{low}$) (91). Tregs can be also classified into two sub-groups according to their development: natural Tregs, which develop during the normal process of T-cell maturation within the thymus and that are characterized by high expression of CD25, co-stimulatory molecule cytotoxic T-lymphocyte antigen 4 (CTLA4) and the tumor-necrosis factor (TNF)-superfamily member GITR (glucocorticoid-induced TNF receptor family-related protein (TNFRSF18); adaptive Tregs, which generate by populations of mature T cells under certain conditions of antigenic stimulation and show variable levels of CD25 expression depending on the disease setting and the site of regulatory activity (92). Treg cell mechanism of action involves immunosuppressive activities against other T cell subsets, B cells, macrophages, DCs and NK cells and the release in the microenvironment of immunosuppressive cytokines such as IL-10, IL-35, and TGF-β to prevent T-cell proliferation and maturation of antigen presenting cells (93). Treg cells may also secrete granzymes and perforins (94) and express CTLA-4, which may inhibit the activity of DCs (95). The pilot study by Levitsky et al. has shown that, in liver transplant patients, monotherapy with SRL resulted in a higher percentage of Tregs in peripheral

blood compared to non-SRL monotherapy (96). These findings are consistent with the observation that the expression of FoxP3 requires IL-2, whose gene transcription is blocked by CNI but not by SRL. These data were confirmed in a subsequent study that showed that RAPA, but not CsA, promotes the induction of Tregs rather than inhibiting their function (97) (**Figure 1**). Of particular relevance in terms of potential increased risk of cancer development is the effect that RAPA has on Tregs. In fact, it has been demonstrated that immature dendritic cells (iDCs) treated with low-doses of RAPA and injected intravenously in rats are able to selectively expand $CD4^+CD25^+Foxp3^+$ Tregs (98). RAPA was also shown to preferentially promote the expansion of $CD4^+CD25^{high}FOXP3^+$ Tregs as compared to the $CD4^+CD25^{neg}FOXP3^+$ Treg subset (75). Intriguingly, RAPA enhances the expression of CXCR4, the ligand of stromal-derived-factor-1 (SDF-1), which is constitutively expressed in the bone marrow, suggesting that this drug may promote the development of Tregs with distinct homing properties (99).

The reduced risk of skin cancer development observed in kidney transplant recipients treated with SRL as compared to those receiving CNIs was associated with different effects exerted by the two drugs on T cell populations infiltrating the skin. It has been shown that the treatment with SRL significantly increased the absolute number of $CD4^+$ T cells, memory $CD8^+$ and $CD4^+$ T cells, and Treg cells in the sun-exposed skin compared to non-sun-exposed (100). Notably, no differences were found in the absolute number of any T cell subset were observed in the blood, suggesting that the percentage of T cell subsets detectable in the blood does not always accurately reflect the percentage of T-cell subsets in the skin of kidney transplant recipients.

Th17 cells are characterized by their ability to produce pro-inflammatory cytokines, including IL-17A, IL-17F, and IL-22, and are critical for host defense against pathogens but have also been implicated in causing autoimmune disorders and cancer, although their role in carcinogenesis is less well defined (101). Besides decreasing the proportion and function of $CD4^+$ Tregs, CNIs up-regulate Th17 cell-associated pathways (102), which are involved in allograft rejection and may also contribute to the enhanced risk of *de novo* malignancies in SOT patients treated with these drugs. Available data indicate that the replacement of TAC therapy to SRL therapy suppresses Th17 activity and up-regulates the percentage of Treg cells in kidney transplant recipients. SRL inhibited Ser705 phosphorylation of STAT3 in CD4+ T cells, which promotes a differentiation switch toward Treg cells rather than to Th17 cells. The drug was also shown to induce a downregulation of IL-17 and an increased expression of Foxp3 in Th17 cells. Therefore, the Th17/Treg ratio modulation induced by conversion from TAC to SRL promotes a better control of the allograft (103) and may also contribute to the decreased risk of cancer associated with the use of SRL.

An increasing number of studies have focused the attention to a recently described subpopulation of T cells, the tissue resident memory T (Trm) cells, a subset of non-circulating lymphocytes that reside in multiple peripheral tissue sites, including lung, intestine, liver and skin. Trm cells are characterized by the expression of CD103, CCR7, CD28, and IL-7R and are CD45RA-CD69-. Although Trm cells were initially considered as early immune effectors in infectious diseases, recent studies highlighted their role in mediating therapeutically relevant immune responses against cancer (104). In the setting of SOT transplantation, available data point to an important role of Trm in the control of common chronic viral infections and site-specific acute infections (105), suggesting that these cells may contribute to some aspects of graft tolerance. Conversely, Trm cells can potentially mediate anti-allograft responses through their strong immunostimulatory abilities (106). The possible implications of Trm cells in the risk of cancer in SOT patients treated with immunosuppressive drugs remain to be elucidated. Nevertheless, the notion that CNI and mTOR inhibitors mainly target the early activation phases of T lymphocytes suggest that these drugs may have limited effect on Trm cells because of their pre-activated phenotype (107).

NK Cells

NK cells are key components of innate immune system are able to mediate cell lysis without prior stimulation by antigens. Their activation is closely dependent on the balance between the expression of inhibitory molecules and triggering of activatory NK cell receptors by their cognate ligands. While in hematopoietic stem cell transplantation NK cells have been shown to play a significant role in the graft-vs.-leukemic effect, the role of NK cells in SOT is controversial due to conflicting clinical and pre-clinical data. In fact, NK cells were shown to worsen T-cell responses during allograft rejection, but also to promote tolerance induction under treatment with immunosuppressive drugs (108). In a murine kidney transplant model based on hybrid resistance, NK cells were shown to mediate long-term allograft injury even in the absence of T and B cells (109). Preclinical data indicate that immunosuppressive agents may affect the number and function of NK cells. In an elegant work, Aislin Meehan et al. have shown that treatment of PBMCs from healthy donors with different immunosuppressive drugs resulted in impairment of NK cell function that varied according to the type of drug investigated and the dose used. At concentrations used in the clinical setting, CsA and Prednisolone caused a significant reduction in NK cell expression of CD107 (a degranulation marker indicative of cytotoxic activity) and the production of IFN-γ (64). The activity of NK cells is also inhibited by treatment with RAPA, as shown by the group of Wai et al. who analyzed the proliferation and potential cytotoxicity of rat NK cell lines in the presence of different types of immunosuppressive drugs. NK cell number and function remained stable in graft recipients treated with CsA and FK506, whereas RAPA significantly inhibited proliferation and cytotoxicity of NK cells (68). On the contrary, Monteau et al. demonstrated that both CsA and TAC have a strong negative impact on degranulation and IFN-γ production of by NK cells *in vitro* (110). Overall, available data seem to indicate that the effect of immunosuppressive drugs on NK cells is not as strong as that induced on T lymphocytes, at least *in vivo* (108). Considering the emerging contributory role of NK cells in the control of primary tumors and particularly of cancer metastasis (111), it will be of relevance to monitor NK cell number and function in SOT patients at risk of *de novo* malignancy.

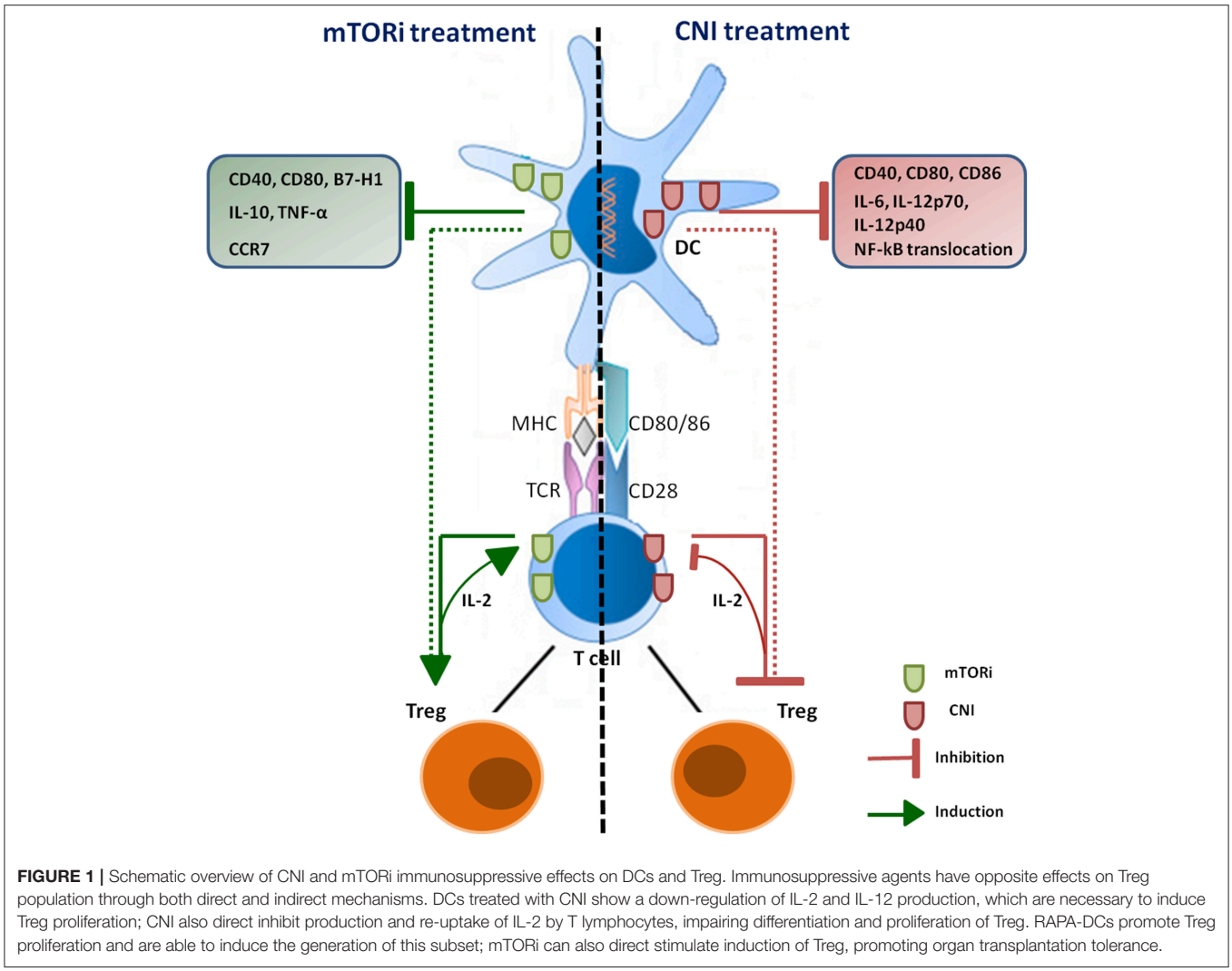

FIGURE 1 | Schematic overview of CNI and mTORi immunosuppressive effects on DCs and Treg. Immunosuppressive agents have opposite effects on Treg population through both direct and indirect mechanisms. DCs treated with CNI show a down-regulation of IL-2 and IL-12 production, which are necessary to induce Treg proliferation; CNI also direct inhibit production and re-uptake of IL-2 by T lymphocytes, impairing differentiation and proliferation of Treg. RAPA-DCs promote Treg proliferation and are able to induce the generation of this subset; mTORi can also direct stimulate induction of Treg, promoting organ transplantation tolerance.

Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) are innate cells that play a pivotal role in inhibiting T-cell dependent responses. MDSCs are not a terminally differentiated cell population and are characterized by $CD33^+$ expression, whereas CD3, CD14, CD19, CD56, and HLA-DR are usually negative. In mice, MDSCs consist of two large groups of cells termed granulocytic or polymorphonuclear (PMN-MDSCs) characterized by $CD11b^+Ly6G^+Ly6C^{lo}$ expression, and monocytic (M-MDSCs) characterized by $CD11b^+Ly6G^-Ly6C^{hi}$ expression (112). Also in human, two main groups of MDSCs have been identified: granulocyte MDSCs (G-MDSCs, $CD33^+CD11b^+CD14^-CD15^+$) and monocytic MDSCs (M-MDSCs, $CD33^+CD11b^+CD14^+CD15^{-/low}$) (112, 113). M-MDCSs contributes to production of inflammatory cytokines and growth factors, which may have a strong immunosuppressive effect, including the inhibition of T-cell proliferation (112, 114), and impaired maturation and development of DCs. In fact, DCs generated in the presence of MDSCs were found to be less effective in antigen uptake, migration and induction of IFN-γ production by T cells (115). Furthermore, MDSCs are able to down-regulate the production of IL-12 by macrophages while increasing IL-10 production in response to cell-cell contact (116). Experiments carried out in mouse models demonstrated that MDSCs identified with phenotypic biomarkers were able to inhibit proliferation, but not activation, of effector T cells and to induce apoptosis in a contact-dependent manner. Interestingly, $CD4^+CD25^{high}FoxP3^+$ Treg cells were insensitive *in vitro* to MDSC-mediated suppression. These results suggest that increasing numbers of MDSCs can inhibit alloreactive T-cell proliferation *in vivo* and that these cells may participate in the maintenance phase of tolerance (114). In an analysis of a cohort of 50 kidney transplant patients, Meng et al confirmed that the number of MDSCs was associated with long-term graft survival and showed that this population regulates the imbalance between Tregs and Th17 cells (117). Moreover, in an experimental mouse model of transplantation, Garcia et al. showed that MDSC were associated with an increased accumulation of Foxp3-expressing Tregs in the allografts of transplant recipient mice following tolerogenic treatment (118). The impact of CsA on the

myeloid population was investigated in a skin transplantation study showing that CsA can stimulate the accumulation of $CD11b^+Gr1^+$ cells (MDSCs in mice) by improving the immune responses through the NFAT pathway, also promoting the differentiation into $CD4^+$ and $CD8^+$ T cells. In a murine cardiac transplantation model, RAPA treatment led the recruitment of MDSCs, which also expanded as a consequence of the effects of the treatment on the mTOR pathway (119). In addition, inhibition of the mTOR signaling was shown to promote a shift of MDSCs toward the G-MDSCs subtype (120). A recent study in human kidney transplantation demonstrated that $CD33^+CD11b^+HLA\text{-}DR^-$ MDSCs were able to expand Tregs *in vitro*, through the release of TGF-β and IL-10 (121). RAPA was also shown to decrease M-MDSCs differentiation from myeloid progenitors by blocking the glycolytic metabolic pathway (76). An excessive expansion of MDSCs promoted by immunosuppressive drugs may contribute to increase the risk of cancer in SOT patients. In fact, MDCSs constitute a critical component of the immune suppressive niche characterizing tumor microenvironment, where these cells may promote immune escape and malignant progression by affecting both the innate and adaptive immune responses (122). Studies carried out so far in the SOT setting focused mainly on the role of MDSCs in the induction of tolerance and allograft control and no data are currently available with regard to the possible correlation between expansion/overactivity of MDSCs and risk of the novo malignancy in these patients. Some indirect clues derived from a study carried out in a mouse neuroblastoma-bearing chimeras, which showed that adoptive recipient leukocyte infusion enhanced anti-tumor responses of allogeneic bone marrow transplantation. These anti-tumor effects, however, were counteracted by expansion of host MDSCs pointing to a relevant role of these immunosuppressive cells in limiting the efficacy of anti-tumor immunity in the transplant setting (123).

Macrophages

Macrophages were identified by Elie Metchnikoff as an essential component of innate immune system that forms the first line of defense against pathogens (124). According to their differentiation status and functional role in the immune system, macrophages are conventionally classified into M1 and M2 subtypes, although these cells has the ability to differentiate into a variety of phenotypes in response to different stimuli from the microenvironment (125). The M1 phenotype can be identified by the overexpression of surface molecules such as MHC-II and CD86, and increased ability to present antigens and kill intracellular pathogens. *In vitro*, classical activation can be induced by stimulating macrophages with IFN-γ and LPS, causing TNFα production, associated with microbicide activity, production of pro-inflammatory cytokines and cellular immunity. Macrophages then undergo a process of activation toward a pro-inflammatory phenotype, increasing their ability to kill intracellular pathogens and contributing to the progression of the inflammatory process. M2 macrophages are generated in the presence of the anti-inflammatory cytokines IL-4, IL-10, and IL-13 and participate in tissue remodeling and long term repair (126). In addition to the host defense role, macrophages play an important role in homeostasis, and pathological processes such as obesity and malignancy (127). In particular, they show a more immunosuppressive phenotype characterized by impaired antigen presentation to T cells and production of cytokines that stimulate a Th2 response (128). Evidence accumulated so far however indicates that the binary classification of macrophages in M1 and M2 subtypes is an oversimplification that does not account for the marked plasticity of these cells that may acquire a broad and continuous spectrum of different phenotypes, with M1 and M2 representing the two extremes (128). Examples of non-M1/M2 cells are: $CD169^+$ macrophages, detected in bone marrow, lymph node, liver, and spleen, and mainly involved in erythropoiesis and immune regulation; macrophages expressing TCRαβ or TCRγδ identified in inflammatory and infectious diseases; a novel subtype of tumor-associated macrophages characterized by an M2-like immunosuppressive gene profile and expressing a novel receptor "macrophage receptor with collagenous structure" (MARCO), detected in mouse tumor models of mammary carcinoma, colon cancer and B16 melanoma (128). The function of these non-M1/M2 macrophages however remain far from being fully characterized. In the SOT setting, macrophages play a controversial role: the presence of $CD86^+$ macrophages is associated with acute rejection in kidney transplants (129) and in mouse models of heart transplant (130). Likewise, macrophages may exert anti-inflammatory responses and immunosuppressive effects that help maintain the peripheral tolerance, being able to produce IL-10 and TGF-β, involved in the resolution of graft inflammation (131). The use of immunosuppressive drugs has also an impact on the macrophage compartment: the use of CsA and TAC was associated with a decrease in the production of inflammatory mediators, such as IL-2 and TNF-α (69). Moreover, CNIs were shown to promote the differentiation of macrophages to the M2 phenotype (132). CsA was shown to impair the phagocytosis activity of macrophages and enhance the severity of infectious diseases (133). mTORi were shown to significantly decrease the production of chemokines induced by LPS, such as MCP-1, IL-8, RANTES, MIP-1α, and MIP-1β and their combination with glucocorticoids increased the production of the anti-inflammatory cytokine IL-10 through the STAT3 transcription factor (134). mTORi could also induce macrophage apoptosis, mainly affecting the M2 rather than M1 subset (77). It has also been documented that the administration of MMF reduces the synthesis of nucleotides by inhibiting the inosine monophosphate dehydrogenase pathway (135), decreases the production of IL-1β, IL-10, TNF-α (134) and the expression of adhesion molecules (136) by macrophages. Studies aiming at assessing the role of macrophages in SOT patients with cancer are scarce. No significant difference in the density of tumor-associated macrophages was observed in SCCs from SOT recipients as compared to SCC of non-transplant patients. These findings seem to rule out that the density of these cells may contribute to a worse prognosis of invasive SCC in transplant patients. Intriguingly, however, the density of tumor-associated macrophages infiltrating SCC *in situ* was markedly lower than that detected in non-transplant patients (137). It remains to be elucidated whether this

is the result of direct effects of immunosuppressive drugs and whether this decreased infiltration of macrophage contributes to the enhanced invasiveness of SCC in SOT patients.

Dendritic Cells

DCs constitute a heterogeneous population of APCs that mediates critical connections between innate and adaptive immunity (138). These cells play a pivotal role in antigen processing and presentation resulting in the induction of effective immune responses against pathogens and tumor cells. Notably, these cells are the most powerful APCs, being able to orchestrate a primary immune response but also to induce immune tolerance (138). In particular, DCs play a central role in priming naive T and B cells, the first critical steps in the induction of an antigen-specific immune response. DCs develop from $CD34^+$ bone-marrow progenitor cells or from $CD14^+$ monocytes and differentiate into immature DCs (iDCs), which are functionally specialized to take up exogenous antigens. After recognition, exogenous antigens are internalized and the activated DCs migrate to the draining lymph node, where they can induce an adaptive immune response (139). This successful outcome requires the processing of antigens and their loading in the form of small peptides on the main histocompatibility complex (MHC) molecules. Peptides loaded on MHC-II molecules are recognized by antigen-specific $CD4^+$ T helper cells, while peptides loaded on MHC-I molecules are recognized by antigen-specific $CD8^+$ T lymphocytes. The presentation of internalized antigens on MHC-I molecules is defined as cross-presentation, a crucial process in the induction of effective adaptive immune responses against tumors and viruses that do not infect DC directly and that may induce peripheral tolerance (140). The MHC-I pathway is normally used to present endogenous antigens and cross-presentation is particularly important because it allows DCs to present through the MHC-I pathway also exogenous antigens, which are usually mainly presented by MHC-II molecules (141). Antigen cross-presentation involves two main pathways: (1) the vacuolar pathway, in which the processing/loading takes place within the endo/lysosomal compartment and (2) the endosome pathway, in which the internalized antigens are transported from the endosome to the cytosol where they are degraded by the proteasome (142). The co-stimulatory CD40/CD40L axis along with the danger signal provided by an exogenous antigen are catalysts for DC licensing. Therefore, exogenous antigen cross presentation and the consequent activation of naive $CD8^+$ cytotoxic T cells, provide the immune system with an important mechanism for generating immunity to viruses while preserving tolerance to self (143).

DCs exist in two differentiation states, each of which has distinct phenotypic, morphological and functional characteristics: immature dendritic cells (iDCs) and mature dendritic cells (mDCs). iDCs phenotypically show high expression of CCR1, CCR5, CCR6, and CD68, while the expression of CCR7, CD86, CD80, CD40, and CD83 is low. These cells are able to capture and process the antigens they encounter and subsequently evolve to mDCs, which are characterized by high expression levels of CD83, CD86, CD49, CD80, CCR7, MHC-II, CD1a, and CD11c (144).

Another classification of DCs is based on different DC subpopulations: classical DCs (cDCs), plasmacytoid DCs (pDCs), monocyte-derived DCs (moDCs), and Langherans cells (LCs). cDCs are professional APCs that play a key role in shaping appropriate adaptive immune responses (145). There are two major subsets of cDC: cDC1, which are $HLADR^+CD11c^+CD9\alpha^+$ and $CD24^+$ or $CD103^+$ and require the transcription factors IRF8 (146), Batf3 (147), Nfil3 (148), and Bcl6 (149) for their development. These cells also express the $CD141^+$ or blood DC antigen 3 (BDCA3). This population was found in spleen, tonsil, liver, lung and skin. Similar to mouse DCs, human cDC1 $CD141^+$ express TLR3, but unlike the mouse subset, they lack the TLR8 and TLR9. This population is particularly efficient at cross-presentation of cellular antigens and are highly competent to stimulating allogenic or autologous $CD4^+$ T cell immune responses. The second major cDC subset, cDC2, expresses $HLADR^+$ $CD11c^{hi}$ and the IRF4 transcription factor. This subset is present in lymphoid tissues and CD141 molecule is expressed by splenic cCD2. Human blood cDC2 express high levels TLR2 and TLR8 and very low levels of TLR4. They are able, without TRL activation, to cross-present soluble antigens, a process that may be enhanced bafilomycin-mediated inhibition of endosomal acidification (145, 150). From an ontogenetic point of view, available data are consistent with the existence of common DC progenitors that give rise to pDCs and intermediate precursors of cDCs (pre-cDCs) that may differentiate into $CD1c^+$ (BDCA-1) or $CD141^+$ (BDCA-3) cDCs. In human peripheral blood mononuclear cells, the $HLA\text{-}DR^+CD14^-CD11b^-$ subpopulation includes the $CD1c^+$ DC subset ($CD172\alpha^+$ and $IRF4^+$), the $CD141^{high}$ DC subset ($Clec9A^+$, $XCR1^+$, $IRF8^+$, and $TLR3^+$), and the pDC subset ($BDCA\text{-}2^+$, $BDCA\text{-}4^+$, and $CD123^{bright}$).

pDCs correspond to a subset of immature $CD11c^-$ population distinct from classical myeloid $CD11c^+$ DCs. Pre-pDCs express CD4 but lack T-cell receptor alpha (TCRα), TCRβ, TCRγ, TCRδ, or CD3 chains and are negative for B-cell lineage (CD19, CD21) or myeloid (CD13, CD14, CD33) markers. This population is characterized by the production of type I interferon during viral infection as they express constitutively IRF7. pDCs circulate in the blood and in peripheral organs and are strongly dependent on Fltl3 ligand (Flt3L), a potent endogenous DC growth factor, for their development and function. After maturation in response to TLR-ligation or CD40-engagement, pDCs became capable to present antigens to T cells and preferentially, induce generation of a unique type of $CD8^+$ Treg cells (151).

LCs are the DCs of the epidermidis, although they may be also present in other stratified epithelia, such as the mucosal oral and vaginal epithelium. LCs share with cDCs common myeloid or monocytic progenitors. LCs are stellate cells expressing MHC-II molecules and the integrin αX chain CD11c. They express also CD1a and CD1c, two MHC-I-related molecules involved in the presentation of lipid antigens. This population is considered sentinel tissue-resident DCs and their development is independent of the Flt3/Flt3L axis stimulation (152).

moDCs are phenotypically difficult to differentiate from cDCs because they share the expression of CD11c, CD11b, and MHC-II, but they express the Fc-gamma receptor 1 (FcγRI) (153). These cells also express CD1α, which is characteristic of the DCs derived from monocytes. Their mature phenotype is characterized by the loss of CD14, a marker of differentiated monocytes and by an increase in the expression of CD1α, MHC-II, CD83, and CD86. moDCs can be successfully grown in the presence of GMC-SF and IL-4 and are capable of induce effective antigen-specific immune responses (154).

Immunosuppressive drugs employed in the management of allograft rejection in SOT patients can influence the phenotypic and functional characteristics of DCs, suggesting that their impaired function may also play a role in the development of tumors or in promoting the reactivation of oncogenic viral infections in immunosuppressed patients. MMF was shown to down-regulate co-stimulatory and adhesion molecules, such as CD40, CD54, CD80, and CD86, in human monocyte-derived DC *in vitro*, a decreased production of TNF-α, IL-10, IL-12, and IL-18, as well as less efficient stimulation of alloreactive T cells (65). Similarly, the 1α,25-Dihydroxyvitamin D3 (1,25(OH)2D3), i.e., the active form of vitamin D3, inhibits the differentiation and maturation of human DCs, leading to down-regulated expression of CD40, CD80, and CD86 costimulatory molecules and inhibition of alloreactive T cell activation (155). Interestingly, Gregori et al. showed that short-term treatment of transplant recipient mice with 1,25(OH)2D3 in combination with MMF induced tolerance to islet allografts and expanded $CD4^+CD25^+$ Treg cells that were shown to adoptively transfer transplantation tolerance. Moreover, these DCs displayed a tolerogenic phenotype characterized by down-regulation of CD40, CD80, and CD86 and reduced IL-12 production (156).

The effects of CNIs on DCs are well-recognized and several groups have investigated the impact of these drugs on mouse and human DCs. TAC and CsA were found to inhibit the allostimulatory capacity of *in vitro*-generated myeloid DCs without affecting DC maturation (70). On the contrary, the pioneering work of Lee et al has shown how the treatment with CsA impaired the allostimulatory capacity of *in vitro* generated mouse bone marrow-derived DCs by downregulating CD40, CD80, and CD86 expression associated with reduced nuclear translocation of NF-κB, a transcription factor promoting DC maturation (157, 158). In addition, CsA inhibited DC-dependent production of INF-γ, IL-2, and IL- 4 by T cells, and IL-6, IL-12p40, and IL-12p70 by DCs (72). Recently, it was demonstrated that DCs generated in the presence of CsA lose their ability to induce Treg proliferation, with a strong reduction of IL-12 secretion and particularly of IL-2, which is necessary for the proliferation of $CD4^+CD25^+Foxp3^+$ T cells (159), thus having a negative impact on this population (160). Moreover, TAC was shown to inhibit the ability of DCs to stimulate T cells and to decrease the production CXCL10 and IL-12. Although IL-12 production by DC was impaired by TAC, these cells did not promote Th2 development as T cells stimulated by TAC-treated DCs produced less interferon IFN-γ, IL-4, and IL-10 (161) (**Figure 1**).

In mouse models, RAPA strongly impacts DCs maturation and function resulting in the induction of well-defined phenotypic characteristics of these cells. Data accumulated so far indicate that RAPA is able to impair maturation of DCs, reduce DCs costimulatory molecule upregulation, decrease the production of pro-inflammatory cytokines, inhibit T cells stimulatory capacity (78) and promote the selectively development of Treg cells in animal models of solid organ transplantation (162) (**Figure 1**). Mouse and human DCs treated with RAPA have an immature phenotype with low levels of CD80 and CD40 receptors and with a decreased expression of B7-H1, the PD-L ligand, a negative regulator of T cells activation and inducer of peripheral tolerance (163).

During the maturation process, DCs downregulate the CCR1 and CCR5 receptors and upregulate the expression of CCR7, which promotes the DCs migration from the peripheral tissues to the sites where they will encounter the naive T cells for their priming (144). In both mouse and human DCs, immunosuppressive drugs have divergent effects on the modulation of chemokine receptors (CKR) in maturing DC, important in regulating DCs localization and homing (164). In an elegant study, Sordi et al. showed how the use of immunosuppressive drugs may interfere with the generation of effective immune responses by affecting DC function. In particular, these authors investigated the functional relevance of CKR in the process of DC maturation both *in vitro* and *in vivo*. CsA and TAC were shown to slightly modulate the expression of CCR7 but without affecting the function of this CKR. In contrast, RAPA increased the expression of CCR7 at the mRNA and protein level and enhanced the *in vitro* migration of human DCs to CCL19 and of mouse DCs to lymph nodes *in vivo,* suggesting that it could be due to the inhibition of endogenous IL-10 production (165), which has an inhibitory activity on DCs maturation (166).

RAPA-treated alloantigen-pulsed DCs were shown to induce antigen-specific regulation and prolong experimental heart allograft survival. Taner et al. demonstrated that RAPA-exposed DC loaded with donor cell lysates and their adoptive infusion prior to experimental heart transplantation prolonged fully MHC mismatched murine heart allograft survival (167). Notably, RAPA-treated DCs were also shown to stimulate the generation of antigen-specific $Foxp3^+$ Treg cells thereby promoting organ transplant tolerance (168). The induction of tolerance by RAPA is further enhanced by the combination with Flt3L, which promotes the generation of tolerogenic, immunosuppressive DCs along with the production of $CD25^+Foxp3^+$ Treg cells and IL-10 secretion (169).

T cell responses are modulated by cytokines secreted by mDCs including pro-inflammatory cytokines, such as IL-12 and INF-α, and anti-inflammatory cytokines, such as IL-10. DCs treated with RAPA have a distinct cytokine secretion profile at different stages of maturation. In iDCs, RAPA reduced IL-10 and IL-12 production after LPS stimulation and increased apoptosis, while mDCs are resistant to RAPA-induced apoptosis, but they also show decreased production of IL-10 and TNF-α (79). A recent study demonstrated that relevant concentrations

of RAPA (20 ng/ml) inhibit the ability of both TLR7- and TLR9-activated pDCs to stimulate the production of IFN-γ and IL-10 by allogeneic T cells. On the contrary, RAPA-treated TLR7-activated pDCs were capable of stimulating the activation of naive and memory T cells, while also stimulating the generation and proliferation of $CD4^+$ $FoxP3^+$ Treg cells (80).

In recent years, tolerogenic DCs (t-DCs) attracted a growing interest for the development of new strategies to prevent and control allograft rejection after SOT. Enhanced generation of t-DCs, however, may negatively impact the induction of antitumor immune responses in this setting. There are two main critical factors that could shape DC functions into immunologic or tolerogenic: the stage of differentiation and the environmental factors that DCs encounter. Indeed, t-DCs comprise most immature DCs and DCs with different maturation states. One of the major hallmarks of t-DCs is their ability to induce tolerogenic responses to a much greater extent and in a shorter time as compared with immature DCs. t-DCs exert a unique immune surveillance function and normally express low levels of MHC and costimulatory molecules (CD80, CD86, CD40, OX40L) on their surface, and display a low capacity of activating T cells, which is potentially associated with T cell anergy and increased Treg cell generation. Moreover, t-DC can express high levels of inhibitory molecules such as programmed death ligand (PD-L)-1 and PD-L2, leukocyte Ig-like transcripts (ILTs) 2,34, HLA-G and galectins that may contribute to their tolerogenic potential (170–172).

Another subpopulation of t-DC was shown to have a semi-mature phenotype characterized by the high expression of MHC class II and B7 costimulatory molecules and a low production of proinflammatory cytokines, such as IL-1β, IL-6, IL-12p40, and IL-12p70 or TNF-α. However, mature DCs can also became tolerogenic. Indeed, in a subset of both mature and immature monocyte-derived DCs, Munn DH et al., demonstrated the expression of indoleamine 2,3-dioxygenase (IDO), a negative regulator of T-cell proliferation. The authors hypothesized that the presence of IL-10 during DC maturation (a regulatory cytokine associated with the development of tolerogenic DCs) prevented IFN-γ-induced down-regulation of IDO, resulting in sustained expression of functional IDO even in mature, IFN-γ-activated DCs (173). IDO and t-DC induction were also found to be associated with spontaneous renal allograft acceptance (174).

Gregori et al identified a novel subset of t-DC, named DC-10, which secretes high levels of IL-10, express ILT4 and HLA-G, and have the specific function to induce Type 1 T regulatory (Tr1) cells which are critical in maintaining tolerance to self- and non-self-antigens (172). There is also a unique subset of human DC, characterized by high expression of CD11b and low expression of MHC class II, which can negatively regulate immune responses. These DC have high expression of Fas that can inhibit $CD4^+$ T-cell proliferation and produce IL-10 and IP-10 via ERK-mediated inactivation of GSK-3 and subsequent up-regulation of β-catenin (175). pDC were also found to exert tolerogenic functions. In particular, these cells display heterogeneous properties with regard to antigen presentation, maturation and expression of different costimulatory molecules. Indeed, pDCs do not induce strong T-cell responses, but they were found to induce IL-10-producing regulatory Tr1 cells *in vitro* (176). Moreover, Abe M et al. reported the ability of pre-pDC to prolong vascularized organ graft survival promoting tolerance (177). A subset of pDCs expressing CCR9 was identified by Hadeiba et al. in resting secondary lymphoid tissues. This population was a potent enhancer of Treg cell function and effectively prevented acute graft-vs.-host disease induced by allogeneic CD4+ donor T cells. In support of the role of pDC in tolerance induction, some other studies suggested a possible functional correlation between the enhanced presence of pDC and the elevated frequency of Treg cells (178) with successful withdrawal of immunosuppression in liver transplant tolerance (179).

Immunosuppressive factors and molecules expressed by T cells can also shape DC into a more tolerogenic state. Indeed, IL-10-producing Treg cells suppressed DC maturation and prevented Th1 cell differentiation. Moreover, interaction between t-DCs and Tregs is essential for the establishment of immune tolerance induced by apoptotic cell administration. Indeed, Wu et al. demonstrated that t-DCs promote the expansion of Tregs via PD-L1 on their surface, and Tregs facilitate t-DCs to sustain tolerogenic state via IL-10 and TGF-β (180). Another example of DC-tolerance induction by T cells occurs via $Foxp3^+$ Tregs that inhibit the expression of MHC-II molecules in DC that were not able to make cognate interactions with CD4+ T cells (181). In addition, regulatory DCs can selectively recruit Th1 cells and inhibit Th1 proliferation, and promote the generation of IL-4-producing alternative memory CD4 T cells with suppressive activity (182).

The possible direct role of immunosuppressive drugs on DC function in SOT patients with or without cancer is difficult to assess. The extent and quality of T-cell responses specific for epitopes of tumor-associated antigens or oncogenic viruses may constitute a surrogate marker of the function of DC in various transplant settings. This possibility is best exemplified by the clinical relevance of the monitoring of EBV-specific T-cell responses in transplanted patients at risk to develop EBV-associated PTLD (183).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In the last decade, the number of patients undergoing solid organ transplantation and those living and aging with a functional transplant have been progressively increased. This situation is posing new challenges in terms of optimization of immunosuppressive regimens to prevent allograft rejection while reducing the incidence of *de novo* malignancies. This issue is of increasing clinical relevance considering that the occurrence of a *de novo* malignancy in this setting is associated with a poor prognosis. In fact, tumors in transplanted patients may be more frequently diagnosed at advanced stages, suggesting a more aggressive behavior of the disease in immunocompromised patients (184). Moreover, several studies reported decreased overall survival in transplanted patients (185–187). A recent study demonstrated that the all-cancer 10-year survival in a

large cohort of Italian liver transplant patients experiencing a malignancy was significantly lower as compared to that of liver transplant recipients without cancer (43 vs. 70%, HR = 4.66). The difference in survival was observed for both early and late mortality, although the effect was more pronounced in the first year after cancer diagnosis (188). These findings clearly point to the need to perform close oncologic monitoring during the post-transplant follow-up in order to ensure early cancer diagnosis and to improve survival. Indeed, a study carried out in more than 7,000 SOT patients indicated that rigorous cancer screening programs were effective in diagnosing at early stages lung, breast and prostate cancers (189). In addition to the conventional clinical follow up approaches for an early diagnosis of cancer, strategies able to identify transplanted patients at increased risk to develop a *de novo* malignancy are needed. Quantification of EBV DNA load in the blood, coupled with assessment of EBV-specific T-cell responses proved to be useful to identify transplanted patients at risk of impending EBV-associated PTLD (190). Nevertheless, for the large majority cancers arising after SOT, no suitable biomarkers are available to estimate the risk of *de novo* malignancy in this setting. A better understanding of the various mechanisms through which the different immunosuppressive regimens impair cancer immunosurveillance is required to develop suitable and effective immunomonitoring strategies for SOT patients. As described above, the immunosuppressants used to prevent allograft rejection have a heterogeneous and often complex impact on the function of immune cells. Dissection of this heterogeneity will be useful to understand why some drugs are associated with a decreased risk of a *de novo* malignancy as compared to others, as shown for mTORi (191, 192). Notably, these drugs used at immunosuppressive doses were also shown to efficiently synergize with an optimal oncological treatment to improve survival of patients with *de novo* carcinoma (193).

In perspective, a more thorough characterization of the effects induced by immunosuppressive drugs on the various DC subsets may give insights on the mechanisms underlying the different ability of these drugs to affect DC-dependent anti-tumor immune response and tolerance. Advanced monitoring of the effects induced by immunosuppressive regimens on the number and function of other immune cells, particularly Tregs and MDSCs, may be also relevant in this setting. This can be achieved by exploiting the power of new technologies such as mass cytometry, which allows for a high-dimensional single-cell analysis of the immune system through the simultaneous measurement of over 40 markers on individual cells (194). This technology has been recently proposed for the assessment of immune reconstitution after hematopoietic stem cell transplantation (195) and may be also useful if applied to SOT patients monitoring to simultaneously assess the phenotypic and functional features of multiple immune cell populations. Considering that the repertoire of B- and T-lymphocyte antigen receptors undergo significant changes during cancer development, the exploitation of novel methods for analyzing or evaluating these immune repertoires may facilitate the development of diagnostic and monitoring tools also in the SOT setting. Indeed, the high-throughput sequencing of immune repertoire technology, which provides a robust tool for deep sequencing repertoires of B- and T-lymphocyte antigen receptors, has been applied to the identification of tumor biomarkers and the development of immunotherapeutics for cancers (196). Integrating these novel technologic approaches with existing immune monitoring techniques will allow a better understanding of immune regulation in SOT recipients and lead to new opportunities to improve patient outcome.

AUTHOR CONTRIBUTIONS

MC and RD designed and wrote the manuscript. All the authors revised and approved the manuscript.

FUNDING

This study was supported by grants from National Breast Cancer Foundation (IIRS_18_047), Cancer Council Queensland (APP1145758), Brain Tumor Charity (495168), Brain Foundation, and Cure Brain Cancer Foundation.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 28 February 2019
doi: 10.3389/fimmu.2019.00116

Ultra-Sensitive *CSF3R* Deep Sequencing in Patients With Severe Congenital Neutropenia

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Primary Immunodeficiencies, a section of the journal Frontiers in Immunology

Received: *30 September 2018*
Accepted: *15 January 2019*
Published: *28 February 2019*

Citation:
Klimiankou M, Uenalan M, Kandabarau S, Nustede R, Steiert I, Mellor-Heineke S, Zeidler C, Skokowa J and Welte K (2019) Ultra-Sensitive CSF3R Deep Sequencing in Patients With Severe Congenital Neutropenia. Front. Immunol. 10:116. doi: 10.3389/fimmu.2019.00116

High frequency of acquired *CSF3R* (colony stimulating factor 3 receptor, granulocyte) mutations has been described in patients with severe congenital neutropenia (CN) at pre-leukemia stage and overt acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS). Here, we report the establishment of an ultra-sensitive deep sequencing of a *CSF3R* segment encoding the intracellular "critical region" of the G-CSFR known to be mutated in CN-MDS/AML patients. Using this method, we achieved a mutant allele frequency (MAF) detection rate of 0.01%. We detected *CSF3R* mutations in CN patients with different genetic backgrounds, but not in patients with other types of bone marrow failure syndromes chronically treated with G-CSF (e.g., Shwachman-Diamond Syndrome). Comparison of *CSF3R* deep sequencing results of DNA and cDNA from the bone marrow and peripheral blood cells revealed the highest sensitivity of cDNA from the peripheral blood polymorphonuclear neutrophils. This approach enables the identification of low-frequency *CSF3R* mutant clones, increases sensitivity, and earlier detection of *CSF3R* mutations acquired during the course of leukemogenic evolution of pre-leukemia HSCs of CN patients. We suggest application of sequencing of the entire CSF3R gene at diagnosis to identify patients with inherited lost-of-function *CSF3R* mutations and annual ultra-deep sequencing of the critical region of *CSF3R* to monitor acquisition of *CSF3R* mutations.

Keywords: severe congenital neutropenia, G-CSFR mutations, leukemogenesis, deep-sequencing, pre-leukemia

INTRODUCTION

Our current understanding of the pathological mechanisms responsible for severe congenital neutropenia (CN) suggests that it is a heterogeneous group of disorders with a common hematological and clinical phenotype characterized by a maturation arrest of myelopoiesis at the promyelocyte/myelocyte stage, resulting in a peripheral blood absolute neutrophil counts (ANCs) of <500 per microliter and early onset of bacterial infections. Although CN patients produce physiological amounts of G-CSF (granulocyte colony-stimulating factor), their myeloid precursor cells fail to differentiate normally into mature neutrophils; therefore, these patients

require long-term treatment with pharmacological dosages of recombinant human G-CSF (rhG-CSF, Filgrastim). The number of G-CSF receptors (G-CSFRs) on myeloid precursor cells of CN patients is rather elevated suggesting that signaling pathways downstream of the G-CSFR are defective (1).

Over the last 10 years, remarkable progress has been achieved in identifying CN causing gene mutations. Mutations in the HAX1 (HCLS1-associated protein X-1) gene have been identified as the underlying genetic cause of the Kostmann syndrome, a subtype of autosomal recessive CN (2). Genetic analyses of autosomal dominant and sporadic cases of CN indicate that the majority of the ethnical European patient population harbor *ELANE* mutations encoding neutrophil elastase (elastase 2) (3). Interestingly, patients with cyclic neutropenia (CyN) also harbor mutations within the ELANE gene, even in the same nucleotide position (4, 5). In addition, mutations at a number of rarely affected genes, among them e.g., *G6PC3* (glucose 6 phosphatase, catalytic, 3) (6), *GFI1* (growth factor independent 1) (7), *TAZ* (tafazzin) (8), *WAS* (Wiskott-Aldrich syndrome) (9) and *JAGN1* (Jagunal Homolog 1) (10) have been identified in CN (11).

Various acquired point mutations in the intracellular domain of G-CSFR have been described. These mutations introduces premature stop codons, resulting in the truncated G-CSFR (12–19). Transfection of the mutated G-CSF receptor with truncated intracellular part into murine cell lines induced hyper-proliferative responses to G-CSF (12). These effects are also seen following co-expression of wild-type and truncated receptors; this so-called dominant-negative effect mirrors patient findings in cases where only one allele is mutated. Intriguingly, there is a high incidence of transformation to myelodysplasia (MDS) or acute myeloid leukemia (AML) in patients who harbor acquired *CSF3R* mutations, suggesting that these mutations are involved in the development of leukemia (19). Our hypothesis is that *CSF3R* mutations arise in hematopoietic stem cells by selective pressure and are present at a low level until this cell clone becomes dominant through the continuous rhG-CSF treatment and acquisition of additional mutations in a leukemia-associated genes, such as *RUNX1* (runt-related transcription factor 1) (20).

Several investigators reported the identification of acquired *CSF3R* mutations in CN patients. Mutation frequencies and detection methods varied dramatically between these studies (19, 21, 22). To date, many investigators have directly sequenced PCR fragments of the intracytoplasmic domain of the G-CSFR. Using the classic Sanger sequencing method, at least 15–20% of the cells investigated must harbor mutations to yield positive results; thus, this method does not allow detection of small sub-clones of *CSF3R*-mutated cells. We recently explored this problem in detail, reporting results obtained by sequencing multiple clones prepared from PCR fragments of the intracytoplasmic region of the G-CSFR from individual CN patients. Using up to 45 clones generated from mRNA of each patient and propagated as recombinant plasmids in *Escherichia coli*, we found that ∼5% of RNA harboring *CSF3R* mutations could be detected (19).

Next-generation sequencing has significantly improved our ability to uncover genetic alterations in the genome. This novel approach allows the detection of low-abundance genetic aberrations, making it useful for the detection and monitoring of initial genetic lesions in AML at an early stage of leukemogenesis. Together with the sensitive detection of low-frequency minor mutant alleles, deep sequencing enables an accurate determination of allele frequencies.

We applied the sensitive deep sequencing of PCR products of the critical region of *CSF3R*, with more than 900,000 depth to detect the presence of *CSF3R* mutations during the course of leukemogenesis. We also investigated the influence of *CSF3R* mutations and single-nucleotide polymorphisms (SNPs) within *CSF3R* on G-CSF responsiveness in CN patients.

MATERIALS AND METHODS

Patients and Controls

CN patients were diagnosed based on results of peripheral blood ANC values $<0.5 \times 10^9$/l within 3 months, examinations of bone marrow aspirates, a history of recurrent severe infections, and negative results for granulocyte-specific antibodies. All patients with a clinical diagnosis of CN were screened for mutations in *ELANE*, *HAX1*, *JAGN*1, and *G6PC3*. In the case of negative result, NGS bone marrow failure syndromes gene panel was performed.

In total, DNA from 54 CN, 17 CyN, 25 SDS, 19 CN-MDS/AML patients as well as 16 idiopatic neutropenia and 7 autoimmune neutropenia (AiN) patients was subjected of *CSF3R* DNA deep sequencing. Additionally, we sequenced groups of patients with clinical diagnoses unrelated to neutropenia, like pediatric *de novo* CML ($n = 14$), *de novo* AML ($n = 10$). We also used BM sample from healthy donors without ($n = 11$) or with ($n = 2$) rhG-CSFR treatment (**Table S1**). *CSF3R* deep sequencing of cDNA samples was performed using RNA isolated from 68 CN, 12 CyN, 13 SDS, 5 CN-MDS/AML, 15 idiopathic, and 2 AiN patients (**Table 1**). Nine patients with inherited syndromes associated with severe neutropenia (Cohen syndrome, WHIM syndrome, GSD-1b, Pearson syndrome, Barth syndrome, DBA, Hermansky-Pudlak syndrome) (**Table 1**) were also included in the study. On average more than 2 samples per CN patient were typically collected during 1–3 years of observation time and were available for *CSF3R* deep sequencing.

Bone marrow and blood samples from patients were collected in association with an annual follow-up recommended by the Severe Chronic Neutropenia International Registry. Study approval was obtained from the Ethical Review Board of the Medical Faculty, University of Tübingen. Informed consent was collected in accordance with the Declaration of Helsinki.

Nucleic Acid Isolation

Bone marrow mononuclear cells (BM-MNCs) and polymorphonuclear cells (BM-PMNs) as well as peripheral blood mononuclear cells (PB-MNCs) were isolated by Ficoll-Hypaque gradient centrifugation (Amersham Biosciences, UK). Peripheral blood polymorphonuclear cells (PB-PMNs) were isolated using Polymorphprep (AXIS-SHIELD PoC AS, Norway). $CD34^+$ and $CD33^+$ cells were separated from BM-MNCs by means of positive selection after incubation with corresponding MicroBead Kit (Miltenyi Biotec) according to a standard protocol. The purity of the isolated $CD34^+$ and $CD33^+$ cells was more than 80%, as evaluated by flow cytometry. RNA

TABLE 1 | Prevalence of *CSF3R* acquired mutations in studied groups using cDNA deep sequencing.

Study groups	Number of patients, cDNA	Patients with acquired *CSF3R* mutations, cDNA
CN:	68	32 (47.1%)
ELANE-CN	38	20 (52.6%)
HAX1-CN	20	9 (45%)
G6PC3-CN	3	1 (33.3%)
WASP-CN	1	1 (100%)
JAGN1-CN	4	1 (25 %)
CSF3R-CN	2	0
CN, genetically unclassified	28	16 (57.1%)
CyN	12	2 (16.7%)
Shwachman-Diamond syndrome (SDS)	13	0
CN-MDS/AML	5	4 (80%)
Idiopathic neutropenia	15	0
Autoimmune neutropenia	2	0
Others*	9	0
Total number of patients	152	54 (35.5%)

**Cohen syndrome, WIHM syndrome, GSD-1b, Pearson syndrome, Barth syndrome, Hermansky-Pudlak syndrome, Diamond-Blackfan syndrome.*

was extracted using an RNeasy Mini Kit (Qiagen, Germany) according the manufacturers' protocols. For DNA purification, DNeasy Blood and Tissue Kit (Qiagen, Germany) was used.

Mutational Screening

Mutational screening of *ELANE*, *HAX1*, *JAGN1, G6PC3*, and *CSF3R* by Sanger sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3130 genetic analyzer (Applied Biosystems, USA) according to a standard sequencing protocol. Primer sequences are listed in **Table S2**. In cooperation with CeGaT (Germany) samples of CN patients negative for mutations in *ELANE*, *HAX1*, *JAGN1, G6PC3*, and *CSF3R* were subjected to gene panel sequencing approach for identification of potential mutations in 230 genes known to be associated with inherited hematological disorders.

CSF3R DNA Deep Sequencing

DNA was extracted from patients and volunteers BM and PB as described above. Fragments of the CSF3R gene (4.4, 5.0, and 5.4 kb) were amplified by long-range PCR. PCR products were designed to cover all exons and introns of the CSF3R gene except intron 3 which was excluded because of low conservation among vertebrates and high enrichment on repetitive and low complexity DNA sequences. For all patients, the sequencing of all 3 long-range PCR product was performed only once. In all subsequent DNA samples 5.0 kb PCR product that includes a critical region of *CSF3R* was sequenced.

PCR products were used as a template for generating a fragment library with a Covaris S2 sonicator (Covaris, Inc., USA). Amplification of 4.4- and 5.0-kb fragments was performed using a Phusion High-Fidelity DNA Polymerase kit (Thermo Fisher Scientific Inc., USA) and a touchdown PCR approach (**Tables S3, S5**). The 5.4 kb fragment was amplified using an Advantage Genomic LA Polymerase Mix (Clontech Laboratories, Inc., CA, USA) (**Tables S4, S5**). Sonicated DNA fragments were size-selected using the Agencourt AMPure XP Bead Reagent (Beckman Coulter, Inc., USA). The 5500 SOLiD Fragment Library Enzyme Module (PN 4464413; Life Technologies, USA) was used to perform blunting/end polishing, dA-tailing, ligation of adaptors and barcodes to size-selected DNA. The Agencourt AMPure XP Reagent (Beckman Coulter, Inc., USA) was used for purification of the ligated DNA. After emulsion PCR and bead enrichment, 3′-modified beads were deposited onto a glass slide and sequenced by ligation using a 5500XL SOLiD Sequencer (Life Technologies, USA).

For the pre-processing, the input sequences underwent *De Novo* Error Correction for SOLiD data using the SOLiD Accuracy Enhancement Tool (Life Technologies, USA). Reads aligned by NovoAlignCS (novocraft.com) were collected as SAM files and then applied to our custom next-generation sequencing pipeline (V0.03), which includes the following workflow: SAMtools (23) for BAM file generation, Picard Tools for duplicate removal, Genome Analysis Toolkit for indel realignment (Broad Institute, USA), SAMtools for variant calling, and AnnTools (24) for annotating detected single-nucleotide variants. Only variants with a Phred quality score >20 were accepted.

For sequencing of the complete CSF3R gene, we chose a mutant allele frequency (MAF) of 0.02 as the default threshold for candidate calls and 0.007 if the number of reads exceeded more than 5000x depth of coverage at a mutation position.

CSF3R cDNA Deep Sequencing

RNA was extracted from PB-MNCs, PB-PMNs, BM-MNCs, and BM-PMNs as described above. RNA purity and yield was measured on Nanodrop. Reverse transcription was performed by Omniscript RT Kit (Qiagen, Germany) according the manufacturers' protocol. Fragments of the CSF3R gene (236 and 198 bp) were amplified by PCR (**Tables S6, S7**). The PCR fragments were size-selected and purified using the Agencourt AMPure XP Bead Reagent (Beckman Coulter, USA). Before library preparation, PCR products were run on Bioanalyzer (Agilent, USA) to control purity.

Sequencing libraries were prepared according to 16S Metagenomic Sequencing Library Preparation Protocol from Illumina (Part#15044223Rev.B). Index PCR was performed using the Nextera XT Index Kit (Illumina, USA). For cleanup, AMPure XP Beads (Beckman, USA) were used. Libraries were quantified with Qubit HS Kit (Thermo Fisher Scientific, USA) and analyzed with Bioanalyser HS DNA Kit (Agilent, USA). Sequencing was performed on Illumina sequencer (NovaSeq 6000) with 2x100 bp sequencing mode using NovaSeq 5000/6000 S2 Flow Cell and appr. 0.6 Gb output per sample.

Demultiplexing, sequencing reads trimming, alignment, variant calling and annotation was done by NGS provider CeGaT (Germany). Demultiplexing of the sequencing reads was performed with Illumina bcl2fastq (2.19). Adapters were trimmed with Skewer (version 0.2.2) (25). Trimmed raw reads were aligned to the human reference genome (hg19) using the Burrows-Wheeler Aligner (BWA-mem version 0.7.17-cegat) (26). Low frequency variants were detected applying LoFreq

(Version 2.1.2) (27). Mapping quality was determined by Burrows-Wheeler Aligner. Positions with coverage ≥ 10.000 and mapping quality of at least 15 (Phred-Score) were included for the variant calling used for the analysis. The quality of FASTQ files was analyzed with FastQC (version 0.11.5-cegat) (28). Plots were created using ggplot2 (29) in R (30). MAF of 0.001 was selected as the default threshold for candidate calls.

Statistical Analysis

Statistical analyses were performed using R (30), Excel (Microsoft, USA) and Prism 7 version 7.03 (GraphPad Software, USA).

RESULTS

Deep Sequencing of Whole *CSF3R* DNA as Well as of Critical *CSF3R* Region cDNA

For deep sequencing analysis, we either sequenced PCR products of the whole CSF3R gene (except intron 3) or amplified cDNA of the intracellular region of G-CSFR that was reported to be mutated in CN and CN-MDS/AML patients. This region is lying between 715 and 787 amino acid positions (NP_000751.3) and included four conserved tyrosine residues, (Y727, Y752, Y767, and Y787) that act as docking sites for SH2 domain-containing proteins (**Figure 1A**) (31, 32).

The median age of patients at the time of sample collection was 13 years (25th percentile = 5.32, 75th percentile = 17.02) for cDNA samples and 12 years (25th percentile = 5.6, 75th percentile = 20.11) for DNA samples. In total, we sequenced 276 DNA samples and 289 cDNA samples. Patients included into the study are presented in **Table 1** and **Table S1**.

In keeping with a previous report (19), we found that 73.7% (14/19) of CN patients at the AML or MDS stage acquired *CS3FR* mutations. We detected a *CSF3R* mutation in 1 out of 10 pediatric *de novo* AML patients and no mutations were found in 14 pediatric CML patients (**Table S1**).

The level of inter-run reproducibility was estimated by re-sequencing DNA samples from 11 CN patients, in which 13 *CSF3R* mutations were detected with MAF values from 2 to 19%. The re-sequencing results showed a high level of MAF concordance (R^2 = 0.89) with consistent detection of all 13 mutations in a subsequent run (**Figure 1B**).

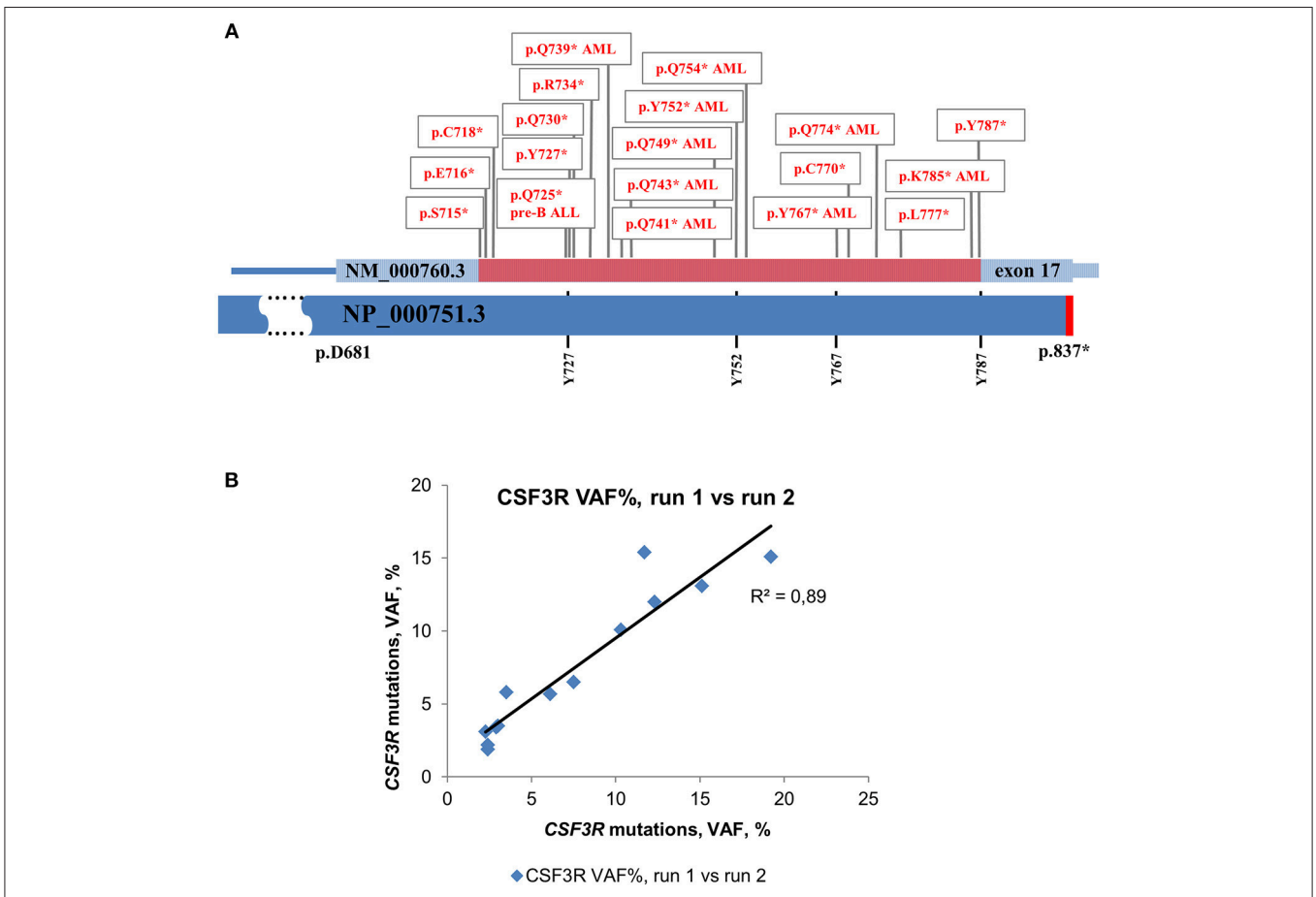

FIGURE 1 | Distribution of acquired mutations in exon 17 of CSF3R in CN and CN-MDS/AML patients. Schematic representation of exon 17 of the CSF3R gene along with protein sequence (NP_000751.3) showing four conserved tyrosine residues. The amino acid positions corresponding to exon 17 are indicated. The intracellular critical region of *CSF3R* is marked in red. Amino acid (AA) positions of the CSF3R gene mutations are confined to the region between amino acid residues 715 and 787. Mutations in G-CSFR associated with leukemia and MDS in CN are denoted by "AML" or "pre-B ALL", *- stop codon. **(B)** Inter-run reproducibility between *CSF3R* DNA deep sequencing runs. Inter-run reproducibility was assessed by sequencing of 13 *CSF3R* mutations from 11 CN patients in two separate runs.

By sequencing of DNA samples, we identified *CSF3R* mutations in 20.4% (11/54) of patients in the genetically defined CN group, and in 28.5% (2/7) of genetically unclassified CN patients. The high frequency of *CSF3R* mutations was observed in CN patients harboring *HAX1* or *ELANE* mutations (30% (3/10) and 22.2% (8/36), respectively).

Notably, using deep sequencing of cDNA samples (**Table 1**), we detected *CSF3R* mutations in almost a half of CN patients (47.1% (32/68) in genetically defined CN group and 57.1% (16/28) in unclassified CN). We also detected *CSF3R* mutations in two CyN patients. No *CSF3R* mutations were found in other studied groups (**Table 1**). An eighty percent (4/5) of CN-MDS/AML patients were positive for *CSF3R* mutations (**Table 1**).

Using *CSF3R* cDNA deep sequencing, 163 mutant clones were identified in 77 samples of genetically defined CN patients with an average MAF of 0.051 and a median MAF 0.018. In genetically unclassified CN patients, we scored 48 *CSF3R* mutant clones in 28 positive samples with an average MAF of 0.074 and a median MAF of 0.025. No significant difference in MAF was observed between genetically defined and unclassified CN groups (Mann-Whitney *U*-test: $U = 3{,}240$, $P > 0.99$) (**Figure 2A**).

No Correlation Between Acquisition of *CSF3R* Mutation and Therapeutic G-CSF Dose

For 102 patients, for whom information on rhG-CSF treatment was available, 96 received rhG-CSF treatment and were evaluated for association of acquired *CSF3R* mutation with the dose of rhG-CSF. We compared 40 patients harboring *CSF3R* mutations (12 *ELANE*-CN, 9 *HAX1*-CN, 1 *JAGN1*-CN, 3 CN-MDS/AML, 13 genetically unclassified CN and 2 CyN patients) with 54 patients without *CSF3R* mutations (10 *ELANE*-CN, 9 *HAX1*-CN, 2 *G6PC3*-CN, 1 *JAGN1*-CN, 1 CN-MDS/AML, 11 genetically unclassified CN, 7 CyN, 6 SDS, 1 idiopathic neutropenia patients as well as 6 patients with inherited syndromes associated with severe neutropenia (3 Cohen syndrome, 1 Barth syndrome, 1 WHIM, 1 GSDIb). Patients with germ-line *CSF3R* mutations ($n = 2$) were excluded from the analysis. Interestingly, we observed no difference in the rhG-CSF dose required to achieve more than 1,000 neutrophils/μl between patients with and without acquired *CSF3R* mutations (Mann-Whitney *U*-test: $U = 890$, $P = 0.1473$) (**Figure 2B**).

Highest Sensitivity of Detection of *CSF3R* Mutations Was Observed in cDNA of PB-PMNs

We compared MAF of *CSF3R* mutations between cDNA and DNA isolated from different cell fractions of five CN patients and detected the highest MAF in PB-PMNs of all studied CN patients (**Figures 3A–E**), as compared to BM-MNCs, BM-PMNs, and PB-MNCs. As expected, the highest sensitivity was achieved by sequencing of cDNA samples. Based on these observations, we suggest sequencing cDNA of PB-PMNs, in order to achieve detection of low-frequency cell clones with mutated *CSF3R*.

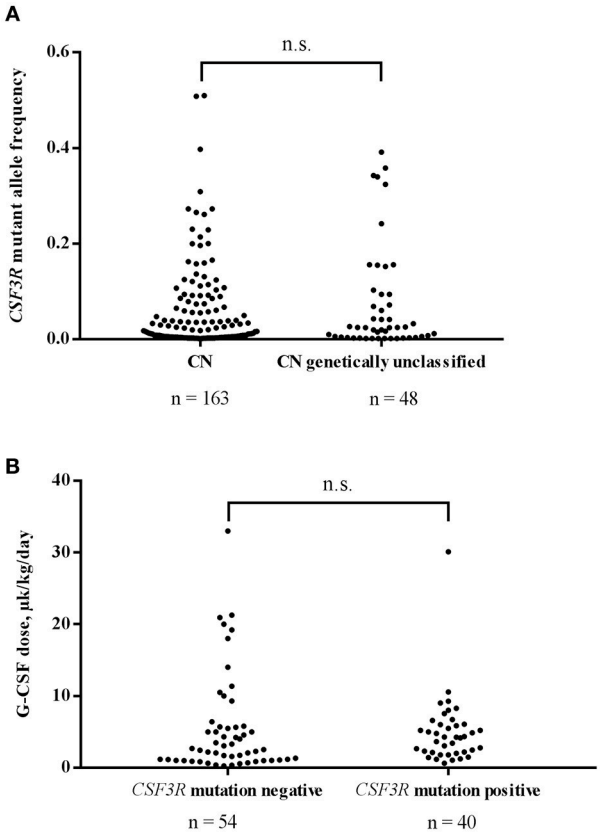

FIGURE 2 | (A) Comparison of *CSF3R* mutant allele frequencies in CN and genetically unclassified CN patients. No significant difference in MAF of *CSF3R* mutations between CN ($n = 163$) and genetically unclassified CN ($n = 48$) groups was observed; n.s.- not significant. **(B)** The acquisition of *CSF3R* mutations is not associated with higher rhG-CSF doses. Patients were divided into 2 groups based on the presence ($n = 40$) or absence ($n = 54$) of *CSF3R* mutations detected by cDNA deep-sequencing. rhG-CSF doses at the time of sample collection are plotted for the both groups; n.s., not significant.

Time Course and Numbers of *CSF3R* Mutations in CN Patients

In some CN patients, we identified multiple *CSF3R* mutations (**Figure 4**). Acquisition of multiple *CSF3R* mutations was not dependent on the type of CN-causing mutations. The percentage of cells expressing mutant *CSF3R* was varying during the years of observation with increasing MAF for some clones, but also decreasing MAF for other clones (**Figure 4**). Multiple *CSF3R* mutant clones were detected using sequencing of either cDNA (**Figures 4A–D**), or DNA (**Figure 4E**). Interestingly, in one *ELANE*-CN patient, all PB-PMNs acquired *CSF3R* mutation at the position p.Q749* (**Figure 4A**). However, only a minor proportion of patient's bone marrow $CD34^+$ hematopoietic stem cells had this mutation (5.45% of total $CD34^+$ cells and 12.08% of G-CSFR expressing $CD34^+$ cells) (**Figure 4A**).

For five CN patients with multiple *CSF3R* mutations, we investigated whether mutations were present in the same allele.

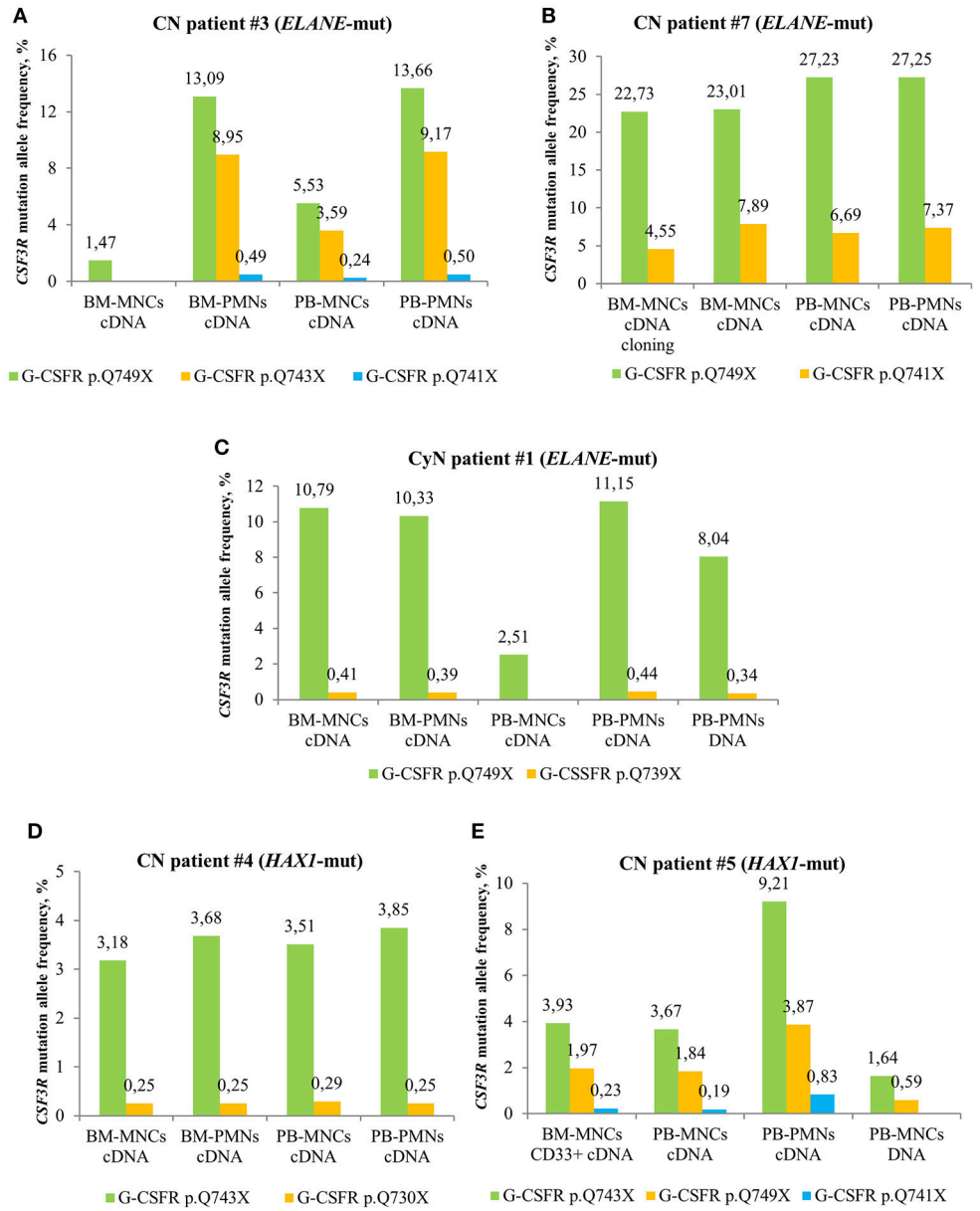

FIGURE 3 | Comparison of deep sequencing results in different types of peripheral blood (PB) and bone marrow (BM) samples of CN and CyN patients. Frequency of *CSF3R* mutant clones in different types of PB and BM samples from *ELANE*-CN patients **(A,B)**, CyN patient **(C)** and *HAX1*-CN patients (**D,E**) was analyzed using cDNA deep sequencing, as described in Material and Methods section. The *CSF3R* mutant clones are indicated based on the relative amino acid positions of mutations.

Interestingly, all non-sense mutations in *CSF3R* were found to belong to different alleles (**data not shown**).

Low Frequency SNPs in the Coding Region of *CSF3R*

We next asked whether genetic polymorphisms in the CSF3R gene affect G-CSF response and, in turn, promote leukemogenic transformation in CN patients. We made a correspondence matrix with all identified SNPs for 83 patients with the available information on the therapeutic rhG-CSF dose. To select patients grouped by SNP combinations, we used hierarchical clustering with Jaccard distances and one-way ANOVA and did not find any significant differences in rhG-CSF dose between groups.

We analyzed the nucleotide variants identified in coding region of *CSF3R*. Among 598 genetic variants listed in public databases, 94 are missense and nearly 25% of them have minor allele frequency (MAF) <0.05. Six SNPs are associated with destructive effects (**Table S8**). In our patient's cohorts, we identified 13 SNPs with MAF <0.05 and 3 novel variants

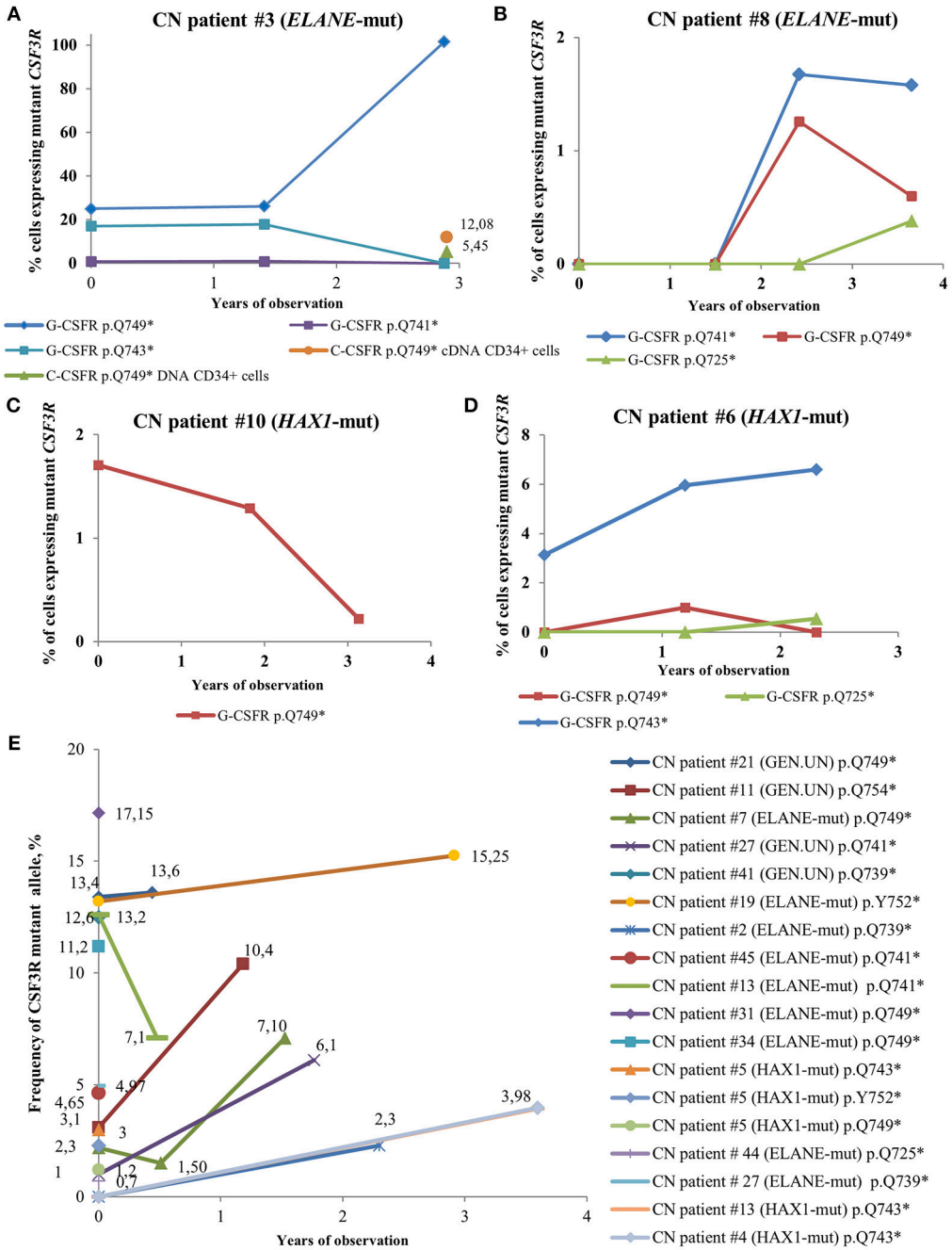

FIGURE 4 | Time-course of *CSF3R* mutations in sequential samples from CN patients detected by cDNA and DNA deep sequencing. **(A–D)** The percentage of cells expressing mutant *CSF3R* at different time points (in years), starting from the date of the first *CSF3R* mutation analysis in a given patient, is plotted. All mutations are considered to be heterozygous. The *CSF3R* mutant clones are indicated based on the relative amino acid positions of mutation sites. The number of cells with *CSF3R* mutation is estimated to be twice the number of reads supporting a mutant allele, *- stop codon. **(E)** Time-course of *CSF3R* mutations occurrence and frequency of mutant alleles in DNA samples from CN patients detected by *CSF3R* DNA deep sequencing. The frequency of mutant alleles at different time points (in years) in a given patient is plotted. The number of cells with *CSF3R* mutation is estimated to be twice the number of reads supporting a mutant allele, *- stop codon.

(**Tables S9**, **S10**). The most frequently detected variants with low MAF were p.D510H (9/173) and p.D320N (9/173) (**Table S10**). The p.D510H SNP was predicted to have damaging effects on protein functions by 4 out of 6 *in silico* prediction algorithms (**Table S9**). The therapeutic rhG-CSF doses in CN patients carrying this SNP varied between 0.2 and 11.3 μg/kg/day. The p.D320N SNP, which is located in the conserved cytokine receptor homology domain, was predicted to be benign by all

six prediction algorithms. Interestingly, in one CyN patient we detected a novel nucleotide polymorphism at p.M222T in the fibronectin type III like domain of the G-CSF receptor which was predicted to have the severe effects on protein function by 4 different algorithms (**Table S9**).

We identified one CN patient with p.R311C SNP, that have a novel variant, p.Q114* in *CSF3R* and no mutations in *ELANE*, *HAX1*, *G6PC3*, and *GFI1*. Of all identified rare SNPs, p.R311C had the most severe effects on protein structure and function (**Table S9**). CN patient with p.R311C and p.Q114* SNPs is responding to high dose of rhG-CSF (median dose 40 μg/kg/day) and the role of these variants as potential causative factors for neutropenia cannot be excluded. Additionally, in one CN patient with no response to G-CSF bi-allelic loss-of-function *CSF3R* mutations: the heterozygous SNP p.W547* and a novel mutation at the 3′ splice-acceptor site of intron 8 c.998-2A>T were detected (**Table S10**) (33). Except one CN patient with p.D510H SNP, no other G-CSF poor or non-responders had genetic alterations in the CSF3R gene. Four rare SNPs (p.M231T, p.Q346R, p.E405K, and p.A750T) were identified in CN patients who required a G-CSF dose <2 μg/kg/day, and one SNP (p.E149D) was detected in a CN patient who was not treated with G-CSF. Intriguingly, 1 CML and 1 CN patients carried a p.E808K SNP, which is known to correlate with high-risk of MDS development (34).

DISCUSSION

We demonstrated for the first time the establishment of sensitive NGS technology that allows identification of low-frequency cell clones with acquired *CSF3R* mutations across the CSF3R gene in a large group of patients with different types of CN. We found, that ultra-deep sequencing of cDNA markedly increased the sensitivity and identification of *CSF3R* mutant clones with MAF of 0.001. Much higher sensitivity of *CSF3R* cDNA over DNA deep sequencing can be explained by in average 900-fold increase of sequencing depth and selection of G-CSFR expressing cell population. We speculate that further increase of the sensitivity may lead to identification of *CSF3R* mutations virtually in all *ELANE*-CN and *HAX1*-CN patients. Our findings also suggest, that HSC clones with acquired *CSF3R* mutations are not leukemic, but pre-leukemic clones with an increased susceptibility to secondary leukemogenic events (e.g., *RUNX1* mutation, trisomy 21) and overt MDS or AML. We detected no correlation between the presence of *CSF3R* mutations and disease severity or G-CSF dose required to achieve sufficient neutrophil counts.

Previous studies have reported contradictory findings on the safety of G-CSF treatment (35–37). This is an important issue, since G-CSF is used not only to treat different types of CN, but is also applied for chemotherapy-induced neutropenia and to induce mobilization of $CD34^+$ stem and progenitor cells for autologous or allogeneic transplantation. To determine whether long-term G-CSF treatment *per se* induces acquisition of *CSF3R* mutations or is a CN-specific phenomenon, we examined the association between G-CSF treatment and acquisition of *CSF3R* mutations. There was no difference in therapeutic G-CSF dose between patients with or without acquired *CSF3R* mutations. Intriguingly, we detected no acquired *CSF3R* mutations in a large group of SDS patients who were on long-term G-CSF treatment. This is in accordance with the report of Xia et al. who demonstrated that clonal hematopoiesis due to mutations in *TP53*, but not *CSF3R* was present in patients with SDS but was not detected in healthy controls or patients with CN (38). We also found no acquired *CSF3R* mutations in G-CSF treated healthy individuals, although the group was small and G-CSF treatment was short-term. Acquired *CSF3R* mutations were detected exclusively in CN patients. These observations strongly argue for the safety of G-CSF therapy. Most likely, intracellular molecular defects specific for disturbed granulopoiesis in CN patients (39) and not G-CSF therapy *per se*, is responsible for the acquisition of *CSF3R* mutations in some HSC clones and clonal overgrowth of these clones.

We have shown in this report and in earlier studies (19) that ∼80% of CN patients who developed leukemia harbor acquired *CSF3R* mutations, and that all leukemic cells in these patients are affected by these mutations. Our *CSF3R* sequencing approach allows direct evaluation of the frequency of *CSF3R* mutant cells, which is not possible with Sanger sequencing and possible to a certain extent with Sanger sequencing of cloned PCR products. The evidence of clonal hematopoiesis in CN patients without signs of malignant transformation suggest that secondary promoting mutations (e.g., in *RUNX1, ASXL1, SUZ12*, or *EP300*) (20, 40) are necessary to transform the *CSF3R* mutant clone in bone marrow. By annual monitoring of *CSF3R* mutations in CN patients using the deep sequencing we were able to trace pre-leukemic clones over a long period of time. Sequencing additional genes known to be associated with leukemogenesis in CN (e.g., *RUNX1*) might help to identify early stages of malignant transformation and could have a decisive role in guiding clinical decisions.

The time between acquisition of *CSF3R* mutations and overt leukemia vary substantial, some patients carry *CSF3R* mutations for more than 10 years. The persistence of mutated clones for years in CN patients without overt leukemia is intriguing. Interestingly, sequential analyses of the same patients at multiple time points led to increase in detection of *CSF3R* mutations to 34% (19). In our study, we found that almost 50% of CN patients acquired *CSF3R* mutations. Based on these data, we hypothesize that a sub-clone of pre-leukemic cells carrying *CSF3R* mutations at a frequency below our detection limit may be present for a long time and evolves to a frequency detectable by the deep-sequencing method in response to e.g., ER stress through unfolded protein response (UPR) caused by germline mutations. This is also supported by the fact, that some clones harboring *CSF3R* mutations evolved and disappeared again below the limit of detection but are most likely not absent completely (19, 20).

Interestingly, acquisition of *CSF3R* mutations in CN patients was independent of the inherited mutational status (**Table 1**). These data are consistent with our previous observations that components of the G-CSF receptor signaling such as the transcription factors LEF-1 and C/EBPα are severely diminished

in myeloid progenitor cells of CN patients harboring *ELANE* or *HAX1* mutations (41).

All acquired mutations identified by us in CN and CN-MDS/AML patients using deep-sequencing technology were located in region encoding the critical intra-cytoplasmic domain of the G-CSFR which is known as the only region mutated in CN-MDS/AML patients (19, 42). This region has four conserved tyrosine residues essential for appropriate activation and suppression of G-CSFR signaling by STAT3 (signal transducer and activator of transcription 3) and SOCS3 (suppressor of cytokine signaling 3), respectively (31). The absence of these conserved tyrosine residues in cells expressing truncated G-CSFR might lead to a strong proliferative advantage (43–45).

We and others have identified CN patients with germline bi-allelic *CSF3R* mutations in the extracellular domain of the receptor that lead to G-CSF non-responsiveness (33, 46). We suggest sequencing the entire coding sequence of *CSF3R* in G-CSF "non-responders." All CN patients regardless of their mutation status, except SDS patients, should undergo annual deep sequencing of the critical intra-cellular region of *CSF3R*.

Interestingly, in one of ten pediatric *de novo* AML patients, we also identified a non-sense *CSF3R* mutation that was located in the critical region of the G-CSFR that affected one of four essential tyrosine residues (Y787). No mutations were observed in pediatric CML patients; however, one CML patient carried the rare SNP, p.E808K, which is known to be correlated with the development of high-risk MDS (34). *CSF3R* mutations in *de novo* AML patients are very rare, and mutations leading to the absence of functionally important tyrosine residues had not been previously described (47–49). Further studies of larger cohorts of AML and CML patients will be required to evaluate the role of *CSF3R* mutations and rare SNPs in the development of *de novo* AML and CML.

Up to now, the role of SNPs in *CSF3R* in CN patients was unclear. By sequencing the entire CSF3R gene, we were able to identify and analyze rare SNPs. We identified 13 dbSNPs with MAF <0.05, 2 novel variants in the coding region and one variant at an intron-exon junction (**Table S10**). However, there were no clear correlations between the presence of distinct rare SNPs and type of neutropenia, G-CSF dose requirement, leukemia progression, or disease severity. Only 2 of 10 CN patients with a poor or no G-CSF response had a rare SNP, effectively excluding intrinsic defects in *CSF3R* as a main cause of poor/non-responsiveness, at least in this studied group.

In summary, we have reported the establishment of sensitive *CSF3R* deep sequencing for assessing *CSF3R* mutation status in neutropenia patients. We suggest sequencing of cDNA of PB-PMNs to monitor hematopoietic clones that acquired *CSF3R* mutations over years. We found that acquisition of *CSF3R* mutations is a CN-specific phenomenon and is not present in patients with other types of neutropenia chronically treated with rhG-CSF.

AUTHOR CONTRIBUTIONS

JS and KW designed and supervised the study. MK and IS performed the main experiments. MU, SK, and MK analyzed deep-seq data. RN helped with sample preparation for NGS. JS, KW, and MK analyzed the data and wrote the manuscript. CZ and SM-H provided patient's material and clinical data.

ACKNOWLEDGMENTS

We thank the physicians of the Severe Chronic Neutropenia International Registry for providing patient material. We thank study subjects for their cooperation. This work was supported by the Madeleine-Schickedanz Kinderkrebsstiftung, Fortüne Programm of the Tübingen University, Else Kröner Fresenius Foundation (project 2017_A45). We also acknowledge support by Deutsche Forschungsgemeinschaft (DFG) and Open Access Publishing Fund of University of Tübingen.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00116/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 12 February 2019
doi: 10.3389/fimmu.2018.03136

Primary Immunodeficiency and Cancer Predisposition Revisited: Embedding Two Closely Related Concepts Into an Integrative Conceptual Framework

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Common understanding suggests that the normal function of a "healthy" immune system safe-guards and protects against the development of malignancies, whereas a genetically impaired one might increase the likelihood of their manifestation. This view is primarily based on and apparently supported by an increased incidence of such diseases in patients with specific forms of immunodeficiencies that are caused by high penetrant gene defects. As I will review and discuss herein, such constellations merely represent the tip of an iceberg. The overall situation is by far more varied and complex, especially if one takes into account the growing difficulties to define what actually constitutes an immunodeficiency and what defines a cancer predisposition. The enormous advances in genome sequencing, in bioinformatic analyses and in the functional *in vitro* and *in vivo* assessment of novel findings together with the availability of large databases provide us with a wealth of information that steadily increases the number of sequence variants that concur with clinically more or less recognizable immunological problems and their consequences. Since many of the newly identified hard-core defects are exceedingly rare, their tumor predisposing effect is difficult to ascertain. The analyses of large data sets, on the other hand, continuously supply us with low penetrant variants that, at least in statistical terms, are clearly tumor predisposing, although their specific relevance for the respective carriers still needs to be carefully assessed on an individual basis. Finally, defects and variants that affect the same gene families and pathways in both a constitutional and somatic setting underscore the fact that immunodeficiencies and cancer predisposition can be viewed as two closely related errors of development. Depending on the particular genetic and/or environmental context as well as the respective stage of development, the same changes can have either a neutral, predisposing and, in some instances, even a protective effect. To understand the interaction between the immune system, be it "normal" or "deficient" and tumor predisposition and development on a systemic level, one therefore needs to focus on the structure and dynamic functional organization of the entire immune system rather than on its isolated individual components alone.

Keywords: primary immunodeficiency, cancer predisposition, down syndrome, childhood leukemia, immune editing, immune activation, inflammation, microbiome

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Primary Immunodeficiencies, a section of the journal Frontiers in Immunology

Received: *31 October 2018*
Accepted: *19 December 2018*
Published: *12 February 2019*

Citation:
Haas OA (2019) Primary Immunodeficiency and Cancer Predisposition Revisited: Embedding Two Closely Related Concepts Into an Integrative Conceptual Framework. Front. Immunol. 9:3136. doi: 10.3389/fimmu.2018.03136

INTRODUCTION

The neoplastic transformation of cells and their subsequent successful clonal expansion and progression into clinically apparent hematologic malignancies and solid tumors is a complex multifactorial process. On the one hand, this process requires changes in the respective cells' genetic program that modify their metabolism and performance and consequently alter their normal differentiation, replicative, and survival capacity. On the other hand, these cells have to learn to adapt themselves and to exploit external deterministic physiological stimuli as well as to flexibly react to a plethora of stochastic environmental challenges (1, 2). This, in turn, defines their capability to achieve successful interactions with and survival strategies within their normal surrounding tissue. With its interactive network of cells, humoral factors, and cytokines, the immune system plays a fundamental role in the recognition of and protection against any internal or external threads, be it abnormal cells, foreign tissues or infections agents. Inborn genetic defects or dysfunctions of the one or the other immune system components may thus unsettle the intricate physiological balance and maintenance of a body's functional homeostasis and thereby diminish its preventive capability or even promote the formation of neoplastic diseases in a proactive manner.

The recent methodological advances in deciphering the composition and structure of the human genome allow us now to identify virtually any DNA sequence alterations in a hitherto unimaginable fast and detailed manner. Various such technologies have in the meantime become invaluable diagnostic mutation screening tools that help to identify clear-cut disease-associated genetic defects in inborn errors of the immune system but also more elusive variants that may participate in the predisposition to malignant diseases in children. These developments are addressed in a large number of original publications as well as in many excellent reviews of these subjects (3–16). Rather than reiterating what has already extensively been written about, I intend to provide a more conceptional framework of this subject and focus my attention on often neglected and less well-appreciated fundamental facts and phenomena, which I consider particular relevant for an in-depth appreciation and understanding of this topic and which I will supplement with some specific examples that illustrate the developments and progress in this field.

To begin with, we first need to (re)define the current view and understanding of "primary immunodeficiency" as well "genetic predisposition and susceptibility."

PRIMARY IMMUNODEFICIENCY SYNDROMES (PID)

The immune system is composed of highly specialized cells, tissues, organs and soluble factors that interact in a complex way to ensure an organism's immune defense. According to the current definition, PID are thus a group of diseases, which are caused by heritable DNA sequence alterations that impair the quantitative or qualitative function of cellular or humoral components of the adaptive or innate immune system (17). The spectrum of their clinical, often intimately interrelated symptoms includes developmental disorders, autoinflammation, chronic inflammation, autoimmunity, neoplasms as well as serious, recurrent, or unusual infections (18, 19). Initially, the diagnosis of these conditions was based on abnormal laboratory parameters and clinical problems, in particular recurrent, severe or unusual infections that in certain groups of patients occasionally concurred with familial clustering, syndromic features, radiation sensitivity and also a certain propensity to develop particular types of malignancies. With the advent of *in vitro* testing and immunophenotyping technologies, it became possible to better define and differentiate certain categories as well as to characterize even subtle cellular and humoral functional deviances already to a certain extent. In the early days of the molecular genetic era, the respective responsible genes were then identified in cases with highly penetrant genetic traits, which instigated a first, albeit restricted diagnostic mutation screening. With the introduction of more sophisticated sequencing technologies, the discovery of causative genetic defects increased steadily in parallel with the refined dissection, delineation, and definition of such immunodeficiency syndromes. The recent 2017 update of the "Primary Immunodeficiency Committee" of the "International Union of Immunological Societies" thus recognizes 344 genetic defects that define 354 distinct disorders of immunity in nine categories (20, 21). Some of these monogenetic disorders are extremely rare and were so far identified in single families only.

This compilation together with the commonly unconsidered use of the term PID leaves the impression that one indeed knows what the term PID stands for. It is therefore intriguing to note and especially important to point out that there is actually no clear consensus about its definition (22). The reason for this now newly flaring-up debate is the recognition that the perception of immunodeficiency has so far clearly focused only on the most obvious and clinically striking disorders in both adaptive and innate immunity that affect the lympho- and hematopoietic system. With the increasing appreciation that also non-hematopoietic cells and tissues participate in a significant manner in the immune defense this view is currently changing and necessitates an expansion of this concept. For instance, keratinocytes, endothelial cells, and fibroblast secrete as much and as many cytokines as hematopoietic cells do and can thus use their intrinsic pathways for protection against infectious agents also in a similar fashion. Another example are neurons and oligodendrocytes, which are similar essential and sufficient guardians against herpes simplex virus I and probably also other infection agents (22).

Another development that one has to consider in this context are the results that derive from the increasingly sophisticated diagnostic work-up of suspicious cases with technologies that enable nowadays the recognition of even clinically not readily apparent quantitative and qualitative deviations of particular cellular and humoral immune system components. As can be appreciated already in a normal setting, such differences are commonly due to and thus correlate with variations on the sequence level, either in form of single nucleotide

polymorphisms/(SNP) alone or in form of definable haplotypes, which can make it more and more difficult to define a physiological norm and, under particular settings, a clear disease-relevant pathological state (23–32). One of the best documented and therefore most instructive example is the context-dependent implications of the highly variable serum levels of the mannan-binding lectin (MBL), the apparently most common deficiency of a humoral component of the innate immune system (33). The respective gene contains 87 different polymorphic sites with a multitude of possible combinations, of which seven common haplotypes stand out. These haplotypes determine the serum levels as well as the configuration and function of the encoded proteins in a predictable manner, although the possible effects are co-determined by the sex and age of the respective carrier, hormonal changes and immune system activation. Moreover, the frequency of the diverse haplotypes varies world-wide in an ethnicity specific manner. Thus, although the magnitude of particular MBL protein levels are clearly recognizable and determined by genetic factors, the ensuing effects, whether a low or high level becomes detrimental or beneficial or whether it remains irrelevant, are strictly context-dependent and therefore difficult to predict or interpret in a given individual.

Based on an estimate that ∼5% of all genes participate in one or the other way in host defense and immune tolerance, it was predicted that with the new sequencing technologies up to 3,000 PIDs will be identified by the year 2021. Even if one considers only a still monogenic scenario with only two types of alleles per locus (i.e., heterozygous vs. homozygous, loss-of function vs. gain-of-function, hypomorphic vs. amorphic as well as various other variations), it is hardly imaginable that one will be able to functionally evaluate and analyze the magnitude of all the possible outcomes in a reasonable manner to make some sense of the ensuing (patho)physiological effects even in an perhaps otherwise well-defined setting (22, 34).

In the end, these reflections leave us with the question how one actually will define primary immunodeficiencies in the future. When, to which extent and in which form do they need to manifest themselves clinically? Will it be sufficient to just view them as pure monogenic disorders or does one eventually also need to consider the contribution of modifying gene, signaling pathway, and cellular networks in a much stronger way?

GENETIC PREDISPOSITION AND SUSCEPTIBILITY

The concept of genetic predisposition and susceptibility, which so far was also based primarily on the clinical perception of disease and inheritance patterns, experiences nowadays a similar reinterpretation and paradigm shift as the one of immunodeficiency. The emergence and continuous improvement of fine-scale and cost-efficient targeted, whole exome and whole genome, methylation as well as RNA sequencing approaches, increase the possibilities to investigate the genetic background of heritable and acquired diseases in a previously unprecedented manner (6, 10). Not only has it become much easier to screen all the eligible genes of already well-recognized conditions for causative mutations, it has also become much simpler to identify novel sequence abnormalities in rare, unusual, or merely suspected cases of immunodeficiencies and cancer predisposition. Thus, the special choice of the appropriate mode to search for and ascertain such genetic factors remains nowadays a matter of intention, clinical opportunities and individual demands that, in particular, is based on patient/family-relevant, gene-related, disease-associated, or population-based aspects (30). In a clinical setting, the direct patient-orientated approach is definitely the most important one. The ascertainment of an inborn genetic cause of a particular disease requires not only its appropriate work-up with the best-fitted mutation screening method but also the careful justification of its significance through the assessment of medical records and family history as well as the clinical and laboratory data of an affected patient (7, 13, 14, 34–47). Especially in those cases in which a cancer-prone condition is recognized already before the onset of a malignant disease, securing the specific genetic cause is essential to guide the necessary clinical measures, such as an appropriate treatment and surveillance program together with a suitable genetic counseling (7, 13, 14, 34–47). However, in many instances a potential predisposing germ-line alteration may only be suspected and searched for at the time a malignant disease is diagnosed. Especially if one screens the neoplastic tissue for disease-specific diagnostic alterations, one cannot avoid coming across inborn genetic errors, not only those in already known genes but occasionally also in novel ones. Distinguishing somatic from inherited defects in tumor tissue alone may turn out quite difficult because both types often affect the same genes, a fact that necessitates the verification of the inborn nature of any such changes by analyzing germ line material in addition. Based on such experiences, it is therefore becoming practice to screen for underlying germ-line defects in a more systematic fashion in form of so called "trio analyses," which in addition to a patient's tumor and germ line DNA also requires the parental ones for comparison (4, 13, 34, 35). An increasing number of publications confirm that this approach is particular rewarding, not only for the sake of the patient and her family but also for scientific reasons. In case a particular gene defect is not already clearly indicative of a specific type of predisposing condition, it may be difficult or virtually impossible to decide whether concomitant immune system derangements at diagnosis are actually the cause or the effect of the respective disease. As I will point out later, more subtle predisposing gene alterations that merely modify the function of a gene, such as single nucleotid polymorphisms (SNPs), may not even exert any easily recognizable effects prior to onset of the malignant disease. Predisposing SNPs were originally discovered by large-scale genome wide association studies (GWAS) in regions of the genome, which are linked with particular disease traits. The biological relevance and functional consequences of some of these variants has in the meantime already been established and confirmed with appropriate experiments and test systems (23, 26, 48, 49).

Our current knowledge of the genetic basis of immunodeficiency and tumor predisposition is primarily based on monogenic disorders. We learned to appreciate the

genetic heterogeneity of these conditions, meaning that single or similar phenotypes can be generated by different genetic mechanisms. *Polygenic* diseases, on the other hand, are caused by the joint contribution of several independent acting or interacting genes, whose individual contribution might be small or even unnoticeable. GWAS together with WGS studies have now allowed us to extend such analyses to the entire genome in a kind of *omnigenic* approach, which means that we will need to learn to cope with the combinatorial effects of a large number of genetic variants, whose individual contribution is not readily apparent (50). In contrast to Mendelian diseases, which are primarily caused by mutations in the protein-coding part of the genome, complex traits are mainly driven by non-coding variants that presumably affect regulatory elements of genes, such as promoters and enhancers. For instance, risk variants for autoimmune diseases show particular enrichment in active chromatin regions of immune cells (51–53).

The "*omnigenic*" model still accepts that only a modest number of "core genes" or pathways are etiologically important for a specific disease and their dysfunction will still have the strongest impact on the disease process (54). However, in this situation the particular risk will be driven by an accumulation of weak and heterogeneous effects of many modifying gene variants, whose specific configuration might even only become relevant in certain cell types and tissues, whereas in others they might remain completely inconsequential (51). The ultimate and most provocative conclusion and interpretation of this "*omnigenic*" model is of course that virtually any variant with regulatory effects in a given tissue is likely to have some (weak) effects on all diseases that are modulated through this particular tissue (51).

Whereas the identification of risk factors in monogenic diseases requires sequencing of specific genes and the careful functional assessment of any unusual sequence variant that pops up, polygenic risk scores of common diseases are statistically determined likelihoods that are calculated from genome-wide SNP patterns. Given the countless possibilities how defective and normal but functionally dissimilar allele variants of one or multiple genes can be combined and co-inherited, it is therefore astonishing that, as reported recently by Khera et al., the risk scores of such common diseases may under particular circumstances nevertheless reach at least the same magnitude as the ones achieved in monogenic diseases (55). Together with the cell- and tissue-specific utilization of the ensuing gene products, these findings provide a ready explanation for the highly variable penetrance of genuine gene defects and, even more so, for functionally modifying variants and, not least, why it is so difficult to foresee their biological consequences even in monogenic disorders (34). In addition, one has of course also to keep in mind that even in instances with a strong predisposing genetic component, the development of malignancies is always a multifactorial process that not only requires a liable genetic architecture but also some probabilistic elements as well as the participation and interaction of a multitude of other intrinsic and extrinsic factors and mechanisms. In case of hematologic malignancies, such cell-intrinsic defects and abnormalities consist of those that affect (I) (lympho- and hematopoietic stem) cell development, differentiation and apoptosis; (II) lymphocyte signaling, cytoskeleton, cytotoxicity and metabolism and (III) chromosome stability as well as DNA repair (3). Cell-extrinsic factors, on the other hand, comprise chronic inflammation; autoimmune- and autoinflammatory diseases, chronic (viral) infections and an impaired tumor surveillance (**Figure 1**).

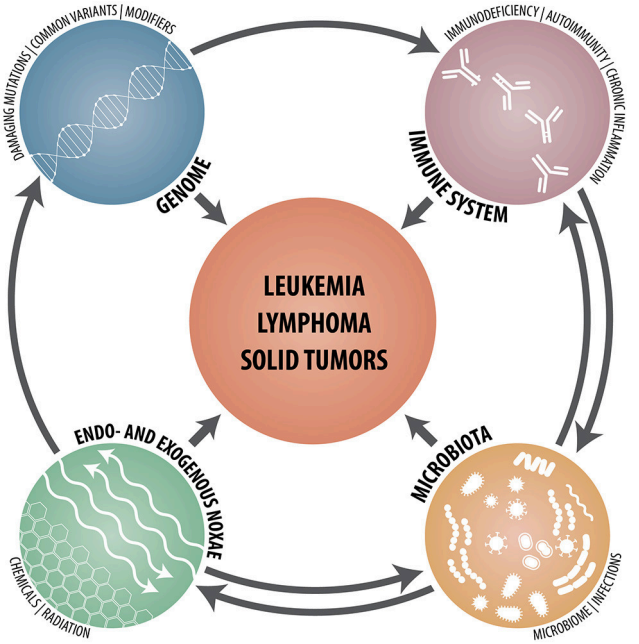

FIGURE 1 | Schematic synopsis of the various genetic, immunological, microbial, and environmental constituents that contribute to and participate in the development of hematologic neoplasms, lymphomas and solid tumors.

SOMATIC MUTATION (SMT) VS. TISSUE ORGANIZATION FIELD (TOFT) THEORY

SMT and TOFT are two apparently competing theories of cancer development. The SMT postulates that cancer is a molecular, gene-based disease that derives from single cells whose autonomous and unrestrained proliferation is driven by the progressive accumulation of accidental and essentially unrelated events (2, 56–58). The TOFT, on the other hand, posits that cancer develops as an adaptive, emergent phenomenon whose fundamental determinants act at the level of tissue and organ homeostasis. In this scenario, inherent genetic constituents as well as a variety of physical, chemical and biological agents, such as cytokines, viruses, chemicals and/or radiation, perturb the functional interaction of diverse cellular modules and subsequently also the organizational state of tissues themselves (58). To a certain extent this process resembles morphogenesis and as such also replicates a tumor's capability to continuously balance novelty with stability and to combine plasticity with robustness (57, 59). The reductionist, bottom-up approach of SMT and the emergentist, top-down approach of TOFT are often considered incompatible because they view the

problem from two different levels of biological complexity. The probably smallest and already generally agreed-upon common centerpiece where these two opinions meet is the tissue micro-environment (57).

Compared to the possibilities of compact tissues, the various closely interconnected humoral and cellular components of the immune system are in a unique situation, because they can exert their action not only in a local microenvironment, but they can also act over and cover the macroenvironment of tissues, organs and even an entire organism in a systemic fashion. Moreover, within a particular context and a respective cellular milieu, components of the immune system can either foster or suppress tumor development. It is thus not surprising that the highly flexible and adaptable immune system, be it normal, impaired, or defective, is one of the major players in the game of tumor predisposition and development (60–64).

The three prevalent and often closely connected complications of PIDs are thus infections, autoimmunity, and malignancies. Nevertheless, it is intriguing to note that except for a few distinct disorders, such as Nijmegen breakage syndrome (NBS), Ataxia telangectasia (AT), and autoimmune lymphoproliferative syndrome (ALPS)-related autoimmune diseases, PIDs do not cluster with malignancies in the human diseaseome network (60, 65). Most of the available information regarding cancer risk derives from specific subtypes that result from defects in genes that regulate DNA repair, cell cycle, apoptosis, bone marrow maturation as well as those that help to protect against virus infections (17, 60). As might be expected, the most common overall hitherto documented forms of malignancies in all these conditions are lymphomas, whereas other neoplasms occur predominantly in a more disorder-typical and -constrained manner (7, 14, 17, 38, 60, 66–72).

"ENVIRONMENT"

Multicellular organisms are organized in a modular fashion with distinct functional units and compartments. Based on the particular level of organization one can thus distinguish various internal as well as external forms of environment. From the perspective of cells, for instance, such environmental shells may constitute specific niches, tissues, organs and the entire organism. The environment of a developing fetus, on the other hand, is provided by the mother. Following birth, the organism becomes embedded in a milieu of beneficial as well as latent pathogenic microorganism and is then openly exposed to the potentially damaging biological, physical, and chemical agents of the outer world, with which it has then to interact in various ways.

Fetal-Maternal Immune System Interactions

With regard to immunology, pregnancy is a particular interesting condition because it requires the constructive co-existence of two genetically and immunological distinct individuals within a single body (73, 74). To succeed, this endeavor requires the beneficial cooperation of a fully developed, but during this period dampened, immune system with a just evolving one that still has to mature and achieve its independence. This interaction requires the temporary reorganization and adaptation of the maternal immune system as well as the acceptance of assistance and cooperation of the fetal one. Maternal immune cells therefore help to "teach" those of the fetus to balance the need of self-defense against that of immune tolerance: too much restraint would lead to lethal infections, whereas too little would lead to autoimmunity (75–77). During this especially vulnerable phase this intricate balance can easily be disturbed, in particular by both cell intrinsic (genetic) as well as cell extrinsic biological factors.

Although a fetus expresses genetically foreign paternal antigens, it coexists in harmony inside the mother because it resides in an immune-privileged cocoon (78). Nevertheless, maternal and fetal cells still traffic through the placenta during the entire gestation period. After birth, surviving cells become then the source of a lifelong micro-chimerism in the corresponding opposite bodies, a phenomenon that under particular circumstances may significantly impact the future life of the mother as well as the child in various positive or negative ways (78, 79). Changes in the number, phenotype or distribution of microchimeric cells, for instance, can have an effect on immune surveillance, tissue repair, autoimmune diseases and tumorigenesis. It has thus been suggested that microchimeric cells may modulate health and disease in a similar way as commensal microorganisms control the susceptibility to various immunological and non-immunological disorders (78, 80).

One of the striking pathological consequences of the bidirectional cell trafficking are particular forms of SCID, in which the accumulation of a significant number of maternal T cells can cause a kind of graft-versus-host disease (GVHD) in the immune incompetent child (81, 82). However, even asymptomatic infiltrations of maternal cells are still an independent predictor for the development of GVHD later in life in case of transplantations (82). Conversely, maternal micro-chimerism in cord blood can mediate a graft-versus-leukemia effect in cord blood transplantation (83). Finally, there are also rare instances of maternal-fetal transmissions of malignancies, such as has been reported for lymphoma, leukemia and melanoma (84).

Within the setting of such pre- and postnatal fetal/maternal immune system interactions, the human leukocyte antigen (HLA) system and, in particular, its paternal component plays a particular important role (78). Exposure to inherited paternal antigens as well as non-inherited maternal antigens during pregnancy can lead to either immunization or tolerization, the sequelae of which can have even consequences decades later, not least in form of an alloimmune response in case of transplantation (85).

HLA System

The HLA system is part of the human major histocompatibility complex (MHC), a region on the short arm of chromosome 6 with some 260 genes that are involved in the immune response. Unsurprisingly, this is also the region of the genome that is associated with the greatest number of diseases with an immune system component, including those "bare lymphocyte" SCID disorders that are caused by deleterious mutations in

certain MHC genes (86–88). As one of the main players in immune system interactions, the HLA system orchestrates the induction, regulation and fine-tuning of immune reactions and, in particular, the selection of the T cell repertoire (89). It is highly polymorphic and comprises more than 15,000 allelic variants (86).

Although specific HLA genotypes do not *per se* predispose to any particular disease in the strict direct sense, they are still highly enriched in and closely associated with distinct forms of inflammatory, autoimmune, and malignant disorders, a fact that not only underlines the central position of this regulatory system in their pathogenesis but also somehow links these otherwise disparate diseases.

HLA genotype patterns are not only associated with distinct sub-types of leukemias and lymphomas, but they can even correlate to some extent with the prognosis and survival of the respective diseases (90, 91). This evidence derived originally from the atypical HLA segregation patterns in leukemic families. They revealed an increase in HLA-identical non-affected sibs, HLA homozygosity, and identical disease-related maternal class II DRB1 haplotypes (90). Together this data is also taken as an indication that ALL is a problem that results from a population level response of HLA to infectious disease. In other words, overrepresented HLA haplotypes can provide some valuable insights into gene environment interactions as well as why and how particular clones are selected that will eventually produce the leukemic cell mass (88, 92–94). According to Greaves, B cell precursor (BCP) ALL evolves in two discrete steps, the *in utero* formation of a preleukemic clone, which is then triggered by a delayed abnormal immune response to common infections, followed by its postnatal conversion to overt leukemia (95–97). In the absence of any direct evidence for a specific causative infectious agent, the respective HLA pattern can thus be used as an indirect proxy measures for a genetically directed immune response and can thereby deliver valuable clues for the involved mechanisms. Based on such investigations, Taylor et al. concluded that BCP ALL is an indirect outcome of a transient auto-immune induced inflammatory molecular mimicry reaction that in turn may also explain why this subtype appears to be associated with delayed infection (88, 92, 93). Other factors that can help tumor cells to escape immune surveillance include somatic mutations that result in structural and functional changes in HLA system components, loss of expression of tumor antigens, lack of co-stimulatory molecules, and production of immunosuppressive cytokines (86). However, even more intriguing is the recent discovery that the HLA class I genotype is also participating in sculpting the entire oncogenic mutation landscape of a neoplasm (98). This is achieved through the continuous elimination of tumor cells with mutations that primarily produce strong antigens, which leads to a selection of cells with mutations that avoid producing such neoantigens (98).

Since the first hit that initiates the formation of the majority of pediatric cancers and leukemias occurs already very early in fetal life, the largest part of their further development still takes place *in utero* (95–97). It is therefore conceivable that fetal/maternal interactions and, in particular, the maternal immune system can influence and modify also the course of the disease in the one or the other way. Because one cannot study these processes directly, one has to rely on the above discussed traces and patterns that remain imprinted especially in the child's immune system after birth and which at least can provide some indirect clues about what had happened prenatally. The essential role of the *in utero* environment is further underlined by the fact that in individuals with a preexistent germline predisposition, secondary leukemia-promoting mutations can only evolve in special niches during distinct stages of organ development. In Down syndrome, for instance, *GATA1* mutations, which emerge exclusively during fetal liver hematopoiesis, cause an accumulation of immature erythro-megakaryocytic precursor cells (99–102). After birth, this pseudo- or preleukemic cell population is usually uncapable to maintain itself any longer and usually collapses within a short period. Although such spontaneous postnatal regressions of embryonic malignancies are quite common, it is not yet clear, whether at all or to which extent this phenomenon can also be attributed, in the strictest or in a broader sense, to the loss of the fetal/maternal interaction or altered activity of the newborns immune system (68, 103).

Disorders of the DNA Repair System

Embryonic malignancies as well as those associated with PIDs are similar unfortunate byproducts of the complex processes that control normal development (102, 104–106). Environmental triggers such as carcinogenic pollutants and radiation play in general only a subordinate pathogenetic role in the development of childhood malignancies (105, 106). Such factors become primarily relevant only in those PID forms that are due to some types of DNA repair deficiencies (60, 71, 107–112). One of the physiological tasks of this system is to orchestrate processes, such as V(D)J recombination, class switch recombination, and somatic hypermutation, which together generate those lymphocyte-specific reorganizations that provide the basis for the adaptive immune system's genetic diversity (71, 108). Therefore, immune deficient patients with a dysfunctional DNA repair, such as those with an AT, NBS, and Bloom syndrome, are prone to develop lymphomas, whereas those with a dysfunctional DNA repair but without immune deficiency, such as xeroderma pigmentosum, Fanconi anemia, Werner syndrome, and Rothmund–Thomson syndrome, will primarily develop other forms of cancers (71). These occur especially in organs with rapidly dividing cells and/or an increased metabolic activity, including the brain, skin, breast, and the gastrointestinal tract. Since particular DNA repair defects produce characteristic mutation patterns and predispose to specific tumor forms in PID patients, it is in turn even possible to infer already from such indicators which DNA recombination processes are impaired (71).

Patients with constitutional mismatch repair deficiency (CMMRD) are prone to develop gastrointestinal, genitourinary and brain tumors, lymphomas, and leukemias (38, 39, 46, 47, 109, 113–116). They may also develop antibody deficiencies of variable severity, although they are neither a constant nor obligatory feature and usually also have no clinical correlate (107).

Genetic defects that disrupt the normal function of the DNA nucleotide excision repair (NER) complex cause at least eight

overlapping phenotypes, such as xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD) (117, 118). NER is responsible for fixing UV-induced lesions, bulky chemical adducts and some forms of oxidative damage (118). The respective complex comprises at least 30 proteins, three of which (XPB, XPD, and TTDA) are also part of the basal transcription factor TFIIH. The most interesting and intriguing of these proteins is XPD, because, as Lehman pointed out so suitably, it is one gene with two functions (DNA repair and transcription) and three diseases (XP, CS, and TTD) (117). Some of the clinical features of these syndromes are quite similar but some are also markedly different (119). Although all three syndromes have an exceptionally sun-sensitive skin, cancers develop only in patients with XP but not in those with CS and TTD (118). CS and TTD, on the other hand, have neurodevelopmental abnormalities and the latter also ichthyosis and brittle hair. To explain this conflicting and somehow mysterious genotype-phenotype discrepancies, Bootsma and Hoeijmakers proposed already quite some time ago that XP would result, if the defect would concern the DNA repair but not the transcriptional function of the complex, whereas, *vice versa*, the developmental TTD-associated problems would arise, if only the transcriptional part would be affected (120).

DNA repair defects may also impair the formation and production of antibodies, which is the defining feature of CVID (19, 121–127). Patients with such a dysfunctional humoral immunity have an increased probability to develop extra-nodal non-Hodgkin B cell and mucosa-associated lymphomas as well as epithelial tumors of the stomach, breast, bladder, and cervix (70, 128–130). In contrast to most PIDs, CVID-associated lymphomas are more common in older people and usually EBV-negative (70, 128–130). Selective IgA deficiency, in particular, goes along with a 7- to 10-fold increase in gastric adenocarcinomas. This risk is most likely related to an inability to clear *Helicobacter pylori* infections and appears to decrease when these bacteria are eradicated (70, 129, 130).

Immunoediting

The immune system is in many, apparently paradoxical ways involved in the manifestation and evolution of malignancies. It can facilitate cellular transformation as well as prevent, promote, control and thus shape their development, phenomena that eventually were summarized under the term "cancer immunoediting" (62, 131–137). The concept of immunoediting evolved from the older and more controversial "cancer immune surveillance" one, which was based on the notion that, analogous to the "non-self" of pathogens, our immune system is also able to discriminate between the "malignant self" of pre-cancerous and cancerous cells and the "self" of normal cells (61, 62, 68). To discriminate cancer cells from normal cells, the immune system pursues two main strategies: T and B cells, which belong to the adaptive immune system, recognize altered self-proteins, whereas natural killer (NK) cells, gamma/delta T cells and macrophages, which are part of the innate immune system, take care of stress-induced self-molecules on transformed cells (61). Still, the necessity to establish an effective antitumor response, goes always hand in hand with the formidable challenge to circumvent the destruction of normal cells and to avoid autoimmunity.

Given the tight interaction between the immune system and neoplastic tissues, one naturally expects, and as Corthay put forward in eight arguments, that individuals with PIDs are more prone to develop tumors than the general population (61). At first sight this notion is well-supported by both animal models and clinical observations (61, 68). The best evidence that this is indeed the case derives from experiments with mice that lack key components of the immune system. They have not only an overall higher tumor incidence, but they are also more susceptible to transplanted or chemical carcinogen-induced tumors (64, 138). At second sight, however, all hitherto available data argues against the long-hold notion that potentially dysfunctional immune surveillance mechanisms indeed increase the general tumor risk in all PID patients. If at all, such processes can only play a subordinate and ancillary role.

Reliable information regarding the general and specific tumor risk of individuals with PIDs derives primarily from three large epidemiological studies from the USA, Australia, and the Netherlands (67, 128, 139). Together these studies comprise more than 5,000 patients with around 300 different forms of PIDs. Compared to the general population and previous estimates, these analyses revealed a surprisingly low, only 2-fold increased, overall tumor risk. However, since the respective risk is primarily confined to and therefore significantly higher in the nine most frequent high-penetrant PIDs, it is conversely also much lower or even absent in most of the other PIDs. Distinct genetic PID defects predispose to and concur with special and often unique types of malignancies, the most common of which are non-Hodgkin lymphomas, leukemias, digestive tract as well as virus-induced cancers (67, 68, 70, 128, 129, 139). This particular distribution patterns can already provide some important clues about the underlying defective, disrupted or impaired immune processes that trigger such disease developments. The two major driving forces that are responsible for the 8- to 10-fold excess lymphoma risk in subjects with PIDs, for instance, are a deficient DNA repair and an inadequate response to viral infections (61, 67, 68, 70). The intriguing part of this story is, however, that the incidence of the most frequent cancers, such as breast, lung, and colon, is in the remaining group of PIDs much lower than that in the general population.

Inadequate Activation and Response of the Immune System

Chronic inflammation, autoinflammation, autoimmunity, and infection-associated overstimulation are closely intertwined derangements of a deficient or compromised immune system (140).

Chronic Inflammation

Inflammation is a physiological response to tissue stressors such as tissue damage and infectious as well as non-infectious agents that ensures the maintenance of tissue homeostasis (141). Under normal circumstances it mitigates infections, clears damaged cells, and initiates tissue repair (142). If this process is not properly terminated, it can cause substantial

collateral damage and contribute to tumor development (143–145). Chronic inflammation plays not only a pivotal role in different stages of tumorigenesis, but it may also impede the response to therapy (63, 145). Along the route to tumorigenesis, intrinsic genetic factors interact with extrinsic immune system and stromal humoral and cellular components to generate a mixed microenvironment that is composed of tumor-promoting and tumor-suppressive factors, including innate NK and adaptive T cells (62, 63, 145, 146). The fact that, despite their dissimilar etiology and physiology, autoimmune and infectious diseases take advantage of the same immunosuppressive pathways and logistics underlines the global relevance and central position of these particular activities (146).

Infection-provoked chronic inflammatory conditions predispose especially but not exclusively to the development of Hodgkin's, Burkitt's, and mucosa-associated lymphoid tissue (MALT) lymphomas (147–149). The best-known associations are those between *Helicobacter pylori* and gastric lymhomas, Chlamydophila psittaci and ocular adnexal lymphomas as well as *Borrelia burgdorferi* and cutaneous MALT lymphomas, while chronic infections with Epstein-Barr virus (EBV) usually predispose to Burkitt- & Hodgkin lymphomas, those with Hepatitis C virus (HCV) to marginal zone lymphomas and those with Hepatitis B virus (HBV) to hepatocellular carcinoma (147, 148). The pathogenetic role of chronic inflammation remains less clear in case of human papillomavirus-, herpes simplex virus 2-, and cytomegalovirus-triggered malignancies (150).

Inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis, provoke especially the development of colorectal cancer (151, 152). Crohn's disease, in particular, is a multifactorial disease whose genetic underpinning encompasses 71 so far recognized susceptibility loci (31). Amongst these are the first recognized monogenic causes of such diseases, namely interleukin-10 (IL-10) and IL-10 receptor (IL-10R) loss of function mutations that are the specific cause of a severe, very early onset type of inflammatory bowel disease (153–156). Affected children have an extremely high probability to develop a unique form of monoclonal EBV-negative diffuse large B cell lymphoma, which is characterized by a constitutive activation of the NF-kappaB pathway and a defective local T cell immune response (153). These findings prompted Neven et al. to postulate that not gut inflammation itself but that the defective IL-10 pathway alone was the responsible pathogenetic trigger. Referring to the fact that all these children received azathioprine, which is a well-known lymphoma risk-increasing factor in adults with inflammatory bowel diseases, they suggested that this immunosuppressive treatment was also the final spark that ignited lymphoma development in these children (153).

Autoimmunity

Autoimmunity is a prominent element in many PIDs, especially in CVIDs, and not least in those, which predispose to malignancies (19, 125, 157–159). Despite their close clinical and genetic interrelationship, autoimmunity, and PIDs were up to now interpreted as two mutually exclusive conditions rather than as two sides of the same coin (157). In consideration of the fact that autoimmunity is the leading symptom in a variety of monogenic disorders that affect T cell development, tolerance, and interferon signaling, complement pathways as well as the resolution of inflammation, this view is changing nowadays (157). The two prototypic examples to illustrate their close interrelationship are the autoimmune lymphoproliferative syndrome (ALPS) and the IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked). In case of ALPS, the respective lymphoproliferation and propensity to develop lymphomas results from apoptosis-impairing germline as well as somatic mutations in the *FAS*, *FASL,* and caspase 10 genes (160–162). Still, mutation carriers have only a <60% chance for disease manifestation (161). The IPEX syndrome, on the other hand, is caused by activating mutations in the *FOXP3* gene (163–165). The encoded transcription factor controls the function of regulatory T cells that are essential for maintaining self-tolerance and immune homeostasis by suppressing aberrant responses such as autoimmunity and allergy (166). Deficiency of cytotoxic T lymphocyte antigen 4 (CTLA-4), which is a crucial inhibitor of T cell response that is also present on regulatory T cells, can therefore generate autoinflammation and autoimmunity in a corresponding fashion (166–169). Moreover, CTLA-4-deficient individuals are at risk to primarily develop EBV-related malignancies (170). Another recently recognized albeit rare cause of autoimmunity are leukemia-predisposing germline mutations in the *IKZF1* gene, which encodes the hematopoietic transcription factor Ikaros (171–175). Finally, one should not forget that autoimmune diseases are also a common problem in one of the most remarkable forms of leukemia-predisposing immunodeficiencies, the Down syndrome (176–178).

Hyperactivation

The inability of cytotoxic lymphocytes to fend off and kill virus-infected or transformed cells leads to an often uncontrollable hyperactivation of the immune system in form of hemophagocytic lymphohistiocytosis (HLH) (179–181). This distinctive clinical feature is the common denominator of a related group of perforin-deficient hyperinflammatory disorders, so called "perforinopathies," that may either be due to rare congenital gene-disrupting mono- or biallelic mutations or, in less severe form, due to functionally impairing hypomorphic alleles (182–186). Familial hemophagocytic lymphohistiocytosis type 2 (FHL2) is caused by biallelic mutations of the perforin gene (*PRF1*) (179). It shares some of its clinical characteristics with those of anaplastic large cell lymphoma (ALCL), which accounts for ∼10 to 15% of all pediatric non-Hodgkin lymphomas (187–190). About a quarter of these lymphoma patients carry only monoallelic *PRF1* mutations but, remarkably, virtually none in *SH2D1A* or *UNC13D*, genes that are implicated in two other forms of FHL (181, 187, 191–193). Moreover, an otherwise common activity-diminishing *PRF1* gene variant (SNP A91V; rs35947132), is also postulated to predispose to the nasal form of NK/T cell lymphoma in adults, which is the most frequent EBV-related NK/T cell malignancy (188).

Microbiome

Human beings are holobiontic meta-organisms (194–197). They are composed of host as well as trillions of viral, fungal, bacterial, and eukaryotic microbes that are collectively referred to as the microbiota or microbiome (194–197). This microbiome is acquired and shaped during the first 2 years of life. It co-evolves with its respective host genome and, under physiological conditions, becomes part of a stable, life-long synergistic homoeostasis (194, 195, 197–200). Because of its tight functional link with and profound effects on the host's immune system in health and disease, the microbiome is therefore already regarded as a complex, polygenic trait (200–202). Environmental, and host-related perturbations of this microbial ecosystem reduce almost invariably its diversity. This is a common finding in many multifactorial inflammatory, autoimmune, metabolic, neoplastic, and neurodegenerative diseases, although it is rarely known whether such a dysbiosis is indeed the cause or the effect of the underlying ailment (197, 203). Nevertheless, the host's immune system is certainly the most important force that shapes the configuration of the normal and dysbiotic microbiome, which, in turn, may of course be a significant cofounding factor in immune-mediated and immune-associated diseases (197). A healthy or dysbiotic microbiota can influence the host innate immune system and it is therefore no surprise that microorganisms are also implicated in the pathogenesis of at least 20% of all human malignancies (204). In a dysbiotic state, alterations in the signature of microbial molecules that are sensed by the host can lead to a different activation state of the immune system. These changes may alter the balance of host cell proliferation and death, guide immune system function and influence metabolism of host-produced factors, ingested food and pharmaceuticals (205). Moreover, they may also drive transformation by affecting genomic stability, resistance to cell death and proliferative signaling (205). Both chronic high-grade as well as lower-grade smoldering inflammatory disorders drive a tumor-permissive milieu, a problem that was extensively studied and confirmed in mice that were deficient in various immunologically relevant genes (199, 205, 206). It is worth noting that such a cancer susceptibility can even be transferred to healthy mice by cohousing, fostering or fecal transplants (195, 199, 207). Since polymorphisms in immunologically relevant genes affect human microbiota composition and cancer predisposition, this observation is therefore also highly relevant for such human diseases (201, 202, 208, 209).

Based on these results, Dzutsev et al. therefore suggested that malignancies can be viewed as systemic diseases that alter the physiological homeostatic interaction of the entire meta-organism (143, 195). Mostly because of its effects on metabolism, cellular proliferation, inflammation, and immunity, the microbiome would interact with their development at virtually every level, including predisposing conditions, initiation, genetic instability, susceptibility to host immune response, progression, comorbidity and, not least, response to therapy (143, 195, 205). In support of this notion, Yamamoto et al reported, for instance, that variation in intestinal microbes between different animal facilities or as a consequence of experimental perturbations profoundly affected the incidence of lymphoma and survival of Atm (ataxia telangiectasia mutated)-deficient mice (210). Another very intriguing and instructive example of how we will one day perhaps be able to exploit particular constituents of the microbiome for medical and therapeutic purposes was recently provided by Bromberg et al. (211). They showed that with the delivery of stool samples from pregnant mice or gavage with isolated B. pseudolongum species to those with cardiac allografts they were able to improve their long-term survival as well as to prevent inflammation and fibrosis in this respective organ (211).

Bona Fide Infections

The IARC classifies 10 microbial agents (7 viruses, 2 parasites, and 1 bacterium) as group 1 human carcinogens (195, 204). Over 90% of all infection-attributed cancers are attributed to *Helicobacter pylori*, HBV and HCV, and human papillomaviruses (HPV) (204). Except for HCV, all human oncogenic viruses encode at least one oncogene and may therefore directly induce neoplastic transformation, although, as alluded to above, infection-associated inflammation and dysbiosis most likely play a likewise significant role in this context. Thus, Plottel et al. distinguish three classes of microbe-induced human malignancies, the first is defined as involving immunologic tissues, the second requires direct microbial interactions with parenchymal cells and the third involves distant effects from local interactions (212).

The likelihood to be either protected or to become infected as well as the infection outcome depends primarily on the diverse conditions that guide the interactions between the respective pathogen and its potential host. These include their specific genetic set-up and functional fitness to invade or defend themselves, as well as on a variety of other factors, such as concomitant infections as well as the age and microbial constitution of the respective host (213–220). To invade host cells, pathogens exploit and hijack particular cell surface proteins. Amongst the docking receptors that were identified so far in case of the Malaria parasite plasmodium falciparum, for instance, are CD55 and structural variants of the GYPA and GYPB genes (220–224). CD55-null erythrocytes, in particular, are refractory to invasion by all isolates of plasmodium falciparum (222). The second example is *CCR5*, which encodes a coreceptor for HIV entry. Consequently, carriers of an otherwise phenotypically and functionally completely inconsequential homozygous 32-bp deletion are resistant to HIV infection (225). Although HIV infections are indisputably the cause of the "acquired" immune deficiency, one can still argue that all these infection-related problems are nevertheless due to a genetically primed primary albeit clinically inapparent susceptibility. The point I want to stress here is that what one currently perceives as a "normal" wild-type or a "defective" susceptible gene variant is merely a matter of choice, frequency, common habit, and/or subjective interpretation. Since the functional consequences of any such variant will always remain context-dependent, one therefore needs to keep in mind that the distinction between "protective" and "defective" can never be an absolute dogma, but always lies in the eyes of the beholder.

Epstein-Barr virus (EBV) is a ubiquitous virus that infects virtually all humans and obligatory leads to a usually

asymptomatic symbiotic lifelong latent persistence (214, 226). Although this EBV latency may provide an evolutionary mutualistic benefit to its hosts as an immune adjuvant that apparently protects against lethal Listeria monocytogenes and *Yersinia pestis* infections (227, 228), the many EBV-associated problems have nowadays become clinically far more relevant and interesting. At present, the literature distinguishes more than 25 EBV-related disease entities, including those that are associated with various forms of immunodeficiencies and those that concur with a high propensity to develop diverse hematopoietic, epithelial, and mesenchymal malignancies (66, 226, 229–234). These disease forms can be roughly divided into reactive EBV-associated lymphoid and histiocytic/dendritic proliferations (including reactive lesions with or without diverse malignant potential), B cell proliferations (including Hodgkin lymphoma and plasma cell neoplasms), T/NK cell proliferations, immunodeficiency-related lymphoid proliferations and histiocytic/dendritic proliferations (66, 226, 229, 231–236). Taken together, EBV contributes to about 1.5% of all cases of human cancer worldwide (229, 237).

The type and incidence of EBV associated diseases varies significantly in different parts of the world, an observation that can be attributed to the different distribution of genetic susceptibility factors, including individual-, HLA-, and ethnicity-specific ones, to environmental- and geographic-specific co-founding influences but also to the existence of particular EBV strains that may produce different disease patterns (27, 66, 153, 213, 214, 217, 218, 229, 230, 232–234, 236, 238–240).

In contrast to the above-mentioned HIV infection, which significantly increases the risk for malignant diseases and especially lymphomas, such a risk is, if at all, by far not as pronounced in case of Malaria (241–243). The only notable exception concerns the concomitant early and sustained infection of Plasmodium falciparum and EBV, which together are the essential pathogenetic ingredients in the endemic form of Burkitt lymphoma in Africa (217–219, 236, 238, 244–246). In this particular combination, the Plasmodium infection destabilizes the genome of rapidly dividing EBV-infested germinal center B cells by eliciting the protracted expression of the activation-induced cytidine deaminase, a mutation-aggravating enzyme (238, 245).

At one point in their life, virtually all humans become infected with EBV, most of them without any acute, severe or lasting health problems. However, in those who do, one can often identify an underlying disease-associated, more or less pronounced genetic susceptibility, which begs for the question whether EBV-associated disease processes indeed afflict also "immunocompetent" individuals, or put the other way around, what in the end will define such an "immune (in)competence" (235).

PREDISPOSITION TO HEMATOLOGIC NEOPLASMS IN CHILDREN

The role of predisposing germline mutations and sequence variants in children and adults with various types of hematologic malignancies was hitherto largely underappreciated, because not all of them concur with nor create any easily recognizable clinical stigmata or suspicious family history. Especially if one screens neoplastic tissues for disease- and/or therapy relevant somatic mutations, it becomes of critical importance to distinguish those from germline ones, because the latter may also have a profound clinical impact as regards choice of therapy, donor selection in case of transplantations, evaluation of comorbidities as well as surveillance strategies (8, 173).

A recent paper by Duan et al. provides an excellent and very comprehensive overview about all the primary immunodeficiencies that in particular predispose to the development of various types of lymphomas and hematologic malignancies (3). The authors compiled more than 60 conditions, which comprised all subgroups of syndromic and non-syndromic cellular and humoral PID as well as defects in phagocytes and innate immunity.

To pack some of the underlying principles and problems into a practical and newsworthy perspective, I will now briefly turn to recent findings in three categories of childhood leukemia.

Constitutional Trisomy 21

Trisomy 21 is not only the most common chromosome abnormality in liveborns but in many aspects also one of the most outstanding and fascinating examples of an immune system disorder, although for a long time the precise nature of the respective immunological derangements remained elusive (178). Since it is not a monogenic ailment, it is also hardly ever viewed as a primary immunodeficiency, although it clearly concurs with multiple distinct immunological and developmental defects that affect the myeloid but also the early and committed B-lymphoid progenitor compartments in second trimester fetal liver (247). I chose this most intriguing and highly instructive example to explain in which way the predisposing risk score of a kind of "polygenic" disposition can easily reach at least the same magnitude as the one that is otherwise only achievable in a monogenic setting (55).The ensuing variable and clinically often inapparent immunological alterations in Down individuals comprise a mild to moderate decrease in T and B cells, impaired mitogen-induced T cell proliferation, reduced specific antibody responses to immunizations as well as defects of neutrophil chemotaxis (248). Affected individuals suffer from various types of autoimmune and autoinflammation diseases, whereas it is still a matter of debate whether they are also more prone to experience more or severer infections than non-Down individuals (176, 178, 248, 249). The first clues that helped to resolve the functional consequences of this immunological conundrum derived from recent transcriptome and proteome analyses. They revealed that the presence of an extra chromosome 21 leads, amongst others, to an overexpression of the four chromosome 21-encoded interferon receptors and, therefore, place this syndrome into the class of interferonopathies. Interferons are normally produced by cells in response to viral or bacterial infections, regulate genes in neighboring cells and shut down the production of proteins, which activate the immune system and thereby prevent the spread of the infection (250, 251). In line with interferonopathies and autoinflammatory conditions, individuals

with Down syndrome display higher levels of many pro-inflammatory cytokines (including IL-6, IL-22, TNF-α, and MCP-1) as well as complement consumption, a state that indicates that the immune system is constantly fighting viral infections that are in fact not there (250). Whether and to which extent such a faulty overreaction may also participate in promoting the development of hematologic neoplasms perhaps in a similar fashion as in genuine virus infection-triggered malignancies, remains to be elucidated.

Individuals with an inborn trisomy 21 have also an extraordinary risk to acquire special forms of hematologic neoplasms in early life, whereas they are otherwise exquisitely protected against the development of any other malignancies (252, 253). Compared to normal age-matched children, the self-limiting transient myeloproliferative disorder (TMD) together with the acute megakaryoblastic leukemia (AML-M7), is ∼150 times and the B cell precursor ALL ∼33 times more common (99, 101, 102, 253, 254). Of particular note are also the absence of infant ALL, the rarity of T-ALL and, compared to normal children, the different distribution pattern of genetic B-ALL subtypes (255).

The occurrence of specific mutations in the receptive precursor cells determine which form of leukemia will eventually develop. In case of TMD, the perturbation of megakaryocyte-erythroid precursor cell differentiation fosters the appearance of a highly specific truncating mutation in exon 2 of the hematopoietic transcription factor GATA1. This in turn provides the receptive cellular and molecular environment for the occurrence of further mutations, primarily in the JAK and RAS signaling pathways as well as in epigenetic regulators and multiple cohesion components, which then facilitate the further progression into AML (99, 101, 104, 254). A reduced lymphoid gene expression in fetal liver hematopoietic precursor cells impairs B-lymphoid development in a similar fashion. The ensuing maturation arrest leads to an ∼10-fold reduction in B cells. The concomitant accumulation of pro-B progenitors (247), on the other hand, increases the likelihood for illegitimate V(D)J recombination-mediated chromosomal rearrangements, in particular *CRLF2* gene fusions, that can be found in approximately half of all Down syndrome BCP ALL cases (99, 104). To explore the potential contribution of chromosome 21-encoded and overexpressed genes, a set of 31 triplicated orthologous human genes were tested in germline mouse models (256). Their presence induced progenitor B cell self-renewal *in vitro*, maturation defects *in vivo* and the development of especially *CRLF2*-rearranged and *JAK2* pathway-activated BCP ALL. Out of these 31 genes, the nucleosome-remodeling protein high mobility group nucleosome-binding domain- containing protein 1 (*HMGN1*), whose protein product suppresses H3K27me3, turned out to be the most relevant candidate. Together with secondary alterations in the *CRLF2*, *JAK2*, *NRAS,* or *KRAS* genes, it promotes both B cell proliferation *in vitro* and the development of B ALL in mice *in vivo* (256).

Given the extraordinary susceptibility and the high incidence of leukemias, one would intuitively expect that both myeloid and lymphoid forms should occasionally occur together by pure chance alone. However, such a coincidence has so far never been reported. On the one hand, this lack of co-occurrence might indicate that the development of a specific type of leukemia requires and is subject to very individual-specific predisposing conditions. On the other hand, it is also in keeping with the fact that these patients virtually never suffer from secondary neoplasms and that they are in a unique and matchless way also protected against the development of any other types of neoplasms (252, 253).

This protective effect also has been put down to a copy number-dependent gene dosage but also context-dependent effect of specific chromosome 21-encoded genes. The presence of three *ETS2* copies, for instance, act as tumor repressor in the ApcMin intestinal cancer mouse model, whereas in the PyMT breast cancer mouse model it functions as tumor promoter, albeit within the non-cancerous stromal cells, where it regulates the expression of genes that produce the extracellular matrix, an essential component for tumor growth and metastasis (257–260). Two other relevant genes are the Down's syndrome candidate region-1 (*DSCR1*), which encodes a suppressor of the vascular endothelial growth factor (VEGF)-mediated angiogenic signaling by the calcineurin pathway, and *DYRK1A*, which encodes a regulator of cell proliferation (261). In mice, the presence of a single extra copy of Dscr1 is sufficient to diminish tumor growth by suppressing the calcineurin pathway and therefore also angiogenesis, an effect that is significantly enhanced by an extra copy of Dyrk1a. For the sake of completeness, one needs to take at least also note of several other trisomic chromosome21-encoded genes whose presence in the stromal compartment helps to reduce tumor angiogenesis, namely the angiogenic inhibitor *ADAMTS1*, the transcription regulator *ERG* and, finally, the endothelial cell-specific genes *JAMB* and *PTTG1IP* (257).

Bone Marrow Failure (BMF), Myelodysplastic Syndromes (MDS), and Myeloid Leukemias

Table 1 provides a comprehensive summary of the various disease entities together with their causative genetic background. For a more in-depth overview I refer the interested reader to recent publications that deal with these individual subjects in detail (8, 60, 158, 264, 265, 268, 269, 272, 275, 283, 299, 300). Herein, I merely select a few instructive examples to highlight some of the intriguing phenomena that are particularly pertinent for the topic discussed herein.

Fanconi Anemia (FA) is not only the most common inherited BMF disorder but, with a 500- to 700-fold higher incidence of head and neck squamous cell carcinomas in older patients, also a highly penetrant cancer susceptibility syndrome (301). Except for the X-linked *FANCB* gene, it is due to bi-allelic mutations that can affect one of 21 genes, which encode various components of the evolutionarily conserved FA/BRCA repair complex. Five of these (*BRCA2, PALB2, RAD51C, SLX4,* and *BACH1*) specifically predispose also to breast cancer. The encoded proteins participate in biochemical pathways that safeguard not only against the effects of alkylating agents and radiation but, probably even more relevant, also against those of endogenous aldehydes, oxidative stress, inflammation,

TABLE 1 | Immunodeficiency syndromes that predispose to the development of bone marrow failure, myelodysplasia and myeloid leukemias.

Disease/Syndrome	Defective genes	Malignancy risk	Remarks	References
Fanconi Anemia (FA)	*FANCA, FANCB, FANCC, FANCD1/BRCA2, FAND2, FANCE, FANCF, FANCG/XRCC9, FANCI/KIAA1794, FANCJ/BRIP1/BACH1, FANCL, FANCM, FANCN/PALB2, FANCO/RAD51C, FANCP/SLX4, FANCQ/ERCC4, FANCR/RAD51, FANCS/BRCA1, FANCT/UBE2T, FANCU/XRCC2, FANCV/REV7/MAD2L2*	MDS, AML, T-ALL, squamous cell carcinomas (head & neck, genitourinary tract), breast cancer	Currently 21 known genes that encode members of the FA/BRCA repair complex	(8, 262–265)
RIBOSOMOPATHIES				
Diamond_Blackfan anemia (DBA)	*RPL3, RPL5, RPL10, RPL10A, RPL11, RPL15, RPL18, RPL19, RPL26, RPL27, RPL31, RPL34, RPL35, RPL35A, RPS7, RPS10, RPS11, RPS15A RPS17, RPS19, RPS24, RPS26, RPS27, RPS28, RPS29, RPLP0, TSR2, GATA1, CECR1*	25% life-long overall risk of 5.4 odds ratio MDS, AML, colon cancer, osteogenic sarcoma, and genital cancer	26 ribosomal genes, 6% phenocopies in non-ribosomal genes, 22% unidentified	(265–269)
Dyskeratosis Congenita (DC)	*ACD, CTC1, DKC1, NAF1, NHP2, NOP10, PARN, POT1, RTEL1, TERC, TERT, TINF2, WRAP53, STN1/OBFC1*	MDS, AML, squamous cell cancers of the head, neck & anogenital region	Telomere-associated ribonucleoprotein (RNP) and shelterin complexes	(265, 269–272)
Shwachman-Diamond-Bodian Syndrome (SDBS),	*SBDS, DNAJC21/HSP40, EFL1*	5% leukemia risk (AML, CML, ALL)	Defective processing of rRNA into ribosome assembly, majority unidentified	(265, 269, 273)
Cartilage hair hypoplasia (CHH)	R*MPR*	Non-Hodgkin lymphoma, basal cell carcinoma	RNA component of RNAse MPR, one single Finnish founder mutation	(265, 269, 274)
Aplastic anemia/ pancytopenia	*MECOM, ERCC6L2*	MDS		(275)
PLATELET DISORDERS				
Amegakaryocytic thrombocytopenia	Mostly *MPL* (thrombopoietin receptor), *RUNX1, ANKRD26, MYH9, PTPN1*	Pancytopenia, leukemia		(265, 276)
Thrombocytopenia absent radius (TAR) syndrome	del(1q21.1) & *RBM8A* SNP	Leukemia (rare)		(265, 277)
Familial thrombocytopenia	*ETV6, RUNX1, DDX41, ANKRD26*	MDS, leukemias		(8)
Congenital neutropenia	*CSF3R, ELANE, G6PC3, GFI1, HAX1, JAGN1, VPS45, WAS*	G-CSF treatment, dose-dependent MDS/AML risk		(264, 278, 279)
GATA2 deficiency (Emberger & Monomac syndrome)	*GATA2*	MDS, AML (monosomy 7)		(275, 280–286)
Mirage & ataxia-pancytopenia syndrome	*SAMD9, SAMD9L*	MDS, AML (monosomy 7)		(273, 287–294)
Rasopathies	*NF1, PTPN11, CBL, NRAS, KRAS*	JMML, ALL		(295–298)

Apart of being present in the germ line, somatic mutations of most of these genes can be commonly encountered in sporadic forms of analogous malignancies.

mitophagy, and virophagy (263, 265, 302, 303). All these factors damage DNA in form of distinct inter-strand DNA crosslinks. The inability to repair these damages is the primary driver of the various biological and clinical problems that define this disease category.

Small aldehydes, such as acetaldehyde and formaldehyde, are not only ubiquitously present in the environment, but also potentially toxic byproducts of the normal cellular metabolism and especially de-methylation reactions (304–306). Given that they provide already a rich source for endogenous inter-strand DNA and protein cross links, one can envisage that the pathogenic manifestations and consequences of FA mutations may also be strongly influenced and modified by the functional capability of aldehyde detoxifying enzymes, such as aldehyde dehydrogenase 2 (ALDH2) and alcohol dehydrogenase 5 (ADH5) (306, 307). In line with this notion, Japanese FA children that carried a functionally deficient ALDH2E504K allele were shown to progress more rapidly to aplastic anemia but not to MDS or AML (307–309). Moreover, malformations were only more severe in two of three homozygous carriers, which indicates that a deficient maternal genetic background might contribute to this outcome (310). Maternal-produced aldehydes diffuse indeed across the placenta and can thus damage the developing embryo's DNA, whereas embryo-derived ones can in turn be detoxified by the maternal organism. An inappropriate *in utero* exposure, such as an excessive maternal ethanol consumption during gestation, would therefore aggravate not only the formation of congenital abnormalities in FA but it provides also an intriguing etiological link to analogous phenotypic changes that define the alcohol embryopathy (308). Whether a disturbed aldehyde detoxifying system might also be causatively involved in the *in utero* initiation of childhood leukemias remains currently a matter of speculation (307).

In addition to stalling and destabilizing DNA replication forks directly, formaldehyde also selectively depletes BRCA2 via proteasomal degradation, a circumstance that poses a special risk for heterozygous *BRCA2* mutation carriers. In these, formaldehyde-induced degradation can decrease the respective protein levels below the otherwise protective one of normal wild-type individuals and thereby potentiate their mutagenic vulnerability (311).

Taken together, these observations have significant implications for risk awareness and avoidance as well as the clinical management of FA patients. On the positive side, they offer new treatment opportunities, for instance in form of ALDH2 agonists and the widely used diabetes drug metformin, which acts as aldehyde scavenger. At least in mouse models, both of them are able to delay the onset of BMF and malignancies as well as improve hematopoiesis (263, 312, 313).

One remarkable feature of many heritable diseases is that somatic mutations can occasionally autocorrect the particular inherited gene defect in the respective cells (314). Such reverting mutations transform a homozygous or combined heterozygous state again back into a heterozygous functionally compensated state, either through somatic recombination, gene conversion or a compensatory mutation (314, 315). Although this phenomenon is well-known in immunodeficiencies, its effects are most probably still underappreciated. In BMF syndromes such an autocorrection can spontaneously improve or even resolve the specific underlying hematopoietic problem. Amongst others, such spontaneous remissions have repeatedly been documented in FA, dyskeratosis congenita, Diamond-Blackfan anemia, Shwachman-Diamond syndrome and, more recently, in the *SAMD9*- and *SAMD9L*-associated Mirage and ataxia-pancytopenia syndromes, respectively (273, 287–293, 301, 316). Heterozygous *SAMD9L* gain-of-function mutations decrease cell proliferation. The loss of the mutation-carrying chromosome 7 in bone marrow cells therefore leads to the development of MDS and acute myeloid leukemias, whereas a compensating duplication of the normal allele in form of an uniparental disomy (UPD) 7 or 7q is able to resolve the cytopenias (287–293).

The somatic appearance of a complete or partial UPD always draws attention to regions that are usually highly relevant for specific disease processes (317, 318). Such UPDs may either contain duplicated gain of function mutations or, as alluded to in the example above, eliminate them (317, 318). A similar important consequence is the mere transformation of a heterozygous to a homozygous state. In case of the HLA-containing region on the short arm of chromosome 6, for instance, it is clearly exerted through a selective pressure, since the loss of one HLA haplotype is an important immune-escape mechanism. It protects neoplastic cells from the immune surveillance machinery and therefore also plays a crucial role for disease recurrence after haploidentical stem cell transplantations (319, 320). Contrariwise, such a haplotype loss is able to shield hematopoietic cells from the destructive effects of autoimmunity, as has been demonstrated in case of aplastic anemia (321). The practical problems that arise from such a hematopoietic revertant mosaicism is that it may lead to an ascertainment bias and cause difficulties in identifying underlying disease-relevant mutations (322).

The formation of such well-adapted clones might suggest that such compensatory mechanisms are rare events. However, there is ample evidence that this is definitely not the case. Rather than being a life-long stable system the genome is a highly dynamic one that is continuously modified and shaped by ongoing mutational processes, which eventually promote the appearance of cell clones and populations with an increased survival fitness. The formation of somatic mosaicism is therefore the rule rather than the exception, as exemplified by Davis et al., who observed a remarkable clonal heterogeneity and diversity of lymphocytes in a patient with a Wiskott-Aldrich syndrome (323). The continuous generation of such cellular variants and the selective pressures they are exposed to is thus not only a characteristic of the exceptional dynamics of neoplastic but also of normal cells populations (324).

B-Cell Precursor Acute Lymphoblastic Leukemia (BCP ALL)

Germline lesions that predispose to BCP ALL in children comprise not only those which cause various cancer prone and chromosomal syndromes but also other genuine gene disrupting defects as well as high and low risk variants. The majority of these

TABLE 2 | Chromosomal locations of GWAS-verified SNPs or genuine germline gene defects that predispose to the development of particular types of childhood ALL.

Chromosome region	Candidate genes	Type	Function	All subset	References
2(q22.3)	Not specified	SNP	–	*ETV6–RUNX1*	(24)
3(q28)	*TP63*	SNP	P53 family of transcription factors	*ETV6-RUNX1*	(325)
7(p12.2)	*IKZF1*	SNP, gene defects	Ikaros family of Zinc finger transcription factors	Not specified	(32, 172, 326, 327)
8(q24.1)	MYC?	SNP	Proto-oncogene, BHLH transcription factor	Not specified	(24, 25)
9(p12)	*PAX5*	Gene defects	Paired box transcription factor	Not specified	(48, 328)
9(p21.3)	*CDKN2A & CDKN2B*	SNP	Cyclin-dependent kinase Inhibitors	Not specified	(329–331)
9(p24.1)	*JAK2*	SNP	Tyrosine kinase	*BCR-ABL1*-like	(49)
10(p12.2)	*PIP4K2A*	SNP	Kinase	Not specified	(329, 332)
10(p14)	*GATA3*	SNP	GATA family of transcription factors	*BCR-ABL1*-like	(332, 333)
10(q21.2)	*ARID5B*	SNP	Transcription coactivator	Hyperdiploid	(26, 32, 326, 327)
10(q26.13)	*LHPP*	SNP	Phosphatase	Not specified	(28)
11(p11.2)	*PTPRJ*	SNP	Family of protein tyrosine phosphatases	*ETV6-RUNX1*	(325)
12(p13.2)	*ETV6*	Gene defects	Proto-oncogene, ETS domain family of transcription factor	Hyperdiploid	(334–338)
12(q23.1)	*ELK3*	SNP	ETS domain family of transcription factor	Not specified	(28)
12(q24.1)	*PTPN11*	Gene defects*	Family of protein tyrosine phosphatases	Hyperdiploid	(297)
14(q11.2)	*CEBPE*	SNP	bZIP transcription factor	Hyperdiploid	(23, 25, 32, 327)
16(p13.3)	*CREBBP*	Gene defects**	Histone acetyltransferase	Hyperdiploid	Haas, unpublished observation
17(p13.1)	*TP53*	Gene defects***	Tumor-suppressor, transcription factor	Hypodiploid	(339–341)
17(q12)	*IKZF3*	SNP	Ikaros family of Zinc finger transcription factors	Not specified	(25)
17(q21.2)	*STAT3*	SNP	Signal transducer and transcription activator	*BCR-ABL1*-like	(49)

*For a more general overview about ALL predisposition syndromes and ALL predisposing RASopathies see Kratz et al. and Cave et al., respectively (62, 243). *Noonan syndromes, Rasopathy, **Rubinstein-Taybi syndrome, ***Li-Fraumeni syndrome.*

affect genes that encode B-cell development and transcription factors as well as components of various signal transduction pathways (**Table 2**) (42, 297, 342).

The development of B lymphocytes, in particular, is coordinated by specific regulatory transcription networks that activate the respective B-lymphoid program and at the same time oppress alternate cell fates (343). Somatic mutations in several of these key regulators, such as *IKZF1*, *TCF3*, *EBF1,* and *PAX5*, induce leukemic transformation by blocking normal B cell differentiation, which then leads to the accumulation of leukemic B-cell precursors. Although their role in leukemogenesis has been known and explored already for quite some time, the awareness that otherwise apparently inconsequential germline variants may also exert a predisposing effect is quite new. The biological relevance and functional consequences of some of these variants has been confirmed in the meantime with appropriate *in vitro* and *in vivo* experiments and test systems (23–26, 28, 332, 333).

Take for instance *PAX5,* a member of the "paired box" family of transcription factors, which encodes the B cell lineage specific activator protein that is expressed at early but not late stages of B-cell differentiation (195). Out of the three up to now reported families with highly penetrant germline variants, 13 carriers developed ALL (48, 328). In the two families, in which the respective information was provided, all unaffected carriers as well as those with ALL had normal immunoglobulin levels and no evidence of an impaired B cell function at diagnosis (328). Moreover, in line with Greaves two step model, leukemia developed in mice only after exposure to common pathogens and the acquisition of second hits in the IL7R/JAK3/STAT5 signaling axis (344).

Another revealing example is *IKZF1*, which encodes IKAROS, a member of a hematopoietic zinc-finger transcription factor family (345–347). The mutational spectrum of human IKZF1-associated diseases ranges from somatic to germline and from haploinsufficient to dominant negative forms (171). Somatic mutations occur in overall 15% of BCP-ALL and especially in prognostic adverse genetic subtypes (348, 349). Heterozygous germline mutations cause two different forms of immunodeficiency. The haploinsufficient, autosomal dominant late onset form of common variable immunodeficiency (CVID)

has an incomplete penetrance, is clinically mild and concurs with a marked decrease in B-cell numbers and immunoglobulin levels as well as autoimmunity (175, 350, 351). The early-onset dominant negative CVID, on the other hand, is characterized by innate and adaptive immune defects of the B, T and myeloid cell lineage (171). Notably, 2/29 of patients with the late onset form developed B-ALL and 1/7 patients as well as another independently reported one with the early onset form developed T-ALL (171, 346, 351). Based on these observations, Churchman and colleagues screened remission samples from 4,963 childhood ALL cases, identified a total of 28 unique *IKZF1* variants in 43 and succeeded to prove a functional consequence in 22 of them (172, 173). Evans et al. even attempted to elucidate the influence of a parental environmental exposure on such leukemia-predisposing risk alleles (352). They found some preliminary albeit hitherto unexplainable evidence that the *IKZF1* risk genotype might have a stronger effect if the mother took folic acid or if the father did not smoke prior to pregnancy (352).

Finally, Auer et al reported a first intriguing example of a "double-hit one-pathway" scenario, in which the biparental inherited combination of two rare germline variants, JAK2 (G571S) and STAT3 (K370R), whose products synergistically interact in the same disease-relevant JAK/STAT signal transduction pathway, is obviously sufficient to induce a Ph-like BCP-ALL (49).

CONCLUDING REMARKS

During their entire development and ongoing existence, both the immune system as well as malignant diseases need to adapt themselves to highly variable and continuously fluctuating environmental conditions, which requires a high flexibility that is largely driven by a combination of interacting antagonistic as well as synergistic deterministic events and regulatory probabilities. "Immunodeficiency" and "tumor susceptibility" are thus two closely intertwined concepts, whose original understanding was based on easily explicable clinical symptoms as well as certain genetic norms. As long as these were based on such more or less simple phenotypic and genotypic features, one did not require any further explanatory definitions. However, the switch from phenotypic to genetic ascertainment programs include now much less obvious disease categories, healthy carriers as well as only vaguely defined potentially predisposed individuals. Although this approach enables of course unprecedented insights into the fine-scale structural and regulatory organization of biological system, the boundaries of classification standards get increasingly blurred, which goes hand in hand with the awareness that it becomes increasingly difficult and virtually impossible to define either of these terms in an unambiguous manner anymore. The more closely one looks, the harder it gets to find genes that are not in one or the other way part of this game.

In his highly recommendable and readable book "Tending Adam's garden," Irun Cohen portrays the immune system as a cognitive system (353). Like its prototypic equivalent, the brain, it learns through individual experience and thereby forms a functionally highly efficient, flexible, and to some extent also redundant interactive structure. As Cohen pointed out, a particular gene may only become essential once the system has organized itself around it so that thereafter the system becomes dependent on it. In case this particular gene is already defective at the beginning, the system often can compensate for this loss and organize itself around an alternative gene, which then becomes the essential one.

Thus, organizational entities depend not only on distinct features of particular sub-units but even more so on their functional interactions. In case of the immune system, such multi-component, self-emergent networks comprise a variety of distinct cellular as well as humoral host constituents, but also a manifold of environmental factors, such as the maternal immune system, the microbiome, infectious agents, as well as physical and chemical agents, that co-govern and modulate, but often also interfere and disrupt its normal development during different stages.

Thus, organizational entities depend perhaps less on the appropriate function of particular genetic sub-units alone but much more on the successful interaction of their cellular and humoral products, an observation that is readily evident in case of genetically determined developmental disorders that can cause both immune system deficiencies as well as malignant diseases.

The essential implication of Cohens' model is that we only may become more successful in curing such disease when we begin to understand the decision-making processes of the immune system rather than that of the effects of individual components alone.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

ACKNOWLEDGMENTS

This paper is dedicated to my late friend and colleague Andreas Heitger, a devoted immunologist, with whom it was always fun to discuss the philosophical part of the activity and behavioral pro and cons of the immune system.

I acknowledge the networking support by the COST Action CA16223 LEukaemia GENe Discovery by data sharing, mining and collaboration (LEGEND) as well as by the IBFM Leukemia & Lymphoma Genetic Predisposition Committee, the Österreichische Kinderkrebsforschung for the continuous funding of my research work and Fikret Rifatbegovic's invaluable help in designing and drawing the Figure.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 22 January 2019
doi: 10.3389/fimmu.2018.03135

CVID-Associated Tumors: Czech Nationwide Study Focused on Epidemiology, Immunology, and Genetic Background in a Cohort of Patients With CVID

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OPEN ACCESS

Edited by:
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Reviewed by:
Roshini Sarah Abraham, Nationwide Children's Hospital, United States
Jolan Eszter Walter, University of South Florida, United States

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Specialty section:
This article was submitted to Primary Immunodeficiencies, a section of the journal Frontiers in Immunology

Received: *11 September 2018*
Accepted: *19 December 2018*
Published: *22 January 2019*

Citation:
Kralickova P, Milota T, Litzman J, Malkusova I, Jilek D, Petanova J, Vydlakova J, Zimulova A, Fronkova E, Svaton M, Kanderova V, Bloomfield M, Parackova Z, Klocperk A, Haviger J, Kalina T and Sediva A (2019) CVID-Associated Tumors: Czech Nationwide Study Focused on Epidemiology, Immunology, and Genetic Background in a Cohort of Patients With CVID. Front. Immunol. 9:3135. doi: 10.3389/fimmu.2018.03135

Background: Common variable immunodeficiency disorder (CVID) is one of the most frequent inborn errors of immunity, increased occurrence of malignancies, particularly lymphomas, and gastric cancers, has long been noted among CVID patients. Multifactorial etiology, including immune dysregulation, infections, chronic inflammation, or genetic background, is suggested to contribute to tumor development. Here, we present the results of the first Czech nationwide study focused on epidemiology, immunology and genetic background in a cohort of CVID patients who also developed tumors

Methods: The cohort consisted of 295 CVID patients followed for 3,070 patient/years. Standardized incidence ratio (SIR) was calculated to determine the risk of cancer, and Risk ratio (RR) was established to evaluate the significance of comorbidities. Moreover, immunophenotyping, including immunoglobulin levels and lymphocyte populations, was assessed. Finally, Whole exome sequencing (WES) was performed in all patients with lymphoma to investigate the genetic background.

Results: Twenty-five malignancies were diagnosed in 22 patients in a cohort of 295 CVID patients. SIR was more than 6 times greater in comparison to the general population. The most common neoplasias were gastric cancers and lymphomas. History of Immune thrombocytopenic purpura (ITP) was established as a potential risk factor, with over 3 times higher risk of cancer development. The B cell count at diagnosis of

lymphoma was reduced in the lymphoma group; moreover, post-treatment B and T cell lymphopenia, associated with poorer outcome, was found in a majority of the patients. Intriguingly, no NK cell depression was observed after the chemotherapy. WES revealed heterogeneous genetic background among CVID patients with tumors, identifying gene variants associated with primary immunodeficiencies (such as CTLA4, PIK3CD, PMS2) and/or increased cancer susceptibility (including BRCA1, RABEP1, EP300, KDM5A).

Conclusions: The incidence of malignancy in our CVID cohort was found to be more than 6 times greater compared to the general population. Gastric cancers and lymphomas were the most frequently diagnosed tumors. ITP was identified as a risk factor for malignancy in CVID patients. WES analysis confirmed a wide genetic heterogeneity among CVID patients. The identified causative or modifying gene variants pointed to errors in mechanisms contributing to both immunodeficiency and malignancy.

Keywords: common variable immunodeficiency, malignancy, lymphoma, gastric cancer, whole exome sequencing

INTRODUCTION

Immune control of tumor development and growth requires a functional immune system capable of complex immune responses necessary for recognition and elimination of malignant cells. As such, inborn errors resulting in immunodeficiencies may convey an increased risk of cancer. In general, the spectrum of malignancies in primary immunodeficiency (PID) patients is clearly biased when compared to malignant diseases in the general population. Malignancies in PIDs tend to be restricted to certain oncological entities, and their pathophysiology is often linked to the mechanism underlying the particular immunodeficiency (1). For example, immunodeficiencies associated with gain-of-function mutations in *PIK3* are associated with a high risk of lymphoma (2). The role of this signaling pathway in cancer genesis and immunodeficiency is validated by therapeutic success of targeted PI3K/mTOR pathway inhibition used in activating *PIK3* syndrome, as well as in malignant diseases (3). Similarly, immunodeficiencies arising from developmental defects of stem cells, myeloid cells or lymphocytes are associated with an increased incidence of leukemia or lymphomas, implying errors in the corresponding pathways (1). Recent advances in understanding of molecular mechanisms underlying primary immunodeficiencies, as well as tumors, has provided evidence for such associations. However, in immunodeficiencies that are not yet precisely defined by their molecular/genetic cause, the situation is more complex. Common variable immunodeficiency disorder (CVID) is one of the most frequent forms of antibody deficiencies; yet, its pathophysiology remains largely unknown. The hallmark of CVID is the impairment of the B cell compartment, typically manifesting as a reduction of mature forms of B cells and expansion of less differentiated stages of B lymphocytes (4). The T cell compartment is also usually skewed in CVID, specifically toward terminally differentiated forms, including senescent cells, typically affecting both CD4and CD8 T cells, which are crucial for anti-tumor immunity (5). The mechanisms of B cell involvement in anti-tumor immunity are largely unknown. B cells may promote both pro-tumourigenic responses (e.g., specific subsets of B cells may produce IL-10 or TGF-beta with immunosuppressive properties, B cells may promote tumor genesis and tumor progression by alteration of the angiogenic and proinflammatory microenvironment), as well as anti-tumourigenic responses (e.g., B cells may enhance cytotoxic T cell activity, indirectly mediate antibody dependent cytotoxic mechanisms or serve as professional antigen-presenting cells, initiating the T cell response) (6). Inborn impairment of the B cell lineage, along with T cell dysregulation, may facilitate the genesis of tumors in CVID patients. Furthermore, the immunologic defect is accentuated by recurrent and chronic infections. Chronic viral infections, particularly EBV, are strongly associated with lymphoproliferative diseases and lymphoma (7). Additionally, chronic inflammatory response, *per se*, represents a risk factor for tumor development, especially in patients genetically predisposed to malignancy.

Efforts made to establish the genetic etiology of CVID have thus far been successful in 2–10% of CVID patients (known CVID-associated gene variants are shown in **Table 1**) (8). Some of the CVID-associated genes represent a clear predisposition to cancer, as described in previously published CVID cohorts; most prominently, these mutations are in genes causing alterations in the NFkB or PI3k pathways or in genes affecting B cell receptors (3, 9, 10).

Overall, the factors contributing to increased incidence of malignancy in CVID are complex, encompassing genetics, immune response dysregulation, infections, inflammation, and perhaps other not yet elucidated mechanisms.

Here, we present the results of a complex study on a Czech national cohort of CVID patients who also presented with malignancy. National epidemiological data were collected, immune profiles were analyzed and, in a subgroup of CVID patients with lymphoma, Whole exome sequencing (WES) was performed.

TABLE 1 | List of genes associated with Common variable immunodeficiency (monogenic causes and modifier genes) and their prevalence, adjusted according to Bogaert et al. (8).

Gene	Prevalence (%)
MONOGENIC CAUSE OF CVID: 2–10%	
PIK3CD	26.74
LRBA	26.74
CTLA4	6.42
NFKB2	5.35
TNFRSF7 (CD27)	4.81
PIK3R1	4.81
ICOS	3.74
CD19	3.74
IL-21R	3.21
IKZF1 (IKAROS)	3.21
PRKCD	2.14
PLCG2	2.14
NFKB1	1.6
CR2 (CD21)	1.7
MODIFIER GENES: UNKNOWN PREVALENCE	
TNFRSF13B (TACI), TNFRSF13C (BAFF-R), MSH2, MSH5, CLECG1, MLH1, RAD50, ORC4L, FCGR2A.	

METHODS

This study was approved by local ethics committee of Motol University Hospital. Written informed consents were obtained from all enrolled patients.

Data Collection

Retrospective clinical and laboratory data of 295 enrolled patients were obtained from medical records of national referral centers for the treatment of adult patients with primary immunodeficiency diseases. The collected data covered the period from 1997 to 2016. They included a total amount and a length of surveillance of all CVID patients fulfilling ESID/PAGID criteria, a number of CVID cancer patients and patient-specific data: year of birth, age at first symptoms associated with CVID and their nature, clinical comorbidities, age at cancer diagnosis, type of cancer, therapeutic regimens, survival rates, cause of death (if applicable), and specific details of cancer diagnostics.

A more detailed set of clinical data was obtained from 11 CVID patients with cancer and from 160 randomly selected cancer-free CVID patients, who represented the reference group. This cohort included 95 females and 65 males with a median age of 48 years (range 19–88). Czech general population data on occurrence of malignancy were obtained from the Czech National Cancer Registry and covered the period from 1994 to 2014 (the last available reports).

Epidemiology

Prevalence and SIR were calculated to express the probability of cancer diagnosis in a CVID cohort compared to the general population. RR was used to assess the significance of comorbidities in CVID patients with cancer. Confidence intervals (95% CIs) were determined for both parameters. All results for which number 1 was beyond the 95% CI were accepted as statistically significant (11).

Immunophenotyping

Serum levels of IgM, IgG, IgA were evaluated by nephelometric method using Image 800 systems (Beckman-Coulter, Brea, CA, USA). Basic lymphocyte subpopulations, including T cells, T helper cells, T cytotoxic cells, B cells and NK cells, were distinguished by FACS based on the expression of specific cell surface membrane markers using fluorochrome-conjugated monoclonal antibodies CD3-FITC, CD4-PE/Cy, CD8-APC/Cy,CD19-APC, CD16-PE, CD56-PE; KOMBITEST, Exbio, Prague, Czech Republic. B cell subpopulations (including CD21low, naive, transitional, marginal zone-like, class-switched cells, and plasmablasts) and T cell subpopulations (recent thymic emigrants-RTE, naïve, central memory-CM, effector memory-EM, effector memory expressing CD45RA-TEMRA, activated T cells) were performed using antibody-fluorochrome conjugates: CD45-APC-H7, CD3-APC, CD4-PerCP-Cy5.5, CD16-PE, CD56-PE, TCRgd-PE-Cy7, CD38-FITC, CD21-APC, IgM-FITC, CD8-Horizon V-500, CD45RA-PE-Cy7 (BD Biosciences, San Jose, CA, USA), CD5-PE,CD8-FITC, CD27-Brilliant Violet 421, IgM-Brilliant Violet 510, IgD-PerCP-Cy5.5, CD4-Brilliant Violet 510, CD62L-Brilliant Violet 421, HLA-DR-PerCP-Cy5.5 (Biolegend, San Diego, CA), CD19-PE-Cy7, CD24-PE, CD24-APC-Alexa Fluor 750, CD8-APC-Alexa Fluor 750 (Beckman Coulter, Miami, FL, USA), CD27-Pacific Blue, CD38-Alexa Fluor 700, CD45RO-FITC, CD31-PE, CD4-Alexa Fluor 700 (Exbio, Vestec, Czech Republic), CCR7-PE (MiltenyiBiotec, BergischGladbach, Germany), CD3-PerCP-Cy5.5 (Affymetrix eBioscience, ThermoFisher Scientific, Waltham, MA, USA), and IgD biotin (SouthernBiotech, Birmingham, AL, USA) followed by Streptavidin-Qdot 605 (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA).

The absolute and relative counts were assessed for all subpopulations. Examinations of the basic subpopulations and immunoglobulin levels were performed at the time of diagnosis of CVID and at the time of diagnosis of lymphoma. The B and T cell subpopulations were measured prior to the genetic testing. All parameters were compared to the control cohort of 20 randomly selected CVID patients without lymphoma. All obtained data were statistically evaluated. A non-parametric Mann-Whitney test was used to compare independent samples, and a non-parametric Wilcoxon matched-pairs signed rank test was used to compare dependent samples; differences of $p < 0.05$ were regarded as significant. Median and 95% CIs were calculated for all analyzed parameters.

CTLA-4 Expression

CTLA-4 expression was assessed in patient with novel mutations using FACS. Intracellular CTLA-4 was detected 16 h following anti-CD3/CD28 stimulation (Dynabeads, Thermo Fisher Scientific, MA, USA) using CTLA4-APC antibody together with the FOXP3 Fix/Perm Buffer set (BioLegend, San Diego, CA, USA) in CD4+CD127dimCD25+ T regulatory cells (Tregs).

CD45-APC-H7, CD4-Brilliant Violet 510, and CD127-Brilliant Violet 421 (BD Biosciences, San Jose, CA, USA), CD25-PE-Cy7 and CD8-FITC (Exbio, Vestec, Czech Republic) antibodies were used for detection of Tregs.

Whole Exome Sequencing

Sequencing libraries were prepared using a SureSelectXT Human All Exon V6+UTR kit (Agilent Technologies, Santa Clara, CA) from DNA isolated from patients' peripheral blood with a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Sequencing was performed by our facility on the NextSeq 500 (Illumina, San Diego, CA) instrument according to the manufacturer's protocols. The reads in resulting Fastq files were aligned against the human reference genome hg19 with BWA (12). Genomic variants were called with samtools and VarScan (13). Variant annotation was performed using SnpEff (14). Variant filtering was performed with Ingenuity® Variant Analysis™ (IVA) software (www.qiagen.com/Ingenuity, QIAGEN). Only variants with read depths of at least 10 and allele frequencies of at least 0.3 were evaluated. Common variants with allele population frequencies of more than 0.1% or homozygous counts of 5 or more in the ExAC or gnomAD databases were filtered out unless reported as disease-causing in the HGMD® (BIOBASE GmbH) or dbSNP databases (15, 16). Variants predicted to have low impact by at least 2 out of 3 scores calculated by SIFT, PolyPhen2, or CADD and present in population databases were also discarded (17–19). Remaining variants were manually evaluated in Integrative Genomics Viewer (http://www.broadinstitute.org/igv) to exclude variants in reads with low mapping quality (20). The analysis was then focused on variants in genes reported as causative for inborn errors of immunity in the last International Union of Immunological Societies (IUIS) guidelines, cancer-predisposition genes in children and in-house lists of genes possibly leading to immune dysregulation based on recent publications and close interactions with causative genes reported by IUIS (21, 22).

RESULTS

Epidemiology and Clinical Manifestation

Our cohort of patients included 295 patients followed for 3,070 patient/years in total. The average ages at the first CVID-related symptoms and at the time of CVID diagnosis in a subgroup of CVID patients with malignancy were 34.2 and 38.3 years, respectively. A total of 25 malignancies were found in 22 patients (7.4% of all included patients) with SIR 6.3 (95% CI: 4.08–9.31). These cases included 6/25 (24.0%) gastric carcinoma (GC): SIR 5.7, 95% CI: 2.08–12.32, 4/25 (16.0%), B cell Non-Hodgkin lymphoma (B-NHL): SIR 5.5, 95% CI: 1.50–14.09, 5/25 (20.0%), Hodgkin lymphoma (HL):SIR 30.0, 95% CI: 9.73–69.93 and 10/24 (41.7%) other cancers (SIR 5.0, 95% CI: 2.40–9.16). These were two cases of spinocellular carcinoma, basocellular carcinoma, and T-cell lymphoma, and a single case each of tonsillar carcinoma, breast carcinoma, renal carcinoma and urine bladder cancer. Cancer duplicity was observed in 3 patients (gastric and tonsillar carcinoma, breast cancer and urothelial carcinoma, spinocellular, and gastric carcinoma). Malignancies were diagnosed in 16 males and 6 females; the average age at diagnosis was 52.3 years (15 years after the diagnosis of CVID and 19 years after the first symptoms). The average ages of manifestation of GC, B-NHL, and HL in CVID cancer group were 55–59, 35–39, and 40–44 years, respectively.

Autoimmune cytopaenias, including Immune thrombocytopenic purpura (ITP) and Autoimmune hemolytic anemia (AIHA), were the most common complications in CVID both with and without malignancy. They were found in 8/22 (36.4%) and 27/160 (16.9%), respectively. Interestingly, a strikingly increased risk of malignancy was detected in a subgroup of CVID patients with a history of ITP (RR 3.52, 95% CI 1.42–7.26). Cumulative risk (RR 4.53, 95% CI 1.23–11.59) observed for B-NHL and HL together was similar. However, no risk increase was observed for isolated HL, B-NHL, and GC, probably due to the small number of cancer events and because the direct association with other documented CVID-related comorbidities was not significant (data not shown).

As mentioned above, lymphomas and GC represent the most frequent malignancies diagnosed in CVID patients in our cohort. While five out of six patients with GC (4 males, 2 females) underwent surgery, one received palliative chemotherapy with FLOX regimen (fluorouracil, leucovorin, oxaliplatin) due to highly progressed disease at the time of diagnosis. Five out of six patients had deceased before the study initiation, 2/6 (33.3%) due to disease progression, 2/6 (33.3%) passed away after achieving more than 10 years survival, and one patient died because of malnutrition due to severe enteropathy 2 years after the diagnosis of lymphoma. All patients suffered from various gastrointestinal complications related to CVID, and the majority of them also had splenomegaly.

All 5 patients (4 males, 1 female) with HL received chemotherapy. The following chemotherapeutic regimens were used: R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone) in 2/5 (40%) of patients, BEACOPP (bleomycine, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisone), eBEACOPP respectively, in 2/5 (40%) and DBVE-PC (adriamycin, bleomycine, vincristine, etoposide, prednisone, cyclophosphamide) in 1 patient (20%). No therapy-associated deaths or unexpected toxicities were noted. One patient died 3 years after treatment because of severe enteropathy, and 4/5 (80%) patients are still surviving today. All patients had previously described splenomegaly, and 3/5 (60%) had lymphadenopathy described prior to cancer diagnosis.

Similarly, all 4 patients (3 males, 1 female) with B-NHL received chemotherapy; no therapy-associated deaths or unexpected toxicities were noted. The following chemotherapeutic regimens were used: R-CHOP/CHOP [rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone in 3/4 (75%) patients−2 with DLBCL (Diffuse large B-cell lymphoma) and in 1 patient with MALT (Mucosa-associated lymphoid tissue] lymphoma. A GMALL (German multicenter ALL) regimen was used in 1 patient with Burkitt lymphoma. Two of these patients had been regularly followed even prior to the diagnosis of B-NHL for lymphadenopathy and previously reported splenomegaly. All patients are still

alive. T-NHL was diagnosed in 2 patients. The clinical features of CVID patients in whom lymphomas were diagnosed are summarized in **Tables 2**, **3**. In this cohort, WES and detailed immunophenotyping were performed as part of further investigation (results presented further).

Immunophenotype

Parameters of cellular immunity were investigated, including T cell (CD4 T helpers as well as CD8 T cytotoxic cells), B cell and NK cell counts. No significant differences were registered between the absolute counts of T cells, T helper cells and T cytotoxic cells at the time of diagnosis of CVID in a cohort of patients with lymphoma compared to those without lymphoma. The T cellcounts were also well within the normal reference ranges (T cells 0.8−2.10E9/l, T helper cells 0.3−2.8E9/l, T cytotoxic cells 0.2−1.0E9/l). Unsurprisingly, the chemotherapeutic regiments for lymphoma led to skewing of T cell numbers (median 0.65E9/l, 95% CI 0.46–0.75 vs. 1.22E9/l, 95% CI 1.07–1.47, $^{***}p = 0.0004$), specifically T helper cells (median 0.34E9/l, 95% CI 0.14–0.36 vs. 0.56E9/l, 95% CI 0.53–0.76, $^{***}p = 0.0004$) and T cytotoxic cells (0.26E9/l, 95% CI 0.14–0.36 vs. 0.54E9/l, 95% CI 0.41–0.64, $^{**}p = 0.006$).

The number of total B cells at the diagnosis of CVID did not differ significantly from the control group of CVID patients without lymphoma and from normal ranges. No significant difference was noted in the serum levels of IgG in the group of CVID patients with lymphoma (median 2.88 g/l, 95% CI 1.83–3.91, normal values 7.65–13.6 g/l) compared to the CVID control group (median 2.02 g/l, 95% CI1.63–3.24). In contrast, the number of total B cells at the diagnosis of lymphoma was reduced in the lymphoma group (median 0.01E9/l, 95% CI 0–0.13 vs. 0.195E9/l, 95% CI 0.16–0.29, $^{**}p = 0.006$). Absolute B cell counts were further depleted by the chemotherapy (median 0.11E9/l, 95% CI 0–0.46 vs. 0.08E9/l, 95% CI 0.03–0.197, $^{*}p = 0.02$). Post-therapeutic B cell lymphopenia (B cells count $\leq$ 0.03E9/l) was found in 6 patients. A complete total B cell count reconstitution was achieved in only 3 patients (median 0.33E9/l, range 0.137–0.654); however, mature forms of B cells, including marginal zone-like, class-switched cells and plasmablasts, remained reduced in these subjects (mean interval after chemotherapy 102 months, range 6–204). The remaining 3 patients failed to re-establish their B cell populations and continued to maintain severely reduced B cell compartments (mean 0.02E9/l, range 0.001–0.08; mean interval after chemotherapy 39 months, range 4–145). Concerning NK cells, their absolute counts were similar to CVID patients without lymphoma and the general population (normal range 0.05–1.0 E9/l) and remained unchanged throughout the disease course. Curiously, no NK cell depression was observed after the chemotherapy. The immunophenotype profiles are summarized in **Figure 1**, and the B cell subpopulations are shown in detail in **Table 4** and **Figure 2**.

Whole Exome Sequencing

WES was performed in 10 out of 11 CVID patients with lymphoma in whom biological material for genetic testing was available. The WES results were divided into 5 groups.

TABLE 2 | Characteristics of the cohort of 11 CVID patients with lymphoma (M, Male; F, Female; LYM, Lymphadenopathy; SPLE, Splenomegaly; ITP, Idiopathic thrombocytopenic purpura; RTI, Respiratory tract infections; DBLCL lymphoma, Diffuse large B-cell lymphoma; MALT lymphoma, Mucosa-associated lymphoid tissue lymphoma; PTCL, Peripheral T-cell lymphoma; N/A, not applicable; Y, Yes; N, No; chemotherapeutic regimens are described in the results).

Patient Nr.	Gender	Age at diagnosis of CVID	Manifestation	IgG serum level	Age at diagnosis of lymphoma	Type of lymphoma	Cause of death	Survival interval	Staging	Therapy
1	M	35 years	RTI	1.9 g/l	57 years	T lymphoma	Infection	0 month	Died before staging	Died before treatment
2	M	53 years	LYM, SPLE	1.15 g/l	64 years	HL	Enteropathy	36 months	IVB	BEACOPP
3	F	41 years	ITP	3.89 g/l	58 years	HL	Alive	4 months	IIA	R-CHOP
4	M	18 years	RTI	2.43 g/l	45 years	DBLCL lymphoma	Alive	9 months	IIA	R-CHOP
5	M	39 years	LYM, SPLE	0.03 g/l	35 years	DBLCL lymphoma	Alive	96 months	IVB	R-CHOP
6	M	37 years	ITP	4.1 g/l	40 years	HL	Alive	12 months	IVA	R-CHOP
7	M	36 years	RTI	4.1 g/l	42 years	HL	Alive	6 months	IIIB	eBEACOPP
8	M	26 years	ITP	4.88 g/l	36 years	Burkitt lymphoma	Alive	25 months	IIIA	B-NHL GMALL
9	F	25 years	RTI	2.88 g/l	36 years	MALT lymphoma	Alive	145 months	IVA	R-CHOP
10	M	11 years	RTI	4.48 g/l	11 years	HL	Alive	204 months	IIIA	DBVE-PC
11	M	25 years	RTI	1.76 g/l	30 years	PTCL	Infection	9 months	IVA	CHOP

TABLE 3 | Characteristics of CVID-related complications in a cohort of 11 patients with lymphoma (ITP, Immune thrombocytopenic purpura; AIHA, Autoimmune hemolytic anemia; RA, Rheumatoid arthritis; LIPS, Lymphocytic interstitial pneumonia; BE, Bronchiectasis; ACOS, Asthma-COPD overlap syndrome; EAA, Exogenous allergic alveolitis; NLH, Nodular lymphoid hyperplasia; IBD, Intestinal bowel disease; CG, Chronic gstritis; Y, Yes; N, No).

Patient Nr.	Autoimmunity	Chronic lung disease	Enteropathy	Granulomatous complications	Lymph-adenopathy	Spleno-megaly
1	AI thyreoiditis	LIPS	NLH	N	N	Y
2	N	BE	Celiac-like disease	N	Y	Y
3	ITP	BE	N	N	Y	Y
4	ITP, AIHA	LIPS	Celiac-like disease	N	Y	Y
5	N	N	N	N	Y	Y
6	ITP	N	N	N	N	Y
7	RA	N	N	N	N	Y
8	ITP, psoriasis	ACOS	N	N	N	Y
9	N	EAA	IBD-like disease	N	Y	N
10	N	BE, NLH	NLH, CG	N	Y	Y
11	N	BE	NLH	N	Y	N

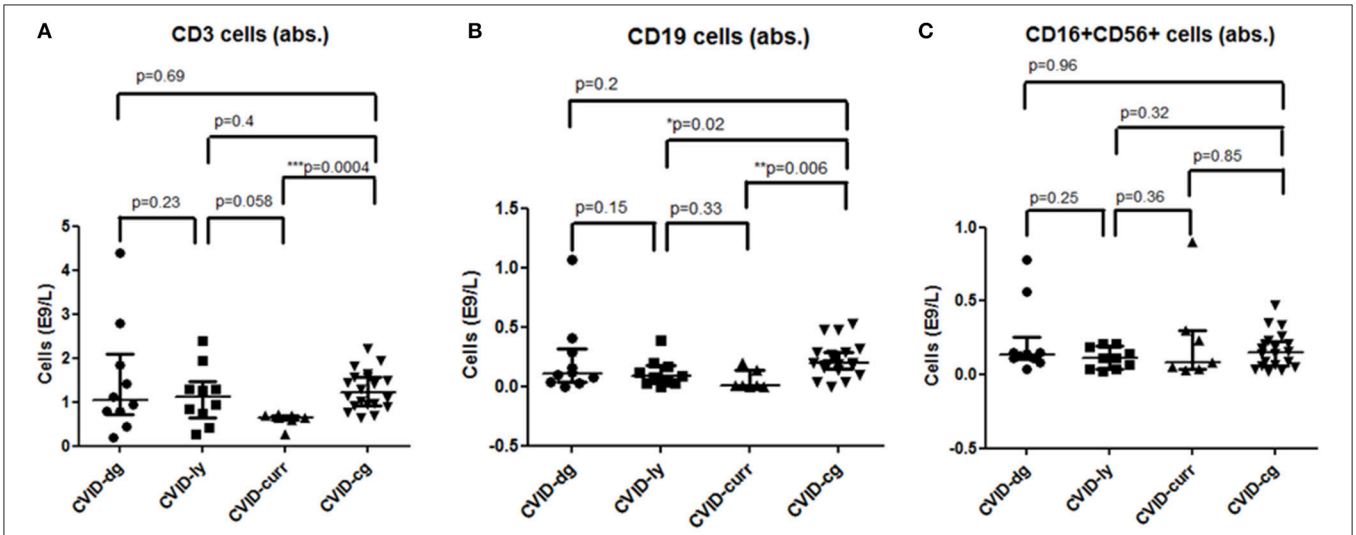

FIGURE 1 | Absolute counts of **(A)** T (CD3+) cells, **(B)** B (CD19+) cells, and **(C)** NK (CD16+, CD56+) cells in a cohort of CVID patients with lymphoma at the time of diagnosis of CVID (CVID-dg), at the time of diagnosis of lymphoma (CVID-ly) and current values (CVID-curr) compared to the control group of CVID patients without lymphoma (CVID-cg);median and 95% CI are shown.

Gene variants previously described in association with CVID or in patients with inborn error of immunity (Group 1) were found in 4 patients. A novel heterozygous missense variant in *CTLA4* was identified inpatient Nr. 9, who developed B—NHL (MALT) at the age of 36 years. She was also followed and treated for lymphadenopathy and enteropathy (with features of IBD-like disease), which is in concordance with the expected phenotype of CTLA4 deficiency. The deleterious effect of the mutation was verified by determination of decreased basal and stimulated (CD3/CD28) expression of CTLA4 protein in the patient's T regulatory cells (CD4+CD127dimCD25+) (**Supplementary Figure 1**).

Another type of B—NHL (DBLCL) was diagnosed in patient Nr. 4 at the age of 45 years. A genetic variant in *PMS2* was found, which has an important role in the mismatch repair system and class switch recombination (23, 24). In addition to the lymphoma, the patient also manifested with a broad spectrum of non-infectious complications, including autoimmune cytopaenias (both AIHA as well as ITP), celiac–like disease and generalized lymphoproliferation, including lymphadenopathy, splenomegaly, and lymphocytic interstitial pneumonia.

The clinical manifestation of patient Nr. 10, who was found to harbor a *PIK3CD* mutation, corresponded with the previously described APDS (activated PI3K-delta syndrome) phenotype due to an activating mutation (2). He has been followed for generalized lymphadenopathy, splenomegaly, and nodular lymphoid hyperplasia (NLH) of the lungs and gastrointestinal tract since his childhood. This patient developed Hodgkin lymphoma at the age of 11 years.

TABLE 4 | B cell subpopulations in CVID patients with lymphoma post-chemotherapy (absolute counts in E9/L; reference values for general population in brackets; (↓), decreased count; (↑), increased count; N/A, value not available).

Patient Nr.	CD21low (0.01–0.02)	Naïve (0.06–0.47)	Transitional (0.0–0.03)	MZ-like (0.01–0.08)	Class-switched (0.02–0.09)
1	N/A	N/A	N/A	N/A	N/A
2	0.0252 (↑)	0.069	0.004	0.009 (↓)	0.002 (↓)
3	N/A	N/A	N/A	N/A	N/A
4	0.001 (↓)	0.005 (↓)	0.002	0 (↓)	0 (↓)
5	0.216 (↑)	0.614 (↑)	0.137 (↑)	0.022	0.001 (↓)
6	0 (↓)	0 (↓)	0	0 (↓)	0 (↓)
7	0.005 (↓)	0.2	0.045 (↑)	0.003 (↓)	0 (↓)
8	0.004 (↓)	0.0076 (↓)	0	0.0013 (↓)	0 (↓)
9	0.0002 (↓)	0.002 (↓)	0	0 (↓)	0 (↓)
10	0.011	0.069 (↓)	0.059 (↑)	0.008 (↓)	0.009 (↓)

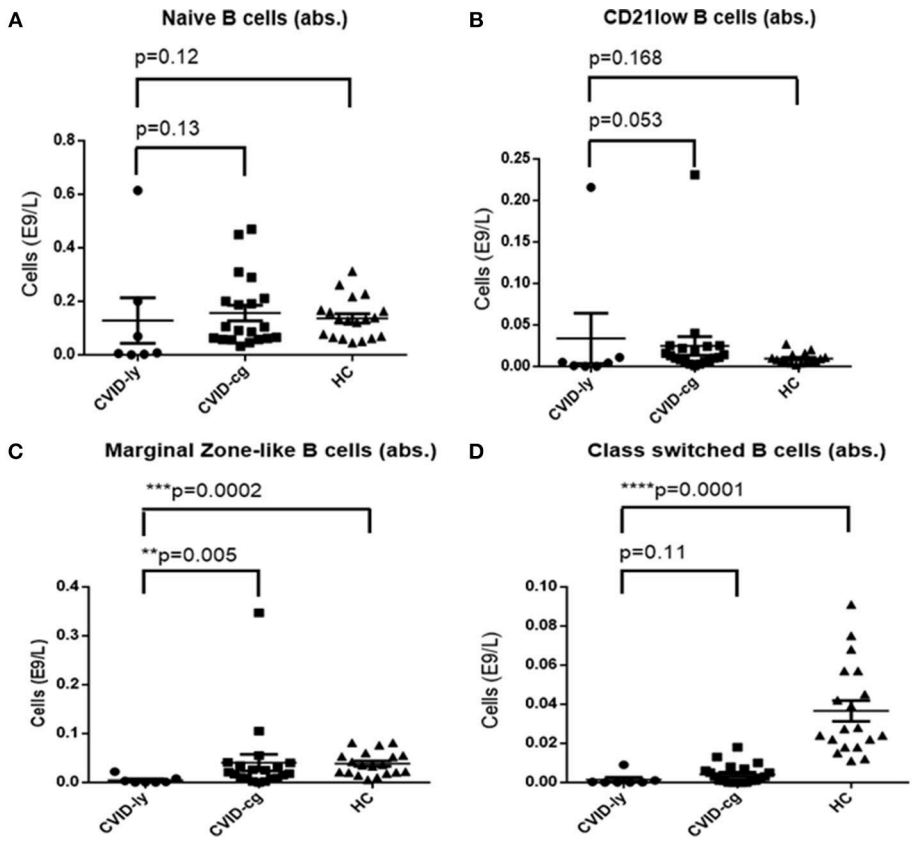

FIGURE 2 | Absolute counts of **(A)** Naïve B cells, **(B)** CD21low B cells, **(C)** Marginal Zone-like B cells, and **(D)** Class-switched B cells in CVID patients with lymphoma upon chemotherapy (CVID-ly) compared to the compared to the control group of CVID patients without lymphoma (CVID-cg) and healthy controls (HC); median and 95% are shown.

Nodular lymphoid hyperplasia and lymphocytic interstitial pneumonia were also noted in patient Nr. 1, who was followed and treated for splenomegaly and autoimmune thyroiditis before the diagnosis of T cell lymphoma, which developed at the age of 57. A gene variant in *TNFRSF13B (TACI)*, known to increase susceptibility to CVID, was found. *TACI* variants are not regarded as disease-causing but rather as modifying (Group 2) (8).

Furthermore, several heterozygous variants were identified in genes associated with known primary immunodeficiencies that are inherited in an autosomal recessive manner, such as *LYST, LRBA, RAG1, EXTL3, and STX11* (Group 4). The clinical phenotype of these patients did not match the respective disease; nevertheless, we report these variants because of their rarity in the healthy population and their potentially damaging character predicted by *in silico* tools. Functional assays, which would

elucidate the impact of these variants on protein function, were not performed, as they exceeded the scope of this study. The tumor DNA was not available for analysis of somatic "second-hit" mutations, which might explain the pathogenesis of some of the malignancies.

Variants in genes previously described in association with cancer susceptibility or as likely to increase the risk of cancer development, such as *BRCA1, RABEP1, EP300, KDM5A,* and others, were found in 6 out of 10 patients. They were divided into variants reported as pathogenic (Group 3) and variants of unknown significance and novel variants predicted as damaging *in-silico* (Group 5).The summary of WES results and a detailed description of the gene variants is presented in **Table 5** and in **Supplementary Table 1**.

DISCUSSION

Immune dysregulation associated with primary immunodeficiencies represents an increased risk of cancer development. We aimed to search for the occurrence of malignant diseases in a nationwide cohort of CVID patients, taking into account relevant epidemiology, immunophenotype, and the genetic background of the patients.

Similarly to published studies, we detected a higher incidence of malignancies among our CVID cohort (25–30). Also in alignment with previous reports, we noted a distinct spectrum of tumors in CVID patients, with Hodgkin and Non-Hodgkin lymphomas and gastric cancers being the most prevalent malignancies (**Table 6** and **Supplementary Table 2**). The overall risk of malignancy was more than 6 times greater in comparison to the general population, while the specific risk of HL was as much as 30 times greater. Curiously, an over 3 times greater risk of malignancy was determined in a subgroup of CVID patients with a history of ITP. Moreover, we noted that the diagnosis of GC (average age 55–59 years vs. 70–74 in general population) and B-NHL (35–39 years vs. 65–69) was established at a much younger age compared to the Czech general population, while HL developed later in life compared to the healthy population (40–44 years vs. 30–34).

Patients with CVID present with a characteristic immunophenotypic profile that is reflected in the diagnostic criteria of CVID. In this context, we specifically searched for potential differences between CVID patients with tumors and CVID patients who did not develop a malignant disease. Malignant hematologic diseases may, in general, reduce lymphocyte counts in up to 60% of patients, and lymphoma in particular may affect an entire spectrum of lymphocyte subpopulations, including CD4+, CD8+, CD19+, and CD56+ cells (31, 32). Nevertheless, in our cohort of CVID patients, we did not observe any significant differences between absolute or relative counts of CD3+, CD4+, CD8+, and CD56+ cells measured at the time of diagnosis of immunodeficiency and those measured at the time of diagnosis of lymphoma. Furthermore, the values of all T cell subpopulations and NK cells were similar to the control group of CVID patients without malignancy. In contrast, chemotherapy regimens had significant impacts on the

TABLE 5 | Summary of whole exome sequencing results performed in CVID patients with lymphoma.

Patient Nr.	Chromo-some	Gene symbol	Transcript variant	Protein variant	Geno- type	SIFT function prediction	SIFT score	Polyphen-2 function prediction	CADD score	ExAC Freq.	GnomAD Freq.	HGMD accession
GROUP 1: VARIANTS IN IUIS-CLASSIFIED GENES WITH LINKS TO CVID												
4	7	*PMS2*	c.1687C>T	p.R563*	Het				34.000	0.002	0.001	CM102799
9	2	*CTLA4*	c.515C>G	p.S172W	Het	Damaging	0.00	Possibly Damaging	28.700			
10	1	*PIK3CD*	c.3061G>A	p.E1021K	Het	Tolerated	0.07	Probably Damaging	31.000		0.000	CM067447
GROUP 2: MODIFIER GENES												
1	17	TACI	c.310T>C	p.C104R	Het	Damaging	0.00	Probably Damaging	25.900	0.321	0.346	CM052924
GROUP 3: CANCER SUSCEPTIBILITY GENES												
3	17	*BRCA1*	c.547+1delG		Het				0.424	0.012	0.009	CD176513
6	17	*BRCA1*	c.5263_5264insC	p.Q652fs*74	Het				35.000	0.016	0.016	CI941841

TABLE 6 | Summary of tumor prevalence and SIR (Standardized Incidence Ratio, median and 95% Confidence Intervals (CIs) shown) in a cohort of 295 CVID patients.

Tumor	Prevalence	SIR (95% CI)
All tumors	25/295 (8.5%)	6.3 (4.08–9.31)
B cell lymphoma (all types)	9/295 (3.0%)	10.1 (4.61–19.12)
B cell non-Hodgkin lymphoma	4/295 (1.4%)	5.50 (1.50–14.09)
Hodgkin lymphoma	5/295 (1.7%)	30.0 (9.73–69.93)
Gastric cancer	6/295 (2.0%)	5.70 (2.08–12.32)
Other types of cancer	10/295 (3.4%)	5.0 (2.40–9.16)

CD4+ and CD8+ cell counts. A similar observation has already been published in patients with lymphomas without underlying primary immunodeficiencies who underwent chemotherapy with an R-CHOP protocol. In the study, a reduction of CD4+ absolute counts to values $<0.343 \times 10^9$/l was declared an independent negative prognostic factor with a significant impact on 5-year progression-free survival and overall survival (33). Indeed, in our CVID cohort with lymphoma, the median level of post-chemotherapy absolute numbers of CD4+ was very low, 0.343×10^9/l, 95% CI 0.14–0.36 (R-CHOP being the regiment used in 5 out of 11 patient), which implies that a CVID population treated with chemotherapy should be prognostically regarded as a higher risk group. Quite unexpectedly, NK cell counts were not affected by the chemotherapy. However, the B cell compartment was profoundly depleted in all CVID patients who underwent chemotherapy. The total B cell count normalized in only 3/10 patients. However, even in those, selective reductions of mature forms (including class-switched, marginal zone-like B cells and plasmablasts) persisted. This observation was in striking contrast to patients with autoimmune diseases receiving anti-CD20 therapy (rituximab), in whom the reconstitution is achieved within 5–9 months in up to 90% of patients (34).

B cell deficiency seems to be the hallmark in CVID patients with lymphoma, as they presented with reduced B cell count even at the time of diagnosis of lymphoma, which decreased and remained persistently lower after chemotherapy. Despite this, neither B cell nor T cell detailed immunophenotyping provided a strong enough predictive tool for assessment of the cancerogenic predisposition of CVID patients. Therefore, we set out to search for possible genetic causes of malignancies in CVID using massive parallel sequencing. Out of 10 patients who were available for testing, we identified gene variants previously classified as associated with CVID in 4 patients, namely, *CTLR4, PIK3CD*, *PMS2,* and *TNFRSF13B*. It is noteworthy that mutations in *CTLA4* and *PIK3CD*, which account for the majority of currently known molecular causes of CVID (8), were both found among our small cohort of CVID patients.

CTLA4 heterozygous mutation was first described as a cause of CVID-like syndrome that displayed a significant overlap with CVID phenotype, including hypogammaglobulinemia, low B cell counts and immune dysregulation with variable organ involvement (35). The clinical and laboratory spectra of *CTLA4* haploinsufficiency were described in detail in a recently published cohort of 133 patients. In this cohort, 8 mutation carriers developed lymphoma, and 3 had gastric cancer. Thus, our finding of a single*CTLA4* mutation among our small cohort corresponds well with this report. Furthermore, this particular patient also presented with IBD-like gastrointestinal disease that was retrospectively reclassified as a CTLA4-related gastrointestinal presentation. Interestingly, the spectrum of tumors found in the above mentioned CTLA4 study was limited to lymphomas and gastric carcinomas, which also correlates with our findings.

PIK3CD is a well-established genetic cause of APDS, activated PI3K-delta syndrome. Similarly toCLTA4, the clinical presentation of APDS overlaps significantly with the CVID phenotype, and a number of patients originally diagnosed with CVID were found to harbor mutations in *PIK3CD*. A large study including 53 patients with APDS reported lymphoma occurrence in 13% of patients (36). Furthermore, somatic mutations of *PIK3* were found in several types of HL and NHL, thus suggesting the role of PI3K signaling in tumorigenesis.

Finally, the *PMS2* protein is involved in complex mechanisms of DNA repair. As such, mutations in *PMS2* are directly associated with an increased risk of cancer (37). At the same time, mutations in *PMS2* were also implicated in class-switch recombination defects and impaired immunoglobulin production (36). Therefore, our finding of *PMS2* mutation among CVID patients with lymphoma corresponds well with these reports.

Overall, we suggest that each of these CVID-associated genes may also convey a predisposition to tumor development.

The role of the *TNFSF13B* molecule, also known as *TACI*, in CVID has long been discussed. *TACI* variants have been found to be associated with autoimmune complications in CVID (38). Moreover, given the involvement of *TACI* in B cell activation, its mutations might contribute to immune dysregulation and lymphoma development (39). However, *TACI* variants are regarded as modifier genes rather than a monogenic cause of CVID.

Apart of the above mentioned genetic findings, we identified several variants in genes involved in lymphogenesis and immune system regulation. Although sufficient data to postulate their role in immune deficiency or tumorigenesis are lacking, we report them along with our results for the sake of completeness, reflecting the previously described roles of heterozygous mutations in PID and possible epistatic roles of various genes in immune dysregulation (40–42).

Finally, we also detected several variants in genes involved in tumor surveillance in our cohort, such as BRCA1 and others listed in **Supplementary Table 1**. These variants were previously reported in patients with a broad spectrum of solid tumors (including breast, ovarian, colorectal cancer, and others) and may therefore represent another contributory mechanism of malignant susceptibility.

CONCLUSION

Malignancies belong to the most severe non-infectious complications of common variable immunodeficiency disorder. The prevalence of malignancy in our CVID cohort was found to be more than 6 times greater than in the general population. The spectrum of cancers was characteristically narrow, involving mostly gastric cancers and lymphomas. Moreover, ITP was elucidated as a novel risk factor for malignancy in CVID patients. Post-treatment T and B cell lymphopaenias, associated with poorer prognosis, were found in a majority of CVID patients who received chemotherapy. Surprisingly, NK cells remained unaffected. WES analysis illustrated a wide heterogeneity of potential genetic background of the oncogenic predisposition among CVID patients and identified several causative or contributing gene variants, pointing toward immune system dysregulation. In the future, modern genetic analytic approaches applied on larger cohorts of CVID patients, along with the use of oncogenomic tools, will undoubtedly enable the identification of other CVID-associated genes with increased risk of cancer and elucidate their roles in tumorigenesis.

AUTHOR CONTRIBUTIONS

PK and TM conceived and designed the study, collected data, and drafted manuscript. JL, IM, DJ, JP, JV, AZ, MB, ZP, and AK collected and provided primary patient data. EF and MS performed analysis and interpretation of data from genetic testing (Whole exome sequencing). VK performed analysis and interpretation of immunophenotyping data. JH provided statistical analysis of the obtained data and its interpretation. TK and AS provided critical revisions of the manuscript and final approval of the version to be published.

ACKNOWLEDGMENTS

This study was supported by grants from the Grant Agency Charles University in Prague (GAUK) nr. 2120217 and the Czech Health Research Council (AZV) nr.NV18-05-00162.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.03135/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 08 January 2019
doi: 10.3389/fimmu.2018.03108

Loss of JAK1 Drives Innate Immune Deficiency

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The Janus kinase—signal transducers and activators of transcription (JAK-STAT) signaling pathway is critical in tuning immune responses and its dysregulation is tightly associated with cancer and immune disorders. Disruption of interleukin (IL)-15/STAT5 signaling pathway due to the loss of IL-15 receptor chains, JAK3 or STAT5 leads to immune deficiencies with natural killer (NK) cell abnormalities. JAK1, together with JAK3 transmits signals downstream of IL-15, but the exact contribution of JAK1 to NK cell biology remains to be elucidated. To study the consequences of JAK1 deficiency in NK cells, we generated mice with conditional deletion of JAK1 in NKp46$^+$ cells (*Jak1$^{fl/fl}$Ncr1Cre*). We show here that deletion of NK cell-intrinsic JAK1 significantly reduced NK cell numbers in the bone marrow and impaired their development. In line, we observed almost a complete loss of NK cells in the spleen, blood, and liver, proving a crucial role of JAK1 in peripheral NK cells. In line, *Jak1$^{fl/+}$Ncr1Cre* mice showed significantly impaired NK cell-mediated tumor surveillance. Our data suggest that JAK2 is not able to compensate for the loss of JAK1 in NK cells. Importantly, conditional deletion of JAK2 in NKp46$^+$ cells had no effect on peripheral NK cells revealing that NK cell-intrinsic JAK2 is dispensable for NK cell survival. In summary, we identified that loss of JAK1 in NK cells drives innate immune deficiency, whereas JAK2 deficiency leaves NK cell numbers and maturation unaltered. We thus propose that in contrast to currently used JAK1/JAK2 inhibitors, the use of JAK2-specific inhibitors would be advantageous for the patients by leaving NK cells intact.

Keywords: JAK-STAT, natural killer cells, JAK1, JAK2, tumor surveillance

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Primary Immunodeficiencies, a section of the journal Frontiers in Immunology

Received: *28 September 2018*
Accepted: *17 December 2018*
Published: *08 January 2019*

Citation:
Witalisz-Siepracka A, Klein K, Prinz D, Leidenfrost N, Schabbauer G, Dohnal A and Sexl V (2019) Loss of JAK1 Drives Innate Immune Deficiency. Front. Immunol. 9:3108. doi: 10.3389/fimmu.2018.03108

INTRODUCTION

Natural killer (NK) cells are innate lymphocytes which recognize and kill virally infected or transformed cells (1). Deficiency of NK cells is a rare but increasingly appreciated subtype of primary immunodeficiency (PID). Classical NK cell deficiency is characterized by the absence of NK cells in the peripheral blood and results in enhanced susceptibility to viral infections (2).

The Janus kinase (JAK)—signal transducer and activator of transcription (STAT) signaling pathway acts downstream of multiple cytokines, growth factors, and hormones thereby critically regulating immune responses (3, 4). Upon binding of a specific ligand to its cognate receptor, conformational changes lead to receptor oligomerization and activation of the receptor-associated JAKs. JAKs auto- and trans-phosphorylate one another and phosphorylate receptor

chains, providing the docking sites for STAT molecules. STATs then undergo JAK-mediated phosphorylation, dimerize, and translocate to the nucleus, where they regulate the transcription of target genes (5). JAK3 and STAT5 are crucial players in transducing the signal downstream of cytokines which utilize γc receptor (6). "Loss-of-function" (LOF) mutations in genes encoding JAK3 (7) or STAT5B (8) lead to PIDs with an NK cell abnormality underlining the importance of the pathway for innate lymphocytes. The immunodeficiency of these patients has been explained by impaired IL-7 and IL-15 responses (6). Importantly, JAK1 has a dominant role over JAK3 in activating STAT5 downstream of γc-containing cytokine receptors (9). It is attractive to speculate that LOF mutations of JAK1 could also result in PID. To date, only one patient harboring JAK1 germline mutations, where JAK1 was reduced but not absent, has been identified and indeed presented with immune suppression (10).

In mice, complete loss of JAK1 leads to perinatal lethality and newborn mice display a strong reduction of thymocytes and B cells (11). These observations were confirmed in adult mice: inducible deletion of JAK1 leads to impairment of hematopoietic stem cells (HSCs) homeostasis and markedly reduces the frequencies of B cells and the $B220^+CD11c^+NK1.1^+$ subset of NK cells (12). However, to date, no study has directly analyzed the effect of loss of JAK1 on conventional NK cells.

The first insights into the contribution of JAK1 to NK cell biology derive from studies using JAK inhibitors—approved drugs for treatment of cancers and autoimmune diseases (13). Both, mice and patients treated with the JAK1/JAK2 inhibitor Ruxolitinib showed reduced NK cell numbers, impaired maturation, and function (14, 15). Since JAK2 has also been implicated in driving NK cell differentiation (14, 16), it remains to be elucidated which of the two kinases is responsible for the observed effects of Ruxolitinib treatment.

Using mice with knockout of *Jak1* or *Jak2* in $NKp46^+$ cells, we show here that JAK2 is dispensable for NK cell survival. In contrast, deletion of JAK1 in mature NK cells leads to NK cell deficiency and loss of one allele of *Jak1* is sufficient to impair tumor growth control. Thus, we identified JAK1 as a key factor for mature NK cells and generated a mouse model of classical NK cell deficiency.

MATERIALS AND METHODS

Mice and Cell Lines

$Jak1^{fl/fl}$ (*C57BL/6N-Jak1*$^{tm1c(EUCOMM)Hmgu/H}$; were kindly provided by Dr. Alexander Dohnal (CCRI, Vienna, Austria). The $Jak1^{tm1c}$ allele of the mutant was generated from mice with the $Jak1^{tm1a}$ knockout first allele (described by International Mouse Phenotyping Consortium https://www.mousephenotype.org) by excision of the lacZ-neo cassette via Flp-recombination. The conditional potential of $Jak1^{fl/fl}$ mice was activated by Cre-recombination and excision of the loxP-flanked exon 3 of *Jak1*. Tissue-specific recombination was induced by cross breeding of $Jak1^{fl/fl}$ or $Jak2^{fl/fl}$ [$Jak2^{tm1Kuw}$; (17)] with B6N-Tg(Ncr1Cre); (18) mice. $Stat5^{fl/fl}$ (19) and $Stat5^{fl/fl}Ncr1Cre$ (18) mice were described before. $Jak1^{fl/fl}$, *Ncr1Cre,* $Stat5^{fl/fl}$, $Stat5^{fl/fl}Ncr1Cre$ mice were on C57B6/N background and $Jak2^{fl/fl}$ were on mixed background. The experimental animals were age-matched (8–12 weeks) and maintained under specific pathogen-free conditions at the University of Veterinary Medicine, Vienna according to Federation for Laboratory Animal Science Associations (FELASA) guidelines (2014). The animal experiments were approved by the Ethics and Animal Welfare Committee of the University of Veterinary Medicine Vienna and the national authority (Austrian Federal Ministry of Science and Research) according to §§ 26ff. of Animal Experiments Act, Tierversuchsgesetz 2012—TVG 2012, under licenses BMWF-68.205/0218-II/3b/2012 and BMBWF-68.205/0174-V/3b/2018 and were conducted according to the guidelines of FELASA and ARRIVE. Throughout the paper $Jak1^{WT}$ refers to pooled data from $Jak1^{fl/+}$ and $Jak1^{fl/fl}$ mice.

The mouse lymphoma cell lines RMA-Rae1 [kindly provided by Prof. A. Cerwenka; (20)] and YAC-1 were cultured in RPMI1640 (Sigma) complete medium containing 10% FCS (Bio & Sell), 100 U/mL penicillin, 100 mg/mL streptomycin (Sigma), and 50 μM 2-mercaptoethanol (Sigma).

In vivo Tumor Model

$Jak1^{fl/+}$ and $Jak1^{fl/+}Ncr1Cre$ mice were injected *s.c.* with 10^6 RMA-Rae1 cells into both flanks and the tumor growth was monitored every other day. Ten days post injection the mice were sacrificed and the tumor weight was determined. For flow cytometric analysis of tumor infiltrating NK cells, tumors were cut into ∼5 mm^2 pieces and the single cell suspension was obtained using gentleMACS™ *Octo Dissociator* (Miltenyi Biotec) with digestion buffer containing Collagenase D (1 mg/mL; Sigma Aldrich) and DNAse I (20 mg/mL; Roche).

NK-Cell Isolation, Expansion, and Stimulation

NK cells were isolated from spleen single-cell suspensions using DX5-labeled MACS beads according to the manufacturer's instructions (Miltenyi Biotec). NK cells were expanded in RPMI1640 complete medium supplemented with 5,000 U/mL rhIL-2 (Proleukin, Novartis) for 7 days. The number of $CD3^-NK1.1^+$ cells was assessed by flow cytometry on day 0, 3, 5, and 7. On day 7 cells were lysed for Western blot analysis. For pSTAT5 analysis 10^6 splenocytes were stimulated with 50 ng/ml rmIL-15 (PeproTech) for 15 min and the cells were fixed in 2% PFA followed by methanol permeabilization and rehydration.

NK-Cell Cytotoxicity Assay

For *in vitro* cytotoxicity assays, DX5-MACS–sorted NK cells were expanded for 7 days in IL-2 as described above and mixed at

Abbreviations: FACS, fluorescence-activated cell sorting; HSCs, hematopoietic stem cells; ILC, innate lymphoid cells; JAK, Janus kinase; LOF, loss of function; NK, natural killer; PID, primary immunodeficiency; SCID, severe combined immune deficiency; STAT, signal transducer and activator of transcription.

indicated effector: target ratios with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, CellTrace CFSE Cell Proliferation Kit) labeled target cells. After 4 h of incubation at 37°C, the cells were stained with Sytox Blue Dead Cell Stain (Thermo Fischer) and the specific target cell lysis was assessed by flow cytometry.

Flow Cytometry

Single cell suspensions were prepared from spleen, bone marrow, or liver. Liver was perfused via the portal vein with 5–10 mL sterile PBS. Separation of lymphocytes was performed using 37.5% percoll (GE Healthcare). For blood analysis, the erythrocytes were lysed using BD FACS Lysing Solution according to manufacturer's protocol (BD Bioscience). The antibodies (clones) targeting following proteins were purchased from eBioscience: CD3 (17A2), CD3e (145-2C11), CD11b (M1/70), CD16/CD32 (93), CD19 (eBio1D3) CD27 (LG.7F9), CD49b (DX5), CD122 (5H4), CD226 (10E5), Gr-1 (RB6-8C5), KLRG1 (2F1), Ly49A (A1), Ly49G2 (eBio4D11), NKG2A/C/E (20d5), NKG2D (CX5), NKp46 (29A1.4), NK1.1 (PK136), and Ter119 (TER-119). CD49a (Ha31/8) and pSTAT5 [47/Stat5(pY694)] antibodies were purchased from BD Pharminogen and pan-Rae1 (186107) was purchased from R&D Systems. Total cell numbers were assessed by flow cytometry using counting beads Count Bright Beads (Invitrogen). Flow cytometry experiments were performed

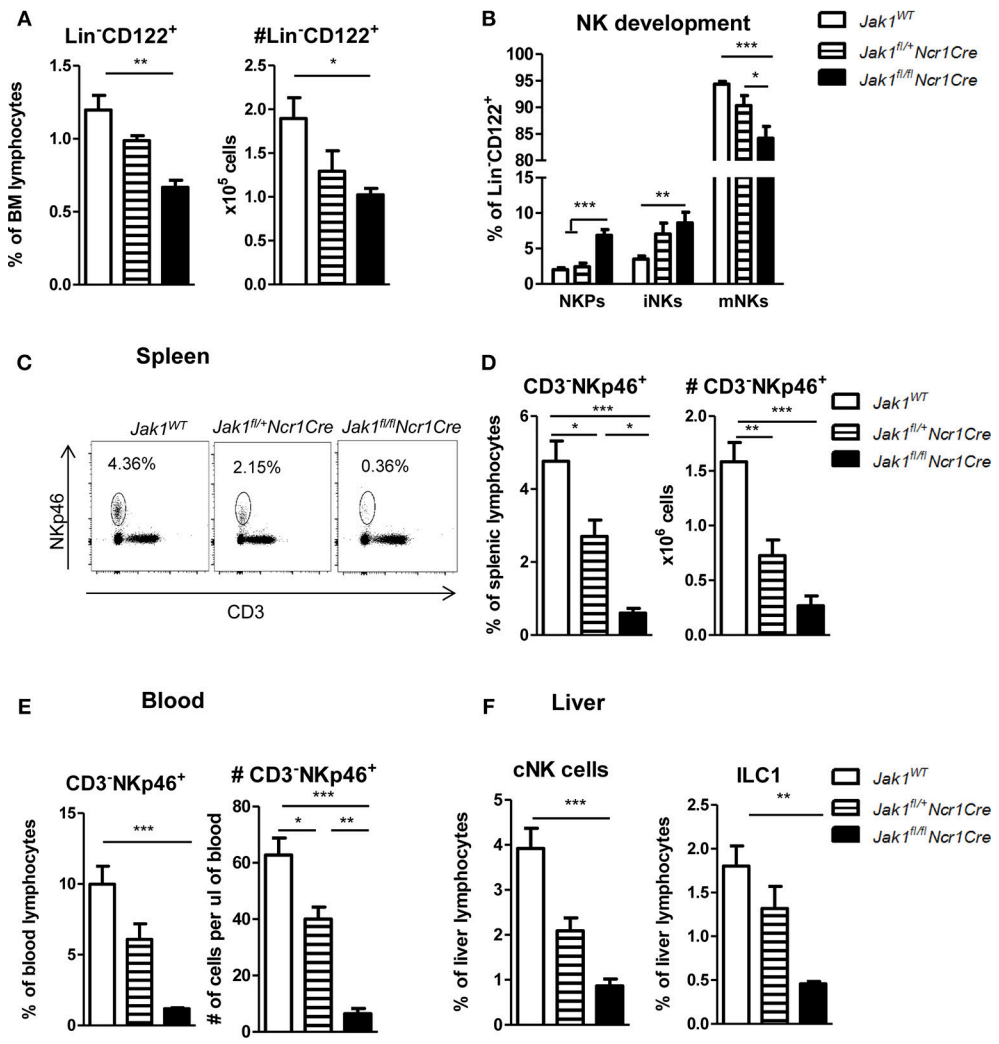

FIGURE 1 | Loss of JAK1 in NKp46$^+$ cells leads to an almost complete absence of peripheral NK cells. **(A)** Frequency (left panel) and total numbers (right panel) of Lin$^-$(CD3$^-$CD19$^-$Ly-6G$^-$Ter119$^-$) CD122$^+$ NK cells in the bone marrow were assessed by flow cytometry. **(B)** Bone marrow Lin$^-$CD122$^+$ cells were further divided into NK precursors (NKPs: NKp46$^-$NK1.1$^-$), immature NK cells (iNKs: NKp46$^-$NK1.1$^+$), and mature NK cells (mNKs: NKp46$^+$NK1.1$^+$). **(C)** Frequency of CD3$^-$NKp46$^+$ NK cells in the spleen was assessed by flow cytometry and representative plots are shown. **(D,E)** Frequency (left panel) and total numbers (right panel) of CD3$^-$NKp46$^+$ NK cells in the **(D)** spleen and **(E)** blood were assessed by flow cytometry. **(F)** Frequency of conventional NK cells (CD3$^-$NK1.1$^+$NKp46$^+$CD49b$^+$, left panel) and ILC1 cells (CD3$^-$NK1.1$^+$NKp46$^+$CD49a$^+$, right panel) was analyzed in the liver of *Jak1*^{WT}, *Jak1*$^{fl/+}$*Ncr1Cre,* and *Jak1*$^{fl/fl}$*Ncr1Cre* mice by flow cytometry. **(A,B,D–F)** Bar graphs represent mean ± SEM of 1–2 independent experiments; $n = 3$–11. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

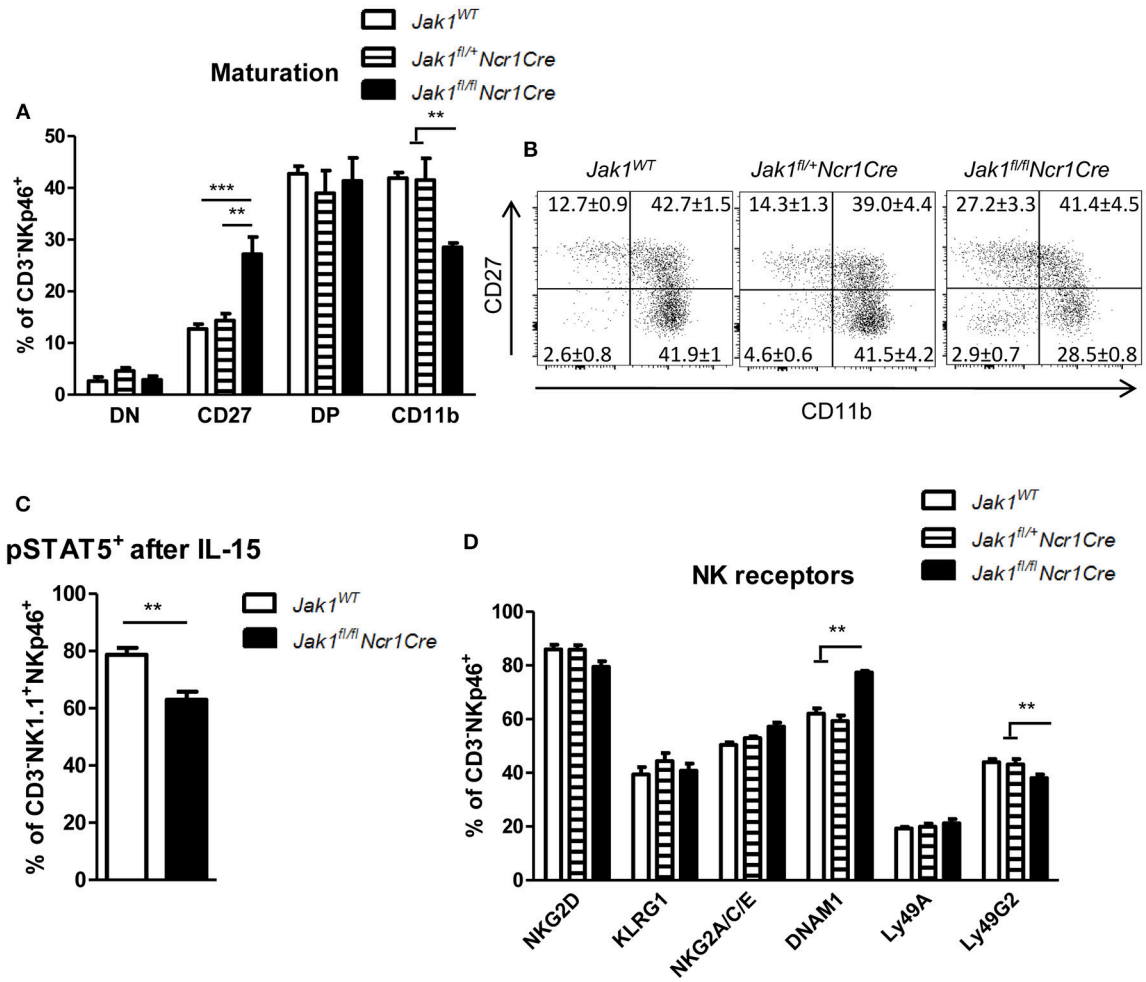

FIGURE 2 | The remaining *Jak1*$^{fl/fl}$*Ncr1Cre* NK cells show an immature phenotype. **(A,B)** Splenic CD3$^-$NKp46$^+$ NK cells were analyzed for expression of CD27 and CD11b by flow cytometry. **(A)** Frequency of cells in each maturation stage is shown: DN (CD27$^-$CD11b$^-$), CD27 (CD27$^+$CD11b$^-$), DP (CD27$^+$CD11b$^+$), CD11b (CD27$^-$CD11b$^+$). The total numbers of cells in each maturation stage are shown in **Figure S1C**. **(B)** Representative plots are shown. **(C)** pSTAT5(Y694)$^+$ cells were analyzed within the CD3$^-$NKp46$^+$NK1.1$^+$ population *ex vivo* after 15 min stimulation with IL-15 by flow cytometry. **(D)** Splenic CD3$^-$NKp46$^+$ NK cells were analyzed for expression of the indicated activating and inhibitory receptors by flow cytometry. The percentage of NK cells positive for each receptor is shown. The median fluorescence intensity data is presented in **Figure S1D**. **(A–D)** Bar graphs and numbers on the plots represent mean ± SEM of 2 independent experiments; $n = 5$–8. $^{**}p < 0.01$, $^{***}p < 0.001$.

on a BD FACSCanto II (BD Bioscience) or Cytoflex (Beckman Coulter) and analyzed using BD FACSDiva V8.0 (BD Bioscience), CytExpert (Beckman Coulter) or FlowJo V10 (FlowJo, LLC) software.

Western Blot

Cell lysis, SDS-PAGE, and Western blots were performed as described previously (21). The detection of chemiluminescence was performed using Clarity Western ECL substrate (BioRad) and the ChemiDocT XRS+ Molecular Imager (BioRad) and analyzed by Image Lab software (BioRad). The following antibodies were used: anti–β-actin (C4, sc-47778) from Santa Cruz as loading control, anti-JAK2 (D2E12; #3230), anti-Perforin (#3693) and anti-JAK1 (#3332) from Cell Signaling Technology.

Statistical Analysis

Unpaired *t*-tests or one-way ANOVA with Tukey post tests were performed using GraphPad Prism version 5.00 (GraphPad Software). The level of significance is indicated for each experiment ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

RESULTS

JAK1 Deletion Reduces NK Cell and ILC1 Numbers in a Dose-Dependent Manner

The JAK1/JAK2 inhibitor Ruxolitinib has been shown to reduce NK cell numbers, maturation, and function (14, 15). To compare and address the contribution of JAK1 and JAK2 for NK cell biology we generated mice with conditional deletion of either JAK1 or JAK2 in NKp46$^+$ cells. We thus crossed *Ncr1Cre* (18)

mice with *Jak1*$^{fl/fl}$ or *Jak2*$^{fl/fl}$ [see Materials and Methods section and (17)] mice, respectively. NK cells develop in the bone marrow from NK cell precursors (NKPs), which are defined as Lin$^-$CD122$^+$NK1.1$^-$NKp46$^-$. They develop into immature NK cells (iNKs) that become NK1.1$^+$ while only mature NK cells (mNKs) are NK1.1$^+$NKp46$^+$ (22). As the *Cre* recombinase expression in *Ncr1Cre* mice is driven by the NKp46 promoter, *Cre*-mediated deletion is restricted to mNK cells. We observed a significant decrease of percentage and total numbers of bone marrow NK cells in *Jak1*$^{fl/fl}$*Ncr1Cre* mice (**Figure 1A**). The Lin$^-$CD122$^+$ NK cells in *Jak1*$^{fl/fl}$*Ncr1Cre* mice showed enriched percentages of NK cell precursors (NKPs) and immature NK cells, while mNKs were significantly reduced in the bone marrow in line with a developmental block at the iNK cell stage preventing progression to mNK cell stage (**Figure 1B**). Deletion of one allele of *Jak1* led to intermediate numbers of bone marrow NK cells (**Figure 1A**). Consistently, NK cell development showed an intermediate phenotype suggesting a *Jak1* gene-dosage effect on NK cell development (**Figure 1B**).

The block in development of bone marrow NK cells translated into drastically reduced numbers of NK cells in the periphery. Loss of JAK1 led to an almost complete deficiency of splenic and blood NK cells (**Figures 1C–E**). In line with a *Jak1* gene dosage effect, *Jak1*$^{fl/+}$*Ncr1Cre* mice displayed reduced NK cell percentages and total numbers to 50% compared to wild-type littermates in the spleen and blood (**Figures 1C–E**). Deletion of the JAK1 downstream effector and transcription factor STAT5 in NKp46$^+$ cells also leads to a reduction of mature NK cells (18). A direct comparison of *Jak1*$^{fl/fl}$*Ncr1Cre* and *Stat5*$^{fl/fl}$*Ncr1Cre* mice revealed that deletion of JAK1 provoked an even more pronounced NK cell deficiency in spleen and blood than deletion of STAT5 (**Figures S1A,B**).

Liver NKp46$^+$ innate lymphocytes comprise two groups of distinct lineages (23). Conventional NK cells (cNK) are characterized by expression of CD49b and circulate freely whereas liver resident type 1 innate lymphocytes (ILC1) are characterized by the expression of CD49a and are restricted to the liver (24). Similarly to spleen and blood, liver cNKs and tissue resident ILC1s were almost completely ablated upon loss of JAK1 (**Figure 1F**). Again the deletion of one allele of *Jak1* resulted in an intermediate abundance of liver innate lymphocytes (**Figure 1F**). In summary, these findings led us to conclude that JAK1 expression in NKp46$^+$ cells is indispensable for NK cell development and maintenance in peripheral organs in a dose-dependent manner.

JAK1 Is Crucial for NK Cell Maturation

In the periphery NK cells undergo maturation steps which are characterized by sequential expression of CD27 and CD11b surface markers (22). One allele of *Jak1* was sufficient to drive NK cell maturation as we did not detect any differences in percentage of cells in each maturation stage between *Jak1*^{WT} and *Jak1*$^{fl/+}$*Ncr1Cre* cells (**Figures 2A,B**). The remaining *Jak1*$^{fl/fl}$*Ncr1Cre* NK cells showed an increase in the immature population (CD27$^+$CD11b$^-$) and a decrease in the mature CD27$^-$CD11b$^+$ population (**Figures 2A,B** and **Figure S1C**). This result suggests that the remaining cells might just have lost

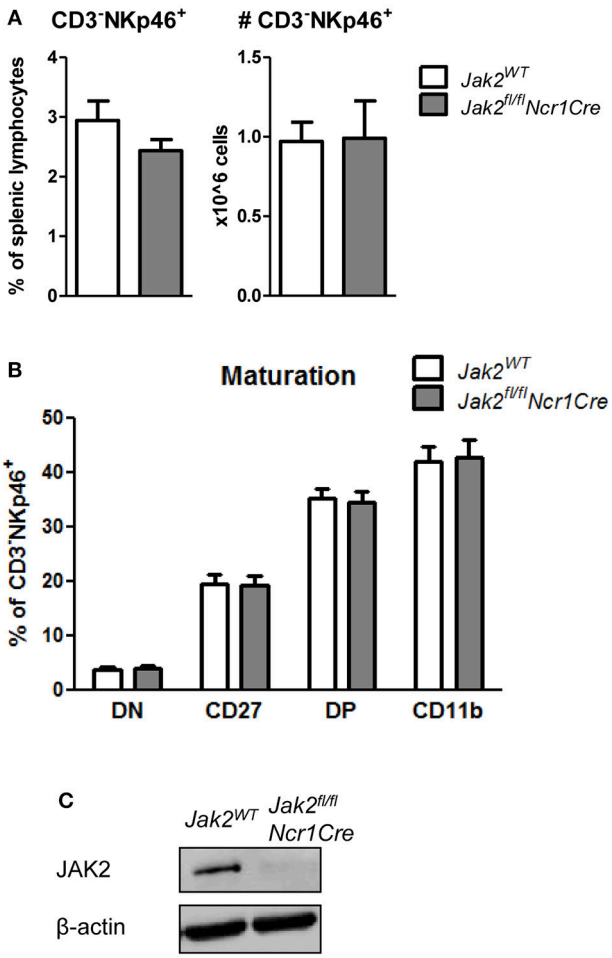

FIGURE 3 | JAK2 is dispensable for NK cell survival and maturation. **(A)** Frequency (left panel) and total numbers (right panel) of CD3$^-$NKp46$^+$ NK cells in the spleens of *Jak2*^{WT} and *Jak2*$^{fl/fl}$*Ncr1Cre* mice were assessed by flow cytometry. **(B)** Splenic CD3$^-$NKp46$^+$ NK cells were analyzed for expression of CD27 and CD11b by flow cytometry. Frequency of cells in each maturation stage is show: DN (CD27$^-$CD11b$^-$), CD27 (CD27$^+$CD11b$^-$), DP (CD27$^+$CD11b$^+$), CD11b (CD27$^-$CD11b$^+$). **(C)** The expression of JAK2 and β-actin was analyzed by Western blot in NK cells upon 6 days of expansion in IL-2. Scans of full blots are available in **Supplementary Material**. **(A,B)** Bar graphs represent mean ± SEM of 2–3 independent experiments; $n = 5$–12.

JAK1 and have not received sufficient IL-15 signaling to fully mature. Indeed, the remaining *Jak1*$^{fl/fl}$*Ncr1Cre* NK cells showed reduced phosphorylation of STAT5 *ex vivo* upon short-term stimulation with IL-15 (**Figure 2C**). NK cell activity is controlled by a balance between activating and inhibitory receptors. Deletion of neither one nor both alleles of *Jak1* had an effect on the percentage of NK cells expressing following activating and inhibitory receptors: KLRG1, NKG2D, NKG2A/C/E, and Ly49A (**Figure 2D**). The most prominent difference was a slight decrease of Ly49G2$^+$ NK cells and an increase in DNAM-1$^+$ NK cells in the *Jak1*$^{fl/fl}$*Ncr1Cre* mice (**Figure 2D**). Similarly, no gross differences were detected in the expression level (MFI) of each receptor, besides an increase in the MFI of DNAM1 and Ly49G2

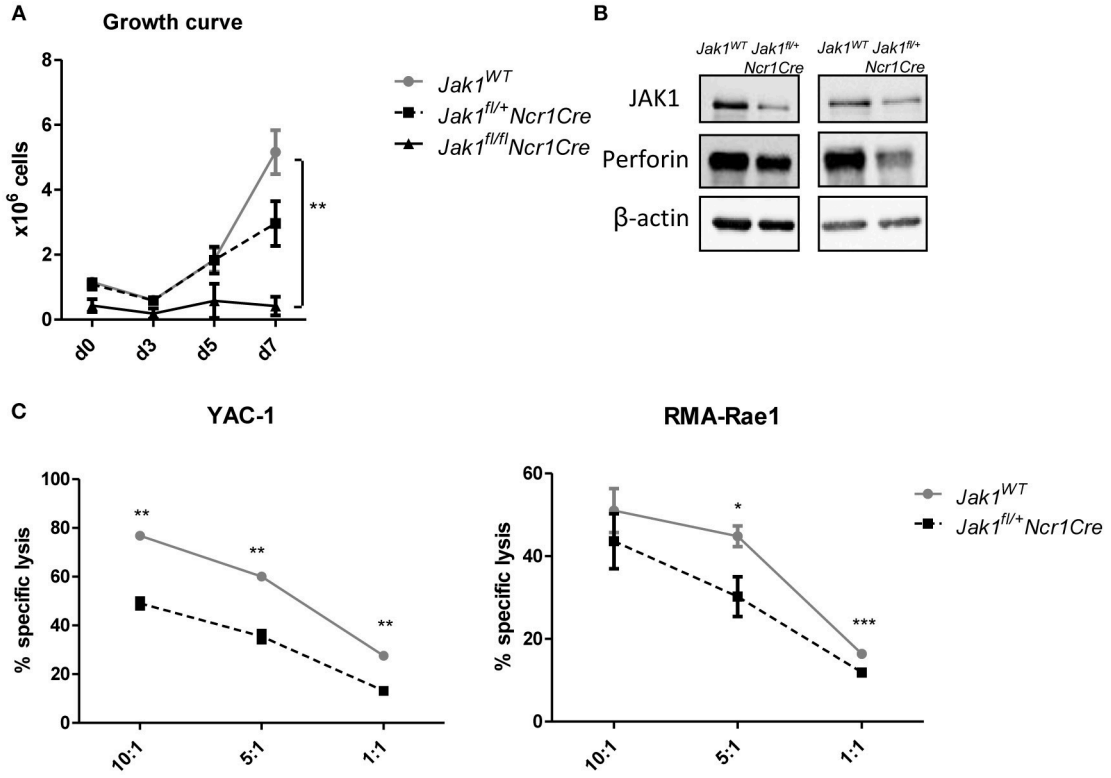

FIGURE 4 | Loss of one allele of *Jak1* impairs NK cell activity**. (A)** NK cells were MACS-purified from spleens and cultured in IL-2 for 7 days. The numbers of living $CD3^-NK1.1^+NKp46^+$ cells were assessed every 2–3 days. The symbols and the error bars present mean ± SEM of 2–4 biological replicates. **(B)** The expression of JAK1, Perforin and β-actin was analyzed in expanded NK cells from two independent experiments by Western blot. Scans of full blots are available in **Supplementary Material**. **(C)** Expanded NK cells from $Jak1^{WT}$ and $Jak1^{fl/+}Ncr1Cr$ mice were mixed with CFSE-stained YAC-1 (left panel) or RMA-Rae1 (right panel) target cells at indicated effector target ratios. The specific lysis was assessed by flow cytometry. For YAC-1 a representative graph of one of two independent experiments is shown. The symbols and the error bars present mean ± SEM of 2 technical replicates. For RMA-Rae1 a mean out of two independent experiments is shown. The symbols and the error bars present mean ± SEM of 2–3 biological replicates. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

(**Figure S1D**). Besides its role as an activating receptor, DNAM-1 expression marks a developmental step; DNAM-1^+ cells give rise to DNAM-1^- cells (25). We reasoned that the changes in DNAM-1^+ reflect the maturation block of $Jak1^{fl/fl}Ncr1Cre$ NK cells.

JAK2 Is Dispensable for NK Cell Survival and Maturation

So far we showed that NK cell-intrinsic JAK1 deletion leads to NK cell deficiency. To elucidate if JAK2 impacts on NK cell survival and maturation, we analyzed splenic NK cells in $Jak2^{fl/fl}Ncr1Cre$ mice and their wild-type littermates. We failed to detect any impact of JAK2 deletion on the frequency or total numbers of $CD3^-NKp46^+$ cells in the spleen (**Figure 3A**). Furthermore, $Jak2^{fl/fl}Ncr1Cre$ mice showed normal NK cell maturation, as similar percentages of $CD27^-CD11b^+$ cells were detected in both genotypes (**Figure 3B**). As the deletion of JAK2 protein in NK cells was very efficient (**Figure 3C**), these data unequivocally define that unlike JAK1, NK cell-intrinsic JAK2 is dispensable for NK cell survival and maturation.

Loss of One Allele of *Jak1* Impairs NK Cell Functionality

To get further insights into how JAK1 regulates NK cell functionality, we analyzed the growth of MACS-purified splenic NK cells from $Jak1^{WT}$, $Jak1^{fl/+}Ncr1Cre$, and $Jak1^{fl/fl}Ncr1Cre$. JAK1-deficient NK cells did not expand, which shows that even under a very high dose of IL-2 other JAKs cannot compensate for the loss of JAK1 (**Figure 4A**). The loss of one allele of *Jak1* resulted in a minor growth deficiency (**Figure 4A**). Western blot analysis confirmed the reduced JAK1 protein expression in expanded $Jak1^{fl/+}Ncr1Cre$ NK cells which was paralleled by reduced levels of perforin (**Figure 4B**). In line, $Jak1^{fl/+}Ncr1Cre$ NK cells displayed an impaired cytotoxic activity against target cell lines YAC-1 and RMA-Rae1 (**Figure 4C**). These results prove that JAK1 is not only indispensable for maintaining NK cells in periphery, but also contributes to their cytotoxic activity.

NK Cell Depletion Induced by Loss of One Allele of *Jak1* Impairs Tumor Surveillance

NK cells are crucial for the early recognition and elimination of transformed cells. To investigate whether NK cell reduction

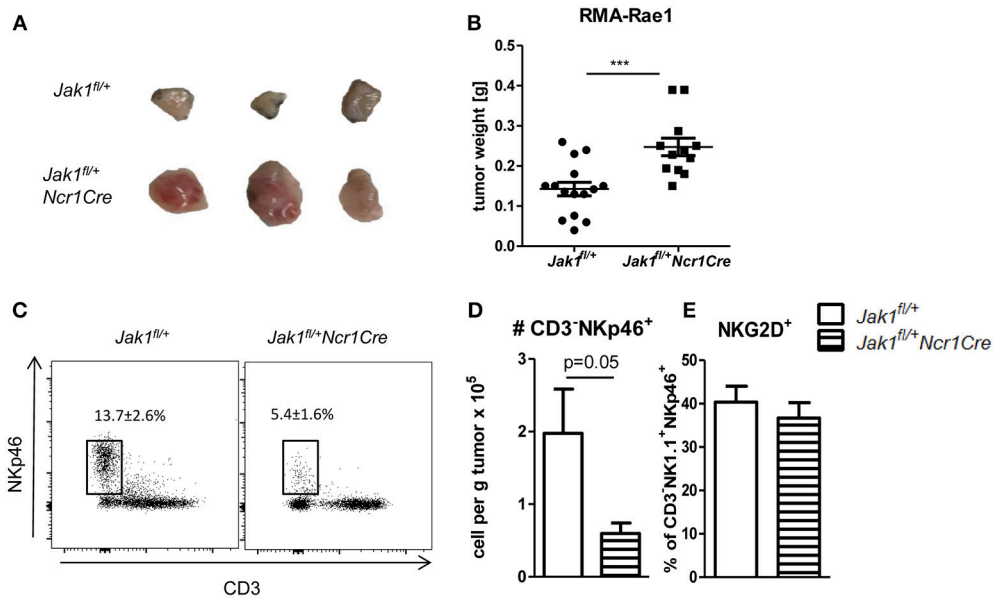

FIGURE 5 | Loss of one allele of *Jak1* impairs tumor surveillance. **(A,B)** *Jak1*$^{fl/+}$ and *Jak1*$^{fl/+}$*Ncr1Cre* mice were injected *s.c.* with 10^6 RMA-Rae1 cells and after 10 days the tumor weight was assessed. Shown are **(A)** representative tumor pictures and **(B)** dot plots with horizontal lines representing mean tumor weights ± SEM from 2 independent experiments; $n = 6$–8 **(C,D)** Tumor infiltrating NK cells were analyzed by flow cytometry. **(C)** Representative plots of tumor infiltrating CD3$^-$NKp46$^+$ cells and their percentage among Rae1$^-$ cells are shown. **(D)** Total numbers of tumor infiltrating CD3$^-$NKp46$^+$ cells per gram of tumor and **(E)** percentages of NKG2D$^+$ NK cells are presented as bar graphs showing mean ± SEM from one experiment $n = 4$–5. ***$p < 0.001$.

and their impaired functionality in *Jak1*$^{fl/+}$*Ncr1Cre* mice results in increased susceptibility to tumor growth and is not compensated by other means, we made use of RMA-Rae1 lymphoma cells. This cell line is a tool to study NK cell-dependent tumor surveillance in a robust and efficient way (20). We subcutaneously transplanted RMA-Rae1 lymphoma cells into both flanks of *Jak1*$^{fl/+}$ and *Jak1*$^{fl/+}$*Ncr1Cre* mice. Lacking one allele of *Jak1* impaired the ability of NK cells to control the tumor growth as illustrated by increased tumor size (**Figure 5A**) and tumor weight in *Jak1*$^{fl/+}$*Ncr1Cre* (**Figure 5B**). In line, these tumors showed significantly reduced NK cell infiltration (**Figures 5C,D**). We failed to detect any difference in the frequency of NKG2D$^+$ tumor infiltrating NK cells (**Figure 5E**), which are crucial for the recognition of RMA-Rae1 tumors. In summary, our results show the reduced NK cell numbers combined with an impaired NK cell functionality in *Jak1*$^{fl/+}$*Ncr1Cre* mice are sufficient to significantly impair tumor growth control *in vivo*.

DISCUSSION

Dysregulation of the JAK-STAT signaling pathway is tightly associated with cancer development as well as immune disorders (7). The first JAK-linked disease discovered was the severe combined immune deficiency (SCID) which was characterized by NK cell abnormalities caused by LOF JAK3 mutations (26, 27). We here show that deletion of JAK1 in NKp46$^+$ cells leads to innate immune deficiency with loss of NK and ILC1 cells in peripheral organs, whereas JAK2 is redundant for NK cell survival and maturation.

Our previous work uncovered that loss of STAT5 in NK cells leads to severe reduction of NK cell numbers in peripheral organs (18). The loss of NK cells in *Stat5*$^{fl/fl}$*Ncr1Cre* mice was rescued by enforced expression of the pro-survival molecule BCL2 (28). This study defined STAT5 as a crucial survival factor for NK cells. STAT5B-deficient mice largely lack NK cells (29), in line with the fact that STAT5 signals downstream of cytokines that are vital for NK cell biology, such as IL-2 or IL-15 (30). IL-15 is crucial for NK cell development and survival as *Il15*$^{-/-}$ mice are largely devoid of peripheral NK cells (31). IL-15 signals via a receptor complex of γc receptor chain, IL-2Rβ, and IL-15rα (32). Knockout mice of each receptor chain prove an absolutely critical role for signals sent downstream of IL-15 for NK cell development (33, 34). To date, the contribution of γc-associated JAK3 to NK cell development has been well-established. Mice with JAK3 deficiency show a similar SCID phenotype as observed in human patients and NK cell development is blocked at the pre-NK progenitor stage (35, 36). Now we place previously underappreciated JAK1 as a crucial part of the IL-15/STAT5 axis in NK cells. *Jak1*$^{fl/fl}$*Ncr1Cre* NK cells show a developmental block at the iNK cell stage and an almost complete loss of NK cells in peripheral organs.

Interestingly, the consequences of JAK1 deletion for NK cells exceed the effects of STAT5-deficiency. Impairment of combined STAT3 and STAT5 activation may underlie the more pronounced loss of NK cells, as STAT3 has been shown to induce expression of the crucial NK cell survival gene *Mcl1* (37, 38). Alternatively,

JAK1 and STAT5 may have different half-lives which account for different frequencies of "just deleters"—NK cells which have just lost the gene but still carry the protein, that may explain differences.

The gene-dosage effect of JAK1-deficiency is reflected in NK cell numbers, while loss of one allele of JAK1 is dispensable for NK cell maturation. This suggests that activated STAT5 is rate limiting for NK cell survival but not maturation. Deletion of one allele of *Jak1* is also sufficient to significantly impair tumor surveillance based on decreased numbers of NK cells combined with a diminished functionality of the remaining NK cells. In accordance with our data, NK cell-specific deletion of the STAT5 target gene *Mcl1* leads to severe NK cell deficiency which causes a significant increase in metastatic burden (38).

JAKs may exhibit redundant functions and compensate for each other. Downstream of interferon γ receptor JAK1 can partially compensate for loss of JAK2 kinase activity (39). JAK2 has also been shown to phosphorylate STAT5 downstream of IL-15 during *in vitro* differentiation of NK cells (16). On the other hand, constitutively active JAK2 can only modestly compensate for the loss of JAK1 in stem cells suggesting a non-redundant role of JAK1 and 2 in HSCs. In line, we observe that in the absence of JAK1, JAK2 fails to compensate in activating STAT5, even under high dose of IL-2 *in vitro*, to allow NK cell survival. It remains to be elucidated whether compensatory effects are achievable by expressing a constitutively active form of JAK2. More importantly, we show that loss of JAK2 in NKp46^{+} cells is dispensable for NK cell survival. JAK2-deficient NK cells are fully mature, proving that NK cell-intrinsic JAK2 is not driving NK cell maturation. This also indicates that the impaired NK cell maturation in *Jak2*$^{fl/fl}$*Mx1Cre* mice (14) is most likely caused by NK cell-extrinsic functions of JAK2. One might speculate that the constitutive deletion of JAK2 alters the cytokine milieu.

Our data provide novel insights into results obtained upon JAK1/JAK2 inhibitor treatment. Inhibition of JAK1/JAK2 reduces NK cell numbers and maturation (14, 15), which according to our data is clearly the effect of inhibiting JAK1 rather than JAK2. We thus propose that the development of JAK2-specific inhibitors may be advantageous as they would leave NK cell-mediated tumor surveillance intact. This might be of particular relevance in the case of JAK2-driven leukemia such as JAK2^{V617F}-induced myeloproliferative neoplasms.

AUTHOR CONTRIBUTIONS

AW-S, KK, DP, and NL performed experiments. AW-S analyzed the data. VS, GS, and AD provided the resources. AW-S and VS wrote the manuscript.

FUNDING

The work was supported by the Austrian Science Fund FWF grant SFB-F61 to VS.

ACKNOWLEDGMENTS

We thank Philipp Jodl for great technical support and Manuela Kindl for maintaining and monitoring the mice. We are grateful to Andrea Hölbl-Kovacic, Dagmar Gotthardt, and Eva Maria Putz for critical feedback on the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.03108/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 07 January 2019
doi: 10.3389/fimmu.2018.02959

Extreme Phenotypes With Identical Mutations: Two Patients With Same Non-sense *NHEJ1* Homozygous Mutation

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Cernunnos/XLF deficiency is a rare primary immunodeficiency classified within the DNA repair defects. Patients present with severe growth retardation, microcephaly, lymphopenia and increased cellular sensitivity to ionizing radiation. Here, we describe two unrelated cases with the same non-sense mutation in the *NHEJ1* gene showing significant differences in clinical presentation and immunological profile but a similar DNA repair defect.

Keywords: XLF/Cernunnos, NHEJ1 mutation, DNA repair, severe combined immunodeficiency, lymphomagenesis, radiosensitive SCID (RS-SCID)

OPEN ACCESS

Edited by:
Andrew R. Gennery, Newcastle University, United Kingdom

Reviewed by:
Silvia Clara Giliani, Università degli Studi di Brescia, Italy
John Bernard Ziegler, Sydney Children's Hospital, Australia

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Specialty section:
This article was submitted to Primary Immunodeficiencies, a section of the journal Frontiers in Immunology

Received: *05 October 2018*
Accepted: *30 November 2018*
Published: *07 January 2019*

Citation:
Recio MJ, Dominguez-Pinilla N, Perrig MS, Rodriguez Vigil-Iturrate C, Salmón-Rodriguez N, Martinez Faci C, Castro-Panete MJ, Blas-Espada J, López-Nevado M, Ruiz-Garcia R, Chaparro-García R, Allende LM and Gonzalez-Granado LI (2019) Extreme Phenotypes With Identical Mutations: Two Patients With Same Non-sense NHEJ1 Homozygous Mutation. Front. Immunol. 9:2959. doi: 10.3389/fimmu.2018.02959

INTRODUCTION

Clinical Presentation and Laboratory Test Results

We report two patients harboring the same homozygous mutation in *NHEJ1* gene. Strikingly, their clinical phenotypes differed markedly. One presented with severe combined immunodeficiency whereas the other only had isolated thrombocytopenia and macrocytosis. Clinical characteristics are summarized in **Table 1**. Both patients presented severe lymphopenia: immunophenotyping of Patient 1 (P1) showed severe T and B-cell lymphopenia and normal to elevated NK cells ($T^-B^-NK^+$ phenotype) in contrast to Patient 2 (P2) who presented a milder immunophenotype. T-cell compartment also showed differences between the two patients: P1 had a more senescent T-cell phenotype with increase in $CD4^+$ and $CD8^+$ effector memory ($CCR7^-CD45RA^-$) and decrease naïve and recent thymic emigrants (RTE) (CD4+CD45RA+CD31+) T cells and a higher proportion of activated T-cells ($CD3^+HLA\text{-}DR^+$) (**Table 2**). Of note, P1 had a severe decrease in CD8+ population mimicking the one reported in ZAP70 or HLA class I deficiencies among patients with combined immunodeficiency/severe combined immunodeficiency.

B cells were severely reduced in both patients. The B-cell profile in P2 showed a normal proportion of naive, unswitched memory, switched memory, transitional, and plasmoblast B-cells. Serum immunoglobulins were reduced in P1. Due to age limitation, specific antibody response was

TABLE 1 | Clinical features of the patients with Cernunnos/XLF deficiency.

		P1	P2
	Origin	Caucasic	Caucasic
	Consanguinity	No	No
Age	Onset	1 m	9 m
	Current	1 year 10 m.	8 year 4 m.
Clinical features	Microcephaly	+	+
	Growth retardation	+	+
	Facial dysmorphism	−	+
Additional clinical features	Neurological manifestations	−	−
	Bone malformation	−	−
	Autoimmunity	−	+
	Cytopenias	−	+ (thrombocytopenia)
Infections	Respiratory tract infections	-	+
	Bacterial and opportunistic infection	−	−
	Urinary tract abnormalities	−	−
	Age at HSCT	4.5 m. and 9 months	7y. 10 m.
Outcome	Status	Alive and well (HSCT)	Alive and well (HSCT)

only tested in P2, showing a specific antibody deficiency: IgG and IgG2 levels against pneumococcus antigen were reduced while IgG levels against tetanus toxoid antigen showed normal values after vaccination. Regarding to the immunophenotype in T and B-cells, P1 had very low TRECs and KRECs copies, whereas P2 had preserved levels (**Table 2**).

The clinical presentation and immunologic features of both patients lead to suspicion of a primary immunodeficiency (PID). Accordingly, an in house targeted NGS sequencing panel for 192 PID related genes (**Table 3**) was performed and revealed a homozygous nucleotide substitution in exon 2 of *NHEJ1* gene (NM_024782, c.169C>T) that affects codon 57 and is predicted to result in a severely truncated protein (p.R57X), this mutational change has been previously described in two Cernunnos /XLF defective patients similar to P2 patient displaying microcephaly and slight lymphopenia (1–6). Sanger sequencing confirmed the variants in the patients which were inherited in autosomal recessive fashion from their healthy parents (**Figure 1**).

Increased sensitivity and decreased double strand breaks (DSB) rejoining to ionizing irradiation (IR) is a feature of Cernunnos /XLF deficient cells. Accordingly, a cell-survival assay revealed an increased IR sensitivity in fibroblasts from P2 similar to that observed in LIG4-deficient fibroblasts (**Figure 2A**). We also analyze DSB rejoining in primary fibroblasts from both patients (P1 and P2) by enumerating the rate of loss of γ-H2AX foci following exposure to γ-IR. The results showed impaired DSB rejoining after treatment with γ-irradiation and etoposide in both patients (**Figure 2B**). These findings are in agreement with previously published results in Cernunnos and LIG4-defective patients (7).

Comparative testing of DNA repair was also performed on T cells from the second patient (P2). Upon exposure of PBMCs to 10 Gy, induction and resolution of DNA damage was measured at various time points and a delayed kinetics of DNA repair were observed in P2 compared to a healthy control (**Figure 2B**).

METHODS

Cell Culture

Primary fibroblasts were grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin.

Immunofluorescence and Antibodies

To characterize the repair capacity of the patient's cells we scored the *in situ* modification of the histone variant H2AX, which is phosphorylated proximal to sites of DNA double-strand breaks. The number of phosphorylated H2AX (γH2AX) foci in a nucleus is reported to be directly proportional to the number of DSBs, and de-phosphorylation coincides with DSB repair. Primary skin fibroblasts were irradiated with ionizing irradiation (^{137}Cs) or treated with 20 mM Etoposide for 1 h. After the indicated treatments, the slides were washed with PBS, fixed using 4% formaldehyde for 10 min at room temperature and permeabilized with 0.5% Triton X-100 for 5 min. Cells were incubated with primary antibodies for 1 h, the slides were washed with PBS and the bound antibodies were revealed by IgG Alexa fluor antibodies (Invitrogen). Nuclei were counterstained with DAPI, and slides were mounted for immunofluorescence. Images were taken with a fluorescent microscopy (Zeiss AxioImages.A1, Carl Zeiss). In a single experiment at least 30 cells per sample were counted.

Survival Assay

Primary skin fibroblasts were irradiated with ionizing radiation (^{137}Cs). After irradiation, the cells were seeded at a density of 1×10^4 cells/mL in T75 flasks in triplicate. To evaluate cell sensitivity to γ-IR (1 and 3 Gy), adherent cells were trypsinized and counted 11 days later.

TABLE 2 | Immunologic features of patients with Cernunnos/XLF deficiency.

Parameter	RefValues (children)	P1	P2
Lymphocyte (n°/μL)	2500–6000	809	879
T CELLS			
CD3+ n°/μL (%)	1400–4300 (52–88)	60 (7)	661 (75)
CD3+TCRab (%)	85–99	5	54
CD3+TCRγδ (%)	2–15	1	16
CD3+HLA–DR+ (%)	0–10	22	7
CD3+TCRαβCD4–CD8- (%)	0–2.5	0.2	0.7
CD4+ n°/μL (%)	1000–2500 (33–55)	53 (7)	304 (35)
CD4+CD45RA+CCR7+(Naïve) (%)	32–82	4.1	45.4
CD4+CD45RA–CCR7+ (CM) (%)	15–30	41.5	28.9
CD4+CD45RA–CCR7– (EM) (%)	8–30	53.9	23.8
CD4+CD45RA+CCR7– (E) (%)	0.4–4	0.4	1.89
CD4+CD45RA+CD31+ (%)	44–60	2	ND
CD8+ n°/μL (%)	400–1400 (17–34)	6 (1)	264 (30)
CD8+CD45RA+CCR7+(Naïve) (%)	30–80	15.3	72.0
CD8+CD45RA−CCR7+ (CM) (%)	3–28	16.2	4.5
CD8+CD45RA–CCR7– (EM) (%)	17–40	59.5	16.7
CD8+CD45RA+CCR7– (TEMRA) (%)	2–15	9	6.8
TRECS (copies/punch)	> 10	< 10	50
NK CELLS			
CD56+CD3– n°/μL (%)	100–650 (2–20)	671 (83)	191 (21.7)
B CELLS			
CD19+ n°/μL (%)	400–1500 (9–28)	49 (6)	22 (2.5)
CD19+CD27+ (%)	7–19	ND	32
CD19+IgD+CD27– (%Naive)	75–89	ND	63
CD19+IgD+CD27+ (%MZ)	2.6–7.1	ND	14.9
CD19+IgD–CD27+ (%SW)	4.5–20	ND	17.10
CD19+CD38hiIgM+ (%Transitional)	3–10	ND	13
Plasmablasts	0.5–5	ND	4.6
KRECS (copies/punch)	>10	<10	100
SERUM IMMUNOGLOBULINS (mg/dl)			
IgG (mg/dL)	600–1230	446	779
IgA (mg/dL)	30–200	18	<6.67
IgM (mg/dL)	50–200	40	109
SPECIFIC ANTIBODIES			
IgG vs. Pneumococcus (mg/dL)	>5.4	ND	2.9
IgG2 vs. Pneumococcus (mg/dL)	>2.4	ND	0.36
IgG vs. Tetanus toxoid (IU/mL)	>0.1	ND	9.10

ND, not determined.

Flow Cytometry

Proportions and lymphocyte count of T-, B-, and NK-cells were determined in blood samples using conjugated mouse anti-human monoclonal antibodies and data were collected by flow cytometry using a Navios Cytometer (Beckman Coulter, Madrid, Spain) and analyzed with Kaluza 1.5a software (Beckman Coulter, Indianapolis IN, US).

PBMCs from patient and healthy controls were irradiated with 10Gy, fixed and stained for CD3, CD19, and phospho-histone H2AX. Mean fluorescence intensities (MFI) of γH2AX were evaluated on gated CD3+ lymphocytes.

Immunoglobulins

Total serum immunoglobulins (IgG, IgA, IgM, and IgE) were measured by nephelometry (Beckman Coulter, Madrid, Spain).

NGS and Sanger Sequencing

Genomic DNA was extracted from EDTA whole blood using a MagNa Pure Compact Nucleic Acid Isolation Kit (Roche, Madrid, Spain). Missense *NHEJ1* mutation was identified by targeted next-generation sequencing with an in-house designed panel of 192 genes involved in primary immunodeficiencies (PID) (Ampliseq, Thermo Fisher, Madrid, Spain) and confirmed

TABLE 3 | Gene-panel related to PID.

ACT1 ADA AICDA AIRE AK2 AP3B1 AP3D1 ATM BCL10 BLNK BTK C3 CARD11 CARD9 CASP10 CASP8 CD127 CD19 CD20 CD21 CD27 CD3D CD3E CD3G CD3Z CD45 CD79A CD79B CD81 CD8A CEBPE CECR1 CLEC7A COPA CORO1A CTLA4 CTPS1 CTSC CXCR4 CYBA CYBB DCLRE1C DKC1 DNMT3B DOCK2 DOCK8 ELANE EVER1 EVER2 FADD FCGR3A FOXN1 FOXP3 G6PC3 GATA2 GFI1 HAX1 HOIL1 ICOS IFNGR1 IFNGR2 IGHM IGLL1 IKAROS IKBA IKBKB IKBKG IL10 IL10RA IL10RB IL12B IL12RB1 IL12RB2 IL17F IL17RA IL17RC IL1RN IL21 IL21R IL2RA IL2RG IL7 IRAK4 IRF3 IRF7 IRF8 ISG15 ITGB2 ITK JAGN1 JAK3 KIND3 KRAS LAMTOR2 LCK LIG4 LPIN2 LRBA LYST MAGT1 MALT1 MAP3K14 MCM4 MEFV MHC2TA MRE11 MST1 MVK MYD88 NCF1 NCF2 NFKB1 NFKB2 NHEJ1 NHP2 NLRC4 NLRP12 NLRP3 NOD2 NOP10 NRAS ORAI1 p40phox PGM3 PIK3CD PIK3R1 PLCG2 PMS2 PNP POLE1 PRF1 PRKDC PSMB8 PSTPIP1 PTPN6 RAB27A RAG1 RAG2 RFX5 RFXANK RFXAP RLTPR RMRP RNF168 RORC RTEL1 SH2D1A SMARCAL1 SP110 SPINK5 STAT1 STAT2 STAT3 STAT5B STIM1 STX11 STXBP2 TAP1 TAP2 TAPBP TBK1 TCF3 TCN2 TERC TERT TINF2 TIRAP TLR3 TMEM173 TNFRSF13B TNFRSF13C TNFRSF1A TNFRSF5 TNFRSF6 TNFSF5 TNFSF6 TRAF3 TRIF TRNT1 TTC7A TWEAK UNC119 UNC13D UNC93B1 UNG VPS45 WAS WIPF1 XIAP XRCC4 ZAP70 ZBTB24

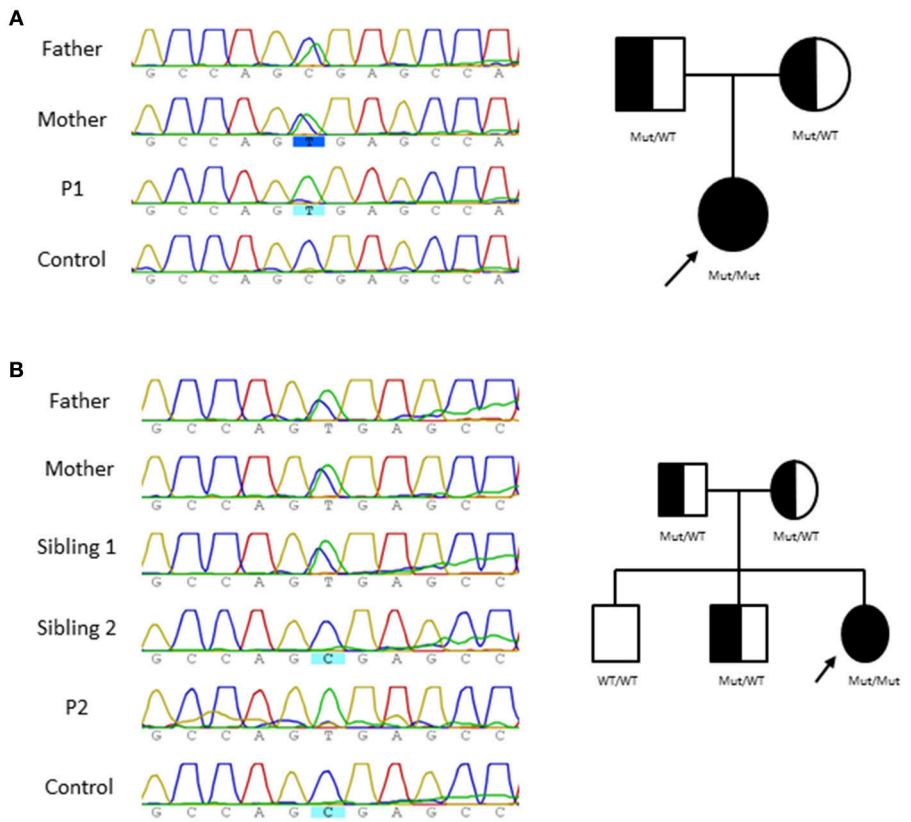

FIGURE 1 | Pedigree and genomic sequence analysis of *NHEJ1* gene. Genomic *NHEJ1* DNA sequence showing c.169C>T mutation and Family tree in P1 **(A)** and P2 **(B)**.

by PCR and Sanger sequencing using an ABI PRISM 3130 genetic analyzer.

TRECs and KRECs

TRECs, KRECs, and beta-actin (ACTB) copy numbers were determined from dried blood spots (DBS, punches of 3.2 mm) using triplex real-time quantitative polymerase chain reaction (RT-PCR) (TIB MOLBIOL) and run in a Light Cycler 480 II from Roche Diagnostics.

Ethics Statement

The protocols of this study were approved by the Institutional Review Board of Hospital Universitario 12 de Octubre (Madrid, Spain) and written informed consent was obtained from all subjects/caregivers in accordance with the Declaration of Helsinki.

BACKGROUND

DNA non-homologous end-joining (NHEJ) is the major DNA double strand break (DSB) repair pathway in mammalian cells. NHEJ also functions during immune development rejoining the programmed DSBs introduced during V(D)J recombination (7). Most of the patients deficient in NHEJ components display radiosensitivity and severe combined immunodeficiency (SCID), a phenotype which has been called radiosensitive SCID (RS-SCID). To date, mutations in five genes encoding components of the NHEJ pathway, *LIG4*

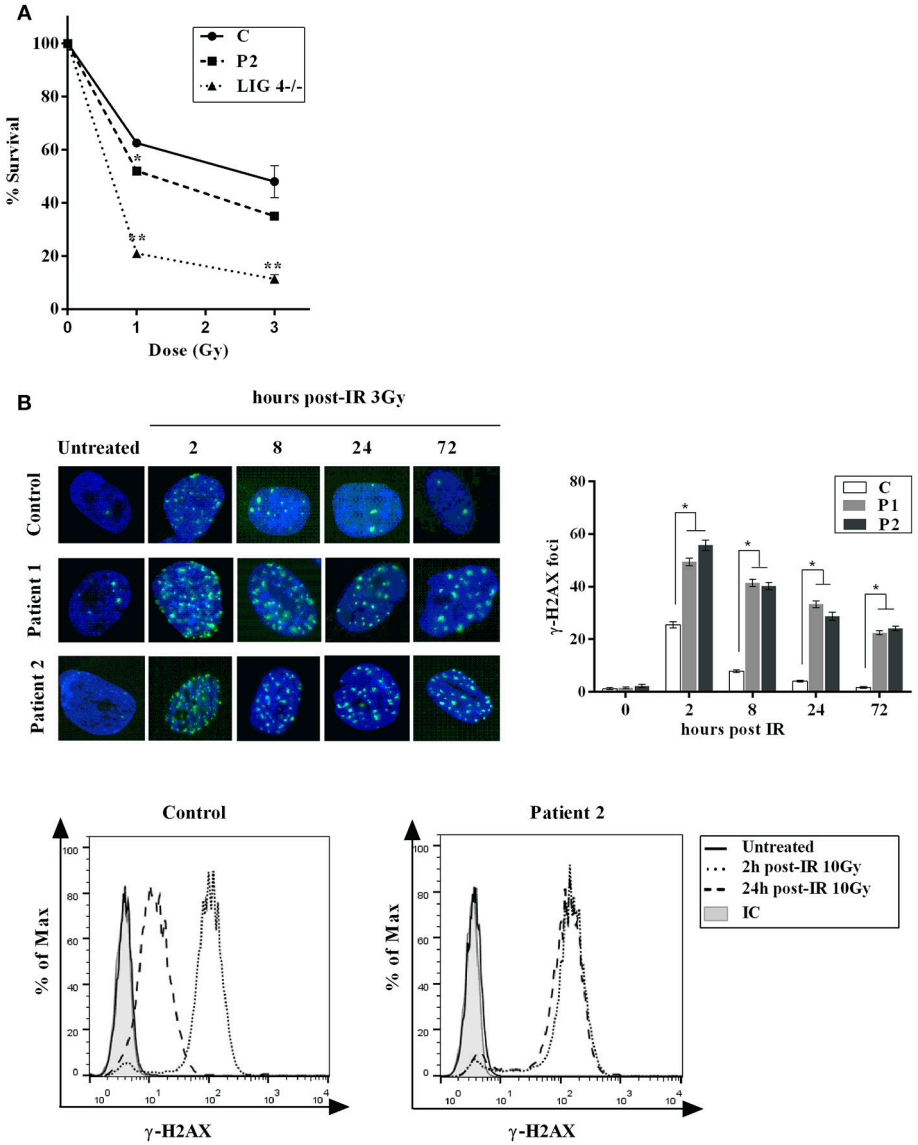

FIGURE 2 | Cellular Response to DNA Damage. **(A)** Clonogenic survival assay in XLF-deficient fibroblasts. Cell survival following IR with γ-rays (1 and 3Gy) was assessed in primary fibroblasts from normal control (C) and patient (P2). LIG4-deficient fibroblasts (LIG4$^{-/-}$) were used as a radiosensitive control. The results represent the mean and standard deviation of two separate experiments and are expressed as percentages of survival cells relative to unirradiated primary fibroblasts. **(B)** Top panel: Primary fibroblasts from control (C) and patients (P1 and P2) were irradiated with 3 Gy and fixed at given time points post-irradiation before staining with anti- γ -H2AX. Nuclei were stained with DAPI. Numbers of γ -H2AX foci per nucleus were determined at indicated time points after irradiation (average number of γ -H2AX foci per nucleus in 30 cells). Error bars represent the SD from 3 independent experiments. Bottom panel: γ-H2AX detection by flow cytometry was performed in PBMCs from P2 and control. Mean fluorescence intensities (MFI) are shown as histograms (unirradiated, 2 and 24 h) compared to isotype (IC). Persistence of γ H2AX signal at 24 h post-treatment in P2 is indicative of a general DNA repair defect. *$p < 0.05$, **$p < 0.01$.

(encoding DNA ligase IV), *NHEJ1* (encoding Cernunnos), *PRKDC* (encoding DNA-PKcs), *DCLRE1C* (encoding Artemis), and *XRCC4* (encoding XRCC4) have been identified in patients. Although RS-SCID represents the extreme phenotype, radiosensitivity coupled with variable immunodeficiency ranging from Omenn's Syndrome to CID has been observed. Additional features are also observed in some patients, including microcephaly and severe growth delay. However, all of these patients are at risk of malignancies, particularly lymphoma (6).

History

Cernunnos, was identified through both cDNA complementation of cells derived from a IR-sensitive immunodeficient patient (1) and through a yeast two-hybrid screen for XRCC4-interacting partners (8). Cernunnos is a homolog of Nej1,

one of the NHEJ factors identified in yeast (9, 10). Data from several studies suggest that although Cernunnos may not be strictly required for NHEJ, the loss of Cernunnos does affect NHEJ and interestingly DNA repair defect in these patients is quite similar to that found in LIG4 syndrome patients (11).

Review of Similar Cases

Cernunnos/XLF deficiency in human results in extreme sensitivity to IR, microcephaly, and growth retardation, but the effect on the immune system is variable (1–4, 8). Mutations in *NHEJ1* have been described previously in 27 patients with clinical features comparable to LIG4 deficiencies. Of contrast, XRCC4 deficiency shares the radiosensitivity and neurological impairment but not overt immunodeficiency (5, 12, 13).

Most of the Cernunnos/XLF deficient patients are hypersensitive to IR and have a significant NHEJ defect (1, 14) similar to LIG4 deficiency, which belong to NHEJ ligation complex; immunodeficiency, however, is milder compared with defects in NHEJ factors involved in hairpin opening as Artemis and DNA-PKcs.

In this study, we reported two patients who present the same c.169C>T mutation in *NHEJ1* gene but different immunologic features (**Table 2**). P2 presented with mild T lymphopenia, hypersensitivity and NHEJ repair defect, typical for patients with Cernunnos/XLF defects (1, 5). On the other hand, P1 presented a more severe phenotype (T-B-); hypersensitivity and NHEJ repair defect, however, was similar to P2. These findings indicate that patients with same *NHEJ1* mutation and DNA repair defect may show great variability in the clinical phenotype.

Patients with homozygous mutations (p.R178X) in *NHEJ1* gene have been previously reported. Two patients died at 1.5 and 4 years (1, 2), while another of the patients remains alive at the age of 8 years (without HSCT) (5). However, none of these patients presented the severe T lymphopenia observed in our first patient.

One explanation for the different degree of lymphopenia found in Cernunnos-deficient patients (P1 and P2) might be the existence of alternative DNA repair proteins that only work to repair DSBs generated during lymphocyte development (3). It has been described that RAG complex and Cernunnos functionally overlap in the repair of DNA breaks during antigen receptor assembly ensuring stabilization of DNA ends after DNA cleavage by RAG (15). ATM and/or ATM-dependent DRR factors, as 53BP1, would contribute to the RAG-DSB stabilization and the recruitment of NHEJ pathway proteins. Severely impaired joining of RAG-generated DSBs in cells that are deficient for Cernunnos and either ATM, 53PBP1or H2AX has been observed (16). Thus, genetic factors such as mutations or polymorphisms in some of these proteins could affect the VDJ recombination process and explain the severe T lymphopenia observed in P1. As Cernunnos is not usually found in the context of a patient with a SCID phenotype we cannot rule out a digenic cause (particularly 53BP1, as other VDJ recombination genes were wild-type in our panel).

DISCUSSION

Diagnosis and Treatment

The assignment of a timely and accurate diagnosis is of paramount importance in the management of patients with defects in DNA repair, as HSCT is the only curative therapy available. Usually the repair defect in these disorders is assessed by immunofluorescence assays of irradiation-induced γ-H2AX foci using skin fibroblasts. Flow cytometry (FC) can be applied as a rapid diagnostic tool for DNA repair disorders (17, 18). Therefore, we have used flow cytometry to analyze PBMCs from P2 and the results showed a DNA repair defect similar to that obtained in skin fibroblasts (data not shown) (19).

Hence, a high throughput, sensitive and reliable assay to quantify γ-H2AX foci in PBMCs isolated from blood samples would be a valuable tool to diagnose these patients and thus allow HSCT without delay.

In addition, it would also be helpful in cancer patients to individualize and to guide the dosing of ionizing radiation (IR) and/ or genotoxic agents to avoid accumulation of cells with genomic instability that could accelerate cancer development.

In the era of newborn screening an abnormal TREC assay should be followed by NGS approach as Cernunnos/XLF deficiency may present early in life as SCID, as other RS-SCID defects (20). Since genetic diagnosis takes time, functional radiosensitivity assays in peripheral blood may lead to the correct diagnosis and avoid exposure to alkylating agents during the conditioning regimen even in the absence of a genetic diagnosis (21).

The patients presented in this work are alive and well and both patients after undergoing HSCT. Of note, P2 has survived to age 7 years. It has been reported that the Cernunnos/XLF deficient patients may survive the first years of life, or even up to 18 years, without HSCT. However, due to the comorbilities that these patients face, it is highly recommended HSCT pre-emptively, rather than expect the appearance of a malignant refractory disease disregarding the age at diagnosis (21).

Given the extreme rarity of the disorder, the appearance of two unrelated homozygous cases in non-consanguineous family with the same mutation seems unlikely. However, we can offer three reasons to discard consanguinity in both kindreds: First. Geographical: Both families are more than 450 km apart each other. Both kindreds deny any consanguinity (even far) ties. Second, Molecular: HLA typing were done in both families. It is shown HLA haplotypes generated by segregation analysis in both families: Family 1, Father: A*30, B*18, DRB1*03 and A*02, B*40, DRB1*08; Mother: A*01, B*37, DRB1*13 and A*-, B*57, DRB1*07. Family 2, Father: A*02, B*39, DRB1*11 and A*29, B*51, DRB1*04; Mother: A*11, B*40, DRB1*04 and A*11, B*07, DRB1*15. In conclusion, both families did not share any HLA haplotype (**Table 4**). Third, No other mutations in PID genes that associate radiosensibility (*LIG4, PRKDC, DCLRE1C, ATM, RNF168*) were found.

TABLE 4 | HLA typing in P1 and P2 families.

HLA	P1 Family			P2 Family		
	P1	**Father**	**Mother**	**P2**	**Father**	**Mother**
HLA-A	A*01, A*30	A*02, A*30	A*01, A*Null	A*02, A*11	A*02, A*29	A*11, A*11
HLA-B	B*18, B*37	B*18, B*40	B*37, B*57	B*07, B*39	B*39, B*51	B*07, B*40
HLA-DRB1	DRB1*03, DRB1*13	DRB1*03, DRB1*08	DRB1*07, DRB1*13	DRB1*11, DRB1*15	DRB1*04, DRB1*11	DRB1*04, DRB1*15

CONCLUDING REMARKS

We report two unrelated cases diagnosed with Cernunnos/XLF deficiency. Both patients showed a similar DNA repair defect and increased cellular sensitivity to ionizing radiation as per *in vitro* assays. However, clinical presentation and immunological profile were extremely different. The first patient showed a senescent phenotype with decreased TRECs, RTE and naive T-cells counts suggesting that the sustained self-renewal of T-cell pool was impaired, while P2 presented only a slight reduction in T-cell counts as it has been reported in Cernunnos/XLF deficient patients reported up to date with normal TRECs/KRECs and IgG levels (22).

AUTHOR CONTRIBUTIONS

JB-E and RR-G performed the laboratory work for this study and computational predictions. MR, RR-G, ML-N, RC-G, MC-P, MP, and JB-E performed the laboratory work for this study. CR, CM, ND-P, NS-R, CR, and LG-G were responsible for the clinical management of the patients. LA, MR, JB-E, and LG-G designed the research and drafted the manuscript. All authors approved the final version of this manuscript.

FUNDING

This research was supported by grants from the Spanish Health Research Fund FIS PI16/2053 to LA and LG-G, PI16/0044 to LG-G, MINECO grants SAF2014-54708-R, SAF2016-81876-REDT, and RTI2018-095673-B-I00. CAM grant B2017/BMD3673. The project has been co-financed with FEDER funds.

ACKNOWLEDGMENTS

We thank both families and Pablo Morales, Isabel Castro, María José Díaz-Madroñero, Marta Martín Cabezudo, and Maria Teresa Fernandez González for their personal and technical assistance and Jaime de Inocencio for critical review of the article.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 04 January 2019
doi: 10.3389/fimmu.2018.03060

Epstein-Barr Virus (EBV)-Related Lymphoproliferative Disorders in Ataxia Telangiectasia: Does ATM Regulate EBV Life Cycle?

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Epstein-Barr virus (EBV) is an ubiquitous herpesvirus with a tropism for epithelial cells (where lytic replication occurs) and B-cells (where latency is maintained). EBV persists throughout life and chronic infection is asymptomatic in most individuals. However, immunocompromised patients may be unable to control EBV infection and are at increased risk of EBV-related malignancies, such as diffuse large B-cell lymphomas or Hodgkin's lymphomas. Ataxia telangiectasia (AT) is a primary immunodeficiency caused by mutations in the *ATM* gene and associated with an increased incidence of cancers, particularly EBV-associated lymphomas. However, the immune deficiency present in AT patients is often too modest to explain the increased incidence of EBV-related malignancies. The ATM defect in these patients could therefore impair the normal regulation of EBV latency in B-cells, thus promoting lymphomagenesis. This suggests that ATM plays a role in the normal regulation of EBV latency. ATM is a serine/threonine kinase involved in multiple cell functions such as DNA damage repair, cell cycle regulation, oxidative stress, and gene expression. ATM is implicated in the lytic cycle of EBV, where EBV uses the activation of DNA damage repair pathway to promote its own replication. ATM regulates the latent cycle of the EBV-related herpesvirus KSHV and MHV68. However, the contribution of ATM in the control of the latent cycle of EBV is not yet known. A better understanding of the regulation of EBV latency could be harnessed in the conception of novel therapeutic strategies in AT and more generally in all ATM deficient EBV-related malignancies.

Keywords: ataxia telangiectasia, Epstein Barr virus, latency, B-cell, primary immune deficiency, lymphomagenesis, hodgkin lymphoma, non-hodgkin lymphoma

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Primary Immunodeficiencies, a section of the journal Frontiers in Immunology

Received: *21 September 2018*
Accepted: *10 December 2018*
Published: *04 January 2019*

Citation:
Tatfi M, Hermine O and Suarez F (2019) Epstein-Barr Virus (EBV)-Related Lymphoproliferative Disorders in Ataxia Telangiectasia: Does ATM Regulate EBV Life Cycle? Front. Immunol. 9:3060. doi: 10.3389/fimmu.2018.03060

INTRODUCTION

Epstein-Barr virus (EBV) is a γ-herpesvirus that infects 95% of adults worldwide. EBV targets epithelial cells where lytic replication occurs, and B-cells where latent infection is established. The distinct phases of EBV infection are carefully controlled throughout the infected host's life, and chronic infection in immunocompetent individuals is mostly asymptomatic (1). Control of chronic EBV infection in immunocompromised patients may fail, leading to lymphoproliferative disorders as well as *bona fide* lymphomas (hereafter referred to as EBV-LPD) (2).

Several primary immune deficiencies (PID) are associated with poor responses to EBV and are also associated with a high risk of EBV-LPD (3). Inherited genetic abnormalities causing PID are often associated with poor or absent EBV-specific cytotoxic T-cell response and studies on PID and their underlying molecular mechanisms have led to a better understanding of immunological and cellular processes that control herpesvirus infection.

EBV infects B-cells both *in vivo* and *in vitro* and can lead to their immortalization. EBV latent genes drive the activation and differentiation of B-cells (4). Deregulation of this complex and dynamic interaction of viral gene expression and cellular activation may lead to cell transformation.

Ataxia telangiectasia (AT) is a rare PID caused by mutations in the *ATM* gene. AT patients are at increased risk of cancer, including EBV-LPD (5). However, the extent of immune compromise in AT is variable, and many patients have only minor immunological alterations (6). ATM is involved in many functions ranging from DNA repair to gene expression. Based on the paradoxical observation that EBV-LPD frequency is increased in ATM patients while they do not exhibit major T-cell deficiency, we raise the hypothesis that the defect of ATM in EBV-infected cells could play a role *per se* in the control of EBV latency, favoring a latent program more prone to lymphomagenesis. We review here the characteristics of AT and discuss the immunological and cellular abnormalities that may confer susceptibility to EBV-related malignancies.

CLINICAL AND IMMUNOLOGICAL FEATURES OF AT

AT is an autosomal recessive disorder caused by biallelic mutations in the *Ataxia-telangiectasia mutated* (*ATM*) gene. Its estimated incidence is about 1/300.000 live births (7). AT was first reported by Syllaba and Henner in 1926 (8), further characterized by Denise Louis-Bar (9), and finally named by Boder and Sedgwick (10).

AT is characterized by progressive neurodegeneration leading to ataxia, oculo-cutaneous telangiectasia, variable degrees of immune deficiency, and susceptibility to cancer. AT is clinically heterogeneous, the classic form starts typically around 4 years of age, most patients becoming wheelchair-bound by the age of 10. Milder forms of AT may appear later and develop slowly. AT patients have a reduced life expectancy with a median survival of 19 to 25 years (11, 12). Mortality is mostly due to respiratory tract infections and cancers (6).

AT patients have a variable immunodeficiency, rarely progressive, with some patients not affected at all. Complete loss of ATM kinase activity leads to a more severe immunologic phenotype. B-cell and T-cell lymphopenia may be present in ∼70% of AT patients (6, 13). Over 60% of patients also have abnormal serum immunoglobulin levels, most notably a deficiency of IgG4 (65%), IgA (63%), and IgG2 (48%) (6).

GENETIC AND MOLECULAR BASIS OF AT

The *ATM* gene (∼160kB) was cloned in 1995 (14). Over 400 mutations of *ATM* have been reported, spanning all 66 exons of the gene (Leiden Open Variation database). Most of these mutations lead to complete loss of ATM protein expression, but missense, and splice mutations can lead to the expression of a protein with residual kinase activity (15).

The *ATM* gene encodes a 350 kDa serine/threonine kinase belonging to the phosphatidylinositol 3-kinase-related kinase (PIKK) family (16). ATM is mostly located in the nucleus, but ∼20% are found in the cytoplasm, mainly in peroxisomes, endosomes, and as soluble proteins (17). ATM is involved in many cellular functions, including cell cycle checkpoint, apoptosis, oxidative stress, mitochondrial metabolism, gene regulation, and telomeres maintenance, but one of its major roles is its involvement in double strand break (DSB) repair (18). DSB can occur by endogenous processes, during replication fork collapse, V-(D)-J recombination or class switching, and can be induced by exogenous factors such as chemotherapy. In the canonical pathway, ATM is partially activated few seconds after DSB, probably after the relaxation of chromatin adjacent to the break. The MRN complex (Mre11/Rad50/Nbs1) recognizes the site of DNA break and in turn recruits ATM (**Figure 1**). Autophosphorylation of the ATM dimer occurs after its association with the MRN complex and precedes the formation of the fully active monomer forms (17).

ATM then phosphorylates H2AX, a variant of the histone H2A family, forming γH2AX foci that serve as a scaffold for the recruitment of DNA repair proteins such as MDC1, 53BP1, and BRCA1. Several other important partners are phosphorylated by ATM, such as ChK2 and p53, which initiate the downstream events of DNA repair and induce cell cycle arrest or apoptosis if DNA repair fails (18). The repair mechanism involves non-homologous end joining, an error-prone process occurring in G1/S phase (19), or homologous recombination, a faithful process in G2/M when sister chromatid is available (20).

As a result, defective DSB repair in AT patients elicit genomic instability that leads to B-cell and T-cell lymphopenia, premature senescence, and cancer. ATM plays a key role in the development of lymphocytes, allowing DSB repair occurring during B-cell, and T-cell differentiation. However, DSB repair may be possible via the alternative end-joining, an error prone, and poorly understood ATM independent pathway, which could explain the modest degree of lymphopenia in AT patients (21). Some lymphoid malignancies, such as mantle cell lymphoma, diffuse large B-cell lymphomas, and Hodgkin lymphomas, are also associated with acquired ATM mutations (22).

Patients with AT are at increased risk of cancer, especially lymphoid malignancies (23). To estimate precisely the risk of cancers in patients with AT, we conducted a retrospective study of cancers of 279 AT patients from the registry of the French National Reference Center for Primary Immune Deficiencies (CEREDIH) (5) and found that 25% of AT patients develop malignancies, the most common of which were aggressive non-Hodgkin's lymphomas (55% of all cancers), followed by Hodgkin's lymphoma (17%), leukemia (16%), and various solid tumors (12%). EBV was associated with 100% of Hodgkin's lymphomas and 50% of B-cell non-Hodgkin's lymphomas.

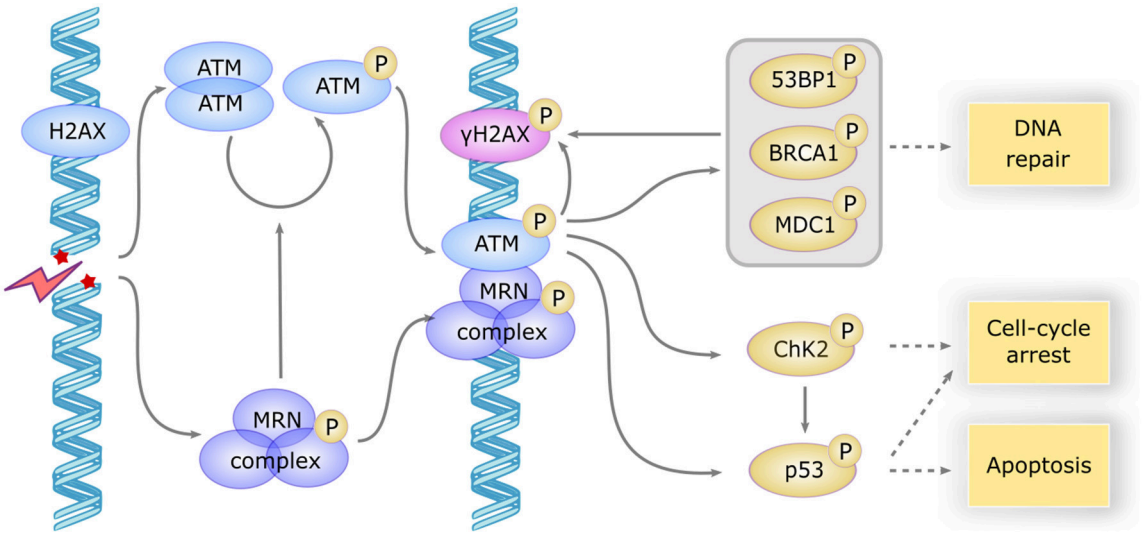

FIGURE 1 | ATM activation and downstream signaling in response to DSB. DSB induces a rapid activation of the ATM dimer and the MRN complex, which in turn induces the autophosphorylation and monomerization of ATM protein. ATM becomes fully active and phosphorylates a large subset of downstream proteins including γH2AX which serves as a scaffold for the recruitment of repair proteins, MDC1, BP53, and BRCA1 involved in DNA repair, CHK2 involved in cell cycle arrest and p53 involved apoptosis induction. CHK2 also phosphorylates P53 to promote cell-cycle arrest or apoptosis.

EPSTEIN-BARR VIRUS

EBV belongs to the *herpesviridae* family of large enveloped double-stranded DNA viruses, and was first identified in Burkitt's lymphoma in the 1960s (24). All herpeviruses have 2 distinct phases of infection, lytic, and latent. The lytic EBV infection occurs in oropharyngeal epithelial cells and leads to viral replication and production of multiple virions that spread into the underlying lymphoid tissues and infect naive B-cells (25). In the latter, EBV enters latency, a phase during which the viral genome is maintained as a nuclear episome, and only a few viral genes are transcribed (26).

Expression of the latency genes is tightly controlled by cellular and viral factors. Cellular immunity is also strongly induced during EBV infection and contributes to the elimination of infected cells expressing immunogenic lytic and latent antigens (27). Analysis of viral gene expression in EBV-associated cancers has led to a model in which EBV transitions through several latency gene expression programs (latency III, II, I) (28). Depending on the stage of latency, cells can express nuclear proteins (EBNA-1,−2,−3a,−3b,−3c, and -LP), latent membrane proteins (LMP-1,−2A, and−2B), non-coding RNAs (EBER-1 and−2), and several microRNAs (4). According to this model, EBV uses different latency programs to exploit B cell maturations pathways in the germinal center, leading the infected B-cell to the long-lived memory B-cell pool (28). The viral proteins LMP1 and LMP2 replicate the signals induced in B cells during the germinal center reaction and cause their proliferation. LMP1 mimics the signal of the activated CD40 (29) and induces EBV-LPD in transgenic mice (30), and LMP2 mimics the signal of the antigen activated B-cell receptor (31) and allow B-cell development even in the absence of normal B-cell receptor signaling (32).

EBV latency genes and non-coding RNAs may have oncogenic properties or interfere with cellular activation pathways leading to proliferation of B-cells as immunoblasts (33). Indeed, *in vitro* EBV infection of naïve B-cells leads to immortalized and fully transformed lymphoblastoïd cell lines in latency III that can induce tumors in nude mice (34). During latency III, EBNA-2 interacts with the target of the Notch pathway RBP-Jκ (35) and recruits co-activators, allowing the transcription of many genes involved in proliferation including *MYC* (36). EBNA-2 also promotes the transcription of all other latency genes, including the EBNA-3 family which gradually replaces EBNA-2 in its association with RBP-Jκ (37). The EBNA-3 family recruits co-repressors and inhibits the transcription of EBNA-2 induced genes. Indeed, prolonged expression of MYC could induce early senescence and be harmful to the virus (38). Infected B-cells migrate to the germinal center and progressively lose the expression of EBNA-2 and EBNA-3, escaping the immune control (latency II). During their transit through the germinal center to finally reach the memory B-cell pool, infected B-cells further restrict the expression of latency genes to express only EBNA1, a viral protein involved in the tethering of the viral episome to the nuclear chromatin (latency I) (39). EBV persists then indefinitely in memory B-cells (40). When these infected cells differentiate into plasma cell following an encounter with their cognate antigen, the transcription factor XBP-1 induced during plasma cell differentiation activates the expression of BZLF1, a viral protein sufficient to induce the lytic cycle (41). Virions are then released into the bloodstream and can re-infect the oropharynx epithelium to perpetuate the infectious cycle.

Occasional lytic replication occurs in the oropharynx of healthy individuals, and evidence shows that the majority of these cells do not complete the full lytic cycle despite BZLF1

expression (42–44). Abortive cycles have also been described in EBV-LPD such as Burkitt lymphomas (45), or diffuse large B-cell lymphomas (46).Several BZLF1-induced viral genes have anti-apoptotic or immunomodulatory properties, allowing the lytic cell to avoid cell-death (47). These genes are also activated during the abortive cycle and growing evidences suggests that these genes may contribute to lymphomagenesis. Indeed, the rate of EBV-LPD induced by EBV infection of humanized mice is severely reduced when BZLF1-deficient virus is used (48).

MECHANISMS UNDERLYING AT SUSCEPTIBILITY TO EBV

AT Immune Dysfunction

Cytotoxic T-cells (CTL) play a major role in controlling the expansion of EBV infected B-cells. Primary EBV infection in young adults leads to infectious mononucleosis associated with a massive CTL expansion (49). The viral EBNA-3 proteins and, to a lesser extent, EBNA-2 induce a potent CTL response (27), which eliminates most infected cells.

Thymic hypoplasia has been described in AT (13), and may be the cause of the various degree of T-cell lymphopenia, especially of the naïve T-cell population (CD3+ CD4+ CD45RA+ and CD3+ CD8+ CD45RA+), found in these patients (50). Similarly, TCR excision circles (TRECs) as a measure of thymic output, can be useful for early diagnosis of AT (51). The naïve T-cell defect may also contribute to the described defect in IFNγ (52) which is important for defense against viruses and bacteria, and immunosurveillance of cancers.

However, severe viral or opportunistic infections are not frequent (6) and most AT patients seem to have an intact T-cell response (53, 54). The vaccine response is also functional, with a totally normal response for some patients and a reduced response for others (6).

Nonetheless, AT patients have recurrent sinopulmonary bacterial infections that seems to increase with age (55). This could be explained by the IgA deficiency associated with an increased risk of chronic rhinosinusitis (56), the IFNγ production deficiency (52), but also by the progressive neurodegeneration; AT patients may have mastication and swallowing difficulties that worsen with age leading to an unintentional inhalation of food (6, 57).

Several observations suggest that γδ T-cells play a role in the control of viral infections. γδ T-cells represent 1–10% of the total T-cells and recognize a distinct range of antigenic targets (58). Infusion of pamidronate (known to activate γδ T-cells) in humanized mice significantly reduced EBV-LPD. AT patients seem to have an increase in the γδ T-cell population (59).

A humoral response is also generated but plays a limited role in the control of EBV infection (60). The NK-cells response is also important in the control of primary infection (61) but the number of NK-cells and their function in AT patients seems normal (62), despite an expansion of the CD56 bright population (CD3- CD16+ CD56+). This population is important for cytokines production but is not sufficient to overcome the IFNγ production defect (52). In summary, these immunological defects in AT patients do not seem sufficient to explain the increased incidence of EBV-LPD.

Some studies have also suggested a role for invariant NK-T cells (iNKT) in EBV control. These cells are restricted by CD1d, a class I MHC-like molecule exposing lipid antigens. Patients with mutations in *SH2D1A* (encoding SAP) or in *BIRC4* (encoding XIAP) have little or no iNKT and are very sensitive to EBV. However, these mutations also affect normal T-cells function, making it unclear if the iNKT defect is responsible for the disease (63, 64). Infused iNKT in immunodeficient mice injected with EBV transformed cells show reduced tumor formation (65). Similarly, a study on EBV-infected peripheral blood mononuclear cells *in vitro* showed higher transformation efficiency when iNKT were previously depleted (66). There has been to date no full exploration of iNKT levels in AT patients, but a small study of 3 patients suggests that AT patients do have iNKT deficiency (67).

While most PID patients with EBV sensitivity have an anti-EBV CTL defect, other PID patients have a specific EBV sensitivity by other mechanisms, such as XMEN (mutations in *MAGT1)* (68), or patients with mutations in *CTPS1* (69). MAGT1 allows a TCR-induced influx of magnesium that activates T-cells (70) and CTPS1 allows CTP synthesis involved in nucleic acids anabolism (69). T-cells from these patients can respond to a standard stimulation of the immune system, but the ability of their T-cells to cope with the overwhelming proliferative stress induced by EBV infection is severely impaired, leading to an EBV specific immune deficiency.

Evidence for a Role of ATM During EBV Infection

As mentioned above there is evidence pointing to an abnormal control of EBV infection in AT patients without unequivocal evidence for an associated cellular immune defect leading to EBV-LPD. In the face of this apparent paradox, a cell intrinsic defect leading to impaired control of EBV latency in B-cells from AT patients, thereby promoting the oncogenic properties of the virus, may be hypothesized. There is indeed some evidence demonstrating the implication of ATM in the lytic and latent cycle of EBV as discussed below.

During the Lytic Cycle

ATM operates in the regulation of the lytic cycle of many viruses including EBV. During this cycle, viral replication generates a large amount of double-stranded linear DNA in the nucleus that are recognized as double strand breaks and thus activate the repair machinery (71). ATM and the MRN complex have been shown to bind the viral genome and recruit other proteins such as RPA, RAD51, and RAD52 that promote replication of the virus. Recent studies have reported inhibition of viral replication after pharmacological inhibition of ATM (72). BGLF4, one of the first viral proteins expressed during the lytic cycle, directly phosphorylates ATM, and H2AX (73). BGLF4 also phosphorylates and activates TIP60 (74), a histone acetyltransferase, which in turn activates ATM (75) (**Figure 2A**).

During lytic replication, ATM activation allows the phosphorylation of P53 and SP1, a transcription factor involved in DNA repair (76). SP1 plays a role in the formation of the

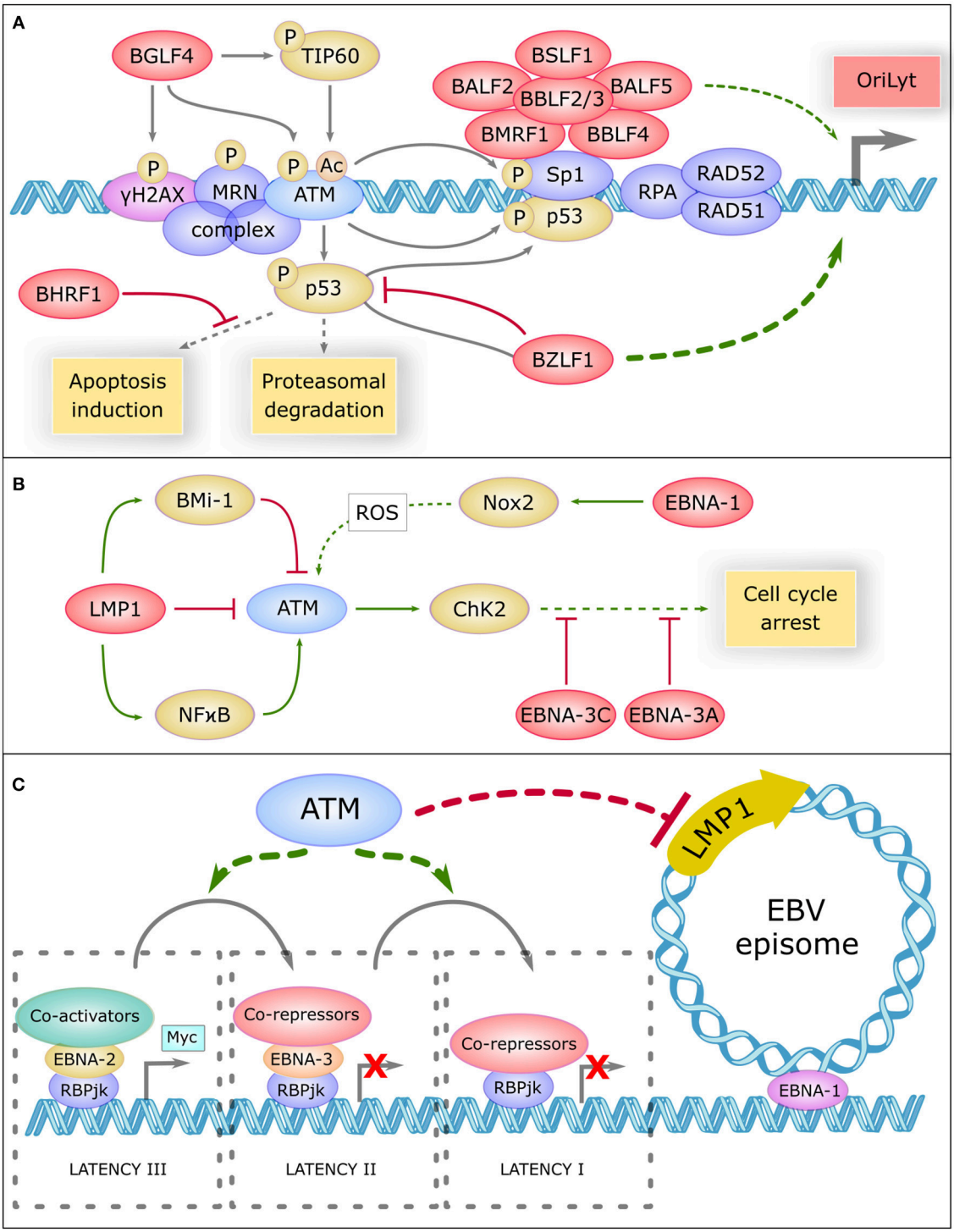

FIGURE 2 | Role Of Atm In Ebv Life Cycle Regulation **(A)** The central role of ATM in the replication compartment of EBV. In the lytic cycle, DNA damage response proteins such as γH2AX, the MRN complex, ATM, SP1, RPA, RAD51, and RAD52 bind the viral genome and promote replication of the virus. Viral proteins are shown in red. BGLF4 phosphorylates H2AX, ATM, and TIP60 which acetylate ATM to promote this replication. ATM phosphorylates and activates Sp1 which is necessary to the formation of the replication compartment comprising a large complex of six core viral replication proteins (BSLF1, BALF2, BBLF2/3, BALF5, BMRF1, and BBLF4). ATM phosphorylates and activates P53, which is inhibited and driven by BZLF1 to the replication compartment. BZLF1 is a major transactivator of the lytic genes promoter OryLyt. P53 binds to Sp1 and promote the activation of OryLyt. P53 is regulated by proteasomal degradation and can induce apoptosis, but BHRF1 inhibits a panel of pro-apoptotic proteins. **(B)** ATM is regulated by EBV during latency. In the latent cycle, LMP1 downregulates ATM, and upregulates Bmi-1 which also

(Continued)

FIGURE 2 | downregulates ATM. On the other hand, LMP1 activates the NFκB pathway which activates ATM. EBNA-1 upregulates NOX2, which generates reactive oxygen species (ROS) that could activate ATM. Once activated, ATM activates CHK2 which promotes cell cycle arrest. However, EBNA-3C and EBNA-3A inhibit many proteins involved in cell cycle control. **(C)** Potential involvement of ATM in the regulation of EBV latency. ATM could be involv ed in inhibiting the expression of certain viral oncogenes, such as the main viral oncogene LMP1. ATM could also favor the progressive restriction of EBV latency, from type III latency to type I. In type III latency, EBNA-2 interacts with the target of the Notch pathway RBP-JK, recruits coactivators and induces the transcription of pro-proliferative genes like Myc. In type II latency, EBNA-3 replaces EBNA-2, and recruits co-repressors, thus preventing prolonged expression of MYC. In type I latency, RBP-JK is associated with corepressors and only EBNA-1 remains expressed, which allows the attachment of EBV episome on cellular chromosomes.

nuclear replication compartment of the virus (72) where a high level of P53 is found. SP1 and P53 form a complex which binds and activates BZLF1 promoter (71), the major viral transactivator of the EBV lytic genes. Other repair proteins present in these compartments such as RPA, RAD51, and RAD52 also appear to be involved in the induction of BZLF1 because their knockdown greatly reduces viral replication (77). In addition, the activity of CyclinA/CDK2 and cyclinE/CDK2 complexes appears enhanced in this context, leading to a prolonged pseudo-S phase environment that promotes replication of the viral DNA (71).

EBV uses ATM activation to facilitate its own replication. But long-lasting activated ATM may promote P53 accumulation and apoptosis induction. During the lytic cycle, the level of P53 is constant despite recurrent activation of the DNA repair pathway, and appears to be regulated by proteasomal degradation (71). In addition, BZLF1 associates with P53, inhibits its transactivating activity and drives it to the EBV replication compartment (71), which greatly limits the ability of P53 to activate pro-apoptotic genes. Even in that case, BHRF1, a viral analog of the BCL-2 protein expressed early during the lytic cycle, inhibits a large panel of pro-apoptotic proteins such as BIM, BID, BAK, or PUMA (78).

During Latency

ATM is also involved in the early steps of EBV latency establishment where it plays a tumor suppressor role. *In vitro*, the early hyperproliferation period of infected B cells is associated with ATM activation, leading to the death of the majority of cells (79). A total of about 3% of infected B-cells survive and become indefinitely proliferating lymphoblasts (80). Some EBV latency proteins have been shown to interact with ATM as well as with other DNA damage related proteins, but the overall implication of ATM in the latent cycle remains to be explored.

LMP1 upregulate BMI-1 in Hodgkin's lymphomas, a Polycomb related protein, and both proteins combine their effects to downregulate ATM expression (81). Similarly, EBV infection of the EBV negative BJAB line showed a defective DNA damage response (82). In addition, biopsies of patients with EBV-positive nasopharyngeal carcinoma (NPC) revealed downregulation of ATM protein levels (83). On the other hand, it has been reported that LMP1 positively regulates ATM in NPC by activating NF-κB pathway (84). This divergence in the effect of LMP1 on ATM expression is unclear and may be due to different LMP1 expression levels or to the use of different cell line types (**Figure 2B**).

EBNA1 upregulates the catalytic subunit of Nox2 in the NADPH oxidase complex, inducing the production of reactive oxygen species that could activate ATM (82). The EBV-infected BJAB cells expressing EBNA1 also show more chromosomal aberrations (82). EBNA3C, a viral protein essential for transformation, has been shown to attenuate DNA damage response pathways in the early steps of transformation. It also inhibits the activity of many proteins involved in cell cycle control, such as P14, P16, P27, CHK2, P53, BUBR1 (85–90). EBNA3A appears to collaborate with EBNA3C in the inhibition of P14 and P16 (85).

There is also evidence pointing to a role of ATM in the regulation of latency of Kaposi's sarcoma associated herpesvirus (KSHV) and Murine γ-herpesvirus 68 (MHV68), two herpesviruses closely related to EBV. During KSHV latency, there is a steady phosphorylation of a small amount of ATM and γH2AX, which play a role in LANA-1 transactivation, the major latency protein (91). During MHV68 latency, ATM plays a role in the transactivation of the LANA-1 analog protein ORF73. Inactivation of ATM significantly reduces the expression of LANA-1 (91) and ORF73 (92), respectively, demonstrating the importance of ATM in the control of KSHV and MHV68 latency.

DISCUSSION

The increased incidence of malignancies in AT has primarily been linked to the genetic instability caused by DNA repair abnormalities. The high rate of association of B-cell malignancies with EBV may be interpreted as the consequence of AT associated immunodeficiency. Most patients however do not present opportunistic infections, indicating the absence of profound cellular immune deficiency. Although data are scarce, AT patients appear to have iNKT deficiency which may participate to the lack of control of viral infections. The contribution of iNKT deficiency in the propensity of AT patients to develop EBV-LPD requires further study.

Beside immune deficiency, ATM could also contribute to AT-associated EBV-related lymphoid malignancies by interfering with the B-cell intrinsic regulation of EBV persistence. ATM is known to play a role during the lytic cycle of EBV by creating the replication compartment of the virus and by promoting its replication. During latency, several viral proteins appear to interfere with ATM expression or with its downstream signaling. However, the effect of ATM on the regulation of viral latency is not yet known. The fact that ATM plays a role in the regulation of latency proteins of EBV-related herpesvirus, such as KSHV or MHV68, suggests that ATM may also be involved in the control of EBV latency.

Recent studies have shown an involvement of ATM in the inhibition of gene expression. Indeed, ATM activation in the vicinity of the DSB promotes the ubiquitination of nearby

H2A histones. This prevents the progress of polymerase II and thus inhibits the transcription of nearby genes (93). In the case of DSB within the nucleolus, ATM allows the blocking of polymerase I and its release of the nucleolus (94). ATM could conceptually also inhibit the transcription of some viral genes, such as the main EBV oncogene LMP1. ATM deficiency in AT patients could therefore release this inhibition, contributing to lymphomagenesis (**Figure 2C**). ATM could also participate in the restriction of EBV latency by promoting the transition from type III to type II latency and/or from type II to type I latency. The large number of EBV-associated Hodgkin's lymphomas, described as being derived from type II-latency-infected B-cells (95), suggests that the restriction of latency may not efficiently occur in AT patients.

Humanized mice are a potent model to study the early stages of EBV infection, establishment of latency III, and the immune system response (96). However, B-cell ontology is not complete in these mice, with little germinal center reactions or BCR maturation, impeding the study of latency II and I. Moreover, lytic infection cannot take place because of the absence of human epithelial cells. Recent advances have greatly improved the ontogeny of B-cells in these mice (97) and could open a new field for EBV study. An infection of these cells by EBV has, to our knowledge, not been yet assessed.

Studies on the mechanisms of EBV-induced lymphomagenesis in AT patients may shed light on the pathways involved in the control of chronic EBV infection. This will have a significant impact on the understanding of the physiopathology of EBV-LPD, even outside of the context of AT. The tumor suppressor role of ATM is highlighted by the frequent somatic mutations of *ATM* in many lymphoid malignancies. This understanding could allow the exploration of new therapeutic targets in these lymphomas, for which there is still no effective treatment targeting EBV, and in patients with AT where the usual therapeutic approaches by cytotoxic agents are limited because of their toxicity in the context of DNA repair abnormalities.

AUTHOR CONTRIBUTIONS

MT performed the research and wrote the manuscript. OH wrote the manuscript. FS supervised the research and wrote the manuscript.

FUNDING

This work was supported by a grant from Fondation ARC pour la recherche sur le cancer (France) and a ministerial doctoral fellowship (France).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 19 December 2018
doi: 10.3389/fimmu.2018.03058

The Pattern of Malignancies in Down Syndrome and Its Potential Context With the Immune System

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The immune surveillance theory of cancer posits that the body's immune system detects and destroys randomly occurring malignant cells. This theory is based on the observation of the increased frequency of malignancies in primary and secondary immunodeficiencies, and is supported by the successful demonstration of immune augmentation in current oncological immune therapy approaches. We review this model in the context of Down syndrome (DS), a condition with a unique tumor profile and various immune defects. Children and adults with DS are more prone to infections due to anatomical reasons and a varying degree of T- and B-cell maturation defects, NK cell dysfunction, and chemotactic or phagocytic abnormalities. However, despite an increased incidence of lymphoblastic and myeloblastic leukemia of infants and children with DS, individuals with DS have a globally decreased incidence of solid tumors as compared to age-adjusted non-DS controls. Additionally, cancers that have been considered "proof of immune therapy principles," such as renal carcinoma, small cell lung carcinoma, and malignant melanoma, are less frequent in adults with DS compared to the general population. Thus, despite the combination of an increased risk of leukemia with detectable immune biological abnormalities and a clinical immunodeficiency, people with DS appear to be protected against many cancers. This observation does not support the immune surveillance theory in the context of DS and indicates a potential tumor-suppressive role for trisomy 21 in non-hematological malignancies.

Keywords: down syndrome, immune surveillance, immune defect, trisomy 21, cancer, cancer incidence, tumor profile, cancer protection

OPEN ACCESS

Edited by:
Khashayarsha Khazaie, Mayo Clinic College of Medicine & Science, United States

Reviewed by:
Kawaljit Kaur, University of California, Los Angeles, United States
Virginia Smith Shapiro, Mayo Clinic, United States

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *06 August 2018*
Accepted: *10 December 2018*
Published: *19 December 2018*

Citation:
Satgé D and Seidel MG (2018) The Pattern of Malignancies in Down Syndrome and Its Potential Context With the Immune System. Front. Immunol. 9:3058. doi: 10.3389/fimmu.2018.03058

INTRODUCTION

According to the cancer immune surveillance theory, the immune system detects and destroys cancer cells that develop randomly in various tissues (1–3). In line with this model, medical conditions with inherited or acquired immune deficiency should also be associated with an excess of all types of cancers: malignant cells would escape surveillance by an impaired immune system and therefore proliferate (4). However, recent reviews of cancer events in individuals with primary immune deficiencies show only a mildly increased frequency of cancers and a particular distribution of cancer types, questioning this model (5, 6).

Here, we review the immune surveillance model in the context of trisomy 21, or Down syndrome (DS), a condition that is extensively studied for its immune defects (7) and unique tumor profile (8). Data on this well-defined genetic condition do not fit with the cancer immune surveillance theory because, despite decreased immune efficiency, people with DS have a reduced incidence of solid malignancies. This conflict raises important questions and offers new avenues to understand the poorly explored topic of natural protection against cancer.

CANCERS AND IMMUNE FUNCTION IN DS

Unique Cancer Distribution in DS

DS, due to a supernumerary chromosome 21, is the most frequent viable chromosome anomaly, with an occurrence of 1 in 700–1,000 live births worldwide. Currently, the life expectancy of people with DS is >50 years (9), permitting evaluation of the occurrence of frequent adult cancers. Although DS was formerly suspected to increase the general cancer risk because of an increased leukemia incidence in childhood, age-adjusted epidemiological studies have established that individuals with DS have a decreased global malignancy burden (10, 11). This is mainly due to reduced frequency of adult solid tumors that account for nearly half of the tumor burden in the general adult population (10–12), but also due to a reduced incidence of many solid tumor types of childhood (13).

Additionally, cancer distribution in DS differs from that in the general population (8). For instance, breast cancer and neural malignancies, such as neuroblastoma (14) and medulloblastoma (15), have a decreased incidence in DS. However, some cancers, especially early childhood leukemia and testicular germ cell tumors in young men (16), only in part attributable to cryptorchidism and testicular microlithiasis, and, to a lesser extent, cancers of the liver and stomach, also appear to be more frequent in individuals with DS than in the general population (10–12) (**Table 1**).

TABLE 1 | Cancer distribution in Down syndrome.

Increased frequency	SIR	Observed/ expected	References
Children			
Acute myeloid leukemia	11.8 (7.11–18.5)		(11)
Acute lymphoid leukemia	13.0 (8.74–18.5)		(11)
Germ cell tumors		5%/1.1%	(13)
Adults			
Testicular cancer	4.8 (1.8–10.4)		(10)
Gastric carcinoma	1.65 (0.33–4.83)		(11)
	1.5 (0.3–4.5)		(10)
Liver carcinoma	1.19 (0.02–6.65)		(11)
	2.4 (0.1–13.2)		(10)
DECREASED FREQUENCY			
Children			
Neuroblastoma and PNETs		0/5.40 ($p = 0.005$)	(14)
Medulloblastoma		1/7.11 ($p = 0.007$)	(15)
Adults			
Breast carcinoma	0.16 (0.03–0.47)		(11)
Lung carcinoma	0.10 (0.00–0.56)		(11)
Prostate carcinoma	0.0 (0.0–0.03)		(11)
Colon carcinoma	0.37 (0.04–1.34)		(11)
ENT and oral carcinoma	0.00 (0.00–1.15)		(11)
Malignant melanoma	0.25 (0.03–0.89)		(11)

SIR, Standardized Incidence Ratio; PNETs, Primitive neuroectodermal tumors; ENT, Ear Nose and Throat; Ref, references.

Impaired Immune Function in DS

DS is the most common recognizable genetic syndrome associated with immune defects (7), which are detectable as early as fetal development (17). Abnormal parameters of the immune system were identified following evidence of frequent respiratory infections responsible for recurrent hospitalizations and frequent otitis media (7, 18, 19). Overall, the risk to die from an infection is 12-fold higher in patients with DS as compared to individuals without DS (20). DS-related immune impairment is complex and varies among individuals, affecting mainly B cells and humoral (including mucosal) immunity, T-cell-mediated immunity, NK cells, and neutrophils (21–26) (**Table 2**). Some features are reminiscent of premature immune senescence (23) and common variable immune deficiency (23–26), leading to immune dysregulation with relative imbalance between pro-inflammatory and anti-inflammatory immune responses. In line with this, people with DS are more prone to autoimmune diseases of the thyroid (Graves disease, Hashimoto thyroiditis), pancreas (type 1 diabetes mellitus), gut (celiac disease), and skin (alopecia areata, vitiligo). These autoimmune manifestations usually appear earlier in life and are more frequently associated in comparison to persons without DS (18).

At least four genes mapping to chromosome 21 are involved in immune functions and have been postulated to account for some of the biological and clinical findings related to immunity in DS: interferon alpha receptor 1 (*IFNAR1*); interferon gamma receptor chain 2 (*IFNGR2*); ICOS ligand (*ICOSLG*), which encodes CD275; and integrin beta chain 2 (*ITGB2*), which encodes CD18. These four genes should theoretically be overexpressed through a gene dosage effect, since three copies are present in DS cells, including leukocytes. However, only CD18 is significantly elevated in individuals with DS (19). Additionally, two other genes on chromosome 21, DS critical region 1 (*DSCR1*) and dual-specificity tyrosine phosphorylation-regulated kinase 1A (*DYRK1A*), are involved in a regulatory circuit that includes nuclear factor of activated T-cells (NFAT) proteins, potentially contributing to a modulation of the immune response (18, 19).

TABLE 2 | Sum of reported immune abnormalities and other factors that potentially contribute to an increased risk of infections in Down syndrome.

Compartment	References*
T CELLS	
Normal or mildly-moderately decreased T cell numbers	(21)
Reduced proportion of naïve T cells	(22)
Increased proportion of T cell receptor γδ+ T cells	(22)
Impaired T cell maturation and memory development	(18)
Normal or decreased mitogen stimulation response (SEB, PHA)	(22)
Impaired functional activity of T regulatory cells	(21)
B CELLS AND HUMORAL IMMUNITY	
Mild–moderate decrease in B cell numbers	(23)
Normal transitional but reduced naïve, effector, and memory B cells	(23)
Activation and adherence defect	(21)
Lower serum levels of IgM, higher serum levels of IgA and IgG; inconsistent reduction of IgG2, reduction of IgA in saliva	(21, 23)
Impaired molecular maturation of IgA and IgM	(23)
Impaired specific antibody production against protein antigens	(24)
Impaired specific antibody production against polysaccharide antigens	(24)
NK CELLS AND INNATE IMMUNITY	
Reduced functionality of NK cells	(25)
PHAGOCYTE NUMBER AND/OR FUNCTION	
Impaired neutrophil chemotaxis and, inconsistently, of phagocytosis	(26)
Non-immunological factors	
Anatomical: laryngo- and/or tracheomalacia, macroglossia, ear abnormalities; obstructive sleep apnea	(27)
Gastro-esophageal reflux and aspiration	(27)

**According to (18, 21, 24, 25, 25, 27) in part reviewed and summarized by Ram and Chinen (7) and Kusters et al. (22); SEB, staphylococcal enterotoxin B; PHA, phytohemagglutinin A.*

NO INCREASED INCIDENCE OF MOST SOLID CANCER TYPES DESPITE INCREASED RISK OF INFECTIONS AND BIOLOGICAL ABNORMALITIES OF THE IMMUNE SYSTEM IN DS

Decreased efficiency of immune cells should result in an increased cancer frequency, because escape from impaired immune surveillance would enable cancer cells to survive and proliferate. Individuals with DS have an increased rate of mortality from infections as compared to the general population. This susceptibility, together with a variety of biological abnormalities of the immune system that are reminiscent of common variable or combined immunodeficiency (CVID or CID, respectively), could prompt the assumption that immune surveillance is impaired. Additionally, mucosal immunity may be impaired and fail to control infections of the gut that contribute to carcinogenesis. In fact, the observation of mildly increased mortality from gastric and liver cancers suggests that extrinsic mechanisms of tumorigenesis such as chronic infection or inflammation, in combination with potentially impaired elimination of tumor cells by the immune system, could be at play (**Figure 1**).

However, individuals with DS have an, age-corrected, decreased frequency of those solid tumor types that comprise half of the total cancer burden of the general population (11), arguing against a role of (globally impaired) immune surveillance. Further, people with DS also have a reduced cancer frequency compared to people with other conditions of intellectual disability, who develop cancers at rates similar to the general population (28, 29). This suggests that the excess of genetic material on the supernumerary chromosome 21 provides protection against certain types of malignancy.

Additionally, other observations fail to support the role of immune surveillance in DS. First, three malignancies that have been considered proof of immune therapy principles—kidney carcinoma, small cell lung carcinoma, and malignant melanoma (5)—are not more frequent in people with DS. On the contrary, kidney cancer, lung cancers including small cell carcinoma, and malignant melanoma have a decreased incidence in DS (11). Second, medulloblastoma, a neural cell embryonal brain malignancy, is rare in children with DS (15). An immune mechanism would hardly explain why, in the same epidemiological study, the frequency of glial malignancies of the brain was not found to be reduced in children with DS (15). Third, nearly 1 out of 20 infants with DS develops a transient myeloproliferative disorder that spontaneously disappears in most affected individuals during the first months of life (30). These spontaneous regressions of premalignant abnormal proliferation occur at a time when the immune system is weak and immature, and it is even more impaired in children with DS.

Even in typically malignancy-prone primary immune deficiencies, an increased risk of leukemia is attributable to an intrinsic mechanism of oncogenesis, in parallel, rather than as a consequence of the immune defect (5). Thus, the increased frequency of leukemia observed in people with DS is unlikely to be due to a lack of immune surveillance. The genetic etiology of myeloid (typically megakaryoblastic) or lymphoblastic (often Philadelphia-like, high-risk) leukemias is complex and beyond the topic of this review. The slightly increased risk of gastric and liver cancers in DS in part reminds of that of patients with predominantly antibody deficiencies such as CVID (31), who also show reduced mucosal immunity, which in turn could facilitate chronic infection, inflammation, and thereby, stochastically, increase the risk of malignant transformation (**Figure 1**). The inconsistently detected defects in T-cellular immunity appear to play a minor role clinically, as the pattern of infections observed in people with DS does not reflect the typical distribution of opportunistic pathogens seen in CID. In general, a large part, although not all, of the increased frequency of infection-related hospitalizations may be due to non-immunological risk factors such as anatomical reasons and their consequences (7). Moreover, for instance, despite the increase frequency of celiac disease in children with DS, we are not aware of a single case of duodenal lymphoma (32).

In summary, the observed clinical and biological abnormalities of the immune system in DS on the one hand, and the reported cancer frequency and unique distribution of

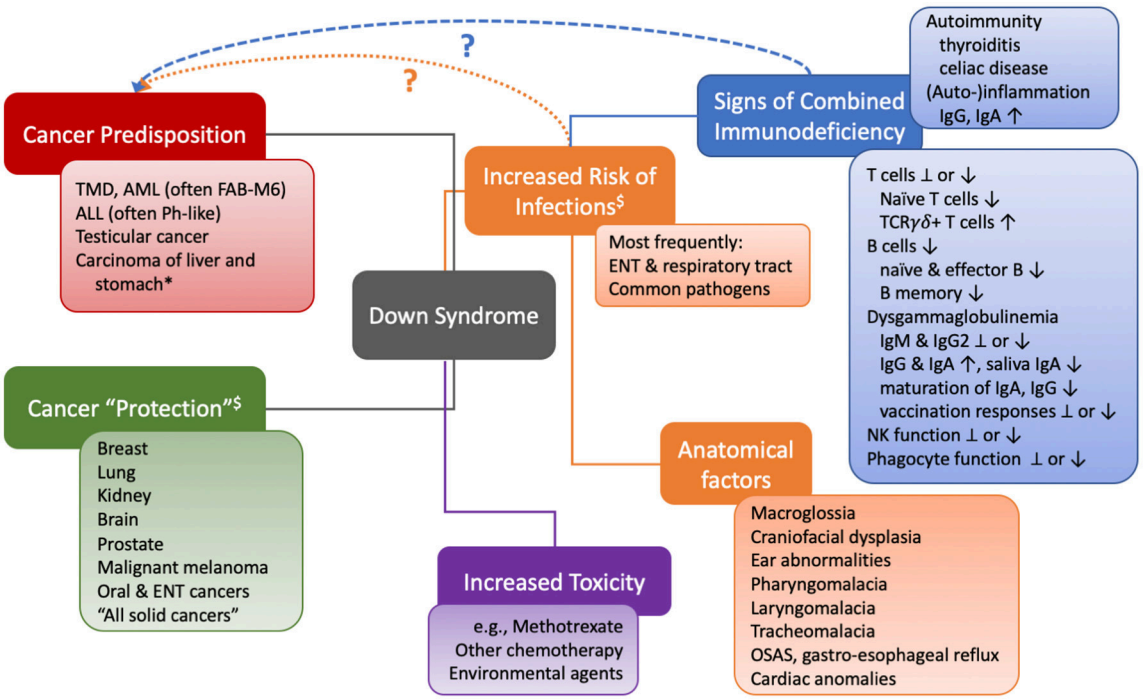

FIGURE 1 | Visual contextualization of cancer risks and the immune system in Down syndrome. *different risk ratios (increased vs. decreased) were detected in different studies. $Caveats: most but not all studies took into account age-matched control cohorts, but not social and environmental factors (smoking, UV, diet, institutionalization, sexual activity…), endocrine differences, aging, or senescence. TMD, transient myeloproliferative disorder; AML, acute myeloid leukemia; FAB-M6, French American British classification M6 (megakaryocytic); ALL, acute lymphoblastic leukemia; Ph-like, Philadelphia chromosome-like signature, often associated with mutations in *IKZF1;* ENT, ear nose throat; TCR, T cell receptor; Ig, immunoglobulin; NK, natural killer cell; OSAS, obstructive sleep apnea syndrome.

malignant disease types on the other hand, suggest that immune surveillance plays little role, if any, in this context (**Figure 1**).

OTHER GENETIC CONDITIONS WITHOUT EFFECTIVE CANCER IMMUNE SURVEILLANCE

Interestingly, trisomy 18, or Edwards syndrome (ES), is also associated with a unique tumor profile. Children with ES have an increased incidence of hepatoblastoma and nephroblastoma compared to children with a normal constitutional karyotype (33). However, extensive review of the literature indicates that hematopoietic malignancies and brain tumors, the two most frequent malignancies in children, are unusually rare in children with ES. Further, similar to DS, the immune system in fetuses with ES shows immunological defects, with a decrease of some B lymphocyte and T lymphocyte subpopulations (34, 35).

In DS and ES, the impaired immune system cannot explain the lower cancer burden and cancer incidence variations because, following the immune surveillance theory, one might expect a globally increased cancer burden. Similar to conditions with primary immune deficiency—such as common variable immune deficiency, X-linked agammaglobulinemia, selective IgA deficiency, X-linked hyper-IgM syndrome, Wiskott Aldrich syndrome, and severe congenital neutropenia (5, 6)—there is no uniform increase in all malignancies, but rather overrepresentation of a narrow spectrum of cancers, including, e.g., lymphomas, digestive tract tumors, and virus-induced tumors. Additionally, primary immunodeficiency diseases have a decreased incidence of some cancers, such as breast, lung, and colon carcinomas (36). A unique general mechanism therefore is unlikely to explain the tumor profiles of these various primary immunodeficiency disorders.

This evidence raises two important questions that largely extend beyond people with DS. First, what is the basic role of the immune system in cancer in non-therapeutic conditions? Given the increasing success of various immunotherapies in modern oncological treatment (37), it is surprising that the frequency and spectrum of malignancies in individuals with primary immune deficiencies does not reflect the corresponding mechanisms of impaired immune surveillance (6). Second, which mechanism(s) protect(s) people with DS so efficiently against the most frequent human solid tumors, particularly carcinomas? Does the presence of a third chromosome 21 offer tumor-suppressive factors?

WHAT IS THE RELATIONSHIP BETWEEN CANCER AND THE IMMUNE SYSTEM IN DS?

Considering cancer immune surveillance, primary immune deficiencies do not exhibit an important excess of all types of cancers, but rather a slight global increase due to a high frequency

of lymphomas and digestive tract or virus-related cancers (36, 38). Lymphomas mostly occur in conditions with cells (lymphocyte precursors) more vulnerable toward transformation due to impaired cell maturation, function, or signaling. Digestive tract or virus-related cancers may be a consequence of microorganism infections and chronic inflammation, potentially facilitated by immunodeficiency and a lack of immune surveillance (extrinsic mechanisms). Although epigenetic and environmental factors such as a different exposure to tobacco of individuals with DS as compared to the general population may play a role, and, similarly, a different diet, intestinal microbiome, or other factors cannot be ruled out, these conditions, for which cancer incidence is based on strong epidemiological data and where the immune function is well documented, challenge the idea of a global immune-mediated protection against cancer. However, additional studies are needed to examine the model of immune surveillance in other conditions and particularly in the general population. These results do not contradict the current therapeutic successes of immune treatment in several cancers (37, 39).

WHAT PROTECTS INDIVIDUALS WITH DS FROM CANCERS?

The broader population of people with intellectual disabilities develops a similar frequency of cancers as the general population (10–12), suggesting that the protection of individuals with DS against cancer must be linked to specific excess of genetic material on the supernumerary chromosome 21 (comprising nearly 300 genes). However, not only aberrantly expressed genes of chromosome 21 that include oncogenes and tumor suppressors, but rather complex interactions between them with genes mapping to other chromosomes lead to modified phenotypes and functions in various tissues and biological processes. Despite increased cancer risk factors—such as being overweight, low physical activity, nulliparity (for breast cancers in women) (28), and accelerated aging—sensitivity of tissues to genotoxic stress, increased DNA damage, and deficient DNA repair (40), many organs and tissues of people with DS are protected against malignant transformation, particularly breast and neural cells (but not glial cells). Thus, the "physiological" state of tissues with trisomy 21 is the result of a modified regulation of many interacting pathways that lead to tumor-protective protection. Analyzing the "interactome" (the signaling pathway-specific transcriptome and proteome) of DS tissues and comparing the exome of cancers in DS with normal DS tissues might therefore represent a possibly more fruitful approach than focusing on the effects of single genes on various functions. Because the observed profile of malignancies is not simply explained by impaired immunosurveillance, other avenues to understand reduced cancer incidence deserve additional attention. For instance, metabolic modifications in relation to the Warburg hypothesis could be considered a key context for reduced cancers in DS (41). Yet, metabolic effects on cancer occurrence have not been studied despite well-documented mitochondrial anomalies in DS (42). Other studies should more fully consider the roles of angiogenesis and stem cell availability (40).

CONCLUSION

The incidence, distribution and clinical course of cancers in children and adults with DS in context with their increased risk of infections and abnormalities in the immune system do not support a model of enhanced immune surveillance providing protection from tumors. Rather, they suggest that other inherent, trisomy 21-linked, mechanisms account for the natural and strong protection against many cancer types, except leukemia and testicular cancer, in this condition. DS therefore offers an interesting condition in which to study how organisms may efficiently be protected against certain malignancies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

This work was supported by the Fondation Jérôme Lejeune (DS). MS is in part supported by the Styrian Children's Cancer Foundation (Steirische Kinderkrebshilfe). Christiane Satgé is acknowledged for her help in preparing the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 12 December 2018
doi: 10.3389/fimmu.2018.02912

Current Understanding and Future Research Priorities in Malignancy Associated With Inborn Errors of Immunity and DNA Repair Disorders: The Perspective of an Interdisciplinary Working Group

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Edited by:
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Specialty section:
This article was submitted to Primary Immunodeficiencies, a section of the journal Frontiers in Immunology

Received: 02 October 2018
Accepted: 27 November 2018
Published: 12 December 2018

Citation:
Bomken S, van der Werff Ten Bosch J, Attarbaschi A, Bacon CM, Borkhardt A, Boztug K, Fischer U, Hauck F, Kuiper RP, Lammens T, Loeffen J, Neven B, Pan-Hammarström Q, Quinti I, Seidel MG, Warnatz K, Wehr C, Lankester AC and Gennery AR (2018) Current Understanding and Future Research Priorities in Malignancy Associated With Inborn Errors of Immunity and DNA Repair Disorders: The Perspective of an Interdisciplinary Working Group. Front. Immunol. 9:2912. doi: 10.3389/fimmu.2018.02912

Patients with inborn errors of immunity or DNA repair defects are at significant risk of developing malignancy and this complication of their underlying condition represents a substantial cause of morbidity and mortality. Whilst this risk is increasingly well-recognized, our understanding of the causative mechanisms remains incomplete. Diagnosing cancer is challenging in the presence of underlying co-morbidities and frequently other inflammatory and lymphoproliferative processes. We lack a structured approach to management despite recognizing the competing challenges of poor

response to therapy and increased risk of toxicity. Finally, clinicians need guidance on how to screen for malignancy in many of these predisposing immunodeficiencies. In order to begin to address these challenges, we brought together representatives of European Immunology and Pediatric Haemato-Oncology to define the current state of our knowledge and identify priorities for clinical and research development. We propose key developmental priorities which our two communities will need to work together to address, collaborating with colleagues around the world.

Keywords: inborn error of immunity, DNA repair defect, cancer, lymphoma, EBV (Epstein-Barr virus), haematopoietic stem cell transplant, chemotherapy, screening

INTRODUCTION

Patients with an inborn error of immunity (IEI) or a DNA repair disorder (DNARD) have a significantly greater risk of developing malignancies than the general population (1) with an overall relative risk varying from 1.4- to 5-fold in registry-based studies (2–5). The risk, however, varies greatly between underlying genetic conditions and within the narrow range of malignancies seen to occur, with the risk of lymphoid malignancies overall being 8–10-fold higher than age matched controls, according to a recent study from the US Immune Deficiency Network (5). Whilst many of the recognized, genetically defined IEI are very rare, disease-specific studies of the more common disorders have shown prevalences of malignancy to range from 8 to 21% in common variable immunodeficiency (6–8) to between 19 and 42% in DNARDs such as ataxia telangiectasia, Nijmegen breakage syndrome and Bloom syndrome (9–13). International collaboration, resulting in growing cohorts of patients with more recently described combined immunodeficiency disorders (14) including CD27 deficiency (15), CD70 deficiency (16), activated PI3Kδ and activated PI3Kδ2 syndromes (17, 18) has demonstrated comparable rates of malignancy, although the risk of case ascertainment bias must be considered in these very rare patient groups.

Across the spectrum of IEI/DNARD, lymphoid malignancies account for 60–70% of diagnoses and disproportionally affect children when compared to control cohorts (11, 13, 19). Teams managing patients with IEI/DNARD will recognize that both clinical and histological diagnosis of malignancy can be challenging and diagnosis may be delayed, especially in the setting of pre-existing non-malignant lymphoproliferation. Similarly, management is often complicated by an increased incidence of infectious comorbidities and severe, even life-threatening, toxicities following conventional chemotherapy or radiotherapy. These factors commonly reduce the intensity of deliverable treatment. This combination of diagnostic challenge, increased comorbidity and toxicity, and reduced therapy intensity results in an inferior outcome for patients, making malignancy a leading cause of death for this group of patients (6, 7).

To improve the management of malignancy in patients affected by IEI/DNARD, we brought together a working group of representatives from both immunology and lymphoid malignancy fields to define the current state of knowledge and identify priorities for research, focusing predominantly on lymphoid malignancy (**Figure 1**). We present here our experience of diagnosing and managing these conditions, with a focus on the unresolved challenges faced when caring for this complex patient group. We discuss how our limited understanding of the molecular basis of oncogenesis must be expanded in order to drive the development of improved diagnostics and more effective and less toxic targeted therapies. From this we have drawn suggested priorities to be addressed through clinical and basic research collaborations. Finally, as our two fields continue to develop an increased understanding of predisposition to malignancy, we will consider the issue of identifying an underlying IEI or DNARD in patients with malignancy as a first clinical presentation.

MECHANISMS OF ONCOGENESIS

There is an established role for a number of infectious agents in the development of both hematological and non-hematological malignancies in sporadic as well as IEI associated cases (16, 22–24). Notably, EBV is present in a significant proportion of lymphoproliferative conditions as well as other soft-tissue tumors (25). Human papillomaviruses are common in epithelial malignancies and *Helicobacter pylori* is associated with both carcinoma and extranodal marginal zone lymphoma of the stomach. Whilst, much is still unknown about the oncogenic processes these microorganisms modulate, particularly in an IEI/DNARD background (26), EBV is known to be directly oncogenic through the LMP1 protein (27). This makes IEI patients with particular susceptibility to EBV, at a very high risk of developing cancer. Nevertheless, as the same infectious agent in two patients with the same underlying condition, even siblings with identical causative mutations, can result in development of different malignancies, causation must be more complex than solely the effect of inadequate control of infection. The additional role of the host immune system in tumor immunosurveillance, a concept supported by the effective introduction of immune checkpoint inhibitor therapies, may well be important, as is consideration of the cell-intrinsic effects of the underlying IEI/DNARD, including dysregulated cellular maturation, cell signaling, apoptosis, and DNA damage responses (21, 28, 29). A well-known example of these phenomena would be the failure

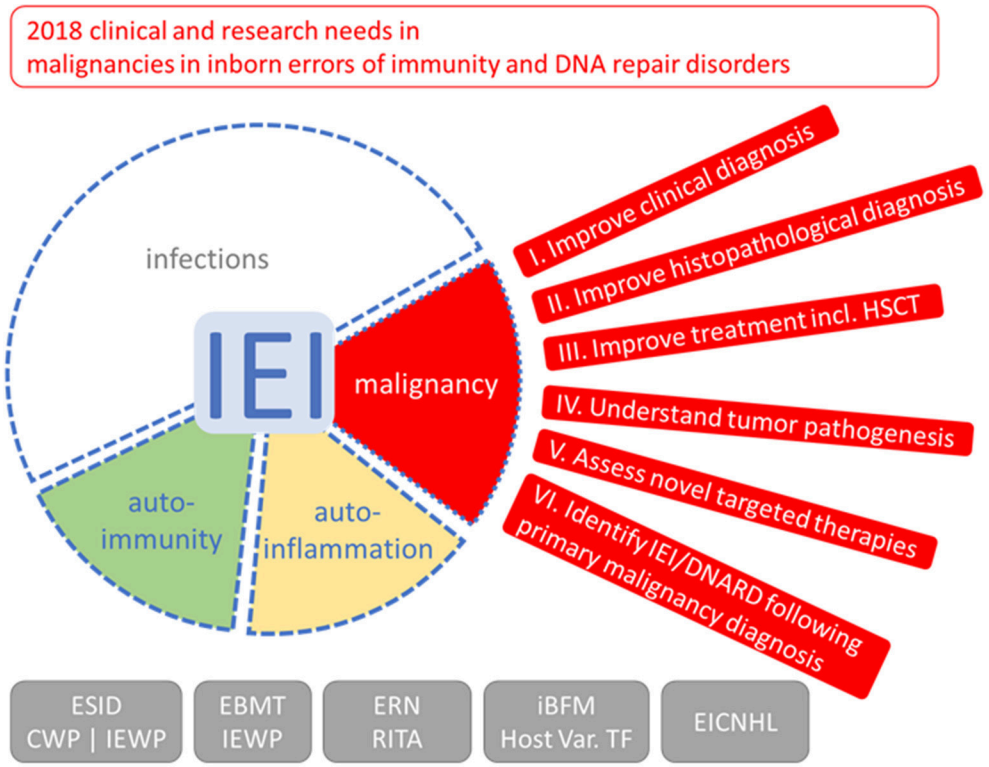

FIGURE 1 | Outlook on clinical and research needs for malignancies in inborn errors of immunity (IEI) and DNA repair disorders (DNARD). Due to the increased risk and unfavorable outcome of malignancies in IEI and DNARD, the clinical working party (CWP) and the inborn errors working party (IEWP) of the European Society for Immunodeficiencies (ESID), of the European group for Blood and Marrow Transplantation (EBMT), the European Reference Network on Immunodeficiency, Autoinflammatory and Autoimmune diseases (ERN-RITA), the host variation task force (Host Var. TF) of the international Berlin-Frankfurt-Münster (iBFM) study group, and the European Intergroup Collaboration for Childhood non-Hodgkin Lymphoma (EICNHL) formulated six topics with needs and tasks for clinical management and clinical, translational, and basic scientific research to increase specific knowledge and improve management of these special malignancies; see main text and boxes for more detailed lists. The red segment depicts an eye with outlook, the spectrum of symptoms of IEI/DNARD, with infections, and, additionally, approximately 25% patients having autoimmune or autoinflammatory symptoms (20) and 4–25% of patients suffering from malignancies (21), is shown with blurred borders indicating that they are not mutually exclusive.

of clonally expanded populations to apoptose in patients with autoimmune lymphoproliferative syndrome–ALPS (30, 31).

Of particular interest is the interplay between these processes in patients with an underlying DNARD. For these patients, a number of potentially oncogenic or selective pressures accumulate, notably (i) DNA damage, especially during attempted B- and T-cell receptor rearrangement, immunoglobulin class switching and somatic hypermutation, (ii) reduced immune repertoire affecting both infectious and tumor immunosurveillance and (iii) dysregulated immune development with potential for (pre-)malignant clonal selection. This is highlighted by the substantially lower rate of tumor EBV carriage in B lymphoid proliferations in patients with ataxia telangiectasia compared to patients with other IEI (11). The relative contributions of these potential oncogenic factors are still not well-understood. Although our view of cancer susceptibility in IEI is evolving, much more in depth research will be required to define the relative importance of each of these potential causative mechanisms (32).

Currently, the cytogenetic and molecular genetic basis of malignancy in patients with IEI/DNARD is incompletely defined, despite the massive increase in genome-wide sequencing and whole transcriptome technologies over the last 15 years. In part this is due to the rarity of the individual underlying patient cohorts. However, there is also a lack of biobanked diagnostic material of sufficient quality to allow the use of advanced molecular techniques. This can, at least in part, be overcome by increasing awareness within immunology and oncology communities, but collaborative patterns of working will also accelerate progress in this area (**Box 1**). Addressing these challenges is important, as an improved understanding of the molecular drivers of IEI-associated malignancy will have significant benefits for patients. Firstly, mechanistic understanding, exemplified by the studies of genomic instability in acute lymphoblastic leukemia (ALL) in patients with ataxia telangiectasia (33), will elucidate causative mechanisms. Secondly, it will allow comparison with sporadic disease, driving rational selection of molecularly targeted

BOX 1 | Developmental priorities for our understanding of oncogenesis.

- Form collaborative networks to provide critical number of tumors and matched constitutional DNA from each defined IEI/DNARD cohort for analysis.
- Establish prospective tumor biobanks with storage of high quality diagnostic material, suitable for development of advanced diagnostic (omic) techniques.
- Develop cytogenetic and molecular biological techniques to allow analysis of historic archived material such as sequencing from formalin fixed paraffin embedded material.
- Associate new molecular knowledge with development of histopathological techniques to aid diagnosis and prognostication.

therapies which have been developed for those counterpart conditions.

MAKING A DIAGNOSIS OF MALIGNANCY IN IEI/DNARD

Despite an increased awareness of the risk of malignancy in patients with an underlying IEI or DNARD, making a definitive diagnosis can be challenging (**Box 2**). Many patients have complex co-morbidities, including inflammatory and infectious conditions often involving atypical organisms, non-neoplastic lymphoproliferation and bone marrow dysfunction. Each of these may mimic a developing malignancy clinically, radiologically or histopathologically. These uncertainties can result in a substantial psychological impact on the patient and family, a factor which must be borne in mind during the diagnostic process.

Whilst radiological imaging can define the location and some features of pathology, current imaging techniques, even advanced techniques such as diffusion weighted imaging, MR spectroscopy and PET/CT, are not capable of definitive differentiation of malignant from non-malignant lesions. Furthermore, for those patients with a DNARD, there is a strong rationale for minimizing the use of ionizing radiation, resulting in understandable hesitance to undertake radiological investigations. However, in order to prevent excess mortality from delayed diagnosis it is important that alternative imaging modalities or limited exposures are used when clinical concerns of a possible malignancy exist.

Similar challenges exist for the majority of existing biomarkers. Very few blood or urine-based investigations are considered adequate for diagnosis of malignancy, with human chorionic gonadotrophin (HCG), alpha-fetoprotein (AFP), urine catecholamines and neurone specific enolase (NSE) being notable exceptions. Whilst these examples are equally valid in patients with IEI, they are infrequently relevant in diagnosis of malignancy in these patients. Cytomorphological and flow cytometric analysis of peripheral blood, bone marrow or effusions can be diagnostic in hematological malignancies, including lymphoblastic lymphomas and Burkitt lymphoma, but is commonly more complex than in patients without an underlying IEI. Other markers of lymphoproliferation, including lactate dehydrogenase, β2-microglobulin, IgM, oligoclonal/monoclonal immunoglobulin bands and serum free light chains, are commonly measured but we lack evidence to guide their use in supporting diagnosis. They may, however, have a role to play in monitoring disease following therapy. Quantitative analysis of EBV in the peripheral blood is also commonly performed, both during the diagnostic process and as a marker of response to therapy. Newer technologies developing the value of so-called liquid biopsies, such as identification of circulating tumor cells and analysis of cell-free tumor DNA, may offer the potential for a more integrative diagnostic test, sampling the "whole" patient, rather than a single lesion and identifying mutations driving malignant transformation (34). To date, these remain in early clinical development in mainstream oncology and have no established role in patients with IEI/DNARD.

Histopathology remains the cornerstone of diagnosing malignancy. However, even this gold standard approach can be challenging in patients with IEI, particularly when investigating for possible lymphoid malignancy. Firstly, judging when to perform an, often invasive, diagnostic investigation requires careful multidisciplinary discussion. Targeting the lesion with the highest likelihood of being diagnostic is a further challenge. If clinically appropriate, a surgical biopsy providing sufficient material for assessment of tissue architecture and ancillary diagnostic techniques is preferred over a needle core biopsy, but even when high quality material is obtained, histological diagnosis is often difficult. Many IEI are associated with non-neoplastic lymphoproliferations which may have specific characteristic features but together constitute a broad spectrum of processes, either inflammatory in etiology or resulting directly from the underlying genetic defect. These lesions may precede or co-exist with lymphoid malignancies and, in many settings, diagnostic boundaries between non-neoplastic and neoplastic lesions are ill-defined and difficult to apply. Existing molecular techniques to assess lymphocyte clonality may aid diagnosis, but these alone cannot provide diagnostic certainty—clonal B-cell and T-cell proliferations falling short of malignancy are not uncommon in IEI (35). Some polyclonal proliferations can be clinically aggressive and some clonal lymphomas respond to immunomodulatory therapies better than to cytotoxic chemotherapy (36). Clonal cytogenetic abnormalities may even occasionally be detected in immunodeficiency-associated lymphoid hyperplasias (37), raising the question of how to define and assess malignant transformation. In the future, personalized genomic medicine may address these challenges, but this must be established on the basis of further detailed research. Integrating these newer sources of information and deciphering diagnostic patterns will require a reference network of specialist pathologists, supported by hematologists and immunologists.

BOX 2 | Developmental priorities for improving diagnosis and classification of malignancies.

- Improve awareness amongst both immunologists and hematologist/oncologists of the increased risk of malignancy in particular patients with IEI/DNARD.
- Improve diagnostic and prognostic biomarkers with investigation and validation of developing technologies where appropriate.
- Prospective study of malignancies in defined cohorts of IEI/DNARD to include pathological and molecular characterization.
- Consensus diagnostic classification with integration of molecular pathological features with consideration of current and future targeted therapies.
- Develop provisional recommendations for screening cohorts at risk with studies to validate the impact of screening, including the impact on affected and unaffected family members.

Lymphomas are the most common malignancy associated with IEI (4, 5). In many cases these are immunohistologically similar to lymphomas in immunocompetent people and can be readily diagnosed according to the WHO Classification of Tumors of Haematopoietic and Lymphoid Tissues (38). However, other lymphoproliferative disorders arising in IEI/DNARD patients are not as easily classified and the WHO Classification does not provide an explicit framework applicable to the full range of IEI/DNARD-associated tumors as it does for the post-transplant lymphoproliferative disorders (PTLD). Nevertheless, as highlighted at a recent Workshop of the Society for Hematopathology/European Association of Haematopathology (39–41), the spectrum of lymphoproliferative disorders is in many ways similar across different immunodeficiency settings including IEI and PTLD, prompting the Workshop Panel recently to propose a unifying three-part nomenclature incorporating the histopathological name of the lesion (e.g., hyperplasia, polymorphic proliferation, lymphoma classified according to the WHO Classification), any viral association, and the underlying immunodeficiency background (42). This proposal has merit in facilitating clinical and biological comparison between related lesions in conceptually similar contexts but the tumor heterogeneity seen across individual patients with the myriad IEI now recognized remains incompletely defined.

Underpinning this heterogeneity is an almost completely unknown genomic landscape that is likely to be different between immunocompromised and immunocompetent individuals, and between individuals with different IEIs, but which must be incorporated into any future pathological schema. The importance of this is clear. With the increasing availability of targeted therapies, both small molecular inhibitors and immunotherapies, understanding the underlying molecular biology of individual diseases is a clinical imperative.

One final, but important, consideration is that of screening for malignancy in patients with known predisposing conditions. This is a very challenging area, with the need to take account of factors including level of risk, location of predominant malignancies and natural history of those malignancies. In essence, each underlying IEI/DNARD must be considered separately, in keeping with a number of existing examples (43, 44), with individual validation of the benefit of screening. This will require the inclusion of large cohorts of patients with proven IEI with analysis of the incidence of all malignancies in each cohort. Despite these challenges, there may be significant benefit to early detection of malignancy, with the potential to limit treatment intensity and therefore restrict the consequent toxicity.

MANAGEMENT OF MALIGNANCY IN IEI/DNARD

In the majority of patients with an underlying IEI/DNARD who develop cancer, treatment should be offered with the intention of curing them of their malignancy. However, managing such a patient must balance offering potentially curative therapy with the risk of severe and even life-threatening toxicity (**Box 3**). This includes not only an increased risk and severity of expected toxicities, such as haemorrhagic cystitis following cyclophosphamide in patients with DNARD, but also unexpected toxicities such as cardiotoxicity and hepatotoxicity as well as deterioration of pre-existing comorbidities, especially of renal and pulmonary function. Examples of excess toxicities are well-documented in many case reports and series (11, 45–48). However, what is less well-appreciated from the literature is the variability in toxicity, even within a single disease cohort, a rare example of which has been summarized for patients with Nijmegen Breakage syndrome (49). One approach which therefore is commonly taken in IEI/DNARD is to apply an initial dose reduction strategy. This allows for subsequent dose escalation in patients not showing severe toxicity, as confirmed by frequent monitoring investigations (13, 47, 50). An alternative approach is the delivery of full-dose chemotherapy, but with increased intervals between cycles to allow optimal recovery from toxicity. Finally, protocol substitutions may be considered, with alternative agents being used in certain circumstances such as the substitution of topoisomerase inhibitors in DNARD patients. Specifically introducing novel, non-genotoxic therapies is a very attractive concept and is discussed further below. The risk is that substitution with less intensive or alternative therapies may result in under treatment and failure to achieve the primary aim, which is the cure of malignancy. Equally, whilst targeted therapies are hoped to carry less risk of toxicity, there is little clinical experience with anything other than rituximab to support this to date.

Many IEI and some examples of DNARD are suitable for allogeneic haematopoietic stem cell transplantation (HSCT) and this may form a core component of the management of their underlying condition. Many centers would consider HSCT in first remission as a consolidative treatment following cytoreductive chemotherapy, with the added benefit of addressing the underlying predisposition disorder. However,

BOX 3 | Developmental priorities for the treatment of malignancy including HSCT.

- Improve understanding of cancer therapy-associated toxicities, including predictive biomarkers for toxicity
- Develop consensus guidelines for initial therapy and dose modification, dependent on underlying IEI/DNARD.
- Prospective studies addressing the timing and delivery of HSCT dependant on underlying IEI/DNARD.
- Prospective studies of the true burden of therapy on subsequent quality of life and life expectancy to guide treatment strategy decision making.
- Develop guidelines for supportive care.

there are currently a number of key unanswered questions in this area, including the state of response required prior to HSCT (partial remission vs. complete remission), optimal conditioning strategy (myeloablative vs. reduced intensity), value as salvage for refractory/recurrent malignancy and the potentially increased risk of secondary, second primary or relapsed malignancy post-transplant.

For those IEI/DNARD where transplantation is not immediately indicated, pre-emptive HSCT might be suitable for patients with a particularly high risk of developing a malignancy. This approach will require improved risk stratification and development of appropriate biomarkers. Furthermore, prolonged follow-up is required to validate outcomes of children whose risk of pediatric malignancy is reduced, but who then may continue to be at risk of other disease manifestations including an adult cancer spectrum. This is particularly true for children with DNARD (51) who, despite the use of modified conditioning regimens, may be at substantial ongoing risk of complications including non-haematopoietic malignancy.

In addition to chemotherapy, which provides the current mainstay of malignancy therapy, and HSCT, a core component of curing underlying IEI, patients require an intensive package of supportive care measures, both to allow maximal intensity of treatment delivery and to ensure minimal deterioration in baseline organ function. Dependent on the specific underlying IEI/DNARD, particular attention should be paid to surveillance for and management of infection, with many clinicians opting to keep a patient hospitalized for the duration of their cancer treatment. Upgraded supportive care strategies include more intensive immunological monitoring and wide-ranging microbiological diagnostics as well as consideration of supportive intravenous immunoglobulin, prophylactic antibiotics and haematopoietic growth factors. Episodes of presumed or confirmed infections require aggressive combinatorial broad spectrum antimicrobials, as with all patients receiving cytotoxic chemotherapy, but the duration of therapy may need to be extended and obligate bactericidal agents may be warranted. Particular attention must be paid to patients' nutrition to maximize recovery as well as to maintain intestinal integrity, minimizing translocation of intestinal organisms. Physiotherapy involvement is critical to reduce the risk of long-term respiratory deterioration from infection and to rapidly identify and manage musculoskeletal and neurological complications. Finally, clinical psychology support provides essential support for patients and families performing complex joint decision making which must address the balances of risks and benefits described above.

NOVEL THERAPIES

The significant burden of toxicity seen in patients with IEI/DNARD following treatment with, particularly genotoxic, chemotherapy argues strongly in favor of the investigation of alternative anti-cancer treatment strategies. Existing alternatives, developed for the mainstream oncology market, rely on a range of effector mechanisms which would need to be carefully evaluated in each underlying IEI. This will be particularly important for the immune modulating therapies including checkpoint inhibitors and CAR-T cell therapies (**Table 1**). This presents substantial challenges, not least the relative infrequency of cancer diagnoses in patients with rare predisposing conditions. Additionally, the complex combinations of pre-existing co-morbidities would make defining tolerability very difficult in an acceptable early phase trial setting. Conducting unbiased clinical trials of novel therapies in specific patient cohorts will therefore be restricted to very few conditions.

Despite these difficulties, the potential impact of targeted drugs is substantial, as has already been seen with the anti-CD20 monoclonal antibody rituximab which has been used routinely as a single agent therapy in the management of PTLD for many years. Whilst achieving cure with single targeted agents has proven uncommon in general hematology/oncology, patients with IEI may prove more amenable to a strategy in which a targeted, low toxicity drug provides cytoreductive therapy prior to curative HSCT. This would still require a detailed understanding of the driving oncogenic mechanisms, biomarkers predicting response to therapy and potential mechanisms of resistance (**Box 4**). Developing a collaborative network of groups able to model the relevant disease process would allow for pre-clinical drug testing prior to developing a clinical strategy. Whilst randomized prospective clinical trials may be unrealistic, the need to investigate alternative therapies in these very high risk patient groups argues in favor of developing common treatment strategies with structured prospective collection of toxicity and outcome data within specific underlying IEI/DNARD cohorts.

SCREENING FOR IEI IN NEW MALIGNANCY CASES

A number of studies have suggested that between 6 and 10% of all childhood cancer cases are the result of an underlying cancer predisposition syndrome (52–54). A significant proportion of these are not previously known to the family, but can be

TABLE 1 | Examples of novel targeted therapeutics with potential application in IEI/DNARD associated malignancy.

Class of agent	Clinical example	Target	Mechanism of action	Relevance in IEI/DNARD
Monoclonal antibody	Rituximab Alemtuzumab Daratumumab Trastuzumab Cetuximab	CD20 CD52 CD38 Her2 EGFR	Direct and indirect cellular toxicity through activation of immune targeting Competitive binding of cell surface molecule and ADCC	
Antibody-drug conjugate	Brentuximab vendotin Inotuzumab ozogamicin	CD30 CD22	Targeted delivery of cytotoxic drug	No residual immune function required
Bi-specific T-cell engaging antibody	Blinatumomab	CD19-CD3	Antigen directed T cell targeting	Require functioning cytotoxic T cells
Immune checkpoint inhibitor	Nivolumab Atezolizumab	PD-1 PD-L1	Inhibit negative regulation of T cell activation	Activity may correlate with hypermutant tumors, common in CMMRD. Require functioning cytotoxic T cell
CAR-T cells	Tisagenlecleucel	CD19	Autologous T-cells expressing chimeric T-cell receptor	Requires autologous T-cell harvest – optimal efficiency likely only in functionally normal T-cells. Allogeneic options in development
Small molecule inhibitor	Ibrutinib/Acalabrutinib Idelalisib Everolimus Trametinib Crizotinib	BTK PI3Kδ mTOR MEK ALK	Inhibits BCR signaling Inhibits PI3K/AKT signaling Inhibits mTOR pathway signaling Inhibits MAPK/ERK signaling Inhibits ALK signaling	Under investigation in sporadic B/T cell malignancies commonly seen in IEI/DNARD.

CMMRD, constitutional mismatch repair deficiency syndrome; TCR, T cell antigen receptor; BCR, B cell antigen receptor.

BOX 4 | Priorities for assessment and application of novel targeted therapies.

- Improve molecular biological understanding of oncogenesis relevant to novel therapeutic approaches.
- Develop models of disease including genetically engineered mouse models and patient-derived xenografts to allow functional molecular investigations and therapy testing.
- Develop common and standardized approaches to dosing, including adjuvant chemotherapy, in order to generate structured case series.
- Prospective collection of immediate and long-term toxicity data.

BOX 5 | Priorities for identification and investigation of suspected IEI/DNARD following cancer diagnosis.

- Develop screening tools suitable for identification of IEI/DNARD at diagnosis of malignancy.
- Develop understanding of immune system status at presentation and under therapy in both sporadic and predisposed cases of malignancy.
- Longitudinal studies of immune reconstitution following treatment with both cytotoxic chemotherapy and immune system targeted therapies.
- Define algorithms for immunological investigations, including use of genomic sequencing.

identified using screening approaches such as exome/genome family trio sequencing with recently identified complex patterns of inheritance identified (55, 56). Consequently, amongst all oncology patients there will be a proportion of patients carrying a previously undiagnosed IEI/DNARD. Given the increased risk of acute toxicity and infection as well as the need to address family counseling, especially with a perspective toward HSCT, identifying and investigating such patients presents an important part of their holistic care (**Box 5**).

In most centers consideration of immunological screening investigations has traditionally been based on the presence of additional clinical features including (i) a personal history of infections, co-morbidities, developmental delay or congenital abnormalities; (ii) a family history of known inherited conditions, strong infectious or cancer history or a consanguineous parental relationship; or (iii) unusual presentations of malignancy. The latter group may include rare tumors such as extranodal marginal zone lymphoma or peripheral T cell lymphoma in a child (57, 58), unusual background histopathological findings, unusual sites of disease (primary central nervous system lymphoma in a child) (59), or unusual characteristic cyto-/molecular genetics, our understanding of which is currently in its infancy (33). Having made a diagnosis of malignancy, the presence or occurrence of unusual infections, in terms of severity, organism or frequency, or of severe or unusual therapy associated toxicity may raise the suspicion of an underlying predisposition syndrome.

An alternative approach which is being used more frequently, and will soon be included in a number of international late

phase oncology clinical trials, is the use of predisposition screening tools for newly diagnosed cancers, most notably in children (60, 61). These have been developed primarily as decision support tools to identify higher risk patients who should be referred to expert genetic counseling and consideration of targeted or genome wide investigation. However, specific screening for immune disorders as a causative underlying cancer predisposition mechanism is rarely included.

For patients in whom an increased concern regarding an underlying IEI/DNARD exists, determining the optimal time and approach to screening can be challenging. Standard laboratory investigations for underlying IEI/DNARD can be hard to interpret at diagnosis of malignancy as a result of the patient's general clinical state, the presence of infection and fever, and, for haematopoietic malignancies, involvement of the bone marrow. Investigation during and immediately following treatment is affected by anti-cancer therapy, including both traditional chemotherapy and agents targeting haematopoietic/lymphoid surface markers including CD20, CD22, CD30, and CD52 (**Table 1**). Direct assessment of the presence of an underlying disorder might involve radiosensitivity studies, DNA damage assays or, increasingly, genetic screening for known IEI/DNARD associated mutations using constitutional DNA samples. With the increasing availability of routine paired cancer/germline and family trio genome sequencing, this approach is likely to become more important and may bypass the challenges of more classical cellular and functional testing. However, implementation of genomic medicine at the clinical level will require dedicated analyses to be developed and probably more widespread training in interpretation and counseling to allow for immediate treatment stratification by clinicians.

CONCLUSION

Despite a growing awareness of the increased risk of malignancy in people with IEI/DNARD, the diagnosis and management of these patients remain poorly understood and thus challenging and are frequently based on correlation with sporadic malignancies rather than dedicated IEI/DNARD specific guidance. In order to improve this situation, diagnostic and clinical teams caring for patients with IEI/DNARD will need to work in collaborative groups and international networks in order to bring together sufficient cases, experience and understanding. This first meeting of ESID, EBMT, RITA, iBFM, and EICNHL represents an example of such a collaborative initiative. Having developed this (non-exhaustive) list of developmental priorities, it is now important to broaden collaborations in order to maximize achievements that will allow improvement in patient care and outcomes.

AUTHOR CONTRIBUTIONS

All authors developed the concept of the current position and edited the manuscript. SB wrote the manuscript. MS created the graphic figure.

ACKNOWLEDGMENTS

MS created the graphic figure depicting our vision of the immediate challenges facing clinicians and scientists working with IEI-associated malignancy. We thank Arjan Lankester for hosting the working group meeting in Leiden, 2017.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 05 November 2018
doi: 10.3389/fimmu.2018.02546

Gastric Cancer Is the Leading Cause of Death in Italian Adult Patients With Common Variable Immunodeficiency

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An increased prevalence of malignant lymphoma and of gastric cancer has been observed in large cohorts of patients with common variable immunodeficiency (CVID), the most frequently symptomatic primary immunodeficiency. Surveillance strategies for cancers in CVID should be defined based on epidemiological data. Risks and mortality for cancers among 455 Italian patients with CVID were compared to cancer incidence data from the Italian Cancer Registry database. CVID patients showed an increased cancer incidence for all sites combined (Obs = 133, SIR = 2.4; 95%CI = 1.7–3.5), due to an excess of non-Hodgkin lymphoma (Obs = 33, SIR = 14.3; 95%CI = 8.4–22.6) and of gastric cancer (Obs = 25; SIR = 6.4; 95%CI = 3.2–12.5). CVID patients with gastric cancer and lymphoma had a worse survival in comparison to cancer-free CVID (HR: 4.8, 95%CI: 4.2–44.4 and HR: 4.2, 95%CI: 2.8–44.4). Similar to what observed in other series, CVID-associated lymphomas were more likely to be of B cell origin and often occurred at extra-nodal sites. We collected the largest case-series of gastric cancers in CVID subjects. In contrast to other reports, gastric cancer was the leading cause of death in CVID. Standardized mortality ratio indicated a 10.1-fold excess mortality among CVID patients with gastric cancer. CVID developed gastric cancer 15 years earlier than the normative population, but they had a similar overall survival. Only CVID diagnosed at early stage gastric cancer survived >24 months. Stomach histology from upper endoscopy performed before cancer onset showed areas of atrophic gastritis, intestinal metaplasia or dysplasia. CVID patients might progress rapidly to an advanced cancer stage as shown by patients developing a III-IV stage gastric cancer within 1 year from an endoscopy without signs of dysplasia. Based on high rate of mortality due to gastric cancer in Italian CVID patients, we hereby suggest a strategy aimed at early diagnosis, based on regular upper endoscopy and on *Helicobacter pylori* infection treatment, recommending an implementation of national guidelines.

Keywords: common variable immunodeficiency: cancer, gastric cancer, lymphoma, IgA, upper endoscopy, risk, guidelines

OPEN ACCESS

Edited by:
Andrew R. Gennery, Newcastle University, United Kingdom

Reviewed by:
Vanessa L. Bryant, Walter and Eliza Hall Institute of Medical Research, Australia
Anastasios E. Germenis, University of Thessaly, Greece

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Specialty section:
This article was submitted to Primary Immunodeficiencies, a section of the journal Frontiers in Immunology

Received: *28 June 2018*
Accepted: *16 October 2018*
Published: *05 November 2018*

Citation:
Pulvirenti F, Pecoraro A, Cinetto F, Milito C, Valente M, Santangeli E, Crescenzi L, Rizzo F, Tabolli S, Spadaro G, Agostini C and Quinti I (2018) Gastric Cancer Is the Leading Cause of Death in Italian Adult Patients With Common Variable Immunodeficiency. Front. Immunol. 9:2546. doi: 10.3389/fimmu.2018.02546

INTRODUCTION

Inherited conditions affecting immune system function are classified as primary immune deficiencies (PID) (1). As the PID life expectancy increased because of improvements in the surveillance, prevention, and treatment, the occurrence of cancer increased (2, 3). In PID, hematological and non-hematological malignancies occur mainly in the fourth to the seventh decades of life while rare case of malignancies are commonly observed in the pediatric population (4). Among PID, an increased prevalence of cancer is recognized in patients affected by common variable immunodeficiency (CVID), the most common symptomatic primary antibody defect. In CVID, the antibody deficiency might derive from decreased diversity of the naive pool, decreased hyper mutation in memory repertoires, an unusual clonal expansion of un-mutated B cells, and from a number of defects in innate and adaptive immune mechanisms (5). Other than sino-pulmonary infections, CVID patients suffer from associated clinical conditions, including autoimmune and inflammatory diseases and neoplasia, mainly lymphoma and gastric cancer (6, 7). Ten years ago, our group described a higher prevalence of lymphoma and gastric carcinomas in the Italian cohort of CVID in comparison to the normative population (8). Five years later, we confirmed this high prevalence rate of lymphoma and gastric carcinomas in a four decades study showing that 21% of adult CVID patients developed cancers. We also observed that deaths from cancer occurred in 10.2%, a percentage double than that reported in a study from a CVID cohort in New York over the same length of time (2, 9). We suggested that the discrepancy in cancer survival, between the two cohorts, might have been due to the high prevalence of deaths for malignancies other than lymphoma in the Italian CVID cohort, and to deaths for gastric cancer. The percentage of patients who died for lymphoma, indeed, was similar in the two studies.

Herein, we analyzed data on the prevalence of hematological and non-hematological malignancies, on cancer risk, on mortality and on survival rate in a cohort of 455 Italian adult CVID patients compared to normative population. Detailed data on CVID patients diagnosed with gastric cancer, histopathology of gastric lesions, cancer outcome and possible associated risk-factors were reported. Based on the high rate of mortality for gastric cancer in Italian CVID patients, we highlight the need of a strategy for an earlier diagnosis and we suggest a new schedule for gastric endoscopy in CVID patients.

METHODS

Study Design

Data on adult CVID patients (>18 years old), regularly followed in three University-based PID referral centers located in Central Italy (Rome), Southern Italy (Naples), and Northern Italy

TABLE 1 | Characteristics of 455 CVID patients enrolled in the study.

Characteristics	All patients	Cancer	Cancer-free
AGE INTERVAL—*n*. (SD)			
18-35 years	75 (16.5)	7 (6.0)	68 (20.0)***
36–50 years	142 (31.2)	29 (25.0)	113 (33.3)
51–65 years	150 (32.9)	45(38.8)	105 (31.0)
66–80 years	82 (18.0)	33 (28.5)	49 (14.5)**
>80 years	6 (1.3)	2 (1.7)	4 (1.2)
Sex (female)—*n*. (%)	235 (51.6)	58 (50.0)	162 (47.8)
Age at CVID diagnosis—mean (SD)	40.1 (15.4)	45.8 (13.2)	38.8 (15.7)***
SERUM IMMUNOGLOBULIN AT DIAGNOSIS (mg/dL)—MEAN (SD)			
IgG	250.3 (172.3)	256.2 (168.6)	248.7 (173.6)
IgA	21.6 (34.2)	19.9 (30.8)	22.1 (35.1)
IgM	25.2 (49.8)	37.0 (89.8)	21.9 (30.4)*
Bronchiectasis	118 (31)	26 (31)	92 (30)
Autoimmunity	130 (28)	27 (28)	103 (29)
Lymphoproliferative	113 (31)	28 (33)	85 (29)
Enteropathy	52 (14)	11 (14)	41 (14)
Time of follow-up person-year—mean (SD)	11.5 (8.9)	11.8 (8.4)	11.4 (9.2)
Patients with cancer—*n*. (%)	116 (25.5)	–	–
Patients with more than one cancer—*n*. (%)	18 (4.0)	–	18(15.5)
Patients alive at the last follow up—*n*. (%)	377 (82.9)	51(44.0)	27 (8.0)

*$p < 0.01$, **$p < 0.001$, and ***$p < 0.0001$ (cancer vs. cancer-free CVID patients). SD, standard deviation; CVID, common variable immunodeficiency; Ig, immunoglobulin. Immunoglobulin normal range (adults): IgG 700–1600 mg/dL; IgA 68–400 mg/dL; IgM 40–259 mg/dL.

TABLE 2 | Prevalence of cancer diagnosis in 455 Italian CVID patients.

Cancer diagnosis—*n*. %	All patients *n*. 455		Female *n*. 235		Male *n*. 220	
	n.	%	*n*.	%	*n*.	%
Non-Hodgkin lymphoma	33	7.3	15	6.4	18	8.2
Gastric cancer	25	5.5	9	3.8	16	7.1
Colorectal cancer	10	2.2	4	1.7	6	2.7
Breast cancer	10	2.2	10	4.3	-	-
Thyroid cancer	6	1.3	3	1.3	3	1.3
Hodgkin lymphoma	5	1.1	2	0.9	3	1.3
Large Granular Lymphocytic Leukemia	5	1.1	3	1.3	2	0.9
Lung cancer	4	0.9	2	0.9	2	0.9
Liver cancer	4	0.9	2	0.9	2	0.9
Uterine cancer, body	4	0.9	4	1.7	–	–
Uterine cancer, cervical	3	0.7	3	1.3	–	–
Prostatic cancer	3	0.7	–	–	3	1.3
Pancreatic cancer	3	0.7	2	0.9	1	0.4
Other blood cancer (CML, polycythemia vera)	3	0.7	1	0.4	2	0.9
Kaposi sarcoma	1	0.2	0	–	1	0.4
Others*	13	3.5	7	3.0	6	2.7

*Others: Bladder cancer, meningioma, melanoma, neuro-endocrine carcinoma, ocular carcinoma, kidney carcinoma, adrenal carcinoma.

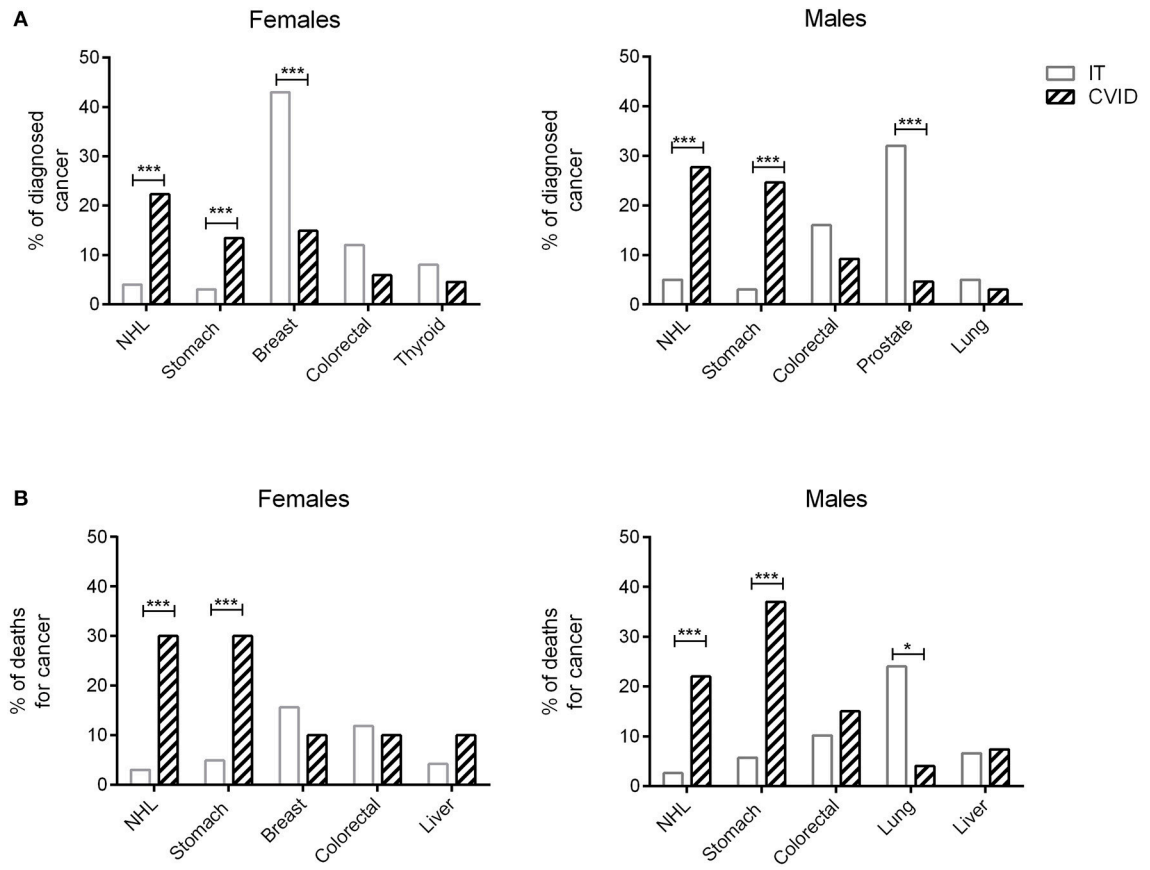

FIGURE 1 | Cancers diagnosis and death for cancer in CVID and in the normative population. Data related to the proportion of the five most frequently diagnosed cancers in male and female CVID patients (dashed bars) are shown in comparison to the normative population (IT, white bars) **(A)**. Proportion of deaths for cancer in male and female CVID patients (dashed bars) are shown in comparison to the normative population (IT, white bars) **(B)**. In CVID, NHL and gastric cancer were the most commonly diagnosed cancers in both sexes, whereas breast cancer and prostate cancer were the most frequently recorded malignancies in Italian normative population. Gastric cancer was the first cause of death for cancer in CVID females and males, followed by NHL; breast and lung cancers were the most common cause of death for cancer in normative population. Data of normative population referred to 2017 AIRTUM report. NHL, non-Hodgkin lymphoma. $^{*}p < 0.01$; $^{***}p < 0.0001$.

(Padua-Treviso) were prospectively collected from 01/01/2001 to 31/12/2017 and retrospectively collected from 01/01/1993 to 31/12/2000. To be considered for analysis, subjects needed to fulfill the 2016 ESID revised criteria (http://esid.org/Working-Parties/Registry/Diagnosis-criteria). A set of variables was recorded for each patient including: gender, date of birth, date of CVID diagnosis, data on cancer diagnosis and histology, date of last follow up visit, vital status information, date and cause of death, CVID-associated diseases (infections, cancer, autoimmunity, unexplained persistent proliferation, and unexplained persistent enteropathy) and *Helicobacter pylori (H. pylori)* status. We excluded from the analysis patients whose data on date cancer occurrence and its outcome and on date of cancer diagnosis, death and last follow-up were lacking. The follow-up period before the occurrence of cancer was calculated since the year of immunodeficiency onset. All subjects were followed until date of death or date of the end of the study (31 December 2017). For the subset of patients who developed cancer, medical records were traced to verify cancer diagnosis, treatments received, clinical complications, and outcome.

AIRTUM Estimated Cancer Incidence

The Associazione Italiana Registro Tumori (AIRTUM) (www.registri-tumori.it) is a coordinated system of population-based cancer registries that collects cancer incidence and survival data from 20 geographic areas throughout Italy, covering 70% of the Italian population (without age restriction). Detailed information is available at http://www.registri-tumori.it/. We used AIRTUM published data to estimate the expected incidence of cancer. Among skin malignancies, melanoma was the only one cancer with data on incidence and mortality reported in the AIRTUM database. For this reason, we did not collected data for not-malignant skin cancer.

Statistical Analysis

Demographics of the CVID database were summarized with descriptive statistics. Sociodemographic and clinical variables

TABLE 3 | Observed (Obs) and Expected (Exp) numbers and Standardized Incidence Ratio (SIR) of cancer among 455 Italian patients with CVID.

	Obs	Exp	SIR	95%CI
CANCER				
All malignant neoplasms	133	55.1	2.4	1.7–3.5
Non-Hodgkin lymphoma	33	2.3	14.3	8.4–22.6
Gastric cancer	25	3.9	6.4	3.2–12.5
Colorectal cancer	10	8.2	1.2	0.0–1.9
Breast cancer	10	10	1	0.7–1.2
Thyroid cancer	5	1.7	2.9	0.0–6.4
Hodgkin Disease	5	0.4	12.5	3.4–22.4
Lung cancer	4	28	0.1	0.2–0.7
Liver cancer	4	2.1	1.9	0.3–5.6
Uterine cancer, body	4	1.2	3.3	0.1–6.5
Uterine cancer, cervical	3	1.2	2.5	0.1–4.8
Prostatic cancer	3	7.1	0.4	0.1–1.0
Pancreatic cancer	3	1.9	1.6	0.3–3.9

TABLE 4 | Cause of death in CVID patients.

Causes of death	*n.*	%
Cancer	47	60.3
Gastric cancer	16	20.5
Non-Hodgkin Lymphoma	14	17.9
Colorectal cancer	6	7.7
Liver cancer	4	5.1
Pancreatic cancer	2	2.6
Breast cancer	2	2.6
Hodgkin Disease	1	1.3
Lung cancer	1	1.3
Uterine cancer	1	1.3
Infections	15	19.2
LRTI (respiratory failure)	12	10.3
Other infections (sepsis, CMV)	3	10.3
Cardiovascular disease	5	6.4
Autoimmune manifestations: AHA, AIH	4	5.1
Others*	7	9.0
Total	78	–

LRTI, lower respiratory tract infections; AHA, autoimmune hemolytic anemia; AIH, autoimmune hepatitis.
**Parkinson disease, cirrhosis, accident, suicide.*

were compared between the patients who developed cancer and cancer-free patients. Statistical analysis was performed using frequency distributions. The X^2 test was used for categorical variables and the *t-test* was used for continuous variables. The observed numbers of cancer cases among CVID were compared with the expected numbers calculated based on AIRTUM data on incidence rates of cancer in 5-year interval to yield the standardized incidence ratio (SIR). "All cancer" and site-specific cancer SIRs were calculated for the entire cohort, and separately for men and women. For mortality analysis, the time since diagnosis was determined using the age at the time of CVID diagnosis or the age at birth. The endpoint used was the time of last known follow-up or the date of death. Probabilities of survival after the diagnosis of CVID and after the diagnosis of cancer were estimated from Kaplan Meier life Table. Mortality rates (crude death rates, CDRs) of the general population were used to calculate the standardized mortality ratio (SMRs). The CDR was obtained from AIRTUM. SMRs were calculated using the formula, SMR = Observed (Obs) deaths/expected (Exp) deaths. We calculated SMRs as incident cases divided by the contributed person-years. However, general population incidence and mortality data for Italy before 2003 were not available, so only cancer and death occurred after 2003 were included in the analysis. Statistical Package for Social Sciences version 15 (SPSS Inc., 233 South Wacker Drive, 11th Floor, Chicago) was used for the analysis. Confidence Intervals (CI95%) were calculated by R-3.4.4 version.

RESULTS

Patients

As of 31 December 2017, 501 subjects with a CVID diagnosis were included in the dataset. We excluded from the analysis 46 subjects who did not satisfy ESID criteria and patients whose date of death, date of cancer occurrence and outcome, and date of last follow-up could not be accurately determined. Data on 455 CIVD patients were included in the analysis. The characteristics of CVID patients enrolled in the study are summarized in **Table 1**. The mean age at last follow-up was 51.1 ± 15.0 years, with a 1:1 female: male ratio. Patients were followed-up for a cumulative period of 5,169 person-years with a mean time of follow-up of 11.5 ± 8.9 years. *H. pylori* status was available in 325/455 patients. *H. pylori* infection by histology was found in 40 patients (12%).

Cancer Prevalence and Risk in CVID Patients

During the study time, 132 separate cancers were diagnosed in 116 patients (25.5%). Eighteen patients (4%) developed more than one cancer. The age at CVID onset was higher for patients who developed cancer in comparison to those who did not (45.9 ± 13.2 vs. 38.1 ± 15.7 yrs, $p < 0.0001$, **Table 1**). The mean age at the first cancer diagnosis was 52.5 ± 13.8 (range: 26–85 yrs). Sixty-seven cancers were diagnosed in women and 65 cancers in men. Malignancies diagnosed were: lymphoma (38; 29%), gastrointestinal cancers (35; 26%), genitourinary cancers (14; 8%), breast cancers (10; 7%), uterine cancers (7; 5.3%), thyroid cancers (6; 4%), lung cancers (4; 3%), liver cancers (4; 3%), prostatic cancer (3; 2%), and pancreatic cancer (3; 2%). The overall and sex-related prevalence of single cancer was summarized in **Table 2**.

Figures 1A,B showed the percentage of the top five diagnosed cancers and the top five fatal cancers seen in CVID in comparison to the normative Italian population. The most common malignancy diagnosed in the AIRTUM database was breast cancer (women only), prostate cancer (men only), lung, and colorectal cancers. The incidence of these cancers was not increased in CVID patients in comparison to the AIRTUM

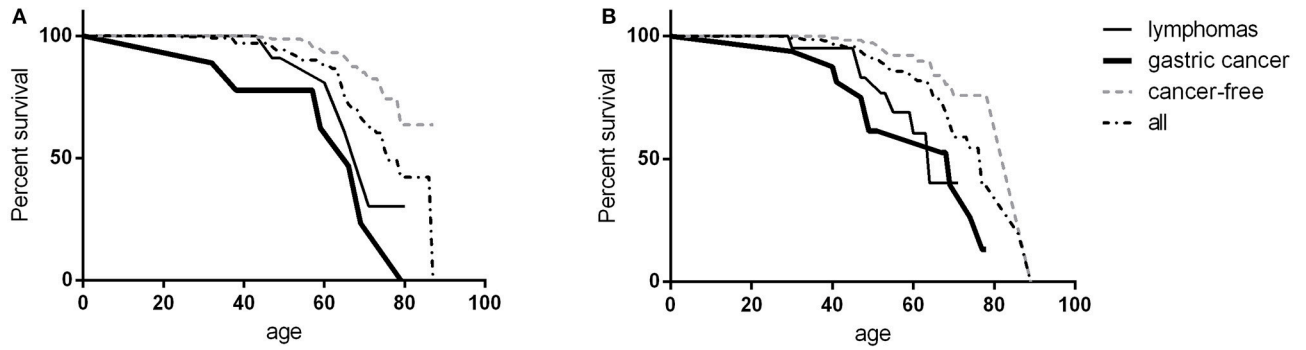

FIGURE 2 | CVID survival. Survival in female **(A)** and male **(B)** CVID participants: data were shown as overall survival (black dashed line), in CVID patients with gastric cancer (black bold line), in patients with lymphoma (black line) and in cancer-free CVID patients (gray dashed line). No survival differences were observed between females and males; CVID subjects with gastric cancer or lymphoma had a worse survival in comparison to cancer-free CVID population.

TABLE 5 | Standardized mortality ratios (SMRs) for cancers causing death in CVID.

Cancers	Obs	Exp	SMR	95%CI
All malignant neoplasm	47	44.5	1.0	0.5–1.6
Gastric cancer	16	2.0	10.1	3.8–16.3
Non-Hodgkin lymphoma	14	0.8	16.5	8.8–31.4
Colorectal cancer	6	2.1	2.8	0.1–6.3
Liver cancer	4	1.8	2.9	0.1–5.9
Pancreatic cancer	2	1.7	1.2	0.6–3.2
Breast cancer	2	7.0	0.3	0.0–0.5
Hodgkin Disease	1	0.1	10.0	0.0–45.2
Lung cancer	1	6.6	0.1	0.3–0.5
Uterine cancer	1	0.3	2.8	0.0–8.3

database (**Table 3**). Ten female CVID patients were diagnosed with breast cancer (Exp: 10.0; SIR: 1.0; 95%CI: 0.7–1.2). Three male CVID patients were diagnosed with prostate cancer (Exp: 7.1; SIR: 0.4, 95%CI: 0.1–1.0). There was no increase in the rates of lung cancer (Obs: 4, Exp: 28.0, SIR 0.1; 95%CI: 0.2–0.7) and colon cancer (Obs: 10; Exp: 8.2; SIR 1.2; 95%CI: 0.0–1.9) among CVID patients vs. normative population (**Table 3**). In contrast to Italian normative population, the most commonly diagnosed malignancies in female and male subjects with CVID were non-Hodgkin lymphoma (NHL) and gastric cancer (**Figure 1A**). The risk for NHL and Hodgkin's lymphoma (HD) was increased by 14.3- and 12.5-fold, respectively, based on 33 and 5 cases observed. The risk for gastric cancer was increased 6.4-fold based on 25 cases observed (**Table 3**).

Survival and Mortality

Three-hundred and seventy-four (82.4%) patients were alive at the end of the study-time. During the study time, we observed 78 deaths in the patient population. Malignancies were the first cause of death, accounting for 60.3% of deaths. Gastric cancer was the leading cause of death (20.5%). Infections accounted for 19.2% of deaths, 80% due to lower tract respiratory infections. Causes of mortality in patients are detailed in **Table 4.** The CVID overall survival (OS) was 85.1% (SE 2.3%) at 60 years and 61.8% (SE 4.2%) at 70 years. No differences were observed between males and females. Cancer-free CVID had a better survival in comparison to those with gastric cancer (Log-Rank $p < 0.0001$, HR: 4.8, 95%CI: 4.2–44.4, **Figure 2A**) and lymphomas (Log-Rank $p = 0.001$, HR: 4.2, 95%CI: 2.8–44.4, **Figure 2B**).

Cancer excess of mortality was expressed as SMRs (**Table 5**). The most fatal cancers in the AIRTUM database were lung, colorectal and breast cancer. We found no significant increase in the mortality for colorectal cancer (Obs: 6; Exp: 2.1; SMR: 2.8, 95%CI: 0.1–6.3) and a significant lower mortality for lung and breast cancers in CVID patients (lung cancer: Obs: 1; Exp: 6.6; SMR: 0.1; 95%CI: 0.3–0.5; breast cancer: Obs; 2; Exp: 7.0; SMR: 0.3; 95%CI: 0.0–0.5, **Table 5**). Moreover, in CVID we found an excess of mortality for NHL (Obs: 14; Exp: 0.8; SMR: 16.5; 95%CI: 8.8–31.4) and gastric cancer (Obs: 16; Exp: 2.0; SMR: 10.1; 95%CI: 3.8–16.3) (**Table 5**).

Gastric Cancer

Gastric cancer was the second most frequent cancer diagnosed in CVID accounting for 18.9% of cancer diagnosed, and the first cause of death. Of the 25 cases of gastric cancer, 16 (64%) occurred in men. The age at cancer diagnosis was 51.8 ± 13.7 years (range: 30–75), 15 years younger than that reported in the Italian population (10). The diagnosis of cancer occurred within 10 years from the CVID diagnosis in two thirds of these patients. CVID subjects with gastric cancer had a similar age at immunodeficiency onset than the entire CVID cohort (40.0 ± 15.0 vs.39.9 ± 15.4, $p = 0.975$). Undetectable IgA (<7 mg/dL) and IgM (<6 mg/dL) serum levels at time of CVID diagnosis were more likely in patients with gastric cancer in comparison to those without that complication (IgA: OR 27.5, 95%CI: 1.5–475.9, $p = 0.027$; IgM: OR 7.4, 95% CI: 1.5–36.1, $p = 0.013$). Seven out of 25 patients had an additional malignancy: three patients were diagnosed with lymphoma, two with colorectal cancer, one with gallbladder cancer, and one with meningioma. In addition, two patients had a multifocal gastric adenocarcinoma treated by two-stage gastrectomy (**Table 6**). One patient (n. 1)

TABLE 6 | Age at PID and at cancer diagnosis, survival, outcome, histology, cancer stage, and cancer treatment in 25 CVID patients with gastric cancer.

ID	Sex	Age at PID diagnosis (years)	Age at cancer diagnosis (years)	Survival after cancer diagnosis (months)	Outcome	Histology	Stage	Treatment	Additional cancer	Enteropathy (before cancer diagnosis)
1	M	39	40	408	Alive	Early gastric cancer, pT1N0 G1	stage I	Gastrectomy (total)	Kaposi sarcoma, colorectal carcinoma	No
2	F	25	31	12	Deceased (cancer)	Gastric adenocarcinoma, NOS	NA	Chemotherapy, NOS	No	Yes
3	F	45	45	252	Alive	Gastric adenocarcinoma, NOS	NA	Gastrectomy, NOS	Meningioma	No
4	F	67	69	120	Deceased (cancer)	Early gastric cancer pT1N0 G2,	stage I	Gastrectomy (total)	Biliary tract carcinoma	NA
5	M	40	49	7	Deceased (cancer)	Gastric adenocarcinoma, NOS	NA	Gastrectomy (total)	No	Yes
6	M	27	51	204	Alive	Gastric adenocarcinoma, intestinal type pT1N0 G2	stage I	Gastrectomy (total)	NHL (duodenal) lymphoma	No
7	M	58	74	36	Deceased (cancer)	Gastric adenocarcinoma, intestinal type, PT1, N0, M0	stage IA	Gastrectomy (subtotal)	No	Yes
8	F	35	45	144	Alive	Gastric adenocarcinoma, NOS pT1bN1 G1	stage IB	Gastrectomy (subtotal)	No	No
9	M	67	67	132	Alive	Gastric adenocarcinoma, NOS		Gastrectomy (subtotal)	Colorectal carcinoma	Yes
10	F	32	38	132	Alive	Early gastric cancer, pT1N0 G2	stage I	Gastrectomy (subtotal)	HD	Yes
11	M	64	67	30	Deceased (cancer)	Gastric adenocarcinoma, intestinal type, PT1, N0, Mx, G3	stage I	Gastrectomy (subtotal), Chemotherapy (lederfolin, xeloda, 5-fluorouracil)	No	Yes
12	M	69	75	24	Deceased (cancer)	Gastric adenocarcinoma, NOS	NA	Supportive	No	Yes
13	F	35	68	12	Deceased (cancer)	Gastric Adenocarcinoma, G3	NA	Supportive	NHL	Yes
14	F	27	38	9	Deceased (cancer)	Gastric adenocarcinoma, NOS	NA	Supportive	No	NA
15	M	30	47	12	Deceased (cancer)	Gastric adenocarcinoma, NOS	stage IIIB	Chemotherapy, NOS	No	Yes
16	M	40	40	84	Alive	Multifocal Gastric Adenocarcinoma pT2bN0 G3 CMV+	stage IB	Gastrectomy (total, two-step)	No	No
17	M	59	68	12	Deceased (cancer)	Gastric adenocarcinoma, NOS	NA	Supportive	No	Yes
18	M	36	48	11	Deceased (respiratory failure)	Gastric adenocarcinoma, NOS	NA	Chemotherapy, NOS	No	NA
19	M	18	40	15	Deceased (cancer)	Gastric adenocarcinoma, intestinal type pT3N3bMx G3	stage IIIC	Gastrectomy (total), Chemotherapy (platinum/5-fluorouracil)	No	Yes
20	F	43	64	24	Deceased (cachexia, meningitis)	Gastric adenocarcinoma, intestinal type pT3N2 G3	stage IIIA	Gastrectomy (subtotal), + Capecitabine	No	No
21	M	22	30	8	Deceased (cancer)	Gastric adenocarcinoma, NOS	stage IV	Supportive	No	No

(Continued)

TABLE 6 | Continued

ID	Sex	Age at PID diagnosis (years)	Age at cancer diagnosis (years)	Survival after cancer diagnosis (months)	Outcome	Histology	Stage	Treatment	Additional cancer	Enteropathy (before cancer diagnosis)
22	M	29	40	7	Deceased (cancer)	Gastric adenocarcinoma, NOS	stage IV	Chemotherapy, NOS	No	Yes
23	M	49	51	15	Alive	Multifocal gastric adenocarcinoma, intestinal type, pT1pN0 G3	stage I	Gastrectomy (total, two-step)	No	No
24	M	47	50	14	Alive	Early gastric cancer, intestinal type, pT1bNx G3	stage I	Gastrectomy	No	No
25	F	46	59	6	Deceased (cancer)	Gastric adenocarcinoma G3	stage IV	Chemotherapy (epirubicine, platinum, 5-fluorouracil)	No	Yes

NOS, not otherwise specified; NA, not available M, Males, F, Females; NHL, Non-Hodgkin lymphoma; HD, Hodgkin disease.

had a positive family history for gastric cancer: three relatives, including his IgA-deficient brother, developed the malignancy. In these two brothers, mutations of *CDH1* gene were not found. Fifteen patients with gastric cancer died during the study time. Overall, the average SMR indicated a 10.1-fold excess mortality among CVID patients with gastric cancer. The 10-year survival probability of the entire cohort of patients with gastric cancer was 25%. Clinical staging was available for 16/25 patients. Patients classified as stage I had a better survival in comparison to those with stage III-IV (HR: 0.01, 95%CI: 0.0–0.1, $p < 0.0001$, **Figure 3**). *H. pylori* status and histology of gastric endoscopic biopsies collected before the diagnosis of cancer was available for 7 patients (**Table 7**): areas of dysplasia were identified in two subjects whereas areas of atrophic gastritis and/or intestinal metaplasia were found in all patients. In the two patients with dysplasia, the following endoscopy revealed a stage I malignancy, 6 months apart (patient n. 23). Patient n. 25 agreed to undergo a further gastroscopy only 15 months apart, which allowed diagnosis of a stage IV gastric cancer. Interesting to note, patient n. 20 and n. 22 developed a high-grade gastric cancer <14 months after the preceding endoscopy whose histology did not show any signs of dysplasia. At least one *H. pylori* detection was significantly related to gastric cancer (43 vs. 13%, OR: 5.3, 95%CI: 1.1–24.8, $p = 0.042$).

Lymphoma

Lymphoma was the most frequent cancer diagnosed in CVID and the second cause of death for cancer (**Tables 2**, **4**). The age at lymphoma diagnosis was 32.8 ± 4.6 years for HD and 52.4 ± 13.1 years for NHL. The age at CVID diagnosis was higher in patients with lymphoma in comparison to those without lymphoma (45.7 ± 12.4 vs. 39.6 ± 15.6, $p = 0.008$). Three patients first presented with lymphoma prior to CVID diagnosis, raising the question if hypogammaglobulinemia might be secondary to the lymphoproliferative disease (**Table 8**). However, the longtime state of antibody defect after the diagnosis and treatment of lymphoma might suggest this possibility. As widely described (2–9, 11), also in our cohort, CVID-associated lymphoma was more likely to be of B cell origin (88.4%) with a predominance of NHL (81.8%). T-cell lymphomas (peripheral T-cell lymphoma, angio-immunoblastic T-cell lymphoma and anaplastic T-cell lymphoma) and one primitive effusion cavity (PEL) lymphoma were also observed (**Table 8**). Similar to what observed in other series of CVID patients about 30% were extra-nodal lymphomas. Patients with lymphoma were more likely to have lymphopenia (lymphocytes < 1,000 cell/mm^3) (OR: 3.0, 95%CI: 1.1–8.3, $p = 0.030$) and polyclonal lymphocytic infiltration phenotype (OR: 2.7, 95%CI: 1.2–6.3, $p = 0.016$) before cancer diagnosis.

DISCUSSION

This longitudinal study on a large cohort of CVID patients over a cumulative period of 5,169 person-years showed that one fourth of patients developed a malignancy. Cancer represents the first cause of death in our patient's population. The most commonly diagnosed malignancies in CVID were NHL and the first cause of death was gastric cancer. The excess of mortality for lymphoma and gastric carcinoma in CVID was increased by more than 10-fold in comparison to normative population. Several studies reported a high frequency of malignancies in CVID patients (2, 3, 6–9, 11–14) with a prevalence ranging from 1.5 to 20.7%. However, only few studies provided SIR, allowing the comparison of data on CVID to data on normative population. These surveys showed an excess of incidence ranging from 4- to 30-fold for NHL and from 3- to 47-fold for gastric cancer (3, 6, 7, 15). Nevertheless, the prevalence other malignancies was not increased, confirming that patients with antibody deficiencies have a narrow range of cancers (6).

Lymphoma is considered as one of the more severe complications of CVID. The prevalence registered in our cohort was similar to that found across the different countries examined (2–9, 11, 12). The histological types reported in the different series of CVID-associated lymphoma were also similar to our findings, with a predominance of non-Hodgkin

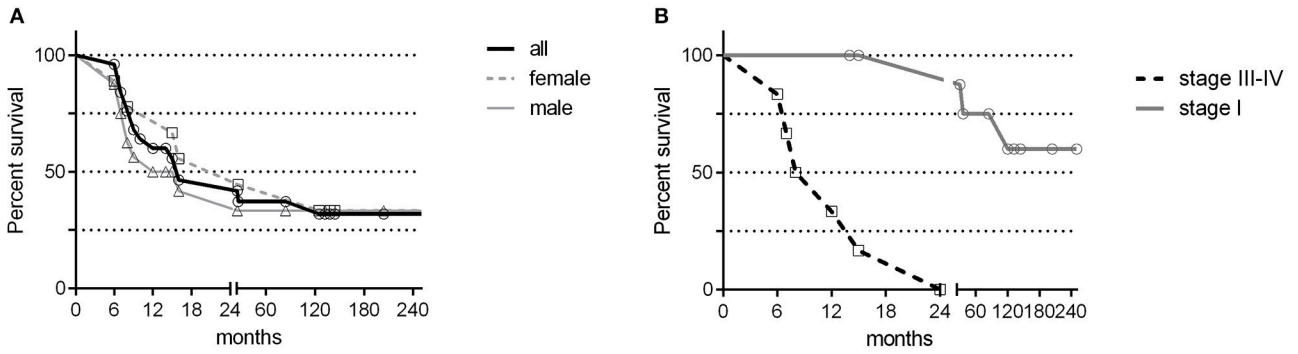

FIGURE 3 | Gastric cancer survival by sex and staging. Survival in the cancer free CVID subjects (black bold line) and in CVID females (gray dashed line) and males (gray line) with gastric cancer was shown in **(A)**. Survival in patients scored as stage I (gray line) and in patients scored as stage III-IV (dashed line) was shown in **(B)**. No difference was observed between CVID females and males with gastric cancer; patients scored as stage I had a better survival in comparison to patients scored as stage III-IV.

TABLE 7 | *H. pylori* status and histology of gastric biopsies from the endoscopy preceding the examination leading to gastric cancer diagnosis in seven CVID patients.

ID	*H. pylori* (pos/neg)	Histology of biopsy taken at the endoscopy preceding the one with gastric cancer diagnosis	Interval between endoscopies (months)	Stage at cancer diagnosis	Outcome
9	pos	Active chronic gastritis, intestinal metaplasia	36	NA	Alive
12	neg	Atrophic gastritis	35	NA	Death
13	pos	Atrophic gastritis	12	NA	Death
20	neg	Active chronic gastritis (moderate) with incomplete intestinal metaplasia	14	Stage IIIA	Death
22	pos	Active chronic gastritis (moderate), intestinal metaplasia	14	Stage IV	Death
23	neg	Atrophic gastritis, high grade dysplasia	6	Stage I	Alive
25	neg	Intestinal metaplasia, high-grade dysplasia	15	Stage IV	Death

NA, not available.

B cell lymphomas, possibly occurring at extra nodal sites. We confirmed the observation by Chapel et al. showing that CVID patients with polyclonal lymphadenopathy phenotype have an increased risk of lymphoid malignancy that generally occurs late in the disease course (16). In addition, we found that CVID patients with lymphopenia had a 3-fold increased risk to develop lymphomas. In CVID patients, diagnosis of lymphoma may be particularly challenging. Immune-histochemical analysis, studies on clonality and molecular studies might be helpful to distinguish reactive from neoplastic lymphoproliferative diseases, even if CVID patients with clonal B cell expansion, who survive without developing an overt lymphoma, have been described (12, 17). Treatment of CVID-associated lymphoma was usually like the treatment of lymphoma in other settings and usually it included rituximab.

Herein, to the best of our knowledge, we collected the largest case-series of gastric cancers in CVID subjects ever described, showing a high prevalence and an excess of mortality for gastric cancer. However, the SIR for gastric cancer was similar to that found across other studies providing this kind of figure (3, 6, 7). This difference might be related to the observation that gastric cancer prevalence may vary significantly within and between countries (18). In comparison to the normative population, CVID patients were on the average 15-years younger at the time of cancer onset. As reported for non-CVID subjects (19), CVID patients with early-stages gastric cancer had a better prognosis in comparison to those with more advanced stage, who died within 2 years since cancer diagnosis. According to our data, chronic atrophic gastritis and extensive intestinal metaplasia are invariably associated with gastric cancer in CVID. Similarly, De Petris et al. (20) showed that these adenocarcinomas were diagnosed at a young age and were of intestinal type. They were also associated with increased numbers of intra-tumoral lymphocytes, paucity of plasma cells and nodular lymphoid hyperplasia, all features suggestive of chronic inflammation of the gastric mucosa.

These observations gave us the chance to suggest the implementation of current screening strategy, aimed to an early diagnosis. The appropriate timing of upper endoscopy in CVID is a matter of debate. In the general population, Rugge et al. suggested performing upper endoscopy every 2 years in subjects with gastritis scored as stage III–IV (10). In CVID, Dhalla et al. (21) suggested to perform upper endoscopy in patients with risk factors for gastric cancer (*H. pylori* positivity, low serum vitamin B12 and iron concentrations) with an interval between the subsequent endoscopic assessment based on histological

TABLE 8 | Characteristics of CVID patients diagnosed with lymphoma.

ID	Sex	Age at cancer onset, years	Age at CVID diagnosis, years	Survival after cancer, months	Outcome (cause)	Histology and stage	Treatment	Additional cancer
6	M	50	37	216	Alive	Diffuse large B cell lymphoma (small bowel)	Chemotherapy NOS	Gastric cancer
10	F	32	42	336	Alive	HD	CHOP, ABV	Gastric cancer
13	F	67	35	12	Deceased (gastric cancer)	Diffuse large B cell lymphoma	NA	Gastric cancer
26	F	47	47	8	Deceased (lymphoma)	NHL not further classified	NA	No
27	M	38	37	120	Alive	Diffuse large B cell lymphoma of small bowel, stage IVE	R-CHOP	No
28	F	50	44	13	Deceased (lymphoma)	T-cell lymphoma (peripheral T cell lymphoma)	CHOP, autologous HSCT, Brentuximab	No
29	F	64	58	12	Deceased (lymphoma)	T-cell lymphoma (angioimmunoblastic T cell lymphoma)	Prednisone	No
30	M	40	47	144	Deceased (lymphoma)	NHL not further classified	NA	No
31	M	48	41	132	Alive	NHL not further classified	NA	No
32	F	74	47	24	Alive	Diffuse large B cell lymphoma (large bowel)	R-CHOP, RTX	No
33	M	53	47	9	Alive	Diffuse large B cell lymphoma (T-cells rich)	R-CHOP	No
34	M	29	30	14	Alive	Diffuse large B cell lymphoma	R-CHOP	No
35	M	58	55	36	Alive	NHL not further classified	Chemotherapy NOS	No
36	M	59	35	12	Deceased (lymphoma)	Cutaneous diffuse large B cell lymphoma leg-type	R-CHOP, radiotherapy	No
37	F	67	43	84	Alive	Marginal Zone Lymphoma (Splenic)	Splenectomy	Lung cancer
38	M	54	47	84	Alive	NHL not further classified	NA	No
39	F	29	28	24	Alive	Lymphoplasmacytic lymphoma	Chemotherapy NOS	No
40	M	47	45	6	Deceased (lymphoma)	NHL not further classified	NA	No
41	F	65	62	8	Deceased (lymphoma)	Diffuse large B cell lymphoma	NA	No
42	F	62	56	12	Alive	Marginal Zone Lymphoma (nodal and extra nodal)	RTX, bendamustine	No
43	M	60	60	36	Alive	Marginal Zone Lymphoma (nodal and extra nodal)	R-CHOP	No
44	M	47	47	72	Alive	Anaplastic T cell Lymphoma ALK- stage IVB (skin)	CHOEP, FEAM and autologous HSCT	No
45	F	67	64	11	Deceased (lymphoma)	NHL not further classified	NA	No
46	F	61	70	168	Deceased (cardiovascular)	Diffuse large B cell lymphoma (small bowel)	Ileocecal resection + R-CHOP	No
47	F	41	38	24	Alive	Marginal Zone Lymphoma	NA	No
48	M	52	56	120	Alive	NHL, not further classified, stage IV	R-FN, R-CHOP	No
49	M	70	59	12	Alive	Marginal Zone Lymphoma (nodal, indolent behavior)	Rituximab + bendamustine	Prostatic cancer
50	M	41	40	15	Alive	Kaposi sarcoma/Primitive effusion lymphoma, HHV8+/EBV+	CDE	No

(Continued)

TABLE 8 | Continued

ID	Sex	Age at cancer onset, years	Age at CVID diagnosis, years	Survival after cancer, months	Outcome (cause)	Histology and stage	Treatment	Additional cancer
51	F	74	62	72	Alive	MALT Lymphoma (gastric)	NA	No
52	M	59	59	60	Deceased (lymphoma)	Diffuse large B cell lymphoma (lung), stage IVB	R-CHOP, R-COMP	No
53	F	45	54	216	Deceased (lymphoma)	NHL not further classified (low grade)	R-CHOP + Etoposide	Uterine cancer, body
54	M	55	55	10	Deceased (lymphoma)	NHL not further classified	NA	No
55	M	36	21	108	Alive	HD, classic type, stage IIIsB	ABVD	No
56	M	30	28	12	Deceased (lymphoma)	HD, classical type, lymphocyte-depleted, stage IV	Chemotherapy NOS	Thyroid cancer
57	F	28	38	12	Deceased (T-cell lymphoma)	HD, mixed-cellularity type	Radiotherapy	Thyroid cancer, angio-immunoblastic T cell lymphoma)
58	M	29	18	8	Alive	HD, sclero-nodular type	ABVD, radiotherapy	No
59	M	39	38	96	Deceased (lymphoma)	HD, classical type, stage IVA	VEBEP, HSCT	No

ABV, Adriamycin, Hydroxydaunorubicin, Bleomycin, Vinblastine; ABVD, Adriamycin, Hydroxydaunorubicin, Bleomycin, Vinblastine, Dacarbazine; CHOEP, Cyclophosphamide, Doxorubicin, Etoposide, Vincristine, Prednisone; CHOP, Cyclophosphamide, Hydroxydaunorubicin, Vincristine, Prednisone; CDE, Cyclophosphamide, Doxorubicin, Etoposide; COPP, Cyclophosphamide, Vincristine, Procarbazine, Prednisone; FEAM, Fotemustine, Etoposide, Cytarabine, Melphalan; R-CHOP, Rituximab, Cyclophosphamide, Hydroxydaunorubicin, Vincristine, Prednisone; FN-R, Rituximab, Fludarabine, Mitoxantrone; HD, Hodgkin disease, NHL, Non-Hodgkin lymphoma, HSCT, Hematopoietic stem cell transplantation; R-COMP, Rituximab, Cyclophosphamide, Vincristine, Myocet, Prednisone; RTX, Rituximab; VEBEP, Etoposide, Epirubucin, Bleomycin, Cyclophosphamide, Prednisolone; NOS, not otherwise specified; NA, not available.

findings: every 1–3 years in CVID patients with metaplasia, every 3 years in patients with atrophic gastritis, and every 6–12 months in those with dysplasia. However, this interval may not be suitable for CVID, who rapidly develop advanced-stage cancer with poor prognosis. In fact, we showed that some CVID developed a high-grade gastric cancer already 12–14 months after an endoscopy showing no histologic signs of dysplasia. This rapid cancer development in CVID was unexpected since no epithelial gastric cancer was identified in patients without signs of dysplasia on a cohort of 1,615 Italian non-CVID followed over a 1–5 years period (10).

H. pylori eradication represents the main strategy to reduce the lifetime risk of gastric cancer since *H. pylori* is widely recognized as the leading cause of gastric cancer (22). In CVID, serological tests are not useful to identify *H. pylori* positive patients and only direct diagnostic methods for *H. pylori* detection should be considered (23). However, follow-up strategies targeted at gastric cancer secondary prevention cannot rely only on *H. pylori* identification, since the eradication of *H. pylori* might not abolish the risk for neoplastic progression (24–26). This is supported by our observation in two CVID patients who developed gastric cancer 1–2 years after a *H. pylori*-negative gastric biopsy.

On the basis of our data, we recommend the implementation of national guidelines based on regular upper endoscopy and on treatment of *H. pylori* infection. We propose to always perform upper endoscopy at the time of CVID diagnosis; to repeat endoscopy every 24 months in patients with normal histology; every 12 months in patients with atrophic gastritis or intestinal metaplasia, and every 6 months in patients with dysplastic lesions. Diagnosis of *H. pylori* infection should be actively ruled out at diagnosis and during the course of CVID disease. The prevalence and the risk of gastric cancer detected in Italian CVID patients might be related to the epidemiology of *H. pylori* infection in our country. Thus, further studies should be undertaken in other countries before they adopt our suggested measures of disease management. However, a careful endoscopic monitoring of gastric cancer should be advisable also in countries with low *H. pylori* prevalence, since the rate of antibiotic-resistant strains is increasing worldwide (22).

Our study has some limitations. First of all, we included in the analysis also retrospective data with possible survival bias. Second, we did not include in the analysis the genetic diagnoses of the cohort. Since it has been shown the risk of gastric cancer was not increased among relatives of CVID patients (7), however, it is possible that cancer morbidity might be related to the immunodeficiency *per se* rather than to family habits or environmental factors, including *H. pylori* sharing. Finally, preliminary data suggested spontaneous gastric cancer in models of NFkappaB1 deficiency (27) and recent papers suggested that significant proportion of CVID patients may harbor haploinsufficient *NFKB1* mutations (28). Additional studies on alterations of gastric mucosal immunity and microbiota and on genetic alterations are needed to better understand the gastric carcinogenesis in CVID patients.

ETHICS STATEMENT

This study was carried out in accordance with the Good Clinical Practice guidelines, the International Conference on

Harmonization guidelines, and the most recent version of the Declaration of Helsinki. The protocol was approved by Ethics Committee of Sapienza University of Rome and Azienda Policlinico Umberto I: Protocollo di osservazionale retrospettivo-prospettico sui soggetti affetti da Immunodeficienza Comune Variabile arruolati nei centri AIEOP/IPINET. Rif. CE:4063 on 04/14/2016.

AUTHOR CONTRIBUTIONS

FP, GS, CA, and IQ: conceived and designed the study; FP, AP, FC, ES, LC, FR, and CM data collection; FP, CM, GS, CA, MV, ST, and IQ: data analysis and interpretation; FP, CM, GS, CA, ST, and IQ: manuscript preparation.

FUNDING

This work was funded by Progetto Ateneo 2016, 2017.

ACKNOWLEDGMENTS

We thank our patients and their families and the Jeffrey Modell Foundation for the continuous support to our Centre. We thank Dr. Raffaella Neri, Dr. Saitto, and Dr. Paci for the manuscript revision.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 01 November 2018
doi: 10.3389/fimmu.2018.02544

Genes at the Crossroad of Primary Immunodeficiencies and Cancer

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Primary immunodeficiencies (PIDs) are a heterogeneous group of inherited disorders affecting one or multiple components of the innate and/or adaptive immune system. Currently, over 300 underlying genetic defects have been discovered. The most common clinical findings in patients with PIDs are infections, autoimmunity, and malignancies. Despite international efforts, the cancer risk associated with PIDs, given the heterogeneous character of this group of diseases, is difficult to estimate. The diverse underlying mechanisms of cancer in PID add another layer of complexity. Treatment of cancer within a context of PID is complicated by serious toxicities and long-term effects, including second malignancies. This review will focus on the little-known crossroad between PID and cancer genes and the value thereof for directing future research on our understanding of cancer in PID and for the identification of early cancer biomarkers in PID patients.

Keywords: primary immunodeficiency, cancer, predisposition, genetics, biomarkers

OPEN ACCESS

Edited by:
Markus G. Seidel,
Medizinische Universität Graz, Austria

Reviewed by:
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Oskar A. Haas,
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Specialty section:
This article was submitted to Primary Immunodeficiencies, a section of the journal Frontiers in Immunology

Received: *28 June 2018*
Accepted: *16 October 2018*
Published: *01 November 2018*

Citation:
Derpoorter C, Bordon V, Laureys G, Haerynck F and Lammens T (2018) Genes at the Crossroad of Primary Immunodeficiencies and Cancer. Front. Immunol. 9:2544. doi: 10.3389/fimmu.2018.02544

INTRODUCTION

Integrity of the immune system is crucial in the defense toward infectious organisms and surveillance on deviating cellular transformations, i.e., development of cancer. Primary immunodeficiency diseases (PIDs) constitute a heterogeneous group of life-threatening heritable genetic disorders in which parts of the human immune system are missing or dysfunctional (1). Per definition, PIDs are thus characterized by an increased susceptibility to infections, autoimmunity, inflammatory organ damage, and malignancy (2–4). During the last two decades, driven by technological advances in next-generation sequencing, progress has been made in defining the genetics of PID (5). Nowadays, more than 300 PID-causing genes are reported (6), classified into eight categories based on the affected immune function.

An increased risk for malignancy in PIDs has been recognized for many years (7–11). Moreover, the presence of a "malignancy" has been acknowledged as a diagnostic criterion for some PIDs by the European Society of Immunodeficiencies (ESID) (https://esid.org) and malignancy is the second leading cause of death in PID patients. In general, an excess of cancer risk in PID patients compared with an age-adjusted population is observed for all cancer types. As "common variable immunodeficiency" (CVID) is the most common PID subtype, incidence results are often focused on this subgroup, revealing a higher incidence for lymphoma and an association with stomach and skin cancer. This increased risk is likely multifactorial and related to viral infections and/or sustained activation and proliferation during chronic infections causing genetic instability in lymphocytes (12, 13). The enhanced risk for gastric cancer has been attributed to

Helicobacter pylori infection, although the exact mechanisms are still unknown (14). Cancer incidences for other PID subtypes are not well-defined, but associations were noted for lymphoma, gastric cancer, skin cancer and/or leukemia in Ataxia Telangiectasia (AT), "diseases of immune dysregulation" and "other well-defined immunodeficiency syndromes" (8, 9, 14–16).

The most commonly accepted theory to explain an enhanced cancer risk in PID patients is based on the reduced cancer surveillance caused by PID mutations (17, 18). This view has recently been challenged (12) and it must be considered that PID genetic defects *per se* alter the risk for malignant transformation through a direct oncogenic effect, exemplified by DNA repair disorders. In addition, PID genes cause altered T- and B-cell functions through impaired V(D)J recombination, class switch recombination and somatic hypermutation, causing chronic viral infections and inflammation (19). Similarly, researchers have shown that Natural Killer T (NKT) cells might play a major role in tumor development in a genetic background susceptible to carcinogenesis (20), as it has been observed that loss of type 1 NKT cells enhances tumor development in $p53^{+/-}$ mice and secondly, NKT cells protect against B-cell lymphoma development in mice (20, 21). A comprehensive overview of the mechanisms that may explain the enhanced risk of cancer is out of scope of this review, and has recently been documented by Hauck et al. (13).

Within this review, we provide a synopsis on the current knowledge about the genetics of malignancies in PID. In addition, we will elaborate on the presence of a largely ill-explored intersection between PID and cancer genes and the importance thereof for guiding future research on our understanding of cancer in PID and for the identification of early cancer biomarkers in PID patients.

INTERSECTION OF PID AND CANCER GENETICS

The study by Neven et al. is unique in extensively documenting molecular and immunophenotypical resemblance between lymphomas in patients with *IL10* and *IL10R* loss-of-function mutations (causing severe early-onset inflammatory bowel disease) and germinal center B-cell diffuse large B-cell lymphoma (GCB DLBCL) (22). Although typical DLBCL mutations were observed (including the mutation p.S219C in *MYD88*), mutations in histone and chromatin modifying genes were completely absent, in contrast to classical DLBCL (22). Additional gene expression profiling revealed some similarities, but also enriched expression of spliceosome pathway genes and genes involved in ubiquitin-mediated proteolysis was present in PID-associated, but not sporadic, DLBCL.

Although broad biological insights into the pathogenesis and characteristics of PID-associated cancers remain scarce, it is notable that many key molecules going awry in PID, have been mentioned independently in the context of carcinogenesis. In order to strengthen these observations, we have visualized the intersection of PID-causing genes (https://esid.org) with true cancer genes (https://cancer.sanger.ac.uk/census) and cancer predisposition genes (23) (**Figure 1**). It is important to note that different mutations in the same gene can lead to varying clinical phenotypes. There is a need to characterize the mutational landscape in sporadic cancer compared to PID-associated cancer and additionally in PID patients with a high cancer risk compared to those with a low risk.

PID AND CANCER PREDISPOSITION GENES

Interestingly, several well-known PID genes are also recognized as cancer predisposition genes, such as *GATA2* and *BLM*. ***GATA2*** is a key transcription factor required for the development and maintenance of hematopoietic stem cells. The phenotype of *GATA2* mutations comprises MonoMAC syndrome (PID associated with disseminated non-tuberculous mycobacterial infections) and familial myelodysplastic syndrome (MDS) (25–27). However, one should note that mutations are documented in different domains according to the clinical phenotypes: MDS/Acute Myeloid Leukemia (AML)-associated mutations are located in the zinc finger motif ZF2, whereas PID-associated mutations mostly before ZF2. Positive testing of germline *GATA2* mutations in leukemia has profound effects on clinical management, such as adapted prophylactic antimicrobial management during therapy (27). Importantly, screening of familial donors for *GATA2* mutations is crucial in the procedure for hematopoietic stem cell transplantation, the only available therapy. Similarly, the ***BLM* gene**, coding for a DNA helicase involved in DNA repair, has a well-described role in both cancer predisposition and immunodeficiency (28). DNA repair is crucial in the generation of B- and T-cell antigen receptors through T-and B-cell-specific V(D)J rearrangements, class switch recombination and/or somatic hypermutation. Defects in *BLM* thus impair lymphocyte development, explaining the immunodeficiency phenotype. In addition, through its role in maintaining genomic stability, an increased cancer risk is observed in these patients (12, 13, 28). *FAS*, *ITK*, *RECQL4*, *CDKN2A*, *WAS*, *SBDS*, *ATM*, *NBN,* and *POLE* are other examples of PID-causing genes involved in genetic cancer predisposition (29–32).

PID AND CANCER GENES

Next to these well-known relations, **Figure 1** also illustrates that several cancer genes, not yet officially recognized within predisposition panels, are also germline mutated in PIDs. As PID is a hallmark of cancer predisposition, one might speculate that several of the genes listed within the cancer gene list and intersecting with the PID list are potentially undiscovered or underexplored cancer predisposition genes (**Figure 1**). This is obviously the case for genes such as *IKZF1*, *TYK2*, *MYD88*. It indeed has been proven that several of the genes known to be somatically mutated in cancer types (i.e., *IKZF1* in leukemia), are found to be germline mutated through i.e., familial cancer studies, and thus getting recognized as cancer predisposition genes (33–35). This indicates that immunologists

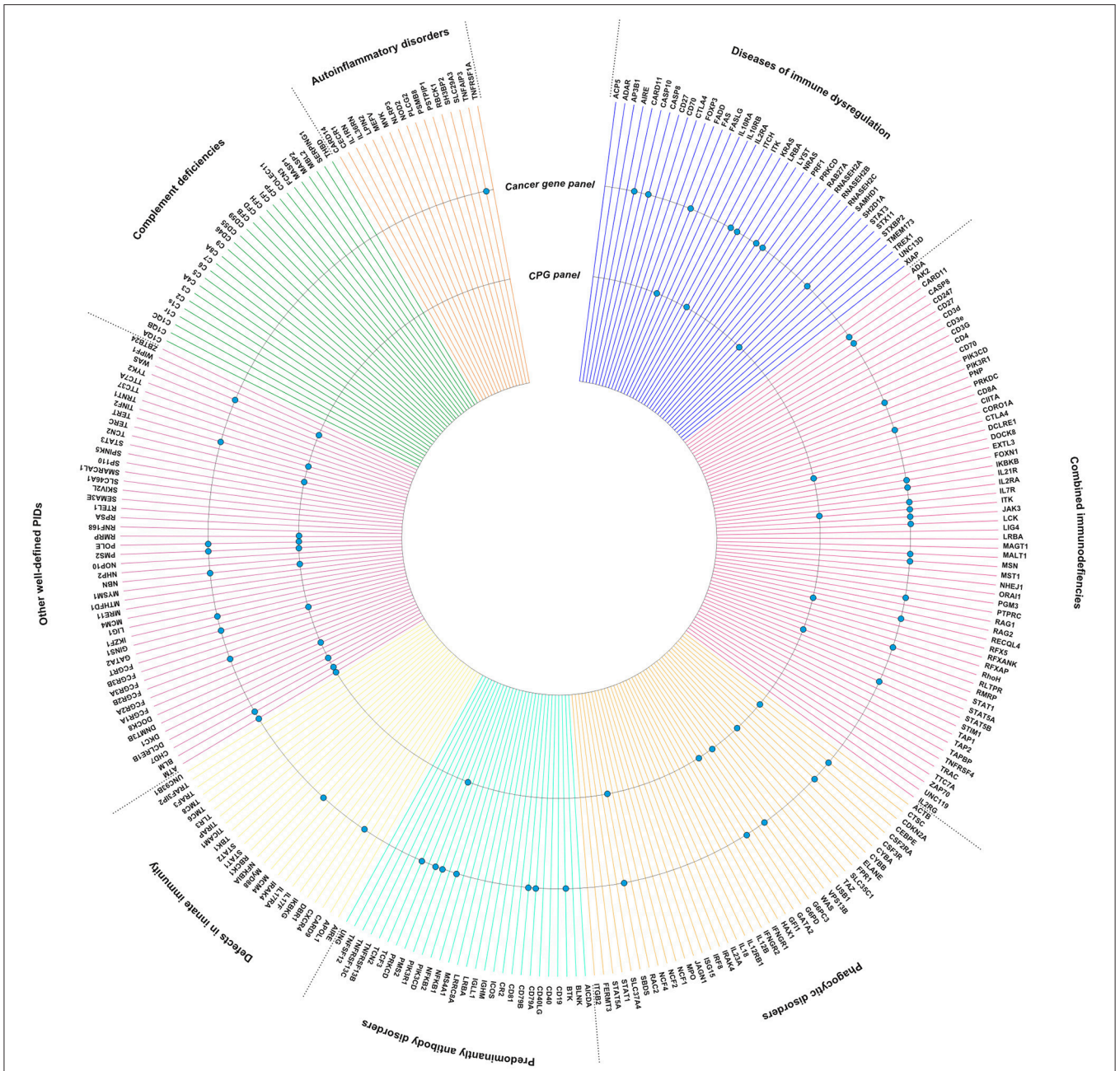

FIGURE 1 | Intersection between PID genes (https://esid.org) and the Cancer Gene Census (CGC), a catalog of genes which contain mutations that have been causally implicated in cancer (https://cancer.sanger.ac.uk/census) (24), or Cancer Predisposition Genes (CPGs) (23). PID genes also listed in the CGC or as CPG are indicated with a dot.

should acknowledge the possibility of an underlying cancer predisposition in PID with those genes affected, while vice versa oncologists should be triggered to evaluate a potential underlying PID upon a novel cancer diagnosis.

Somatic defects in ***IKZF1***, a hematopoietic zinc finger transcription factor, have been linked to acute lymphoblastic leukemia (ALL) for several years and have been proven to harbor negative prognostic effects (36). Recently, *IKZF1* mutations have been identified in familial ALL and in the germline of presumably sporadic cases. These mutations were dispersedly distributed over the whole protein coding sequence and were proven to be functionally damaging, even when not located in one of the functional domains. In the index family, individuals without ALL, but carrying the D186fs mutation in *IKZF1*, had variable lymphopenia and low-normal IgG levels, albeit not defined as immunodeficient (33). Remarkably, germline *IKZF1*

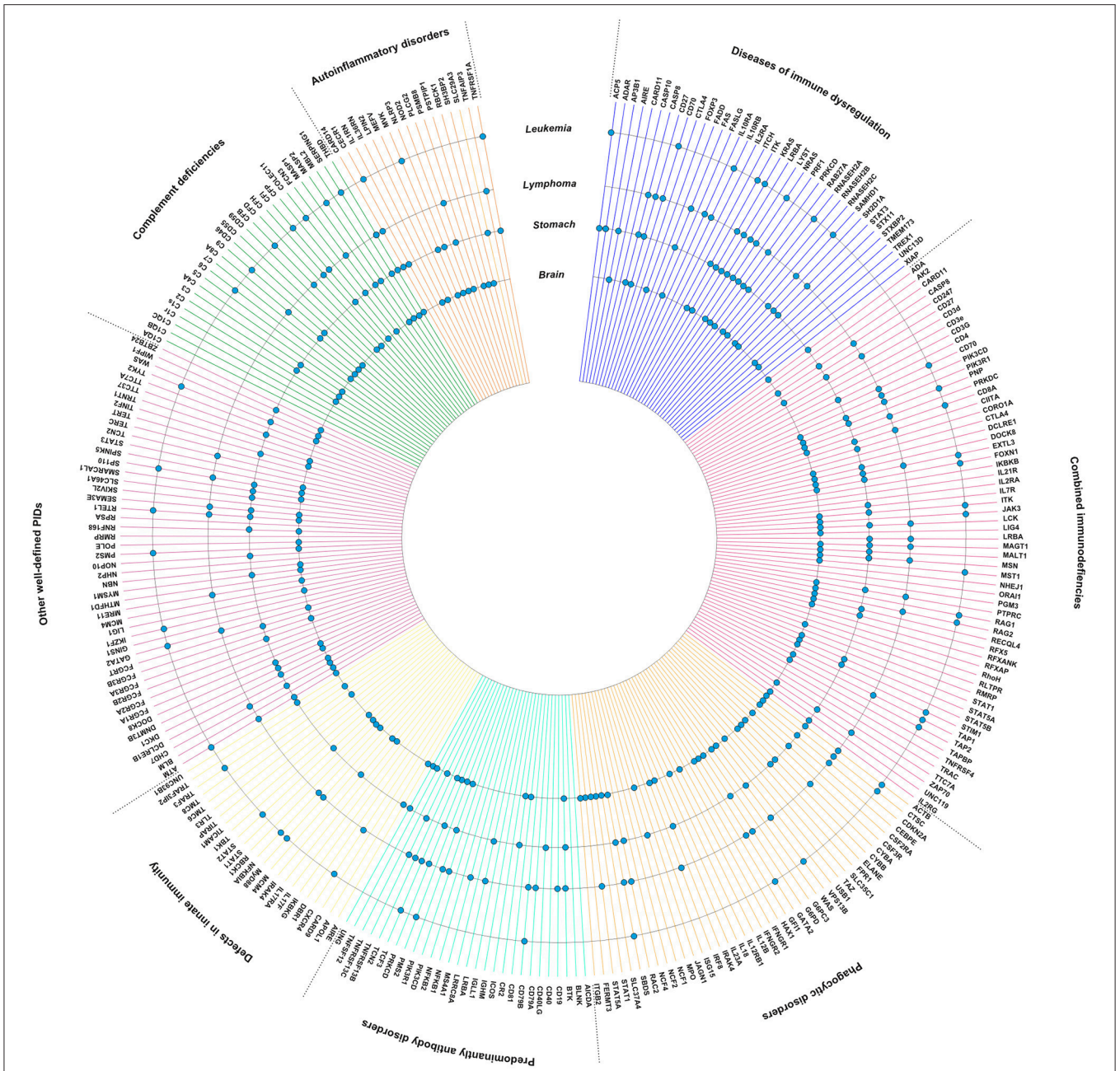

FIGURE 2 | PID genes (https://esid.org) associated with high impact mutations in lymphoma, leukemia, brain tumors, and stomach cancer. Data was generated using the International Cancer Genome Consortium (ICGC) Data Portal, a catalog of genomic abnormalities from over 20,000 tumor genomes (https://dcc.icgc.org/) (51). Donor age at diagnosis was restricted to age categories with an increased risk for PID-associated malignancies (0–59 years) (8, 11). PID genes with high impact mutations found in lymphoma, leukemia, brain tumors, and/or gastric cancer are indicated with a dot.

mutations within the ZF2 DNA binding domain were reported to be associated with an early-onset CVID (37). Similarly, an intersection between PID and cancer genetics can be observed for mutations in several of the **JAK-STAT** signal pathway genes (*STAT3*, *STAT5B*). Indeed, somatic mutations in *STAT* and *JAK* family members have been recognized as important drivers in oncogenesis, especially in different leukemia types (38–40). In addition, germline mutations in JAK-STAT signaling are associated with PIDs. Notably, the PID phenotypes depend on the affected gene and mutation, ranging from mild phenotypes involving *TYK2*, a moderate hyper-IgE syndrome for *STAT3* and severe combined immunodeficiency (SCID) in case of *JAK3* mutations (41). It is only recently, that mutations in *TYK2*, a JAK kinase family member, were found in the germline of patients

presenting with a second primary leukemia, causing constitutive JAK signaling and a propensity for developing leukemia (42).

Also, for ***MYD88*** a role in immunodeficiency and cancer development has been shown. MYD88 deficient animals have an increased risk of gastric cancer upon challenging them with *H. pylori* (43). In humans, deficiency in MYD88 results in impaired TLR signaling (44, 45). These patients have recurrent invasive infections (cellulitis, sepsis, meningitis, osteomyelitis), mainly caused by *Staphylococcus aureus* and *Streptococcus pneumoniae*. In addition, *MYD88* is found to be mutated in several hematologic B-cell malignancies, as Waldenström macroglobulinemia, DLBCL and IgM monoclonal gammopathy (46).

OTHER PID GENES

Importantly, it has to be recognized that several important gaps need to be filled. This is illustrated by the involvement of the ***CTLA4*** in both cancer and PIDs (47–49). Indeed, a survey of 131 affected *CTLA4* mutation carriers shows a cancer prevalence of 12.9%, mainly lymphoma, gastric adenocarcinoma and metastatic melanoma (50). Nevertheless, *CTLA4* has not been mentioned in the cancer census gene list.

Subsequently, we visualized the intersection between PID genes and genes published to be somatically mutated in lymphoma, leukemia, stomach cancer and brain tumors (**Figure 2**). Notably, mutations found in lymphoma are clearly enriched in PID genes involving "primary antibody disorders" (PADs) and "diseases of immune dysregulation," and less frequent in "defects in innate immunity." Although differences are smaller, fewer somatically mutated genes can be observed in PADs and "phagocytic disorders" for lymphoma, and in PADs and "defects in innate immunity" for brain tumors. Of note is the high intersection between PID genes and genes mutated in brain tumors (**Figure 2**). The high level of intersection might partially result from the observation that hypermutation is especially found in brain tumors, specifically the H3.3 or H3.1 K27-wildtype high-grade gliomas with biallelic germline mutations in *MSH6* or *PMS2* (52).

DISCUSSION

The diagnosis and management of cancer in PID patients is cumbersome. Guidelines and techniques for cancer screening within PID are ill-defined and should be evaluated in large international study cohorts. Although our understanding of the mechanisms of cancer development in PID is increasing, the genetic and molecular characteristics of cancers in PID patients remain uncovered. Here, we show that several PID genes are recognized as cancer predisposition genes. In addition, several of the genes listed within the cancer gene list and intersecting with the PID list are potentially undiscovered or underexplored cancer predisposition genes. Although the specific mutations and thus functional impact in both entities might be different, this observation implies that both oncologists and immunologists should be triggered to search for an underlying PID or potential development of cancer, respectively. Importantly, many PID genes might be candidates for further study in cancer research.

Improved understanding in cancer biology has led to the development of immunotherapies. The contribution of germline genetic factors is expected to be higher in pediatric cancers (53, 54) and PIDs. One could question if current immunotherapies might improve clinical outcomes for pediatric cancer. However, studies have illustrated that current inhibitory checkpoint immunotherapies are most efficient for tumors with high mutational load, which is not the case for most pediatric malignancies (55, 56). In this respect, there is a need for novel targets, again highlighting the importance of elucidating the genetics of PID-associated cancers in children, which may contribute to novel targeted treatment.

Importantly, efforts in creating awareness will be crucial to obtain these goals. Together with increasing technological advances (including i.e., testing cancer patients on radiosensitivity), one could expect to see the number of PID patients growing, especially if cancer is the first manifestation. This increasing number unavoidably will impact our view on the cancer landscape and incidence within PID. In addition, PID patient registers should be established/maintained with sufficient information on underlying genetic defects and malignancies or, ideally, an intersection with a national cancer registry. Furthermore, it is of utmost importance to improve the collection of biological material of PID patients with associated malignancy and perform "omic" studies to enhance our knowledge on this specific disease biology, improve on diagnosis and follow-up, and design newer therapeutic options.

CONCLUSION

Despite international efforts, the cancer risk associated with PIDs is difficult to estimate. Furthermore, treatment of cancer within a context of PID is complicated by serious toxicities and long-term effects, including second malignancies. Detailed molecular studies are required to identify common and distinct molecular pathways in PID-associated malignancies vs. sporadic cases and in PID patients with a high cancer risk vs. those with a low risk. These biological insights may allow early molecular recognition of cancer in PID, optimization of existing therapies and the development of targeted therapies, reducing toxicities within this patient population.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study can be found in the ICGC Data Portal (https://dcc.icgc.org/).

AUTHOR CONTRIBUTIONS

TL, CD, and FH conceptualized the study. TL and CD generated the figures. All authors contributed to the writing of the manuscript and approved the final version of the manuscript.

ACKNOWLEDGMENTS

The authors would like to thank vzw Kinderkankerfonds—a non-profit childhood cancer foundation under Belgian law (grant to TL) for supporting this work. CD is a PhD student at Ghent University and this work is submitted in partial fulfillment of the requirement for the PhD.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

CASE REPORT
published: 16 October 2018
doi: 10.3389/fimmu.2018.02400

EBV Negative Lymphoma and Autoimmune Lymphoproliferative Syndrome Like Phenotype Extend the Clinical Spectrum of Primary Immunodeficiency Caused by STK4 Deficiency

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OPEN ACCESS

Edited by:
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Medizinische Universität Graz, Austria

Reviewed by:
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Specialty section:
This article was submitted to Primary Immunodeficiencies, a section of the journal Frontiers in Immunology

Received: 29 June 2018
Accepted: 27 September 2018
Published: 16 October 2018

Citation:
Schipp C, Schlütermann D, Hönscheid A, Nabhani S, Höll J, Oommen PT, Ginzel S, Fleckenstein B, Stork B, Borkhardt A, Stepensky P and Fischer U (2018) EBV Negative Lymphoma and Autoimmune Lymphoproliferative Syndrome Like Phenotype Extend the Clinical Spectrum of Primary Immunodeficiency Caused by STK4 Deficiency. Front. Immunol. 9:2400. doi: 10.3389/fimmu.2018.02400

Serine/threonine kinase 4 (STK4) deficiency is an autosomal recessive genetic condition that leads to primary immunodeficiency (PID) typically characterized by lymphopenia, recurrent infections and *Epstein Barr Virus (EBV)* induced lymphoproliferation and -lymphoma. State-of-the-art treatment regimens consist of prevention or treatment of infections, immunoglobulin substitution (IVIG) and restoration of the immune system by hematopoietic stem cell transplantation. Here, we report on two patients from two consanguineous families of Turkish (patient P1) and Moroccan (patient P2) decent, with PID due to homozygous *STK4* mutations. P1 harbored a previously reported frameshift (c.1103 delT, p.M368RfsX2) and P2 a novel splice donor site mutation (P2; c.525+2 T>G). Both patients presented in childhood with recurrent infections, CD4 lymphopenia and dysregulated immunoglobulin levels. Patient P1 developed a highly malignant B cell lymphoma at the age of 10 years and a second, independent Hodgkin lymphoma 5 years later. To our knowledge she is the first STK4 deficient case reported who developed lymphoma in the absence of detectable EBV or other common viruses. Lymphoma development may be due to the lacking tumor suppressive function of STK4 or the perturbed immune surveillance due to the lack of CD4+ T cells. Our data should raise physicians' awareness of (1) lymphoma proneness of STK4 deficient patients even in the absence of EBV infection and (2) possibly underlying STK4 deficiency in pediatric patients with a history of recurrent infections, CD4 lymphopenia and lymphoma and unknown genetic make-up. Patient P2 experienced recurrent otitis in childhood, but when she presented at the age of 14, she showed clinical and immunological characteristics similar to patients suffering from Autoimmune Lymphoproliferative Syndrome (ALPS): elevated DNT cell number, non-malignant lymphadenopathy and hepatosplenomegaly,

hematolytic anemia, hypergammaglobulinemia. Also patient P1 presented with ALPS-like features (lymphadenopathy, elevated DNT cell number and increased Vitamin B12 levels) and both were initially clinically diagnosed as ALPS-like. Closer examination of P2, however, revealed active EBV infection and genetic testing identified a novel *STK4* mutation. None of the patients harbored typically ALPS-associated mutations of the Fas receptor mediated apoptotic pathway and Fas-mediated apoptosis was not affected. The presented case reports extend the clinical spectrum of STK4 deficiency.

Keywords: primary immunodeficiency, serine/threonine kinase 4 (STK4)-deficiency, autoimmune lymphoproliferative syndrome, lymphoma, epstein barr virus

INTRODUCTION

Deficiency of serine/threonine kinase 4 (STK4), also referred to as mammalian sterile 20-like protein (MST1), is an autosomal recessive primary immunodeficiency (PID) typically characterized by profound CD4 lymphopenia and recurring infections (1–6). STK4 deficiency in humans leads to decreased proliferation, increased susceptibility to apoptosis and dysregulation of the transcription factor Forkhead box protein O1 (FOXO1) and its downstream targets in T cells (1–3). Leukocytes show defective adhesion and chemotaxis (5). Due to the T cell impairment, many STK4-deficient patients experience fulminant EBV infections leading to lymphoproliferation and lymphoma development (1, 2, 5). This EBV associated lymphoproliferation can be observed in various PIDs including T cell defects, e.g., in patients with mutations in the *Interleukin-2-Inducible T Cell Kinase (ITK)* gene or in the *Magnesium Transporter 1 (MAGT1)* gene (7–9). Other disorders associated with lymphoproliferation and EBV infections are X-chromosomal linked lymphoproliferative disorders (SAP deficiency, XIAP deficiency), CD27 deficiency or FAAP24 deficiency (10–14). Interestingly, a recent report showed that STK4 plays a critical role as a tumor suppressor in the development of lymphoma and leukemia (15), putting STK4 deficient patients at an even higher risk to experience malignancies. Here we report on two patients with primary immunodeficiency due to homozygous STK4 mutations that were both initially clinically diagnosed as potentially ALPS like. One of them presented with the first reported case of an EBV negative lymphoma in this patient cohort.

MATERIALS AND METHODS

Patients, Relatives and Healthy Controls

Patients, relatives and healthy controls were enrolled in this study after obtaining written informed consent. All experiments were approved by the Ethical Review Boards of the Hadassah Hebrew University, the Israeli Ministry of Health and the University Hospital Düsseldorf.

Isolation and Cultivation of Mononuclear Cells

Peripheral blood was obtained from the patients, relatives and healthy individuals. Mononuclear cells (PBMC) were isolated using density gradient centrifugation and cultured in medium consisting of RPMI 1640 (Life Technologies, Darmstadt, Germany) and Panserin 401 (PAN-Biotech, Aidenbach, Germany) mixed 1:1, supplemented with 10% fetal calf serum (FCS, GE Healthcare, Little Chalfont Buckinghamshire, UK) and 100 μg gentamycin (Life Technologies) and 30 U/ml IL2 (Miltenyi, Bergisch Gladbach, Germany). For the first 4 days, cells were activated by addition of 7 μg/ml phytohemagglutinine (PHA, Life Technologies).

Transformation of Primary T Cells

T cells from patient P1 and healthy controls were immortalized by transformation with herpes virus saimiri as decribed (16).

Whole-Exome Sequencing and Bioinformatic Analysis

To identify the disease causing mutations next generation sequencing was carried out after targeted enrichment of whole exonic regions from sheared genomic DNA of the patient and family members using the SeqCap EZ Exome Library 2.0 kit (Roche/Nimblegen, Madison, WI). 100 bp single-read sequencing was performed on a HiSeq2000 (Illumina, San Diego, CA). Sequencing data was aligned to the human genome assembly hg19 (GRCh37) using BWA (17). Sequencing data was converted using Samtools (18). Variation calls were obtained employing GATK, HapMap, OmniArray, and dbSNP134 datasets (The Broad Institute, Cambridge, MA). Single nucleotide variations were annotated using the Variant Effect Predictor, based on the Ensemble database (v70). Variations were imported into a proprietary MySQL database driven workbench (termed Single Nucleotide Polymorphism Database, SNuPy).

Validation of the *STK4* Sequence Variations

Validation of *STK4* nucleotide variations were performed by PCR/Sanger sequencing using genomic DNA from the patients and family members. The following primers were used: *STK4* c.1103 delT (P1) forward: 5′-CCT TTT TCT AAT GCG CTG ATG-3′, reverse: 5′- ATC TTT TCC TGG GGT TCA GG-3′; *STK4* c.525+2 T>G (P2) forward: 5′- GGA CCT GAA TAA GTG TTA AAT CTC G−3′, reverse: 5′- TAA GCC TGC ATG AAC CAT GA−3′. DNA fragments were amplified by PCR employing the Phusion High Fidelity PCR Master Mix (NEB, Ipswich, MA), 0.5 μM each primer and 20 ng of template genomic DNA. Cycling conditions: 30 s at 98°C followed by 30

cycles of 7 s at 98°C, 23 s at 60°C, 30 s at 72°C and a final extension of 10 min at 72°C. Sanger sequencing was carried out by a core facility (BMFZ, University Düsseldorf, Germany). The nucleotide variations were visualized using sequencher software (Gene Codes, Ann Arbor, MI).

Real-Time PCR

cDNA preparation was carried out using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantitative Real Time PCR was performed using the QuantiFast SYBR Green PCR Kit (Qiagen) on a CFX Connect machine (Bio-Rad Laboratories GmbH, Munich, Germany) under the following conditions: an initial step of 95°C for 15 min was followed by 40 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s. For STK4, Bcl-2, IL-7RA, Foxo1, and GAPDH we used the QuantiTect Primer Assay (Qiagen, QT00068922, QT00025011, QT00053634, QT00044247, QT00079247). Expression was calculated as fold of control using the ΔΔCt method.

Immunoblotting

Cells were lysed in RIPA buffer containing 1% NP40, 50 mM Tris, pH 7.5, 350 mM NaCl, 0.5 mM EDTA, 2 mM dithiothreitol, protease- and phosphatase- inhibitor cocktail. Proteins were separated on 8.5% polyacrylamide gels, transferred to nitrocellulose membranes and detected by chemiluminescence. The following antibodies were used: STK4 (#3682, Cell Signaling Technology, Leiden, Netherlands), β-Actin (#A2228, Sigma-Aldrich, Taufkirchen, Germany), anti-rabbit and anti-mouse antibody (both from Cell Signaling Technology, #7074S and #7076S).

Cell Proliferation

PBMC were treated with 7 μg/ml phytohemagglutinine (PHA), 5 μg/ml anti-CD3 antibody (BioLegend, San Diego, USA) or 5 μg/ml tetanus toxoid (Sigma-Aldrich) and cultivated for 72 h. Radioactive uptake was quantified after 4 h priming with ^{3}H-thymidine (Perkin Elmer, Rodgau, Germany). Primary T cells from patient P1, family members and healthy control were stimulated with the human T Cell Activation/Expansion Kit (Miltenyi) according to the manufacturer's protocol for up to 4 days. Every day cell count was analyzed with trypan blue (Sigma-Aldrich) solution to control cell proliferation over time.

Apotosis

Activated primary or transformed T cells were stimulated with recombinant Fas ligand (Super-Fas, 50 ng/ml, Enzo Life Sciences, Loerrach, Germany) or 0.5 μM staurosporine (LC Laboratories, Woburn, MA) for 16 h or serum starved for 2 days or left untreated. Apoptosis was analyzed by flow cytometric measurement of Annexin V-FITC (BD Biosciences, Heidelberg, Germany) and propidium iodide (PI, Sigma-Aldrich) staining using a FACSCalibur equipped with CellQuest software according to the manufacturer's instructions (BD Biosciences).

BACKGROUND

We report on two patients. Patient P1, a girl, was born to a consanguineous family of Turkish origin. She had a history of pulmonary valve stenosis, recurring otitis and polyarthritis with massively elevated rheumatoid factors. At the age of 10, she presented in our department to exclude a rheumatoid disorder. Hematological studies showed CD4 lymphopenia with a profound dysregulation of immunoglobulin levels (decreased IgG, increased IgM and IgA). In addition, sonography revealed abdominal masses in the liver and enlarged lymph nodes, which were caused by a highly malignant non-classical B cell lymphoma with intermediary characteristics of diffuse large B cell lymphoma and Burkitt's lymphoma. Diagnostic for infectious disease in blood, liquor and a tumor biopsy returned negative for viral DNA (including CMV, EBV, HHV6, HSV1, and HSV2) and antibody titer analysis of the serum showed no sign of an active viral infection. In addition, immune histochemical analysis of tumor material demonstrated the absence of EBV positive blasts. She was then treated according to NHL-BFM 04 protocol (risk group 2) with an additional BFM-CC block (risk group 3, dosis reduced to 80%). She responded well and achieved a complete tumor remission fast. At the age of 12, she presented again with lymphadenopathy and suspicion of relapse, but pathohistological characterization showed no malignancy at that time. Because immunoglobulin dysregulation, CD4 lymphopenia and recurring infections persisted, targeted sequencing of the PID associated *activation-induced cytidine deaminase (AID)* and *uracil DNA glycosylase (UNG)* genes was carried out, but revealed no mutations.

Patient P2, a girl, was born to a consanguineous family of Arab origin. At the age of 5, she developed sudden onset of nasal bleedings. Complete blood count showed low platelet numbers and she was treated for immune thrombocytopenia with multiple courses of IVIG and steroids. At the age of 7, she developed recurrent chest infections with symptoms of malabsorption clinically diagnosed as cystic fibrosis. Sweat chloride tests showed a chloride level of 46 mmol/L. However, no mutations in the *cystic fibrosis transmembrane conductance regulator* (*CFTR)* gene were found, while a chest computed tomography scan revealed bronchiectasis and pulmonary nodules. The patient also experienced two hemolytic episodes (Hgb: 2.9 g/dL) with a positive direct coombs test. At the age of 14, she presented again at our ward with hematolytic anemia (Hgb: 4.29 g/dL). General examination showed clubbing of the fingers, lymphadenopathy and hepatosplenomegaly. Immunological characterization revealed CD4 lymphopenia, increased level of DNTs (13.2%) and hypergammaglobulinemia leading to the initial clinical diagnosis of ALPS like disease (19). But in addition and not characteristic for ALPS like patients she presented with fulminant EBV infection. After discharge, the patient did not return to our ward and died at the age of 15.

To identify the underlying genetic cause of the disease whole exome sequencing was performed on peripheral blood mononuclear cells (PBMC) of the patients and their family members. Next generation sequencing and data analysis revealed

that both patients harbored homozygous *STK4* mutations segregated from their heterozygous parents. Patient P1 carried a previously described single nucleotide deletion leading to early protein truncation [c.1103 delT, p.M368RfsX2, (2)]. Patient P2 harbored a novel mutation at a splice donor site (c.525+2 T>G). Pedigree analysis revealed that the patients were the only homozygous mutation carriers in their families (**Figure 1A**). *STK4* mutations described in patients are homozygous nonsense or frameshift mutations that occur across the whole coding sequence. The M368RfsX2 that leads to truncation before the SARAH domain is the most C terminal described so far. The SARAH domain of STK4 is involved in signal transduction and homodimerization of STK4. The mutation found in P2 is the first splice site mutation reported in this cohort (**Figure 1B**). Expression analysis of STK4 on mRNA and protein level showed a decrease of *STK4* transcript levels in both patients compared to healthy controls and an STK4 protein deficiency for patient P1 (**Figure 1C**, **Supplemental Figures 1, 2**). Due to scarcity of patient material and lack of transformed cells from patient P2, the effect of the mutation on protein level is unclear. However, the mutation most likely leads to mis-splicing of STK4 within its kinase domain and any generated protein should be functionally deficient.

Functional analysis of primary patient derived cells from P1 showed a phenotype of impaired T cell proliferation (**Figure 2A**) and dysregulated gene expression of FOXO1, IL/RA, and BCL2 (**Figure 2B**). Transformed T cells derived from patient P1 showed increased susceptibility to apoptosis induced by kinase inhibitor staurosporine or serum withdrawal compared to healthy controls (**Figure 2C**). Not typical for ALPS like disease, these cells showed no defect of apoptosis induced via the Fas death receptor pathway (**Supplemental Figure 3**).

To investigate whether lacking or reduced *STK4* expression is a general trait of lymphomas, we analyzed the expression of STK4 in publicly available datasets of 201 healthy tissues and 1,675 lymphomas available in the R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl). We found that STK4 was significantly downregulated in all lymphoma datasets compared to healthy datasets indicating a tumor suppressive role of STK4 (**Supplemental Figure 4, Supplemental Table 1**).

During further follow up patient P1 developed another EBV negative neoplasm, a Hodgkin lymphoma, at the age of 15 years. She received anti-CD20 monoclonal antibody rituximab and chemotherapy, but after one block of OEPA induction therapy (prednisone, doxorubicin, vincristine and etoposide as recommended by the EuroNet-PhL-C2 protocol) she experienced an infection or reactivation of Varicella zoster virus (VZV or human herpesvirus 3, HHV-3) and developed clinical herpes zoster with paraparesis and osteonecrosis of the legs. After treatment with cyclophosphamide and IVIG the neurological situation improved. Consolidation treatment with two modified COPDAC blocks (typically a combination of prednisone, vincristine, cyclophosphamide, and dacarbazine) was modified here by omission of prednisone and vincristine because of osteonecrosis and the neurologic situation. After achieving complete remission she received an allogeneic stem cell transplant from her HLA-matched sister. At the time of manuscript preparation, a few weeks after transplantation, she is tumor free and in good condition.

DISCUSSION

Homozygous *STK4* mutations have been described in PID patients presenting with CD4 lymphopenia and recurring infections (1–6). Here, we report on two additional patients with STK4 deficiency, one of them (P2) harboring the first homozygous splice site mutation (c.525+2 T>G) described in this patient entity and the other carrying a previously reported homozygous frameshift mutation (P1; c.1103 delT, p.M368RfsX2) (**Table 1**) (2). The initial clinical picture of these two cases resembled the phenotype of ALPS like syndromes (lymphadenopathy, increased numbers of DNT cells). This was more exlicit in the case of patient P2 who presented with classical clinical ALPS symptoms including lymphadenopathy, hepatosplenomegaly, highly elevated DNT cell number (13.2%), hemolytic anemia, and hypergammaglobulinemia. However, further clinical work up showed that, not typical for ALPS patients, these symptoms were associated with malignant disease at early age in patient P1 and with EBV infection in patient P2.

The cohort of STK4 deficient patients is highly susceptible to EBV infections. Eight of 15 reported cases so far presented with persistent EBV viremia and five of 15 developed EBV associated lymphoproliferative disorders, primarily affecting B cells (**Table 2**). The impact of active EBV infections on lymphoma development is well-established (20). Therefore, similar to other related disorders associated with EBV infections (such as MAGT1 or ITK deficiency), the lymphoma incidence in STK4 deficient patients is assumed to be increased although the absolute number of reported cases, including the case presented here is still low ($n = 3$). Sherkat et al. reported on a patient who presented first with a B cell lymphoproliferative syndrome and developed later an EBV+ primary cardiac T cell lymphoma, a very rare pediatric malignancy (1, 6). Nehme et al. reported on a case who suffered from an EBV+ Hodgkin B cell lymphoma (2). The patient P1 presented here harbored the same mutation as described by Nehme et al. and developed two independent lymphomas in the course of follow up: an aggressive B cell lymphoma and a Hodgkin lymphoma. Strikingly, both lymphomas were negative for EBV tested in blood, liquor and tumor biopsies. A hit-and-run mechanism of virus infection seems therefore unlikely. This strongly suggests a cancer predisposition mechanism of STK4 mutations independent of EBV infections.

The potential role of STK4 in tumorigenesis was investigated in various studies in mice. Conditional knockout of the mouse gene homolog *Mst1* in intestine or liver resulted in the generation of solid tumors at these sites (21, 22). Pathogenic upregulation of the Hippo signaling pathway was revealed as the tumor driver. Kim et al. (15) reported that *Mst1* knockout mice were prone to leukemia and lymphoma development after mutagen treatment or p53 deletion. Lymphomagenesis was not driven by hippo upregulation under these conditions, but by chromosomal instability. They analyzed publicly available microarray gene expression data of human acute lymphoblastic leukemia and

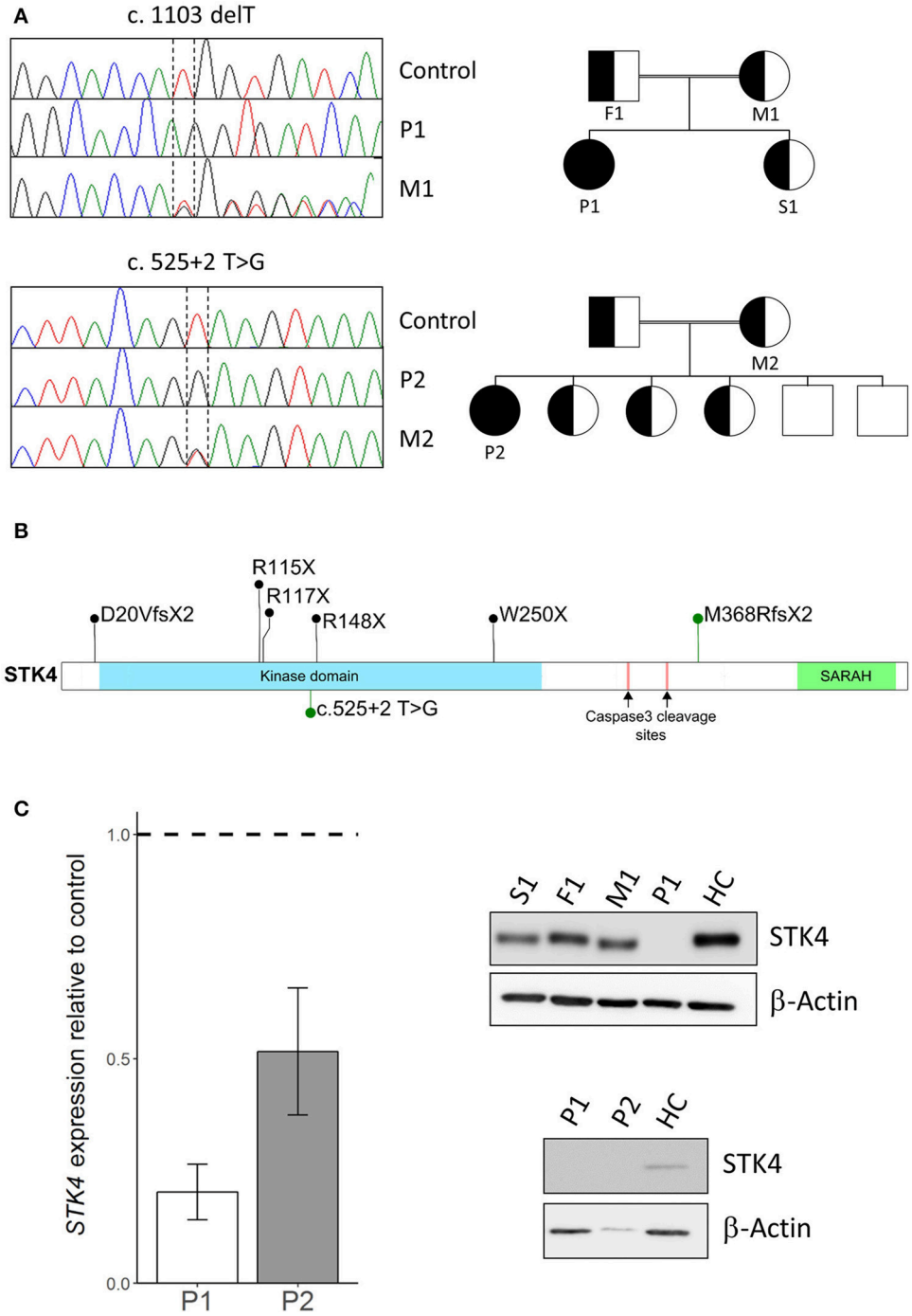

FIGURE 1 | STK4 mutations in two patients. **(A)** Left*:* Sanger sequencing using genomic DNA confirmed homozygous *STK4* mutations in Patient 1 (P1) and Patient 2 (P2). Representative chromatograms of a healthy control, the homozygous patients (P1/P2) and the heterozygous mothers (M1/M2) of each patient are shown. Right*:* Pedigrees of the two consanguineous families are presented. The patients are the only diseased members of the family and the only homozygous mutation carriers. **(B)** Schematic drawing of the STK4 protein. The protein harbors a kinase domain and a SARAH (Sav/Rassf/Hpo) domain that mediates signal transduction and homodimerization of STK4. Two caspase cleavage sites are described that lead to truncation of the protein before the SARAH domain similar to the M368RfsX2 mutation of P1. Mutations identified in our patients are presented in green, other previously reported mutations in black [M368RfsX2, previously also described by (2)] (2–6). **(C)** Left*: STK4* transcript expression is reduced in both patients compared to healthy controls. Presented are levels of *STK4* mRNA expression in whole blood extracts relative to healthy controls assessed by qPCR. Expression was calculated as fold change compared to healthy control using the ΔΔCt method. GAPDH and β-actin expression were used as internal standards. A representative experiment of two is shown. Mean values of an experiment carried out in triplicates and corresponding standard deviations are shown. Right*:* STK4 protein deficiency is analyzed in patient derived transformed T cells of patient 1 and primary cells of patient 2. STK4 protein levels were analyzed by western blot. β-actin was used as a control. Total protein levels of P2 are low due to scarcity of primary patient material.

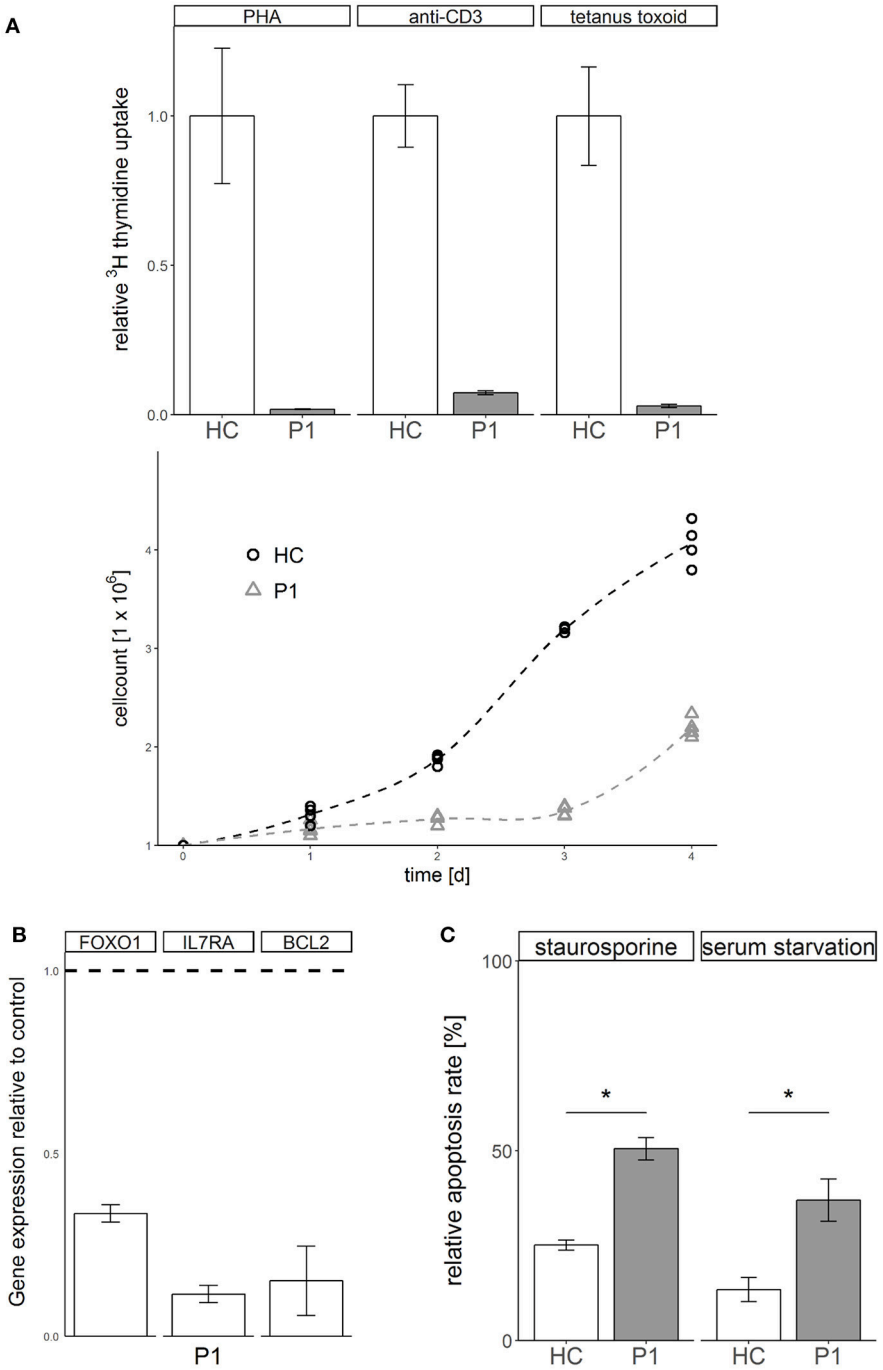

FIGURE 2 | STK4 deficiency results in defective proliferation, gene dysregulation and increased susceptibility to apoptosis. **(A)** Proliferation is defective in STK4 deficient primary cells. Upper*:* Peripheral blood mononuclear cells of patient P1 and a healthy control were treated with the indicated lymphocyte activating compounds. Relative proliferation was quantified by ^{3}H thymidine uptake (measured in counts per minute per 2×10^5 cells) after 72 h. A representative experiment of two is shown. Relative values are given compared to a healthy control (HC, = 1). Mean values of triplicates and corresponding standard deviations are shown. Lower*:* Primary T cells of patient P1 and a healthy control were activated with PHA and cultivated in the presence of IL2. Viable cells were determined by trypan blue staining and counted with a Neubauer chamber. **(B)** STK4 deficiency dysregulates gene expression. Expression of target genes was determined in whole blood from patient P1 relative to healthy controls (dotted line). Expression was calculated as fold change compared to a healthy control using the $\Delta\Delta$Ct method. GAPDH and β-actin expression were used as internal standards. A representative experiment of two is shown. Mean values of an experiment carried out in triplicates and corresponding standard deviations are shown. **(C)** STK4 deficient T cells have a significantly increased apoptosis susceptibility in response to staurosporine or serum starvation compared to healthy control T cells. Primary T cells of patient P1 and a healthy control were stimulated with 0.5 μM staurosporine for 16 h or serum starved for 2 days or left untreated. Apoptosis was analyzed by flow cytometric measurement of Annexin V-FITC and PI staining. Relative apoptosis is presented compared to untreated controls. A representative experiment of two is shown. Mean values of an experiment carried out in duplicates and corresponding standard deviations are shown. *$p < 0.05$.

TABLE 1 | Clinical and immunological characteristics of the patients.

	P1	P2	Reference values
STK4 mutation	c.1103 delT, p.M368RfsX2	c.525+2 T>G	
Age at first presentation	10	5	
Sex	Female	Female	
Hematology	Lymphadenopathy, CD4 lymphopenia, elevated DNT cell number, dysregulated Ig levels, increased Vitamin B12	Lymphadenopathy, hepatosplenomegaly, elevated DNT cell number, CD4 lymphopenia, thrombocytopenia, hemolytic anemia, hypergammaglobulinemia	
Infection	Recurring otitis, VZV/HHV3 infection/reactivation during chemotherapy, negative for CMV, EBV, HHV6, HSV1, and HSV2	Recurrent chest infections, active EBV infection	
Other clinical findings	B cell lymphoma, Hodgkin lymphoma, pulmonary valve stenosis, polyarthritis	Nasal bleedings, bronchiectasis and pulmonary nodules, clubbing of the fingers	
Treatment	1st lymphoma: NHL-BFM 04 protocol (risk group 2), additional CC block (risk group 3, dosis reduced to 80%), 2nd lymphoma: rituximab and chemotherapy: one OEPA and two modified COPDAC blocks, allogeneic hematopoietic stem cell transplantation of matched sibling donor	IVIG, steroids	
IMMUNOLOGICAL PHENOTYPE			
Leukocytes/μL	7400	na	4.1–8.3 × 1000/ μL
Lymphocytes [%]	6	25	34.5–48.2
Lymphocytes absolute	444	na	1.0–5.3 × 1000/μL
CD3+ [%]	71	80	52–78
CD3+CD4+[%]	10	18	25–48
CD3+CD8+[%]	50	50	9.0–35
CD45RA+CD4+[%]	30	na	49.3–72
CD45RO+CD4+[%]	70	na	24.5–44.4
CD45RA+CD8+[%]	48	na	62.3–86.3
CD45RO+CD8+ [%]	52	na	12.2–27.2
TCRαβ+CD4-CD8-[%]	3.7	13.2	<2
CD45+CD127+ (IL7RA) [%]	8.7	na	65.8–89.6
CD20+ [%]	12	na	9.1–21
IgD+CD27-[%]	86	na	9.1–21
IgD+CD27+[%]	6	na	0.85–2.53
IgD-CD27+ [%]	2.4	na	4.1–18.7
CD3-CD56 + [%]	19.7	3	6.0–27
IgG [mg/dL]	147	1814	572–1474
IgA [mg/dL]	504	486	34–305
IgM [mg/dL]	752	33	31–208
Vitamin B12 [pg/mL]	>2000	na	197–866

Cell surface markers were measured and analyzed by flow cytometry on a FACSCalibur using CellQuest software (Becton Dickinson Biosciences, Heidelberg, Germany). Immunoglobulins (Ig) were measured in the serum by ELISA. [CMV, Cytomegalovirus; DNT, double negative T cells; EBV, Epstein Barr virus; HHV3, human herpes virus 3; HHV6, human herpes virus 6; IVIG, intravenous immunoglobulins; NHL-BFM, Non-Hodgkin Lymphoma-Berlin-Frankfurt-Münster, VZV, varicella zoster virus (synonymous for HHV3)].

various human lymphomas from the Oncomine database (www.oncomine.org) that showed a downregulation of *STK4* in these entities. In the present study, we analyzed publicly available datasets of 201 healthy tissues and 1,675 lymphomas available in the R2 platform. We could confirm a significant general downregulation of *STK4* in lymphomas derived from B- T- and NK cells. The mechanisms of STK4 in lymphoma suppression still need to be clarified and will most likely be complex due to the multiple essential functions of STK4 in e.g. apoptosis, autophagy, chromosome stability, cell cycle progression. A perturbed B cell function is indicated by reduced B cell numbers and hypogammaglobulinemia in some STK4 deficient patients. In addition, Dang et al. demonstrated impaired adhesive response to chemokines in patient-derived EBV transformed B cells (5). However, a comprehensive analysis of STK4 deficient B cell function is still lacking. Our own *in vitro* analyses confirmed the effect of STK4 deficiency on lymphocyte function and gene expression established in the previous works.

TABLE 2 | Lymphoproliferation and malignancies reported so far in STK4 deficient patients.

Patient- No.	Persistent EBV viraemia	Lymphoproliferation/ malignancy	References
1	Negative		(1)
2	Negative		(1)
3	Positive	B-lymphoproliferative syndrome/ EBV+ primary cardiac T-cell lymphoma	(1, 6)
4	Positive	EBV+ Hodgkin B-cell lymphoma	(2)
5	Positive	B-lymphoproliferative syndrome	(2)
6	Positive		(2)
7	Negative		(2)
8	Negative		(3)
9	Negative		(4)
10	Negative		(4)
11	Positive	Lymphoproliferative syndrome	(5)
12	Positive	Lymphoproliferative syndrome	(5)
13	Positive		(5)
14	Negative	EBV-B-cell lymphoma and Hodgkin lymphoma	Present study
15	Positive	Lymphoproliferative syndrome	Present study

A perturbed immune surveillance due to CD4 lymphopenia has to be taken into account as an EBV independent mechanism that may promote lymphoma development. In this line, approximately 20% of patients with idiopathic CD4 lymphopenia also develop malignancies, lymphoma being the most common one. EBV infections were remarkably uncommon in this patient entity (∼ 2%) (23). A similar cohort with CD4 lymphopenia is presented by patients with HIV infections. The risk of lymphoma in HIV patients is increased 60–200 fold for non-Hodgkin lymphoma and 8–10 fold for classical Hodgkin lymphoma compared to the healthy population (24).

Our data suggests that patients with STK4 deficiency should be under close lymphoma surveillance even in the absence of EBV infections. In addition, a possibly underlying STK4 deficiency should be suspected and tested in patients with a clinical history of recurrent infections, CD4 lymphopenia and lymphoma and unknown genetic make-up.

AUTHOR CONTRIBUTIONS

CS: Performed laboratory work, designed research, analyzed data, and wrote the paper; DS and AH: Performed laboratory work, designed research, analyzed data, and participated in writing the paper; SN: Performed laboratory work, designed research, and analyzed data; PS, JH and PO: Provided clinical samples and analyzed clinical data; BF: Provided reagents, designed and supervised research; BS: Designed and supervised research, analyzed data; SG: Provided bioinformatic analyses; AB: Designed research and critically reviewed the paper; UF: Designed research, analyzed data, and wrote the paper.

FUNDING

This work was funded by the Katharina Hardt Stiftung, an intramural grant of the Research Commission of the Medical Faculty of Heinrich Heine University Duesseldorf (2016-70) and the Duesseldorf School of Oncology.

ACKNOWLEDGMENTS

The authors wish to thank Monika Schmidt, Bianca Killing, and Katayoun Alemazkour for excellent technical assistance, Michael Gombert for next generation sequencing and data management. We would like to thank our patients and their families, as well as departmental nursing and administrative staff for their commitment to patient care. We would like to thank Prof. Zeev Rotstein, Director of the Hadassah Medical center, for his support of the department and the patients.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02400/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 10 September 2018
doi: 10.3389/fimmu.2018.02012

Increased Risk for Malignancies in 131 Affected *CTLA4* Mutation Carriers

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Edited by:
Andrew R. Gennery, Newcastle University, United Kingdom

Reviewed by:
Silvia Clara Giliani, Università degli Studi di Brescia, Italy
Tomohiro Morio, Tokyo Medical and Dental University, Japan

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Specialty section:
This article was submitted to Primary Immunodeficiencies, a section of the journal Frontiers in Immunology

Received: *20 June 2018*
Accepted: *15 August 2018*
Published: *10 September 2018*

Citation:
Egg D, Schwab C, Gabrysch A, Arkwright PD, Cheesman E, Giulino-Roth L, Neth O, Snapper S, Okada S, Moutschen M, Delvenne P, Pecher A-C, Wolff D, Kim Y-J, Seneviratne S, Kim K-M, Kang J-M, Ojaimi S, McLean C, Warnatz K, Seidl M and Grimbacher B (2018) Increased Risk for Malignancies in 131 Affected CTLA4 Mutation Carriers. Front. Immunol. 9:2012. doi: 10.3389/fimmu.2018.02012

Background: Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) is a negative immune regulator on the surface of T cells. In humans, heterozygous germline mutations in *CTLA4* can cause an immune dysregulation syndrome. The phenotype comprises a broad spectrum of autoinflammatory, autoimmune, and immunodeficient features. An increased frequency of malignancies in primary immunodeficiencies is known, but their incidence in CTLA-4 insufficiency is unknown.

Methods: Clinical manifestations and details of the clinical history were assessed in a worldwide cohort of 184 *CTLA4* mutation carriers. Whenever a malignancy was reported, a malignancy-specific questionnaire was filled.

Results: Among the 184 *CTLA4* mutation carriers, 131 were considered affected, indicating a penetrance of 71.2%. We documented 17 malignancies, which amounts to a cancer prevalence of 12.9% in affected *CTLA4* mutation carriers. There were ten lymphomas, five gastric cancers, one multiple myeloma, and one metastatic melanoma. Seven lymphomas and three gastric cancers were EBV-associated.

Conclusion: Our findings demonstrate an elevated cancer risk for patients with CTLA-4 insufficiency. As more than half of the cancers were EBV-associated, the failure to

control oncogenic viruses seems to be part of the CTLA-4-insufficient phenotype. Hence, lymphoproliferation and EBV viral load in blood should be carefully monitored, especially when immunosuppressing affected *CTLA4* mutation carriers.

Keywords: CTLA4, combined immunodeficiency, primary immunodeficiency, malignancy, cancer predisposition, EBV, CMV

INTRODUCTION

Antibody-mediated autoimmunity, recurrent infections, lymphoproliferation, and lymphoid infiltrations into the lung, the bowel, or the central nervous system, characterize the clinical spectrum of CTLA-4-insufficient patients (1, 2). Furthermore, recent publications reported on malignancies in those patients (3–6).

Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) is an essential negative regulator of T cell-mediated immune responses (7). CTLA-4 competes with CD28 with higher affinity for the binding to CD80 and CD86 (also known as B7-1 and B7-2), two costimulatory molecules on the surface of dendritic cells, activated B cells, and monocytes. CD28 is constitutively expressed on T cells whereas CTLA-4 is mostly stored in vesicles and becomes upregulated in conventional T cells upon activation. Whereas CD28 delivers a positive signal into T cells leading to T cell activation and effector cell differentiation, the ligation of CTLA-4 to CD80 and CD86 delivers a negative signal to T cells, limiting IL-2 production, proliferation, and survival of T cells (8). Thus, heterozygous *CTLA4* germline mutations impair the suppressive function of T cells and cause an immune dysregulation syndrome characterized by an activated immune system with autoimmune features and organ pathology caused by infiltrating effector T cells (2).

Given that patients with heterozygous *CTLA4* mutations have an activated immune system consisting of an expansion of effector T cells and given that anti-CTLA-4 treatment is successfully used in the treatment of certain cancers, one would expect that patients with CTLA-4 insufficiency have a lower risk to develop cancer (2, 6, 9). Hence, our study aimed to assess whether a heterozygous germline mutation in *CTLA4* is associated with a decreased risk to develop cancer.

Abbreviations: ABVD, Adriamycin (Doxorubicin), Bleomycin, Vinblastine, Dacarbazine; allo-HSCT, Allogeneic hematopoietic stem cell transplantation; BMT, Bone marrow transplantation; CIN, Cervical intraepithelial neoplasia; CVID, Common variable immunodeficiency; DA-R-EPOCH, Rituximab, Etoposide, Prednisolone, Oncovin, Cyclophosphamide, Hydroxychloroquine; HSCT, Hematopoietic stem cell transplantation; IGRT, Immunoglobulin replacement therapy; ITP, Immune thrombocytopenia; MS, Multiple Sclerosis; LRBA, Lipopolysaccharide-responsive and beige-like anchor protein; PBSC, Peripheral blood stem cells; PCR, Polymerase chain reaction; PET-CT, Positron-emission tomography and computer tomography; PID, Primary immunodeficiency; PD-1, Programmed cell death protein; R-BEAM, Rituximab, Camustin, Ara C, Etoposid, Melphalan; R-CHOP, Rituximab, Cyclophosphamide, Hydroxychloroquine (Doxorubicin), Vincristine (Oncovin), Prednisolone; R-CVAD, Rituximab, Cyclophosphamide, Vincristine, Adriamycin, Dexamethasone; R-DHAP, Rituximab, Dexamethasone, high Ara-C (Cytarabine), Cisplatin; R-ICE, Rituximab, Ifosfamide, carboplatin, etoposide; VIN, Vulvar intraepithelial neoplasia.

MATERIALS AND METHODS

We have collected a world-wide cohort of 184 *CTLA4* mutation carriers. Of these, 131 were considered affected (mutation carriers were classified as affected if they showed clinically apparent symptoms related to CTLA-4 insufficiency requiring medical care or treatment), indicating a reduced penetrance of the disease of 71.2%. A malignancy-specific questionnaire was provided to collaborating physicians, who had reported any malignancy in their CTLA-4-insufficient patients, to collect data on cancer entity, cancer induction, and treatment outcome. This study was carried out in accordance with the recommendations for studies with human subjects of the scientific committee at the University Medical Center of Freiburg. All physicians confirmed that their patients had signed an informed consent under local ethics-approved protocols and in accordance with the Declaration of Helsinki. The protocol was approved by the scientific committee at the University Medical Center of Freiburg (Approval No. 251/13_KW and 295/13_140782).

The following patients are part of a single-center retrospective analysis of malignancy cases associated with common variable immunodeficiency (CVID) as well: A.III.1 = ID 21, MM.II.1 = ID 7, and JJ.II.1 = ID 13.

All statistics and figures were computed and created by using R (version 3.4.1). Person-time of risk described the time period from date of birth to last follow-up or until an event (cancer or death) occurred and was used to compute life-time incidence rate. Individuals were censored, if follow-up date was missing (six individuals). Incidence rates of malignancies in *CTLA4* wildtype population are in cases per 100,000 persons per year and are age-adjusted to the 2000 US Std Population (19 age groups—Census P25-1130 for US rates), respectively to the SEGI-world standardization for German rates and were arithmetically averaged (10, 11).

RESULTS

We identified 17 patients with a malignancy (**Table 1**). This amounts to a cancer prevalence of 12.9% in patients with CTLA-4 insufficiency in our cohort. Among the 17 cases there were two entities predominant: ten patients with lymphomas and five patients with gastric cancers. Interestingly, seven of ten lymphomas and three of five gastric cancers were EBV-associated. Additionally, one case of multiple myeloma, and one case of metastatic melanoma were reported.

The cumulative person-years of documentation in our cohort of 184 *CTLA4* mutation carriers comprise 3,825 patient-years in total. Taken together, the reported 17 cases result in 4.4 new malignancies per 1,000 patients per year.

TABLE 1 | CTLA-4-insufficient-patients with malignancies, viral association, and treatment details.

Cohort ID	Sex	*CTLA4* Mutation	Type of malignancy	Age of diagnosis of malignancy	CVID diagnosis	EBV pos.	CMV pos.	Other pos.	Treatment				
									Chemotherapy	Surgery	HSCT	Others	Remission
GROUP ONE—LYMPHOMAS													
A.III.1	Female	c.105C>A; p.C35*	Hodgkin lymphoma	30	Yes	Yes	No	No	AVD-Brentuximab	No	No	No	Pending
B.II.3	Male	c.109+1G>T	Hodgkin lymphoma	51	No	Yes	No	No	A(B)VD	No	Yes	No	Yes
H.II.1	Male	c.407C>T; p.P136L	Hodgkin lymphoma	21	No	Yes	No	No	RTX, Vincristine	No	No	No	No
L.II.2	Male	c.437G>T; p.G146V	Hodgkin lymphoma	17	No	Yes	No	No	ABVD	No	Yes	No	Yes
JJ.II.1	Male	c.223C>T; p.R75W	Hodgkin lymphoma	28	Yes	Yes	No	No	ABVD	No	No	No	Yes
MM.II.1	Male	c.530_543del; p.F179Cfs*29	Hodgkin lymphoma	33	Yes	Yes	No	No	ABVD	No	No	No	Yes
K.II.1	Female	c.308G>C; p.C103S	DLBCL	45	No	No	No	No	R-CHOP, R-DHAP, R-BEAM	No	No	No	No
UU.III.3	Female	§	DLBCL	50	No	No	No	No	R-CHOP	No	No	Yes	No
CO.I.1	Male	c.209G>A; p.R70Q	DLBCL	62	Yes	No	No	No	R-CHOP	No	No	No	No
FF.II.1	Male	c.208C>T; p.R70W	Burkitt lymphoma	22	No	No	No	No	R-CVAD, R-ICE, DA-R-EPOCH	No	No	No	No
GROUP TWO—GASTRIC CANCERS													
B.II.4	Female	c.109+1G>T	Gastric adenocarcinoma	40	No	Yes	No	No	No	Yes	No	No	Yes
G.III.2	Male	c.179A>G; p.Y60C	Gastric adenocarcinoma	17	No	No	No	No	No	Yes	No	No	Yes
M.II.3	Male	c.76_77insT; p.F28Sfs*40	Gastric adenocarcinoma	34	Yes	No	Yes	Hp	No	Yes	No	No	No
XX.II.1	Male	c.407C>T; p.P136L	Gastric adenocarcinoma	42	Yes	Yes	Yes	Hp	No	No	No	Yes	Yes
CM.II.2	Female	c.406C>T; p.P136S	Gastric adenocarcinoma	25	Yes	No	No	No	No	Yes	No	No	Yes
GROUP THREE—OTHER MALIGNANCIES													
CM.I.2	Male	c.406C>T; p.P136S	Multiple myeloma	75	No	No	No	No	Bortzezomib, Melphalan	No	No	No	Pending
CZ.II.2	Female	c.410C>T; p.P137L	Metastatic melanoma	24	No	No	No	No	No	Yes	No	Yes	Pending

*HP, Helicobacter pylori; DLBCL, Diffuse large B cell lymphoma; CVID, Common variable immunodeficiency; pos, positive; chemotherapy abbreviations, see Abbreviations section; remission no, deceased; §Deceased prior being genotype. *translation termination codon.*

The median age of onset was 32 years for lymphoma and 34 years for gastric cancer. The Hodgkin lymphomas occurred at a median age of 29 years and the Non-Hodgkin-Lymphomas at a median age of 48 years. The multiple myeloma occurred at the age of 75 years and the melanoma at the age of 24 years. The median age of death was 53 years for DLBCL, 35 years for gastric cancer, and 43 years in total.

Six of the 10 lymphomas were classified as Hodgkin lymphomas, of whom one patient is currently on treatment (A.III.1), four patients reached a complete remission (B.II.3, L.II.2, JJ.II.1, MM.II.1), and one patient died (H.II.1). Two out of four patients who were cured received chemotherapy (A(B)VD protocol) followed by a successful allo-HSCT (B.II.3 and L.II.2).

Four of ten lymphomas were classified as Non-Hodgkin lymphomas (NHL): Three diffuse large B cell lymphomas (DLBCL) in K.II.1, UU.III.3, and CO.I.1, and one Burkitt lymphoma in FF.II.1. All four patients died due to their NHL.

Additionally, one patient (CM.I.2) developed a multiple myeloma and is currently on treatment. All details in **Table 1**.

The five patients with gastric cancer had a long history of autoimmune enteropathy or atrophic gastritis. Three of the gastric carcinomas were EBV-associated, two were positive for CMV and *Helicobacter pylori* (**Table 1**). Three of the patients have previously been published (G.III.2, M.III.3 and B.II.4) (2, 4, 5). For treatment, three out of five patients underwent total gastrectomy, one received subtotal gastrectomy, and one patient (XX.II.1) underwent endoscopic resection and stopped his recent abatacept therapy. The first gastroscopy after 6 months in patient XX.II.1 showed quiescent conditions and gastrectomy might finally be performed when aggravation occurs. So far, remission was reached in four patients, however, patient M.II.3 never fully recovered and died 25 months after gastrectomy.

The two further malignancies comprise one patient (CZ.II.2), who had developed a metastatic melanoma and precancerous cervical and vulvar lesions, indicating decreased control of Human papilloma viruses (HPV). Excision of the melanoma was performed, but suspicious PET-CT avidity prompted further interventions of which results are currently pending. The second patient (CM.I.2) had developed an axillary sarcoma at the age of 60 years, had reached clinical remission, but had presented recently an amyloidosis-associated multiple myeloma at the age of 75 years and is currently on treatment.

Histologically, the hyperinflammatory condition of CTLA-4-insuffient patients often manifests at mucosal surfaces of the gastrointestinal tract but may also damage associated organs as the liver (**Figure 1**). On the other hand, the disturbed immunosurveillance allows neoplastic lymphoproliferations and solid cancers to develop, often promoted by viruses as EBV and CMV (**Figures 2–4**).

CASE REPORTS

Lymphomas

Patient A.III.1 is a 30-year-old female, who presented with antibody deficiency at age 15 and the diagnosis of CTLA-4 insufficiency was made at the age of 27 years. Additional complicating features included CNS involvement, psoriatic skin irritation, arthralgia, and a recurrent enteropathy, treated by steroids and immunoglobulin replacement therapy (IGRT).

By the age of 30 years she had lost 6 kg of weight during 3 months and multiple enlarged lymph nodes on both sides of the diaphragm were detected. Supraclavicular lymph node resection revealed a grade IV EBV-associated Hodgkin lymphoma with mixed cellularity (**Figure 2**). Laboratory findings showed a viral EBV load of 59,000 IU/ml blood.

The EBV viremia was treated with four courses of rituximab. The patient recently received her first course of AVD-Brentuximab and is currently stable.

Patient H.II.1 was a 21-year-old male, who presented with protracted diarrhea, ITP, and AIHA at the age of 10 years. The cytopenias became steroid-dependent and prompted intensive immunosuppression and finally splenectomy at the age of 20 years.

Moreover, he developed lymphocytic CNS lesions and recurrent generalized lymphadenopathies during his adolescence. Repeated biopsies revealed polyclonal cellularity in the lymph nodes and aplasia, fibrosis, and nodular lymphocytic aggregates in the bone marrow, compatible with an autoimmune lymphoproliferative syndrome-phenotype (**Figure 1**). Additional, he suffered from a cholestatic giant cell hepatitis at the age of 17 years (**Figure 1**).

At the age of 21 years, his EBV load had risen from 2,000 copies/ml to 8,400 copies/ml within 4 weeks, accompanied with high fevers and worsening clinical condition. He was admitted to intensive care due to progressive respiratory insufficiency, severe pancytopenia, and severe colitis. Despite immunosuppressive treatment with everolimus and prednisolone, rituximab, and high dose dexamethasone, the patient deteriorated and developed sepsis. Sequential therapy approaches with MMF, ATG, and G-CSF were made and quadruple-therapy for a concomitant atypical tuberculosis was initiated. Nonetheless, his condition worsened and he died 4 months after the onset of his EBV viremia. Pathology revealed post-mortem the diagnosis of an EBV-associated classic Hodgkin lymphoma with bone marrow infiltration (**Figures 1**, **2**).

Patient B.II.3 is a 54-year-old male, in whom CTLA-4 insufficiency was revealed by family screening at the age of 49 (2). Fever, night sweats, and fatigue occurred just a few months later, EBV viral load began to rise, and a generalized lymphadenopathy was detected. In addition, pancytopenia developed in the context of a hemophagocytic syndrome. Laboratory values showed an IL2-receptor load of 44.141 U/ml and an EBV load of 297.000 copies/ml blood. Although a therapy with high dose corticosteroids, rituximab, and etoposide was initiated, his condition aggravated and he developed *Aspergillus fumigatus* sepsis. Aged 51 bone marrow biopsy revealed a classical Hodgkin lymphoma. With an adjusted chemotherapy protocol AVD (bleomycin was excluded due to aspergillosis) the patient reached clinical remission, subsequent bone marrow transplantation was successfully realized and the patient is in complete remission for more than 3 years.

Patient L.II.2 is a 20-year-old male, who initially presented with inguinal and axillary lymphadenopathy and severe

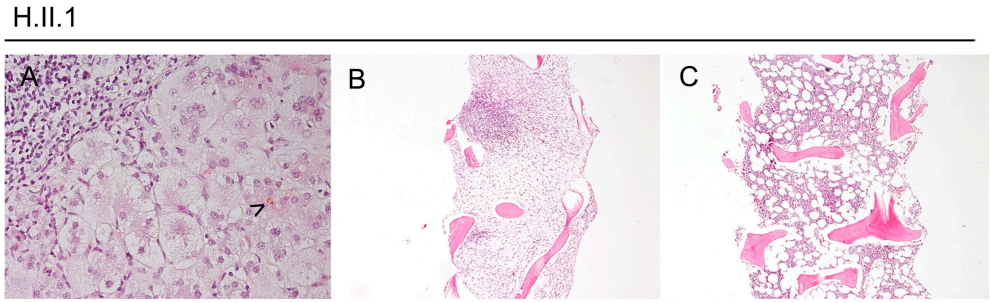

FIGURE 1 | Hyperinflammation, exemplified in a CTLA-4-insufficient-patient. Panel **(A)** shows a liver biopsy taken from patient H.II.1 at the age of 17 years with a cholestatic giant cell hepatitis (bile marked by arrowhead, exemplary). Panels **(B,C)** show bone marrow trephine biopsies with nodular lymphocytic aggregates (of T-cell origin, by IHC). In **(B)**, subtotal replacement of hematopoiesis by fibrosis and stromal edema can be seen.

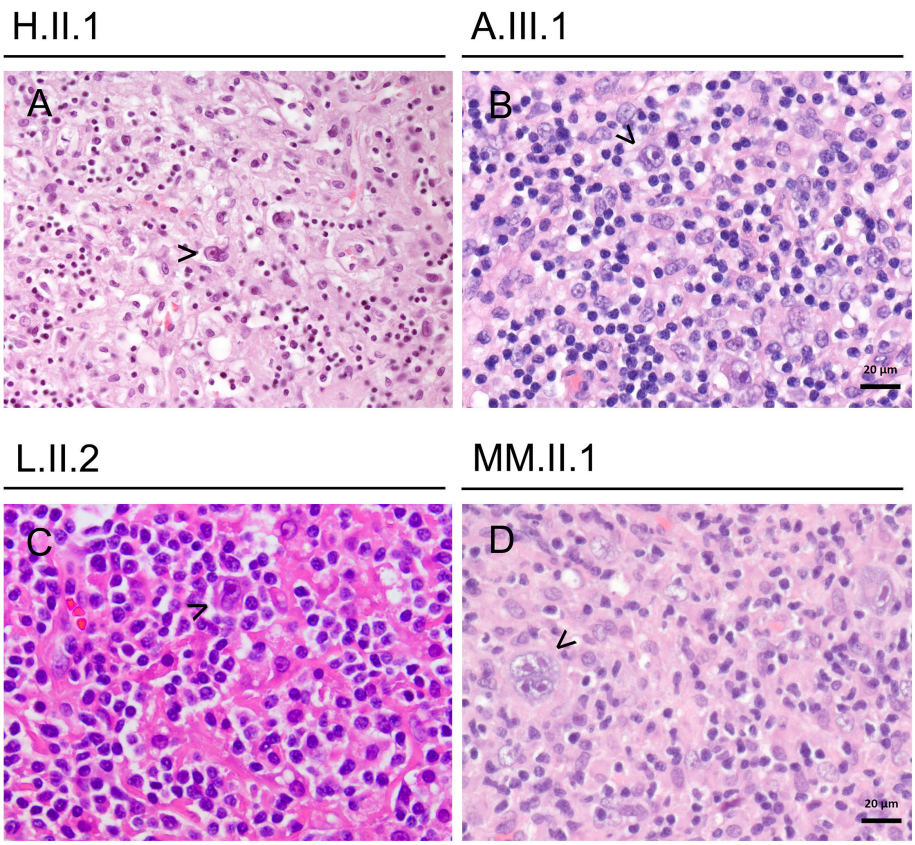

FIGURE 2 | Lymphomas in CTLA-4-insufficient-patients **(A–D)** showing classical Hodgkin lymphomas (mixed cellularity subtype), exemplary Reed-Sternberg cells are highlighted by arrowhead. All cases were EBV-associated.

pancolitis, at the age of 16 years. The diagnosis of lymphocyte-rich Hodgkin lymphoma was established based on an inguinal lymph node biopsy.

Laboratory values remained negative for EBV, but immunohistochemical staining was positive for CD15, CD30, EBV-LMP, and EBV *in situ*-hybridization. Histological work-up showed architectural effacement by a diffuse and partially nodular infiltrate of lymphocytes and histiocytes; these cells were interspersed with Reed-Sternberg cells (**Figure 2**).

The Hodgkin lymphoma was treated with three courses of ABVD chemotherapy (Euronet PHL-C1 2007), the colitis with corticosteroids, sirolimus, and belatacept and the hypogammaglobulinemia with IGRT. PET-CT at the first re-evaluation after 3 months showed, that the lymphoma was now in remission. He underwent matched unrelated bone marrow transplantation with reduced intensity 7 months after diagnosis and is now alive and well 2 years post-BMT.

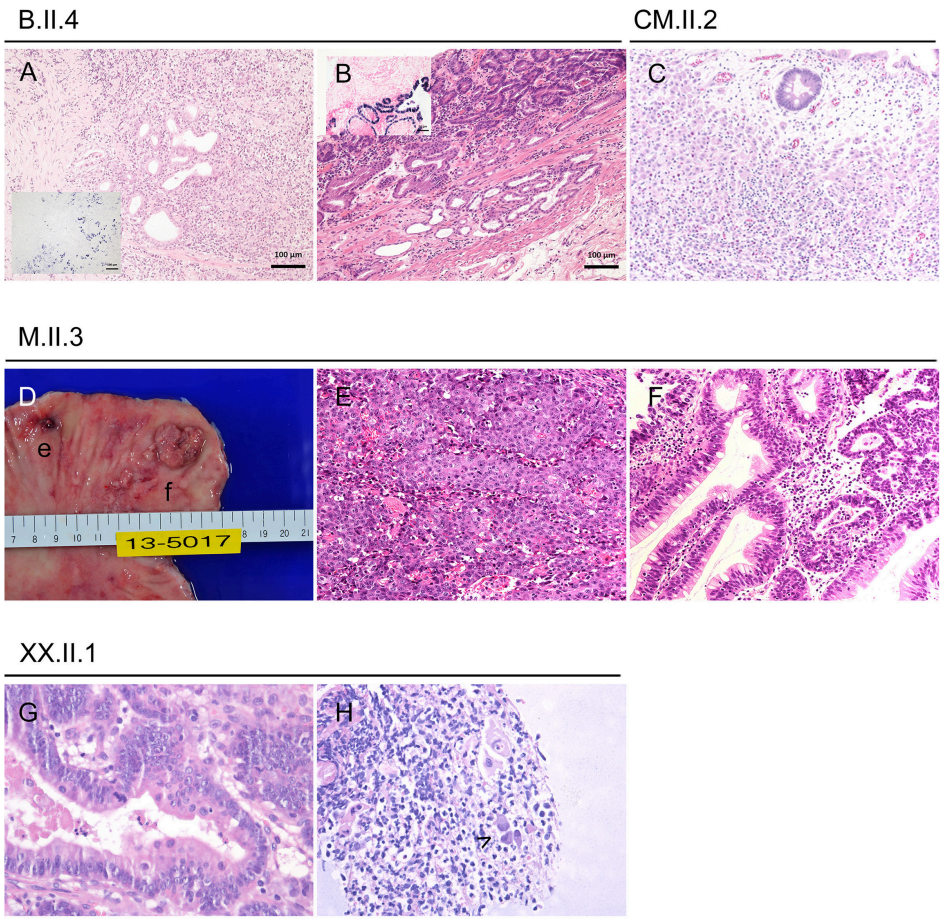

FIGURE 3 | Gastric cancer and precursor lesions in CTLA-4-insufficient-patients. **(A)** Poorly differentiated EBV associated adenocarcinoma at the age of 41 years (inlet displays chromogen *in situ* hybridization of EBV coded mRNA/EBER ISH). **(B)** Same patient at the age of 44 years with a well-differentiated EBV associated adenocarcinoma (inlet with EBER ISH). **(C)** Infiltration of the lamina propria through discohesive carcinoma cells in a 25-years-old patient. (D-F) 34-years-old patient with small poorly differentiated adenocarcinoma (**D**,e for macroscopy; **E** for histology) and larger well-differentiated adenocarcinoma (**D**,f for macroscopy; **F** for histology). **(G,H)** Pyloric biopsies taken at the age of 43 years showing high grade dysplasia **(G)** and viral inclusions (**H**, highlighted by arrowhead).

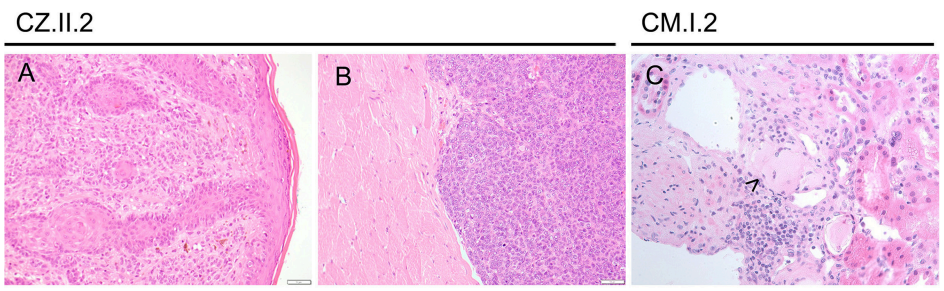

FIGURE 4 | Other malignancies in CTLA-4-insufficient-patients. **(A)** Superficial spreading melanoma of the ear (at the age 24 years) and metastasis (**B**; at the age of 25 years) infiltrating striated muscle fibers. **(C)** Amyloid deposits (highlighted by arrowhead) were immunohistochemically positive for amyloid p in this 75-years-old patient with multiple myeloma.

Patient JJ.II.2 is a 31-year-old male, who presented with antibody deficiency at the age of 10 years (CVID Euroclass B+smB-CD21low TR high). In the course of his illness, he developed recurrent respiratory infections, intermittent cytopenia, renal impairment necessitating dialysis, fluctuating EBV levels, enteropathy, and bilateral granulomatous lesions in

the lungs. At the age of 28 years a heterozygous mutation in *CTLA4* was detected.

A few months later, clinical assessment indicated a weak patient with enlarged inguinal lymph nodes accompanied by intermittent fevers and the diagnosis of a Hodgkin lymphoma with mixed cellularity including bone marrow infiltration was made; EBV-PCR detected a low positive result of 90 copies/ml blood. Complete remission was reached by six cycles of AVD protocol; the patient is alive and well more than 500 days after his initial cancer diagnosis without any signs of recurrence.

Patient MM.II.1 is a 40-year-old male, who initially presented with haemolytic anemia at the age of 14 years and diagnostic workup led to the diagnosis of a CVID (EUROClass B+smB-21low TR norm). Several pneumonias and recurrent autoimmune phenomena with haemolysis and thrombocytopenia occurred and were temporarily controlled by corticosteroids and azathioprine.

After 13 years of clinical remission, MM.II.1 presented with lymphadenopathy and B symptoms at the age of 33 years. His condition deteriorated rapidly and a diagnosis of an EBV-associated classical Hodgkin lymphoma (grade IIIB) was made based on cervical lymph node resection (**Figure 2**). Laboratory values showed an EBV load of <500 copies/ml and a CMV load of <1,000 copies/ml blood. Complete remission was reached with four cycles of ABVD chemotherapy protocol.

During remission, recurrent gastrointestinal irritations and relapses of a past encephalomyelitis occurred intermittently. So far, there are no hints for recurrence of the lymphoma and the patient remains in remission for 7 years.

Patient K.II.1 was a 52-year-old female, who was the first out of four patients with Non-Hodgkin-Lymphoma in our cohort. She was diagnosed with CVID during her twenties and received IGRT for decades. At the ages of 33 and 39 years hypercellularity and lymphocytic infiltrations were found in her bone marrow, but malignant cell growth could be ruled out. However, 6 years later at the age of 45 years, generalized lymph node enlargement appeared accompanied with fevers, night sweats, and weight loss. Cervical lymph node biopsy revealed clonal lymphoproliferation with typical features of a DLBCL with EBV association.

The lymphoma was treated with four courses of rituximab with only partial response, thus she received four cycles of R-CHOP 21 and could reach a complete remission.

After 4 years of remission, she developed recurrent abdominal and retroperitoneal lymphadenopathy, but biopsies negated malignant transformation. At the age of 51 years, her clinical condition deteriorated soon and finally the diagnosis of a non-EBV-associated DLBCL (grade IVb) was made based on an additional diffuse hepatic lesion biopsy. Pathology examination described compact atypical B cell infiltrate with a component of high reactive T cells. The non-EBV-associated DLBCL only responded partially to treatment with two cycles of R-CHOP, two cycles of R-DHAP, as well as one cycle of R-BEAM. In the end, she suffered from CMV-viremia and deceased due to pneumonia and gastric bleeding 7 months following the relapse at the age of 52 years.

Patient UU.III.3 was a 51-year-old female, who had severe and recurrent gastroenteritis for many years. Clinical records are rare and even genotyping could not be made prior her death, but family screening revealed a heterozygous *CTLA4* mutation in five out of seven siblings and in four out of her five children. At the age of 50 years she presented with inguinal lymphadenopathy accompanied with B symptoms and she was diagnosed with DLBCL based on inguinal lymph node resection.

Despite five cycles of R-CHOP, local radiotherapy, and radio-immune-therapy with ibritumomab-tiuxetan, the patient died 13 months after cancer diagnosis.

Patient CO.I.1 was a 62-year-old male, whose CVID diagnosis was first made at the age of 38 years and a heterozygous mutation in *CTLA4* was identified at the age of 61 years following clinical assessment. His clinical history followed a long history of recurrent but steroid-sensitive granulomatous infiltration in kidney, skin, lung, and conjunctivae. Finally, he complained of weight loss and fatigue over months at the age of 62. Biopsy of a hepatic lesion revealed morphological features of a DLBCL germline subtype. Immunohistochemical staining showed atypical lymphoid infiltrates, which were positive for CD20, Bcl6 and Bcl2, and negative staining for CD3, CD5, CD10, MUM-1, TdT, and EBERish.

The lymphoma was treated with three cycles of R-CHOP chemotherapy (two with reduced, one with full intensity), his health deteriorated and he deceased after a short and fulminant sepsis just 3 months after cancer onset.

Patient FF.II.1 was a 23-year-old male, who initially attracted clinical assessment at the age of 6 years and at the age of 16 years with treatment-dependent immune thrombocytopenia (ITP). He presented at age 22 with diffuse lymphadenopathy and in the years prior to his diagnosis he had benign lymphadenopathy with negative biopsies on multiple locations. At the time of his diagnosis, the lesions had increased in size, number, and PET-CT avidity prompting repeated biopsies. Those revealed typical features of a Burkitt lymphoma without EBV association. Laboratory values showed overall lymphopenic levels and negative EBV, CMV, and toxoplasma ranges. Immunohistochemical staining was positive for CD10, CD20, PAX5, c-MYC, and 100% for proliferation index Ki-67.

The lesions were refractory to four cycles of R-Hyper-CVAD and showed only a minimal response to two cycles of R-ICE. Next, he started treatment with rituximab and selinixor on study KPT330, but he was taken off the study because of disease progression with worsening thoracic and retroperitoneal lymphadenopathy. Ultimately, another therapy attempt was started following the DA-R-EPOCH protocol, but nonetheless the patient died of his progressive disease with thoracic and retroperitoneal lymphadenopathy at the age of 23 years.

Gastric Cancers

Patient B.II.4 is a 47-year old female, who had manifested with antibody deficiency, recurrent hypokalaemic periods due to severe diarrhea, and chronic renal failure most likely due to lymphocytic renal infiltrations during her early forties. Her *CTLA4* mutation was found at the age of 43 years by screening of 71 unrelated patients with CVID and enteropathy (2).

Two years prior her CTLA-4 insufficiency diagnosis, at the age of 41 years, she developed an EBV-associated and poorly differentiated gastric adenocarcinoma (type M, **Figure 3**), that was treated by radical mucosal resection. Nonetheless the cancer relapsed 3 years later—again EBV-associated (**Figure 3**)—and the patient finally underwent total gastrectomy at the age of 44 years and reached a complete remission; 19 lymph nodes were negative for cancer. Her clinical condition improved steadily under immunosuppressive therapy and today her intestinal symptoms are well-controlled.

Patient XX.II.1 is a 44-year-old male, who had manifested with a number of hospitalisations due to gastrointestinal symptoms such as appendicitis and severe diarrhea, that prompted many years on budesonide and anti-TNF-α treatment.

Gastroscopy at the age of 42 years showed severe atrophic gastritis with extensive intestinal metaplasia, a well-known risk-factors of gastric cancer (12). The diagnosis of an *in situ* adenocarcinoma was based on two tubular adenomas and extensive endoscopic resection of the lesions was performed (**Figure 3**). Laboratory values showed EBV load of 7,510 UI/ml blood and no CMV level, but PCR of biopsies were positive for EBV, CMV, and in presence of *H. pylori*.

He is receiving sirolimus, ustekinumab, and will start an antibiotic treatment for *H. pylori*. First control by gastroscopy 6 months after diagnosis showed two quiescent tubular adenomas with low grade dysplasia—gastrectomy might finally be performed when aggravation occurs.

Both patients who developed EBV-associated gastric cancer had received abatacept, a CTLA-4-Ig-fusion-protein, which is expected to improve especially the gastrointestinal symptoms in adult autoimmune enteropathy but could raise EBV serum levels (13). In fact, abatacept treatment has to be interrupted in patient B.II.4 after 7 months due to increased serum levels of EBV copies and rituximab treatment was needed, but nonetheless a relapse of gastric cancer developed 2 months after interruption and patient finally underwent total gastrectomy. However, patient B.II.4 resumed abatacept treatment 18 months after her relapse following mucosal healing for her severe CTLA4-associated enteropathy, reporting a repeated sustained clinical recovery of her bowel symptoms under abatacept until today. The second case (XX.II.1) stopped his therapy with abatacept after 3 months when EBV level rose and switched to sirolimus plus ustekinumab to control sustained intestinal symptoms with protracted diarrhea. EBV load decreased within few weeks after interruption of abatacept to <500 copies/ml blood.

Nonetheless both patients will need yearly follow-up by gastroscopy and carefully monitoring of EBV load.

Patient M.II.3 was a 35-year-old male, who suffered from chronic diarrhea since he was 10 years (5). Additional features were bacterial pneumonia and acute hepatitis with uncertain etiology around the age of 24 years, that needed corticosteroid pulse treatment. Around this time, he was diagnosed with CVID and started IGRT.

Acute gastritis mucosal lesions prompted recurrent endoscopies around the age of 28 years, and finally multiple biopsies at the age of 34 years revealed a poorly differentiated adenocarcinoma, a well-differentiated tubular adenocarcinoma and epithelium cells with CMV-associated antigen; no metastasis was found (**Figure 3**).

For treatment, he underwent total gastrectomy without perioperative chemotherapy, but protracted diarrhea and enterocolitis continued postoperatively for months despite immunosuppressive therapy. Recurrence of cancerous lesions has been monitored by gastroscopies, but he never fully recovered and died 25 months after gastrectomy following a *Klebsiella pneumonia*-induced sepsis.

Patient G.III.2 is a 25-year-old male, who had repeated gastrointestinal examinations and recurrent active gastritis over several years, which was initially diagnosed as Crohn's disease at the age of seven years (4). Additionally, he developed trilineage cytopenia and CT scan examination revealed interstitial pulmonary nodules. Despite aggressive immunosuppressive treatment he required partial colectomy at the age of 14 years, which improved his condition together with budesonide and anti-TNF-α treatment. However, his atrophic gastritis progressed and at the age of 17 years endoscopic biopsies revealed an early invasive adenocarcinoma without EBV or CMV association. The patient's treatment consisted of a total gastrectomy. Histopathology of the stomach revealed diffuse marked chronic active gastritis with multifocal areas of low to high grade dysplasia and an early invasive adenocarcinoma; 20 lymph nodes were negative for cancer. Remission was reached, but enterocolitis is still active and he requires intensive immunosuppressive therapy due to the neurological, hematological and respiratory involvement. Nonetheless, the patient is in complete remission for 7 years.

Patient CM.II.2 is a 29-year-old female, who has developed recurrent infections since the age of one year and was diagnosed with CVID at the age of 14 years, treated with IGRT.

At the age of 20 years, assessment of curious paraesthesia and muscle power decrease revealed a vitamin B12 deficiency-associated pernicious anemia. Following this diagnosis, gastroscopy discovered an early gastric adenoma (gastric type) with high grade dysplasia and without EBV or CMV association. Endoscopic submucosal dissection was made and she has been on regular follow-ups, which repetitively confirmed chronic active gastritis. Finally, at the age of 25 years, follow-up gastroscopy revealed a poorly differentiated gastric adenocarcinoma, again without viral association (**Figure 3**).

The treatment consisted of laparoscopic subtotal gastrectomy. Chemotherapy was not realized and the patient does not have any cancer relapse since more than 3 years and is still treated only by IGRT.

Other Malignancies

Patient CM.I.2 is a 75-year-old male, who has been asymptomatic during his life regarding PID-associated symptoms. At the age of 60 years, he presented with an axillary sarcoma and received operative excision without chemotherapy and reached clinical remission. Aged 75, he had developed proteinuria and urine protein electrophoresis revealed a monoclonal peak in

beta globulin region. Finally, bone marrow biopsy verified the diagnosis of multiple myeloma.

Since his sister and niece (CM.II.2) have been diagnosed with CTLA-4 insufficiency a few months prior the patient's cancer diagnosis, genetic testing revealed the same heterozygous variant in *CTLA4*. Additional antibody test showed decreased serum levels.

The patient recently received chemotherapy with bortezomib and melphalan (in reduction due to renal insufficiency). Shortly after the chemotherapy he developed complications including pericardial and pleural effusions, he was admitted to intensive care, but he recovered well.

To continue treatment for multiple myeloma, he was transferred to a central hospital, where a kidney biopsy was made, which was immunohistochemically positive for amyloid p (**Figure 4**).

Patient CZ.II.2 is a 25-year-old female, who was initially diagnosed with relapsing-remitting Multiple Sclerosis (MS) at the age of 19 years and subsequently was noted to be hypogammaglobulinemic at 23 years. Her neurological symptoms were treated with several MS-targeting monoclonal antibodies and immunosuppressive therapies over the years, which were blamed for most of her other autoimmune features including interstitial lung disease, lymphocytic enterocolitis, and cytopenia, until finally a diagnosis of CTLA-4 insufficiency was made at the age of 24 years. She showed additionally impaired control of oncogenic viruses presenting with precancerous CIN II and VIN II.

Her melanoma (left-ear) was first noted on routine skin checks at the age of 24 years and was excised with clear margins (**Figure 4**). Seven months later, she noted an enlarged left post-auricular lymph node. A fine needle aspirate revealed a non-small cell malignancy suggestive of metastatic melanoma, which was confirmed with an excisional lymph node biopsy. PET-CT scan revealed avid lesions scattered in her lungs and brain. However, review of her old imaging suggested that these were more consistent with her previously noted inflammatory lesions.

She underwent a total left neck lymph node dissection, which revealed five involved lymph nodes out of a total of 22, with soft tissue deposits and intramuscular extension, confirming the diagnosis of an aggressive and unresectable stage III metastatic melanoma. Genetic testing revealed an *NRAS* mutation not amenable to BRAF immunotherapy. A follow-up PET-CT scan revealed new lesions involving her right psoas muscle and right axilla, with reductions in the previously noted lesions, on a slightly higher dose of steroids (**Figure 4**). Biopsies of both new lesions revealed granulomatous change, without evidence of melanoma. Recently, the patient has commenced abatacept therapy and remains on methotrexate and prednisolone. She is receiving radiation therapy to her neck and surrounding area.

DISCUSSION

Primary immunodeficiencies (PIDs) are known to confer an increased risk of cancer predisposition (14). This seems to be explained by the impaired immune surveillance of lymphocytes by the defective immune system. For patients with CVID an increased susceptibility to malignancies, such as gastric cancer and particularly lymphomas is known (15, 16). Interestingly, this observation is paralleled by our results in CTLA-4-insufficient patients, showing predominantly lymphoma and gastric cancer. Lymphomas are known to occur in patients with primary immunodeficiencies, possibly as a result of the lymphopenia present in most patients. Gastric cancers often arise from a chronic inflammatory tissue, which is a commonly known risk factor (12). Despite to that, malignancies like the multiple myeloma in older individuals should also be seen in consideration of immune senescence.

Our findings demonstrate an elevated risk for malignancies of 12.9% in affected *CTLA4* mutation carriers. In addition, comparing the risk of cancer between the general population and affected *CTLA4* mutations carriers, affected *CTLA4* mutation carriers had a higher cancer rate per year (**Figure 5**).

The median time of survival since diagnosis of cancer was 361 days, however, this appears to vary dependent on entity (**Figure 6**). In 14 out of 17 patients, the diagnosis of CTLA-4 insufficiency was made prior the diagnosis of cancer. Special adjustment of cancer therapy because of the underlying PID was not reported, except in patient CZ.II.2, where additional immune dysregulatory features necessitated therapy with abatacept.

Lipopolysaccharide-responsive and beige-like anchor protein (LRBA) deficiency seems to be a phenocopy of CTLA-4 insufficiency (17, 18). In LRBA-deficient patients, CTLA-4 is transcribed normally, but impaired membrane trafficking induces an enhanced degradation of CTLA-4 in lysosomes (19). However, only a single report of gastric cancer was recently described for LRBA deficiency (20). The lack of reported malignancies in LRBA deficiency may result of the fact that the phenotype of LRBA deficiency is usually even more severe than that of CTLA-4 insufficiency and patients either die at an early age or receive HSCT, possibly prior to the development of any malignancy.

The reported melanoma in a CTLA-4-insufficient patient (CZ.II.2) is alerting and beyond our present understanding of the functional interaction between immune checkpoints and melanoma development. While the anti-CTLA-4 drug Ipilimumab acts to upregulate antitumor immunity and shows significant improvement in survival in metastatic melanoma, heterozygous *CTLA4* mutations should act similarly (21). Therapy is still pending but PD-1 inhibitors are being considered.

PD-1 blockade plus CTLA-4 insufficiency in patient CZ.II.2 should act like combined immune checkpoint inhibition, that had shown significant benefit on survival and CTLA4-Fc could attempt to decrease immune dysregulation (22). The possible deterioration of metastatic lesions under CTLA4-Fc should be considered, therefore the option of mono-chemotherapy with alloHSCT may be the best option if the patient's condition permits it.

Nine out of 17 patients had steroids (5–20 mg/d), one had additional anti-TNF-α treatment due to inflammatory bowel disease (XX.II.1), one had additional daclizumab due to MS (CZ.II.2), and two had received abatacept (CTLA4-Fc)

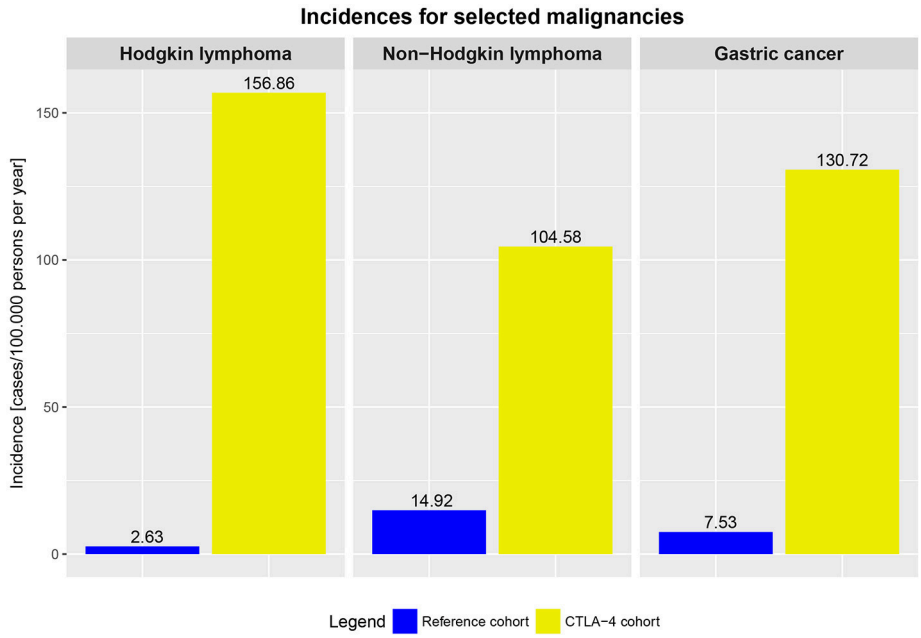

FIGURE 5 | Incidences of selected malignancies in the averaged German and US population (reference cohort, general population, 2009–2014) and incidences in affected *CTLA4* mutation carriers (2009–2017).

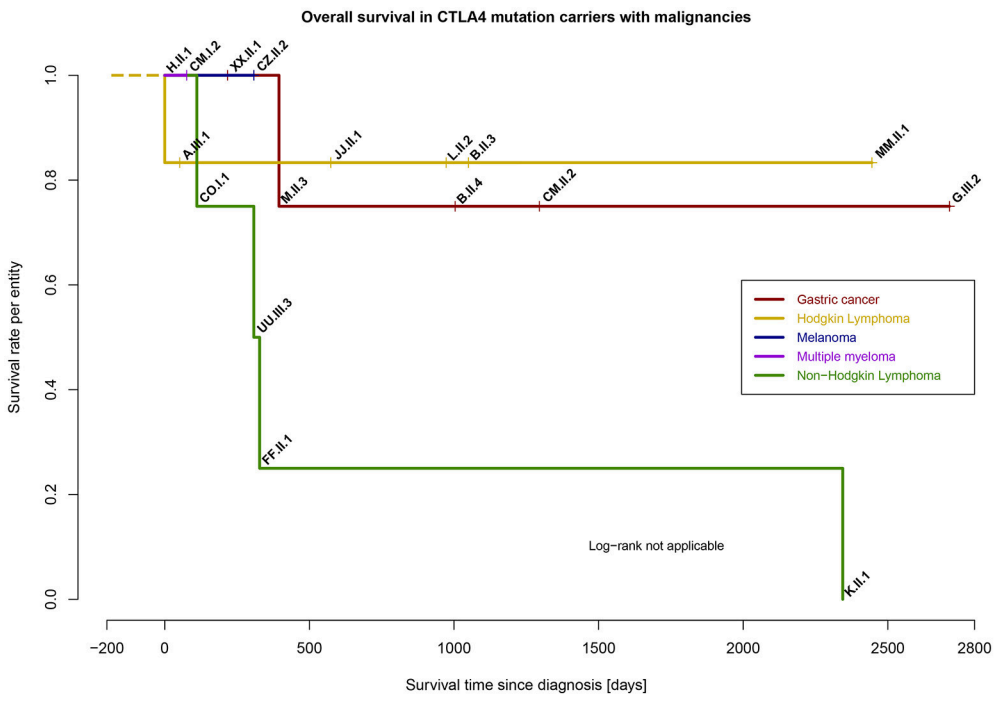

FIGURE 6 | Survival rate per entity of malignancies in *CTLA4* mutation carriers ($n = 17$), time range from day of diagnosis to last follow-up (days).

additionally in order to ameliorate the CTLA-4 insufficiency. The occurrence of cancer in eight of 17 CTLA-4 patients without any immunosuppression indicates that there may be an intrinsic defect to control especially the herpes viruses, EBV, and CMV in CTLA-4 insufficient individuals. However, further studies and observations need to address the question whether or not immunosuppressive therapy may have been a secondary trigger for cancer development.

The increased cancer risk, combined with the observation of viral association, leads us to assume that a defective immune surveillance of chronically virus-infected cells and the reduced elimination of oncogenic viruses leads to deregulated cell growth. Decreased CTLA-4 expression results in uncontrolled proliferation of T cells with a possible overgrowth of autoreactive clones over e.g., EBV-specific T cell clones, as known for persons with HIV infection, but experimental data are missing (23).

As a result, monitoring of EBV and CMV replication in blood in patients with CTLA-4 insufficiency by PCR should be performed.

In general, EBV-infected memory B cells are controlled mainly by Naturel Killer (NK) and CD8+ T cells. The quantity and quality of the CD8+ T cell response to EBV are essential to control the infection (24). In some PID patients an underlying genetic disorder affecting T- and NK cell function results in failure to control EBV infection, predisposing for EBV-associated HLH and EBV-associated cancer (25). Recently, alerting high frequencies of asymptomatic EBV viremia in affected and unaffected individuals with *CTLA4* mutations were described, showing the clinical importance of further research about CTLA-4 signaling and EBV infection (26).

Since the functional outcome of NK and CD8+ T cells depends on a balance between activating and inhibitory signals, costimulatory molecules such as CTLA-4 should be considered to play a critical role in control of EBV infection. NK cells express CTLA-4, resulting in decreased IFN-y secretion upon engagement by the recombinant ligand CD80 (27). Notably, *CTLA4* mutation carrier NK cells show defective effector functions, but normal peripheral maturation (28). Furthermore, EBV-specific CD8+ T cells in EBV-associated DLBCL (*CTLA4* wildtype) are functionally impaired, hence, CTLA-4 insufficiency might push the oncogenic capacity of EBV (29).

Together, changes in the function and clonal diversity of NK and T cells due to CTLA-4 insufficiency might be further augmented by viral persistence and may lead to life-threatening EBV. Whether the increased risk, however, also extends to HPV-induced malignancies [as e.g. seen in ICOS-deficiency (30)] needs to be monitored in affected individuals, even more since similar precancerous lesions had been seen in patient CZ.II.2.

Whether abatacept treatment will decrease the risk to develop cancer in CTLA-4 insufficiency remains to be proven, as the substitution of CTLA4-Fc ameliorates the dysregulated immune response. Until then, HSCT should be the preferred option and should be considered early-on in the treatment strategy of malignancies in the context of CTLA-4 insufficiency.

Cancer predisposition in CTLA-4 insufficiency appears as a result of immune activation with chronic inflammation, failure to control oncogenic viruses or neoplastic cells by immunological means, plus an intrinsic T cell impairment due to the underlying genetic defect. These findings go along with the observation that individuals with PIDs have intrinsic abnormalities of the lymphoid system which result in an increased frequency of malignant cell growth (31). Moreover, recently published data supports this hypothesis of cancer predisposition: Hauck et al. discussed similarly and separated in intrinsic lymphoid abnormalities and extrinsic insufficient cancer immunosurveillance with failure to eliminate oncogenic viruses and chronic tissue inflammation (32). However, the spectrum of malignancies in patients with PIDs as well as in CTLA-4 insufficiency appears to be restricted and derived from the same molecular defect as the immunodeficiency itself; either in the same cell type that has been primarily affected by the immunodeficiency or in another cell type in which malignant transformation could occur secondarily and indirectly facilitated by the underlying PID (32).

AUTHOR CONTRIBUTIONS

DE and CS, data collection and writing of the manuscript; AG, data collection; PA, LG-R, Y-JK, MM, ON, SOj, SOk A-CP, SSe, SSn, KW, and DW, contribution of patient data; PD, MS, EC, K-MK, J-MK, CM contribution of histologic images; BG, patient care, conception of the study, and writing of the manuscript.

FUNDING

The financial support for the conduct of the research came from the German Ministry of Education and Research (BMBF, grants # 01E01303 and 01ZX1306F), and the German Research Society (DFG; SFB1160–IMPATH). The article processing charge was funded by the German Research Foundation (DFG) and the University of Freiburg in the funding programme Open Access Publishing. The grant agencies had no role in study design, the collection, analysis, and interpretation of data, the writing of the report, nor in the decision to submit the paper for publication. The authors are responsible for the content of this research.

ACKNOWLEDGMENTS

The authors would like to acknowledge the help of Dr. Veronika Reiser and Dr. Henrik Heiland for biostatistics advice.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 02 July 2018
doi: 10.3389/fimmu.2018.01506

No Overt Clinical Immunodeficiency Despite Immune Biological Abnormalities in Patients With Constitutional Mismatch Repair Deficiency

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OPEN ACCESS

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Specialty section:
This article was submitted to Primary Immunodeficiencies, a section of the journal Frontiers in Immunology

Received: *23 March 2018*
Accepted: *18 June 2018*
Published: *02 July 2018*

Citation:
Tesch VK, IJspeert H, Raicht A, Rueda D, Dominguez-Pinilla N, Allende LM, Colas C, Rosenbaum T, Ilencikova D, Baris HN, Nathrath MHM, Suerink M, Januszkiewicz-Lewandowska D, Ragab I, Azizi AA, Wenzel SS, Zschocke J, Schwinger W, Kloor M, Blattmann C, Brugieres L, van der Burg M, Wimmer K and Seidel MG (2018) No Overt Clinical Immunodeficiency Despite Immune Biological Abnormalities in Patients With Constitutional Mismatch Repair Deficiency. Front. Immunol. 9:1506. doi: 10.3389/fimmu.2018.01506

Immunoglobulin class-switch recombination (CSR) and somatic hypermutations (SHMs) are prerequisites for antibody and immunoglobulin receptor maturation and adaptive immune diversity. The mismatch repair (MMR) machinery, consisting of homologs of MutSα, MutLα, and MutSβ (MSH2/MSH6, MLH1/PMS2, and MSH2/MSH3, respectively) and other proteins, is involved in CSR, primarily acting as a backup for nonhomologous end-joining repair of activation-induced cytidine deaminase-induced DNA mismatches and, furthermore, in addition to error-prone polymerases, in the repair of SHM-induced DNA breaks. A varying degree of antibody formation defect, from IgA or selective IgG subclass deficiency to common variable immunodeficiency and hyper-IgM syndrome, has been detected in a small number of patients with constitutional mismatch repair deficiency (CMMRD) due to biallelic loss-of-function mutations in one of the MMR genes (*PMS2, MSH6, MLH1, or MSH2*). To elucidate the clinical relevance of a presumed primary immunodeficiency (PID) in CMMRD, we systematically collected clinical history

and laboratory data of a cohort of 15 consecutive, unrelated patients (10 not previously reported) with homozygous/compound heterozygous mutations in *PMS2* (*n* = 8), *MSH6* (*n* = 5), and *MLH1* (*n* = 2), most of whom manifested with typical malignancies during childhood. Detailed descriptions of their genotypes, phenotypes, and family histories are provided. Importantly, none of the patients showed any clinical warning signs of PID (infections, immune dysregulation, inflammation, failure to thrive, etc.). Furthermore, we could not detect uniform or specific patterns of laboratory abnormalities. The concentration of IgM was increased in 3 out of 12, reduced in 3 out of 12, and normal in 6 out of 12 patients, while concentrations of IgG and IgG subclasses, except IgG4, and of IgA, and specific antibody formation were normal in most. Class-switched B memory cells were reduced in 5 out of 12 patients, and in 9 out of 12 also the $CD38^{hi}IgM^{-}$ plasmablasts were reduced. Furthermore, results of next generation sequencing-based analyses of antigen-selected B-cell receptor rearrangements showed a significantly reduced frequency of SHM and an increased number of rearranged immunoglobulin heavy chain (IGH) transcripts that use IGHG3, IGHG1, and IGHA1 subclasses. T cell subsets and receptor repertoires were unaffected. Together, neither clinical nor routine immunological laboratory parameters were consistently suggestive of PID in these CMMRD patients, but previously shown abnormalities in SHM and rearranged heavy chain transcripts were confirmed.

Keywords: primary immunodeficiency, hyper-IgM syndrome, DNA repair defect, mismatch repair, somatic hypermutation, class-switch recombination, IgA deficiency, IgG subclass deficiency

INTRODUCTION

Biallelic germline mutations in a mismatch repair (MMR) gene result in a condition referred to as *Constitutional Mismatch Repair Deficiency Syndrome* (CMMRD; OMIM #276300). This rare, devastating, cancer predisposition syndrome overlaps with the autosomal recessive form of Turcot's syndrome, a condition characterized by the co-occurrence of multiple adenomatous colon polyps with an increased risk of colorectal cancer and of brain tumors (1). In addition, individuals with CMMRD have a very high risk of developing hematological and other malignancies starting in early childhood [reviewed in Ref. (2)]. Often, patients with CMMRD show café-au-lait macules (CALMs) and other signs reminiscent of neurofibromatosis type 1 (NF1) which is of diagnostic importance (3). For the clinical diagnosis of CMMRD and tumor surveillance in affected patients, recent consensus reports provide helpful diagnostic scores and screening guidelines (4–7).

The main function of the MMR system is repairing replication errors that escape the proofreading activity of the polymerases [reviewed in Ref. (8)]. In addition, the MMR system is involved (i) in immunoglobulin class-switch recombination (CSR) in that it recognizes activation-induced cytidine deaminase- (AID) catalyzed conversion of cytidines to uridines in DNA switch regions and (ii) in somatic hypermutation (SHM) [reviewed in Ref. (9)]. Both processes are needed for B cell maturation and for diversification and specification of the mammalian immunoglobulin repertoire. Defects of CSR are the molecular basis of hyper-IgM syndromes, which are primary immunodeficiencies (PIDs) with a predominant antibody formation defect associated with decreased IgG, IgA, and IgE, and normal or increased concentrations of IgM (9–11). With these functions, the MMR system constitutes a link between the immune system and tumor suppression (12).

Various levels of immunodeficiency were detected in single CMMRD patients or small patient series, supporting the hypothesis that the MMR machinery contributes to immunoglobulin CSR and SHM. IgA deficiency or common variable immunodeficiency (CVID) was first reported in one MSH2- and three MSH6-deficient patients (13–15). Further analyses focused on defects related to CSR and allowed the identification of three PMS2- and eight MSH6-deficient individuals with biallelic loss-of-function mutations, who presented variable degrees of hyper-IgM-like features and clear defects of CSR *in vitro* and *in vivo* (16, 17). In addition, larger screens for single nucleotide polymorphisms within MMR genes in selected patient cohorts with IgA deficiency or with CVID led to the identification of certain monoallelic *MSH5*, *MLH1*, and *MSH2* variants which could be linked to these PIDs (18, 19). Together, the results of these studies suggested that CMMRD consistently entails a PID.

The risk of malignancies is higher in most primary immune deficiency and dysregulation disorders (PID), but the mechanisms and frequencies of malignant transformation vary according to the different categories of PID (20). In CMMRD, any impairment of the immune system might be critical for the evolution of malignancies, since it would compromise tumor immune surveillance, which could accelerate tumorigenesis in addition to the remarkably increased mutation rates that are intrinsic to cells with MMR deficiency. Because previous studies reported varying degrees of immunodeficiency in patients with CMMRD that might render

them less responsive to oncological immune therapy such as, e.g., checkpoint inhibition, the clarification of whether CMMRD patients suffered from PID has potential implications for future oncologic immune treatment strategies. On the other hand, a uniform pattern of clinical symptoms such as warning signs suggestive of PID or laboratory immunological abnormalities could facilitate early diagnosis of CMMRD. Furthermore, immunodeficiency secondary to chemotherapy might be aggravated in these individuals, requiring additional caution and supportive measures.

The present systematic analysis of PID in CMMRD addressed the *in vivo* cellular, humoral, and clinical immune phenotypes of CMMRD patients from Europe and the Middle East.

RESULTS

Fifteen consecutive, unrelated patients with a genetically confirmed diagnosis of CMMRD reported from nine countries were included in this study (11 females, 4 males; age at inclusion: 1–38 years, median age 9 years; age at first malignancy: 0.7–22 years, median age 5 years). Five of these patients were included in previous studies, while data of the remaining 10 patients were not published previously. **Table 1** summarizes the patients' genotypes, clinical presentations, and family histories.

In line with previous observations, homozygous ($n = 7$) or compound heterozygous ($n = 1$) *PMS2* germline mutations were present in more than half of the patients; consanguinity was reported by five of the parents (**Table 1**). Two of the four novel patients with PMS2-deficiency (P5 and P12) had truncating mutations affecting both *PMS2* alleles and the other two (P9 and P16) were homozygous for splice mutations leading to aberrant out-of-frame transcripts. Six of PMS2-deficient patients had a recent history of high-grade malignant glioma, and one had a recent history of Burkitt's lymphoma. In one patient with glioblastoma, acute lymphoblastic leukemia (ALL), and in another one, T-cell Non-Hodgkin's lymphoma (T-NHL) had preceded the brain tumor by 1 and 5 years, respectively. Two patients had metachronous LS-associated carcinomas, and three patients also had bowel adenomas.

Five patients had *MSH6* mutations. Two patients from reportedly consanguineous parents were each homozygous for a truncating *MSH6* mutation. Three patients were compound heterozygous for two different *MSH6* mutations. Interestingly, one of these patients (P13) had a *de novo* mutation that was absent in both genetically confirmed parents, while the second mutation was maternally inherited. While this patient had two different truncating mutations, the other two patients (P2 and P14) had one truncating and one missense mutation. Both missense *MSH6* mutations (p.Asp439Gly and p.Tyr994Asn) are so far unreported, but could be classified as likely pathogenic at least in the context of CMMRD according to ACMG guidelines (25). The tumor spectrum of MSH6-deficient patients included Wilms tumor, two medulloblastomas, and two NHL, which relapsed in one patient and was preceded by a B-cell ALL in the other patient (**Table 1**). One of the MSH6-deficient patients had bowel adenomas at 10 years of age. With a median age of 6.5 (range 4–7) years, none of the other patients had bowel adenomas or LS-associated tumors.

One of two patients with biallelic mutations in *MLH1* was from consanguineous parents. This patient (P8) carried the known missense mutation p.Ala111Val classified as likely pathogenic by the InSiGHT Variant Interpretation Committee. The other patient (P7) was compound heterozygous for the known missense mutation p.Ala21Glu classified as pathogenic by the InSiGHT Variant Interpretation Committee and the missense variant p.Val716Met which is classified as benign variant by the InSiGHT Variant Interpretation Committee. Of note, both patients (P7 and P8) showed in a germline microsatellite instability (gMSI) assay elevated gMSI ratios as did all PMS2-deficient individuals tested for this feature and which is highly specific for CMMRD (26). Although, we cannot exclude that not the detected variant p.Val716Met but a different *MLH1* mutation is responsible for CMMRD in patient P7, it is noteworthy, that this variant has already previously been discussed to be potentially responsible in combination with a stop-mutation for the colorectal cancer in a 12-year-old boy (27). Both MLH1-deficient patients (P7 and P8) had T-NHL at a very early age (8 months and 1 year), and P7 developed another lymphoma (B-NHL) at 12 years of age, a borderline phylloides tumor at 16 and a glioblastoma at 21. She also had multiple bowel adenomas removed.

Two patients (P3 and P10) were from families with previously diagnosed CMMRD patients. One patient (P11) was diagnosed with CMMRD prior to tumor development (24). All 12 remaining index patients in the families fulfilled the C4CMMRD criteria for the clinical suspicion of CMMRD when they had their first tumor with a mean of 5.5 (range 3–7) C4CMMRD scoring points (5). Non-malignant features indicative of CMMRD according to the C4CMMRD scoring system were present in 13 out of 15 patients. Two or more CALM, hyper- and/or hypopigmented skin patches were noted in 13 patients, and cerebral cavernoma/hamartoma were present in three. Furthermore, vascular anomalies of the skin (hemangioma and venocapillary malformation) were reported in three patients, and one patient had a hepatic hemangioma. Parents of eight patients reported consanguinity. Family histories of LS or LS-associated carcinomas in the first, second, or third degree relatives were reported for five patients, and three had siblings with CMMRD-associated cancers (**Table 1**).

To define the clinical immunodeficiency associated with CMMRD, we first assessed the clinical immunological parameters. Patients' clinical history data were obtained using a study questionnaire, which is provided in the Online Repository (Figure S1 in Supplementary Material; see Patients and Methods). Data were retrieved from 14 out of 15 patients and showed no clinical signs of PID (**Table 2**). A filled questionnaire was not available from P6, but her physician-reported history did not show any signs of PID. Only 2 out of 14 patients (P2 and P3; aged 4 and 6 years) were reported to have 4–7 infections of the ear-nose-throat tract or simple viral infections per year, which is within the physiological range at that age (**Table 2**). For none of the other patients an increased number of infections were reported. P3 had a history of bronchial asthma, with an IgE within the normal range (**Table 2**).

TABLE 1 | Characteristics of 15 patients with CMMRD.

Patient (reference)	Age (years) at study inclusion[a]	Genotype (mutated gene, mutation at cDNA level, mutation at protein level)	First symptom or malignancy (age, years)[b]; C4CMMRD points at first tumor diagnosis	History of clinical immunodeficiency or dysregulation[c]	Family tumors (age, years)	Parental consanguinity (as reported by parents)	Nonmalignant features	Premalignancies	Hematological malignoma (age, years)	Brain tumor (age, years)	LS-associated cancer (age, years)	Others
P1 (21)	38	PMS2 c.[137 G>T]; [137 G>T]; p.[Ser46Ile]; [Ser46Ile]	CRC (22); 6	Negative	LS family, mother: CRC (46)	Yes	Adenoma sebaceum, hepatic hemangioma	Dysplastic adenomata (colon)	–	Glioblastoma (34)	CRC (22) Duodenal Ca (36) Endometrial Ca (35)	
P2 (unpublished)	4	MSH6 c.[467C>G]; [1316A>G]; p.[Ser156*]; [Asp439Gly]	Anaplastic medulloblastoma (4); 5	Negative	Paternal grandfather: CRC (50), maternal cousin: AML[h] (11)	No	CALM, freckling, ash-leaf spots; hemangioma, non-therapy-induced cavernoma		–	Anaplastic medulloblastoma (4)	–	–
P3 (22)	6	MSH6 c.[3261dupC]; [3261dupC]; p.[Phe1088Leufs*5]; [Phe1088Leufs*5]	CALM, T-NHL (3); 6	Negative	Two cousins affected with CMMRD-related malignancies	Yes	~10× CALM (generalized), bilateral frontal venous angioma; supra- and infratentorial hamartoma		T-NHL (3) T-NHL relapse [as T-ALL] (6)	–	–	–
P5 (unpublished)	10	PMS2 c.[634C>T]; [1239del]; p.[Gln212*]; [Asp414Thrfs*34]	Glioblastoma (9); 4	Negative	No	No	CALM		–	Glioblastoma (9)	–	–
P6 (23)	26	PMS2 c.[2192T>G]; [2192T>G]; p.[Leu731*]; [Leu731*]	CRC (20); 7	n.a.	Maternal grandfather: CRC (40)	Yes	CALM	Villous adenoma (small bowel)	–	Low grade diffuse astrocytoma (23) → high grade (26)[d]	CRC (20) Papilla Vateri Ca (22)	–
P7 (unpublished)	21	MLH1 c.[62C>A]; [2146G>A]; p.[Ala21Glu]; [Val716Met][e]	T-NHL (1); 6	Negative	LS family, mother: CRC (40), maternal aunt: CRC (50), maternal grandfather: CRC (64)	No	1× CALM, cerebral cavernoma, varicosis, vascular malformation with pigmentation disorder right calf	9 adenomas (small, large bowel)	T-NHL (1) B-NHL (12)	Glioblastoma (21)	–	Borderline phylloides tumor (16)
P8 (unpublished)	1	MLH1 c.[332C>T]; [332C>T]; p.[Ala111Val]; [Ala111Val]	T-NHL (7 months); 6	Negative	Maternal grandmother: CRC/ breast cancer; paternal uncle: ColonCa (34)	Yes	CALM		T-NHL (7 months)	–	–	–
P9 (unpublished)	3	PMS2 c.[2007-2A>G]; [2007-2A>G][f]	ALL (2); 6	Negative	Brother: CRC (12)	Yes	CALM		ALL (2)	Glioblastoma (3)	–	–

TABLE 1 | Continued

Patient (reference)	Age (years) at study inclusion[a]	Genotype (mutated gene, mutation at cDNA level, mutation at protein level)	First symptom or malignancy (age, years)[b]; C4CMMRD points at first tumor diagnosis	History of clinical immunodeficiency or dysregulation[c]	Family tumors (age, years)	Parental consanguinity (as reported by parents)	Nonmalignant features	Premalignancies	Hematological malignoma (age, years)	Brain tumor (age, years)	LS-associated cancer (age, years)	Others
P10 (unpublished)	7	MSH6 c.[2653A>T]; [2653A>T]; p.[Lys885*]; [Lys885*]	Wilms tumor (5); 6	Negative	Maternal uncle: B-NHL; affected siblings-CMMRD	Yes	CALM		–	–	–	Wilms tumor (5)
P11 (24)	7	PMS2 c.[2444C>T]; [2444C>T]; p.[Ser815Leu]; [Ser815Leu]	CALM (7); n.a.	Negative	No	Yes	CALM		–	–	–	–
P12 (unpublished)	13	PMS2 c.[1515delG]; [1515delG]; p.[Phe506fs]; [Phe506fs]	B-cell Burkitt lymphoma (13); 6	Negative	No	No	CALM Venocapillary malformation	Multiple dysplastic colonic adenomatous polyps	B-cell Burkitt lymphoma (13)	–	–	–
P13 (unpublished)	10	MSH6 c.[1135_1139delAGAGA]; [2277_2293del]; p.[Arg379*]; [Glu760Profs*6]	B-ALL (3); 3	Negative	No	No	CALM (>6); Spitz naevus; hypopigmented areas; MRI signal alterations reminiscent of NF1-FASI; colitis chronica	Tubulous adenoma, low grade dysplasia	B-ALL; T-NHL (3; 7)	–	–	–
P14 (unpublished)	7	MSH6 c.[2238dupT]; [2980T>A]; p.[Leu747Serfs*9]; [Tyr994Asn]	Medulloblastoma (6); 3	Negative	No	No	CALM	–		Medulloblastoma (6 years)		
P15 (21)	12	PMS2 c.[2007-2A>G]; [2007-2A>G][f]	T-NHL (4); 4	Negative	No	No	CALM (>5), hypopigmented macules	–	T-NHL (4)	Glioblastoma (9)		
P16 (unpublished)	9	PMS2 c.[1145-31_1145-13del]; [1145-31_1145-13del]; p.[Asn383*; Gly382Valfs*19]; [Asn383*; Gly382Valfs*19][g]	Glioblastoma (8); 4	Negative	Paternal grandfather: prostate carcinoma (>70 years); one sister died from NHL (4 years)	Yes	– (no signs of NF1)			Glioblastoma (8)		

[a]*At one time point within 4 years of patient recruitment, when blood sampling for immunological analyses was undertaken.*
[b]*At first malignancy.*
[c]*Defined by immunological warning signs as assessed by a questionnaire (Figure S1 in Supplementary Material) with data shown in* ***Table 2****.*
[d]*Counted as one malignancy.*
[e]*p.Val716Met is classified a benign variant according to the InSiGHT Variant Interpretation Committee and it cannot be excluded that this patient carries a different pathogenic mutation on this allele.*
[f]*In a different patient, it was shown that the mutation c.2007-2A>G leads to the following two aberrant transcripts: r.2007_2023del (p.Ser669Argfs*9) and r.2007_2174del (p.Ser669_Ala725delinsArg).*
[g]*cDNA-sequencing showed two aberrant transcripts: r.1144_1145insGATAGTCCACGTTTGCTTAG (p.Asn383Ter) and r.1145_2006del (p.Gly382Valfs*19).*
[h]*In this family member, a germline mutation in CEBPa associated with a predisposition toward myeloid malignancies was detected.*
CRC, colorectal carcinoma; LS, Lynch syndrome; CALM, café-au-lait macule (spots); T-NHL, T-cell Non-Hodgkin's Lymphoma; ALL, acute lymphoblastic leukemia; NF1-FASI, NF1-associated foci of abnormal signal intensity; CMMRD, constitutional mismatch repair deficiency.

TABLE 2 | History of clinical signs of immunodeficiency or immune dysregulation of 14 (out of 15)[a] patients with CMMRD.

	P1	P2	P3	P5	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16
Immunodeficiency warning signs														
Family history of immunodeficiency	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Hospitalization for infections	No	No	Only during chemo	No	No	Only during chemo	Only during chemo	Only during chemo	No	No	No	Yes (in Syria)	No	No
Reactions/complications after live vaccines	No	No	No	No	No	n.a.	No	No	No	No	No	No	No	n.d.
Pathological healing of the navel	No	No	No	No	No	No	No	No	No	No	No	n.a.	No	n.d.
Delayed growth during childhood/reduced thriving	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Infections														
Frequency (per year)	0	7	4–5	Normal	Normal	Increased since chemo	Increased since chemo	Increased during chemo	Normal	Normal	Normal	1	Normal	Normal
Severity	n.a.	Simple viral	Simple viral, simple bacterial; history of bronchial asthma	Simple viral	Simple viral	Complicated viral complicated bacterial	Invasive fungal	Simple viral and invasive fungal	n.a.	n.a.	n.a.	Complicated viral	Simple viral	Simple viral
Localization	n.a.	Respiratory tract	ENT, bacterial: lungs	ENT	ENT	Lungs bacteremia	Skin	n.d.	n.a.	n.a.	n.a.	Bone marrow, liver	n.a.	n.a.
Infectious agents	n.a.	n.d.	During chemo: *Staphylococcus aureus*, MRSA, *Aspergillus fumigatus*	n.d.	Normal spectrum	During chemo: opportunistic	During chemo: opportunistic, *Candida albicans*	During chemo: Herpes simplex, *C. albicans*	n.a.	n.a.	n.a.	Parvovirus HCV	n.a.	n.a.
Response to antibiotics	n.a.	Normal	Delayed, during chemo	n.a.	Normal	Delayed	n.d.	Normal	n.a.	Normal	Normal	Normal	n.a.	n.a.
Requirement of corticosteroids or immunosuppression	Only as oncological treatment	No	Yes, during chemo	No	No	Yes, during chemo	n.d.	No	No	Yes, during chemo	No	No	n.a.	No
Autoimmunity/immune dysregulation														
Granuloma	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Autoimmunity	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Autoinflammation/recurrent fever	No	No	No	No	No	No	No	No	No	No		No	n.a.	No
Lymphoproliferation/splenomegaly	No	No	Yes, mild axillary	No	No	No	No	No	No	No	Yes	No	No	No
Hepatopathy, cholangitis, *Cryptosporidium* infection	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Inflammatory bowel disease	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Interpretation regarding primary immunodeficiency (PID)	No signs	No signs	No signs	No signs	No signs	No signs	No signs	No signs	No signs	No signs	No signs	No signs	No signs	No signs

[a]*No immunological history data available from patient P6.*

ENT, ear-nose-throat tract; chemo, cytostatic chemotherapy; MRSA, multidrug resistant Staphylococcus aureus; CMMRD, constitutional mismatch repair deficiency.

Next, we sought to determine whether there was any consistent abnormality detectable in the routine parameters of the cellular immune system. To this end, an analysis resembling an extended, routine, diagnostic workup of any suspected, combined immunodeficiency was performed. Due to the role of the MMR machinery in CSR and SHM, we focused on the B cell maturation stages. But also, T cell subsets and the T cell receptor repertoire were analyzed. **Tables 3** and **4** show the raw data for the most relevant B and T cell subsets together with the corresponding normal ranges (28, 29). Patients are grouped according to their genotype (i.e., mutated MMR gene) and sorted according to age. Importantly, no uniform pattern of variation from the norm was identified within the cellular immune system of CMMRD patients as a result of the routine PID diagnostic FACS analysis, including an analysis of memory B cell subsets. Class-switched memory B cells were reduced to a varying degree in 5 and normal in 7 out of 12 patients. Still, we detected a clear trend of reduced $CD38^{hi}IgM^{-}$ plasmablasts (measured as a percent of B cells in 12 patients: normal in 3 patients, reduced in 7, absent in 2, and an increased in none) and a relative increase of $CD21^{low}CD38^{low}$ (activated) B cells in 6 of 12 patients across all three genotypes analyzed (**Table 3**). As expected, values of the following lymphocyte subsets were unremarkable with mild, inconsistent variations: T cells and T cell subsets including $CD4^{+}$ and $CD8^{+}$ T cells, T cell receptor alpha/beta (TCRab)-positive $CD4^{-}CD8^{-}CD3^{+}$ double negative T cells, TCRgamma/delta as well as naïve $CD45RA^{+}CD4^{+}$ (**Table 4**) and naïve $CD8^{+}$ T cells (not shown), activated T cells (not shown), and NK cells and monocytes (**Table 4**). Furthermore, the results of the TCR repertoire (spectratyping), assessed by conducting quantitative sequencing of 24 TCR Vbeta family fragments from CD4 and CD8 T cells, was unremarkable in all individuals tested ($n = 14$; not shown). Overall, apart from a reduction in the number of class-switched B memory cells in 5 out of 12 individuals and of class-switched plasmablasts detected in most (9 out of 12), no abnormality of the cellular immune system was consistently found among these CMMRD patients.

Abnormal parameters of humoral immunity and B cell function such as IgG subclass or IgA deficiency, hypogammaglobulinemia, and hyper-IgM syndrome are expected in CMMRD as they were shown to occur in patients with defective CSR (16, 30, 31). Thus, we examined the results of the quantitative immunoglobulin isotypes and subclass analyses (**Figure 1**) and of the specific antibody formation capacity (Table S1 in Supplementary Material). These serological analyses were conducted at the patients' local hospitals, and they were not conducted for all patients. The results were quite heterogeneous, with mostly normal, but some reduced and some increased immunoglobulin concentrations. Importantly, we did not observe a consistent reduction of IgA, IgG, or any IgG subclass except for IgG4 (in four out of seven tested patients). However, the interpretation of IgG4 subclass deficiency is limited, because normal ranges indicate that IgG4 might be undetectable throughout preschool age and still very low (0.05 g/l) even in healthy adults (32). IgG was mildly to moderately reduced in 4 out of 12 tested patients, all of whom were ≥10 years of age

TABLE 3 | Quantification of B cell subsets of CMMRD patients.

	CD19/µL	$CD19^{+}CD27^{+}IgD^{+}$ (ncsBm) %B	$CD19^{+}CD27^{+}IgD^{-}$ (csBm) %B	$CD19^{+}CD27^{-}IgD^{+}$ (naive) %B	$CD21^{lo}CD38^{lo}$ (activ) %B	$CD38^{hi}IgM^{hi}$ (trans) %B	$CD38^{hi}IgM^{-}$ (csPlasmablasts) %B
P9 (PMS2; 3 years)[a]	*7*	–	–	–	–	–	–
P11 (PMS2; 7 years)	548	15.21	4.52	73.87	8.29	5.6	**0.15**
P16 (PMS2; 8 years)	**213**	7.31	10.47	76.92	6.32	8.07	0.53
P5 (PMS2; 10 years)	**193**	13.85	9.88	74.75	6.84	8.22	**0**
P12 (PMS2; 13 years)	**775**	5.31	7.30	78.54	**22.32**	4.24	0.45
P15 (PMS2; 14 years)	**168.5**	5.1	5.37	80.81	**69.43**	4.7	**0.14**
P6 (PMS2; 26 years)	**1,079**	**3.3**	**4.67**	**84.29**	**32.54**	1.39	**0**
P1 (PMS2; 38 years)	317	**6.75**	**1.93**	**89.29**	**84.21**	**0.73**	**0.1**
P2 (MSH6; 4 years)	**57**	**0.35**	**1.06**	**93.47**	**80.91**	5.78	**0.35**
P3 (MSH6; 6 years)	*46.5*	*10*	*30*	*0*	*6.67*	*0*	*0*
P10 (MSH6; 7 years)	**179**	3.21	5.02	**90.54**	5.14	20.09	0.65
P14 (MSH6; 7 years)	582	4.71	5.54	84.49	9.25	7.99	**0.23**
P13 (MSH6; 10 years)	**195**	**1.95**	**2.26**	93.46	8.02	3.61	**0.04**
P8 (MLH1; 1 year)[a]	***8***	*8*	*4*	***80***	–	–	–
P7 (MLH1; 21 years)	342	**3.15**	**2.14**	**92.24**	**62.95**	1.29	**0.18**
Controls/reference values (5th–95th percentile)							
1 year[b] ($n = 26$)	700–1,300	3.25–10.75	1–5	83.25–93.75	1–11	1–25	0.4–3.6
2–3 years[b] ($n = 38$)	700–1,300	4.9–14.2	2.9–9.2	74.7–90.5	1–11	1–25	0.4–3.6
4–5 years[b] ($n = 38$)	700–1,300	7–15.2	3.9–16.2	69.9–85.6	1.11	1–25	0.4–3.6
6–10 years[b] ($n = 38$)	300–500	2.93–19	3.85–16.5	63.1–89.15	1–11	1–25	0.4–3.6
11–18 years[b] ($n = 22$)	300–500	5.05–17.95	4–22.8	60.15–88.95	1–11	1–25	0.4–3.6
19–61 years[b] ($n = 54$)	300–500	7.4–32.5	6.5–29.1	42.6–82.3	1–11	1–25	0.4–3.6

Samples are sorted according to gene defect and age.

[a]*Data for patients P3, P8, and P9 were excluded from B cell analyses due to their recent treatment with rituximab (P8 and P9) and/or chemotherapy (P8 and P3) (italic).*

[b]*Reference values for ncsBm, csBm, and naïve B-cells were taken from Huck et al. (29) for the age groups 1 year, 2–3 years, 4–5 years, 6–10 years, 11–18 years; and from Warnatz and Schlesier (28) for the age group 19–61 years and csPlasmablasts.*

*Reduced values are printed in **red boldface**.*

*Increased values are printed in **green boldface**.*

CMMRD, constitutional mismatch repair deficiency.

TABLE 4 | Quantification of T cell subsets and other peripheral blood mononuclear cells of CMMRD patients.

	CD3⁺/μL	CD3⁺CD4⁺	CD3⁺CD8⁺	CD4⁺CD45RA⁺ (%CD3)	TCRab⁺CD4⁻CD8⁻CD56⁻ (%CD3)	TCRgd⁺ (%CD3)	NK/μL	Mono/μL	Stem cells in PB/μL
Age-specific normal range[a]	**1,400–2,000**	**700–1,100**	**600–900**	**>11–30% (age-dep.)**	**<2%**	**<11–15% (age-dep.)**	**200–300**	**400–1,000**	
P9 (PMS2; 3 years)	1,839 [1,800–3,000]	1,088 [1,000–1,800]	613 [800–1,500]	36.64	0.09	3.17	252	**2,254**	13
P11 (PMS2; 7 years)	**5,139**	**3,209**	**1,748**	53.81	0.06	7.21	566	609	3
P16 (PMS2; 8 years)	**1,161**	**529**	606	18.68	0.05	10.82	337	**323**	4
P5 (PMS2; 10 years)	**954**	**543**	**377**	77.22	0.20	5.93	**140**	400	1
P12 (PMS2; 13 years)	**2,864**	**1,361**	**1,258**	31.01	0.23	5.98	247	**185**	0
P15 (PMS2; 14 years)	1,786	**112**	755	30.25	0.13	5.37	278	684	0
P6 (PMS2; 26 years)	**2,481**	**1,485**	**948**	n.d.	n.d.	n.d.	**189**	**1,664**	14
P1 (PMS2; 38 years)	**2,053**	**1,342**	639	21.75	n.d.	1.7	266	**320**	n.d.
P2 (MSH6; 4 years)	**644** [1,800–3,000]	**483** [1,000–1,800]	**189** [800–1,500]	60.8	0.15	3.29	**124**	735	0
P3 (MSH6; 6 years)	*833*	*266*	*556*	*32.79*	*0*	*26.21*	*276*	*398*	*n.d.*
P10 (MSH6; 7 years)	**1,123**	**405**	681	36.30	0.20	7.10	**120**	427	1
P14 (MSH6; 7 years)	1,697	903	754	22.54	0.52	4.69	253	**386**	1
P13 (MSH6; 10 years)	**454**	**216**	**196**	**9.55**	0.12	3.52	**72**	444	1
P8 (MLH1; 1 year)[b]	71 [1,800–3,000]	53 [1,000–1,800]	15 [800–1,500]	39.93	0	5.41	24	374	2
P7 (MLH1; 21 years)	**1,378**	776	**538**	19.91	0.21	3.41	**323**	690	1

Samples are sorted according to gene defect and age.

[a]*Except otherwise stated [square brackets].*

[b]*Data of patients P3 and P8 were excluded from T cell analyses due to preceding chemotherapy (italic).*

Reduced values are printed in ***red boldface****.*

Increased values are printed in ***green boldface****.*

TCRab, T cell receptor alpha/beta; CMMRD, constitutional mismatch repair deficiency.

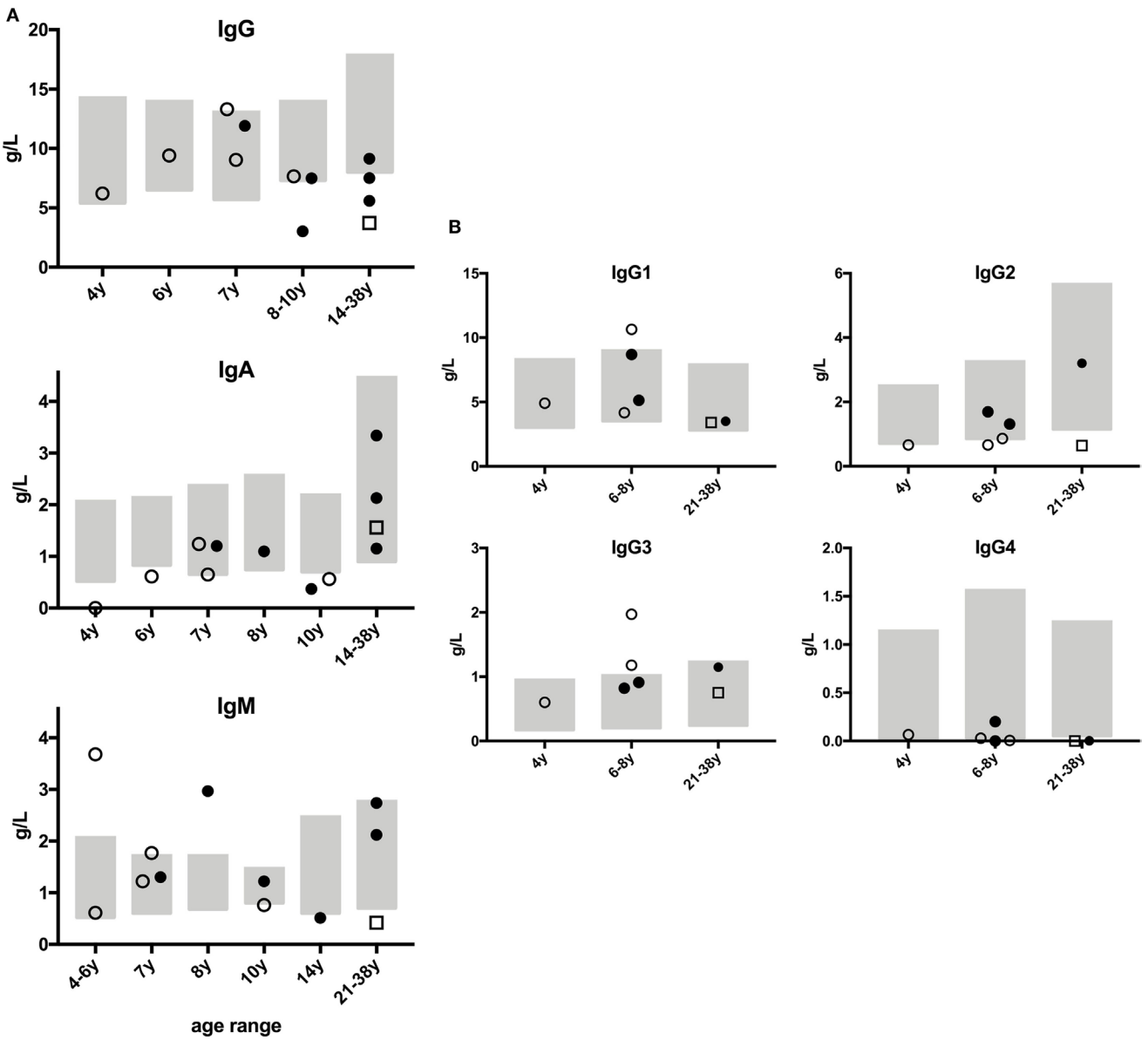

FIGURE 1 | Immunoglobulin concentrations of patients with constitutional mismatch repair deficiency. Humoral immunologic analyses were performed locally at the patients' hospitals, and the results were sent to the study center. None of the patients had received therapeutic (i.v. or s.c.) immunoglobulins within the last 6 months prior to analysis. Results from PMS2-deficient patients are shown as full dots, from MSH6-deficient patients as open dots, and from MLH1-deficient patient as open square. Age-specific normal ranges are shown as gray boxes. **(A)** Panels show serum concentrations of IgG, IgA, and IgM in g/dl, with age-specific, normal ranges. **(B)** Graphs show IgG subclass analyses, available from seven patients, related to age-specific, normal ranges to facilitate interpretation. Out of 15 patients, no serological data were available for P12, and those for P8 and P9 were excluded due to their having received prior chemo- or rituximab therapy.

(**Figure 1A**). IgM was increased in 3 out of 12 of the tested patients (P2, P14, P16; **Figure 1A**). Of the three patients with increased IgM, one (P2) also had IgA deficiency and two (P2 and P14) a borderline reduction of IgG2. The third patient (P16) had remarkably increased IgM, but normal IgA and IgG subclass, values. P14, in whom IgM concentrations were only slightly increased, displayed a remarkable increase in IgG1 and IgG3 (**Figure 1**). Of note, three patients had reduced concentrations of IgM (P7, P13, P15; **Figure 1A**), and six individuals had IgM concentrations within the normal range. IgE was detectable within the normal range in three and nearly absent or undetectable in four individuals (not shown). Antibody concentrations against vaccination antigens or childhood infections (such as against diphtheria toxin, tetanus toxoid, hepatitis B virus, rubeola, morbilli, haemophilus influenza B polysaccharide, pneumococcal polysaccharide, varicella zoster virus, Epstein–Barr virus, cytomegalovirus; without recent therapeutic administration of immunoglobulins) tested positive for at least some of the tested antibodies in all patients (Table

S1 in Supplementary Material). Of note, all three patients with increased IgM concentrations had detectable levels of specific antibody formation against protein and polysaccharide antigens (P2, P14, P16; Table S1 in Supplementary Material). Despite the limitation that the vaccination and infection histories were not assessed in detail, compromising the interpretation of specific antibody concentrations, these results do not support a specific antibody formation defect in CMMRD patients. On the contrary, patients with a known history of infection or current infection at the time of analysis displayed an adequately increased concentration of specific antibodies (Table S1 in Supplementary Material). The results of the attempt to detect antinuclear antibodies were inconclusive with three patients showing negative results and two (albeit asymptomatic) patients who tested low-level positive. Together, these data do not support the hypothesis that patients with CMMRD regularly have a clinically relevant humoral immunodeficiency.

After B cells have encountered antigen, they migrate to the germinal center where they undergo SHM, CSR, and selection. To study the effect of MMR deficiency on these processes at the molecular level, we analyzed the B-cell receptor repertoire in five CMMRD patients using next generation sequencing of IGHG and IGHA transcripts derived from antigen-selected B cells. We obtained 253–568 unique immunoglobulin heavy chain (IGH) rearrangements per patient and compared them to age-matched healthy controls (HC) (Table S4 in Supplementary Material). Interestingly, the median frequency of SHM was significantly reduced in both IGHG and IGHA transcripts in all patients (**Figure 2A**). In addition, we analyzed the subclass distribution in the IGHG and IGHA transcripts. This distribution in the IGHG transcripts was altered in four of the five CMMRD patients, who hardly had IGHG transcripts that used the IGHG2 or IGHG4 subclasses (**Figure 2B**). Also, in the IGHA transcripts, fewer transcripts with the IGHA2 subclass were present in four of the five CMMRD patients as compared to the HC. The IGHG2 and IGHG4 constant genes are located more distal in the IGH locus, further away from VDJ rearrangement compared to the IGHG3 and IGHG1 constant gene, and are used during sequential switching during a single immune response or after consecutive germinal center response. A reduction in these subclasses is often seen in patients with a CSR defect, like patients with Ataxia telangiectasia (33), and might indicate a defect in CSR or a disturbed immune response. Given the fact that the frequency of SHM is also reduced it is likely that B-cells in germinal center fail to undergo a second round of affinity maturation and therefore do not make it until switching toward the distal constant regions [see also Ref. (34)].

When B cells differentiate from naïve B cells to memory B cells, they are strongly selected against B cells that have a long complementary determining region (CDR3), and against B cells

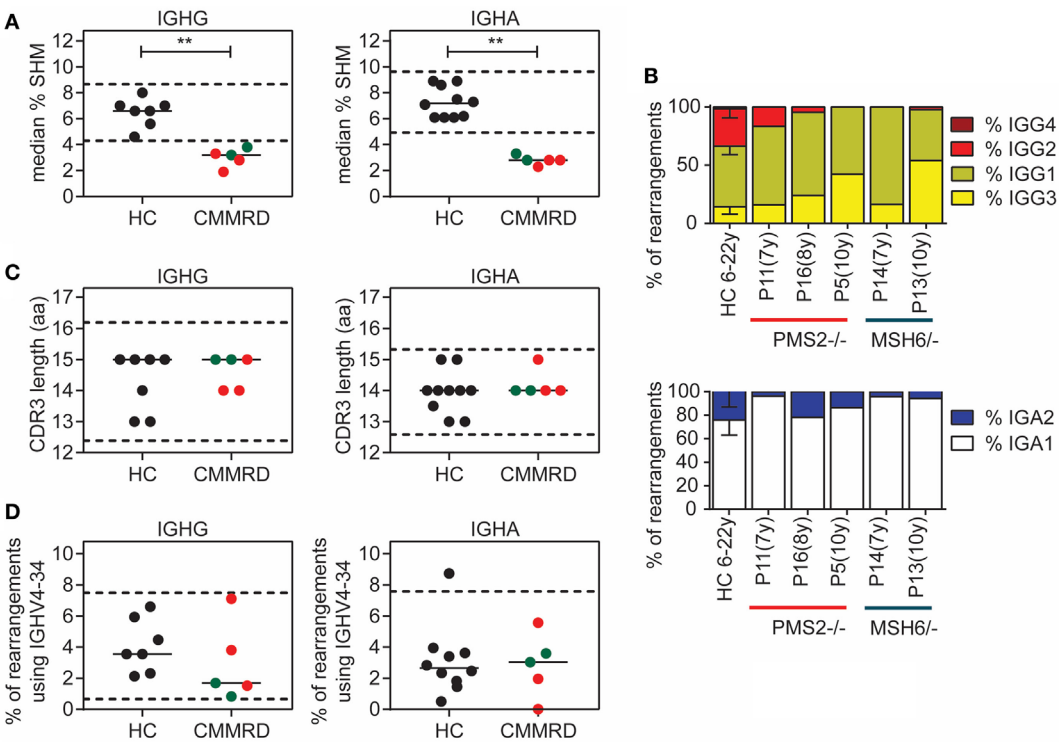

FIGURE 2 | Analysis of somatic hypermutation (SHM) and class-switch recombination (CSR) in B-cell receptor rearrangements. Detailed analysis of B-cell receptor rearrangements showed significantly decreased frequency of SHM in patients with constitutional mismatch repair deficiency (CMMRD) compared to healthy controls (HC) **(A)**. In addition, the frequency of rearrangements that have the IGHG3, IGHG1, and IGHA1 subclass were increased in the CMMRD patients, suggesting a possible defect in CSR **(B)**. Selection against B cells with a long complementary determining region (CDR3) **(C)**, and B-cell that use the IGHV4-34 gene **(D)** was normal in the CMMRD patients. Red dots indicate MSH6-deficient patients and green dots indicate the PMS2-deficient patients. $^{**}P < 0.01$ and $^{***}P < 0.0005$.

that express the IGHV4–34 gene, because they are both associated with autoreactive B cells (35, 36). In the CMMRD patients, both the CDR3 length and the frequency of rearrangements that use the IGHV4–34 gene was normal (**Figures 2C,D**), suggesting that the selection against autoreactive B cells is normal and not affected by the MMR deficiency.

DISCUSSION

This study included 10 hitherto unpublished and 5 recently reported, unrelated patients with CMMRD. Overall, the genotypes and clinical presentations of these 15 patients, with respect to the pattern of malignancies, age-of-onset, and the spectrum of non-malignant symptoms, are in agreement with previous findings. Interestingly, two of the patients had extended skin areas with vascular malformations, a feature that has not (yet) been included in the C4CMMRD scoring system (5). Patient P10 is the second CMMRD patient who has been reported with Wilms tumor (nephroblastoma), and patient P7 is the first CMMRD patient with a reported phylloides tumor. With respect to potential genotype–phenotype correlations, it is noteworthy that two MSH6-deficient patients (P2 and P14) had a medulloblastoma. Because six of the ten previously published CMMRD cases with medulloblastoma were also MSH6-deficient, and given that only approximately 20% of CMMRD patients are due to biallelic *MSH6* mutations, this suggests an over-representation of medulloblastoma among MSH6-deficient individuals. A high-grade glioma was diagnosed in seven patients, six of whom carried biallelic *PMS2* mutations. Among the patients with *PMS2* mutations, two (P1 and P6) had been diagnosed with colorectal cancer as first malignancy only in their early twenties, supporting the notion of less penetrant forms of CMMRD especially in PMS2-deficient individuals. Consistent with a higher penetrance of biallelic *MLH1* mutations, both patients with MLH1 deficiency (P7 and P8) had T-NHL in infancy. Nevertheless, one of them (P7) survived this as well as three additional, malignant tumors. We also report here the family history and data for the first CMMRD patient (P13) with one inherited and one *de novo MSH6* mutation which is absent in both parents.

In the present study, we tested whether CMMRD is associated with a clinically relevant PID with a predominant antibody deficiency or hyper-IgM-like syndrome and a consistent impairment of B memory formation, readily detectable by clinical history taking, physical examination, and routine immunological analyses. This hypothesis was based on the known role of the MMR machinery in the immunoglobulin CSR and SHM in humans and mice (8, 9, 11, 18, 37–44), and previous findings in CMMRD patients (13–17). In contrast to earlier studies, in which MMR genes and their function were examined in selected PID patient cohorts, we took a more clinical and unbiased approach, attempting to confirm and describe or exclude a manifest and relevant PID in a series of consecutively registered patients with CMMRD. Following this systematic analysis, only inconsistent laboratory abnormalities, partially recapitulating previous human and mouse data, were found within extended routine analyses of the cellular and humoral immune system, and these lacked any clinical correlation in terms of a symptomatic PID syndrome. None of the 15 patients showed any of the clinical warning signs of PID according to the international guidelines (45, 46). Although 3 out of 12 tested patients showed mildly to moderately increased IgM concentrations (1.77–3.68 g/dl with widely varying age-specific normal ranges), their IgG and specific antibody formation was intact, and only two of them had an accompanying borderline IgG2 reduction and one, an IgA deficiency. On the other hand, 3 out of 12 patients had decreased IgM concentrations, and most showed normal IgG and IgG subclass concentrations except IgG4. Unfortunately, a complete immunological laboratory analysis was performed in only 12 out of 15 patients. Recent chemo- or immunosuppressive therapy prevented the laboratory analyses in three patients, and the extended serological data could not be obtained from the patient's local hospital in one patient. Although hyper-IgM syndrome cannot be excluded on mere humoral parameters, clearly, the majority of the analyzed patients did not have serological laboratory results that suggested hyper-IgM syndrome (e.g., increased IgM and simultaneously decreased IgG and/or IgA) or a symptomatic, specific antibody formation defect. The fact that IgG concentrations appeared to be reduced more frequently in adolescents and young adults (from 10 years of age and above; **Figure 1A**, upper panel) might indicate an age-dependent subclinical aggravation of the laboratory immune phenotype. By contrast, IgA concentrations were higher in these individuals than in younger patients (**Figure 1A**, center panel). These findings are too inconsistent to support, but might be in line with, the hypothesis that long-lived IgG- and/or IgA-producing plasma cells gained relevance with age in this context (9, 47). Furthermore, the number of patients is too little to confirm this observed trend of age-dependence or to suspect a genotype–phenotype correlation, especially since older patients in our study cohort included more individuals with PMS2 deficiency than individuals with other MMR deficiencies. Accordingly, a potential conclusion of a higher degree of immunodeficiency in PMS2-deficient as compared to MSH6-deficient patients, as suggested by the findings in previous studies showing that three out of nine PMS2-deficient patients were symptomatic and needed IgG substitution, as compared to none out of eight MSH6-deficient individuals (16, 17), cannot be corroborated by our data. Because our study comprised an unselected collection of patients consecutively registered with CMMRD within a given time frame, we can exclude a selection bias toward or against immunodeficiency. Also, due to the recruitment modality, an environmental or epigenetic bias is unlikely in the presented cohort. Nevertheless, despite the lack of a clinical correlate such as bacterial infections that could be ascribed to antibody deficiency in our cohort, our data confirm previous studies insofar as a proportion of CMMRD patients had immune biological abnormalities such as reduced class-switched and non-class-switched memory B cells and varying alterations in immunoglobulin subtypes, and we detected decreased numbers of class-switched $CD38^{hi}IgM^{-}$ plasmablasts in most individuals, which is together indicative for a sub-optimal germinal center reaction.

The data suggest a more redundant role for single components of the MMR system *in vivo* or for the MMR machinery as a whole in human, switched isotype, specific antibody formation, and B cell differentiation. These components could be substituted,

e.g., by the base excision repair pathway (9, 11), rather than by other DNA repair mechanisms such as the MRE11–RAD50–NBS1 complex or by ATM and NHEJ (10). The findings in this cohort of CMMRD patients are reminiscent of the immune phenotype of patients with XRCC4 deficiency. XRCC4 is a binding partner of LIG4 and component of the NHEJ pathway. XRCC4-deficient individuals show a junctional immunoglobulin diversification defect but have normal immunoglobulin concentrations and lack clinical signs of immunodeficiency (48).

Autoimmune diseases are typical findings in patients with combined immunodeficiency syndromes including CD40/CD40L deficiency (hyper-IgM syndromes type 3 and 1, respectively) and in predominant antibody formation disorders such as CVID. Furthermore, systemic lupus erythematosus (SLE) is a frequent finding in a subgroup of complement deficiencies (49) and SLE has been previously described in at least three independent MSH6-deficient patients (17, 50, 51). We detected antinuclear antibodies in two of the five tested individuals in this cohort (P6, 26 years old and P11, 7 years old), both PMS2-deficient and asymptomatic with regards to autoimmunity. Due to the relatively high prevalence of antinuclear antibody (ANA) positivity (>10%) in the general pediatric and adolescent populations (52) and the small number of patients with CMMRD who have been tested for ANA to date, these findings should not be overvalued. Nevertheless, the association between CMMRD and ANA positivity and the risk of developing SLE should be further investigated, and at least a baseline ANA screening should be considered from the age of adolescence and onward.

Mismatch repair plays an important role in the resolution of the uracil:guanine mismatches that are introduced during SHM and CSR. Although on a cellular level no differences were found in the B-cell compartment, detailed molecular analysis of B-cell receptor rearrangements derived from antigen-selected B cells showed a clear reduction in the frequency of SHM and alterations in the IGHG and IGHA subclass distribution. These effects on SHM and CSR can have multiple causes, like a defect in T:B cell interaction, or an intrinsic defect in the SHM and CSR process itself. Based on the previous studies showing that MMR has a role in SHM and CSR, it is most likely that the defect observed in the CMMRD patients in SHM and CSR is caused by an intrinsic defect in these processes. This, however, did not result in constant changes in the frequency of memory B cells, and only in 3 out of 15 patients the level of IgM was mildly to moderately increased.

Taken together with previous findings our results suggest that, although IgG2/4 subclass deficiency, IgA deficiency, or—rarely—more severe phenotypes of antibody formation, B cell class switch, maturation, and memory formation defects may be found in patients with CMMRD, they are neither constant nor obligatory diagnostic hallmarks of this syndrome and tend to lack a clinical correlate.

PATIENTS AND METHODS

Patient and Data Acquisition

Patient identification was achieved through the network of human geneticists and (pediatric) oncologists working throughout Europe and the Middle East who were informed about the study at conferences and *via* personal communication from 2014 to 2017. Most of participating physicians were partners of the consortium "Care for CMMRD (C4CMMRD)." Of the 19 patients who were originally included in the analysis as they were evaluated for CMMRD on the basis of their phenotypic and oncologic features, four had to be excluded since CMMRD was excluded. Two of these patients had a diagnosis of Lynch syndrome (each one had a heterozygous mutation in *PMS2* and *MSH2*) and two had other cancer prone conditions. Results from three patients had to be excluded from some of the analyses because they had recently received chemo- and/or B cell-depleting (anti-CD20) immune therapy (P3, P8, and P9, respectively). Importantly, in all other patients, blood sampling was undertaken before chemo-, steroid or immunosuppressive treatment or until an adequate interval after therapy, with readiness to ensure immune reconstitution, reflected in part by normal complete blood counts (not shown), monocyte, NK, and T cell subset analyses (**Table 3**), and confirmed by the physician in charge prior to lab analyses. None of the patients had received therapeutic immunoglobulins prior to inclusion and blood sampling. To obtain clinical history data with a focus on immunodeficiency, we designed a questionnaire interrogating the most relevant facts regarding the patients' histories and clinical statuses including infections according to the "extended clinical warning signs for PID" (45, 46), inpatient or intravenous antibiotic treatment, failure to thrive, signs of immune dysregulation such as autoimmunity and inflammation, etc. (Figure S1 in Supplementary Material). Extended routine immunologic laboratory data were obtained by conducting retrospective chart reviews guided by the study questionnaire and by collecting results from recommended immunological analyses that were justified by previous reports on a varying degree of humoral immunodeficiency and impaired B cell maturation in defects of CSR (16, 17, 30, 31, 53). The study was performed in compliance with current guidelines for good clinical practice and the Declaration of Helsinki with an IRB approval (29-178 ex 16/17) from the Medical University Graz (IRB00002556).

Genetic Laboratory Analyses

Standard molecular genetic testing included for all novel patients fully or partially analyzed at the Division of Human Genetics at the Medical University Innsbruck (P2, P7, P12, P13, P14, and P16) mutation analysis and concomitantly gMSI analysis of peripheral blood lymphocyte DNA as previously described by Ingham et al. (26). Increased gMSI ratios indicate biallelic mutations in *PMS2*, *MLH1*, or *MSH2*, while biallelic *MSH6* mutations escape the detection by this assay. For mutation analysis, all exonic coding and flanking intronic regions of the MMR and the EPCAM gene were enriched by using hybridization-based TruSightCancer panel (Illumina) and sequenced on a MiSeq platform (Illumina). Sequence data were analyzed with the SeqNext Software (JSI) and all variants present in ≥5% of the reads were classified according to the consensus recommendations of the American College of Medical Genetics (25) as (likely) pathogenic (denoted mutations), unclassified, or (likely) irrelevant variants. Quantitative analysis of the sequence data with respect to copy number variations (CNVs) was performed with the CNV tool of the SeqNext

Software (JSI) and in parallel with the CNV Detective Software cnMOPS (54). CMMRD patients with a variant of unknown significance (VUS; or a monoallelic mutation) in one of the MMR genes were additionally analyzed by direct cDNA-sequencing of the entire coding sequence to assess for potential splice effects of VUS and/or to uncover/exclude (other) mutations that escaped the detection of massive parallel sequencing (55). This analysis was so far not possible for patient P7. For the detection of *PMS2* CNVs multiplex ligation-dependent probe amplification (MLPA) analysis using the SALSA MLPA-Kit P008-B1 (MRC Holland) was performed according to Wernstedt et al. (56).

Mutation analysis for patients P8, P9, and P10 was performed at the Hereditary Cancer Laboratory at the University Hospital Doce de Octubre (Madrid, Spain). Here, all exonic coding and flanking intronic regions of the MMR genes were amplified using a custom designed primer panel with Ion AmpliSeq Library Kit 2.0 reagent (ThermoFisher) and sequenced on an Ion PGM System (ThermoFisher). Data were analyzed using Ion Reporter software (ThermoFisher).

All detected mutations were confirmed in a second DNA-sample that was extracted from an independently extracted blood sample. Nucleotide positions were numbered according to the recommendations of the Human Genome Variation Society (57) with the A of the start codon ATG in exon 1 representing the nucleotide position c.1 using the reference numbers NM_000249.3 for *MLH1*, NM_000251.2 for *MSH2*, NM_000179.2 for *MSH6*, and NM_000535.5 for *PMS*2.

Immunologic Laboratory Analyses

In addition to routine chemistry and clinical immunology laboratory analyses, special immunologic analyses were performed centrally in Graz, Austria, which included flow cytometry performed on a Cytomics FC500 flow cytometer (Beckman Coulter, Brea, Calif) with a panel of mAbs from Beckman Coulter (Vienna, Austria), Becton Dickinson (Vienna, Austria), Dako (Glostrup, Denmark), and Miltenyi Biotech (Vienna, Austria and Bergisch Gladbach, Germany). Analysis of TCR V beta diversity (spectratyping) was performed as follows: RNA from enriched subsets was extracted using RNeasy Protect Mini Kit (Qiagen, Hilden, Germany) following manufacturer instructions. The amount of RNA was determined with Eppendorf Biophotometer plus (Eppendorf, Hamburg, Germany). Finally, a concentration of 1 µg RNA was used for reverse transcription with a First Strand cDNA Synthesis Kit for RT-PCR AMV (Roche, Vienna, Austria), carried out following manufacturer instructions. cDNA was diluted 1:5 for PCR using AmpliTaq Gold™ DNA Polymerase (Applied Biosystems, Vienna, Austria), 1× PCR Gold Buffer (Applied Biosystems, Vienna, Austria), 2.5 mM $MgCl_2$ (Applied Biosystems, Vienna, Austria), 0.4 mM dNTP Polymerization Mix (GE Healthcare, Vienna, Austria), 0.5 µM TCR C β 5′FAM labeled primer (Ingenetix, Vienna, Austria), and 0.5 µM unlabeled TCR V β primer (Ingenetix, Vienna, Austria) according to Monteiro et al. (58). This resulted in 25 reactions per sample. Cycle conditions were a denaturation step at 94°C for 6 min, 35 cycles at 94°C for 1 min each, 59°C for 1 min, and 72°C for 1 min, with a final annealing step at 72°C for 7 min. After amplification, 1 µl of PCR-product was supplemented with 0.5 µl of GeneSCan™-500 ROX™ Size Standard (Applied Biosystems, Vienna, Austria) and 12 µl HI-DI Formamide (Applied Biosystems, Vienna, Austria). Electrophoresis was performed with a 3130 Genetic Analyzer (Applied Biosystems, Vienna Austria) and 3130 Data Collection Software. Analyses were conducted using the GeneScan® Software (Applied Biosystems, Vienna Austria). Calculations included peak count (Complexity score) and single peak area as percentages of whole peak area.

Next Generation Sequencing of the B-Cell Repertoire

Peripheral blood mononuclear cells were isolated from peripheral blood using Ficoll, and mRNA was isolated using the GenElute Mammalian total RNA miniprep kit from Sigma Aldrich (St. Louis, MO, USA). cDNA was created from 2 µg RNA using the Superscript II reverse transcriptase kit from Invitrogen (Paisley, UK). IGH transcripts were amplified in a multiplex PCR using the forward VH1-6 FR1 (BIOMED-2) primers and either the CgCH or the IGHA reverse primer which were adapted with a multiplex identifier sequence to be able to multiplex the PCR products (59–61). PCR products were purified by gel extraction (Qiagen, Valencia, CA, USA) and Agencourt AMPure XP beads (Beckman Coulter, Fullerton, CA, USA). The PCR products were sequenced on the 454 GS junior using the Lib-A V2 kit (Roche). The raw data were demultiplexed, 40 nt trimmed at the 5′ and 3′ side to remove the primer sequence, and converted to fasta files using ARGalaxy (62). The fasta files were uploaded in IMGT High-V-Quest (version 1.5.6) (63) for alignment with the reference sequences. Subsequently, the IMGT output files were analyzed using ARGalaxy (62). To obtain unique rearrangements and reduce the presence of errors in the sequences, only sequences present two or more times (based on CDR1–CDR3 nucleotide sequence) were included once in the analysis. In addition, incomplete sequences or sequences containing an ambiguous "*n*" base were excluded. Since the data from the CMMRD patients were very clonal (determined using Change-O) (64), we only included one sequence per clone in the analysis. Samples that contained less than 45 unique IGH rearrangements were excluded from the analysis. Data from the CMMRD patients was compared to 10 HC (6–22 years of age), which were previously published (36). Details on the number of sequences obtained after filtering can be found in Table S2 in Supplementary Material.

Data Presentation

Due to the small patient number, only a descriptive data analysis was performed. Figures were designed using Prism 7.0c (GraphPad software, La Jolla, CA, USA).

ETHICS STATEMENT

The study was performed in compliance with current guidelines for good clinical practice and the Declaration of Helsinki with an IRB approval (29-178 ex 16/17) from the Medical University Graz (IRB00002556). Extended routine immunologic laboratory data were obtained by conducting retrospective chart reviews guided by the study questionnaire and by collecting results from

recommended immunological analyses that were justified by previous reports on a varying degree of humoral immunodeficiency and impaired B cell maturation in defects of class-switch recombination (16, 17, 30, 31, 53).

AUTHOR CONTRIBUTIONS

MS and KW designed the study. KW, CC, and LB organized patient identification and study inclusion. VT wrote the first draft of the manuscript and the tables; MS designed the figures. MS and KW edited the final version of the manuscript, which all authors approved to. MS, KW, and VT organized the documentation of clinical and laboratory data and the transfer of blood samples to (reference) laboratories. MS, AR, WS, MK, HI, and MB designed and performed immunological laboratory analyses that were not conducted locally. KW, SW, and JZ organized and performed molecular genetic tests that were not performed locally and reference analyses. DR, CC, TR, DI, HI, HB, MN, MS, DJ-L, IR, AA, CB, ND-P, LA, and LB cared for the patients, recorded their medical histories, collected laboratory data, and are active partners of the "Care 4 CMMRD" network.

ACKNOWLEDGMENTS

The authors highly appreciate the cooperation of participating patients and their families, as well as of medical staff at the points of care, who helped us to obtain clinical and laboratory data. Biomedical assistants Barbara Egner and Barbara Leopold (Graz) are thanked for conducting extended routine laboratory analyses, and members of the C4CMMRD consortium are thanked for their critical discussion of the data. MS and the *Research Unit for Pediatric Hematology and Immunology* are in part financed by the Styrian Children's Cancer Aid foundation (*Steirische Kinderkrebshilfe*). Furthermore, the authors thank Dr. Sara Crockett for scientific editing. The research for this manuscript was (in part) performed by a grant from the Jeffrey Modell Foundation Translation Research program (to MB).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01506/full#supplementary-material.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 24 May 2018
doi: 10.3389/fimmu.2018.01149

A Tumor Profile in Primary Immune Deficiencies Challenges the Cancer Immune Surveillance Concept

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Under the concept of cancer immune surveillance, individuals with primary immune deficiencies would be expected to develop many more malignancies and show an excess of all types of cancers, compared to people with a normal immune system. A review of the nine most frequent and best-documented human conditions with primary immune deficiency reveals a 1.6- to 2.3-fold global increase of cancer in the largest epidemiological studies. However, the spectrum of cancer types with higher frequencies is narrow, limited mainly to lymphoma, digestive tract cancers, and virus-induced cancers. Increased lymphoma is also reported in animal models of immune deficiency. Overstimulation of leukocytes, chronic inflammation, and viruses explain this tumor profile. This raises the question of cancers being foreign organisms or tissues. Organisms, such as bacteria, viruses, and parasites as well as non-compatible grafts are seen as foreign (non-self) and identified and destroyed or rejected by the body (self). As cancer cells rarely show strong (and unique) surface antibodies, their recognition and elimination by the immune system is theoretically questionable, challenging the immune surveillance concept. In the neonatal period, the immune system is weak, but spontaneous regression and good outcomes occur for some cancers, suggesting that non-immune factors are effective in controlling cancer. The idea of cancer as a group of cells that must be destroyed and eliminated appears instead as a legacy of methods and paradigms in microbiological medicine. As an alternative approach, cancer cells could be considered part of the body and could be controlled by an embryonic and neonatal environment.

Keywords: primary immune deficiencies, immune surveillance, cancer incidence, tumor profile, cancer, lymphoma, gastrointestinal cancer, virus-induced cancer

OPEN ACCESS

Edited by:
Andrew R. Gennery, Newcastle University, United Kingdom

Reviewed by:
Shigeaki Nonoyama, National Defense Medical College, Japan
Tomohiro Morio, Tokyo Medical and Dental University, Japan

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Specialty section:
This article was submitted to Primary Immunodeficiencies, a section of the journal Frontiers in Immunology

Received: *11 January 2018*
Accepted: *07 May 2018*
Published: *24 May 2018*

Citation:
Satgé D (2018) A Tumor Profile in Primary Immune Deficiencies Challenges the Cancer Immune Surveillance Concept. Front. Immunol. 9:1149. doi: 10.3389/fimmu.2018.01149

INTRODUCTION

According to the concept of immune surveillance proposed by Burnet (1), the immune system identifies and destroys nascent cancers (2, 3). This model is based on the idea that cancers, which are produced by an organism's cells, present specific antigens recognized by the immune system. Accordingly, compared to normal individuals, individuals with weakened or deficient immune systems should develop an excess of all types of cancers, since white blood cells in immune-compromised patients are less able to recognize and destroy malignant cells.

In this study, the concept of immune surveillance was examined in nine of the most well-known and well-characterized primary immune deficiencies (PIDs) chosen from 300 conditions

each with a simple gene defect in the context of immune impairment (4). The immune surveillance concept predicts a global increase in cancer in these PIDs, but instead these conditions associated with a narrow spectrum of particular malignancies. This suggests that several immune processes, such as overstimulation of immune cells, viral infections, reactions to impaired bone marrow tissue, and chronic inflammation, influence cancer in PIDs. The particular distribution of cancers in primary immune-deficient humans and in murine models challenges the concept of immune surveillance as the major cause of increased cancer frequency in these diseases. This observation raises the question of the strategic place of immune mechanisms in diseases that do not arise from intrinsically foreign tissue.

MATERIALS AND METHODS

Nine PIDs were selected from 300 immune deficiency diseases with single gene defects (4), on the basis of their frequency and sufficiently documented cancer occurrences. Less frequent conditions were included if significantly associated with cancers. The diseases were chosen from the most frequently cited conditions in recent cancer and PID epidemiological studies (4–7) and from the most frequently cited conditions in PID and cancer reviews (8–11).

We excluded diseases deemed not well defined for a sufficiently long period (preventing assessment of a number of people with precisely the same condition) and diseases with a chromosome breakage-DNA repair defect (to prevent bias due to their effects on oncogenesis). The following conditions were retained, selective IgA deficiency, common variable immune deficiency (CVID), X-linked agammaglobulinemia, X-linked hyper-IgM syndrome, cartilage-hair hypoplasia, Warts,-hypogammaglobulinemia,-infections, and myelokathexis (WHIM) syndrome, severe congenital neutropenia, and natural killer (NK) cell deficiency. For each condition, a search in PubMed was conducted, cross-searching the name of the disease with the words "cancer" and "malignancies." Articles were selected if their title indicated information on health problems, cancer occurrence, and cancer frequency, or the functional or structural impact of the disease on tissues. Articles reporting *in vitro* experimental data were excluded.

RESULTS

From the 1,112 identified articles, 223 abstracts were selected for reading, 152 articles were read in full, and 80 articles and book chapters were included in the bibliography.

Table 1 displays cancer distributions in the nine chosen PID conditions (4–6, 9, 10, 12–44).

TABLE 1 | Frequency of nine inherited diseases with primary immune deficiency and their cancer risk.

Diseases	Disease frequency	Global cancer risk (fold increase) and/or prevalence (%)	Over-represented cancers	Comments
Selective IgA deficiency (5, 12–15)	1/143–1/965	×1.31	Gastric cancers (×1.64–5.4) Lymphoma × (1.68–2.6)	Lung cancer decreased
CVID (4–6, 16, 17)	1/10,000–100,000	×1.19–3	Lymphoma (×8.4–18.6) Gastric carcinoma (×5–16.2)	No virus-related cancers[a] Decrease frequency of these cancers usual in children and adults
X-linked agammaglobulinemia (10, 18–21)	1/200,000	Increased	Gastric carcinoma Intestinal carcinoma	Chronic infections *Helicobacter pylori*
X-linked hyper-IgM syndrome (22–25)	1/1,000,000	Increased	Liver carcinoma Gallbladder carcinoma Pancreas carcinoma	Frequent cholecystitis Hepatitis
Wiskott–Aldrich syndrome (26–29)	1/10,000–10,000	Increased	Lymphoma CNS lymphoma	Virus-related cancers (EBV infections)
Cartilage-hair hypoplasia (30–33)	NA[b]	×7 45% at 65 years	Lymphoma (×90) Skin cancer (×33)	
WHIM syndrome (9, 34, 37)	1/4,000,000	30% at 40 years	Lymphoma Genital and ENT squamous cell carcinoma	Viral infections
Severe congenital neutropenia (38–40)	1/100,000	31%	Acute myeloid leukemia (AML) and myelodysplastic syndrome	Bone marrow failure G-CSF treatment
Natural killer cell deficiency (41, 44)	NA	Increased	Acute myeloid leukemia, carcinoma of genital organs and skin cancer, and breast carcinoma	Myelodysplasia, HPV, and EBV infections, GATA2 interacts with GATA3[c]

NA, not available; EBV, Epstein–Barr virus; HPV, human papilloma virus; CVID, common variable immune deficiency; ENT, ear nose and throat.

[a]*EBV, HPV.*

[b]*1/1, 000–2,000 in Amish people in USA and 1/23,000 in Finland, very rare in other countries.*

[c]*GATA 3 interacts with GATA 2 which is involved in the growth control of breast cancer.*

Cancer Frequency in PIDs

Based on the concept of immune surveillance, people with PIDs who are less able to eliminate cancer at its early stages should develop an excess of malignancies, particularly an excess of the most frequent cancers, leukemia, brain tumors, lymphomas, embryonic tumors, and germ cell tumors in childhood and colon, breast, lung, and prostate cancers in adults. The nine PIDs chosen for this review, based on their frequency and documented cancer occurrence, account for a large proportion of all PID patients. For instance, seven of the nine diseases per the United States Immune Deficiency NETwork accounted for 56% (2,047/3,658) of enrolled conditions (4). These diseases are also the most frequent conditions cited in reviews on PID (8, 10, 11), if we exclude DNA repair defects. Thus, the chosen conditions are truly representative for analysis of PID-associated cancer frequency. A recently published series of studies of patients with primary immune deficiency, which included large cohorts and a comparison group, from either Australia (6), the Netherlands (7), or the USA (4), indicate that PID causes a significant increased risk of all cancers of 1.6-, 2.3-, or 1.42-fold, respectively, compared to the general population. This is much lower than the approximately 10,000-fold (45) increase estimated in past reports.

Cancer Distribution in PIDs

Despite this global increased risk, the PID-cancer profile observed in the two largest studies, together including 4,790 patients, contained strikingly few cancer types. The Australian patients presented an excess of non-Hodgkin lymphoma (SIR 8.82), leukemia (SIR 5.36), and stomach cancer (SIR 6.10). Tumors were predominantly associated with antibody deficiencies (6). In the US study, the authors observed a 10-fold or 8.340-fold excess of lymphoma for men and women, respectively, but no increase for the most frequent common solid malignancies: prostate, lung, breast, and colon cancer (4).

A closer look at cancers observed in the nine selected conditions using epidemiological studies as well as institutional experience, series, and case reports allows for more precise tumor profiling. There is a global tumor profile for the nine conditions, and concurrently, a tumor profile for each condition. An excess of lymphoma occurred in the most diverse set of conditions, associating with five PIDs; however, liver and biliary tract cancer only associated with one. The majority of over-represented cancers were associated with either two or three conditions. Each condition in turn was associated with a narrow, sometimes very narrow, spectrum of cancers. For example, severe congenital neutropenia associated only with leukemia (40), and X-linked agammaglobulinemia associated only with gastric and intestinal carcinoma (19).

On the other hand, some tumors were rare in some of these diseases. In selective IgA deficiency, deaths from lung cancer were more than twofold less frequent than in the general population (14). In the whole group of PIDs, the most frequent cancers in the USA—breast, lung, and colon cancer—had a lower incidence of 0.41, 0.48, and 0.19, respectively, for women; for men, colon and lung cancer incidences were 1 and 0.62, respectively, compared to the general population. Interestingly, these are the most frequent epithelial malignancies, suggesting an underrepresentation of these types of cancers in the largest available study conducted (4). In CVID, lung, breast, and colon cancer had a lower incidence compared to that of the general population (4). Thus, the incidence of most solid tumors in PIDs is not significantly higher than in the general population. This is well shown by these epidemiological studies which took into account the reduced life expectancy (due to infection-associated sequelae or autoimmunity), for instance in CIVD, Wiskott–Aldrich syndrome, X-linked agammaglobulinemia, and X-linked hyper IgM syndrome, which calculated age-adjusted cancer incidence (4) and adjusted evaluation for 5-year age group (6). Some PIDs, outside of the nine selected, are not associated with malignancies. In contrast, they appear to have global decreased cancer incidences. This is the case for X-linked chronic granulomatous disease where no cancer was registered among 326 patients (4). Globally, cancer distribution in PID does not seem to result from a unique general mechanism applying to every disease. This does not fit with a defective immune surveillance model. The cancer distribution rather suggests that different mechanisms are involved, leading to special and sometimes unique tumor profiles.

Possible Mechanisms

The largest and most recent study of patients with PID-associated cancers points to a restricted role of the immune system in protection from cancer (4).

Lymphoma

Lymphoma is unquestionably the most over-represented malignancy in PID, with an approximate 8- to 13-fold increased risk compared to that of the general population (4, 6, 7). These are mainly B cell lymphoma occurring in extranodal sites, often in patients with preceding polyclonal lymphocyte infiltrations (11). As emphasized by Hauck et al., in PIDs, cancer (lymphoma) affects cells altered by the deficiency itself (lymphocytes) (46). This increase in lymphoma has been linked to recurrent and persistent viral and bacterial infections, which characterize a great majority of PIDs sometimes in an antigen-specific manner (32, 47, 48). The overstimulated defective lymphoid tissue leads to lymphoid hyperplasia observed in 50% of patients with CVID. They show lympho-adenopathies and splenomegaly (17), a state favoring lymphoma transformation.

Leukemia

An increased incidence of leukemia, presenting as abnormal bone marrow, is observed in some PIDs. In severe congenital neutropenia, patients experience bone marrow failure (40). The bone marrow of NK cell-deficient patients is characterized by multilineage dysplasia (44). In these conditions, hematopoietic lineages could be the target of chronic overstimulation to counterbalance the impaired production of white cells. Elevated intrinsic G-CSF observed in severe congenital neutropenia also increases the risk for acute myeloid leukemia and myelodysplastic syndrome (MDS), in addition to the treatment with G-CSF (38). Reviewing chronic myelocytic leukemia (CML) in immune deficiency, Gale and Opelz found no global excess of CML in patients

with immune deficiency. They suggested that failure of immune suppression *per se* does not explain most cases of CML and conclude that immune surveillance does no contribute significantly toward preventing new cases of CML (49). The role of immune surveillance could as well be questioned for other PID-associated leukemias.

Digestive Tract Cancers

Stomach cancer is over-represented in epidemiological studies on malignancies in PID, especially in humoral defects such as CVID and selective IgA deficiency (4–6). In conditions with humoral defects, the impaired immune system permits recurring *Helicobacter pylori* gastric colonization, which leads to gastritis, and, for some patients, to severe atrophy and intestinal metaplasia which are two major risk factors for gastric adenocarcinoma (16, 17). An increased incidence of intestinal cancer has been found in patients with X-linked agammaglobulinemia. In this disease, a team observed inflammatory bowel diseases and infectious enteritis, which are risk factors for cancer, for 11.3% of patients (21). Patients with X-linked hyper-IgM syndrome who present frequent protracted diarrhea also develop more colon cancer (23). Additionally, patients with X-linked hyper-IgM syndrome are much more prone to hepatitis and cholangitis, mainly linked to persistent infection of *Cryptosporidium Parvum*, and are anticipated to develop cancer in organs altered by chronic inflammation and cirrhosis (22).

Viral Infections

Cancers related to oncogenic viral infections account for 12% of cancers in humans and an even higher percentage in patients with primary or acquired immune deficiencies (9, 50). Cancers of the genital, anal, and oropharyngeal regions and of the skin (50, 51) are more frequent in those PIDs that are vulnerable to human papilloma virus (HPV) infections, particularly patients with NK cell deficiency who develop an excess of cervix-uterine HPV-related dysplasia and cancer as well as oropharyngeal squamous cell carcinomas and skin carcinomas (44). Genital and oral HPV-related squamous cell carcinomas are also observed in patients with WHIM syndrome (35, 36). Epstein–Barr virus (EBV) infections cause lymphoproliferative diseases and lymphoma, which are also observed in WHIM syndrome (34, 37), and in cartilage–hair hypoplasia (32). Additionally, EBV causes EBV-smooth muscle tumors, which have been reported in liver, adrenals, and smooth muscle of patients with NK cell deficiencies (42, 44). Significantly, in PIDs there is an excess of malignancies stemming from organs targeted by viruses but no excess of cancers in other tissues.

The molecular pathogenesis of PIDs associated with malignancy cannot be developed for each clinical condition included in **Table 1**; this is beyond the scope of the current article. NK cell deficiency, for example, has been associated with mutations in five genes, *GATA2*, *MCM4*, *RTLLE1*, *IRF8*, and *FCGR3A* (51). At the same time, *GATA2* deficiency has been linked to four clinical syndromes: NK cell deficiency, but also monocytopenia and mycobacterial infection syndrome, familial MDS and Emberger syndrome (44). *GATA2* is a transcription factor highly expressed in immature hematopoietic cells. The gene is necessary for survival and renewal of hematopoietic cells. It is critical for genesis and function of hematopoietic stem cells and thus blood cell lineage (44). Myeloid malignancy in *GATA2* deficiency is related to differentiation arrest and in part to a novel function of the mutated gene. Currently, it remains unclear how germline *GATA2* loss-of-function mutations result in myeloid neoplasms (52). In this context the hypothesis of an overstimulation of myeloid cells could be suggested.

DISCUSSION

The Cancer Distribution in Animal Models of Immune Deficiency Is Similar to That of PID Patients

As animal models are considered to support the concept of immune surveillance, it is interesting to compare the spontaneous tumor occurrence in immune deficient mice to that in PID patients. An early study showed no difference in the incidence of spontaneous lung adenoma between athymic-nude mice, which are deficient for T cells, and immunocompetent mice (53). More recent works using immunodeficient mouse strains with defects in perforin, interferon gamma, recombination activating gene (*Rag2*), signal transducer, and activator of transcription 1 (*Stat1*), and other genes reported an increased cancer incidence; however, the distribution of cancer resembled that observed in humans with PID. In a review of 11 strains, eight showed an excess of lymphoma and one an excess of plasmacytoma, either alone (6) or associated with other malignancies (3). Only two strains showed an excess of carcinomas and no excess of lymphoma (2). One team who studied spontaneous tumors in mice lacking the tumor necrosis factor (TNF)-apoptosis-inducing ligand (TRAIL) concluded that TRAIL-R did not protect against mammary cancers or against colon cancers, but did protect against lymphoid malignancies, which affected more than 25% of the deficient mice (54). An ongoing process of immune activation through IL6 upregulation has been proposed to explain plasma cell hyperplasia followed by plasmacytoma in mice lacking the interleukin-12 receptor Beta2 (55).

The literature describing mouse models of immune deficiency report increased incidence of lymphoma, while carcinomas are rare, as seen in human PID (see above). Notably, for the two mouse strains with only carcinomas, the tumor distribution is quite narrow. Mice lacking *Rag2* developed mainly intestinal adenomas and colon carcinomas (56), which usually occur following intestinal infection (57). Mice lacking both *Rag2* and *Stat1* developed an excess of colon cancer and breast carcinomas (56). As *STAT1* is involved in breast cancer pathways (58), the increase in breast cancer is likely due to STAT1's role in tumorigenesis instead of immune surveillance. Similarly, a mouse model with a deficiency of granulocyte-macrophage colony stimulating factor exhibited an excess of both lymphoma and solid tumors. Interestingly, mice on antimicrobial therapy developed neither lymphoma nor solid malignancies compared to non-treated mice ($p < 0.001$). As the treated mice showed a marked reduction of both chronic infection and cancers,

the authors proposed a role for chronic infection in the onset of malignancies (59). Thus, carcinomas observed in immune-deficient mouse strains are likely the consequence of specific processes affected by the deficient gene, for example, tissue damage secondary to unresolved inflammation, instead of a general decrease in immune surveillance, which should cause an excess of many types of carcinomas.

Additional Observations Challenging Immune Surveillance

Other clinical observations indicate that immune defects increase the incidence of lymphoma instead of all cancers and cast doubt on the protective role of the immune system in cancer. First, the excess of lymphoma (but not other cancers) observed in Nijmegen breakage syndrome is associated with chronic stimulation of defective lymphocytes (60). In fact, only conditions with a chromosome breakage-DNA repair defect presenting with an immune deficiency, i.e., ataxia telangiectasia, Nijmegen breakage syndrome, and Bloom syndrome, develop an excess of lymphoma. In contrast, conditions with a chromosome breakage-DNA repair defect without immune deficiency, such as xeroderma pigmentosum, Fanconi anemia, Werner syndrome, and Rothmund–Thomson syndrome, develop other types of cancers. Second, although malignant melanoma, renal cell carcinoma, and non-small cell lung carcinoma have historically been considered as proof of immune therapy principles, these two cancers are not found more frequently in PIDs than in the general population (46). This is particularly clear in the nine conditions presented in **Table 1**. Third, although the immune system is weak and immature in neonates and young infants (61), there is no increased incidence of cancer at this period (62). In fact, a notable number of various neonatal cancers, some very aggressive, e.g., neuroblastoma, leukemia, and pontine glioma, may undergo spontaneous regression (62–64). Furthermore, in neonates, a cure for sarcoma is possible despite incomplete tumor resection (65). For this reason, the neonatal period was dubbed, three decades ago, the "oncogenic grace period" (66). Fourth, although people with Down syndrome have mildly to moderately weakened immune systems favoring pneumonias and opportunistic infections (67), adults with Down syndrome experience half the burden of solid tumors observed in the general population, notably showing a reduced frequency in the most common cancers: breast, prostate, and lung (68, 69).

Is Cancer a Foreign Organism?

As the immune system's role is to identify and destroy foreign organisms, bacteria, viruses, and parasites, the concept of cancer immune surveillance presupposes that cancer tissues are foreign (non-self) to the body of the patient (self). In this theoretical framework, cancer would be a foreign biological process characterized by genetic modifications. However, it is a genetic modification (gene or chromosome mutation) sufficient to consider a tissue foreign to the body? We should then consider that a person who has a constitutional genetic mosaicism as partly composed of "non-self" tissues. For instance, a woman with mosaic Turner syndrome with 20% 46, XX cells and 80% 45, X0 could be considered to be composed of 80% foreign tissue. A genetic point of view is really debatable for considering that a mutated tissue is foreign to the body. Even if they are genetically modified and even if they become uncontrolled by the integrating and coordinating system of the body, cancer tissues remain composed of body cells.

If cancers are recognized as foreign structures, the body would naturally reject them as it does for bacteria, viruses, parasites, and non-compatible grafts. Foreign tissues induce antibody production by the immune system, but tumor-specific antigens are not common in human tumors. Nonetheless, mouse models show that antigen expression is important for the process of recognizing and eliminating cancer cells (70). However, tumor reactive antibodies mirror tissue damage and reflect a response to tissue necrosis, rather than targeting a specific cell type (71). The observation that cancer cells, excepting those of virus-induced malignancies (70, 71), do not usually harbor strong specific antigens which suggests that cancers are not considered foreign by the body. Cancer could as well be seen as a diseased tissue. Some researchers propose that cancers can be considered as abnormal organs that develop in a manner similar to that of normal organs (72). Moreover, specific immune factors, such as a given major cytokine-like interferon gamma, or major immune effector cells, such as CD4 T lymphocytes, have both pro- and anti-tumoral effects (73, 74). These and other puzzling observations (75) suggest that the immune system has a complex reaction to tumor cells and does not always and systematically protect against malignancies.

Approaching cancer as a foreign organism implies that the main tumor and any tumor cells must be destroyed to cure the patient, similar to treatments aimed at killing bacteria, viruses, and parasites. This point of view seems a legacy of concepts and paradigms developed during the nineteenth and twentieth centuries with the progress of microbial medicine (76). We should not remain constrained by a framework that has been very effective for microbiology but narrows our understanding of oncogenesis. It is time to open our minds to other currently neglected approaches. We may question how best to fight against a disease composed of our own cells, which have escaped bodily control and gone awry. We may also question current cancer treatments that destroy normal cells likely because cancer is not so different from normal tissue.

Cancer could also be considered as a developmental disease, leading us to search for the processes that protect against malignancy during the embryonic and neonatal periods. The observation that embryonic carcinoma cells can incorporate into a normal blastocyst and produce a normal mouse composed of a mixture of normal cells (the host) and normalized cancer cells (the incorporated teratocarcinoma) (77) and that cancer cells in the presence of normal tissue may reverse to a normal phenotype (78) enlarges our perspective on research for cancer control and treatment. While the present study does not discuss the effectiveness of immune therapy and the progresses in treatment (79), we suggest that immune surveillance is probably not, in natural conditions, as effective as expected.

This review has some limitations, since only nine conditions were included. However, these conditions account for more than

half of cancers reported in the largest epidemiological study on cancer in PIDs (4). Epidemiological studies are high-quality works, where the biological diagnosis is difficult to challenge. Isolated case reports and series are well documented, published in high-quality journals. Thus, repetitive errors on tumor diagnosis are unlikely.

CONCLUSION

From the presented data we may draw the following conclusions: (1) The increased risk in frequency of cancer in PIDs is moderate, nearly twofold, much less than formally estimated. (2) The spectrum of cancer in PIDs is narrow. (3) Over-represented malignancies are mainly lymphoma, digestive tract tumors, and cutaneous-mucus carcinoma, which can be explained by an overstimulation of lymphocytes, chronic digestive infection, and cutaneous-mucus viral infections. (4) Animal models of immune deficiency develop a similar spectrum of malignancies as observed in human. Thus, since a defect of immune surveillance should theoretically favor all types of cancers, the data raise the hypothesis that immune surveillance does not play a major role in the increase of cancer in PIDs. While analyzing proposed mechanisms of oncogenesis and unmasking discrepancies with the immune surveillance model, three aspects of oncogenesis were emphasized, the overstimulation of a tissue leading to cancer, the role of tissue inflammation and fibrosis, and the possibility of cancer control by the body as it is observed during the neonatal period.

From an immediate point of view, it is disappointing to find that the role of the immune system is not, in a typical condition (i.e., non therapeutic conditions, particularly when excluding CAR T-cell therapy and immune checkpoint therapy), as important as expected. From another point of view, it is very exciting news implying that each PID with its specific excess or decrease of cancers has something to tell us about precise aspects of carcinogenesis. It is thus necessary to precisely identify the cancer types that occur and to articulate these data to functional and tissue alterations linked to the disease. These all provide many pathways for understanding cancer under precise conditions and possibly to fight against neoplasia.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

ACKNOWLEDGMENTS

A grant from the Fondation Jérôme Lejeune supports the study. Christiane Satgé is acknowledged for the preparation of the manuscript.

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