frontiers in Immunology

REVIEW
published: 19 April 2018
doi: 10.3389/fimmu.2018.00800

Improving the Efficiency of Vγ9Vδ2 T-Cell Immunotherapy in Cancer

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Increasing immunological knowledge and advances in techniques lay the ground for more efficient and broader application of immunotherapies. gamma delta (γδ) T-cells possess multiple favorable anti-tumor characteristics, making them promising candidates to be used in cellular and combination therapies of cancer. They recognize malignant cells, infiltrate tumors, and depict strong cytotoxic and pro-inflammatory activity. Here, we focus on human Vγ9Vδ2 T-cells, the most abundant γδ T-cell subpopulation in the blood, which are able to inhibit cancer progression in various models *in vitro* and *in vivo*. For therapeutic use they can be cultured and manipulated *ex vivo* and in the following adoptively transferred to patients, as well as directly stimulated to propagate *in vivo*. In clinical studies, Vγ9Vδ2 T-cells repeatedly demonstrated a low toxicity profile but hitherto only the modest therapeutic efficacy. This review provides a comprehensive summary of established and newer strategies for the enhancement of Vγ9Vδ2 T-cell anti-tumor functions. We discuss data of studies exploring methods for the sensitization of malignant cells, the improvement of recognition mechanisms and cytotoxic activity of Vγ9Vδ2 T-cells. Main aspects are the tumor cell metabolism, antibody-dependent cell-mediated cytotoxicity, antibody constructs, as well as activating and inhibitory receptors like NKG2D and immune checkpoint molecules. Several concepts show promising results *in vitro*, now awaiting translation to *in vivo* models and clinical studies. Given the array of research and encouraging findings in this area, this review aims at optimizing future investigations, specifically targeting the unanswered questions.

Keywords: gamma delta T-cell, cancer immunotherapy, tumor metabolism, ADCC, NKG2D, immune checkpoints, programmed cell death protein 1, vascular endothelial growth factor

OPEN ACCESS

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Specialty section:
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

Received: *31 January 2018*
Accepted: *03 April 2018*
Published: *19 April 2018*

Citation:
Hoeres T, Smetak M, Pretscher D and Wilhelm M (2018) Improving the Efficiency of Vγ9Vδ2 T-Cell Immunotherapy in Cancer. Front. Immunol. 9:800. doi: 10.3389/fimmu.2018.00800

INTRODUCTION

Following the discovery in the 1980s, gamma delta (γδ) T-cells have become increasingly recognized as important players in natural host defense against infections and malignancies. Early evidence of an anti-tumor functionality of γδ T-cells came from the experiments in mice (1) and it is now well established (2). In humans, γδ T-cells can be found in various cancer tissue samples [e.g., melanoma (3, 4) and epithelial tumors (5–11)]. More recently, analysis of microarray data also described patterns of γδ T-cells in a large collection of malignancies (12) and a prior extensive gene expression study demonstrated that γδ T-cell infiltration into tumors represents a positive prognostic marker in many types of cancer (13). Offering some hints for a functional role in tumor rejection, γδ T-cell infiltration in melanoma, colorectal cancer, and lung tumors were found to be associated with lower stage and lack in metastasis. Additionally, γδ T-cells extracted from such cancer tissues were able to kill malignant cells *in vitro* (4, 14, 15). In cancer patients, γδ T-cells were also repeatedly found reduced or defective and depicted a diminished proliferative capacity (16–18)

and exhaustion (19–23). Patients with higher γδ T-cell count following allogenic stem cell transplantation for acute leukemia had a significant survival advantage (24). In connection with their suspected function in natural tumor defense, the utilization of γδ T-cells has become a promising concept in the field of cancer immunotherapy.

Definition

γδ T-cells express variables Vγ and Vδ chains (25, 26) as part of a T-cell receptor (TCR) complex that is structurally and functionally distinctive from the major histocompatibility complex (MHC) binding TCR of αβ T-cells (27). In humans, it is feasible to further divide γδ T-cells into "Vδ2" and "non-Vδ2 cells," the latter consisting of mostly Vδ1- and rarely Vδ3- or Vδ5-chain expressing cells. Despite unrestricted and the theoretically high combinatory diversity (28), the Vδ2 chain is found preferentially paired with the Vγ9 chain (29). These Vγ9Vδ2 T-cells account for approximately 5% of peripheral blood T-cells, representing the dominant γδ T-cell subpopulation in this compartment in healthy human adults (30). Interestingly, the preferential appearance of Vγ9- and Vδ2-chains develops in the fetus (31), but the overall clonal repertoire of blood γδ T-cells is further contracting after birth (32). The latter is probably a response to a uniform stimulus, like a ubiquitous pathogen or conserved stress molecule (33).

Functional Aspects

Genetic and functional studies indicate that γδ T-cells have developed and act as an intermediate between the innate and the adaptive immune system. Features representative of an innate phenotype is their ability to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) and phagocytosis and to rapidly react toward pathogen-specific antigens without prior differentiation or expansion (28). Notably, the gene expression signature of Vγ9Vδ2 T-cells was characterized as a hybrid of αβ and NK-cells (34). Typical characteristics of the adaptive immune system, found in γδ T-cells, are their capabilities for somatic recombination of receptor genes, memory formation (35), and professional antigen presentation (36). Unlike αβ T-cells, γδ T-cells respond directly to proteins and non-peptide antigens (37) and are therefore not MHC restricted (38). At least some γδ T-cell specific antigens display evolutionary conserved molecular patterns, found in microbial pathogens and "induced self-antigens," which become upregulated by cellular stress, infections, and transformation (28). Following the observation on stimulatory effects of certain non-peptide mycobacterial components on Vγ9Vδ2 T-cells (39, 40), the responsible substances could be isolated and characterized and are commonly termed as phosphoantigens (PAgs) (41). We consider PAgs the primary trigger of Vγ9Vδ2 γδ T-cell activation and discuss them in greater detail in the following. However, Vγ9Vδ2 γδ T-cells may also respond to other antigens and ligands *via* TCR and (co-)receptors (42).

Vγ9Vδ2 T-Cells in Cancer Immunotherapy

Subsets of Vγ9Vδ2 T-cells can be defined analyzing the expression of surface markers (e.g., CD27, CD45RA, CCR7, and CD16) or regarding their dominant cytokine production and correlate with functional differences like proliferative capacity or cytotoxic potential (43, 44). It has been extensively demonstrated *in vitro* (45–55) and using *in vivo* models (22, 56–68) that γδ T-cells are able to recognize various tumor cells and exert strong anti-tumor effects. Tumor growth is inhibited *via* different mechanisms including the release of pro-inflammatory cytokines, granzymes and perforin, and the engagement of apoptosis inducing receptors (69).

Several drugs and treatment concepts might improve the activity of Vγ9Vδ2 T-cells against cancer. Most candidates are still at a pre-clinical stage, some were tested in animal models, and very few went into clinical tests so far. Although Vδ1+ cells shown promising results pre clinically (70), all previous clinical trials focused on the usage of Vγ9Vδ2 T-cells. Reasons for the earlier therapeutic employment of Vγ9Vδ2 T-cells include their relatively high abundance in the peripheral blood and the possibility to efficiently culture them *ex vivo* or to stimulate and expand them *in vivo* using amino-bisphosphonates (N-BP) or synthetic PAgs (45), as discussed later.

Here, we divide the existing clinical studies according to the used strategy into two main groups: (1) *in vivo* activation (17, 18, 23, 71–74) and (2) adoptive cell transfer strategies (75–84). In the latter case, the adoptively transferred cells originally were extracted, activated, and cultured autologous blood cells. Varieties include the transfer of processed haploidentical cell preparations with subsequent *in vivo* stimulation (82), as well as local administration of cultured cells into the tumor or the peritoneal cavity (85, 86). Well organized and comprehensive analyses of the performed clinical studies involving γδ T-cells have recently been published by others (45, 87, 88) and an overview is given in **Table 1**.

Outline

Much has been learned by studying γδ T-cells from animals, especially those from mice. However, there are major distributional, structural, and functional differences between the species, especially the lack of Vγ9Vδ2 T-cells or functional homologs in mice (91, 92). In this review, we focus on human γδ T-cells, their anti-tumor capabilities, and strategies for improving the effectiveness of Vγ9Vδ2 T-cells in cancer immunotherapy. Current publications contain additional information on the topics not covered here, especially the biology of non-Vδ2 cells (93) and their role in cancer and cancer therapy (2). We also refer to more detailed literature regarding the differences of rodent and human γδ T-cells (28), γδ T-cells acting as professional antigen-presenting cells (36), concerning B-cell help (94) and potential use as a vaccine (95), cell ontogeny (33), phylogenetic aspects (28, 42), genetically modified γδ T-cells (e.g., CARs) (96, 97), as well as molecular details of receptor signaling (98, 99). We discuss approaches especially that aim to sensitize target cells and the local interaction of tumor and effector cells in connection with the underlying mechanisms.

TARGETING THE CELLULAR METABOLISM

Survival and growth of cancer cells are connected to specific metabolic alterations which have been considered a distinctive

TABLE 1 | Clinical studies.

Reference	Year	Disease	*N*	Reported outcome	Systemic therapy/comments
***In vivo* stimulation**					
Wilhelm et al. (18)	2003	MM, indolent, lymphomas	19	16% PR, 16% SD	+PAM +IL-2/response correlates with *in vitro* expansion
Dieli et al. (23)	2007	HRPC	18	16% PR, 27% SD	+ZOL +IL-2
Bennouna et al. (73)	2010	RCC, GYN-, GI-cancers	28	42% SD	+BrHPP +IL-2
Laurent et al. (89) abstract only	2010	Follicular lymhoma	45	26% CR, 18% PR	+BrHPP +IL-2 +RTX
Meraviglia et al. (71)	2010	Breast cancer	10	10% PR, 20% SD	+ZOL +IL-2/response correlates with *in vivo* expansion
Lang et al. (74)	2011	RCC	12	16% SD	+ZOL +IL-2
Kunzmann et al. (72)	2012	RCC, melanoma, AML	21	16–42% SD AML: 25% PR	+ZOL +IL-2
Pressey et al. (17)	2016	Neuroblastoma	4	25% SD, 75% PD	+ZOL +IL-2
Adoptive transfer					
Kobayashi et al. (78)	2007	RCC	7	Delayed tumor doubling times in 4/7 patients	–
Bennouna et al. (75)	2008	RCC	10	60% SD	–
Abe et al. (80)	2009	MM	6	66% SD	–
Nakajima et al. (81)	2010	Lung cancer	10	30% SD	–
Kobayashi (79)	2011	RCC	11	9% CR, 45% SD	+ZOL +IL-2
Nicol et al. (84)	2011	Solid tumors	18	16% SD, 16% PR and CR	+ZOL +other tumor-specific treatments
Noguchi et al. (77)	2011	Solid tumors	25	12% SD, 12% PR	+other tumor-specific treatments
Sakamoto et al. (76)	2011	Lung cancer	15	40% SD	–
Cui et al. (86)	2014	HCC	62	Longer PFS and OS	–/in addition to radiofrequency ablation
Wilhelm et al. (82)	2014	Hematological malignancies	4	75% CR	+ZOL +IL-2 +Chemo/*in vivo* stimulation following transfer of haploidentical cells
Wada et al. (85)	2014	Gastric cancer	7	Reduction in ascites in 2/7 patients	–/intraperitoneal administration of γδ T-cells
Aoki et al. (90)	2017	Pancreatic cancer—adjuvant	28	Higher recurrence free survival in patients with sustained higher γδ T-cell numbers	+Chemo

AML, acute myeloid leukemia; BrHPP, bromohydrin pyrophosphate; Chemo, chemotherapy; CR, complete remission, GI, gastrointestinal; GYN, gynecological; HCC, hepatocellular carcinoma; HRPC, hormone refractory prostate cancer; MM, multiple myeloma; N, number of patients; OS, overall survival; PAM, pamidronic acid; PD, progressive disease; PFS, progression free survival; PR, partial remission; RCC, renal cell carcinoma; RTX, rituximab; SD, stable disease; ZOL, zoledronic acid.

"hallmark of cancer" (100). Most prominent example of such adaptation is the "Warburg effect," the preferential utilization of aerobic glycolysis by various tumor cells, described by Warburg in 1924 (101). Obvious elements of this phenotype are the inhibition of oxidative phosphorylation despite sufficient oxygenation, an elevated glucose consumption, and an increased production of lactic acid (LA). Changes in the tumor metabolism can be complex and beside glucose metabolism also affect lipid and amino acid pathways (102). Correspondingly, our idea of Vγ9Vδ2 T-cell natural anti-tumor functions is based on their ability to distinguish normal and transformed cells due to their metabolic phenotype. In particular, they might recognize an intrinsic overproduction of PAgs arising from isoprenoid biosynthesis in tumor cells.

Many PAgs are naturally occurring prenyl-pyrophosphates (41) originating from isopentenyl pyrophosphate (IPP) of the eukaryotic mevalonate pathway as well as those generated in the microbial non-mevalonate (also termed as MEP or DOX-P) pathway (103). A dysregulated mevalonate pathway, conjoined with a higher abundance of mevalonate pathway products was described in certain malignant cell types (104, 105) and may indeed be important to support the survival of malignant cells (106). PAg accumulation has been explained by increased buildup, especially of IPP due to upregulation of the gate-keeping enzyme 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (107) and other mevalonate pathway enzymes (104). We currently lack sufficient information to decide if a dysregulated mevalonate pathway associated with increased PAgs is indeed a "general hallmark of tumorigenesis" rather than an outlier. In any case, several therapeutic concepts focus on Vγ9Vδ2 T-cells' metabolic sensor and potent effector mechanisms.

N-BPs and PAgs

Activation of Vγ9Vδ2 T-cells with PAgs and N-BPs is the most commonly used strategy for *in vitro* research and both *in vivo* stimulation as well as application of adoptive cell therapy. The potency of the individual PAg molecule to elicit response from Vγ9Vδ2 T-cells differs (108) and is especially high for microbial (E)-4-hydroxy-3-methyl-butenyl pyrophosphate (HMBPP), certain synthetic compounds like bromohydrin pyrophosphate (BrHPP) (109) or nucleotides derived from HMBPP (110). However, so far only BrHPP and N-BPs have been used clinically. N-BPs were found to trigger activation and expansion of Vγ9Vδ2 T-cells as well as their interferon-γ (IFN-γ) release (46, 111) and were later recognized as indirect acting PAgs (112). This class of substances is structurally related to direct PAgs, but acts by inhibition of farnesyl diphosphate synthase and the accumulation of upstream metabolites like the direct PAg IPP (113). In immunotherapy N-BPs serve a double purpose. First, they sensitize target cells, rendering many primarily resistant tumor

cells vulnerable to γδ T-cell mediated attack (114). Second, they induce expansion of γδ T-cells *in vivo* and *in vitro*. The degree of inhibition of farnesyl diphosphate synthase thereby correlates well with important anti-tumor functions of Vγ9Vδ2 T-cells over various tumor cell lines (115). Apart from sensitization of tumor cells, N-BPs exert additional direct anti-neoplastic effects, like an increased production of toxic mevalonate pathway products and a decrease of essential downstream metabolites (113, 116).

Ex Vivo Culture and *In Vivo* Models

Potent natural and synthetic PAgs, like the patented drug BrHPP (termed as IPH1101 or Phosphostim®) can be used for effective *in vitro* (117) and *in vivo* (22, 75) expansion of Vγ9Vδ2 T-cells.

Protocols for *ex vivo* culture of human Vγ9Vδ2 T-cells vary regarding the culturing conditions, timing and dosage of used N-BPs or PAgs, and added co-stimulators like IL-2 (88, 118) and may result in different phenotypes and effector cell characteristics. Zoledronic acid (ZOL) is a potent N-BP and commonly used about 1 μM *in vitro*, a concentration also in the range of the peak plasma level following a single standard dose of 4 mg intravenously (88). Repetitive administration of exogenous IL-2 is commonly used as it drives proliferation of PAg stimulated Vγ9Vδ2 T-cells resulting in an increased yield (63, 67). Results of *in vitro* expansion are highly donor dependent and may also predict the respective *in vivo* expansion efficacy, which can be additionally restricted in cancer patients (18). Currently, an optimal dose of ZOL as well IL-2 has not been determined *in vivo* (88) and a recent study indicated that the efficacy of ZOL stimulation depends on drug concentration and duration of exposure with an individual optimum (67).

The ability to recognize the PAgs is linked to germline-encoded regions of the γδ TCR (119) and so far functionally only described in primates (120). Even though homologs sequences of human Vγ9 and Vδ2 genes were recently described in other species, such as alpaca and armadillo (121, 122). As wild type mice lack PAg-responding γδ T-cells the *in vivo* expansion of human Vγ9Vδ2 T-cells has been studied using xenograft mice (57, 123) or cynomolgus monkeys (59). Results from such models show that sensitizing tumor cells with N-BPs, combined with adoptive transfer of *ex vivo* expanded human Vγ9Vδ2 T-cells with or without exogenous IL-2 administration is feasible and induces moderate anti-tumor responses (58, 65, 66, 68, 124). The role for additional systemic application of N-BPs in context with adoptive cell transfer strategies remains uncertain. On one side it has been reported to promote engraftment of *ex vivo* stimulated and adoptively transferred human cells in mice (124), on the other side there are indications that repetitive application of these drugs *in vivo* induces Vγ9Vδ2 T-cells exhaustion (23, 71, 74).

Clinical Experience

One may speculate that the observed anti-tumor effects of N-BPs or high-dose IL-2 monotherapy as well as allogenic stem cell transplantation are influenced by γδ T-cells without being recognized as such (125–127). Implementation of clinical Vγ9Vδ2 T-cell studies benefited from the fact that side effects and pharmacological profiles of N-BPs and IL-2 monotherapy were already known. IL-2 is established as an effective treatment for several types of cancer for about 30 years (128) and N-BPs are widely used for osteoporosis, hypercalcemia, and the treatment of bone metastasis (125). The first prospective trial focusing on the *in vivo* stimulation of anti-tumor functions by γδ T-cells used the N-BP pamidronic acid (18), later studies the more potent ZOL (17, 23, 71, 72, 74) in combination with IL-2. These N-BPs have also been used to stimulate Vγ9Vδ2 T-cells *ex vivo* for adoptive cell therapy (76, 77, 80, 81, 83). Additionally, a few studies applying adoptive cell transfer included the systemic administration of ZOL with (79, 82) or without additional IL-2 (84). Taken together the clinical studies involving the use of N-BPs to increase the anti-tumor effects of Vγ9Vδ2 T-cells in different types of malignancies depicted a tolerable toxicity but revealed inconsistent responses and overall only a modest efficacy (compare **Table 1**).

Similarly, BrHPP was tested in early clinical studies with small success, for both *ex vivo* stimulation and consecutive adoptive transfer of cells in combination with IL-2 in metastatic renal cell carcinoma (75) and for *in vivo* stimulation targeting solid tumors (73). A strategy combining BrHPP stimulation and the tumor targeting antibodies rituximab (RTX) (89) is discussed separately.

Current Obstacles

Several reasons might explain the limited therapeutic effectiveness of both N-BPs and synthetic PAgs *in vivo*. Maybe most importantly N-BPs and synthetic PAgs lack cancer specificity regarding uptake or molecular targeting and also affect other cells. Also, N-BPs and BrHPP both have short plasma half-life periods (22, 67). BrHPP is quickly degraded by plasma phosphatases and common N-BPs cannot passively cross the plasma membrane, and is preferentially rooted to the bone due to their calcium binding characteristics (112). Cancer cells in other compartments are those that lack adequate active transport mechanisms might therefore not be affected. It is established that monocytes/macrophage type cells take up N-BPs *via* fluid endocytosis and induce activation of Vγ9Vδ2 T-cells (129, 130). Unfortunately ZOL also induces killing of human macrophages (131) and, additionally, uptake of N-BP by neutrophils impairs γδ T-cell proliferation *via* production of reactive oxygen species (132). Indeed treatment with N-BP can decrease circulating γδ T-cell count (133) and repetitive stimulation with BrHPP lead to progressive exhaustion of γδ T-cell activation and expansion *in vivo* (22). A new strategy to stimulate Vγ9Vδ2 T-cells and avoid exhaustion might be the application of an attenuated, live vaccine with genetically engineered metabolic profile that overproduces HMBPP. Adapting traits of a bacterial infection with *Salmonella enterica* indeed elicited a prolonged Vγ9Vδ2 T-cell immunity in monkeys (134). A different concept to increase N-BP concentration in the tumor tissue is to administer drugs (and *ex vivo* stimulated cells) locally (135). Nevertheless, this is not a working concept for systemic diseases. It also has to be taken into account that although commonly well tolerated, N-BPs and exogenous IL-2 have considerable and dose limiting toxicities, including inflammatory and cytokine reactions, osteonecrosis of the bone, and hypocalcemia (128, 136).

Modified PAgs and N-BPs

The development of new direct and indirect PAgs may overcome pharmacodynamic restrictions and improve clinical efficacy (112). Newly designed PAgs (137) and bisphosphonate prodrugs (138, 139) have chemically masked phosphate groups, allowing these compounds to enter cells without the need for active transmembrane transport (140) and should not accumulate in the bone. Following intracellular uptake they are converted to their active forms, which are potent stimulators of Vγ9Vδ2 T-cells and sensitize different tumor cell lines toward γδ T-cell anti-tumor effects *in vitro* (138–140). Bisphosphonate prodrugs already depicted some effect in combination with adoptive cell transfer in an animal model of bladder carcinoma and human fibrosarcoma (138, 139).

Nano-technology based carriers for N-BP delivery (141) as well as lipophilic bisphosphonate (60, 142, 143) and synthetic nucleotide pyrophosphates (110) are additional pharmacotherapeutic strategies that may improve Vγ9Vδ2 T-cell immunotherapy in the future.

Butyrophilin 3A (BTN3A)

More recently, Butyrophilin 3A (BTN3A, CD277) was described as essential for γδ T-cell activation by direct PAgs (144, 145). BTN3A belongs to the important B7 family of co-stimulatory molecules (146) and consists of three isoforms: BTN3A1, BTN3A2, and BTN3A3. BTN3A2 differs as it lacks an intracellular B30.2 domain that is needed for PAg recognition. However, when using the mouse anti-human-CD277 antibody clone 20.1 directed against an extracellular domain, all BTN3A isoforms support Vγ9Vδ2 T-cell activation (144). The molecular details of signal transduction are a current research topic and matter of debate, especially regarding two different models: originally, the "antigen presenting model" by Vavassori et al. (145) assuming that CD277 and the TCR interact directly following PAg binding to an extracellular CD277 domain. Recent experimental evidence rather supports a second, so called "allosteric model" by Harly et al., postulating that PAgs interact with the intracellular B30.2 domain of CD277 (147) either directly (148) or indirectly (149, 150) and induce a conformational change that is transferred to the extracellular parts of the CD277 molecule (147, 151). PAg sensing may additionally involve molecules like Rho-GTPase (152) or Periplakin and is modulated by mechanisms enabling transmembranous PAg transport or *via* hydrolyzation of PAgs by ecto-ATPase CD39 (106, 153).

Development of mouse anti-human-CD277 antibodies has been very useful in deciphering the activation processes of Vγ9Vδ2 T-cells (144, 154) and also holds therapeutic potential. The mode of action of these antibodies was proven to be downstream and independent of IPP (144, 149). Furthermore, activating anti-CD277 clone 20.1 has similar but not identical stimulatory capabilities compared with PAg stimulation (155) and might be restricted to certain Vγ9Vδ2 T-cells with specific complementarity-determining region sequences of the TCR (156). Still, anti-CD277 antibodies might outperform N-BPs or other metabolic sensitizers in target cells that fail to internalize drugs or which have decreased mevalonate pathway activity. It was shown that anti-CD277 antibodies enhance anti-tumor functions of Vγ9Vδ2 T-cells *in vitro* (144) and in a xenotransplant mouse model of human acute myeloid leukemia (AML) (157). We also observed that primary chronic lymphatic leukemia (CLL) cells are hardly affected by ZOL sensitization become lysed by Vγ9Vδ2 T-cells following their incubation with activating anti-CD277 antibody (158). Unfortunately, antibodies with a murine background seem inappropriate for clinical use and development of a humanized version or a human homolog of the clone 20.1 antibody has not been reported. A further drawback is the widespread expression of the CD277 molecule in human tissues (146, 159), which is why additional strategies for enhancement of selectivity might be required. One solution could be the development of antibody constructs combining both CD277 activating and tumor-antigen specificity.

Other Agents

Therapeutic specificity might also be achieved by targeting tumor cell specific metabolic alterations. Therefore, we tested whether the pyruvate dehydrogenase activator dichloroacetate (DCA) might improve Vγ9Vδ2 T-cell anti-tumor functions *in vitro*. DCA inhibits aerobic glycolysis, malignant cell proliferation and indirectly facilitates mitochondrial oxidative decarboxylation of pyruvate to acetyl-coenzyme A (160). Indeed, we found that DCA + ZOL treated leukemia cell lines induce higher IFN-γ production by Vγ9Vδ2 T-cells compared with ZOL treatment alone. We also suspected that DCA increases the supply of metabolites upstream of IPP and therefore increases PAg accumulation when combined with ZOL (161). Still, alternative explanations are possible as DCA decreases tumor cells' LA production (160) and LA can directly inhibit several immune functions. Tumor LA efflux is, therefore, an attractive target and could be targeted by inhibition of lactate transporters and nonsteroidal anti-inflammatory drugs (NSAID) (101). Concerning potential anti-tumor effects of NSAIDs, the use of indomethacin as well as specific cyclooxygenase-2 inhibitors resulted in an increase of Vγ9Vδ2 T-cell dependent tumor cell lysis. If this observation is connected to LA release has not been investigated but was attributed to the inhibition of prostaglandin effects (162). Finally, the enzymes CD39 and CD73 that regulate ATP/adenosine balance and thereby the function of immune cells might represent interesting targets for immunotherapy (163). Here, CD39 might be of special interest in the context of Vγ9Vδ2 T-cell therapy as it was shown to be capable of PAg hydrolyzation (164).

Summary

Adoptive transfer of *ex vivo* cultured cells and various combinations of N-BPs, BrHPP, and IL-2 have demonstrated clinical effects but are rather disappointing compared to the promising pre-clinical results. The discrepancy suggests that the *in vivo* characteristics of stimulated Vγ9Vδ2 T-cells are still insufficiently understood. To overcome the current limitations, we need to learn more about differentiation and functionality of PAg activated γδ T-cells, its subpopulations and migration patterns. PAgs and N-BPs with improved pharmacokinetics and potency are very promising new developments, but their toxicity profile and clinical effectiveness have yet to be established. A breakthrough

would be the development of PAg or N-BP analogs with strong molecular tumor cell specificity.

Beside these innovations, we should search for additional tumor-specific transport mechanisms and metabolic peculiarities. A good example for the exploitation of a "metabolic weak spot" in cancer is the use of asparaginase in acute lymphatic leukemia (165). We need to identify such targets in the context of γδ T-cell sensing and will hopefully be able to design specific and effective compounds at least for certain types of cancer. Finally, we should consider the metabolic needs of immune cells as well. They may also rely on mevalonate pathway products or upregulate aerobic glycolysis following activation (166) and therefore become negatively affected by certain therapeutic interventions.

TARGETING ACTIVATING AND INHIBITORY RECEPTORS

NKG2D and Its Ligands

In innate immune responses mediated by NK-cells, NKG2D serves as a primary activating receptor and ligand binding triggers cytotoxicity and cytokine production (167–169). In humans, one NKG2D homodimer assembles with four DAP10 adaptor proteins that become phosphorylated upon ligand binding and activation (170). Ligands from distinctive families, the MHC class I polypeptide related sequence A (MICA) and B (MICB) and the cytomegalovirus UL16-binding protein (ULBP) family bind NKG2D even though they share little sequence similarity (171). The expression of NKG2D ligands (NKG2DL) is induced or upregulated primarily in tissues of epithelial origin, as a result of cellular stresses such as viral infection, malignant transformation, or classical heat shock (172, 173). All NKG2DLs are not functionally equivalent and can enable immune cells to recognize of a broad range of different types of infections and indicate malignant transformation in different tissues (170, 171, 174).

NKG2D is also expressed by γδ T-cells and provides important (co-)stimulatory signals in T-cell-mediated immune responses by amplifying T-cell cytokine production, proliferation, and cytotoxicity *in vitro* (52, 98, 169, 175). The NKG2D pathway is also relevant in the context of N-BP treatment and the expression of ULBP1 was found correlated with the sensitivity of AML blasts toward TCR-mediated killing by Vγ9Vδ2 T-cells (114). Additionally, the results of Wrobel et al. indicated that the NKG2D pathway is involved in anti-tumor effects of γδ T-cells against melanoma and various epithelial cancers (55).

MICA-Polymorphism and Soluble MIC (sMIC)

The general concept is that cell stress and transformation increase the expression of MICA antigens and activate immune cells *via* NKG2D. However, MICA is a highly polymorphic human stress antigen and Shafi et al. showed that MICA coding sequence polymorphisms substantially affected RNA and protein expression (176). Some examined individuals showed better response to higher, others to lower MICA expression, and challenging the concept of an invariable direct correlation between stress molecule abundance and immune cell activation (176, 177).

Tumors also adopt evasion strategies, like shedding of free or the exosome form of MICA/MICB. These released molecules can inhibit immune effector cells due to interaction with NKG2D (178). Märten et al. found elevated levels of sMIC levels in sera of patients with pancreatic carcinoma correlated with tumor stage. The cytotoxic response of immune toward tumor cells was found impaired with in the presence of high sMIC levels but restored by neutralization of sMIC (179).

Temozolomide (TMZ) and Other Chemotherapeutics

Glioblastoma multiforme (GBM) is an extremely aggressive brain tumor, which is not very sensitive to either classical chemotherapy or immunotherapeutic approaches. Lamb et al. showed that *ex vivo* expanded γδ T-cells recognize malignant glioma *via* NKG2DL and lyse glioma cell lines and primary GBM specimens. Additionally TMZ, a DNA methylating chemotherapeutic agent licensed for GBM therapy, increased NKG2DL also on TMZ-resistant glioma cells. They also demonstrated that immune effector cells can be genetically modified to resist the toxicity of TMZ without changing their phenotype or their cytotoxicity against GBM target cells (180). Similarly, Chitadze et al. investigated the NKG2DL system in different GBM cell lines and confirmed that TMZ increased the cell surface expression of NKG2DL and sensitizes GBM cells to γδ T-cell mediated lysis. TMZ might therefore enhance the potential of adoptive transfer of *ex vivo* expanded γδ T-cells for glioblastoma treatment (181, 182).

Dacarbazine is a cytotoxic drug used for treatment of Hodgkin's lymphoma and melanoma. Although dacarbazine does not directly affect immune cells, it triggers the upregulation of NKG2DL on tumor cells, leading to NK-cell activation and IFN-γ secretion in mice and humans (183). Apart from TMZ and dacarbazine, studies suggest that other chemotherapeutics, like fluorouracil, doxorubicin, or vincristine sensitize tumor cell lines toward a NKG2D-dependent cytotoxic activity of Vγ9Vδ2 T-cell (184, 185). This could be a target cell or drug specific phenomenon as we were unable to boost γδ T-cell induced lysis of several leukemia cell lines with other cytostatic drugs (186).

Bortezomib and Epigenetic Drugs

Niu et al. reported that multiple myeloma (MM) cells can be sensitized toward killing by γδ T-cells and NK-cells using low-dose bortezomib. Additionally, bortezomib increases the expression of NKG2D and induces apoptosis of MM-cells, but not γδ T-cells and NK-cells (187). Treatment with 5-azacytidine, its derivate decitabine or histone deacetylase inhibitors may also increase the expression of NKG2DL in different types of malignancies prompting Bhat et al. to consider those epigenetic drugs a promising approach in γδ T-cell immunotherapy (188). Suzuki et al. evaluated possible additive effects of valproic acid (VPA), a histone deacetylase inhibitor, on γδ T-cell mediated cytotoxicity against bladder cancer cell lines TCCSUP and 253J (189). VPA did increase expression of NKG2DL and sensitivity toward cytolysis by γδ T-cells for both cancer cell types, whereas ZOL pre-treatment was only effective against TCCSUP. 253J cells were preferentially engaged *via* NKG2D-NKG2DL interaction, while TCCSUP cells were mainly recognized through the γδ TCR (189). Chávez-Blanco et al. showed that hydralazine in combination

with VPA increase the expression of MICA and MICB ligands by target cells, as well as NK-cell cytotoxicity *via* NKG2D. Additionally it reduces the shedding of MIC molecules to the supernatant (190). Satwani et al. incubated acute lymphoblastic leukemia and non-Hodgkin lymphoma cell lines for 24 h with 10 ng/mL of romidepsin (191). They demonstrated an approximately 50- to 1,300-fold increase in the number of cells positive for the surface expression of MICA/B in these cell lines. They further demonstrated a significant increase in NK-cell-mediated *in vitro* cytotoxicity (191).

Inhibitory Receptors

The development of immune checkpoint inhibitors targeting the cytotoxic T-lymphocyte-associated Protein 4 (CTLA4) or programmed cell death protein 1 (PD-1) and its ligand (PD-L1) has substantially extended the possibilities of immunotherapy. These substances are able to induce enduring remissions in a considerable subset of patients with treatment refractory types of cancer, for example melanoma, non-small cell lung cancer, and Hodgkin's lymphoma (192). Considering their clinical significance, relatively little is known about the role of γδ T-cells in immune checkpoint therapy and also regarding the role of inhibitory axes for γδ T-cell biology.

Programmed Cell Death Protein 1

Programmed cell death protein 1 is a key inhibitory receptor in inflammation, responsible for induction of tolerance, and immunosuppression in cancer (193). Following interaction with its ligands programmed death-ligand 1/2, the PD-1 receptor inhibits TCR and PI3K/AKT signaling and decreases proliferation and IL-2 release (194). It is interesting that both the PD-Ls and the CTLA4 ligands (CD80 and CD86) are members of the B7 family of proteins and therefore interrelated to BTN3A/CD277. Several types of malignancies have a relevant susceptibility to therapeutic PD-1/PD-L1 blockade, but it is barely predictable which individual patient will respond. The initially assumed direct relationship between tumor cell expression of PD-Ls and response rate following therapeutic PD-1 blockade might not be universally valid and the strength of PD-1 dependent immunosuppression is influenced by the topographic organization of the tumor microenvironment (195).

An early *in vitro* study addressed the expression profile and functionality of PD-1/PD-L1 in γδ T-cells following stimulation with HMBPP and suggested that the PD-1/PD-L1 axis is important for regulation of anti-tumor mechanisms of γδ T-cells (196). Later it was found that PD-1 expression is more frequent on Vδ1, compared with Vδ2 T-cells (197) and equably distributed over several functionally distinctive subsets of Vγ9Vδ2 T-cells (44). A report that *ex vivo* cultivated Vδ2 T-cells depict stable, low cell surface expression of PD-1 following adoptive transfer (198) might fit the observations that PD-1 is only temporarily upregulated following *in vitro* stimulation as it has been reported both for HMBPP and ZOL (196, 198). Vδ2 T-cells derived from neonates may behave differently as they depict prolonged PD-1 expression following activation and function as a regulator of tumor necrosis factor-α (TNF-α) production and cell degranulation, both being part of fetal inflammatory response (199).

Programmed cell death protein 1 expression might contribute to insufficient expansion of Vγ9Vδ2 T-cells in cancer patients, as a diminished response to PAg stimulation was demonstrated in bone marrow derived Vγ9Vδ2 T-cells from patients with MM. Such cells depicted a significantly increased PD-1 expression and were located in proximity to PD-L1+ MM-cells and myeloid-derived suppressor cells (200). Additional treatment with PD-1 antibody resulted in a twofold increase in proliferative response and an increased mobilization of CD107a following ZOL stimulation *in vitro* (200). Beside the bone marrow of MM patients, PD-1 positive γδ T-cells were also found in neuroblastoma infiltrated bone marrow (201).

Other Inhibitory Receptors

Alongside PD-1 several other inhibitory molecules are currently investigated regarding their function in limiting anti-tumor responses and potential therapeutic prospects (202). This is of special interest as there are indications for compensatory upregulation of alternative inhibitory receptors during anti-PD-1 therapy (203). Examples are the B- and T-lymphocyte attenuator (BTLA), CTLA4, T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), and lymphocyte activation gene-3 (LAG-3) and their respective ligands.

B- and T-lymphocyte attenuator was suggested to inhibit late phases of immune reactions and has structural and functional similarities to PD-1 and CTLA4 (204). It is expressed on Vγ9Vδ2 T-cells and engagement by its ligand, the herpesvirus entry mediator, reduced activation, proliferation, and anti-lymphoma response (205). Differing from PD-1 expression kinetics (196, 198), BTLA is initially downregulated following stimulation with PAgs but upregulated upon IL-7 treatment (205).

Compared with PD-1 and BTLA, even less is known concerning the functional implications of CTLA4, LAG-3, and TIM-3 on γδ T-cells. Melanoma patients with a higher ratio of Vδ1 to total γδ T-cells had poorer overall survival and *vice versa* higher frequencies of Vδ2 cells were associated with longer survival in a study using CTLA4 inhibitory antibody ipilimumab (16). Expression of LAG-3 indicates inhibition of PD-1 + T-cells in the tumor tissue and poorer prognosis in follicular lymphoma (206). From studies examining distinctive T-cell populations, we know that CTLA4 can inhibit T-cell activity *via* signaling mechanisms distinctive from PD-1 (207), but we still lack mechanistic studies conclusively demonstrating CTLA4 expression and function for Vγ9Vδ2 T-cells. In women with pre-eclampsia γδ T-cells with low TIM-3 expression depict a higher IFN-γ production (208) and in the context of malaria infection a high TIM-3 level was found correlated with reduced pro-inflammatory cytokine production (209). Similar to anti-PD-L1 antibodies, the inhibition of the TIM-3 ligand galactine-9 that is expressed by γδ T-cells, increases tissue infiltration by αβ T-cells in a pancreatic tumor model (5).

Summary

The referred data provide interesting prospects to enhance immunotherapy by means of modulating the expression of NKG2DL. Even though several of the referred effects were shown for NK-cells, these strategies might also apply for sensitizing tumor

cells toward γδ T-cell dependent cytotoxicity. Negative aspects like possible adverse effects on immune cell functionality or tumor escape mechanisms like sMIC and MICA-polymorphism need to be considered in future studies.

The physiological relevance of the currently known inhibitory receptors for γδ T-cells biology remains vague and additional observational and experimental studies are required. Based on the current evidence we assume that PD-1 is important for regulation of Vγ9Vδ2 T-cell functionality under specific conditions only, for example in an immunosuppressive tumor microenvironment. In inflammation and in the tumor microenvironment, γδ T-cells can become inhibited *via* PD-1 and also inhibit other PD-1 + immune cells *via* PD-L1 expression (5, 210). However, inhibitory effects of PD-1 may be overruled upon strong (co-) stimulation, for example *via* the TCR or with IL-2. Beside the local tissue distribution of receptors and ligands, expression kinetics are important to understand the function of the inhibitory receptors for immune homeostasis. Unfortunately, many studies do not distinguish whether tissue infiltrating T-cells are αβ or γδ T-cells in the first place. Combination therapy of adoptive transfer or *in vivo* stimulation of γδ T-cells with PD-1, PD-L1, CTLA4, or BTLA antibodies therefore seems feasible but the pre-clinical rational is currently not well established.

ADCC AND ANTIBODY CONSTRUCTS

Cytotoxicity of γδ T-cells against target cells can be significantly enhanced using specific monoclonal antibodies (mAbs) that induce ADCC. ADCC of γδ T-cells is thought to depend on Fc-γ receptor III (CD16) as it has been demonstrated that anti-CD19 antibody triggered CD107a, IFN-γ, and TNF-α expression is correlated to the amount of CD16+ γδ T-cells in an *in vitro* cytotoxicity assay (211). Furthermore, γδ T-cell mediated ADCC increases with higher numbers of CD16+ γδ T-cells (212) and was found inhibited with CD16 blocking antibodies (213). CD16 expression is usually low in unstimulated γδ T-cells, but increases following activation, for example with PAgs (213, 214).

B-Cell Malignancies

Rituximab

Several lymphoma and B-cell lineage leukemia subtypes were studied using stimulated γδ T-cells in combination with monoclonal anti-CD20 antibodies (212–216). Tokuyama et al. found RTX to increase the killing of several lymphoma cell lines and to improve ADCC of γδ T-cells against CLL and autologous follicular lymphoma cells (213). Furthermore, BrHPP stimulated γδ T-cells demonstrated stronger CD107a expression and increased ADCC toward individual B-cell lymphoma cell lines and patient CLL cells in combination with anti-CD20 antibodies (214). One single clinical phase I/IIa study used RTX plus BrHPP and IL-2 for *in vivo* stimulation of γδ T-cells in patients with relapsed follicular lymphoma (89). Altogether, 45 patients were treated according to protocol and the treatment was generally well tolerated, with low grade pyrexia being the most common side effect (89). Despite the 45% overall response rate (26% complete response) (89), it seems like development of BrHPP containing therapies is no longer pursued by the company in charge.

Second Generation Anti-CD20 Antibodies and Anti-CD52

The newer anti-CD20 antibodies ofatumumab and obinutuzumab were also tested regarding the efficacy inducing ADCC in connection with γδ T-cells (215). Obinutuzumab is an Fc engineered type II monoclonal antibody (217) and causes an increased secretion of perforin and IFN-γ compared to RTX and ofatumumab. Accordingly, the highest ADCC against B-cell lymphoma cell lines and primary follicular lymphoma cells was found for obinutuzumab (215). Similar to anti-CD20 antibodies, Gertner-Dardenne found alemtuzumab, an anti-CD52 antibody, to increase γδ T-cell dependent ADCC against lymphoma cell lines (214).

Solid Tumors

Breast Cancer

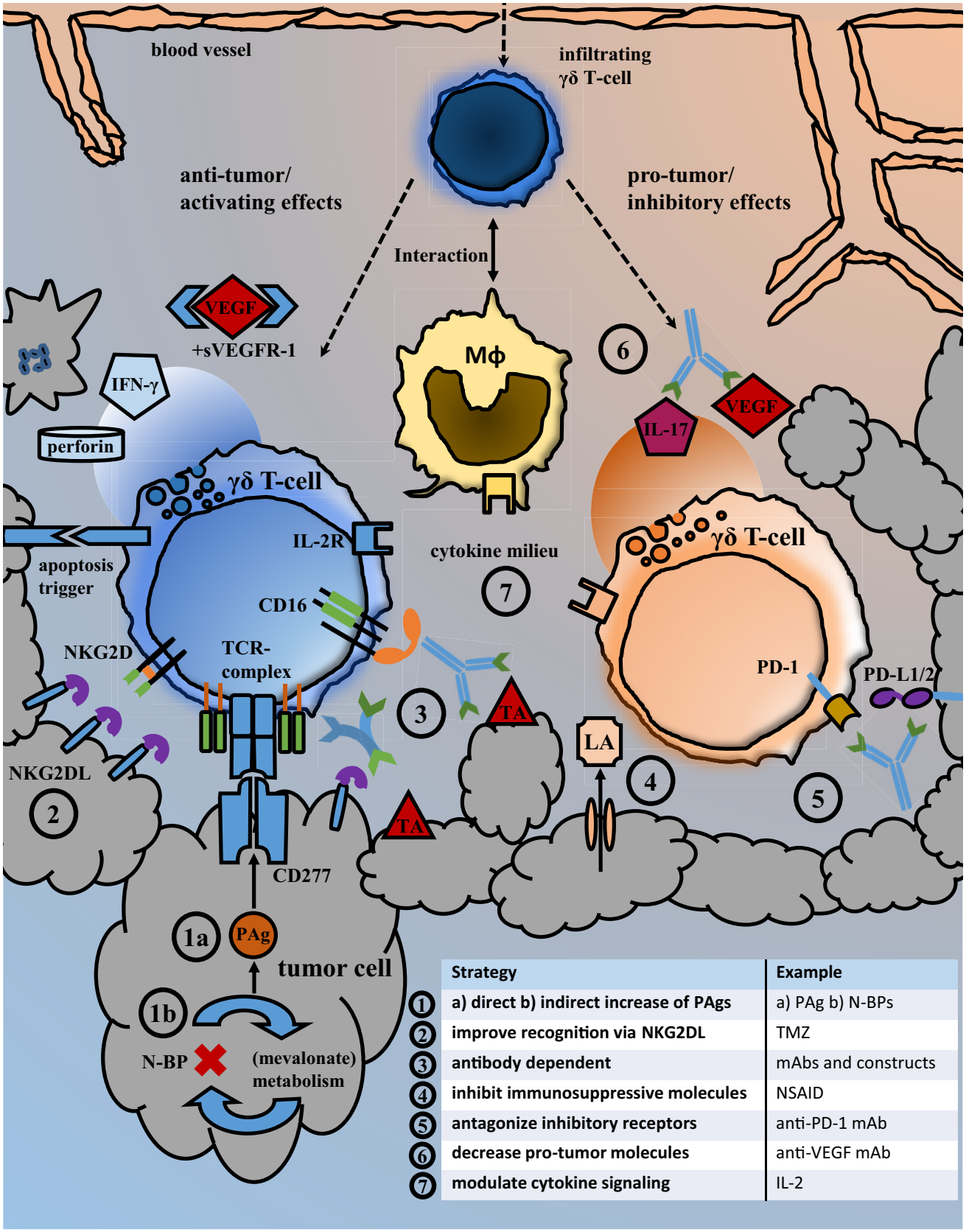
Two groups investigated whether the human epidermal growth factor receptor 2 (HER2/neu) specific antibody trastuzumab enhances γδ T-cell dependent ADCC toward breast cancer cell lines *in vitro* (63, 213). The addition of trastuzumab greatly increased lysis of HER2/neu overexpressing cell lines, whereas there was no change in a HER2/neu negative cell line (213). The extent of ADCC was increased with higher density of HER2/neu expression. Anti-tumor activity was confirmed in an animal model with SCID Beige mice. Here, the tumor growth was more efficiently inhibited by a combination treatment with γδ T-cells and trastuzumab compared to treatment with trastuzumab or γδ T-cells alone (63).

Neuroblastoma and Ewing's Sarcoma

Both in neuroblastoma and in Ewing's sarcoma, the disialoganglioside specific antibody ch14.18/CHO increased γδ T-cell mediated ADCC *in vitro* (124, 218). This finding was confirmed in an advanced immunodeficient mouse model, where *ex vivo* stimulated and adoptively transferred γδ T-cells with simultaneous administration of ch14.18/CHO antibody impaired tumor growth more efficiently than single antibody or sole γδ T-cells treatment (124).

Antibody Constructs and Nanobodies

Antibody constructs have been studied in both lymphoma and solid tumor models. Seidel et al. used the Fc modified CD19 antibody 4G7SDIE as a backbone for bispecific CD19-CD16 and CD19-CD3 antibody constructs (211). Although no direct comparison between unaltered antibodies and the antibody constructs was made, the constructs proofed active in inducing cytotoxic reactions by γδ T-cells. Schiller et al. went one step further and engineered a so called "single chain triplebody," called SPM-1, that consists of three single chain antibody fragments (CD19-CD19-CD16) (219). Indeed, SPM-1 induced a higher lysis compared to 4G7SDIE. A comparable approach is a recombinant construct consisting of a CD20 single-chain fragment variable (scFV) linked to MICA or ULBP2 which enhances cytotoxicity of stimulated γδ T-cells against CD20+ lymphoma cell lines and primary CLL cells *via* NKG2D (220). Oberg et al. designed two bispecific antibodies that bind either CD3 or the Vγ9 TCR-chain on γδ T-cells and Her2/neu expressed



	Strategy	Example
1	a) direct b) indirect increase of PAgs	a) PAg b) N-BPs
2	improve recognition via NKG2DL	TMZ
3	antibody dependent	mAbs and constructs
4	inhibit immunosuppressive molecules	NSAID
5	antagonize inhibitory receptors	anti-PD-1 mAb
6	decrease pro-tumor molecules	anti-VEGF mAb
7	modulate cytokine signaling	IL-2

FIGURE 1 | Continued

FIGURE 1 | Strategies for the inhibition of pro-tumor and the enhancement of anti-tumor effects. Overview of the local tumor microenvironment that illustrates important immune cell interactions and exemplary types of therapeutic interventions facilitating anti-tumor activity. Following their migration from blood to tissue, γδ T-cells may interact with macrophages and exhibit local pro- but also anti-tumor effects. Possible therapeutic strategies aiming to improve the recognition and killing of cancer cells by γδ T-cells as well as those intended to antagonize immunosuppressive receptor signaling and molecules are listed under points 1–7. Abbreviations: BrHPP, bromohydrin pyrophosphate; DCA, dichloroacetate; (G)M-CSFR, (granulocyte-)macrophage colony-stimulating factor receptor; IFN-γ, interferon-γ; IL-2R, interleukin-2 receptor; LA, lactic acid; mAb, monoclonal antibody; Mφ, macrophage/monocyte lineage cell; N-BP, amino-bisphosphonates; NKG2DL, NKG2D ligands; NSAID, nonsteroidal anti-inflammatory drugs; PAg, phosphoantigens; PD-1, programmed cell death protein 1; PD-L1/2, programmed death-ligand 1/2; sVEGFR-1, soluble vascular endothelial growth factor receptor; TA, tumor antigen; TCR-complex, T-cell receptor complex; TMZ, temozolomide; VEGF, vascular endothelial growth factor.

by pancreatic adenocarcinoma cells (221). Both antibodies enhanced γδ T-cell mediated cytotoxicity and adoptive transfer of γδ T-cells combined with [(HER2)2xVγ9] antibody therapy inhibited growth of pancreatic cancer in a SCID Beige mouse model (221). Furthermore, Hoh et al. demonstrated improved anti-tumor effects against hepatocellular carcinoma and hepatoblastoma cells with MT110, an epithelial cell adhesion molecule EpCAM/CD3 bispecific T-cell engager antibody, compared to the anti-EpCAM antibody adecatumumab (222). Zhang et al. utilized a bifunctional fusion protein (anti-CD3 single-scFV/-NKG2D) that binds NKG2DL+ tumor cells and recruits and stimulates T-cells *via* CD3 (223). This fusion protein was able to stimulate IFN-γ production by T-cells, increased cytotoxic reaction against NKG2DL+ tumor cells *in vitro* and promoted survival in a murine lymphoma model (223).

Another innovative approach is the use of so called nanobodies, a single heavy chain fragment. They bind highly selective to the Vγ9Vδ2 chain and elicited either inhibiting or activating reactions from γδ T-cells (224, 225). Although no data on cytotoxic features against tumor cells are available, it seems to be a promising approach to a selective modulation of Vγ9Vδ2 T-cell activity.

Summary

Monoclonal antibodies combine high target specificity with a favorable toxicity profile, but often depict limited activity when used as single agents. Therefore, combination with γδ T-cells is a promising concept for cancer immunotherapy. There are many mAbs for various hematological and non-hematological malignancies in clinical use already and more are currently in pre-clinical or early clinical development. Several such mAbs are promising combination partners as they show a uniformly strong enhancement in γδ T-cell mediated cytotoxicity. However, results of the only clinical study in this regard, which used RTX plus *in vivo* stimulation of γδ T-cells fell short of expectations. With the advent of new and Fc optimized antibodies and more specifically stimulated γδ T-cells, a higher effectivity might be achievable.

COUNTERACTING PRO-TUMOR EFFECTS

The local interplay of malignant, immune and stroma cells *via* direct cellular interactions and soluble factors characterizes the tumor microenvironment. Under these conditions, infiltrating immune cells can be suppressed and therapeutic activation may even unfold unintended tumor-promoting effects. Beside macrophages and regulatory T-cells (70, 96), IL-17-producing γδ T-cells (γδ T17 cells) are often suggested as important local mediators of tumor progression as repetitively demonstrated in animal models (226–228). It is possible to induce IL-17 production in human cells γδ T-cells *in vitro* (229) and γδ T17 cells were described in the human tumor microenvironments (7, 230) where they have been found inversely correlated with survival and associated with increased stage in breast (6) and colorectal cancer (7). It is important to note that not all studies differentiated between Vδ2 and non-Vδ2 cells or other γδ T-cell subclasses but it seems likely that both, Vδ2 but mainly the non-Vδ2 cells produce IL-17 (7). Direct proof is lacking, but it has been suggested that γδ T-cells can be changed toward an IL-17 producing phenotype by means of the tumor microenvironment (229, 231). Beside IL-17, vascular endothelial growth factor (VEGF) and granulocyte-macrophage colony-stimulating factor are predominately recognized as pro-tumor factors in the microenvironment, but it may not be reasonable to attribute an exclusive pro- or anti-tumor effect to any signal protein, cytokine, cell type or receptor-ligand interaction. For example VEGF facilitates neo-angiogenesis and immunosuppressive effects (232, 233) but also promotes tissue trafficking of different leukocytes (234, 235). The use of immunostimulatory drugs can induce unexpected changes in VEGF levels, as we observed an increase in VEGF serum levels following treatment with ZOL plus low-dose IL-2 in cancer patients (72). Pro-angiogenic factors like VEGF play an important pro-tumor role and predict poor clinical response to certain types of immunotherapy (72, 236). We recently described that following stimulation with IL-2 local lymphocyte-monocyte interactions regulate VEGF homeostasis *via* release of VEGF and soluble VEGF receptor 1 in a time-dependent manner *in vitro* (237). Potential pro-tumor factors and cells could be additionally targeted in combination with γδ T-cell therapy, for example *via* VEGF or IL-17 antagonists. VEGF antibodies are already widely used as cancer therapeutics making clinical studies investigating such a combination therapy feasible. The modest clinical effects of anti-angiogenic strategies call for a more fundamental analysis of VEGF signaling in the tumor microenvironment and the contribution of immune cells to these processes. The same also applies for other factors like IL-17.

Finally, both pro- and anti-tumor effects are mediated locally, as a consequence the *in vivo* efficacy of Vγ9Vδ2 T-cells will depend on their ability to infiltrate into the relevant tissues. Unfortunately we have little information concerning the capacity of activated γδ T-cells to reach the tumor in humans. One single clinical study demonstrated that autologous, *ex vivo* stimulated γδ T-cells predominately migrate to lung, liver and

spleen and could also be detected in individual tumor sites (84). Whether or not an effector cell is capable of tissue homing might be predicted by expression of chemokine receptors, selectins and other cell adhesion molecules. Expression of these molecules however depends on γδ T-cells subpopulation and differentiation status (43, 238, 239).

CONCLUSION

The results from pre-clinical research and individual clinical responses to γδ T-cell therapy encourage to carry on studying γδ-T-cell biology and aim to improve γδ T-cell related anti-cancer therapies. The question is, how the manifold observations on cellular mechanisms can help to establish better anti-cancer strategies and which drugs have an actual translational perspective. An overview on current γδ T-cell dependent therapeutic strategies and immune cell interactions in the tumor microenvironment is given in **Figure 1**. The use of mAb in combination with activated γδ T-cells is strikingly effective *in vitro*. Still the results from *in vivo* experiments did not always keep up with such expectations and the results of the only clinical trial did not proof superior to mAb monotherapy. We will need a thorough understanding of Vγ9Vδ2 T-cell subpopulations and their functional differences and must learn how to influence differentiation and prevent exhaustion. Our knowledge regarding the migration and tissue infiltration of Vγ9Vδ2 T-cells *in vivo* is still sparse, as is the understanding of pro- and anti-tumor mechanisms and cellular interactions in the tumor microenvironment. The establishment of better models could help deciphering those local and time-dependent processes. While the relevance of metabolic changes for immune and cancer cell function is now increasingly acknowledged, we need to learn how immune cells detect and respond to such changes. Reactivity to PAg by Vγ9Vδ2 T-cell may serve as an example, but we should be able to target even more specific tumor characteristics with cellular or combination therapy in the future.

AUTHOR CONTRIBUTIONS

TH wrote the manuscript and prepared the figure. MS and DP drafted sections and edited the manuscript. MW structured and edited the manuscript. All authors read and approved the submitted version.

FUNDING

TH and DP were supported by "Verein Hilfe für Krebskranke e.V." and a scholarship from the "W. Lutz Stiftung."

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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.