IRON METABOLISM AT THE CROSSROAD OF INNATE IMMUNE RESPONSE AND CANCER PROGRESSION

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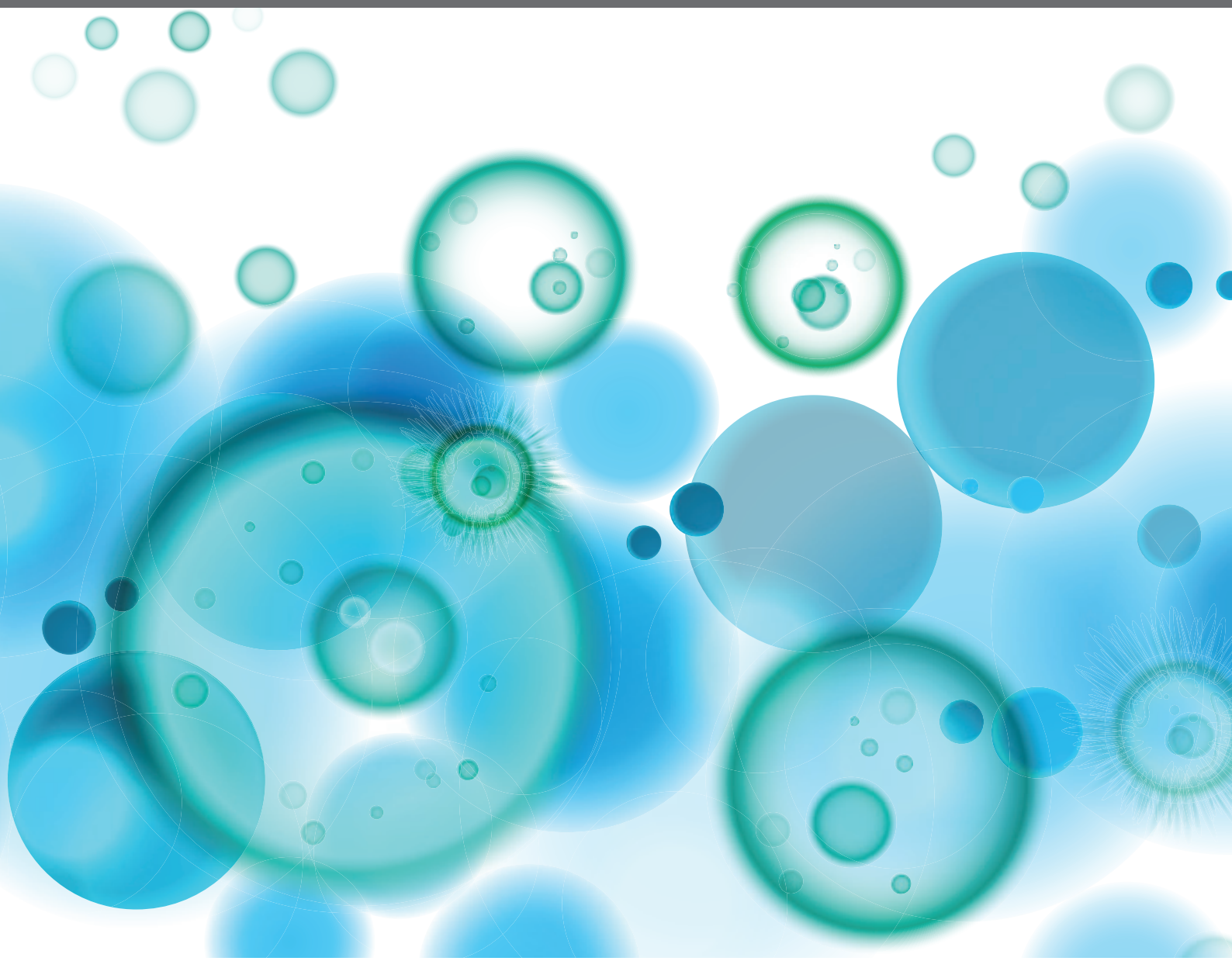
PUBLISHED IN: Frontiers in Immunology and Frontiers in Oncology



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ISSN 1664-8714
ISBN 978-2-88974-400-8
DOI 10.3389/978-2-88974-400-8

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IRON METABOLISM AT THE CROSSROAD OF INNATE IMMUNE RESPONSE AND CANCER PROGRESSION

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Citation: Zacchi, P., Jung, M., Recalcati, S., eds. (2022). Iron Metabolism at the Crossroad of Innate Immune Response and Cancer Progression. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-400-8

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frontiers in Immunology

EDITORIAL
published: 05 January 2022
doi: 10.3389/fimmu.2021.832886

Editorial: Iron Metabolism at the Crossroad of Innate Immune Response and Cancer Progression

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Keywords: iron metabolism, cancer biology, innate immunity, macrophages, ferroptosis

The Editorial on the Research Topic

Iron Metabolism at the Crossroad of Innate Immune Response and Cancer Progression

Iron is a vital nutrient indispensable for the activity of many proteins and enzymes sustaining essential cellular processes such as oxygen transport, energy production, DNA synthesis and repair, cell growth and death, detoxification and host defence (1). Given its high redox activity iron can cause cellular toxicity upon reactive oxygen species (ROS) production *via* Fenton chemistry, being potentially mutagenic (2). Therefore, systemic and cellular iron availability must be tightly regulated.

Iron metabolism is often deregulated in malignant cells, due to their higher metabolic demand cancer cells develop to fuel proliferation, survival, motility and invasion (reviewed in 3). To this goal cancer cells not only enhance their iron import pathways while reducing export, but they also affect how iron is handled by innate and adaptive immune cells, such as macrophages, neutrophils and T cells (4). The innate immune cells are responsible for initiating inflammatory responses aimed at defending body against pathogens but also malignant cells. Therefore, immune cells sequester iron in order to subtract it from tumor cells availability (5). If the inflammatory stimulus persists and becomes chronic, a dangerous interplay between cancer cells and infiltrating leukocytes is established, leading to a drastic change in their polarization toward an immunosuppressive and tumor-supportive phenotype (6). These changes are intimately associated with alterations in iron handling at both tumor and systemic levels, and macrophages, the sentinels of the innate immunity, can be considered the master regulator of all these complex and interconnected events (7).

In this Research Topic, two reviews manuscripts explore the multifaceted role macrophages play in promoting or hampering tumor development based on their ability to influence and be influenced by cancer cells, and how these reciprocal interactions shape iron metabolism.

Liang and Ferrara illustrate extensively the different mechanisms macrophages residing in the tumor microenvironment (TME) put in place, instructed by cancer cells, to become sources of iron and iron-related proteins requested for tumor outgrowth and the signalling pathways involved in these processes. They also include the contribution of neutrophils as iron-donor component of the TME. Neutrophils infiltrating cancerous lesions have long been considered a mere bystander since it was hard to believe that such short-lived leukocytes could perform relevant roles on a chronic and progressive disease like cancer (8). Many recent studies have challenged this view, demonstrating that neutrophils are extremely plastic and can undergo "alternative activation" upon exposure to various cues found in the TME, driving either anti-tumor or pro-tumor functions (9).

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *10 December 2021*
Accepted: *13 December 2021*
Published: *05 January 2022*

Citation:
Zacchi P and Recalcati S (2022) Editorial: Iron Metabolism at the Crossroad of Innate Immune Response and Cancer Progression. Front. Immunol. 12:832886. doi: 10.3389/fimmu.2021.832886

Therefore, their contribution in cancer development and progression cannot be neglected, neither their active involvement in iron dysregulation.

Another review by DeRosa and Leftin examines how tumor-associated macrophages decline systemic iron metabolism alterations with changes in the iron content of the TME and how these changes, shaping their iron polarization phenotypes, impact on the efficacy of the immune response against cancer. They describe as "iron curtain" the characteristic distribution of iron-loaded TAMs that creates a physical border at the tumor front, which would allow TAMs to exert control over metabolic flux and immune response. There are still several issues that need further investigation. It is still poorly understood how spatial heterogeneity in TME affects the way iron is exchanged between cancer cells and macrophages, alters macrophage communication with other tumor associated cell types and how systemic iron dynamics impact on macrophage plasticity during the different steps of cancer progression. These questions need to be carefully addressed to design new anti-cancer therapies targeting the immune-metabolic axis.

Then Weiler and Nairz, in their Hypothesis and Theory contribution, comprehensively address the multifactorial pathophysiology of cancer induced anaemia (CIA). Mechanistically, CIA represents a cytokine-mediated disorder arising from the complex communication established between cancer cells and the immune system. Again, macrophages are of critical importance since their activation makes them source of many proinflammatory cytokines (e.g., IFN-γ, TNF-α, IL-1, and IL-6) that lead to insufficient erythropoiesis mainly through two mechanisms: iron restriction and functional impairment of erythropoietic progenitors. In most cases, this condition negatively impacts on the efficacy of anti-cancer treatments and therefore on patient survival. Several strategies can be adopted to ameliorate CIA, such as hepcidin antagonism, iron supplementation and erythropoiesis-stimulating agents (ESA), but the unintended effects that CIA-directed therapies may exert on TAMs in particular, and other tumor infiltrating leukocytes, are still poorly characterized. Further mechanistic insights need to be provided in order to unveil secondary effects of CIA treatments that may negatively impact on the course of the disease.

In the continuing theme Tymoszuk et al. demonstrate that intravenous iron supplements for curing CIA significantly hampered the T-cell mediated immune response against a murine implanted mammary carcinomas cancer. Key effector cells in antitumor immunity are cytotoxic CD8+ T cells and of CD4+ T helper cells type 1, these latter sustaining the activation, expansion and cell killing activities of the CD8+ T cells (10). This correlates with the fact that most of tumor neo-antigens arising from somatic mutations are presented by the major histocompatibility complex (MHC) class I (11). The authors showed that iron supply has detrimental effects CD8+ T cells proliferation, cytokines production and degranulation. Several mechanisms could be involved, such as ROS-dependent cell death of tumor infiltrating lymphocytes, iron-dependent impairment of T cell receptor signalling or damping of co-stimulatory pathways. Therefore, iron supplementation in cancer patients should be carefully pondered, especially in those treated with immunotherapies.

Weber et al., in their review article, tackle another important aspect of iron dysregulation in patients affected by Myelodysplastic syndromes (MDS), a heterogeneous group of myeloid neoplasms characterized by inefficient hematopoiesis and a risk of progression to acute myeloid leukemia (AML). Anemia and thrombocytopenia are common presenting features in these affected patients and transfusion supportive care is the therapeutic option usually applied to ameliorate the quality of life. Unfortunately, this treatment promotes a secondary iron overload in the bone marrow, while the first arising from disease-dependent insufficient erythropoiesis. This condition is accompanied by ROS production which may contribute to leukemogenesis. In the extreme, iron dependent overwhelming accumulation of ROS could be exploited to promote cell death *via* a novel form of regulated cell death strictly dependent on iron metabolism called ferroptosis (12). This process is driven by the lethal accumulation of lipid peroxidation (13), and multiple genes have been identified as modulators, drivers or markers for this type of iron-dependent cell death in diverse types of cancer.

In Hepatocellular carcinoma (HCC), a highly aggressive cancer with limited therapeutic interventions, accumulating evidence unveiled that ferroptosis performs a key role in regulating the development and progression of this malignancy, the immune status and the anti-tumor response (14). The paper of Liu et al. identified and validated two heterogenous ferroptosis subtypes: the first one was characterized by low expression of Ferroportin releated genes (FRGs) and high load of innate and adaptive cells, vice-versa the second group has an opposite phenotype. Based on FRGs expression, cells infiltration, immune escape mechanisms, genome-driven events and clinical outcomes of the two ferroptosis subtypes, they proposed a scoring system termed ferroptosis related risk score (FRRS), which is expected to reliably assess prognosis and to improve the clinical management of HCC.

The review by Aksan et al. focuses on another aspect of iron genotoxic potential, mostly attributed and studied under iron overload conditions, but found tumorigenic also under iron deficiency context. This is the case of the colorectal cancer, whose pathogenesis has been linked to reduced iron intake and low systemic iron levels. Being iron an essential cofactor for the full performance of a wide variety of enzymes involved in DNA replication and repair, microRNA biogenesis and anti-oxidant systems, its insufficient supply is expected to impair cell mediated immunity and immunosurveillance, activities strictly dependent on iron status (15). Therefore, iron deficiency as well as iron surplus can be considered two sides of the same coin, both negatively impacting on tumorigenesis, cancer progression and clinical outcomes.

The last two papers, from Fan et al. and Zacchi et al. deal with the function and clinical significance of the expression of two key players of cellular iron export in lung cancer pathogenesis and prognosis, namely hepcidin and hephaestin (HEPH). As previously mentioned, hepatic hepcidin regulates systemic iron availability by suppressing intestinal iron absorption and iron

egress from macrophages upon down-regulation of the only known mammalian iron exporter ferroportin (16). Hepcidin synthesis can also occur at extrahepatic location upon diverse stimuli such as iron excess, hypoxia and inflammatory cytokines and cancer cells can locally produce it to sustain their iron-utilization phenotype (17). Even though the functional significance of this local hepcidin production is still poorly understood, Fan et al., based on mRNA expression dataset, found a positive correlation between hepcidin expression and the infiltration levels of lymphocytic cells, neutrophils, macrophages, and dendritic cells. Since patients with high hepcidin expression exhibited a markedly worse survival rate than those with low expression, this may render hepcidin a novel immune-related actor in lung cancer and an independent prognostic biomarker.

In the same cancer context, Zacchi et al., by means of bioinformatics, studied the expression and prognostic value of HEPH, a ferroxidase functionally coupled with ferroportin, that promotes iron export *via* ferrous iron oxidation into its ferric form. HEPH emerged to reside mostly on stromal cellular elements, such as endothelial cells and fibroblasts, key players of the tumorigenic process. Upregulation of HEPH expression correlates with a better outcome as low expression of hepcidin, since both conditions decrease intracellular free iron concentration, known to boost cell proliferation.

In summary, in this Research Topic, leading scientists provided a current state of the art on the role of iron metabolism as a player connecting cancer and immune cells, and its contribution to tumor progression.

AUTHOR CONTRIBUTIONS

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

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frontiers in Oncology

ORIGINAL RESEARCH
published: 04 December 2020
doi: 10.3389/fonc.2020.584477

Iron Supplementation Interferes With Immune Therapy of Murine Mammary Carcinoma by Inhibiting Anti-Tumor T Cell Function

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OPEN ACCESS

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

Received: 17 July 2020
Accepted: 03 November 2020
Published: 04 December 2020

Citation:
Tymoszuk P, Nairz M, Brigo N, Petzer V, Heeke S, Kircher B, Hermann-Kleiter N, Klepsch V, Theurl I, Weiss G and Pfeifhofer-Obermair C (2020) Iron Supplementation Interferes With Immune Therapy of Murine Mammary Carcinoma by Inhibiting Anti-Tumor T Cell Function. Front. Oncol. 10:584477. doi: 10.3389/fonc.2020.584477

Iron is both, an essential compound for many metabolic processes, and iron deficiency can impact on the proliferation of cells including lymphocytes but also tumor cells. On the other hand, excess iron-catalyzed radical formation can induce cellular toxicity which has been previously demonstrated for T cells in hereditary iron overload. Despite these interconnections, little is known on the effects of clinically approved intravenous iron supplements for curing cancer-related anemia, on T cell differentiation, tumor proliferation, anti-tumor T cell responses and, of clinical importance, on efficacy of cancer immunotherapies. Herein, we analyzed the effects of intravenous iron supplementation on T cell function and on the effectiveness of anti-cancer chemotherapy with IL-2/doxorubicin or immunotherapy with checkpoint-inhibitor anti-PD-L1 in C57Bl/6N female mice with implanted E0771 mammary carcinomas. We found that iron application resulted to an increased availability of iron in the tumor microenvironment and stimulation of tumor growth. In parallel, iron application inhibited the activation, expansion and survival of cytotoxic $CD8^+$ T cells and of $CD4^+$ T helper cells type 1 and significantly reduced the efficacy of the investigated anti-cancer treatments. Our results indicate that iron administration has a tumor growth promoting effect and impairs anti-cancer responses of tumor infiltrating T lymphocytes along with a reduced efficacy of anti-cancer therapies. Iron supplementation in cancer patients, especially in those treated with immunotherapies in a curative setting, may be thus used cautiously and prospective studies have to clarify the impact of such intervention on the outcome of patients.

Keywords: T cell, immunotherapy, cancer prognosis, iron, immune checkpoint, mammary carcinoma

INTRODUCTION

Because of its high redox activity iron is a key component of several enzymatic processes. Virtually every cell of the body, including malignant cells, requires iron for its metabolism and proliferation. Especially, the production of hemoglobin during erythropoiesis consumes about 20–30 mg of iron per day and additional iron is needed for the synthesis of several enzymes. Most iron is provided by macrophages which ingest aged or damaged red blood cells (1). After phagocytosis, the heme of erythrocyte hemoglobin is mobilized to the cytoplasm, degraded by heme oxygenase 1, and molecular iron is exported from the macrophage *via* the iron-exporter ferroportin-1 to the circulation, a process which is negatively controlled by the hormone hepcidin (2). Iron in the circulation is transported bound to transferrin and is taken up by metabolically active and dividing cells *via* transferrin receptor-1 (3). The uptake of iron *via* transferrin receptor-1 is thus of highest relevance for the differentiation of rapidly dividing cells such as erythroblasts and lymphocytes (4, 5). As a consequence, mutations in the gene coding for transferrin receptor-1, *TFRC*, can cause combined immunodeficiency characterized by impaired function of B and T lymphocytes (6). On the other hand, an excess of intracellular iron in cells has to be stored within ferritin to avoid toxicity of labile iron *via* catalysis of hydroxyl radical formation (7, 8). Since iron is crucial for both microbes and mammalian cells, iron homeostasis undergoes subtle changes during infection and inflammatory processes resulting in sequestration of the metal within macrophages, thereby reducing circulating iron pools and making the metal less available for pathogens. This process, termed nutritional immunity, is mediated by various cytokines and hepcidin, whose expression gets upregulated upon multiple inflammatory and danger signals (9). Such alterations of iron homeostasis also occur in association with other inflammatory processes including cancer (10) characterized by normal or high iron stores as reflected by increased levels of ferritin whereas circulating iron levels and saturation of transferrin with iron are low. This functional iron deficiency causes iron limitation of erythroid progenitor cells and contributes to the development of so called anemia of inflammation (AI) or anemia of chronic disease (ACD) or anemia of cancer (11). In addition, this also limits iron availability for cancer but also for immune cells such as lymphocytes and may thus impact on anti-cancer immune effector function and even on the efficacy of anti-tumor immunotherapy. There is evidence from literature that this can be traced back to effects of iron on immune and cancer cell proliferation and differentiation, innate immune function and regulation of cellular metabolic processes including mitochondrial activity and micro RNA processing (10, 12–15).

Abbreviations: ACD, anemia of chronic disease; CFSE, Carboxyfluorescein succinimidyl ester; Ctrl, control; DCFDA, 2′,7′-Dichlorodihydrofluorescein diacetate; DOX, doxorubicin; ESA, erythropoiesis stimulating agents; IL-2, interleukin-2; IFNg, interferon gamma; NAC, N-acetyl cysteine; NTBI, non-transferrin bound iron; PD-L1, programmed death-ligand 1; RBC, red blood cell; ROS, reactive oxygen species; TBI, transferrin bound iron; Tc, cytotoxic T cell; Th, helper T cell; Treg, regulatory T cell.

Breast cancer is the most common type of cancer in women worldwide and, despite the enormous progress in diagnosis and treatment, it still represents one of the main causes of cancer-related death. Several studies have shown a link between dysregulation of iron metabolism and progression of breast cancer (16, 17). Particularly, spatio-temporal accumulation of iron in the tumor-microenvironment was linked to an increased cancer risk and poor outcome, respectively (18, 19). Mechanistically, apart from the effects of iron on immune function, the metal can stimulate cancer metabolism, alter iron dependent redox balance, which increases mutation rates, organelle damage, loss of tumor suppressors, oncogene expression and triggers pro-oncogenic signaling like Wnt and NFκB pathways (20–22).

Tumor growth and progression can be both enhanced and inhibited by cells of the immune system including T cells by a process which is called immunosurveillance (23). T lymphocytes as components of the adaptive immune system can destroy tumor cells *in situ*. The predominant tumor infiltrating lymphocytes are $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells, and regulatory T cells (24). $CD4^+$ T cells are classified into T_H1 cells secreting proinflammatory cytokines like IFNγ and IL-2, whereas T_H2 cells secrete IL-4, IL-5, IL-10, and IL-13. T_H2 cytokines induce T cell anergy and lead to an increase of humoral B cell function (25, 26). The primary role of $CD4^+$ helper T cells in tumor response is to assist in the activation of $CD8^+$ T cell mediated cell killing. Most tumor cells are positive for MHC class I, but negative for MHC class II, which makes the primary anti-tumor response dependent on $CD8^+$ cytotoxic T cells (27). In cancer patients a tumor response involving $CD8^+$ T cells, T_H1 $CD4^+$ T cells, and IFNγ producing natural killer cells is associated with a better prognosis (28). In contrast, a B cell and T_H2 polarized response can promote tumor development and progression (28). Immunosuppressive effects of iron on the T cell response have been described. Iron can trigger $CD4^+$ differentiation towards a T_H2 phenotype (14, 29) and impact on $CD8^+$ cell numbers (30). A similar impairment of T cell function has been observed in individuals with hereditary or transfusion mediated iron overload (31, 32).

Of note, individuals carrying the homozygous *HFE* C282Y mutation, the most common cause for hereditary hemochromatosis, are at increased risk of developing cancer, including breast cancer (19). Whether this is a direct consequence of iron toxicity or related to quantitative or qualitative alterations in T cell subsets remains unknown (33).

In spite of the direct effects of iron on tumor cells and anti-tumor immunity, the impact of intravenous iron preparations used for treatment of cancer related anemia towards the further clinical course and outcome of cancer along with their impact on specific cancer therapy is still unknown (34, 35). On the one hand, the functional iron deficiency caused by tumor-accompanying inflammation may be regarded as a measure to limit tumor progression, on the other hand, iron deficiency and ACD may result in suboptimal delivery of iron needed for immune cell function.

Herein we demonstrate that isomaltosoide, an iron formulation used for correction of iron deficiency in humans, negatively impacts on the efficacy of cancer immunotherapy and combined

IL-2/doxorubicin chemo-immunotherapy in a murine E0771 mammary carcinoma model. *In vivo*, iron supplementation led to accelerated cancer growth and impaired efficacy of the investigated therapy protocols along with diminished tumor infiltration by cellular effectors of anti-tumor response, T_H1 and cytotoxic T cells. Mechanistically we show that iron, both in transferrin-bound and non-transferrin bound form, dramatically brakes $CD4^+$ and $CD8^+$ T cell proliferation and cytokine production and promotes cell death.

MATERIAL AND METHODS

Cell Line

E0771 mouse adenocarcinoma cells (obtained from ATCC) were maintained in DMEM (Dulbecco`s Modified Eagles`s Medium; PAN Biotech) plus 10% fetal calf serum (FCS; Biochrom) plus 1% penicillin/streptomycin (Lonza) plus 2 mM L-glutamine (Lonza) at 37°C, plus 5% carbon dioxide (36).

Mice

Female C57Bl/6N mice (obtained from Charles River) had free access to food and water and were housed according to institutional and governmental guidelines in the animal facility of the Medical University of Innsbruck with a 12-hour light-dark cycle and an average temperature of 20°C ± 1°C. Animals were kept on a standard rodent diet (SNIFF, Soest, Germany). Blood was taken through the facial vein and blood counts were measured with a VetABC Animal Blood Counter. Animal experiments were approved by the Austrian Federal Ministry of Science and Research (BMWF-66.011/0117-WF/V/3b/2017) according to the directive 2010/63/EU.

Implantation of Tumors

C57Bl/6 derived E0771 adenocarcinoma cells were washed twice in PBS and 2.5×10^5 cells injected into one of the inguinal mammary glands into 8–12 weeks old female C57Bl/6N mice under short-term inhalation anesthesia with isoflurane. Three days after tumor implantation mice were given intravenously 2 mg elementary iron in the form of iron isomaltoside (Monofer; Pharmacosmos) or PBS. Tumor growth was monitored weekly by caliper measurements of length (l) and width (w). Tumor volume was calculated with the formula $V = lw^2\pi/6$. Three weeks after tumor implantation mice were sacrificed by cervical dislocation, and tumors were isolated by surgical excision.

Tumor Therapy

For checkpoint immunotherapy, tumor-bearing mice were intraperitoneally administered anti-mouse PD-L1 antibodies (0.5 mg/animal, clone10F.9G2; BioXCell) every third day starting from day 1 after tumor implantation. For chemo-immunotherapy, doxorubicin (5 mg/kg, Accord) was administered intraperitoneally into tumor-bearing mice once on day eight after tumor implantation and recombinant murine IL-2 (100,000 IU per animal, Peprotech) daily starting on day nine after tumor implantation (37).

Isolation of Tumor-Infiltrating Lymphocytes

Tumor tissue was minced and digested with Liberase TM (0.15 Wünsch-Units/ml, Roche) and 10 µg/ml DNaseI (Roche) in FCS-free RPMI-1640 (PAN Biotech) medium with constant mixing (250 rpm), at 37°C for 1 h. Tumor cell suspension was collected through a 100 µm cell strainer into a tube containing RPMI-1640 (PAN Biotech) plus 10% FCS (Biochrom) plus 1% penicillin/streptomycin (Lonza) plus 2 mM glutamine (Lonza) and centrifuged at 300*g* for 5 min. Red blood cells were lysed by incubation in ACK buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA) for 2 min at room temperature. Cell suspension was filtered through a 40 µm cell strainer and used for flow cytometry staining.

Flow Cytometry Analysis

Flow cytometry staining was performed with panels of antibodies specific for activated/memory T cells (αCD3-Biotin, αCD4-FITC, αCD8-APCeF780, αCD62L-PeCy7, αCD44-APC; all from BioLegend) in PBS with 0.5% FCS 2 mM EDTA for 15 min. For intracellular staining cells will be stimulated with a mix containing 10 µg/ml Brefeldin A (Sigma), 50 ng/ml PDBu (Sigma) and 500 ng/ml ionomycin (Sigma) in RPMI-1640 (PAN Biotech) plus 10% FCS (Biochrom) plus 1% penicillin/streptomycin (Lonza) plus 2 mM L-glutamine (Lonza) for 4 h. The cells were then formalin-fixed, permeabilized (0.05% Triton X-100 in PBS) and stained for cytokines (αIL-2-PE, αIFNγ-PeCy7), and transcription factors (αFOXP3-FITC) or perforin (αPerforin-APC) for 1 h. All antibodies were from Biolegend. Cells were analyzed with Gallios and Cytoflex S flow cytometers (Beckman Coulter) and FlowJo Software (Beckton Dickinson).

Splenocyte Cell Culture

Spleens were isolated from tumor-naive female C57Bl/6N mice. After lysis of erythrocytes using the Mouse Erythrocyte Lysing Kit (R&D Systems) 2.5×10^5 splenocytes per well were then seeded in a 96-well round bottom plate and stimulated with 4 µg/ml plate-bound or 1 µg/ml soluble rat anti-mouse CD3 (clone 17A2; BD Pharmingen). Ferric chloride $FeCl_3$ (Sigma Aldrich), ferric sulfate $Fe_2(SO_4)_3$ (Sigma Aldrich), ferric citrate $FeC_6H_5O_7$ (Sigma Aldrich), and holo-transferrin were added at concentrations of 2.5µM, 5 µM, 10 µM and 20 µM elementary iron. Splenocytes were cultured in RPMI-1640 medium (PAN Biotech) supplemented with 10% FCS (Biochrom), 2% sodium pyruvate (Sigma), 1× non-essential amino acids (Gibco), 0.01% β-mercaptoethanol (Roth), 1% penicillin/streptomycin (Lonza) and 2 mM L-glutamine (Lonza).

BrdU Labeling of Splenocytes

Splenocytes were cultured as described before and pulsed with 10 µM BrdU (Sigma-Aldrich) 4 h before harvesting. Intracellular staining for BrDU with surface co-staining for CD3, CD4 and CD8 was performed with BrdU Flow Kit (BD) according to the manufacturers` instructions and cells were analyzed with flow cytometry. Iron sources ferric chloride $FeCl_3$, ferric sulfate

$Fe_2(SO_4)_3$, ferric citrate $FeC_6H_5O_7$, and holo-transferrin were added at indicated concentrations.

CFSE Labeling of Splenocytes

Before culture splenocytes were washed twice with PBS and labeled with 2.5 μM CFSE (eBioscience) in PBS for 10 min at 37°C, followed by a wash with RPMI-1640 (PAN Biotech) supplemented with 10% FCS (Biochrom). CFSE dilution after 96 h of culture was measured with flow cytometry. Where indicated, the ferroptosis inhibitor Ferrostatin-1 (1 μM, Sigma), cytoplasmic ROS scavenger NAC (N-acetylocysteine, 10 mM, Sigma), necroptosis inhibitor Necrostatin-1 (30 μM, Sigma), mitochondrial ROS scavenger MitoTEMPO (20 μM, Sigma), or the caspsase-3-inhibitor z-DEVD-FMK (20 μM, BD) were added. Iron was added in the form of 5 μM ferric citrate.

Iron Measurement

Tissue iron was quantified using a colorimetric method with bathophenanthroline disulfonic acid (38). In brief, organ lysates were hydrolyzed with acid for 24 h at 65°C, mixed with a colorimetric solution containing sodium acetate, bathophenanthroline disulfonic acid and l-ascorbic acid and absorbance at 539 nm was measured. The iron content of the organ was calculated from a standard curve and normalized to the protein content of the lysate determined by the Bradford method.

ROS Measurement

Splenocytes were cultured as described before. For the determination of mitochondrial and cytoplasmic ROS, cells were stained with 2.5 μM MitoSOX (Thermofisher) and 2.5 μM DCFDA (Sigma), respectively, for 30 min at 37°C and analyzed by flow cytometry. Splenocytes stimulated for 4 h with the inhibitor of mitochondrial oxidative phosphorylation rotenone (2.5 μM, Sigma) served as a positive control for cytoplasmic and mitochondrial ROS.

Statistics

Statistical analysis was performed with GraphPad Prism 7 and R programming suite (version 3.6.3) with a tidyverse package bundle and ggplot2 graphics library. If not stated otherwise, data are plotted as mean with SEM presented as bars and whiskers and single animals/observations presented as points or symbols. Normality of variable distribution was assessed by Shapiro–Wilk test and visual inspection of the quantile–quantile plots. Statistical significance for two-group comparisons was determined by a two-tailed T-test for normally distributed variables and by the Mann–Whitney U test for non-normally distributed variables. Statistical significance for comparisons of more groups/factors was analyzed by one- or two-way ANOVA, as appropriate, with Tuckey post-hoc test.

Differences in tumor growth rate (**Figure 1**) (1) between the untreated tumor bearers and treatment groups (iron alone, immunotherapy alone, and iron with immunotherapy) and (2) between the immunotherapy- and immunotherapy/iron-treated animals were analyzed with separate mixed-effect multiple linear regression models (fixed effects: time point and therapy group: time point interaction, random effect: individual animal, R packages lme4 and lmer test). Regression estimates for the therapy group: time point interaction term was assumed to model differences in tumor growth rate (1) between the untreated animals and the respective therapy regimen and (2) between the immunotherapy and immunotherapy/iron group.

Statistical significance for differences in T cell counts in cultures stimulated with iron, ROS scavengers or inhibitors of cell death (**Figure 8**) was determined with mixed-effect linear modeling (fixed effects: iron, cell death/ROS inhibitor and the iron: cell death/ROS inhibitor interaction; random effect: cell donor). The estimate of the iron: cell death/ROS inhibitor interaction term was deemed the measure of reversal of iron effects on T cell expansion.

In linear modeling, statistical significance for the regression estimates was determined by a two-tailed T-test (estimate ≠ 0;

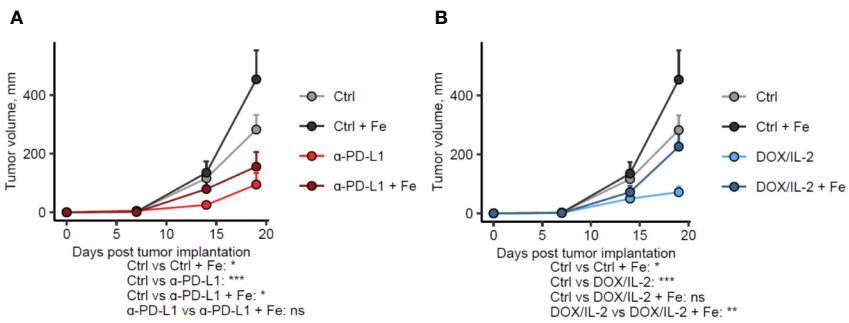

FIGURE 1 | Administration of iron negatively influences the efficacy of different immunotherapies. Female C57Bl/6 mice were subcutaneously implanted with E0771 cells (2.5×10^5 cells per animal), supplemented with intravenous iron isomaltoside (Fe, 2 mg elementary iron per animal) 3 days after tumor implantation and treated with anti-PD-L1 **(A)** or IL-2 and doxorubicin **(B)** as described in *Materials and Methods*. Therapy-naive: n = 17, therapy-naive/iron: n = 5, anti-PD-L1: n = 14, anti-PD-L1/iron: n = 17, IL-2/doxorubicin: n = 13, IL-2/doxorubicin/iron: n = 14. Tumor volume was determined weekly by caliper measurements. Statistical significance was determined by mixed-effect multiple linear regression (fixed effects: time point and time point: treatment group interaction, random effect: individual animal). Group means with SEM are presented. P values were corrected for multiple comparisons with Benjamini–Hochberg method. P values for differences in growth rate between the untreated control and the given group and for the differences in growth rate between the immunotherapy and immunotherapy/iron groups (the time point: treatment group interaction term estimates) are presented under the plots. ns: not significant, *p < 0.05, **p < 0.01, ***p < 0.001.

degrees of freedom calculated with Satterthwaite formula, package ImerTest) and corrected for multiple comparisons with the Benjamini–Hochberg method.

Specific statistical data analyzed in main figures:

Figures 1A, B: Mixed-effect multiple linear regression (fixed effects: therapy group and therapy group: timepoint interaction, random effect: individual animal). P values for the time:therapy interaction model terms are shown in the plots.

Figure 2: Two-way ANOVA (**A**), (**B**), (**C**), (**D**) ns.

Figure 3B: 2-way ANOVA: treatment IL-2 + doxo, $F_{(1, 41)}$ = 22 P <0.0001; iron, $F_{(1, 41)}$ = 3.2 ns; treatment:iron interaction, $F_{(1, 41)}$ = 6.9, P = 0.012; treatment aPD-L1, $F_{(1, 45)}$ = 25, P <0.0001; iron, $F_{(1, 45)}$ = 3.5, ns; treatment:iron interaction, $F_{(1, 45)}$ = 6.2, P = 0.016; Tukey`s post test results presented in the plots.

Figure 3C: 2-way ANOVA: treatment IL-2 + doxo, $F_{(1, 52)}$ = 1.7, ns; iron, $F_{(1, 52)}$ = 6.6, P = 0.013; treatment:iron interaction, $F_{(1, 52)}$ = 7.5, P = 0.0086; treatment aPD-L1, $F_{(1, 46)}$ = 0.00030, ns; iron, $F_{(1, 46)}$ = 4.3, P = 0.044; treatment:iron interaction, $F_{(1, 46)}$ = 4.9, P = 0.032; Tukey`s post test results presented in the plots.

Figure 4A: 2-way ANOVA: treatment IL-2 + doxo, $F_{(1, 54)}$ = 6.55, P = 0.014; iron, $F_{(1, 54)}$ = 3.1, ns; treatment:iron interaction, $F_{(1, 54)}$ = 1.4, ns; treatment aPD-L1, $F_{(1, 49)}$ = 1.8, ns; iron, $F_{(1, 49)}$ = 1.2, ns; treatment:iron interaction, $F_{(1, 49)}$ = 1.3, ns; Tukey`s post test results presented in the plots.

Figure 4B: 2-way ANOVA: treatment IL-2 + doxo, $F_{(1, 59)}$ = 0.96, ns; iron, $F_{(1, 59)}$ = 0.0091, ns; treatment:iron interaction, $F_{(1, 59)}$ = 0.080, ns; treatment aPD-L1, $F_{(1, 54)}$ = 5.5, ns; iron, $F_{(1, 54)}$ = 0.27, ns; treatment:iron interaction, $F_{(1, 54)}$ = 0.024, ns; Tukey`s post test results presented in the plots.

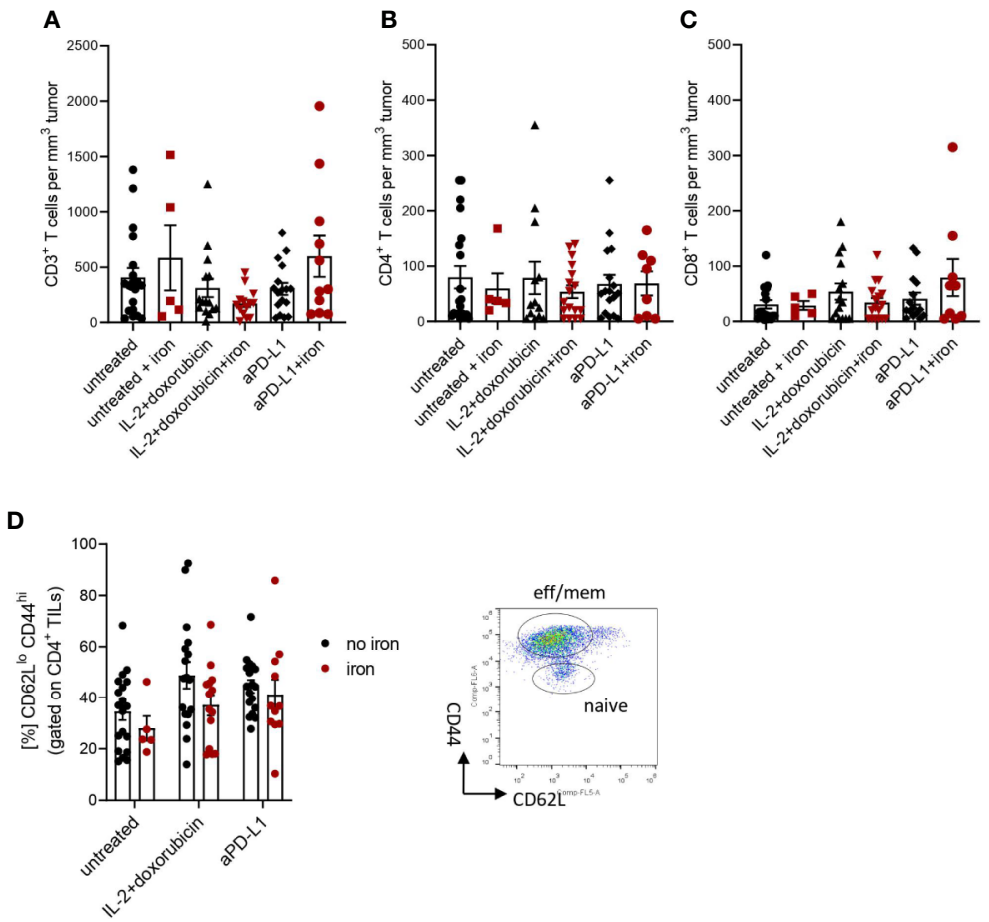

FIGURE 2 | Administration of intravenous iron has no influence on the numbers of effector $CD3^+$ **(A)**, $CD4^+$ **(B)**, $CD8^+$ **(C)** and effector-memory **(D)** tumor infiltrating lymphocytes in different immunotherapeutic settings. Naive TILs were identified as $CD62L^{hi}CD44^{lo}$, effector-memory TILs were described as $CD62L^{lo}CD44^{hi}$ in tumors 21 days post implantation. Mean with SEM is presented in the plots. Statistical significance was determined by 2-way ANOVA. untreated n = 20, untreated + iron n = 5, IL-2 + doxorubicin n = 15, IL-2 + doxorubicin + iron n = 14, aPD-L1 n = 17, aPD-L1 + iron n = 11. The results of ANOVA are presented in *Materials and Methods*/Specific statistical data analysed in main figures.

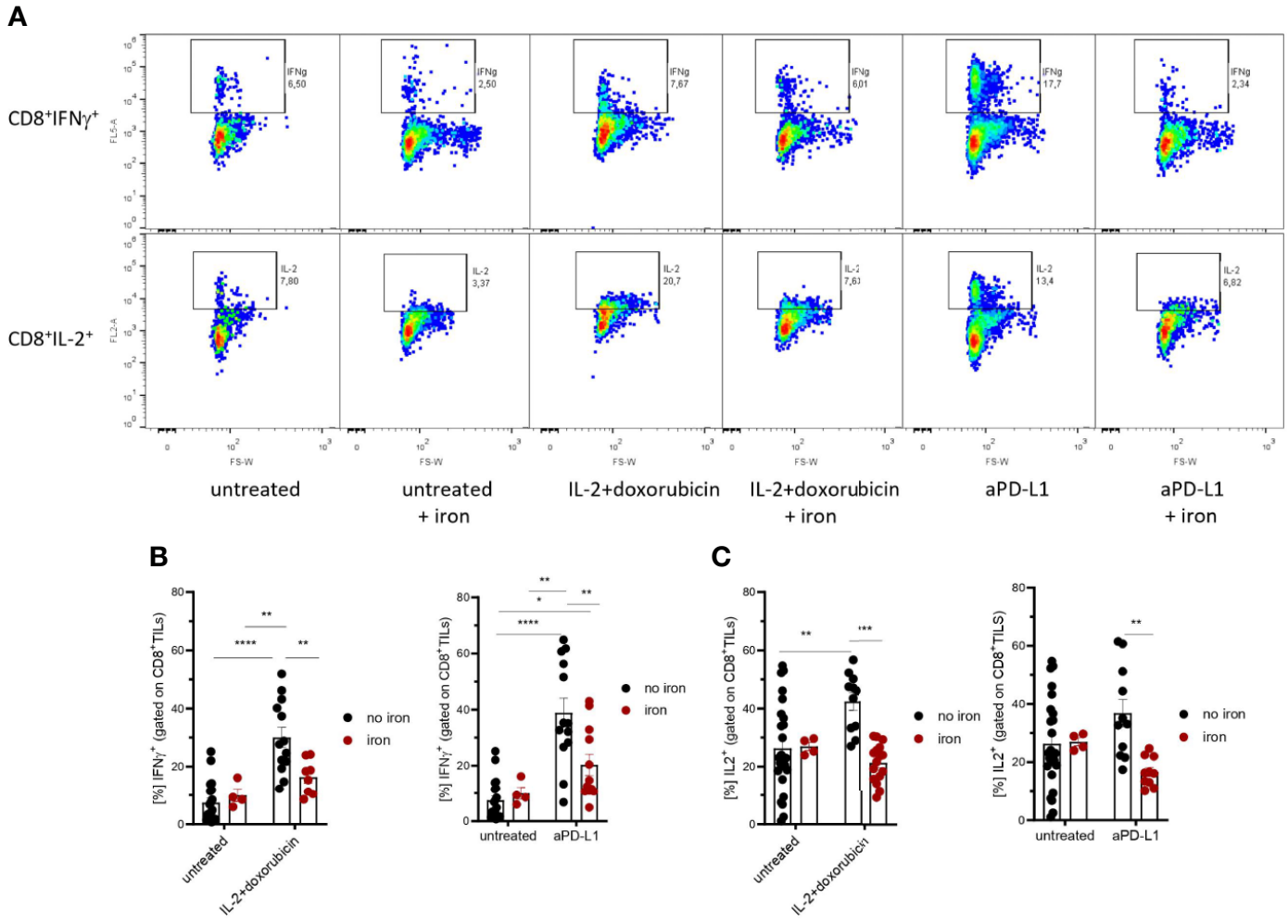

FIGURE 3 | Effects of intravenous iron on functional T cell subsets. Intravenous iron supplementation significantly reduces the function of $CD8^+$ tumor infiltrating lymphocytes **(A–C)**. Representative plots are shown (mean ± SEM). Statistical significance was determined by 2-way ANOVA. The results of Tuckey post-hoc-test are presented in the plots: ns: not significant, *p < 0.05, ** p < 0.01, ***p < 0.001, ****p < 0.0001. untreated n = 20, untreated + iron n = 4, IL-2 + doxorubicin n = 13, IL-2 + doxorubicin + iron n = 8, aPD-L1 n = 13, aPD-L1 + iron n = 12. The results of ANOVA are presented in *Materials and Methods*/Specific statistical data analyzed in main figures.

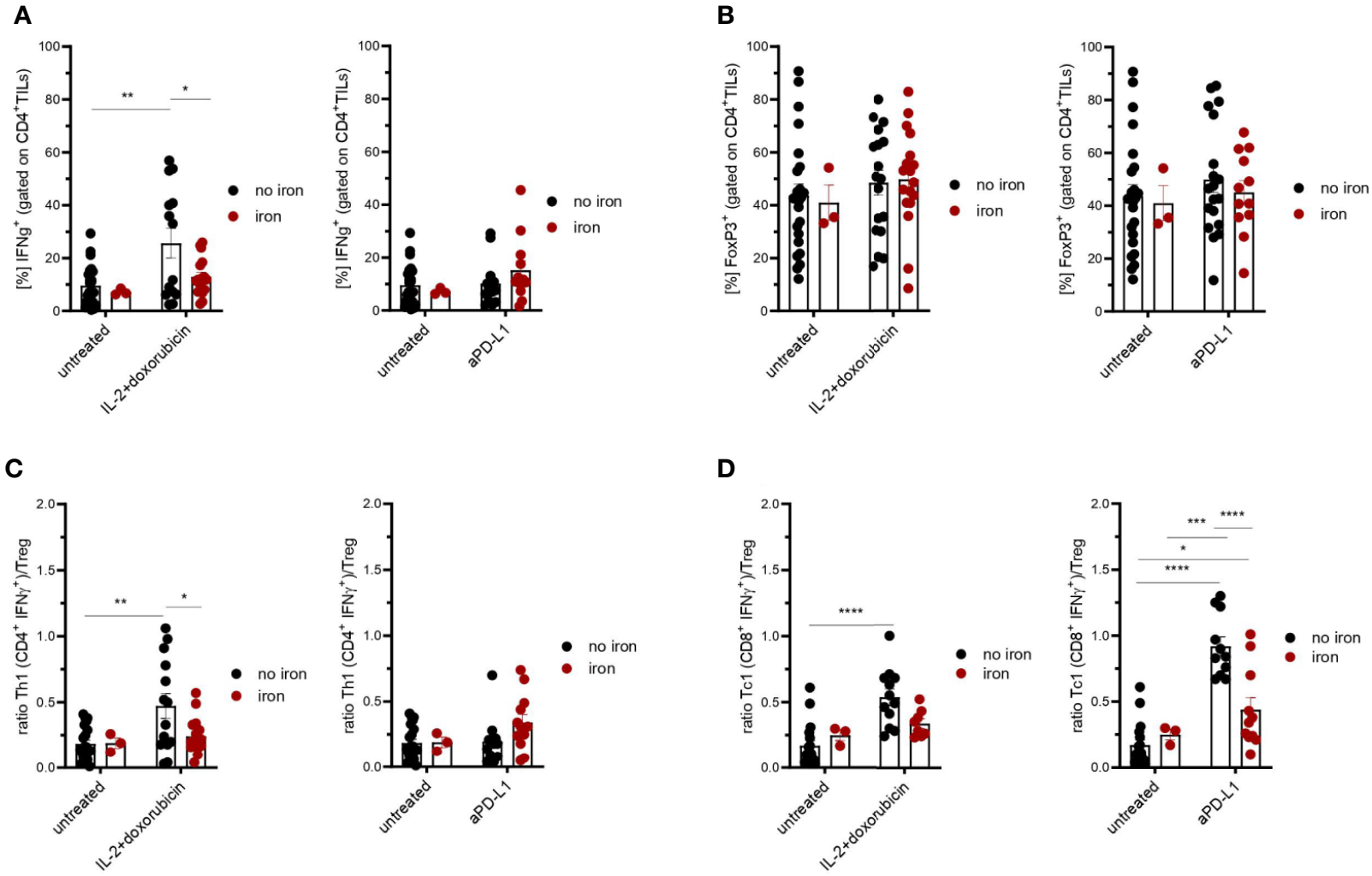

FIGURE 4 | Effects of intravenous iron on $CD4^+$ TILs **(A)**, regulatory T cells **(B)** and Th1/Treg or Tc1/Treg ratios **(C, D)** in immunotherapy and chemoimmunotherapy of mouse mammary carcinomas. Ratios Th1/Treg **(C)** and Tc1/Treg **(D)** were calculated. Representative plots are shown (mean ± SEM). Statistical significance was determined by 2-way ANOVA. The results of Tuckey post-hoc-test are presented in the plots: ns: not significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. untreated n = 24, untreated + iron n = 3, IL-2 + doxorubicin n = 14, IL-2 + doxorubicin + iron n = 17, aPD-L1 n = 13, aPD-L1 + iron n = 12. The results of ANOVA are presented in *Materials and Methods*/Specific statistical data analyzed in main figures.

Figure 4C: 2-way ANOVA: treatment IL-2 + doxo, $F_{(1, 50)} = 5.0$, P = 0.030; iron, $F_{(1, 50)} = 2.2$, ns; treatment:iron interaction, $F_{(1, 50)} = 2.4$, ns; treatment aPD-L1, $F_{(1, 45)} = 1.9$, ns; iron, $F_{(1, 45)} = 1.6$, ns; treatment:iron interaction, $F_{(1, 45)} = 1.4$ ns; Tukey`s post test results presented in the plots.

Figure 4D: 2-way ANOVA: treatment IL-2 + doxo, $F_{(1, 36)} = 12$, P = 0.0017; iron, $F_{(1, 36)} = 0.80$, ns; treatment:iron interaction, $F_{(1, 36)} = 4.4$, P = 0.042; treatment aPD-L1, $F_{(1, 38)} = 30$, P <0.0001; iron, $F_{(1, 38)} = 5.5$, ns; treatment:iron interaction, $F_{(1, 38)} = 11$, P = 0.0022; Tukey`s post test results presented in the plots.

Figure 5: one-way ANOVA for particular iron forms, ANOVA p values presented in the plot legends.

Figure 6A: two-tailed T test to compare the percentages of $CFSE^{hi}$, $CFSE^{med}$ and $CFSE^{lo}$ cells between control- and iron-stimulated cultures, corrected for multiple comparisons with Benjamini–Hochberg method. P values presented in the pie plot.

Figure 6B: two-tailed T test, p values presented in the plot.

Figure 6C: two-way ANOVA: iron, $F_{(1, 15)} = 7.2$, p = 0.017; T cell–target ratio, $F_{(1, 15)} = 2.1$, ns; iron: T cell–target ratio interaction, $F_{(1, 15)} = 0.99$, ns; Tukey`s post test results presented in the plots.

Figure 7: Two-tailed T test for control–iron comparisons, p values presented in the plots.

Figure 8: Mixed-effect multiple linear regression (fixed effects: fixed effects: iron, cell death/ROS inhibitor and the iron: cell death/ROS inhibitor interaction, random effect: cell donor). P values for the iron: cell death/ROS inhibitor interaction interaction model terms are shown in the Forest plots.

Chromium release assay: Murine B16/OVA melanoma cells as target cells were cultivated in DMEM medium (PAN Biotech) supplemented with 10% FCS (Biochrom), 1% penicillin/streptomycin (Lonza) and 2 mM L-glutamine (Lonza). The assay was performed as described (39). Briefly, 2×10^6 target cells were labeled with 200 µCi $Na_2Cr^{51}O_4$ (specific activity 300 to 500 Ci/g chromate; Hartmann Analytik) for 1 h at 37°C, washed once, and resuspended at a concentration of 5×10^4/ml in medium. As effector cells $CD8^+$ T cells were isolated from spleens of C57BL/6-Tg(TcraTcrb) 1,100 Mjb/Crl mice (OTI mice) with the help of the MagniSort Mouse CD8 T cell Kit (Thermo Fisher Scientific). 2.5×10^6 cells/ml were then seeded in a 96 well U-bottom plate (Falcon) and stimulated with 1 µg/ml soluble rat anti-mouse CD3 (clone 17A2; BD Pharmingen) and 1 µg/ml hamster anti-mouseCD28 (clone 37.51; BD Pharmingen) in RPMI-1640 medium (PAN Biotech) supplemented with 10% FCS (Biochrom), 2% sodium pyruvate (Sigma), 1× non-essential aminoacids (Gibco), 0.01% β-mercaptoethanol (Roth), 1% penicillin/streptomycin (Lonza) and 2 mM L-glutamine (Lonza). After 24 h $CD8^+$ T cells were primed for 1 h with 1 µg/ml OVA (257–264) (Anaspec). About 20 µl of target cells (5×10^3) were incubated with 200 µl of various amounts of effector cells with effector:target (E:T) ratios ranging from 30:1 to 7.5:1. After 4 h of incubation in a humidified 5% CO_2/95% air atmosphere, 100 µl of the culture supernatant were counted with a gamma-scintillation counter. Results are presented as percentage of specific lysis.

RESULTS

The administration of intravenous iron is an established therapy for cancer-related anemia but its effects on the underlying malignancy, anti-tumor immunity and efficacy of tumor immunotherapy remain incompletely understood. We thus investigated the effects of intravenous administration of a clinically applicable iron preparation, ferric isomaltoside, in the implantable E0771 mouse mammary carcinoma model. Of note, mice bearing E0771 neoplasms display mild impairment of erythropoiesis as demonstrated by a significantly reduced blood hemoglobin content and hematocrit as compared with tumor-free mice (**Supplementary Figure 1**), hence, in part, mimicking cancer-anemia phenotype observed at a substantial percent of breast cancer patients.

Iron concentrations used for *in vivo* iron studies in mice differ a lot (0.27–35 mg per mouse) and furthermore the basal metabolic rate per gram body weight in mice is higher than in humans (40). Therefore, we used a supra-clinical dose of 2 mg per mouse (approx. 100 mg/kg, corresponding to 6–8 g in humans), which was found to cause a significant accumulation of iron in the canonical iron-storage organs, spleen and liver, in tumor-free animals (spleen P = 0.033; liver P = 0.0013, **Supplementary Figures 2A, B**). To investigate, if such iron supplementation may cause a similar iron accumulation in the tumor tissue, we implanted E0771 adenocarcinoma cells into wildtype C57Bl/6N female hosts followed by intravenous administration of ferric isomaltoside 3 days after tumor implantation. As shown in **Supplementary Figure 2C,** we could not observe any increase of tissue iron measured with the colorimetric, bathophenanthroline disulfonic acid-based assay in the neoplastic tissue on day 21 post implantation arguing against an overt iron overload in the tumor like in the liver and spleen. mRNA levels of transferrin receptor 1 (TFR1 or CD71) are tightly negatively regulated by biologically active intracellular iron (41) and, thus, cell surface levels of the protein may be used as a sensitive surrogate marker for gauging iron availability in the tumor microenvironment. Interestingly, both $CD45^-$ tumor epithelial cells as well as $CD45^+$ tumor-infiltrating leukocytes isolated from the iron-treated E0771 tumor mice demonstrated significantly decreased cell surface levels of CD71 (tumor epithelium and leukocytes) and percentages of CD71-positive cells (tumor epithelium) indicative of a better availability of reactive iron in the tumor milieu upon systemic intravenous iron supplementation (**Supplementary Figure 2D**).Our data indicate that intravenous iron accumulates in the spleen and liver without altering the total iron content of the tumor tissue and increasing the local intracellular availability of reactive iron in the malignant tissue.

As a therapy, mice were either treated with immunotherapy in the form of repeated anti-PD-L1 antibody injections every third day, starting at tumor implantation, or chemo-immunotherapy in the form of single doxorubicin injection followed by daily

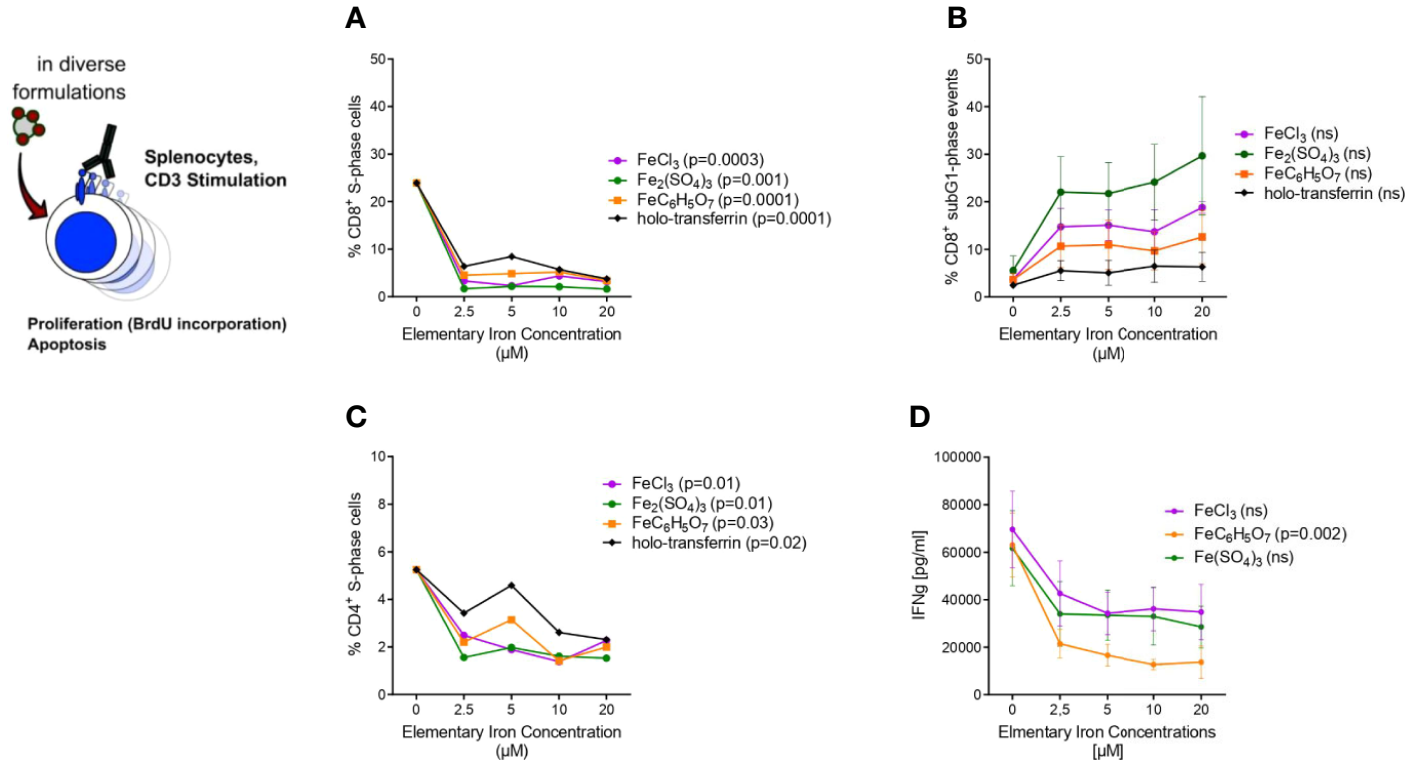

FIGURE 5 | Transferrin bound and non-transferrin bound iron impairs T cells proliferation and promotes apoptosis. Splenocytes isolated from tumor-naive C57Bl/6N mice were stimulated with plate-bound anti-CD3 antibodies and supplemented with iron in the form of holo-transferrin (transferrin bound iron, TBI), ferric chloride $FeCl_3$, ferric sulfate $Fe_2(SO_4)_3$, or ferric citrate $FeC_6H_5O_7$ (non-transferrin bound iron, NTBI). BrdU incorporation and cell cycle distribution in $CD4^+$ and $CD8^+$ T cells was measured by flow cytometry **(A–C)** and IFNγ concentration in culture supernatant was determined by Multiplex 72 h after culture start **(D)**. Statistical significance was assessed by one-way ANOVA for each iron source. Each point represents mean with SEM from n = 3 independent experiments.

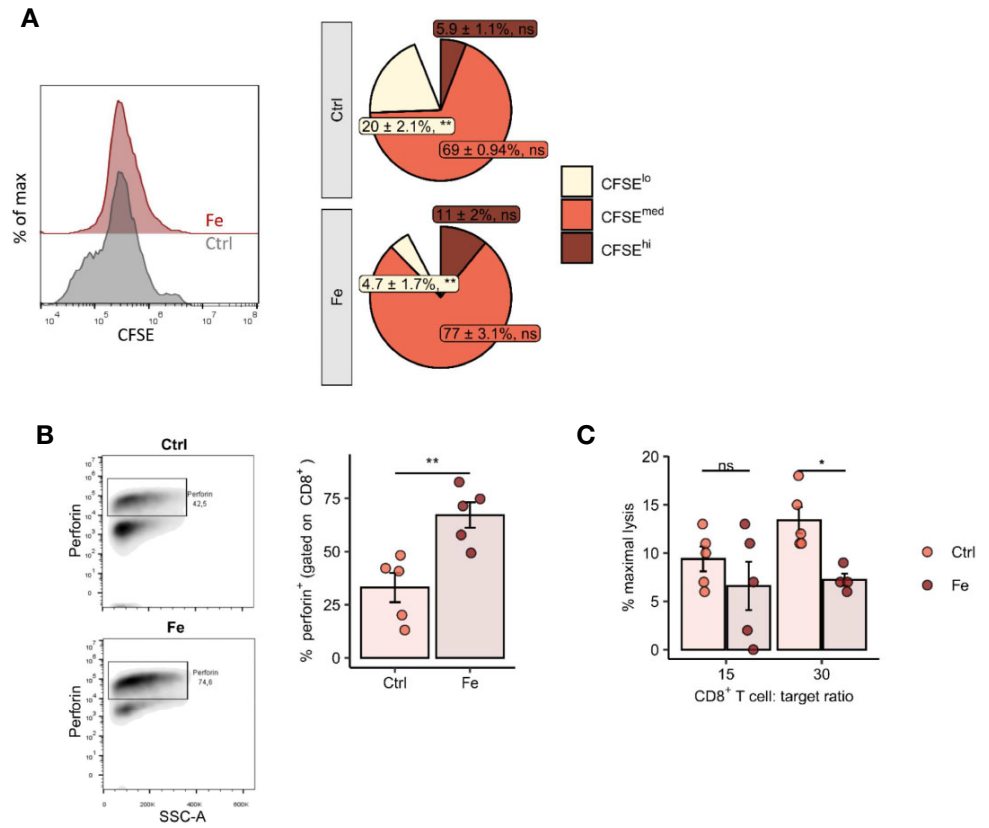

FIGURE 6 | *In vitro* addition of iron to splenocytes decreases the number of proliferating $CD8^+$ T cells (CFSE low) **(A)**, negatively affects perforin degranulation in $CD8^+$ cytotoxic T cells **(B)** and significantly reduces the $CD8^+$ T cell dependent lysis of target cells **(C)**. **(A, B)** Splenocytes isolated from tumor-naive C57Bl/6N mice were stimulated with plate-bound anti-CD3 antibodies and iron in form of iron citrate ($FeC_6H_5O_7$; non-transferrin bound iron, NTBI) was added. Proliferation of $CD8^+$ T cells was measured by flow cytometry depending on CFSE 72h after culture start. Data are presented as Pie Plots (mean ± SEM) n = 4. Perforin was stained intracellularly as described in *Materials and Methods* n=5. Statistical significance was determined by a two-tailed T-test and corrected for multiple comparisons with the Benjamini–Hochberg method. **(C)** The capability of iron treated and non-iron treated $CD8^+$ T cells to lyse target cells was measured with a chromium release assay as described in *Material and Methods*. Representative flow cytometry results and summary plots are shown (mean ± SEM). Statistical significance was determined by 2-way ANOVA. The results of Tuckey post-hoc-test are presented in the plots: ns: not significant, *p < 0.05, **: p < 0.01. control, Fe (ratio 15:1) n = 5, control, Fe (ratio 30:1) n = 4. The results of ANOVA are presented in *Materials and Methods*/Specific statistical data analyzed in main figures.

administration of highly dosed IL-2 starting on day 8 after tumor implantation.

Checkpoint immunotherapy with anti-PD-L1 or combined chemo-immunotherapy with IL-2 and doxorubicin significantly reduced tumor growth as compared with therapy-naive mice (p = 0.00031 and p = 0.00011, respectively), whereas iron supplementation without any therapy led to a significantly faster tumor progression (p = 0.039) for comparison with therapy- and iron-naïve animals. In addition, intravenous iron supplementation led to a substantial albeit not significant reduction of the therapeutic effects of checkpoint anti-PD-L1 therapy (**Figure 1A,** p = 0.016 for the therapy naive–anti-PD-L1/iron group comparison and p = 0.10 for the anti-PD-L1–anti-PD-L1/iron group comparison) and to a significant reduction of the efficacy of IL-2/doxorubicin therapy (**Figure 1B**, p = 0.15 for the therapy naive–IL-2/doxorubicin/iron group comparison and p = 0.0032 for the IL-2/doxorubicin–IL-2/doxorubicin/iron group comparison). We then studied whether the impaired therapeutic effect of both therapies upon iron loading is linked to the function of tumor infiltrating lymphocytes, such as $CD8^+$ cytotoxic T cells (Tc1), which are the responsible subset for effective anti-tumor T cell response, and/or $CD4^+$ T helper cells which are needed as assist to ensure full functionality of $CD8^+$ cytotoxic T cells.

Therefore, tumor infiltrating T cells were isolated. Interestingly, we could not detect any significant, iron- or cancer therapy-dependent differences in the numbers of $CD3^+$, $CD4^+$ and $CD8^+$ tumor infiltrating lymphocytes per mm^3 tumor (**Figures 2A–C** respectively). Although the percentage of $CD4^+$ effector-memory cells ($CD4^+CD44^{hi}CD62L^{lo}$) was consistently reduced in mice receiving intravenous iron, these changes were not statistically significant (**Figure 2D**). However, when we further studied the function of tumor infiltrating lymphocytes, we found that intravenous iron supplementation significantly reduced the production of cytokines IL-2 and IFNγ by tumor $CD8^+$ cytotoxic T cells, indicating iron-dependent reduced functionality of these cells (**Figures 3A, B, C**). Of note, also $CD4^+$ T helper cells in our

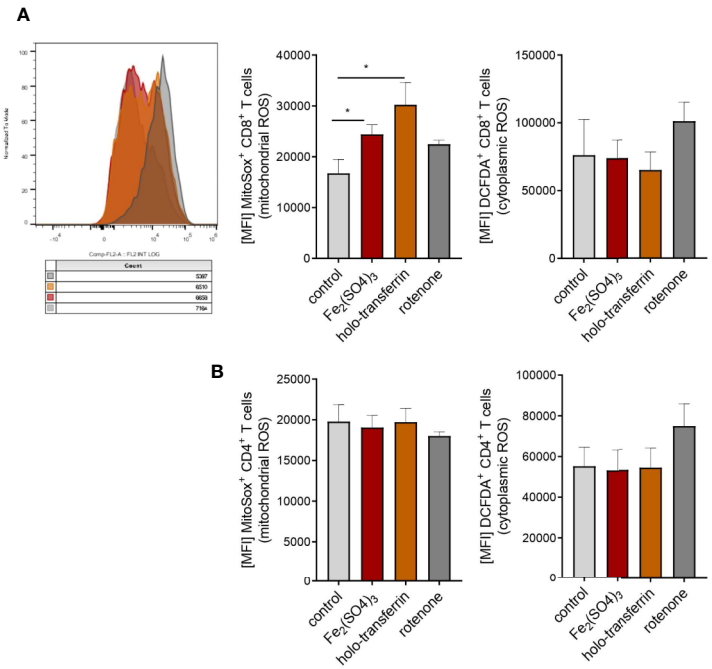

FIGURE 7 | Iron administration to splenocytes leads to oxidative stress and increased production of mitochondrial reactive oxygen species (ROS). Splenocytes were isolated from tumor-naive C57Bl/6N female mice and cultured in 96 well plates coated with anti-CD3. $Fe_2(SO_4)_3$ and holo-transferrin were added as NTBI and TBI, the inhibitor of oxidative phosphorylation rotenone was used as a positive control for ROS formation. After 24h DCFDA$^+$ and MitoSox$^+$ CD8$^+$ T cells **(A)** and CD4$^+$ T cells **(B)** were analysed by flow cytometry. DCFDA is defined as indicator for cytoplasmic ROS, MitoSox for mitochondrial ROS. Statistical significance was determined by Student`s t-test. Representative flow cytometry results and summary plots are shown (mean±SEM; n = 3).

tumor model showed reduced functionality as reflected by reduced production of IFNγ. However, this effect could only be demonstrated in IL-2/doxorubicin treatment (**Figure 4A**). Of interest, we could not find any differences in the percentage of regulatory T cells (CD4$^+$FoxP3$^+$) (**Figure 4B**). Although T_H1/Treg ratios were found significantly lowered by iron solely for the IL-2/doxorubicin protocols, the highly significantly diminished Tc1/Treg ratios upon iron supplementation could be observed for both treatments, most of all in anti-PD-L1 treatment (**Figures 4C, D**).

Based on these observations, we asked whether iron generally influences proliferation and cytokine production of CD8$^+$ cytotoxic T cells and CD4$^+$ T helper cells. In the body fluids, iron can generally exist in two forms: as transferrin-bound iron (TBI), when iron concentration does not exceed binding capacities of transferrin, and as chemically reactive, potentially toxic non-transferrin-bound iron (NTBI), when the concentration of iron is higher than the binding capacity of transferrin (42). Importantly, both forms can be taken up by T cells (43, 44). We isolated splenocytes from tumor-naive C57Bl/6N female mice and stimulated them with anti-CD3 antibodies. To induce NTBI, we supplemented the culture with 5 µM ferric iron, a concentration shown by us and others to generate measurable NTBI (43, 45), in the form of salts ferric chloride ($FeCl_3$), ferric sulfate ($Fe_2(SO_4)_3$), and ferric citrate ($FeC_6H_5O_7$). Holo-transferrin was added as a source of TBI. Iron, both in its physiological TBI form as well as NTBI, halted proliferation of both CD8$^+$ cytotoxic T cells and CD4$^+$ T helper cells as shown by a dramatically reduced fraction of S-phase cells (CD8$^+$ S-phase cells $FeCl_3$ p = 0.0003, $Fe_2(SO_4)_3$ p = 0.001, $FeC_6H_5O_7$ p = 0.0001, holo-transferrin p = 0.0001; CD4$^+$ S-phase cells $FeCl_3$ p = 0.01, $Fe_2(SO_4)_3$ p = 0.01, $FeC_6H_5O_7$ p = 0.03, holo-transferrin p = 0.02 and promoted apoptosis measured by sub-G1 fractions (**Figures 5A–C**). This phenomenon was paralleled by a strongly decreased production of the key anti-tumor cytokine IFNγ by CD4$^+$ cells ($FeC_6H_5O_7$ p = 0.002) (**Figure 5D**). Importantly, the detrimental effects of iron on T cell expansion were corroborated by the results of another proliferation assay employing dilution of the fluorescent CFSE dye (CFSE low population p = 0.0055) (**Figure 6A**). CFSE is a widely used method to monitor lymphocyte proliferation due to the progressive halving of CFSE fluorescence within daughter cells following each cell division (46).

Following these observations, we tested the effect of iron on the cellular levels on the turnover of the cytolytic protein perforin in *in vitro* iron or non-iron supplemented splenocytes. Perforin is found in the granules of CD8$^+$ cytotoxic T cells and is centrally involved in anti-cancer immune function whereby perforin binds to the cell membrane of target cells, forming a pore allowing for granzyme B injection and killing of the target cell (47). We found increased intracellular perforin in iron-stimulated CD8$^+$ splenocytes as compared to splenocytes without iron supplementation indicating that perforin is retained in CD8$^+$ cells (p = 0.006) (**Figure 6B**). Moreover, performing chromium release assays, we could demonstrate that CD8$^+$ T cells incubated with iron significantly attenuate their ability to kill target cells

compared to $CD8^+$ T cells without iron application ($CD8^+$ T cell: target cell ratio 30:1 p = 0.017) (**Figure 6C**).

The main mechanism of toxicity of chemically reactive cellular iron relies on the excellent redox properties of the element culminating in the generation of reactive oxygen species (1, 42). In line with that, we found a significant accumulation of mitochondrial ROS in $CD8^+$ T cells in splenocyte cultures supplemented with TBI or NTBI ($CD8^+$ T cells ferric sulfate p <0.05; holo-transferrin p <0.05) as measured by the fluorescent dye MitoSOX. In turn, cytoplasmic ROS formation detected by the DCFDA dye was unaltered by iron stimulation (**Figure 7A**). Interestingly, these effects could not be observed in the $CD4^+$ T cells fraction in the same culture (**Figure 7B**). The iron-dependent effect on T cell growth was reversed by the addition of MitoTempo, a mitochondria specific anti-oxidant (iron:cell death/ROS inhibitor interaction $CD4^+$ p = 0.009, $CD8^+$ p = 0.0026). Other cell death and stress inhibitors like Ferrostatin-1 (inhibits ferroptosis), the cytoplasmic ROS scavenger NAC (cell death/ROS inhibitor), Necrostatin (inhibits necroptosis), or the Casp3i z-DEVD-FMK (inhibits apoptosis; cell death/ROS) showed no significant effects in regard to reversal of iron-mediated impairment of T cell proliferation (**Figures 8A, B**). These results suggest that iron exposition negatively impacts on T cell function by inhibiting $CD8^+$ cytotoxic T cells degranulation and perforin-mediated killing of target cells as well as on IFNγ formation by $CD4^+$ and $CD8^+$ T cells. In addition, iron exposure induces mitochondrial ROS causing growth arrest and cell death of those lymphocytes. This is in line with the reduced efficacy of cancer immunotherapy in iron-administered animals as described herein.

Taken together, increased iron concentration in the tumor milieu caused by intravenous iron supplementation hampers activation, expansion, survival and functionality of the two key effectors of anti-tumor immunity, $CD8^+$ cytotoxic T cells and $CD4^+$ T helper cells (**Figure 9**). Our results indicate strong immunosuppressive effects of iron on anti-tumor immunity and on the efficacy of immune-therapies for cancer.

DISCUSSION

Patients with breast cancer and other malignant diseases often develop functional iron deficiency or overt anemia as a consequence of their underlying disease (10, 11, 48). Anemia per se may negatively affect cardiovascular function and quality

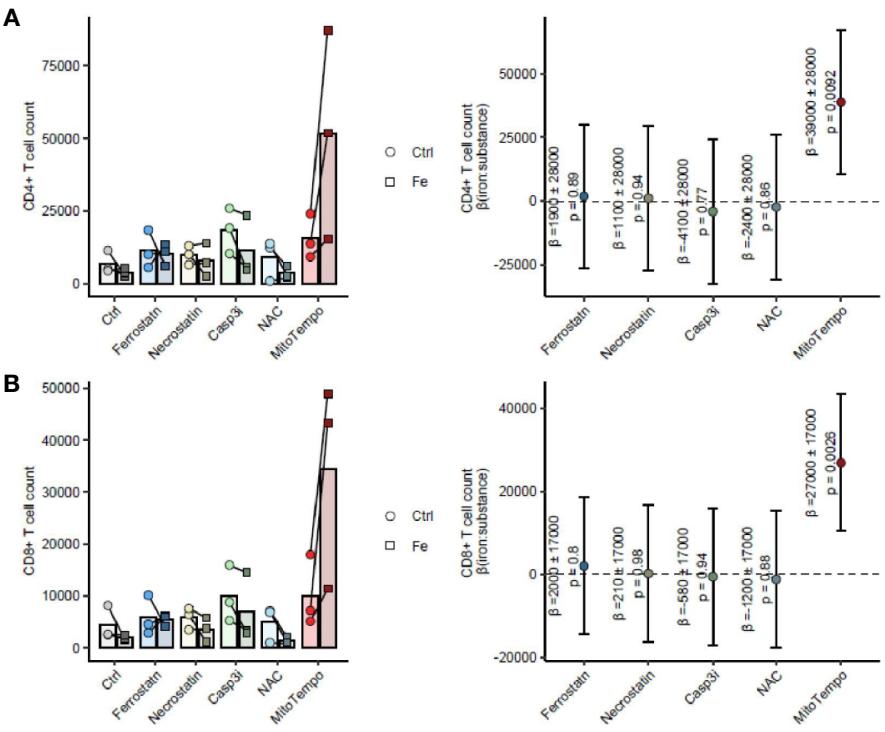

FIGURE 8 | The mitochondrial ROS scavenger MitoTempo reverses the iron-mediated inhibition of T cell growth. Splenocytes were isolated from tumor-naive C57Bl/6 mice (n = 3 separate cell donors) and cultured for 72 h in presence of 1 μg/ml activating anti-CD3 antibody and the inhibitors of ferroptosis (Ferrostatin: 1 μM), necroptosis (Necrostatin: 30 μM), apoptosis (Casp3i, z-DEVD-FMK: 20 μM) or cytoplasmic (NAC, N-acetylcysteine, 10 mM) or mitochondrial (MitoTempo, 20 μM) ROS scavengers. $CD4^+$ T cells **(A)** and $CD8^+$ T cells **(B)** were enumerated by flow cytometry. Statistical significance for reversal of the iron-mediated inhibition of T cell growth measured as the positive interaction of iron and cell death/ROS inhibitor was assessed by mixed-effect linear regression (fixed effects: iron, cell death/ROS inhibitor and the iron: cell death/ROS inhibitor interaction; random effect: cell donor). Left panels: cell counts are presented as points, lines connect data for the same cell donor; right panels: forest plots showing the regression coefficients (beta) of the iron:cell death/ROS inhibitor interaction as points and 95% confidence intervals as error bars.

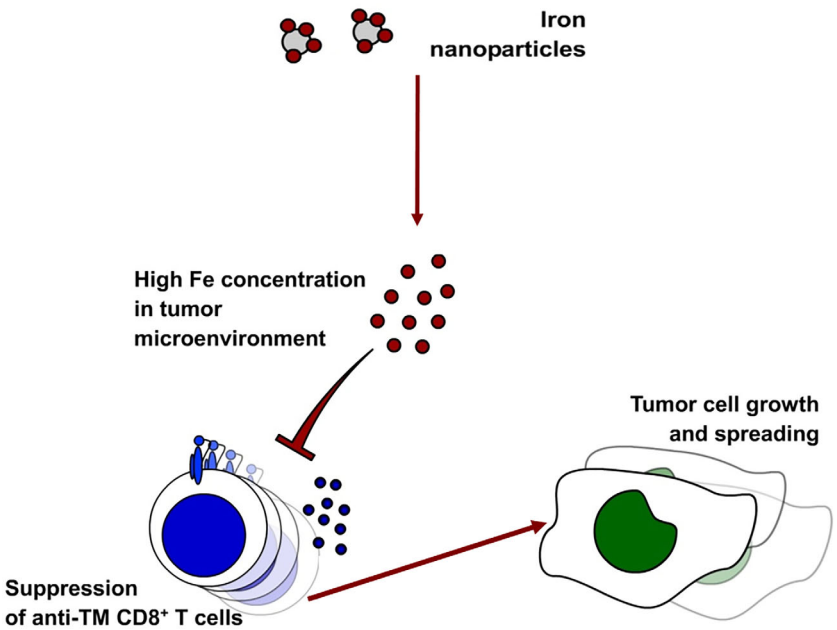

FIGURE 9 | Administration of intravenous iron in the form of ferric isomaltose leads to higher iron concentrations in the tumor milieu. This leads to the inhibition of anti-tumor $CD8^+$ T cells.

of life in affected patients, so that physicians frequently see the necessity to correct anemia by different treatments (34, 35). Intravenous iron administration is one of the treatment options for ACD in oncologic patients specifically if they suffer from absolute iron deficiency with low serum ferritin levels (49) which often coexists based on chronic blood losses and which aggravates the severity of ACD (50, 51). In parallel, intravenous iron preparations improve response rates to and exert dose-sparing effects on the use of erythropoiesis stimulating agents (ESA) (52, 53). This is relevant because treatments with high doses of ESA have been linked to tumor progression as the erythropoietin receptors are expressed on cancer cells including mammary carcinoma, whereas erythropoietin inhibits pro-inflammatory immune responses of innate immune cells which may hamper cancer control (54–57). While the effects of iron treatment on the hematological response have been well studied, no data are available on the effect of such intervention on the clinical course of the underlying tumor disease including end point data (11, 58). Principally, there are several ways by which iron administration may affect the clinical course of breast cancer. First, iron may have direct effects on either the division or the death of breast cancer cells. Consequently, iron can either sustain tumor cell metabolism and promote their proliferation (16, 59) or may sensitize cancer cells to ferroptosis, especially in the context of anti-tumor therapies (60). Second, the administration of iron may impact on the immune control of the tumor and either stimulate or inhibit the activity of distinct immune pathways against malignant cells. Third, iron may affect the susceptibility of tumors cells to immune- or chemotherapy in different ways, either by aggravating radical formation and cancer cell apoptosis/ferroptosis or by inducing their proliferation thereby making them more sensitive to the effects of anti-proliferative agents.

We designed our study to investigate the effects of iron isomaltoside, a clinically approved intravenous iron compound, on the course of disease, therapeutic efficacy of anti-cancer immunotherapies and anti-tumor response of $CD4^+$ and $CD8^+$ tumor infiltrating T cells in the E0771 breast cancer model (61). Blood counts of untreated tumor-bearing mice point out significantly lower hemoglobin concentrations 21 days after tumor implantation as compared with tumor free-mice, referring to a mild impairment of erythropoiesis, which, partly, recapitulates cancer-associated anemia found in a substancial percent of breast cancer patients.

Our results obtained *in vivo* show that iron isomaltoside accelerates tumor progression in therapy-naive mice as compared with iron-untreated tumor bearers. Of practical relevance for cancer treatment, it also significantly diminished the efficacy of the IL-2/doxorubicin chemo-immunotherapy treatment regimen and substantially, yet not significantly, aggravated the effects of the anti-PD-L1 treatment. Notably, $CD8^+$ $IFN\gamma^+$ T cell-mediated anti-tumor response poses one of the mechanisms of action of doxorubicin therapy as demonstrated by us previously (62) and is of critical importance for the anti-PD-L1 immune checkpoint therapy (63, 64). Our results show significant reduction of this T cell population in the tumor tissue of mice treated with either therapy regimen combined with iron, suggesting that inhibition of anti-tumor T cell response poses the common mechanism of the detrimental action of iron supplementation. Another argument for the common mode of action is the cross-talk between the immune

checkpoint pathways and signaling induced by IL-2 and doxorubicin. In breast cancer, $CD8^+$ T cell numbers correlate with PD-L1 expression (65, 66) because tumor-infiltrating $CD8^+$ T cells carry PD-L1 (67). Furthermore, $CD8^+$ T cells are required to mediate the anti-tumor effect of PD-L1 blockade against cancer cells, as shown in a mouse model of malignant melanoma (68). In addition, doxorubicin co-administered with cisplatin (the latter not used in our study) upregulates PD-L1 expression in breast cancer (69) and PD-L1 inhibition in combination with IL-2 has synergistic effects on $CD8^+$ T cells, suggesting that these two therapies may activate converging pathways (70). Moreover, the PD-L1 and IL-2 pathways are interconnected: On the one hand, the PD-1/PD-L1 interaction inhibits IL-2 production. On the other hand, exogenous IL-2 is known to overcome the inhibitory effects of this interaction (71). Taken together, it is reasonable to assume that iron impairs the anti-tumor effects of anti-PD-L1 antibodies and of IL-2/doxorubicin immunochemotherapy mainly by impairing $CD8^+$ T cell functions.

This hypothesis is further supported by the results of *in vitro* experiments clearly demonstrating that increased iron supply, not only as potentially toxic NTBI but also in its physiological transferrin-bound form impairs $CD8^+$ T cell proliferation, cytokine production and degranulation. Of interest, the effects of iron isomaltoside on $CD4^+$ T cells were less pronounced supporting the fact that the main function of $CD4^+$ T cells in the tumor setting is the initiation and maintenance of $CD8^+$ tumor infiltrating killer cells or rather to shape the anti-tumor response in spleens and lymph nodes. In line, the administration of iron isomaltoside had consistent yet not significant effects on effector/memory tumor infiltrating lymphocyte populations. In contrast, numbers of $FoxP3^+$ $CD4^+$ Tregs were comparable across treatment arms suggesting that the adverse effects of iron towards effector T cell populations where direct rather than indirect and Treg-mediated (72, 73).

Our observations raise the question of how iron may impair tumor infiltrating lymphocyte responses in breast cancer-bearing mice. First, iron may impair the proliferation, differentiation or maturation of naïve tumor infiltrating lymphocytes by mitochondrial ROS generation resulting in cell death as indicated by our *in vitro* data. Notably, such a process may take place both in the spleen, which, together with the liver, represent the major storage organ upon ferric isomaltoside treatment, and in the tumor milieu displaying improved iron availability as demonstrated by reduced CD71 levels on the bona-fide neoplastic epithelium. The tendency towards reduced numbers of $CD4^+$ effector T cells following iron isomaltoside administration presented in **Figure 2D** may in fact reflect such iron-mediated cell death happening locally in the tumor microenvironment. Second, iron may impair T cell receptor signaling and thus T cell activation. The *in vitro* data on the increased cell death, reduced proliferation and impaired IFNγ production of iron-exposed $CD4^+$ and $CD8^+$ T cells upon CD3 stimulation support this hypothesis. Third, it is feasible to assume that co-stimulatory pathways are undermined by high iron levels in the tumor microenvironment. Yet, in our *in vitro* system, we did not activate CD28 or other co-stimulatory pathways or study putative effects of iron on down-stream signaling events. Fourth, high iron concentrations in the microenvironment of tumor infiltrating lymphocytes may impair IFNγ output by direct negative effects on the transcription or translation of cytokine genes and mRNAs, respectively (74). This would be in line with the negative effects of iron on IFNγ signaling and IFNγ inducible pathways in macrophages, which impact also on T_H1/T_H2 cell differentiation (29, 75, 76). In summary, the administration of iron to mice with mammary carcinoma exacerbated the disease and impaired the therapeutic response to cancer-immunotherapy. Further studies are underway to characterize the molecular mechanisms by which iron administration impacts on anti-tumor T cell responses in our clinically relevant breast cancer model. Nonetheless, iron administration to cancer patients may have multiple adverse effects on the course of the underlying malignant disease. Therefore, prospective trials are needed which investigate those most important questions beyond the correction of hemoglobin levels.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Federal Ministry Republic of Austria Education, Science and Research.

AUTHOR CONTRIBUTIONS

PT participated in the study design, data collection and analysis, and drafted the manuscript. MN, VP, SH, BK, NHK, and VK participated in the data collection and revised the manuscript, NB and IT revised the manuscript. GW and CPO participated in the study design, data collection, analysis, data interpretation, and manuscript preparation. All authors contributed to the article and approved the submitted version.

FUNDING

Authors MN and IT are currently receiving a grant from the Austrian Research Fund FWF (P33062, P28302, respectively), author GW received a grant by the Christian Doppler Society and an ERA-NET grant by the FWF (EPICROSS, I-3321), and author CP-O was supported by the Austrian Cancer Society/Tirol (P17006). Authors NB and VP were supported by the doctoral college project W1253 HOROS. For the remaining authors none were declared.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.584477/full#supplementary-material

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frontiers in Immunology

REVIEW
published: 12 February 2021
doi: 10.3389/fimmu.2020.626812

Iron Metabolism in the Tumor Microenvironment: Contributions of Innate Immune Cells

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Cells of the innate immune system are a major component of the tumor microenvironment. They play complex and multifaceted roles in the regulation of cancer initiation, growth, metastasis and responses to therapeutics. Innate immune cells like neutrophils and macrophages are recruited to cancerous tissues by chemotactic molecules released by cancer cells and cancer-associated stromal cells. Once they reach the tumor, they can be instructed by a network of proteins, nucleic acids and metabolites to exert protumoral or antitumoral functions. Altered iron metabolism is a feature of cancer. Epidemiological studies suggest that increased presence of iron and/or iron binding proteins is associated with increased risks of cancer development. It has been shown that iron metabolism is involved in shaping the immune landscapes in inflammatory/infectious diseases and cancer-associated inflammation. In this article, we will dissect the contribution of macrophages and neutrophils to dysregulated iron metabolism in malignant cells and its impact on cancer growth and metastasis. The mechanisms involved in regulating the actions of macrophages and neutrophils will also be discussed. Moreover, we will examine the effects of iron metabolism on the phenotypes of innate immune cells. Both iron chelating and overloading agents are being explored in cancer treatment. This review highlights alternative strategies for management of iron content in cancer cells by targeting the iron donation and modulation properties of macrophages and neutrophils in the tumor microenvironment.

Keywords: iron, neutrophils, macrophage, cancer, metastasis

OPEN ACCESS

Edited by:
Stefania Recalcati,
University of Milan, Italy

Reviewed by:
Keehoon Jung,
Seoul National University, South Korea
Shahram Salek-Ardakani,
Pfizer, United States

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *06 November 2020*
Accepted: *30 December 2020*
Published: *12 February 2021*

Citation:
Liang W and Ferrara N (2021) Iron Metabolism in the Tumor Microenvironment: Contributions of Innate Immune Cells. Front. Immunol. 11:626812. doi: 10.3389/fimmu.2020.626812

INTRODUCTION

Innate immune cells such as neutrophils and macrophages are the host's first line of defense against invading pathogens and are responsible for initiating inflammatory responses. Recruitment and activation of adaptive immune cells to/at the infection sites are crucial steps. Cancer-associated inflammation is correlated with poor patient survival and therapeutic outcomes and is listed among the hallmarks of cancer (1). Indeed, extensive efforts have been devoted to elucidating the contribution of the innate immune system in these events. Previous studies have shown that innate immune cells, such as tumor-associated macrophages (TAM) and neutrophils, can facilitate cancer cell growth and metastasis, induce tumor angiogenesis, suppress antitumor immune

response and modulate response to anticancer therapies (2, 3). These complex effects are mediated by a network of cytokines, chemokines, growth factors and enzymes released by innate immune cells that act directly on cancer cells and components of the tumor microenvironment (TME) or through cell-cell contacts between innate immune cells and cancer cells or other stromal cells (2, 3).

Recent evidence has revealed a remarkable plasticity in the metabolism of innate immune cells recruited to the TME (4). These findings perhaps are not surprising given the unique features (e.g., low pH, limited supplies of nutrients and oxygen) concomitant with aberrant accumulation of metabolism-modulating molecules in the TME. Interestingly, cancer cells can utilize metabolic byproducts from innate immune cells and other stromal cells to support cancer growth and promote drug resistance (5–8). Further understanding of how dysregulated cell metabolism in innate immune cells affects cancer behaviors is of great interest and may uncover new therapeutic avenues.

Iron is an essential element for all organisms. Its ability to be oxidized and reduced makes it ideal for transporting electrons and functioning as a co-factor in a variety of biochemical reactions in DNA synthesis (9), mitochondria respiration (10), host defenses (11) and cell signaling (12). On the other hand, this unique property may result in formation of reactive oxygen species (ROS) that have detrimental effects on genomic stability and may induce malignant transformation (13). As a result, iron metabolism is one of the key factors deciding the fates of normal and malignant cells.

This review examines the crosstalk between cancer cells and innate immune cells from the perspective of iron metabolism. We will review the evidence on how innate immune cells contribute to the dysregulated iron metabolism in cancer cells and how this process is regulated by iron metabolism and signaling molecules in the TME. We will also analyze the potential clinical benefits for therapeutic targeting the iron donation and modulation properties of macrophages and neutrophils.

IRON METABOLISM IN CANCER CELLS

Iron metabolism and homeostasis under physiological conditions have been reviewed in details elsewhere (12, 14). Briefly, dietary iron enters the body through absorption by divalent metal transporter 1 (DMT1) expressed on enterocytes at the duodenum of the small intestine. It can then be released to circulation through the only known iron exporter ferroportin. In the systemic circulation, the majority of the iron is bound by an iron-transporting protein named transferrin that is mainly synthesized by hepatocytes. The iron-bound transferrin (holo-transferrin) recognizes the ubiquitously expressed transferrin receptor 1 (Tfr1) or tissue-specific transferrin receptor 2 (Tfr2) and enters cells through clathrin-mediated endocytosis. Non-transferrin bound iron also exists in extracellular spaces and can be taken up by cells through transferrin receptor-independent mechanisms (e.g., DMT1). In endosomes, iron is dissociated from transferrin, reduced by six-transmembrane epithelial antigen of the prostate (STEAP) proteins and released to cytoplasm by DMT1, while transferrin receptors are mostly recycled back to the plasma membrane. Once inside the cell, iron may enter mitochondria and nucleus to participate in a series of biochemical reactions. It may also be stored in ferritin and a labile iron pool (LIP) or exported by ferroportin. Hepcidin, an iron-regulatory peptide mainly synthesized by liver, directly binds to ferroportin, resulting in the internalization and degradation of ferroportin and reduced iron export. Cellular iron homeostasis is regulated through binding of the iron regulatory proteins (IRP1 and IRP2) to the iron-responsive element (IRE) located in the untranslated region (UTR) of target mRNAs involved in iron metabolism. When intracellular iron is low, IRPs binds to IRE of Tfr1, DMT1, ferritin and ferroportin mRNAs, resulting in increased expression of Tfr1 and DMT1 and decreased expression of ferritin and ferroportin. When intracellular iron is high, IRPs is dissociated from IRE, which suppresses expression of Tfr1 and DMT1 yet permits expression of ferritin and ferroportin.

Due to increased proliferation rate and synthetic/metabolic activities commonly associated with malignancy, iron demand in cancer cells is high. To ensure ample supply, the iron uptake machinery in cancer cells is usually enhanced, while export is repressed. Transferrin synthesis acts as an autocrine mechanism supporting iron supply and growth of cancer cells (15). Overexpression of Tfr1 is frequently found in malignant tissues and is associated with worse patient survival (16–18). Inhibition of Tfr1 expression significantly blocks tumor growth and metastasis (19, 20). Moreover, upregulation of other proteins involved in iron import, such as DMT1 and duodenal cytochrome b (DCYTB), has been reported in cancer cells (21, 22). Furthermore, STEAP family members, which are highly expressed in a variety of cancer cells, can facilitate iron uptake (23–26). Conversely, expression of ferroportin is reduced in cancer cells, relative to their normal counterparts and is associated with poor patient survival (27, 28). Overexpression of ferroportin results in suppressed tumor growth (27). Elevated levels of hepcidin are found in different cancer types, further restricting ferroportin-mediated iron export and favoring iron sequestering in cancer cells (28–31). In addition, high IRP2 levels contribute to cancer cell proliferation and survival and correlate with poor patient survival (32–35).

IRON LEVELS IN THE TME ARE REGULATED BY INNATE IMMUNE CELLS

Emerging evidence suggests that innate immune cells such as macrophages and neutrophils contribute to dysregulated iron metabolism in cancer cells. Specifically, macrophages and neutrophils residing in the TME can either serve as sources of iron and iron-related proteins (**Figure 1**) or release factors that activate signaling pathways in control of iron metabolism in cancer cells (**Figure 2**).

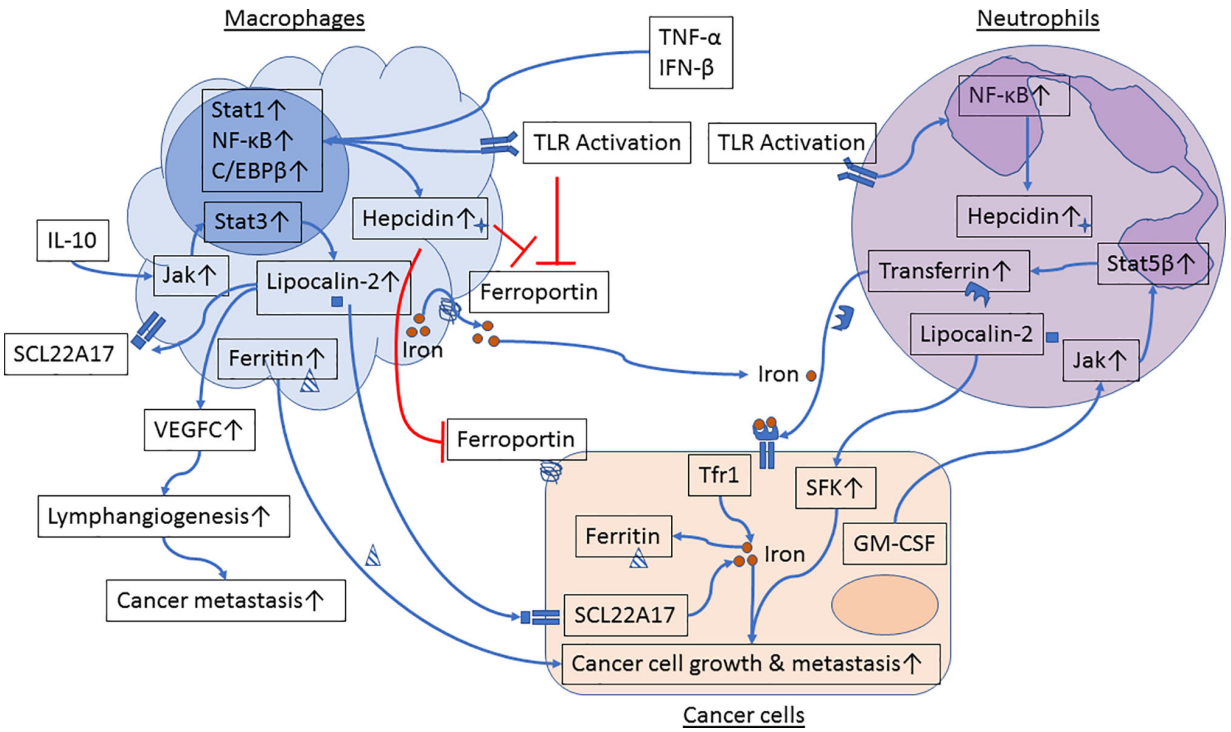

FIGURE 1 | Iron metabolism in the crosstalk between cancer cells and macrophages or neutrophils. Proinflammatory cytokines and Toll-like receptor (TLR) agonists induce activation of Stat1, NF-κB and C/EBPβ in macrophages, resulting in upregulation of hepcidin and inhibition of ferroportin. In parallel, engagement of TLRs further inhibits ferroportin expression. Reduced ferroportin levels in macrophages limit iron transport from macrophages to cancer cells. IL-10 activates the Jak/Stat3 signaling pathway and upregulates expression of lipocalin-2 in macrophages. Lipocalin-2 released from macrophages can bind to its receptor in cancer cells and in macrophages to stimulate cancer cell growth and M2 polarization, respectively. Lipocalin-2 can also induce VEGFC expression, resulting in promotion of lymphangiogenesis and cancer metastasis. Moreover, macrophage-secreted ferritin directly stimulates cancer cell growth. TLR engagement induces activation of NF-κB and hepcidin expression in neutrophils. GM-CSF, produced by metastatic tumor cells, induces activation of the Jak/Stat5β signaling pathway and transferrin synthesis in neutrophils. Neutrophil-derived transferrin can promote growth of metastatic tumor cells. In addition, lipocalin-2 released by neutrophils induces activation of Src family kinases (SFK) in prostate cancer cells and enhances cancer cell migration and metastasis.

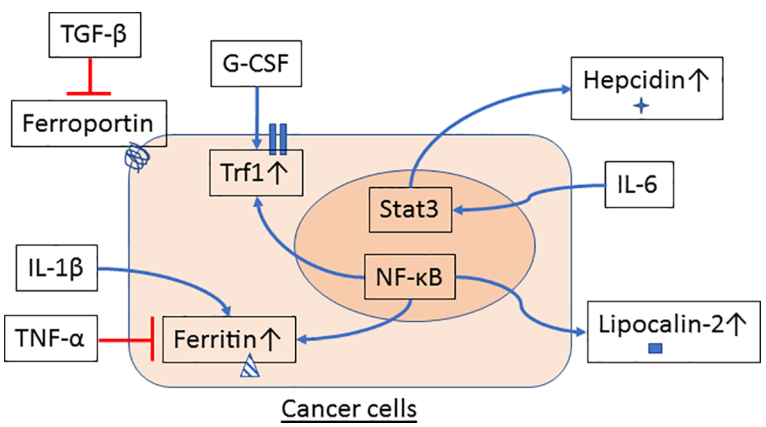

FIGURE 2 | Innate immune cells signal iron metabolism in cancer cells. Cytokines and growth factors are released by macrophages and neutrophils in the tumor microenvironment (TME) and may affect the regulation of iron metabolism in cancer cells. TGF-β reduces ferroportin while G-CSF induces Tfr1 gene expression. Expression of ferritin is inhibited by TNF-α but increased by IL-1β. IL-6 stimulates Stat3 activation and hepcidin expression in hepatocytes and hepatoma cells. NF-κB, a transcription factor that can be activated by a variety of proinflammatory cytokines and factors, also participates in the regulation of gene expression of ferritin, Tfr1 and lipocalin-2 in cancer cells.

Macrophage and Neutrophils as Sources of Iron in the TME

Macrophages are a group of innate immune system cells with high plasticity and are frequently found in the TME. TAM can stimulate cancer cell growth, angiogenesis and metastasis, suppress anticancer immunity and render cancer resistance to therapies (36, 37). In response to different stimuli, macrophages can be polarized to M1 (classically activated, proinflammatory) or M2 (alternatively activated, anti-inflammatory) subtypes (38). Interestingly, it has been reported that M1 macrophages display high expression of ferritin and low expression of ferroportin, favoring iron sequestration in macrophages (39, 40). M2 macrophages, on the other hand, show low expression of ferritin and high expression of ferroportin, representing an iron-releasing phenotype (39, 40). Since TAM shares many features with M2 macrophages (37), it is plausible that TAM acts as a source of iron in the TME and promotes cancer growth through an iron-dependent mechanism. In fact, analysis of primary tumors and axillary lymph nodes from breast cancer patients revealed a substantial iron reservoir in stromal inflammatory cells (41). Concomitantly, while breast cancer cells display an "iron-utilization" phenotype characterized by increased expression of hepcidin and Tfr1 and decreased expression of ferritin, macrophages in primary tumors and metastasized lymph nodes manifest an "iron-donor" phenotype characterized by increased expression of ferroportin and ferritin (41). Further supporting this notion, another group examined the mitogenic effects of M1 and M2 macrophages on cancer cells and found that compared to M1 macrophages, M2 macrophage-conditioned medium has significantly greater ability to stimulate cancer cell proliferation (40). Incubation of macrophages with an iron chelator inhibits M2 macrophage conditioned medium-induced cancer cell proliferation (40). Interestingly, comparison of the mitogenic response toward M1 and M2 macrophage-conditioned media from a patient with "loss of function" ferroportin mutation showed no differences in cancer cell proliferation (40). These results suggest that the greater mitogenic activity of M2 macrophages on cancer cells is at least in part mediated by ferroportin-controlled iron release from macrophages. Additionally, M2 macrophages stimulate MCF-7 breast cancer cell growth and migration by an iron-dependent mechanism (42). Coculture with iron-loaded monocytes recapitulates the effects of iron treatment on rendering myeloma cells resistance to bortezomib (43).

Neutrophils are the most abundant immune cells in humans. Cytokines and growth factors secreted by tumor cells can stimulate neutrophil differentiation, proliferation, mobilization and release from the bone marrow (2, 44). Elevated neutrophil numbers in the systemic circulation and in tumor tissues are frequently reported in tumor patients and often correlate with poor survival (2). Like macrophages, neutrophils can be programed by tumor-released factors to exert a variety of protumoral functions by acting on cancer cells, endothelial cells, other immune cells and the extracellular matrix (2). Proteins involved in iron uptake, storage and export are expressed not only in macrophages but also in neutrophils (45). In fact, iron plays an important role in neutrophils' defense against invading pathogens (45). Iron participates in generating the oxidative burst that is required to kill phagocytosed microbes in neutrophils (45). Moreover, neutrophils produce large amounts of lipocalin-2 and lactoferrin, both of which are iron-scavenging proteins and thus limit microbial growth (45).

Direct evidence that neutrophils are a source of iron in the TME is lacking. Yet, previous work showed that in a rat model mimicking human Alpha-1-antitrypsin (A1AT) deficiency, intratracheal administering of neutrophil elastase (NE) (a serine protease mainly synthesized by neutrophils), increases iron content in the bronchoalveolar lavage (46). NE degrades iron-containing proteins like ferritin in the extracellular space, increasing iron availability and uptake into human airway epithelial cells (46). Whether such mechanism exists in the TME and whether it contributes to increases iron uptake by tumor cells warrant further investigation.

Macrophage and Neutrophils as Sources of Iron-Related Proteins in the TME

Lipocalin-2 is an acute phase protein that can be synthesized by a variety of cell types including epithelial cells, macrophages and neutrophils (45). Lipocalin-2 binds to bacterial or mammalian siderophores loaded with iron (47), which has two potential consequences, possibly depending on the stages of inflammation. At early stages of inflammation, binding of lipocalin-2 to the iron-siderophore complex sequesters iron from uptake by bacteria, limiting bacterial growth and thus mediating the antimicrobial function of lipocalin-2. On the other hand, the iron-siderophore-lipocalin-2 complex can serve as an iron donor and stimulate epithelial cell proliferation, an event likely occurring during the resolution phase of inflammation when epithelial cell proliferation is needed to mediate tissue repair. Given the resemblance of wound healing to cancer development, it is tempting to speculate that lipocalin-2 derived from TAM or neutrophils stimulates cancer cell growth by an iron-dependent mechanism. In fact, enhanced expression of lipocalin-2 has been documented in multiple cancer types and was associated with poor patient survival (48–52). *In vitro*, iron-loaded lipocalin-2 promotes spheroid growth of cancer cells, whereas iron-free lipocalin-2 inhibits it (48).

Macrophage-derived lipocalin-2 induces proliferation, epithelial-mesenchymal transition and metastatic potential in MCF-7 human breast cancer cells (53, 54). Besides cancer cells, lipocalin-2 can enhance the protumoral functions of tumor-associated stromal cells. Lipocalin-2, released by macrophages, induces VEGFC production, lymphangiogenesis and metastasis (55). Moreover, apoptotic tumor cells stimulate expression of lipocalin-2 in macrophages and polarization of these macrophages to M2 phenotypes (56). It remains to be determined whether the aforementioned effects are dependent on the iron donation function of lipocalin-2. In another study, lipocalin-2 was found to be predominantly expressed in TAM and to act as a paracrine factor that supplies iron, thus stimulating proliferation of cancer cells (57). Lipocalin-2

deficiency in TAM inhibits tumor growth, which can be reversed by iron supplement (58). According to a published report, iron demand increased as tumors progressed to the metastatic stage, and this was met by TAM-derived lipocalin-2 and inhibited by lipocalin-2 antibody neutralization (57). Together, these results identify TAM-derived lipocalin-2 as a promising therapeutic target for inhibiting the tumor-supporting functions of TAM. Lipocalin-2 is also secreted by neutrophils as a component of secondary granules and deficiency of lipocalin-2 impairs the chemotaxis ability of neutrophils (59). A recent report indicates that CXCL1 secreted by myofibroblasts recruits neutrophils to the TME. Neutrophil-derived lipocalin-2 induces activation of Src family kinases in prostate cancer cells and promotes cancer cell migration and metastasis (60). Whether such action is dependent on the iron-binding function of lipocalin-2 remains to be determined. Lipocalin-2 can be expressed by other cell types including cancer cells. Interestingly, a recent study reports that in the metastatic microenvironment of cerebrospinal fluid, macrophages do not express lipocalin-2 but rather produce inflammatory cytokines that induce lipocalin-2 expression in cancer cells (61). Expression of lipocalin-2 and its receptor, SCL22A17, by cancer cells was essential for cancer cell growth at metastatic sites (61).

Though typically viewed as an intracellular iron storage protein, ferritin is present in the serum. Indeed, serum ferritin has been reported to be a diagnostic and prognostic marker for inflammatory diseases and cancer (62–66). Both hepatocytes and macrophages have been implicated as sources of serum ferritin (67, 68). The releasing mechanisms and the roles of serum ferritin in inflammatory diseases and cancer are largely uncharacterized. It was shown that ferritin released by erythrophagocytosing Kupffer cells is loaded with iron and can mediate iron transport from Kupffer cells to hepatocytes (69). Moreover, in the absence of transferrin, ferritin synthesized and secreted by macrophages may serve as a source of iron for co-cultured erythroid precursors (68). These results corroborate the potential of macrophage-secreted ferritin as an iron donor for other cell types. Nonetheless, whether such hypothesis can be extended to the TME where TAM and cancer cells co-exist remains to be determined. Interestingly, TAM has been shown to be a predominant source of extracellular ferritin and TAM-secreted ferritin can act as paracrine factor that promotes cancer cell proliferation, angiogenesis and immunosuppression (70). Still, it should be pointed out that TAM-secreted ferritin can stimulate cancer cell proliferation *via* iron-independent mechanisms (71). Further studies are warranted to dissect which functions mediated by TAM-secreted ferritin are iron-dependent or independent.

Hepcidin expression in macrophages and neutrophils can be further enhanced by proinflammatory cytokines like TNF-α and IFN-β, depending on the context; or by engagement of Toll-like receptors (TLR) (72–75). Inflammation-induced hepcidin expression in macrophages is mediated by activation of the Stat1 and NF-κB pathways and induction of C/EBPβ expression (73). Secreted hepcidin can then act as an autocrine factor that inhibits ferroportin function and iron release from these innate immune cells (76). Given the abundance of endogenous TLR agonists in the TME, it will be of great interest to determine whether hepcidin released by macrophages and neutrophils in the TME, in response to endogenous TLR agonists, inhibits ferroportin on cancer cells as a paracrine factor and increases intracellular iron content and thereby cancer cell proliferation.

Transferrin and its receptor Tfr1 are the major route for cellular iron uptake. We previously reported that transferrin is expressed by neutrophils, but not cancer cells, in the metastatic microenvironment and that it mediates neutrophil-dependent mitogenic effects on cancer cells (19). Depletion of neutrophils reduced transferrin levels in the metastatic microenvironment and inhibited metastasis in mouse models (19). GM-CSF, derived mainly from metastatic tumor cells, selectively induces transferrin gene expression in neutrophils through the Jak/Stat5β pathway (19). Blockade of GM-CSF or inhibition of Jak kinases inhibits neutrophil transferrin expression and disrupted the paracrine loop between metastatic cancer cells and neutrophils, resulting in reduced metastasis (19). This work highlighted the potential of neutrophils in modulating iron metabolism in cancer cells and validated the targeting strategies for blocking prometastatic functions of neutrophils.

Iron Metabolism-Modulating Signaling: Inputs From Macrophages and Neutrophils

Besides directly supplying iron and iron-related proteins, macrophages and neutrophils may affect iron metabolism in cancer cells through release of cytokines, chemokines and growth factors that act on cancer cells and induce changes in signaling events that regulate iron metabolism. Treatment of breast cancer cells with TGF-β, a cytokine known to be produced by TAM, reduces ferroportin expression in cancer cells (77). G-CSF, a hematopoietic growth factor that can be expressed by macrophages, induces Tfr1 expression in human myeloid leukemia cell lines (78). TNF-α can be expressed by TAM and neutrophils and treatment with TNF-α results in reduced ferritin expression in prostate cancer cells (79). TAM and neutrophils are known to produce IL-1β. Treatment of hepatoma cells with IL-1β increases ferritin expression (80). IL-6 is expressed by TAM and neutrophils and stimulates hepcidin expression in hepatocytes (81, 82). Augmented levels of hepcidin inhibits ferroportin on cancer cells and increases iron content in cancer cells (31, 83).

Numerous reports have shown that NF-κB and Stat3, two essential transcription factors regulating the inflammatory responses and immune landscape in the TME, also play key roles in controlling gene expression of iron metabolism proteins (84–88). In fact, many of the products from TAM and neutrophils in the TME are known to upregulate or downregulate activation of NF-κB and Stat3 in cancer cells (89, 90), further highlighting the crosstalk between tumor-associated innate immunity and dysregulated iron metabolism in cancer cells.

IRON METABOLISM AND MACROPHAGE POLARIZATION

Iron metabolism is thought to contribute to macrophage polarization, although there are conflicting reports on the precise effects. For example, Agoro et al (91) reported that iron-rich diet induces M2 polarization in liver and peritoneal macrophages and suppresses the proinflammatory M1 phenotypes in mice. Addition of iron to cultured macrophages inhibited expression of M1 costimulatory proteins and prevented LPS-induced NF-κB p65 nuclear translocation and expression of iNOS, IL-1β, IL-6, IL-12 and TNFα (91). The notion that iron overload favors M2 over M1 polarization was also supported by other studies (92, 93). Yet, opposite conclusions were reached by others as it was reported that treatment of bone marrow-derived macrophages with iron induces expression of M1 markers and reduces IL-4-induced M2 markers (94). Dietary iron overload in mice leads to M1 polarization of hepatic macrophages (94). Consistent with these findings, another group reported that iron supplement promotes M1 polarization in mechanisms depending on ROS production and p53 acetylation (95). Exposure of TAM to hemolytic red blood cells or iron nanoparticles increases iron content, M1 marker expression and antitumor activities of macrophages (96, 97). Moreover, lung cancer patients with positive iron staining in tumor tissues had better overall survival associated with higher expression of markers of M1 macrophages (98). These conflicting findings highlight the complex nature of macrophage polarization even in the context of iron metabolism. They also raise some concern for the use of iron overloading or chelating strategies to switch macrophage polarization for treatment of inflammatory diseases and cancer.

IRON MODULATING THERAPY: BEYOND IRON CHELATORS

The markedly elevated demand of iron by cancer cells suggests that the use of iron chelators may be an effective anti-cancer strategy. Indeed, in preclinical models iron chelators inhibited activation of signaling pathways important for cell proliferation and survival and suppress tumor growth and metastasis (99). However, these agents demonstrated only modest therapeutic benefits when tested in cancer patients (99). While continuing efforts are ongoing to improve the bioavailability of and design combination therapies with the iron chelators, different iron-modulating strategies are warranted. The fact that innate immune cells can serve as a source of iron and iron-related proteins in the TME provided a clear rationale for target discovery and validation.

Inhibition of Secretion of Iron and Iron-Related Proteins by Innate Immune Cells

Despite the fact that iron is critically required by cancer cells at different stages of tumorigenesis, altering iron levels and cellular uptake/storage/utilization/export machineries at systemic levels is in principle detrimental, since maintaining iron homeostasis is essential for normal physiological processes. One alternative could be to identify local sources of iron and iron-related proteins in the TME that can be pharmacologically targeted.

Our laboratory has been investigating the role of neutrophils in tumor angiogenesis and in resistance to anti-angiogenic therapies with VEGF inhibitors and these efforts led to the identification of neutrophil-derived angiogenic factors such as Bv8/PROK2 (100–102). We next sought to elucidate the nature of neutrophil-derived factors that directly promote growth of metastatic tumor cells. By employing a proteomic/functional approach, we unexpectedly identified transferrin as the major mitogen for tumor cells secreted by neutrophils (19). Depletion of neutrophils inhibited lung metastasis and transferrin production in the metastatic microenvironment. Transferrin expression by neutrophils was induced by tumor derived GM-CSF (19). The mechanism (Jak/Stat5β) by which GM-CSF induces transferrin expression is unique to neutrophils and is not shared by other cell types (19). In this case, one can expect that therapeutic agents targeting the GM-CSF/Jak/Stat5β signaling pathway (e.g., GM-CSF neutralizing antibodies or Jak kinase inhibitors) lowers transferrin levels specifically in the neutrophil-dominant metastatic microenvironment and inhibits cancer metastasis. Such strategies should spare transferrin production by other cellular sources and leave transferrin-mediated physiological iron homeostasis untouched.

Evidence reviewed above indicates that expression of lipocalin-2 in macrophages or neutrophils can promote cancer cell growth, induce M2 polarization of macrophages and is required for neutrophil chemotaxis. Therefore, blocking lipocalin-2 production from macrophages and neutrophils might simultaneously target cancer cells and cancer-associated myeloid cells. Previous work found that IL-10, an immunosuppressive cytokine that enhances the tumor-supporting functions of macrophages, induces lipocalin-2 expression in macrophages *via* activation of the Jak/Stat3 signaling pathway (53). Pharmacological inhibition of Jak or Stat3 suppresses IL-10-induced lipocalin-2 mRNA and protein levels (53). A variety of inhibitors of Jak kinases or Stat3 have entered clinical trials for cancer treatment, owing primary to the multifaceted functions of the Jak/Stat3 pathway in facilitating cancer cell proliferation, survival and metastasis, tumor angiogenesis and immunosuppression (89). It will be of great interest to determine whether such inhibitors suppress lipocalin-2 production from patient tumor-associated macrophages and neutrophils and reduce iron content in patient tumor cells and whether these effects contribute to the anticancer efficacy.

Iron exiting macrophages through ferroportin can become available for uptake by cancer cells in the TME. Reducing ferroportin expression levels in TAM thus represents a promising strategy for cancer treatment. Numerous studies have shown that stimulation of various TLRs decreases ferroportin expression, associated with increased intracellular iron concentrations in macrophages (103–105). Currently, TLR agonists are being tested in clinical trials for their ability to orchestrate anticancer immunity (106). The anticancer activities

are largely attributed to TLR-mediated maturation and activation of antigen-presenting cells and follow-up activation of adaptive immune responses against cancer cells (107). It remains to be determined, in both preclinical and clinical settings, whether TLR agonists modulate the iron-donation phenotypes of TAM and reduce iron contents in cancer cells and whether such effects contribute to their anticancer activities. Moreover, TLR activation induces hepcidin expression in macrophages and neutrophils (103, 104, 108). Therefore, treatment with TLR agonist might pack a "one-two punch" by decreasing ferroportin expression and inducing hepcidin release to act as an autocrine/paracrine factor to further inhibit ferroportin-mediated iron release from macrophages and neutrophils.

Ferroptosis-Inducing Therapy

Ferroptosis is a newly-identified, nonapoptotic form of regulated cell death, the hallmark of which is the iron-dependent accumulation of lipid hydroperoxides (109, 110). High iron levels, inhibition of glutathione peroxidase 4 (GPX4) and glutathione synthesis, starvation of cysteine, chemotherapy and targeted therapy are known to induce ferroptosis (109, 110). It has been hypothesized that cancer cells, compared to their normal counterparts, are more susceptible to ferroptosis for the following reasons: 1) Intracellular iron levels are higher in cancer cells due to upregulated iron uptake and downregulated export mechanisms. 2) Oxidative stress is more severe in cancer cells due to activation and/or mutation of oncogenic pathways, increased metabolic activities and hypoxia in the TME. As such, therapeutic approaches that induce ferroptosis are being actively investigated for cancer treatment. A recent study, using a hyperactivated transcriptional coactivator with PDZ-binding motif-driven mouse glioblastoma model, indicates that tumor-associated neutrophils induce ferroptosis in cocultured tumor cells and necrosis in tumor tissues, leading to the enhanced cancer aggressiveness and reduced mouse survival (111). Rescue of ferroptosis through GPX4 overexpression or ACSL4 silencing in tumor cells was reported to alleviate neutrophil-induced tumor cell killing and necrosis, inhibited tumor aggressiveness and improved mouse survival (111).

One interesting question is whether cancer cell death by ferroptosis is immunogenic and whether it can modulate the phenotypes of TAM and neutrophils in the TAM. Whereas direct evidence for such a link is lacking, it was recently shown that cancer cells undergoing ferroptosis release HMGB1 (112), an endogenous TLR agonist and a known activator of immunogenic cell death (113). In fact, ferroptotic cell death after heart transplantation stimulates neutrophil recruitment through a TLR4-dependent mechanism (114).

Ferroptosis also takes place in macrophages. Numerous reports confirmed that iron overload induces macrophage ferroptosis *in vitro* and *in vivo (*115–117). Interestingly, M1 macrophages, compared to M2 macrophages that resemble TAM, are more resistant to ferroptosis (117). Therefore, ferroptosis-inducing agents may selectively target the tumor-supporting TAM, while sparing the tumor-suppressive M1 macrophages. Inhibition of GPX4 induces ferroptosis in a variety of cell types including cancer cells (118). However, a recent study found that GPX4 deficiency does not affect survival of macrophages and neutrophils in mice (119). Instead, GPX4 deficiency induces oxidative stress and H_2O_2 release in myeloid cells, which promotes tumorigenesis by triggering genome-wide mutations in intestinal epithelial cells (119). This work argues against using GPX4 inhibitors, at least by itself, for inducing ferroptosis in macrophage and neutrophils. Future studies are warranted to determine the signaling pathways in regulation of ferroptosis in TAM and neutrophils in the context of cancer and whether other therapeutic approaches, alone or in combination, can eliminate TAM and neutrophils by a ferroptosis-dependent mechanism.

CONCLUDING REMARKS AND OUTSTANDING QUESTIONS

The evidence reviewed in this article supports the notion that innate immune cells like macrophages and neutrophils promote cancer development and progression through multiple iron-dependent mechanisms. The dysregulated iron metabolism in the TME may also affect the phenotypes of innate immune cells. Though somewhat speculative, it is possible that cancer cells, equipped with hyperfunctional iron uptake and downregulated export machineries, deprive the TME of iron and produce iron metabolism-related byproducts (e.g., ROS), to boost the protumoral functions or to suppress the anticancer activities of innate immune cells.

Questions to be addressed by future studies are: what is the cause of the inconsistent results on iron-induced macrophage polarization? Given largely shared expression of iron-related proteins with macrophages, can neutrophils contribute to the release of iron and iron-related proteins and aberrantly accumulated iron levels in cancer cells, to the same extent as macrophages? Does release of iron or iron-related proteins from innate immune cells act on other tumor-associated stromal cells like T cells, fibroblasts and endothelial cells and affect their functions? What are the resistance mechanisms of innate immune cells to GPX4 inhibitor-induced ferroptosis? Does iron metabolism in TAM and neutrophils mediate response or resistance of tumor cells to chemo-, targeted- and immunotherapies; are also highly warranted.

The gained knowledge will deepen our understanding of the role of iron metabolism in the crosstalk between macrophages/neutrophils and cancer cells or other stromal cells in the TME and should facilitate the identification of novel targets for disrupting such crosstalk.

AUTHOR CONTRIBUTIONS

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

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Conflict of Interest: WL was employed by the company BioDuro LLC.

The remaining 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.

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frontiers in Immunology

REVIEW
published: 19 February 2021
doi: 10.3389/fimmu.2020.627662

The Clinical Significance of Iron Overload and Iron Metabolism in Myelodysplastic Syndrome and Acute Myeloid Leukemia

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: 09 November 2020
Accepted: 31 December 2020
Published: 19 February 2021

Citation:
Weber S, Parmon A, Kurrle N, Schnütgen F and Serve H (2021) The Clinical Significance of Iron Overload and Iron Metabolism in Myelodysplastic Syndrome and Acute Myeloid Leukemia. Front. Immunol. 11:627662. doi: 10.3389/fimmu.2020.627662

Myelodysplasticsyndrome (MDS) and acute myeloid leukemia (AML) are clonal hematopoietic stem cell diseases leading to an insufficient formation of functional blood cells. Disease-immanent factors as insufficient erythropoiesis and treatment-related factors as recurrent treatment with red blood cell transfusions frequently lead to systemic iron overload in MDS and AML patients. In addition, alterations of function and expression of proteins associated with iron metabolism are increasingly recognized to be pathogenetic factors and potential vulnerabilities of these diseases. Iron is known to be involved in multiple intracellular and extracellular processes. It is essential for cell metabolism as well as for cell proliferation and closely linked to the formation of reactive oxygen species. Therefore, iron can influence the course of clonal myeloid disorders, the leukemic environment and the occurrence as well as the defense of infections. Imbalances of iron homeostasis may induce cell death of normal but also of malignant cells. New potential treatment strategies utilizing the importance of the iron homeostasis include iron chelation, modulation of proteins involved in iron metabolism, induction of leukemic cell death *via* ferroptosis and exploitation of iron proteins for the delivery of antileukemic drugs. Here, we provide an overview of some of the latest findings about the function, the prognostic impact and potential treatment strategies of iron in patients with MDS and AML.

Keywords: myelodysplastic syndrome, acute myeloid leukemia, iron overload, reactive oxygen species, microenvironment, iron chelation

Abbreviations: AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; DFS, disease-free survival; GvHD, graft-versus-host disease; HFE, hereditary hemochromatosis protein; HSCT, hematopoietic stem cell transplantation; ICT, iron chelation therapy; IRE, iron-responsive elements; IRP, iron-responsive element binding protein; LCI, labile cellular iron; LPI, labile plasma iron; MDS, myelodysplastic syndrome; NRM, non-relapse mortality; NTBI, non-transferrin-bound iron; OS, overall survival; PFS, progressive-free survival; PUFA, polyunsaturated fatty acid; RFS, relapse-free survival; ROS, reactive oxygen species; TAM, tumor-associated macrophage; TCA, tricarboxylic acid cycle; TFR, transferrin receptor.

INTRODUCTION

Myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) represent heterogeneous clonal hematopoietic stem cells disorders. MDS is characterized by dysplasia of hematopoietic cells, AML by uncontrolled proliferation of poorly differentiated hematopoietic cells (blasts). Both diseases lead to insufficient hematopoiesis. Chronic fatigue due to anemia, bleeding due to thrombocytopenia and infection due to neutropenia are typical consequences. MDS bone marrow is prone to leukemic transformation with approximately 30% of MDS patients developing secondary AML over time (1). AML, being the most common acute leukemia in adults, is a disease that in most cases needs immediate treatment to avoid death within months or even weeks. Although our knowledge about the molecular drivers of AML is rapidly increasing, and recently resulted in the development of novel drugs and of molecularly informed treatment stratification, the 5-year overall survival (OS) rate is still below 30% (2).

MDS and AML patients may develop primary iron overload arising from insufficient erythropoiesis (3). Repeated transfusions, which aim at ameliorating the symptoms of anemia, often lead to secondary iron overload. Iron overload in MDS and AML patients may lead to multiple cellular and systemic changes and therefore plays a crucial role in these hematologic malignancies (**Figure 1**). Besides the importance of iron and proteins involved in iron metabolism for multiple cellular functions, iron is tightly connected to the production of reactive oxygen species (ROS) and can lead to cell death when in excess (4). Iron overload in the bone marrow and other tissues can result in alterations of the microenvironment and contribute to increased morbidity (5). In this respect, iron has been demonstrated to participate in aggravating the symptoms of MDS and AML patients by contributing to bone marrow failure (6). Excess iron can also alter the components of the immune system and result in an increased susceptibility to various infections (7). Therefore, serum and cellular iron levels have a prognostic value at initial diagnosis, might influence the response to chemotherapy and predict the outcome after hematopoietic stem cell transplantation (HSCT) (8–10). The involvement of iron in diverse metabolic processes and its special necessity for malignant cells makes it an interesting therapeutic target (11).

In this review, we will first give an overview of the molecular basis of iron metabolism and its role in hematopoiesis. We will then focus on the altered iron metabolism in MDS and AML patients including clinical consequences. Subsequently, we will elucidate the effect of iron overload on the pathophysiology of MDS and AML, clinical consequences of the altered iron metabolism and its use as a potential target for therapy.

IRON HOMEOSTASIS AND ITS ROLE FOR NORMAL HEMATOPOIESIS

Iron is an essential micronutrient for fundamental metabolic processes in all cells and organisms and is therefore a crucial

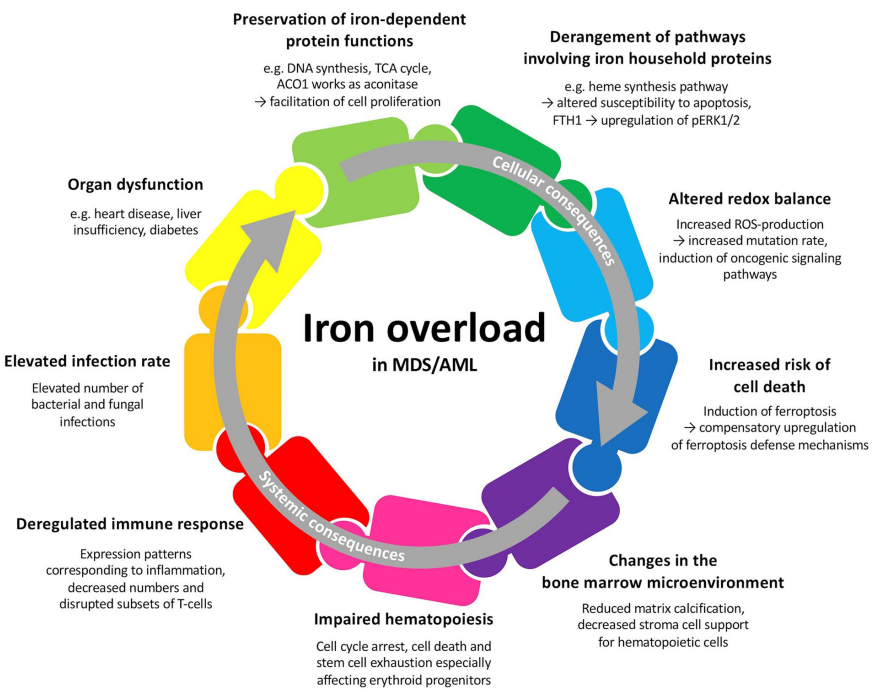

FIGURE 1 | Potential cellular and systemic consequences of iron overload in patients with MDS or AML. Many of these factors are interwoven and may all together contribute to patient outcome.

element for terrestrial life. A vital iron-binding protein of the human body is hemoglobin, which is crucial for the transport, storage and distribution of oxygen. Hemoglobin in circulating erythrocytes and erythroid precursors in the bone marrow contains about two thirds of the total body iron (12). Besides, iron is bound to myoglobin in the muscles. Iron is also part of prosthetic groups such as in cytochrome proteins and Fe-S clusters due to its ability to facilitate electron transfer. Thereby, it is essential for the function of the citric acid cycle (TCA), the respiratory chain, DNA synthesis and DNA repair.

Systemic iron homeostasis is maintained by a balance of iron uptake, recycling and loss (**Figure 2A**). Nutritional iron is mainly available as ferric iron, which can be reduced by ferrireductases. Subsequently, ferrous iron can be internalized into enterocytes *via* need-oriented gastrointestinal active transport mechanisms by the divalent metal ion transporter (SLC11A2). Iron may also be internalized through siderophore-associated binding to lipocalin-2 (LCN2) and subsequent endocytosis (13). Moreover, nutritional heme and possibly also ferritin can be absorbed by enterocytes *via* mechanisms not fully determined yet (14). Efflux of iron across the basolateral membrane into the bloodstream *via* ferroportin (SLC40A1), the only known iron exporter, is usually followed by its oxidation to ferric iron by the membrane-bound ferroxidase hephaestin. Ferric iron can be loaded to transferrin (TF) and then be used for the needs of the body. Excess iron is stored *via* ferritin (FTH and FTL) mainly in the liver. The body loses iron *via* exfoliation of cells on the inner and outer surfaces of the body with stool, urine, sweat and blood loss in menstruating women, but there are no physiological active excretion mechanisms to release an excess of iron in mammals and humans and the iron excretion cannot physiologically be increased beyond these values. High iron levels lead to systemic secretion of hepcidin, the most relevant regulator of the systemic iron metabolism, by the liver.

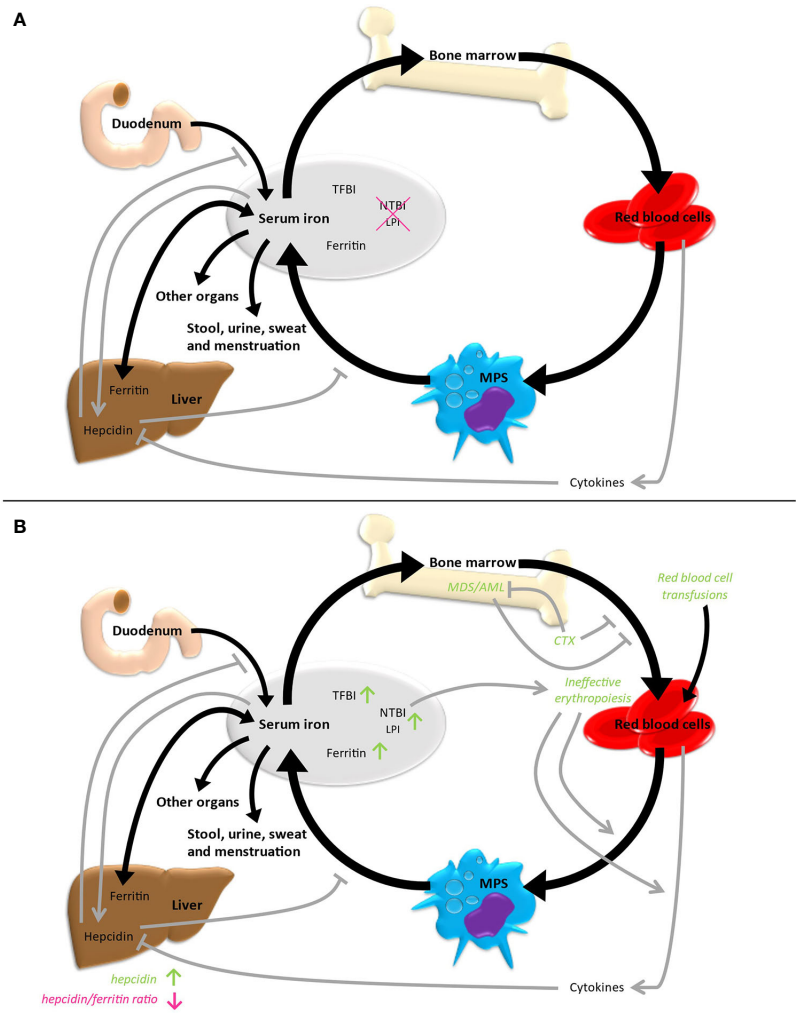

FIGURE 2 | Iron metabolism under physiological conditions **(A)** and in case of MDS/AML **(B)**. Black arrows indicate direct iron metabolism, gray arrows represent regulatory mechanisms. LPI, labile plasma iron; MPS, mononuclear phagocyte system; NTBI, non-transferrin-bound iron; TFBI, transferrin-bound iron.

Hepcidin binds to ferroportin on enterocytes and iron-storing cells like macrophages, resulting in an internalization and degradation of the hepcidin-ferroportin complex and thus effectively shuts down nutritional iron absorption and iron release from internal iron storage. Hepcidin expression is controlled by regulatory feedback mechanisms that involve active erythropoiesis: erythroblast-derived erythroferrone (ERFE), growth differentiation factor 11 (GDF11), growth differentiation factor 15 (GDF15) and twisted gastrulation protein homolog 1 (TWSG1) have been shown to influence hepatic hepcidin secretion, thus linking erythropoietic iron demand to iron supply (15–18).

Overall, only 4% to 10% of the daily iron need is supplied by uptake of nutritional iron, whereas the majority of iron gets recycled by different cell types originating from the bone marrow. Cells within the mononuclear phagocyte system (MPS) remove senescent blood cells *via* phagocytosis and digestion. Afterwards, iron is released into the blood, from where it is transported by transferrin back to the bone marrow for recurrent use in hematopoiesis. About ten times the amount of serum transferrin iron is recycled through this bone marrow-MPS-bone marrow cycle per day (19).

Intracellular iron acquisition is provided by ferrous iron importers (SLC11A2, SLC39A8, SLC39A14) or by binding of diferric transferrin to the cell surface transferrin receptors (TFR: TFRC and TFR2α) resulting in an internalization of the complex by clathrin-mediated endocytosis. Acidification of the endosome results in the release of ferric iron from transferrin. Additionally, circulating FTH can bind transferrin-independently to TFRC and be internalized in this way (20). Endosomal ferric iron is reduced to ferrous iron *via* ferrireductases. Ferrous iron can then be transported to the cytosol, where it represents the labile cellular iron (LCI) pool. This non-bound, redox-active and chelatable iron pool can be utilized in cellular metabolic processes, or, when in excess, be stored in ferritin or excreted *via* ferroportin. NCOA4 can mediate ferritinophagy, while it is degraded *via* HERC2 ubiquitination-mediated induction of the proteasomal degradation machinery in the presence of iron (21). Intracellular iron proteins are post-transcriptionally regulated by the IRP/IREs regulatory network. Therefore, mRNAs of regulated proteins harbor specific hairpin stem loops, called iron-responsive elements (IRE), situated in the 3' or 5' untranslated regions. In iron-deplete cells, the iron-responsive element binding proteins ACO1 and IREB2 bind to the IREs of specific mRNAs resulting in mRNA stabilization or translational repression of these mRNAs. In this way, they modulate the expression of iron-regulating proteins, which subsequently leads to an increase of the labile iron pool. In iron-replete cells, ACO1 works instead as aconitase in the TCA cycle and IREB2 undergoes SCF^{FBXL5} E3 ubiquitin ligase mediated ubiquitination and proteasomal degradation (22).

Both, iron deficiency and iron overload lead to impaired hematopoietic functions. Iron deficiency resulting in microcytic anemia due to impaired hemoglobin production is a common nutritional deficiency disorder affecting especially women and children worldwide. As a consequence of iron overload, dysplastic changes and detrimental effects on erythroblast differentiation and maturation resulting in a reduction of the proliferative capacity of erythropoiesis and of erythroblast apoptosis *in vitro* have been described (3, 23). Additionally, iron overload has been shown to induce growth arrest and cell death due to oxidative stress *via* ROS-mediated activation of p38MAPK, JNK and p53 pathways in immature hematopoietic cells (24, 25). Thereby, the IRP/IRE regulatory network is essential in maintaining hematopoietic stem cells in their physiological self-renewal process. While Ireb2(-/-) mice develop microcytic anemia, deletion of Fbxl5 in murine hematopoietic stem cells leads to impaired hematopoiesis due to Ireb2 overexpression and subsequent iron overload (26, 27).

ELEVATED IRON LEVELS IN MDS AND AML PATIENTS

Measurement of a patient's iron status is difficult due to various pitfalls of the available methods. Most commonly, iron status is measured based on serum iron indicators such as serum ferritin, transferrin saturation and soluble transferrin receptor (sTFR). However, the results may be influenced by external factors including inflammation, growth factors and organ dysfunctions (28). In case of acute iron overload, exceedance of the transferrin binding capacity leads to detectable amounts of non-transferrin-bound iron (NTBI) in the serum. A subfraction of NTBI is chemically labile plasma iron (LPI), which is toxic due to its redox-activity and can cause oxidative damage to cellular membranes, proteins and DNA (29). NTBI including LPI are loosely bound to serum components as albumin and citrate (30). Thereby, the presence and dynamics of active iron forms as NTBI and LPI may be accountable for direct toxic effects, whereas steady iron markers as ferritin may reflect mainly systemic changes in iron metabolism. Iron overload can also be measured *via* organ biopsies or imaging methods as biomagnetic susceptometry or magnetic resonance imaging (MRI) although these methods are rarely applied due to their invasiveness, costs or insufficient validation (31).

Using this variety of methods, over the years several characteristics of an altered iron metabolism in MDS and AML patients have been found together indicating a state of iron overload in these diseases (**Figure 2B**).

The most common reason for iron overload in patients with hematologic diseases is the administration of multiple red blood cell transfusions representing a massive excess of iron uptake with about 200 mg of iron in one unit of packed red blood cells (32). Transfusion-associated iron is processed by hepatic and splenic macrophages, which recycle heme iron from erythrocytes and release it into the extracellular space, thereby increasing the serum iron pool.

Independent of red blood cell transfusions, disease-immanent factors can contribute to the iron overload phenotype. Dysplastic ineffective erythropoiesis is one of the cardinal manifestations of

MDS, leading to an insufficient production of mature erythrocytes and potentially to a higher turnover of erythroid progenitors. This insufficient erythropoiesis leads to the secretion of hepcidin-suppressing cytokines and thus might result in further iron overload. A vicious cycle is formed, in which primary bone marrow dysfunction causes iron overload, which in turn amplifies bone marrow dysfunction. The presence of this mechanism is supported by data from Cui et al., who found elevated hepcidin and ferritin levels, but a reduced hepcidin/ferritin ratio compared to healthy controls in a study including 107 MDS patients without prior transfusions (33). In the same study, elevated ferritin levels correlated with decreased proliferative potential of erythropoiesis *ex vivo*. However, the extent of these mechanisms seems to differ between MDS subtypes. MDS subtypes with a high presence of ring sideroblasts (RARS, corresponding to MDS-RS according to the present WHO classification) as a morphological correlate of iron-loaded mitochondria, have been shown to have the lowest hepcidin/ferritin ratio (34, 35). Therefore, inefficient erythropoiesis might be more prominent in these subtypes than in other MDS patients. Correspondingly, MDS-RS is typically associated with a mutation in the splicing factor gene *SF3B1*. An *SF3B1* mutation was recently identified by Bondu et al. to lead to the expression of an alternative *ERFE* transcript, which suppresses hepcidin transcription and thereby provides an explanation for the increased iron load especially in these patients (36). The European MDS registry (EUMDS) investigated the occurrence of iron overload in MDS patients prospectively (37). Here, clinical data and iron metabolism-associated parameters including serum levels of ferritin, transferrin, hepcidin, GDF15, sTFR, NTBI and LPI were analyzed in newly diagnosed lower-risk MDS patients from 148 centers in 16 countries in Europe and Israel since 2008. The results indicate that the above-mentioned concept of primary, disease-immanent iron overload may not be of strong relevance for the majority of MDS patients: markers of iron overload were elevated over all MDS subtypes. However, occurrence specifically correlated with transfusion-dependent MDS and with the MDS-RS subtype.

During chemotherapy and foremost during hematopoietic stem cell transplantation (HSCT) the iron homeostasis might be further disturbed as a result of erythroid cell lysis and suppressed erythropoiesis. This theory matches data from the German prospective multicenter study ALLIVE including 22 MDS and 90 AML patients and some smaller studies, which show an increase in NTBI and LIP levels during allogeneic HSCT (38–40).

During the course of AML, signs of iron overload have also been described. Frequently, serum ferritin is elevated at initial AML diagnosis. The extent correlates with the leukemic burden, normalizes in remission, and increasing levels may signify a relapse (41). Increased hepcidin serum levels at diagnosis and pre- as well as post-HSCT were described in two small cohorts including exclusively or mostly AML patients (42, 43). However, hepcidin and ferritin are acute-phase proteins and might not only indicate iron overload but may also reflect a state of inflammation. Correspondingly, ferritin and hepcidin serum levels in one of these studies correlated with serum levels of CRP and IL-6 (42). In another study, ferritin levels were also elevated in CRP-low patients and ferritin and hepcidin levels correlated with the number of blood transfusions (43). Overall, valid data including definitive measures of iron overload and investigations in the systemic iron state in AML are missing. Specifically, there are no data available from investigating the interplay of insufficient hematopoiesis and iron metabolism in AML. Presumably, ineffective erythropoiesis due to dysplastic changes applies only to an AML subgroup (especially AML with myelodysplasia-related changes), whereas in other AML subtypes, insufficient erythropoiesis may rather be driven by other pathomechanisms as the suppression of erythropoiesis by inflammatory cytokines (44).

Signs of iron overload show a prognostic impact in both, MDS and AML patients in many studies. One of the open questions in the field is, whether iron overload is just a consequence of increased transfusion frequency, which is a well-known measure of disease severity, which would explain the worse prognosis, or, whether iron overload *per se* has a negative impact on the course of the disease. In both diseases, the degree of transfusion dependency was associated with a worse patient outcome (45–48). However, high levels of LPI were associated with inferior overall and progression-free survival in lower-risk MDS patients irrespective of the transfusion status in the study of the European MDS registry (37). The ALLIVE study revealed that in patients undergoing allogeneic HSCT, pretransplant NTBI was associated with an increased incidence of non-relapse mortality and a worse overall survival, which is hard to explain by the pretransplant disease severity alone (38). Besides, high serum ferritin levels at AML diagnosis were associated with a worse outcome (9, 49, 50). The same is true for ferritin levels before and after allogeneic HSCT in cohorts including mainly MDS and AML patients (51, 52). Data on the prognostic impact of the liver iron content measured by MRI for patients receiving allogeneic HSCT are ambiguous. While a meta-analysis of four studies with mixed patient cohorts including overall 50% AML and 16% MDS patients found that increased liver iron was not indicative for bad patient outcome (53), the ALLIVE study showed an association of high pretransplant liver iron with increased early non-relapse mortality (NRM) (38). Despite different compositions of the patient cohorts with older, more severely iron-overloaded patients in the ALLIVE study, the role for liver iron overload in NRM remains inconclusive.

Taking the data on the prognostic impact of different iron overload markers together, the overall correlation with patient outcome is striking. However, it is difficult to exclude that this is merely the reflection of disease severity. Despite these doubts, clinical correlation data and studies on the consequences of iron overload from other diseases, led to the widespread recommendation to treat transfusion-induced iron overload in patients with hematological malignancies. Several therapeutic options are available that will be reviewed in section *Therapies Aiming at Iron Metabolism as a Possible Target in MDS and AML*.

POTENTIAL ROLES OF IRON-RELATED INTRACELLULAR PROTEINS IN AML AND MDS

To further understand the iron metabolism in MDS and AML, investigating the role of iron-related intracellular proteins might help explaining the interplay between iron and essential intracellular networks in MDS and AML cells.

Expression of iron-importing proteins might be an indicator for the iron need of the cells. It has been appreciated for almost 40 years that AML cells strongly bind to transferrin (54). In humans, two transferrin-binding receptors have been identified: TFRC is a ubiquitously expressed high affinity receptor and TFR2α is restricted to certain cell types as hepatocytes and erythroblasts and has an approximately 25-fold lower affinity for transferrin than TFRC (55, 56). The alternative TFR2 isoform, TFR2β, lacks the transmembrane and cytoplasmic domain but might be involved in the regulation of iron efflux in the MPS (57). Overexpression of TFRC was demonstrated in AML cells (58–60) and supports the hypothesis of a higher iron consumption of these cells. Thereby, TFRC expression was higher in undifferentiated than in more differentiated AML subtypes and decreased with terminal differentiation (59, 61). Neither high *TFRC* mRNA nor TFRC protein levels in AML cells correlated with patient outcome although a correlation was found with increased anemia, thrombopenia and complex cytogenetics (62, 63). On the contrary, higher *TFR2* mRNA levels in bone marrow samples were surprisingly associated with a favorable outcome in AML and MDS patients (64, 65). However, the increase of TFR2 mRNA levels in MDS and AML bone marrow samples were shown to roughly correlate with the proportion of erythroid cells in the marrow and might therefore only to a minor extent reflect the expression of MDS or AML cells themselves (13, 66). This association with the erythroid cell number might be the explanation for the favorable outcome. Deducing from these data, higher TFRC expression of AML cells might reflect an undifferentiated blast status whereas higher TFR2 mRNA expression in the bone marrow of MDS and AML patients might be a marker for the number of erythroid cells. However, there is also evidence for a need of higher iron amounts due to overall higher TFRC expression and the necessity of TFR for leukemic cell growth as shown in TFR antibody studies described in section *Perspectives*.

Only recently, the roles of LCN2 and BDH2 have attracted attention in MDS and AML patients. LCN2 can bind to siderophores and thereby lead to iron internalization *via* endocytosis or to the secretion of iron *via* endosome recycling thereby potentially enabling iron overload or iron deficiency (67). BDH2 catalyzes the rate-limiting step for the formation of the mammalian siderophore 2,5-dihydroxybenzoic acid (68). This might facilitate LCN2-mediated iron uptake but also prevent iron overload in the cytoplasm and iron depletion in mitochondria. In cytogenetically normal AML patients, *LCN2* mRNA was reduced (69). Thereby, high *LCN2* mRNA expression in the bone marrow was associated with a favorable outcome especially in combination with wild-type *FLT3* showing an enhanced apoptosis under hydrogen peroxide and cytarabine treatment whereas showing a protective effect under DFO treatment. On the contrary, *BDH2* overexpression has been associated with poor overall survival in cytogenetically normal AML (70) and with elevated ferritin levels as well as an increased risk for progression to leukemia in MDS (71). As further mechanistical analyses are missing, it can only be speculated that in this case *LCN2* overexpressing cells might have an increased LPI pool predisposing them to oxidative stress, whereas *BDH2* overexpression might prevent cytoplasmatic iron overload. Further studies validating these results and unraveling the underling mechanisms are highly needed.

The intracellular conversion of insoluble ferric to soluble ferrous iron is mediated by ferric reductases including STEAP protein members. Although STEAP1 has no iron reducing function, it co-localizes with transferrin and TFRC suggesting also a role in iron homeostasis. In AML, *STEAP1* was shown to be overexpressed and associated with an adverse OS (72).

Systemically elevated levels of the iron storage protein ferritin suggest a role for intracellular ferritin levels in MDS and AML as well. FTH1 was reported to be expressed particularly in erythroid blasts measured by immunohistochemistry (73). In another study, *FTH1* and *FTL* mRNA overexpression and FTH1 protein overexpression measured by immunoblot were shown in AML primary cells compared to peripheral mononuclear cells (9). The presence of ferritin may reduce the LPI pool and therefore prevent ROS formation. In line with this, a decreased *in vitro* cytotoxic activity of cytarabine was detected in FTH1 overexpressing AML. Additionally, analyses of the erythroleukemia cell line K562 indicate that *FTH1* expression might prevent ROS-induced protein misfolding (74) and ROS-induced activation of the HIF1A/CXCR4 pathway leading to an epithelial-to-mesenchymal transition (EMT)-like phenotype (75). Besides, FTH1 might regulate RAF1 downregulation and activate pERK1/2 through downregulation of the expression of distinct microRNAs (76). Therefore, intracellular ferritin expression might play a role in MDS and AML especially in erythroid blasts on many levels.

Expression of the iron exporter FPT is also suggested to reduce the LPI pool and thereby the formation of ROS. Low *FPT* levels in AML cells correlated with good risk cytogenetics, increased sensitivity to cytarabine treatment and favorable outcomes (10) but a causal relationship could not be deduced from this data.

Overall, several changes in proteins associated with iron metabolism have been detected in MDS and AML cells. Mutually, the iron status and these proteins as well as several intracellular signaling pathways influence each other. Thereby, especially proteins directly regulating the intracellular iron pool seem to have an impact on cell viability and patient outcome.

IRON AND ROS HOMEOSTASIS IN LEUKEMOGENESIS

Iron and ROS homeostasis are closely entangled. Iron contributes to ROS formation by the production of hydroxyl

radicals *via* the Haber-Weiss and Fenton reaction. Moreover, iron is involved in indirect ROS production. Multiple iron-containing enzymes and those which require iron as an indispensable cofactor contribute to ROS production under normal conditions (77). So, as an important component of the respiratory chain iron is involved in the formation of mitochondrial ROS during oxidative phosphorylation (78). Vice versa, ROS can interact with iron sulfur clusters ([4Fe-4S]), turning them into their inactive form ([3Fe-4S]+). This leads to a switch in the function of the iron-sulfur cluster protein ACO1 from its role as aconitase in the TCA cycle to its function as an IRE-binding protein regulating the expression of various proteins involved in iron metabolism and other pathways (79).

Elevated ROS levels have been detected in MDS and AML patients compared to controls (80, 81). Moreover, iron overload is accompanied by increased ROS levels in this patient cohort (82–84). Therefore, iron may contribute to leukemogenesis *via* its effect on the ROS homeostasis.

Due to this connection, iron overload has been discussed to be involved in mutagenesis and leukemic transformation. Highly reactive hydroxyl radicals can directly interact with DNA leading to DNA damage (85). Moreover, ROS can stimulate the generation of lipid peroxyl radicals especially of polyunsaturated fatty acids (PUFAs) leading to reactive aldehydes that are mutagenic and genotoxic (86). In a mouse model for myelodysplastic syndrome using *NUP98-HOXD13* (NHD13) transgenic mice, increased levels of ROS were detected in bone marrow nucleated cells accompanied by increased DNA double strand breaks supporting a connection between ROS and malignant transformation (87). In this line, a 5-year prospective registry study including 599 MDS patients revealed a deceased rate of progression to AML in patients treated with iron chelators (60). On the contrary, an earlier meta-analysis of Zeidan et al. did not confirm differences in the progression of MDS to AML with or without administration of iron chelators (88). Thereby, analyses might differ due to different MDS subgroups, observation periods and a potential selection bias for patients with longer predicted survival receiving iron chelation. Deducing from these results, leukemic transformation as a result of iron overload is a valid hypothesis but data are still ambiguous and more prospective trials are required. Possibly, disease related risk factors in MDS may overcome the influence of iron overload on progression to AML. The fact that mutations in the hereditary hemochromatosis protein (HFE) have not been found to increase the risk of AML (89, 90) may also indicate that *de novo* AML development is not induced by systemic iron overload.

ROS is known to highly influence hematopoiesis including hematopoietic stem cell state and function (91, 92). ROS is also involved in the regulation of various intracellular processes and signaling pathways (e.g. NF-κB, MAPK, PI3K-Akt, ubiquitination) as it is able to interact directly with proteins, ions and other molecules (93). Therefore, ROS might also influence stemness and proliferation of MDS and AML cells. Many molecular lesions related to MDS and AML development including mutations in *FLT3*, *NRAS/KRAS* and *IDH1/2* affect intracellular ROS production, thus potentially promoting ROS-mediated oncogenic signaling (94). Therefore, iron might impact intracellular signaling and cell fate decisions also by its influence on intracellular ROS signaling. Indeed, iron and associated proteins are involved in some of these signaling pathways as described in section *Potential Roles of Iron-Related Intracellular Proteins in AML and MDS*. However, studies further investigating this theory are needed.

In the extreme, iron overload with subsequent overwhelming accumulation of ROS can lead to ferroptosis, a non-apoptotic form of programmed cell death dependent on iron that differs from other regulated cell death mechanisms as apoptosis. First labeled by Dixon in 2012, ferroptosis is the consequence of a reduced antioxidant defense leading to uncontrolled lipid peroxidation and subsequent oxidative cell death (95). Depending on the activation of ROS-connected signaling pathways, cells are at a different risk for ferroptosis. Treatment of NRAS-Q61L mutated AML cells with the ferroptosis-inducing molecule erastin resulted in enhanced ROS levels and cytosolic translocation of HMGB1 leading to cell death, whereas this effect was not seen in unmutated cell lines (96). Importantly, the effect was iron-dependent and *HMGB1* knock-down lead to lower expression of TFRC.

Leukemic cells seem especially exposed to iron overload with the risk of undergoing ferroptosis. This indicates that they may have gained some ferroptosis evasion strategies. Indeed, Hole et al. could show that higher levels of NOX-derived ROS (ROS) in AML blasts were tolerated by evading oxidative stress response through suppression of p38MAPK signaling (97). Additionally, glutathione peroxidases, which can protect cells from oxidative damage by reducing lipid hydroperoxides and free hydrogen peroxide are overexpressed in AML patient samples and associated with an adverse OS (98). Moreover, Yusuf et al. show a dependency of murine and human AML cells on ALDH3A2, which can detoxify fatty aldehydes and thereby prevent oxidative damage due to lipid peroxidation (99). In mouse models, reduction of Aldh3a2 induced ferroptosis in leukemic cells and was synergistically lethal combined with the inhibitor of glutathione peroxidase 4 (GPX4) RSL3, whereas it was dispensable for normal hematopoiesis. In this line, the transcription factor NFE2L2 also seems to strengthen the oxidative stress defense in leukemic cells by regulating the expression of many antioxidative proteins especially in case of additional chemotherapeutic treatment (100, 101). Parallelly, NFE2L2 also regulates the expression of iron-related proteins as FTH1, FTL and HMXO1 again supporting a close connection between ROS and iron homeostasis. All these findings support the hypothesis that AML cells might benefit from the toleration of higher iron and ROS levels. To which extent iron is involved in this pathomechanism and if this is also the case for MDS cells has to be further elucidated.

IRON AND THE MICROENVIRONMENT

Hematopoietic and leukemic blasts reside and proliferate in bone marrow niches interacting with their microenvironment. The microenvironment including mesenchymal cells, endothelial cells, sympathetic neurons, other hematopoietic and immune cells and the extracellular matrix is considered to be a key regulator of MDS and AML pathogenesis and recurrence (25, 102). Leukemic cells

seem to adopt the bone marrow microenvironment according to their needs and suppress normal hematopoiesis *via* secretion of cytokines, microRNAs and exosomes.

Excess iron in AML and MDS patients is deposited in various organs including the bone marrow thereby altering the composition of the hematopoietic niche and potentially leading to hematopoietic niche defects. A murine iron overload model revealed elevated ROS levels and increased bone resorption leading to changes in the bone microarchitecture with trabecular and cortical thinning of the bone (103). This loss of bone substance seems related to changes in the bone marrow mesenchymal stem cells (BM-MSCs). Several alterations in the bone marrow stroma cell number and composition have been reported which concur in the fact that iron overload reduces the differentiation into osteoblasts relative to other cell subtypes and reduces matrix calcification (103–105). Cheng et al. could also demonstrate a ROS-mediated cell death of mesenchymal cells due to iron overload mediated by the AMPK/MFF/DNM1L pathway triggering mitochondrial fragmentation and reducing ATP production (106). The alterations of the mesenchymal cell compartment were also shown to influence their supporting function for hematopoiesis. Thereby, the expression of several adhesion molecules and cytokine secretion was altered in bone marrow stroma cells under overload conditions impairing their capacity to support hematopoietic cells growth (24, 105, 107). This might also be important for transplant engraftment during HSCT, as transplantation from normal donor mice to mice with iron overload resulted in a delayed hematopoietic reconstitution (107). Therefore, the effects of iron overload on bone marrow structure and mesenchymal cells might attribute to the defective hematopoiesis found in MDS and AML patients.

Macrophages in the bone marrow of MDS patients were shown to have higher FTH expression (108). Additionally, expression of HMOX1 in macrophages was associated with an adverse patient outcome. In the microenvironment of solid tumors, tumor-associated macrophages (TAMs) are thought to contribute to tumor progression *via* delivery of iron to the tumor cells by an iron-release macrophage phenotype (109). However, it has not been investigated if there might be a similar role of leukemia-associated macrophages.

Normal cells of the hematopoietic and especially erythropoietic system are also highly affected by changes in the iron homeostasis as already described in section *Iron Homeostasis and its Role for Normal Hematopoiesis*. Morbidity and mortality in iron overloaded MDS and AML patients might therefore largely be explained by the toxicity of iron to these cells. In a murine iron overload model using RUNX1S291fs-induced MDS mice, the survival of iron overloaded MDS mice decreased as a result of an impaired frequency and colony-forming capacity of normal hematopoietic stem cells (110).

The iron household in the bone marrow might also affect endothelial cells and the vascular architecture. Cellular iron deficiency increases VEGF-induced angiogenesis (111, 112). Moreover, it has been shown that ferritin promotes the assembly of endothelial cells by antagonizing the antiangiogenic effects of cleaved high molecular weight kininogen (113).

Beside the bone marrow, an altered iron metabolism can also impact other organs. Iron overload due to multiple transfusions has been demonstrated to be toxic to various organs as liver, heart, pancreas, thyroid and pituitary gland leading to an increased morbidity and mortality (114). The influence of organ iron overload on patient outcome in MDS and AML patients is not fully determined yet. As described in section *Elevated Iron Levels in MDS and AML Patients*, data on a potential correlation of liver iron overload with NRM are ambiguous.

IRON, INFLAMMATION, AND INFECTION

Iron and proteins related with iron overload are closely connected to local or systemic inflammation and might also influence the occurrence of infections by effects on the immune system and various pathogens.

Patients with AML and MDS are often immunocompromised due to a suppression of normal hematopoiesis by the disease and bone marrow toxicity of applied chemotherapies. Additionally, the patients frequently undergo multiple medical interventions including placements of catheters, which further increase the risk of inflammation and infection. Patients receiving an allogeneic HSCT have also a risk of inflammation due to a Graft-versus-Host Disease (GvHD) and need immunosuppressive therapy.

Inflammatory stimuli lead to an upregulation of hepcidin and other acute-phase proteins as ferritin and caeruloplasmin as well as a down-regulation of negative acute-phase-proteins as transferrin. The resulting downregulation of available plasma iron may withhold iron from pathogenic microorganisms and protect healthy tissues from ROS damage at the site of infection. Many microorganisms require iron for electron transport, glycolysis, genome synthesis and defense, making it an essential nutrient. Excess iron has shown to stimulate the growth of many gram-positive and gram-negative bacteria, fungi and single-celled eukaryotes as well as the replication of viruses (115, 116). Correspondingly, patients with hemochromatosis or hemo globinopathies are at increased risk for infectious diseases due to iron overload (117, 118).

In patients undergoing allogeneic HSCT, high pre-transplant ferritin levels have been associated with an increased risk for invasive fungal pneumonia (119, 120) and hepatosplenic candidiasis (121). Patients suffering from mucormycosis in allogeneic HSCT recipients were found to have a severe iron overload compared with a matched control population (122). Moreover, early bacterial infections in allogeneic HSCT recipients were increased in patients with elevated pre-transplant hepcidin levels (123). A large metanalysis demonstrated a higher incidence of blood stream infections, a lower incidence of chronic GvHD and no effect concerning acute GvHD to be associated with high-pretransplant ferritin levels (52). Additionally, Pullarkat et al. reported in a prospective study, that iron overload measured by pre-transplant ferritin was a risk factor for mortality and blood stream infections but also for acute GvHD (124). Thereby, all these studies point towards a prognostic impact of iron overload markers as ferritin and hepcidin for fungal and bacterial

infections as well as for the occurrence of GvHD. Although an elevation of these markers was measured before the onset of the diseases, a bias for patients that were already initially prone to inflammation and infection cannot be excluded.

The function of cells belonging to the immune system may also be influenced by iron homeostasis. In MDS patients with iron overload measured by elevated ferritin and transferrin saturation, Chen et al. found a lower percentage of CD3+ T-cells and disrupted T-cell subsets accompanied by higher ROS-levels in these cells (125). Using a murine iron overload model, Chen et al. showed that iron overload could reduce peripheral T-cells, decrease Th1/Th2 as well as Tc1/Tc2 ratio and increase CD4/CD8 ratio as well as the fraction of regulatory T-cells by inducing ROS-mediated oxidative stress and apoptosis of T-lymphocytes. The impact of these alterations on the anti-leukemic defense, inflammation and infection as well as patient outcome is yet unclear.

THERAPIES AIMING AT IRON METABOLISM AS A POSSIBLE TARGET IN MDS AND AML

Features of iron overload, a differential iron metabolism and changes in proteins associated with iron have been found in MDS and AML patients. Markers of iron overload correlate with a worse prognosis in both patient cohorts. There is a rational for potential pathomechanisms explaining detrimental effects on the patient outcome by consequences of the altered iron metabolism. However, markers of iron overload are in many ways subject to the chicken-and-egg problem making it impossible to discriminate between cause and consequence. Therefore, interventional studies might cast light on the causative impact of the altered iron metabolism.

Iron-targeting strategies are based on the differential iron metabolism in case of MDS and AML compared to normal circumstances constituting a potential vulnerability in these diseases. Therapeutic strategies aiming at iron metabolism as a possible target in MDS and AML can be roughly distributed in four approaches: reduction of iron required for cellular functions *via* iron chelation, modulation of proteins involved in iron metabolism, induction of ferroptosis und exploitation of iron proteins for the delivery of antileukemic drugs (**Figure 3**). Thereby, most studies have been conducted using iron chelators, whereas the other approaches are in the early stages of development.

Iron Chelation

Iron overload, whether or not caused by an impaired underlying, dysregulated mechanisms or by multiple red blood cell transfusions, has been demonstrated to influence many

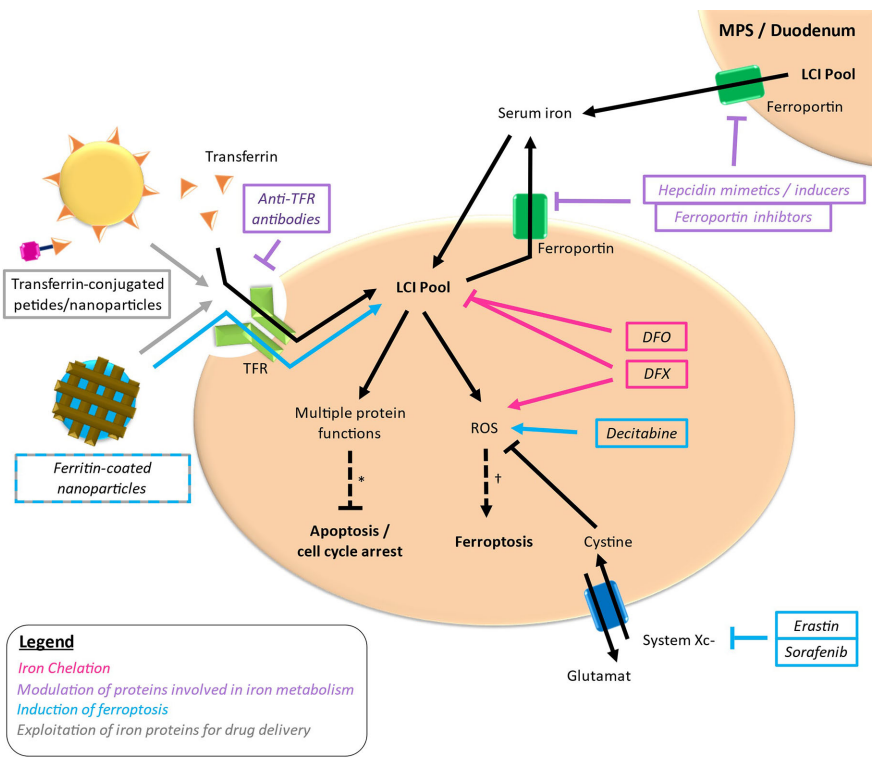

FIGURE 3 | Targets of different drugs interfering with iron metabolism. Simplified outline with colored arrows indicating the respective way of action. *loss of important protein functions can induce apoptosis and cell cycle arrest. †excessive ROS production leading to lipid peroxidation can lead to ferroptosis. LCI, labile cellular iron; TFR, transferrin receptor.

intracellular and systemic processes. The reduction of iron overload thus seems like an obvious therapeutic strategy to correct prognostically unfavorable effects.

Chelators can bind metal ions and afterwards be excreted as water-soluble complexes. By reducing NTBI, LPI and LCI pools, iron chelators may influence enzyme functions depending on iron, and influence ROS homeostasis. Therefore, iron chelation therapy (ICT) offers a rational therapeutic option in the treatment of patients with iron overload aiming at an induction of an antileukemic effect and a reduction of secondary organ dysfunctions and infections. So far, there are three iron chelators approved by the European Commission/ EMA for the treatment of patients with iron overload: Deferoxamine (DFO) administered parenterally and the orally available deferiprone (DFP) and deferasirox (DFX). Whereas DFP is approved only for patients with thalassemia major, DFO and DFX have broader indications including iron overload in MDS and AML patients.

Iron chelators seem to act by various mechanisms. Deferoxamine (DFO) was shown to negatively affect DNA synthesis and reduce cell growth in the leukemic cell line K562 by impairing the activity of ribonucleotide reductase (126). Ribonucleotide reductase catalyzes the formation of deoxyribonucleotides and needs iron as a cofactor to build a tyrosyl radical crucial for its function. DFO was shown to inhibit the enzyme activity by depletion of the LCI pools necessary to regenerate the active enzyme (127). Moreover, iron chelators can affect ROS homeostasis in two opposite directions leading to either ROS depletion or ROS promotion (128). The ROS depleting effect is suggested to depend on diminished free labile iron levels (129), whereas the ROS promoting effect may be facilitated by an iron-mediated free radical generation through the iron-chelator-complex (130, 131) or by a potentially iron-unrelated induction of ROS signaling (132). The effect of ICT on ROS seems thereby to depend on the binding-characteristics of the chelator, the time of treatment and the used concentration (133). Both mechanisms seem to play a role in ICT activity. The ROS-promoting activity has been suggested to participate in the effect of DFX in AML cells (128, 133). On the contrary, oxidative stress was reduced under long-term DFX treatment in MDS patients with iron overload (134, 135). ICT is also reported to enhance the effect of other antiproliferative drugs. *In vitro* and *in vivo* studies showed an increased antileukemic effect for the combination of DFO and cytarabine (136), DFX and decitabine (137) as well as DFO and doxorubicin (138). A potential mode of action for the combination of DFX with doxorubicin might be an increase of the intracellular calcium resulting in an improved sensitivity to chemotherapy in leukemia cell lines (138). Moreover, ICT has been found to modulate different signaling pathways including a repression of mTOR and NF-kB signaling pathways, which might also explain a potential synergistic effect with other drugs (139, 140). Iron chelators were also shown to act synergistically with differentiating agents in the treatment of AML (133). Thereby, iron chelation led to ROS production, activation of MAPK pathways and also induced expression and phosphorylation of the vitamin D3 receptor (VDR) leading to blast differentiation *in vitro*, *in vivo* and also in one patient with secondary AML treated with DFX and vitamin D3 after relapse of the disease (133). Deducing from these results, the mechanism of ICT action might not solely be the iron-deprivation but rather also a modulation of ROS homeostasis and intracellular signaling. A relation of the latter effects with the iron-modulating activity seems likely, but iron-independent effects of the ICT cannot be excluded. The diverse effects might not only depend on the way of chelator administration but also on the status of the treated cells.

Clinically, there is some evidence from *post-hoc* analyses in cohorts of low/intermediate-1 risk MDS patients with iron overload that iron chelators as DFX may improve hematological parameters after administration over at least one year in a small proportion of the patients (141–145). An increase of hemoglobin, platelets and/or neutrophils was observed in 11%–22% of the patients with a few multilineage improvements and a few transfusion independencies. Thereby, the data of List et al. suggest a possible correlation between the amount of ferritin reduction by iron chelators and hematological response (143). In a retrospective analysis of 182 patients with MDS with various subtypes, the multivariate analysis revealed a significant benefit in OS for patients receiving ICT with 140.9 months vs 36.3 months ($p=0.0008$) in case of refractory anemia (RA or, according to the present WHO classification: MDS-RS), 133.4 months vs 73.3 months ($p=0.02$) in case of refractory anemia with ring sideroblasts (RARS/RARS-t, corresponding to MDS-RS according to the present WHO classification) and no difference for refractory cytopenia with multilineage dysplasia (RCMD/ RCMD-RS, corresponding to MDS-MLD according to the present WHO classification) (146). The latter indicates that not all subtypes of MDS may benefit from ICT. It should also be noted that ICT seems to have the largest effects in subtypes which were suspected to suffer more from primary iron overload, MDS-RS and MDS-RA but not MDS-MLD, as marked by a reduced hepcidin/ferritin ratio described in section *Elevated Iron Levels in MDS and AML Patients*.

A recent systematic review and meta-analysis by Zeidan et al. included nine studies (4 prospective and 5 retrospective) with a total of 2450 patients with particularly low-risk MDS of whom 38.4% received ICT (88). Patients with ICT had a lower mortality and longer OS compared to no ICT with a pooled estimate of the ratio median OS of 2.1 years, suggesting that iron chelation therapy might double the OS in MDS. Additionally, there were some hints at a correlation between dose intensity of ICT and OS. Two of the reported studies compared patients with high adequate ICT to no ICT showing a highly significant survival advantage for patients with a higher adequate dose, but comparing any degree of ICT with no ICT, the OS benefit was less pronounced (88, 147, 148). In the study by Rose et al., adequate ICT was associated with median OS of 124 months compared to 85 months for ICT ($p < 0.001$) (147). Similar results were described by Delforge regarding OS with adequate ICT and no adequate ICT ($p = 0.001$) but not between weak ICT and no ICT (148). Hereby, adequate chelation was defined for DFO

subcutaneously (40 mg/kg/day in slow infusion over 8–12 h for at least 3 days per week), DFX (20–30 mg/kg/day p.o.) or DFP (30–75 mg/kg/day p.o.); weak chelation treatment was considered to be less than 3 g per week of DFO. The question whether there are any differences regarding the efficacy between the iron chelators cannot be answered finally due to a lack of randomized trials. However, the compliance of DFX might be better than that of DFO or DFP due to the oral mode of administration and the less frequent occurrence of side effects resulting in a continued application and more remarkable reduction of iron overload (149–153). Gastrointestinal adverse events and neutropenia were more frequently observed in DFP than in DFO (149, 150).

Randomized trials in MDS looking for the clinical benefit using iron chelators in patients with excessive iron overload are highly needed. Recently, Angelucci et al. published data from the randomized clinical study TELESTO (154). Here, 225 patients with low- to intermediate-1 risk MDS were treated with DFX versus placebo in a 2:1 randomization. The event-free survival (EFS) was prolonged with 3.9 years in the DFX versus 3.0 years in the placebo arm (HR 0.64). Although the study is limited by an amendment from a planned phase 3 trial with 630 patients to a phase 2 trial with 225 patients and different follow-up times between the groups, the data again support a benefit of iron chelation on the clinical outcome.

There are some weak hints that iron chelation also has positive effects after allogeneic HSCT on hematological reconstitution, but the number of patients reported is limited. So, in a rather small cohort of eight patients with incomplete hematological reconstitution after allogenic HSCT, treatment with DFX led to hematological improvements with a subsequent loss of transfusion dependency in all patients within a maximum of 30 days (155). Moreover, Cho et al. propose an enhanced graft-versus-leukemia (GvL) effect leading to a lower incidence of relapse, an improvement of DFS and OS, while the incidence of chronic GvHD by DFX treatment post-transplant increases (156). The data, however, are limited due to their retrospective analysis.

Besides the iron chelators mentioned above, there are also new iron chelators and other substances with iron-chelating properties under investigation. In a phase 2 study, triapine, forming a potentially redox active iron complex and known to inhibit the M2 subunit of the ribonucleotide reductase, showed clinical activity when administered sequentially with fludarabine in patients with accelerated myeloproliferative diseases and secondary AML (157, 158). Ciclopirox olamine, an antimycotic agent with iron chelation activity, showed a hematologic improvement in 2 out of 23 patients with relapsed or refractory hematologic diseases in a phase 1 study (159). Moreover, eltrombopag, a thrombopoietin receptor agonist approved for the treatment of idiopathic thrombocytopenic purpura and aplastic anemia, has also shown to be an efficient iron chelator, mobilizing iron and reducing ROS working synergistically with other iron chelators *in vitro* (160). In a mechanistic study on HSCs, eltrombopag stimulated hematopoiesis at the stem cell level through iron chelation-mediated reprogramming (161). Randomized placebo-controlled phase 1/2 data revealed a reduction of clinically relevant thrombocytopenic events upon eltrombopag treatment in MDS and AML patients (162, 163). On the contrary, a subsequent randomized phase 2 trial investigated the receipt of eltrombopag during standard induction therapy in AML patients and found no clinical benefit of eltrombopag but rather a tendency for increased severe adverse events (164).

The clinical data demonstrate activity of ICT in the treatment of low/intermediate-1 risk MDS patients with iron overload suggesting a potency of ICT as an additional treatment option. The other way around, it can be deduced that iron overload in these patients might be accountable for a worse patient outcome. Thereby, ICT seems to specifically improve the hematopoietic response. There is only limited data on the effect of ICT on leukemic cells themselves and on the role of ICT in AML. Deducing from some preclinical studies, ICT might here influence intracellular signaling and ROS homeostasis specifically in combination with other drugs.

Modulation of Proteins Involved in Iron Metabolism

Many different proteins are involved in iron metabolism and have demonstrated differential expression in MDS and AML cells as described in section *Potential Roles of Iron-Related Intracellular Proteins in AML* and *MDS*. Targeting these proteins therefore represents another potential treatment approach.

Considering that malignant cells need iron for proliferation and that TFR was demonstrated to be expressed on the surface of AML cells, it was tested if inhibition of the TFR may lead to an antiproliferative effect due to a decreased iron import. Indeed, various TFR antibodies showed inhibition of DNA synthesis and a subsequent growth inhibition of AML cells *in vitro* and a reduction of tumor growth in mouse models (126, 165–168). The effect of different TFR antibodies was even enhanced when used in combination (169). However, as TFR is also expressed on normal cells of the hematopoietic system and TFR antibodies have shown to impair growth of normal hematopoietic cells as well (165), bone marrow toxicity is thought to be an important side effect of the treatment. Despite this fact, administration of the TFR antibody 42/6 in patients with refractory cancer including lymphoma patients was well tolerated in a phase 1 trial (170). Clinical data for the treatment of MDS and AML patients are missing.

Hepcidin as regulator of systemic iron provides another reasonable antileukemic target with the aim to reduce overall iron load and subsequent toxic effects on organs as heart, liver and bone marrow. Hepcidin as a potential target of iron-homeostasis has been investigated in iron overload situations but without specific data for MDS and AML. Synthetic hepcidin mimetics such as PTG-300 or LJPC-401 have been reported to reduce serum iron levels and to be well-tolerated in phase 1 trials in healthy subjects and patients with iron overload, although the clinical relevance has still to be determined in ongoing studies (171, 172). Various other hepcidin targeting agents, for instance humanized monoclonal antibodies (LY2787106; 12B9m), the

anticalin (PRS-080), and Lexaptepid Pegol (NOX-H94) have been tested in preclinical models or early in-human trials as reviewed by Crielaard et al., but failed major efficacy so that further development was stopped (173). Matripase-2 (MT2A), a transmembrane serine protease predominantly expressed in hepatocytes suppresses the expression of hepatic hepcidin by cleaving the membrane hemojuvelin into an inactive form (174). Antisense DNA (IONIS-TMPRSS6-LRx) or liposomal siRNA (ALN-TMP) as well as some protease inhibitors have demonstrated specific MT-2 inhibiting activity with the potential to reduce secondary anemia in patients with iron overload in preclinical models (173–176). Targeting the hepcidin-ferroportin pathway by inhibiting the bone morphogenic protein BMP6, which stimulates hepcidin expression in the liver or the iron exporter ferroportin *via* the monoclonal antibodies, LY3113593 and LY2928057, has not been further investigated beyond a phase 1 study (177). Therefore, data on the role of the hepcidin-ferroportin axis as a potential therapeutic target were mostly negative, further studies of MT-2 inhibitors have to be awaited.

Induction of Ferroptosis

In contrast to influencing the course of the disease in MDS and AML by reducing iron overload, enhancing iron overload to induce ferroptosis represents an opposing but alterative mechanism. There are various agents acting as inhibitors or inducers of ferroptosis: Iron chelators, lipophilic antioxidants, inhibitors of lipid peroxidation and depletion of PUFAs inhibit ferroptosis, whereas ferroptosis is induced by the accumulation of iron or PUFA-phospholipids and by the depletion of endogenous inhibitors such as GSH, NADPH, GPX4 or vitamin E (178).

Erastin is a ferroptosis inducer acting on multiple levels. It inhibits the cysteine/glutamate antiporter system Xc-, thereby revoking cysteine import and thus reducing glutathione synthesis. It activates TP53, which can also inhibit system Xc, and it induces the opening of voltage-dependent anion channels (VDACs), thereby inducing mitochondrial dysfunction (179). The activation of ferroptosis by erastin promotes chaperone-mediated autophagy and the degradation of glutathione peroxidase 4 (GPX4) (180). In AML cell lines, erastin has shown a dose-dependent mixed-type of cell death, including ferroptosis, and enhanced the antileukemic effect of cytarabine and doxorubicin (156). Besides, the tyrosine kinase inhibitor sorafenib, which is approved for the treatment of liver renal and thyroid carcinoma and also showed efficacy in AML patients with FLT3-ITD (181, 182), also inhibits the system Xc^- (183).

Other ferroptosis inducers have shown antileukemic activity in AML cells as well: Dihydroartemisinin (DHA) was shown to induce ferroptosis of AML cells by leading to accelerated degradation of ferritin and increasing LPI (184). Besides, the frequently used antileukemic drug decitabine has recently suggested to induce ferroptosis (185). Treatment of MDS/AML cell lines with decitabine increased ROS levels by reducing GSH and GPX4 activity. Ferroptosis inducers enhanced the effect of decitabine, whereas ferroptosis inhibitors abrogated the effect. As iron chelators also potentiated the effect of decitabine, this is another hint that treatment effects may be mediated by ROS and might also be influenced by the intracellular iron household.

The data suggest a potential use of ferroptosis inducers in the treatment of AML, although clinical data are missing. There are not enough data to estimate the role of ferroptosis induction in MDS.

Exploitation of Iron Proteins for Targeted Drug Delivery

Another attempt to specifically target malignant cells is to use the TFR as target protein for the delivery of another tumor-specific cargo. Covalent conjugates of the ferroptosis inducing agent artemisinin and a transferrin-receptor targeting peptide combined ferroptosis induction and targeted delivery and revealed antileukemic affectivity *in vitro* (186). Thereby, artemisinin could be co-internalized with receptor-bound transferrin and could use the iron deliberated by transferrin to generate cytotoxic ROS. Moreover, transferrin-conjugated nanoparticles have shown potential in the delivery of antileukemic drugs: Transferrin-conjugated lipid nanoparticles delivering an antisense oligonucleotide targeting *BCL2* mRNA induced caspase-dependent apoptosis in AML cell lines and suppressed tumor growth of human AML xenograft tumors in mice (187, 188). Transferrin-conjugated liposomal nanoparticles containing antagomiR-126 resulted in reduction of leukemic stems cells in an AML mouse model (189). Additionally, transferrin-conjugated nanoparticles delivering doxorubicin showed cytotoxicity in myeloid leukemia cells *in vitro* and *in vivo* (190, 191). Also, transferrin-conjugated polymeric nanoparticles delivering edelfosine and lipid-based nanoparticles delivering etoposide revealed antileukemic activity *in vitro* (192, 193).

Ferritin can also be used as a protein cage for the delivery of other molecules due to its tertiary structure (194). As FTH can be bound and uptaken by TFRC (20), this provides another way of directed targeting. Ferritin nanovesicles delivering cytochrome C induced apoptosis in a promyelocytic AML cell line (195). Delivery of cytarabine in form of Fe_3O_4@SiO_2-cytarabine nanoparticles increased the cytotoxic effect of cytarabine alone about two orders of magnitude in cell lines (196). The combination of erastin and rapamycin, an inducer of autophagy, with ferritin as a nanodrug showed increased inhibition of tumor growth compared to the drugs administered separately (197). Besides, use of iron saturated ferritin as a component of nanoparticles may also contribute to ferroptosis induction. The intravenous iron preparation ferumoxytol has also shown to increase ROS and thereby induce ferroptosis in patient derived xenografts from primary AML samples with low ferroportin (198). Furthermore, nanoparticles using Fenton reactions to improve ferroptosis are under investigation (199).

Taken together, a couple of possible therapeutic agents have been developed that hijack iron proteins for target delivery. Their effectivity has been demonstrated *in vitro* and *in vivo*. Clinical studies have to further evaluate their use in patients.

PERSPECTIVES

In this review, we demonstrated the clinical significance of iron homeostasis in MDS and AML patients. Iron metabolism has been shown to impact multiple intracellular functions, the production of ROS, the microenvironment as well as the susceptibility to infections. Markers of iron overload were demonstrated to have prognostic relevance although the impact of an altered iron metabolism on patient outcome in MDS and AML is still under debate as markers of iron overload are highly influenced by inflammatory signals and complicate the detection of causative associations. Supporting a partially causative connection between iron metabolism and patient outcome, therapeutics addressing the iron balance as ICT were found to improve the outcome especially in low/intermediate-1 risk MDS patients. As recurrent red blood cell transfusions constitute the major source of secondary iron overload in MDS and AML patients, a more restrictive application should be considered. Moreover, various agents targeting proteins involved in iron homeostasis or inducing ferroptosis are investigated preclinically or are in early clinical development. With a more detailed understanding of the pathophysiology of MDS and AML in the context of iron, future development of new iron-targeting strategies may lead to better patient outcomes. Therefore, basic research further investigating the processes involved in iron homeostasis linked with redox balance and leukemia is inevitable. Moreover, clinical studies analyzing reliable markers for pathophysiological active iron overload and prospective studies exploring function of iron-homeostasis targeting drugs are essential. Especially the combination of iron-homeostasis targeting drugs with other antileukemic agents constitutes a promising approach due to potential synergistic effects and should therefore be further elucidated.

AUTHOR CONTRIBUTIONS

Conceptualization was done by SW, AP, and HS. Investigation and writing of the draft was done by SW and AP. Review and editing of the draft was done by NK, FS, and HS. Supervision was done by HS. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the German Cancer Consortium (DKTK) (SW, HS), the Alfred and Angeliga Gutermuth-Stiftung (SW), the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation)—SFB1177 TP E07 (HS) and SFB815 TP A10 (HS, NK) and by the LOEWE Center Frankfurt Cancer Institute (FCI) funded by the Hessen State Ministry for Higher Education, Research and the Arts [III L 5-519/03/03.001-(0015)].

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Conflict of Interest: 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.

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frontiers in Oncology

ORIGINAL RESEARCH
published: 02 March 2021
doi: 10.3389/fonc.2021.619242

The Identification and Validation of Two Heterogenous Subtypes and a Risk Signature Based on Ferroptosis in Hepatocellular Carcinoma

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OPEN ACCESS

Edited by:
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University of Trieste, Italy

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

Received: *19 October 2020*
Accepted: *18 January 2021*
Published: *02 March 2021*

Citation:
Liu Z, Wang L, Liu L, Lu T, Jiao D, Sun Y and Han X (2021) The Identification and Validation of Two Heterogenous Subtypes and a Risk Signature Based on Ferroptosis in Hepatocellular Carcinoma. Front. Oncol. 11:619242. doi: 10.3389/fonc.2021.619242

Background: Ferroptosis is essential for tumorigenesis and progression of hepatocellular carcinoma (HCC). The heterogeneity of ferroptosis and its relationship with tumor microenvironment (TME) have still remain elusive.

Methods: Based on 74 ferroptosis related genes (FRGs) and 3,933 HCC samples from 32 datasets, we comprehensively explored the heterogenous ferroptosis subtypes. The clinical significance, functional status, immune infiltration, immune escape mechanisms, and genomic alterations of different subtypes were further investigated.

Results: We identified and validated two heterogeneous ferroptosis subtypes: C1 was metabolism^{low}immunity^{high} subtype and C2 was metabolism^{high}immunity^{low} subtype. Compared to C2, C1 owned worse prognosis, and C1 tended to occur in the patients with clinical characteristics such as younger, female, advanced stage, higher grade, vascular invasion. C1 and C2 were more sensitive to immunotherapy and sorafenib, respectively. The immune escape mechanisms of C1 might be accumulating more immunosuppressive cells, inhibitory cytokines, and immune checkpoints, while C2 was mainly associated with inferior immunogenicity, defecting in antigen presentation, and lacking leukocytes. In addition, C1 was characterized by BAP1 mutation, MYC amplification, and SCD1 methylation, while C2 was characterized by the significant alterations in cell cycle and chromatin remodeling processes. We also constructed and validated a robust and promising signature termed ferroptosis related risk score (FRRS) for assessing prognosis and immunotherapy.

Conclusion: We identified and validated two heterogeneous ferroptosis subtypes and a reliable risk signature which used to assess prognosis and immunotherapy. Our results facilitated the understood of ferroptosis as well as clinical management and precise therapy of HCC.

Keywords: ferroptosis, hepatocellular carcinoma, tumor microenvironment, molecular subtype, immunotherapy

INTRODUCTION

Primary liver cancer is the sixth most prevalent malignant tumor worldwide and ranks fourth among the causes of tumor-related deaths, with approximately 840,000 new cases each year (1). Hepatocellular carcinoma (HCC) is major histological type (75–85%) and characterized by high invasiveness and mortality rate (1). Surgical resection is mainly performed for early HCC, but the 5-year recurrence rate is up to 70%, and most patients relapse within 2 years after surgery (2). Patients with unresectable HCC usually receive the multi-kinase inhibitors such as sorafenib and lenvatinib, but drug-resistance and adverse reactions limit the survival benefit (3). In recent years, although great progress in immunotherapy represented by immune checkpoint inhibitors (ICI), only 25% of patients have durable responses (4, 5). Even when combined with other treatment modalities such as local ablation and transcatheter arterial chemoembolization (TACE), the 5-year survival rate of patients is only 18% (6). Therefore, there is still a long way to improve the therapeutic effect of HCC patients.

Ferroptosis is a newly discovered pattern of programmed cell death characterized by iron-dependent lipid peroxidation and accumulation of reactive oxygen species (ROS), distinguished from typical apoptosis, autophagy, and programmed necrosis (7). Sorafenib, as the first-line drug for advanced HCC, could inhibit cystine-glutamate antiporter (system Xc^-), and further lead to ferroptosis due to glutathione (GSH) depletion. Our previous studies confirmed that haloperidol could enhance sorafenib-induced ferroptosis in HCC (8), moreover, sigma-1 receptor can antagonize the ferroptosis in HCC, and non-coding RNAs further regulated the process (9, 10). In addition to iron metabolism, lipid metabolism also plays a pivotal part in ferroptosis. Ou and colleagues found that low density lipoprotein docosahexaenoic acid nanoparticles could induce ferroptosis through glutathione peroxidase-4 (GPX4) inactivation, GSH depletion, and lipid peroxidation, thereby significantly inhibiting the growth of HCC (11). The above suggests that ferroptosis play an essential role in the progression as well as treatment of HCC, and further mining mechanisms will help the development of new therapeutic strategies.

The cancer immunoediting theory suggests that the tumor microenvironment (TME) can identify the body's dead cells (mainly apoptotic cells) and then clear them by immune system (12). Were ferroptosis cells the same as apoptotic cells? Wen and colleagues found that ferroptosis cancer cells could release high mobility group box 1 (HMGB1) of the damage-associated molecular pattern molecules (DAMPs) family in an autophagy-dependent manner, and then HMGB1 could elicit an inflammatory response upon recognition by pattern recognition receptors (13). Interestingly, previous study demonstrated that tumor cells with autophagy-dependent ferroptosis could release KRAS protein, which was further packaged into exosomes to promote tumor-associated macrophage (TAM) polarization to exert immunosuppressive effects (14). Recent study also found that GPX4 was essential for the survival and expansion of newly activated T cells. The lipid peroxidation of T cells could promote ferroptosis and further contributing to their low immune response rates to infection (15). Nevertheless, most of these scattered studies focused on the link between ferroptosis and individual immune cell, the interaction between TME and ferroptosis have yet to be further deciphered.

With the deepening of ferroptosis studies, its anti-tumor effect has gradually aroused much interest. Wang and colleagues found that CD8+ T cells activated by anti-PD-1 therapy enhanced the lipid peroxidation of tumor cells by releasing interferon gamma (IFN-γ), while the enhanced ferroptosis response could further elevate the immune efficacy (16). In recent years, the advantages of various new materials in cancer prevention and treatment have gradually emerged. Previous studies demonstrated that manganese-doped silica nanoparticle (MnMSN) can deplete GSH, and on-demand drug release can be achieved by loading sorafenib into MnMSN, while dual induction of ferroptosis is achieved by depletion of GSH and inhibition of intracellular GSH synthesis, showing efficient anti-HCC activity (17). Jiang and colleagues observed that a platelet membrane-camouflaged magnetic nanoparticle could sensitize ferroptosis by inhibiting system Xc^-, which lead to immunosuppressive M2 TAM reversely polarize to the anti-tumor M1 phenotype, further increasing response to immunotherapy (18). Therefore, the more exploration of the ferroptosis heterogeneity might facilitate the target treatment in HCC.

In the present research, we collected a total of 3,933 HCC samples from 32 datasets for analysis. Based on the expression of ferroptosis related genes (FRGs), we identified and validated two heterogeneous subtypes, high and low ferroptosis subtypes, and the two subtypes displayed specific clinical outcomes, immune escape mechanisms, and genomics driver events, respectively. Besides, we developed and validated a prognosis signature termed ferroptosis related risk score (FRRS), FRRS demonstrated outstanding advantages in predicting prognosis and response to immunotherapy. Overall, our work may deepen the understanding of ferroptosis, as well as provide a basis and reference for the clinical management and targeted therapy of HCC.

METHODS AND MATERIALS

Data Source and Processing

The workflow of our study was shown in **Figure S1**. We retrieved eligible datasets from GEO (Gene Expression Omnibus), the Cancer Genome Atlas (TCGA), and the International Cancer Genome Consortium (ICGC) using the following criteria: (1) data was acquired using microarray platforms detecting >10,000 genes; (2) the probe-to-gene mapping annotations were clear; (3) there were >=30 patients in each dataset; (4) patients with primary liver cancer were retained; (5) untreated patients; (6) samples taken after intervention (e.g. after cancer resection) were excluded.

A total of 3,933 eligible HCC samples were enrolled from 32 meta datasets including GSE102079, GSE107170, GSE109211, GSE112790, GSE116174, GSE121248, GSE14323, NCI (National Cancer Institute) cohort (GSE14520), GSE16757, GSE19977, GSE20017, GSE25097, GSE36376, GSE36411, GSE39791,

GSE43619, GSE45436, GSE46444, GSE50579, GSE54236, GSE57957, GSE62043, GSE62232, GSE63898, GSE64041, GSE76297, GSE76427, GSE84005, GSE87630, GSE9843, TCGA-LIHC, and ICGC-LIRI-JP. Among them, only NCI, TCGA-LIHC, and ICGC-LIRI-JP datasets possessed completely clinical and prognosis information (**Table S1**). All expression data was log-2 transformed because gene expression data is often heavily right-skewed in the linear scale. We took the gene intersection of all datasets and retained the common 8,731 genes; and all other genes can be considered "missing" for at least one cohort. To our knowledge, there are no guidelines for handling missing data in multicohort studies. However, guidelines for randomized clinical trials recommend skipping imputation and using only observed data when more than 40% of the data is missing. In this study, we served 30 meta cohorts from GEO database as the discovery cohort, and TCGA-LIHC and ICGC-LIRI-JP datasets as two independent validation cohorts.

The rma function implemented in affy package was employed to normalize the raw data from Affymetrix, and normalized matrix files of the other microarrays from other platforms were directly downloaded. Batch correction was performed using the combat algorithm implemented in SVA package. The RNA-seq data (FPKM normalized) of TCGA-LIHC cohort was obtained from the UCSC-Xena database and was further transformed to log2 (TPM+1). The RNA-seq data of ICGC-LIRI-JP dataset was retrieved from the ICGC data portal. Subsequently, we transformed the expression data into z-score in both discovery and validation cohorts. The corresponding clinical information were obtained from GEO, UCSC, and ICGC databases. The somatic mutation, copy number variation (CNV), and DNA methylation data in TCGA-LIHC were all downloaded from the TCGA portal. We calculated or recruited the tumor mutation burden (TMB), single nucleotide variants (SNV) and indel neoantigen load, microsatellite instability (MSI), cancer testis antigen (CTA) scores, and TCR/BCR diversity from Thorsson et al. study (19).

Identification of the Ferroptosis Subtypes of HCC

After a detailed literature research, we selected a total of 74 FRGs (**Table S2**). According to the FRGs expression, we performed consensus clustering in the discovery cohort *via* ConsensusClusterPlus package (20). The method was set to Kmeans algorithm based on the Euclidean distance, 1,000 times iteration, and taking 80% of the samples for each iteration. The number of clusters was set from 2 to 9, and the optimal number was determined by the cumulative distribution function (CDF) of the consensus score and the proportion of ambiguous clustering (PAC) (21). The NbClust package was applied to further verify the optimal number (22). Principal component analysis (PCA) was used to distinguish different subtype information in two-dimensional space.

Validation of the Ferroptosis Subtypes

We further quantitatively assessed the stability and reproducibility of proposed subtypes in the discovery and validation cohorts with in group proportion (IGP) statistic (23). IGP was defined as the proportion of the nearest neighbors of a certain subtype sample that were also assigned to the same subtype. A high IGP indicated that samples of this subtype were reproducible partitioned. To measure the IGP, we first calculated the centroid of each subtype in the discovery cohort. Each sample in the TCGA and ICGC validation cohorts was assigned to a certain subtype with the highest Pearson correlation coefficient between centroid and sample. The permutation in the clusterRepro package was set to 2000.

Functional Analysis and Immune Cell Infiltration Assessment

The gene set variation analysis (GSVA) was performed to identify specific pathways of each subtype (24). We downloaded Hallmark and KEGG gene sets from the Molecular Signatures Database and further transformed the gene expression matrix into gene set matrix using the GSVA package. Afterwards, we performed gene sets difference analysis using the limma package and the screening threshold were set to |log2 fold change (FC)| >0.2 and adjusted P-value <0.05. Adjusted P-value was obtained from the Benjamini–Hochberg multiple test correction.

Referring to Charoentong et al. study (25), we obtained the markers of 23 immune cells including: innate immune cells (activated dendritic cells, CD56+ natural killer cells, CD56− natural killer cells, eosinophils, immature dendritic cells, macrophages, mast cells, MDSC, monocytes, natural killer cells, neutrophils, and plasmacytoid dendritic cells) and adaptive immune cells (activated B cells, activated CD4+ T cells, activated CD8+ T cells, Gamma delta T cells, immature B cells, natural killer T cells, Treg cells, follicular helper T cells, Th1 cells, Th2 cells, and Th17 cells). Endothelial cells and fibroblasts, also the important components of TME, played a crucial role in tumor inflammation, angiogenesis, invasion, and metastasis. The markers of endothelial cell and fibroblast were retrieved from the MCP-counter (26) (**Table S3**). Based on these markers, we applied the single sample gene set enrichment analysis (ssGSEA) algorithm to evaluate the infiltration abundance of 25 TME cells.

Assessing Clinical Significance of the Ferroptosis Subtypes

We compared the differences between the two subtypes in age, gender, Body Mass Index (BMI), AJCC stage, grade and vascular invasion, and estimated relapse-free survival (RFS) and overall survival (OS) by the Kaplan-Meier survival analysis. Afterwards, we applied the pRRophetic package to predict the sensitivity to sorafenib in both discovery and validation cohorts (27). The IC50 (half maximal inhibitory concentration) values of the two subtypes were estimated by ridge regression, the smaller its IC50, the more sensitive it was to the drug. In addition, we also utilized TIDE web tool (http://tide.dfci.harvard.edu) to predict the sensitivity of the two subtypes to immunotherapy (28). TIDE algorithm was a computational method to model two primary mechanisms of tumor immune evasion: the induction of T cell dysfunction in tumors with high infiltration of cytotoxic T lymphocytes (CTL) and the prevention of T cell infiltration

in tumors with low CTL level. The Subclass mapping algorithm was used to evaluate the similarity of gene expression patterns between the two subtypes and immunotherapy-sensitive/insensitive populations (29).

Deciphering the Genomic Variation Landscape of the Two Subtypes

We identified significantly mutated genes (SMGs) in the two subtypes using MutSigCV 1.41 software, and genes with q values <0.05 were retained to further analysis. The MutationalPatterns package was applied to extract the mutational signatures of each subtype, and non-negative matrix factorization (NMF) determined the optimal number of mutational signatures. It turned out that the optimal number was 3 in both subtypes (**Figures S8K, L**). We then calculated the cosine similarity metrics between these extracted mutational signatures and 30 mutational signatures from the COSMIC database, and named after the most similar COSMIC signature. The GISTIC 2.0 software in GenePattern was applied to identify significantly amplified or deleted broad and focal segments. The global methylation level (GML) was retrieved from Jung et al. study (30). Moreover, we performed the following procedure to identify epigenetically silenced genes (ESGs): (1) excluding the CpG sites methylated in normal tissues (mean β-value of >0.2); (2) the DNA methylation data was divided into the methylation group and unmethylation group, according to the cutoff (β-value = 0.3), and further removed the probe that less than 10% of the tumor samples in the methylated group; (3) for each probe, if the difference between the corresponding gene mean expression in the unmethylated group and that in the methylated group was >1.64 standard deviations of the unmethylated group, the probe would be labeled as epigenetically silenced; (4) when multiple probes were assigned to the same gene, the gene with more than half of the corresponding probes were labeled as epigenetically silenced, and identified as ESG.

Generation of Ferroptosis Related Risk Score

We applied the limma package to identify differentially expressed genes (DEGs) between the two subtypes, setting the thresholds: |log2 FC| > 1 and adjusted P-value < 0.05. Adjusted P-value was obtained from the Benjamini–Hochberg multiple test correction. Combined with the previously obtained significant CNV associated genes (CAGs), SMGs and ESGs, we used Venn diagram to illustrate the relationship among the four gene sets, and then selected genes present in at least two gene sets for further analysis. A univariate Cox regression analysis revealed the prognosis value of these genes. The genes with statistically significant ($p < 0.05$) were incorporated into multivariate Cox regression analysis. Afterwards, we constructed the ferroptosis related signature using stepwise regression, and selected the optimal model when the AIC (Akaike Information Criterion) score was the smallest. This optimal model was as follows:

$$risk\ score = \sum(Expression(gene) * coef(gene))$$

where expression (gene) denoted the expression level of a gene and coef (gene) represented its regression coefficient. We named the signature the ferroptosis-related risk score (FRRS). The HCC samples were categorized into high and low FRRS groups according to the optimal cut-off value determined by the survminer package. Then, we performed Kaplan-Meier analysis of FRRS in three independent cohorts: TCGA, ICGC, and NCI, and further assessed the predictive accuracy of model with Concordance index (C-index).

Collection of Immunotherapy Cohorts and Biomarkers

We systematically collected immunotherapeutic cohorts that were publicly available and had expression data and complete clinical information, and three cohorts finally enrolled in our study: (1) advanced urothelial cancer patients who received the intervention of anti-PD-L1 antibody atezolizumab (IMvigor210 cohort) (31); (2) metastatic melanoma treated with anti-PD-1 antibody pembrolizumab (GSE78220 cohort) (32); (3) melanomas received adoptive T cell therapy (GSE100797 cohort) (33). According to the RECIST v1.1 criterion, patients whose treatment effectiveness could not be assessed were excluded. The complete response and partial response were regarded as immunotherapy response, the stable disease and progressive disease were regarded as immunotherapy non-response. The normalized expression data was further transformed into z-score. We evaluated the predictive performance of FRRS in three immunotherapy cohorts, and compared FRRS with seven other known biomarkers, including TMB, TIDE, MSI score, Merck18, IFGN, CD8, and CD274 (28, 34–36) (**Table S4**). The receiver operator characteristic (ROC) curves and the area under the ROC curve (AUC) were applied to estimate the predictive accuracy of each biomarker.

Statistical Analysis

The Pearson's chi-squared test or Fisher's exact test was employed to compare categorical variables. Continuous variables were compared between two groups through Wilcoxon rank-sum test or T test. Survival analysis including Kaplan-Meier and Cox regression analysis was performed by survival R package. The optimal cut-off value was determined by survminer R package. The ROC for predicting immunotherapy was performed by pROC R package. All P value were two-side, with $p < 0.05$ as statistically significance. The whole data processing, statistical analysis, and plotting were conducted in R 3.6.3 software.

RESULTS

Genomic Variation Landscape of FRGs in HCC

We retrieved 74 FRGs from previous literatures and KEGG pathways (**Table S2**). The multi-omics landscape of FRGs were summarized from the TCGA-LIHC cohort (**Figure 1**). According to these genes, we can separate tumor tissue from normal tissue distinctly (**Figure S2A**). Most of FRGs displayed significant expression differences between tumor and normal tissues. For instance, SLC7A11, CDKN2A, and ALOX15 were up-regulated in HCC, while PTGS2, CFTR, and GLS2 were down-regulated. Further studies observed infrequent mutations of FRGs and widespread copy number variations (CNVs), which

suggested that CNVs might play a dominant role in the regulation of FRGs relative to mutation. For example, EGLN1, ENPP2, and MUC1 focused on amplification of copy number, whereas SLC39A14, ALOX15, and ACSL1 preferred deletion. Besides, the DNA methylation also displayed a broad regulatory effect on FRGs, such as ACSL1, ACSL5, and SCD. Univariate Cox regression analysis further demonstrated that most of FRGs played a protective role in HCC, which in line with the protective biological function of FRGs (**Figure 1**).

Identification and Validation of the Ferroptosis Subtypes

A total of 3,327 samples from 30 GEO datasets were defined as the discovery cohort, and further divided into k groups (k = 2 ~ 9) *via* ConsensusClusterPlus R package. We found that k = 2 was optimal choice according to the CDF curve of the consensus score (**Figures 2A, B**). The PAC and NbClust methods further verified the result (**Figures 2C** and **S2B**). The principal component analysis of 74 FRGs expression showed significant separation between two clusters (**Figure 2D**). To ensure the reliability and stability of the clustering results from the meta cohorts, we further performed IGP analysis in two independent cohorts. The results exhibited that the IGP values of C1 was 90.3% and C2 was 92.9% in the TCGA cohort, while was 88.4% and 91.7% in the ICGC cohort (all $p < 0.001$). The NbClust also indicated it was optimal to split into two clusters in both cohorts (**Figures S2C, D**).

Compared to C1, most of FRGs were significantly up-regulated in the C2 (**Figure 2E**). Recent studies revealed that ferroptosis can induce tumor-specific immune responses and enhance the effect of immunotherapy (18, 37). Further correlation analysis suggested intense correlations between 74 FRGs and TME cells in HCC (**Figure S2E**). We then explored the differences of TME cells infiltration in the two subtypes. It turned out C1 exhibited a higher overall level of infiltration (**Figure 2F**). In addition to display superior immune activated cells (e.g., CD 4+/CD8+ T cells), C1 also showed higher abundance of immunosuppressive cells (e.g., Treg, MDSC, Th17 cell, and fibroblast) (**Figure 2G**). The above implied that ferroptosis may have a profound impact on TME in HCC. To further clarify the biological characteristics of the two subtypes, we performed GSVA enrichment analysis using Hallmark and KEGG gene sets. As illustrated, C1 was observably enriched in inflammation related pathways, such as allograft rejection, inflammatory response, and T cell receptor signaling pathway; while C2 was predominantly associated with metabolism related pathways, such as oxidative phosphorylation, fatty acid metabolism, bile acid metabolism, and amino acid metabolism (**Figures 2H, I**). The similar results were obtained from the TCGA and ICGC cohorts (**Figures S3, S4**). Overall, the two subtypes were defined as follows: 1) $metabolism^{low}immunity^{high}$ type (LMHI): low levels of FRGs expression and inflammation-related pathways enrichment as well as high abundant of immune cells infiltration; 2) $metabolism^{high}immunity^{low}$ type (HMLI): high levels of FRGs expression and metabolism-related pathways enrichment as well as low abundant of immune cells infiltration.

Clinical Characteristics of the Ferroptosis Subtypes

The clinical significance of two subtypes were further explored. Survival analysis revealed C2 had a better OS and RFS relative to C1 in three cohorts (**Figures 3A–E**). Previous studies indicated sorafenib could induce ferroptosis by inhibiting System Xc^- (38). We thus predicted the sensitivity of two subtypes to sorafenib using the pRRophetic package, and the result prompted that C2 was more likely to benefit from sorafenib (**Figure 3F** and **Figures S5A, D**). Besides, the previous analysis displayed C1 possessed superior immune cells infiltration, the checkpoint molecules (e.g., PD-L1 and CTLA-4) also were over-expressed in C1 (**Figure 3G**). These results hinted C1 may be more sensitive to immunotherapy. Therefore, we further assessed the effectiveness of immunotherapy on both subtypes. Using the TIDE web tool, C1 displayed a higher response compared to C2, and similar results was obtained in the two validation cohorts (**Figure 3H** and **Figures S5B, E**). Moreover, the Submap algorithm were applied to evaluate the similarity of expression profiles between the two subtypes and 47 pretreated patients with comprehensive immunotherapy information, and the results indicated C1 was significantly related to patients responding to anti-PD-1 treatment, and similar results was obtained in the two validation cohorts (**Figure 3I** and **Figures S5C, F**). In addition, we also observed that patients in C1 subtype was significantly associated with the features such as younger, female, advanced stage, higher grade, and vascular invasion (**Figures 3J–N**). There was no difference of BMI between two subtypes (**Figure 3O**).

Potential Extrinsic Immune Escape Mechanism of the Two Subtypes

We questioned whether the effect of ferroptosis on HCC could cause the differences in immune escape mechanisms between the two subtypes. Therefore, we first researched the extrinsic immune escape mechanism (12). Previous publications have shown that extrinsic immune escape mainly includes four aspects: lack of leukocytes, massive immunosuppressive cells, high concentrations of immunosuppressive cytokines, and increase in fibroblasts (39).

According to the above results, we summarized the abundance distribution of TME cells in the two subtypes. As shown in **Figure 4A**, the abundance of immunosuppressive cells and fibroblasts in C1 were superior, while C2 demonstrated a lack of innate immune cells and adaptive immune cells. In addition, the infiltration levels of immunosuppressive cells such as MDSC, Treg, Th17, and fibroblasts were also higher in C1 (**Figures 4B–E**). Consistent with these results, C1 also exhibited an increase in chemokines, interleukins, interferons, and other important cytokines and their receptors, such as CCL5 (recruiting MDSC to migrate to tumor areas), IL-10 (a cytokine synthesis inhibitor), and TGF-β3 (having a wide range of immunosuppressive activities) (40–42) (**Figure 4F** and **Figures S6A, B**). Overall, we speculated that the aggregation of immunosuppressive cells, fibroblasts, and the high concentrations of immunosuppressive cytokines might lead to the extrinsic immune escape of C1, while C2 was mainly related to immune cells defects.

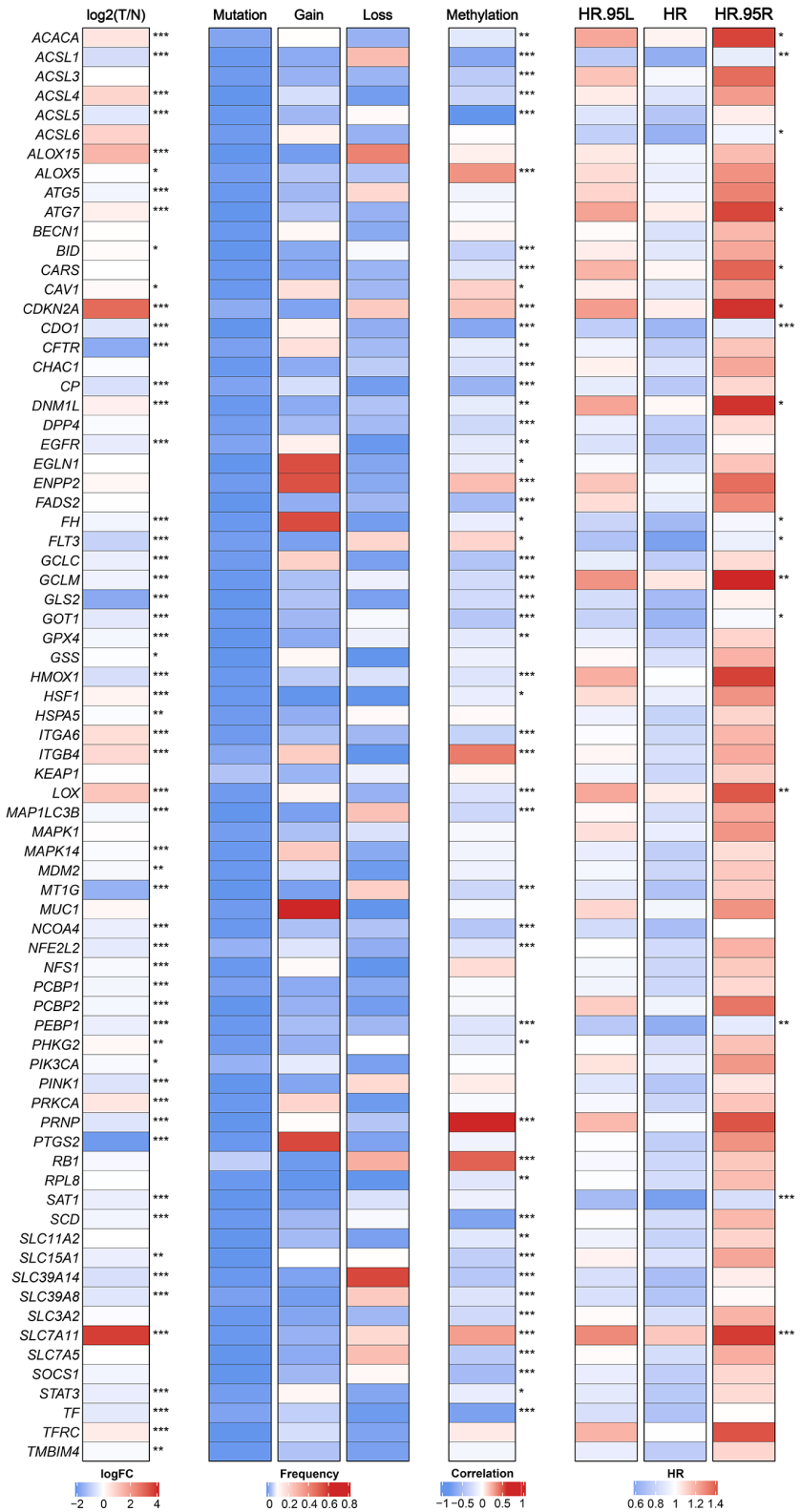

FIGURE 1 | The expression, genomic variation and hazard ratios of FRGs in TCGA-LIHC. From left to right panel, the expression difference of FRGs in tumor tissues compared with normal tissues, the mutation and copy number variation frequency of FRGs, the correlation of DNA methylation modifications and expression for FRGs, and univariate Cox regression analysis presented hazard ratios of FRGs. *p < 0.05, **p < 0.01, ***p < 0.001.

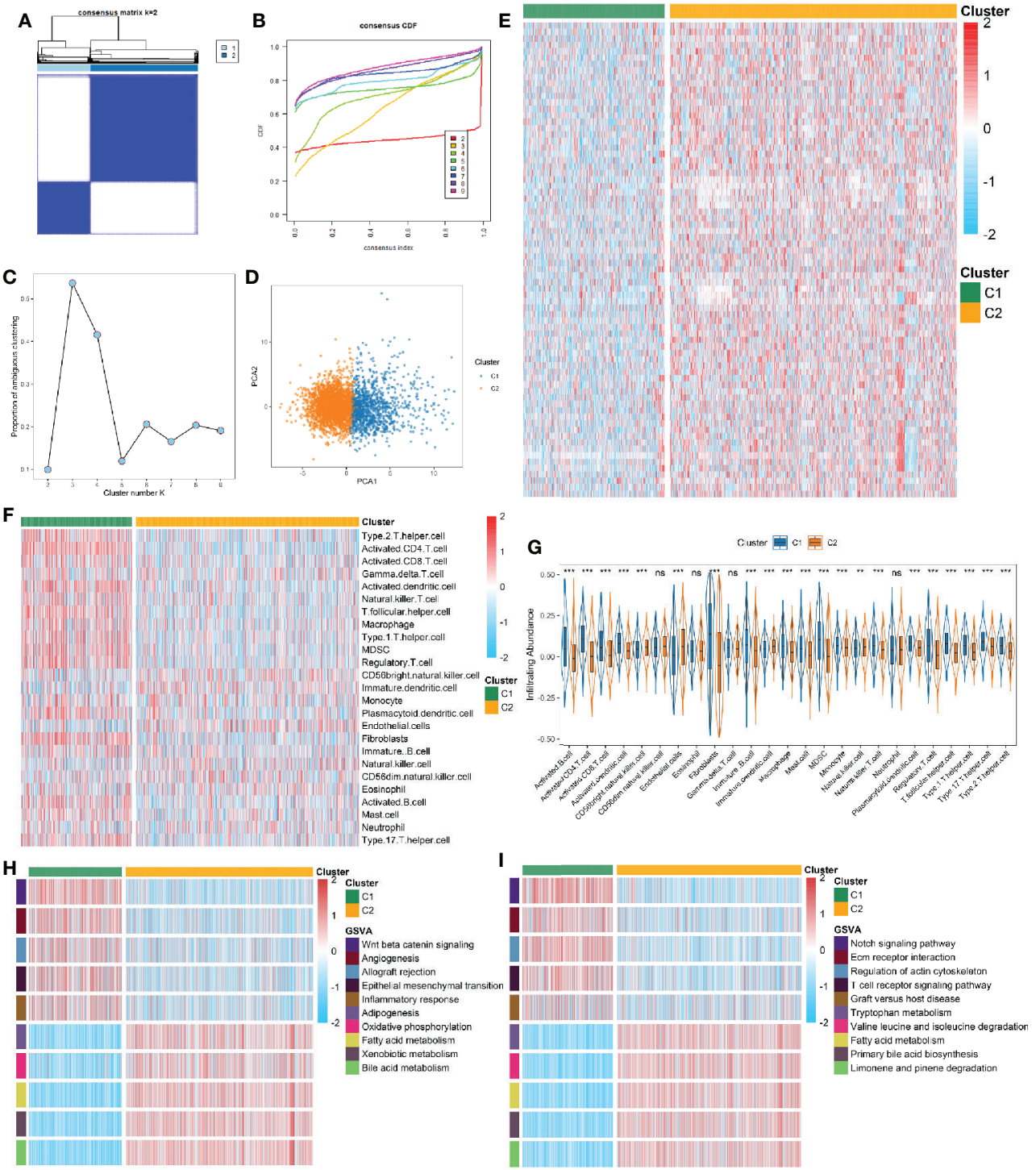

FIGURE 2 | **(A)** The consensus score matrix of all samples when k = 2. A higher consensus score between two samples indicates they are more likely to be grouped into the same cluster in different iterations. **(B)** The cumulative distribution functions of consensus matrix for each k (indicated by colors). **(C)** The proportion of ambiguous clustering (PAC) score, a low value of PAC implies a flat middle segment, allowing conjecture of the optimal k (k = 2) by the lowest PAC. **(D)** Two-dimensional principle component plot by the expression of 74 FRGs in the two subtypes. The orange dots represented C1, and blue dots represented C2. **(E)** The expression heatmap of 74 FRGs in the two subtypes. **(F)** The heatmap of immune cells in the two subtypes. **(G)** The infiltration difference of TME cells between the two subtypes. The asterisks represented the statistical p value (^{ns}P > 0.05; **P < 0.01; ***P < 0.001). **(H, I)** GSVA enrichment analysis revealed activated Hallmark **(H)** and KEGG **(I)** pathways of the two subtypes.

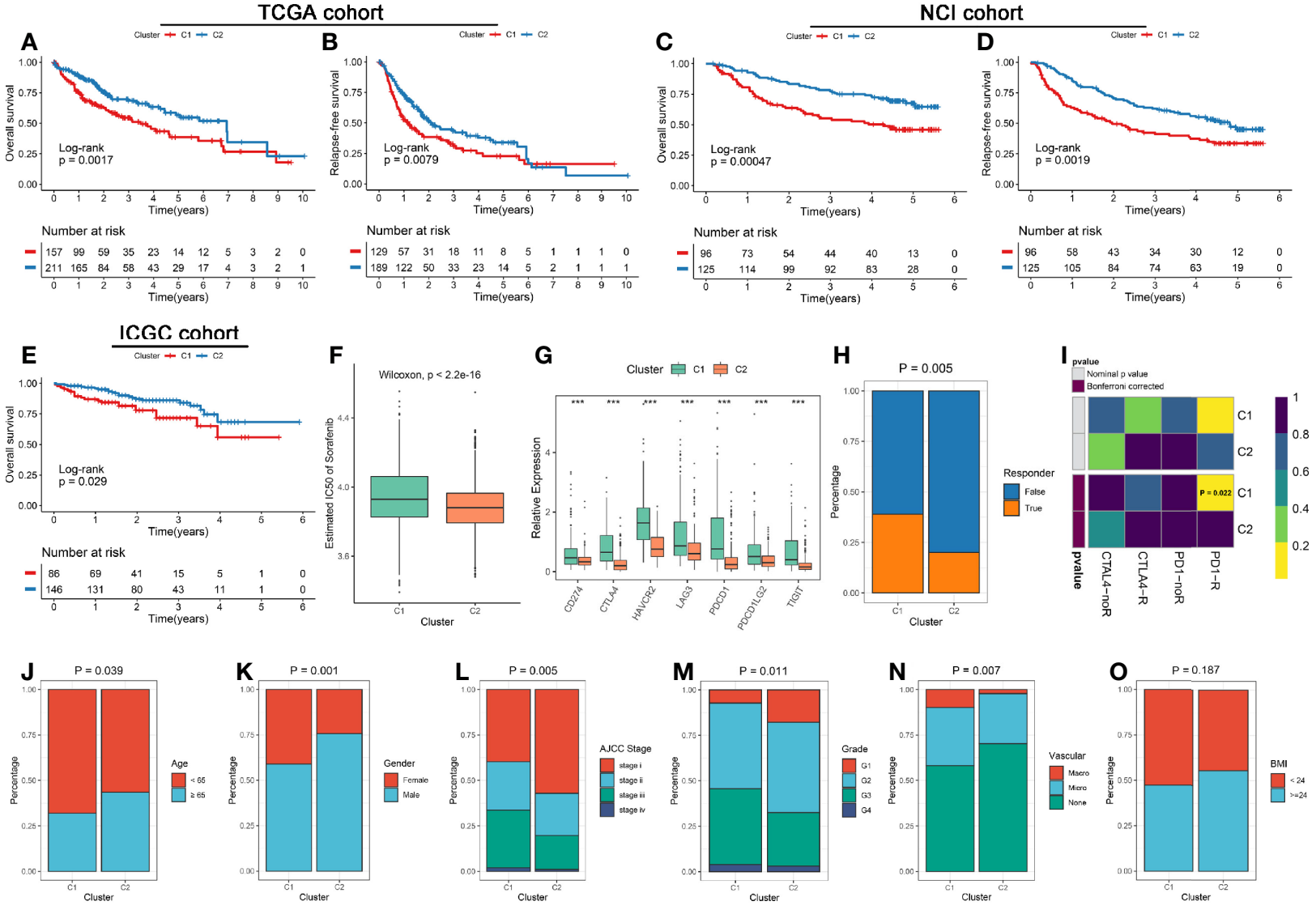

FIGURE 3 | **(A, B)** Kaplan-Meier analysis for OS **(A)** and RFS **(B)** of the two subtypes in the TCGA cohort. **(C, D)** Kaplan-Meier analysis for OS **(C)** and RFS **(D)** of the two subtypes in the NCI cohort. **(E)** Kaplan-Meier analysis for OS in the ICGC cohorts. **(F)** The estimated IC50 of sorafenib between the two subtypes in the discovery cohorts. **(G)** Comparison of ICP molecules expression between the two subtypes. The asterisks represented the statistical p value (***P < 0.001). **(H)** The TIDE algorithm was used to predict the sensitivity of the two subtypes to immunotherapy in the discovery cohorts. **(I)** Submap analysis of the two subtypes and 47 pretreated patients with comprehensive immunotherapy annotations in the discovery cohorts. For Submap analysis, a smaller p-value implied a more similarity of paired expression profiles. **(J–O)** Composition percentage of the two subtypes in clinical characteristics such as age **(J)**, gender **(K)**, BMI **(L)**, AJCC stage **(M)**, grade **(N)**, and vascular invasion **(O)**.

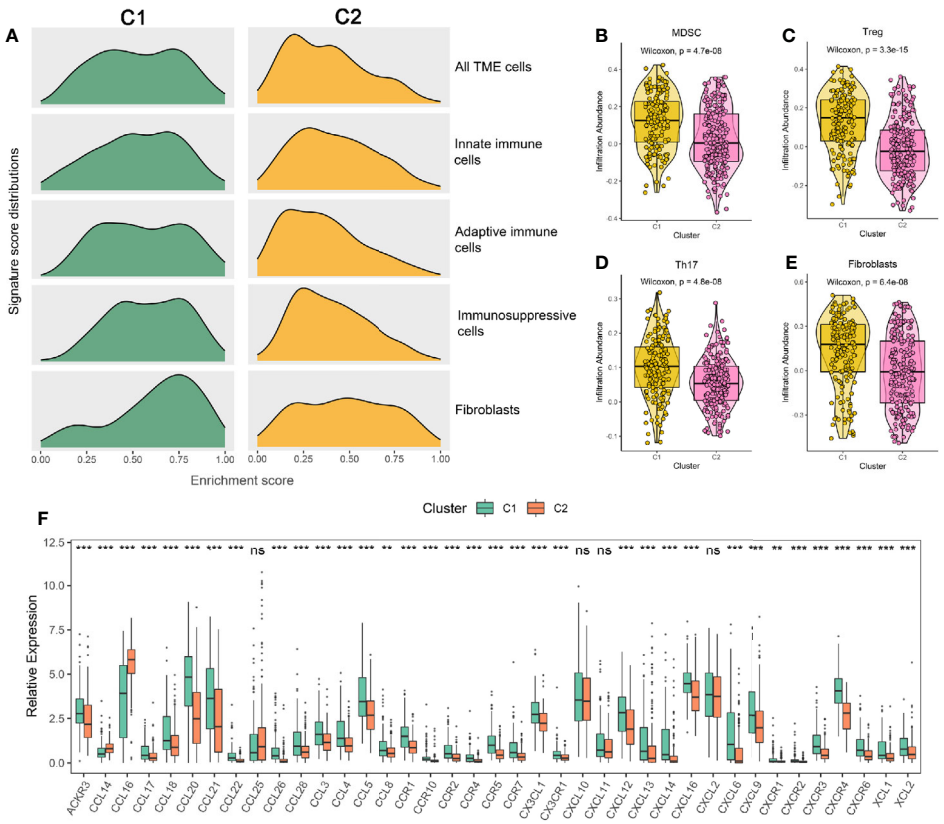

FIGURE 4 | **(A)** Signature score distributions of five cell subsets between the two subtypes. **(B–E)** Comparison of MDSC **(B)**, Treg **(C)**, Th17 **(D)**, and fibroblasts **(E)** between the two subtypes. **(F)** The relative expression levels of chemokines and their ligands of the two subtypes. The asterisks represented the statistical p value ($^{ns}P > 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

Potential Intrinsic Immune Escape Mechanism of the Two Subtypes

We next investigated the potential intrinsic immune escape mechanism in HCC, including the following three aspects: antigen presentation capacity, expression of immune checkpoints (ICPs), and tumor immunogenicity (12). Compared to C1, the expression of MHC and APS were significantly lower in C2, suggesting that defective antigen presentation capacity might be an intrinsic immune escape mechanism for C2 (**Figure 5A** and **Figures S7A, B**). Subsequently, we explored the expression and regulatory patterns of the immune checkpoints in the two subtypes. C1 displayed the higher expression of costimulatory and coinhibitory molecules, which implied that C1 might overexpress immune checkpoints (e.g., CTLA4, CD274, PDCD1) to evade the immune elimination after immune activation (**Figure 5A** and **Figures S7C, D**). Notably, the expression difference of ICPs were not derived from mutation, but were strongly associated with CNV and methylation. For example, TNFSF4, TNSF18 and CD48 focus on amplification, whereas TNFSF13 possessed a high frequency of deletion (**Figure 5A**). The DNA methylation of CD28, CD27, and LAG3 obviously negatively regulated their expression, implying epigenetic silencing (**Figure 5A**). Therefore, CNV and methylation modification might play a dominant role in regulating ICPs compared to mutation, which pointed a new direction for the development of immune checkpoint inhibitors (ICIs).

Afterwards, we focused on evaluating eight indicators related to HCC immunogenicity. As the main source of tumor-specific antigens (43), TMB, neoantigen load (including SNV neoantigens and indel neoantigens), and MSI status had no significant difference between the two subtypes, while C1 displayed the higher CTA score (**Figures 5B–E** and **Figure S7E**). Besides, we found that C1 has evidently higher CNV load in the level of focal, chromosomal arm and base, respectively (**Figures 5F–I** and **Figure S7F**). In line with this, the TCR/BCR diversity were superior in C1 (**Figures 5J, K** and **Figures S7G, H**). These results suggested C1 possessed higher immunogenicity relative to C2, and CNV may dominate the differences in immunogenicity of the two subtypes.

Comprehensive and Integrative Genomic Characterization of the Two Subtypes

Based on the MutSigCV algorithm, a total of nine SMGs was identified in the two subtypes (**Figure 6A** and **Figures S8A, I**). We observed the mutation of these genes had an influenced on their expression such as CTNNB1, AXIN1, and RB1. Univariate

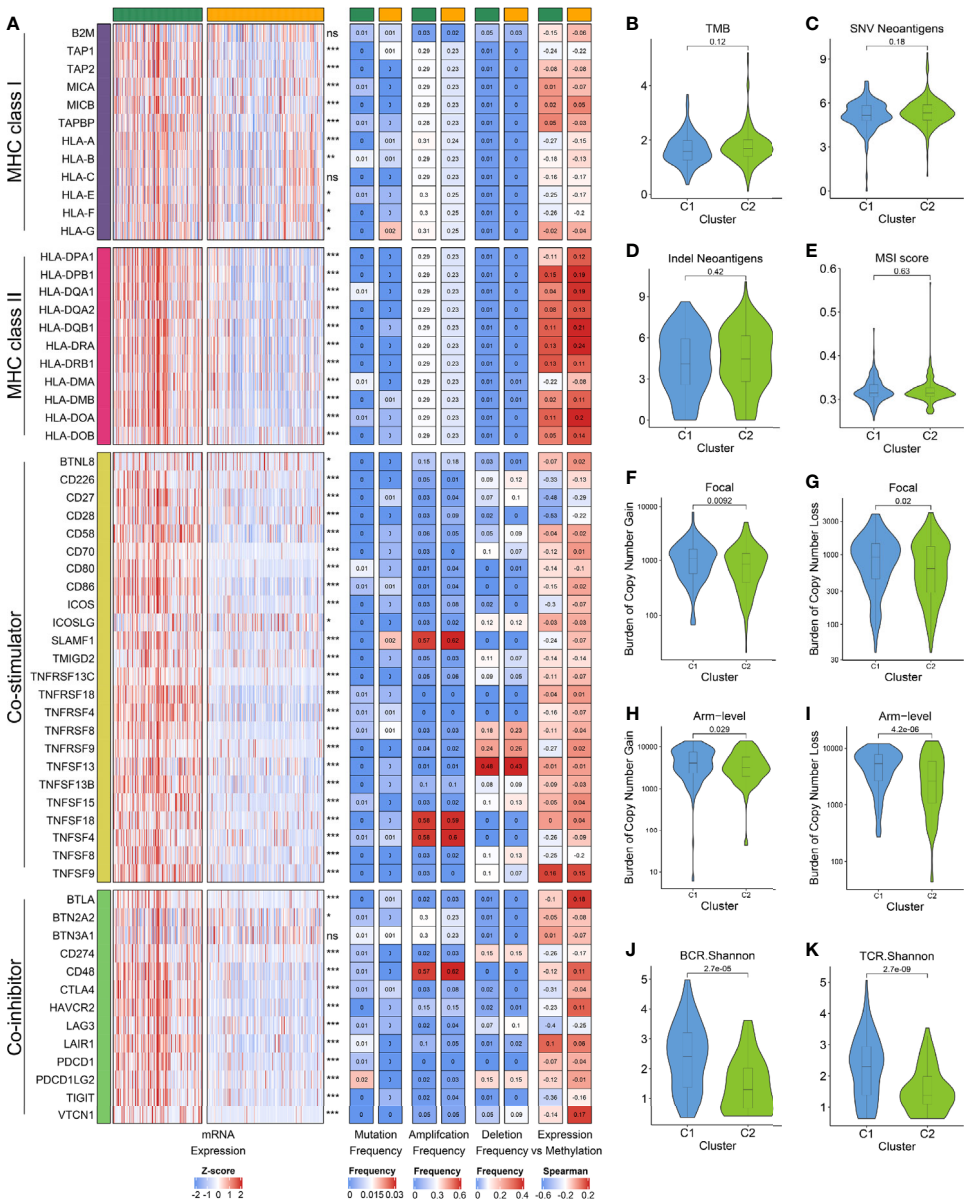

FIGURE 5 | **(A)** From left to right: mRNA expression; mutation frequency; amplification frequency; deletion frequency, and expression *versus* methylation (gene expression correlation with DNA methylation β value) for MHC molecules, co-stimulators and co-inhibitors in the two subtypes. **(B–E)** Comparison of the two subtypes in four immunogenicity associated indicators such as TMB **(B)**, SNV neoantigens **(C)**, indel neoantigens **(D)**, and MSI score **(E)**. **(F–I)** Comparison of the two subtypes in focal **(F, G)** and broad **(H, I)** CNV burden. **(J, K)** The distribution of TCR **(J)** and BCR **(K)** diversity in the two subtypes.

Cox regression further revealed the prognostic value of SMGs (**Figure S8J**). The two subtypes shared five common SMGs including TP53, CTNNB1, ALB, RB1 and AXIN1, suggesting their mutations were prevalent in HCC. Specifically, tumor suppressor BAP1 was a SMG of C1, while SMGs related to chromatin remodeling such as ARID1A, ACVR2A, and CDKN2A mainly occurred in C2 (44, 45). In addition, we further explored the mutation signatures of the two subtypes and found that signature 6 (associated with defective DNA mismatch repair) and signature 22 (had a history of exposure to aristolochic acid) presented in both subtypes, but with different proportions (**Figures 6B–E**). Notably, we also discovered that signature 24 associated with aflatoxin was specifically presented in C1, whereas age-related signature 5 only existed in C2 (**Figures 6B, C**). Overall, C1 was mainly dominant in signature 6 and signature 22, while signature 5 had a master proportion in C2, indicating that different leading carcinogenic factors in the two subtypes (**Figures 6D, E**).

GISTIC2.0 was utilized to define recurrently amplified and deleted regions in the two subtypes (**Figure 6F** and **Table S5**).

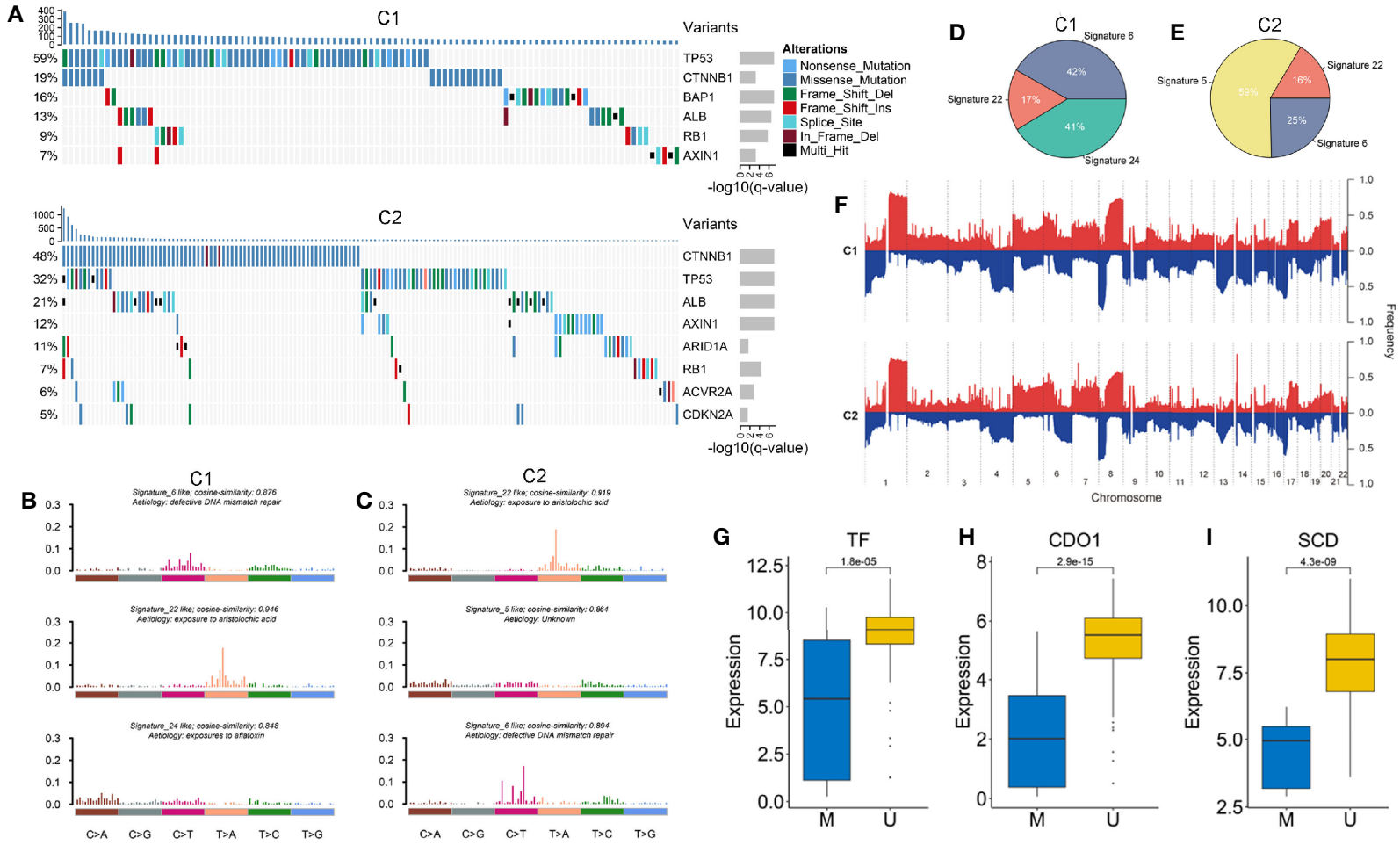

FIGURE 6 | **(A)** The waterfall plot of significantly mutation genes in the two subtypes. Each column represented individual patients. The upper barplot showed TMB, the number on the left showed the proportion of samples with mutations. The right barplot indicated the mutation frequency in each gene. **(B, C)** The three mutation signatures with the highest cosine similarity to COSMIC signatures in C1 **(B)** and C2 **(C)**. The etiology of each signature and the cosine similarity between the original and the reconstructed mutation signatures were indicated. **(D, E)** The pie charts showed the proportion of the three mutation signatures contributing to the mutations spectrum of C1 **(D)** and C2 **(E)**. **(F)** The copy number variations of the two subtypes. **(G–I)** The expression difference of three ESGs including TF, CDO1 and SCD between the methylated and unmethylated groups.

The results showed that the two subtypes had frequent CNVs in the regions where oncogenes and tumor suppressor genes (e.g. MYC and TP63), as well as cell cycle regulators (e.g. CDK3, CDK8, and MAPK11) were located, which indicated the CNVs might have a profound impact on the tumorigenesis and progression of HCC. We observed recurrent focal CNVs in C1 included amplifications containing 8q24.21 (MYC) and 1p11.2 (NOTCH2) and deletion of 10q23.1(GRID1). Recurring focal CNVs in C2 included amplifications of 6p21.1 (VEGFA) and 17q25.1 (CDK3), and deletion of 3q28 (TP63), 13q13.3 (BRCA2, CDK8), and 22q13.33 (MAPK11). These specific CNVs might contribute to formation of the two subtypes.

We further investigated methylation modification in the two subtypes and found that C1 had a higher GML than C2 (**Figure S9A**). Next, we identified 30 and 17 ESGs from C1 and C2, respectively (**Figures S9B, C**). Among them, we observed that the expression levels of three FRGs (e.g. TF, CDO1, and SCD) were significantly lower in methylated group (**Figures 6G–I**). Notably, both subtypes possessed a common ESG, HOXA3, which was associated with focal adhesion and ECM-receptor (46). We also discovered some specific ESGs such as ACOX2 and SCD that played a crucial role in lipid metabolism only appeared in C1. This might explain that C1 was a hypometabolic status. Whereas WIPF3 and LAMA3 that associated with pathogen infection and inflammatory diseases specifically presented in C2. These ESGs might lead to defects in TME cells and cytokines in C2.

A Novel Prognostic and Immunotherapy Biomarker: FRRS

We identified 1,023 DEGs between the two subtypes (**Figure S10A**). GO enrichment analysis showed that these genes were strongly correlated with extracellular matrix organization and organic acid transport, and KEGG pathway analysis revealed that cytokine-cytokine receptor interaction, bile secretion, and Wnt signaling pathway were significantly enriched (**Figure S10B**). Among the four gene sets including DEGs, SMGs, CAGs, and ESGs (**Figure S10C**), we selected 33 genes that were present in at least two of the four categories for further study (**Table S6**). Univariate COX regression analysis indicated that six genes had predominant prognostic significance ($p < 0.05$). Next, we enrolled the six genes ($p < 0.05$) for multivariate COX regression analysis, a stepwise regression approach was applied. Based on the smallest AIC value, we determined the best model: FRRS = 0.348 * Expression (SLC16A3) − 0.151 * Expression (CPS1). Survival analysis exhibited patients with high FRRS had a worse prognosis (HR: 2.511 [2.145–2.876] in the TCGA cohort, 1.542 [1.236–1.847] in the ICGC cohort, and 1.614 [1.351–1.877] in the NCI cohort) (**Figures 7A–C** and **Figure S11A**). The concordance index (C-index) analysis also confirmed that FRRS had high accuracy in the three independent cohorts of TCGA, ICGC, and NCI (C-index = 0.785; 0.716; 0.733; respectively; **Figure S11B**). Combined with clinical factors, we observed FRRS was an independent prognosis factor in HCC through multivariate Cox regression analysis (**Figure 7D**).

Although immunotherapy represented by ICIs has been gradually recognized as a promising tumor treatment, only a small number of patients can benefit from it (47). We explored the biological characteristics of FRRS related to immunotherapy response, and found that FRRS was significantly positively correlated with the expression of ICP molecules such as HAVCR2, CTLA4, and PDCD1, as well as the infiltration patterns of Treg cells and MDSC (**Figures S11C, D**). Thus, we included three immunotherapy cohorts to further investigated whether FRRS could predict responsiveness of the patients to immunotherapy. In line with the above, patients with high FRRS showed an unfavorable survival in these three cohorts (**Figures 7E, H, K**). In addition, patients who were clinically responsive to immunotherapy showed lower FRRS, suggesting that patients with lower FRRS were more likely to benefit from immunotherapy (**Figures 7F, I, L**). The area under the curve (AUC) for the ROC curve was used to measure the accuracy of FRRS in predicting the response to immunotherapy. These results strongly suggested that FRRS was a reliable biomarker (IMvigor210: AUC = 0.769; GSE78220: AUC = 0.778; GSE100197: AUC = 0.942; **Figures 7G–M**). Then we calculated seven widely used immunotherapy biomarkers, including TMB, TIDE, MSI score, Merck18, IFGN, CD8, and CD274. In all three cohorts, FRRS afforded greater accuracy in the prediction of immunotherapy (**Figures 7N–P**). Notably, TIDE performed worse in predicting response to immunotherapy in the IMvigor210 cohort and GSE100797 cohort (AUC = 0.52 and 0.54; respectively), although the predictive power of FRRS in the GSE78220 cohort is slightly lower than that of TIDE (**Figures 7N–P**). Overall, our study strongly confirmed that FRRS can be used to assess the prognosis and immunotherapy response of patients, and outperformed widely used biomarkers.

DISCUSSION

Ferroptosis, as a recently recognized programmed cell death modality, has been confirmed to be significantly associated with tumor progression, immune status, and anti-tumor response, and its role in HCC has gradually attracted people's attention (48, 49). Our study identified and validated two heterogeneous ferroptosis subtypes in HCC. C1 possessed low levels of FRGs expression and high abundance of innate and adaptive immune cells, and were closely associated with inflammation, which was defined as the metabolism^{low}immunity^{high} subtype. C2 expressed high FRGs expression but lacked infiltrating immune cells, presented a metabolism-related functional characteristic, which was defined as the metabolism^{high}immunity^{low} subtype. We also validated the stability and reproducibility of the two subtypes in two independent cohorts. The two subtypes also exhibited heterogeneity in immune escape mechanisms, genome-driven events, and clinical outcomes (**Table 1**). In addition, based on the two subtypes, we proposed a prognosis signature: FRRS, which was an independent prognosis factor for HCC. Further immunotherapy prediction also indicated FRRS might be a promising immunotherapy marker. These results facilitated the understood of ferroptosis as well as clinical management and precise therapy of HCC.

The two subtypes demonstrated distinct clinical characteristics. We observed C1 owned worse OS and RFS relative to C2.

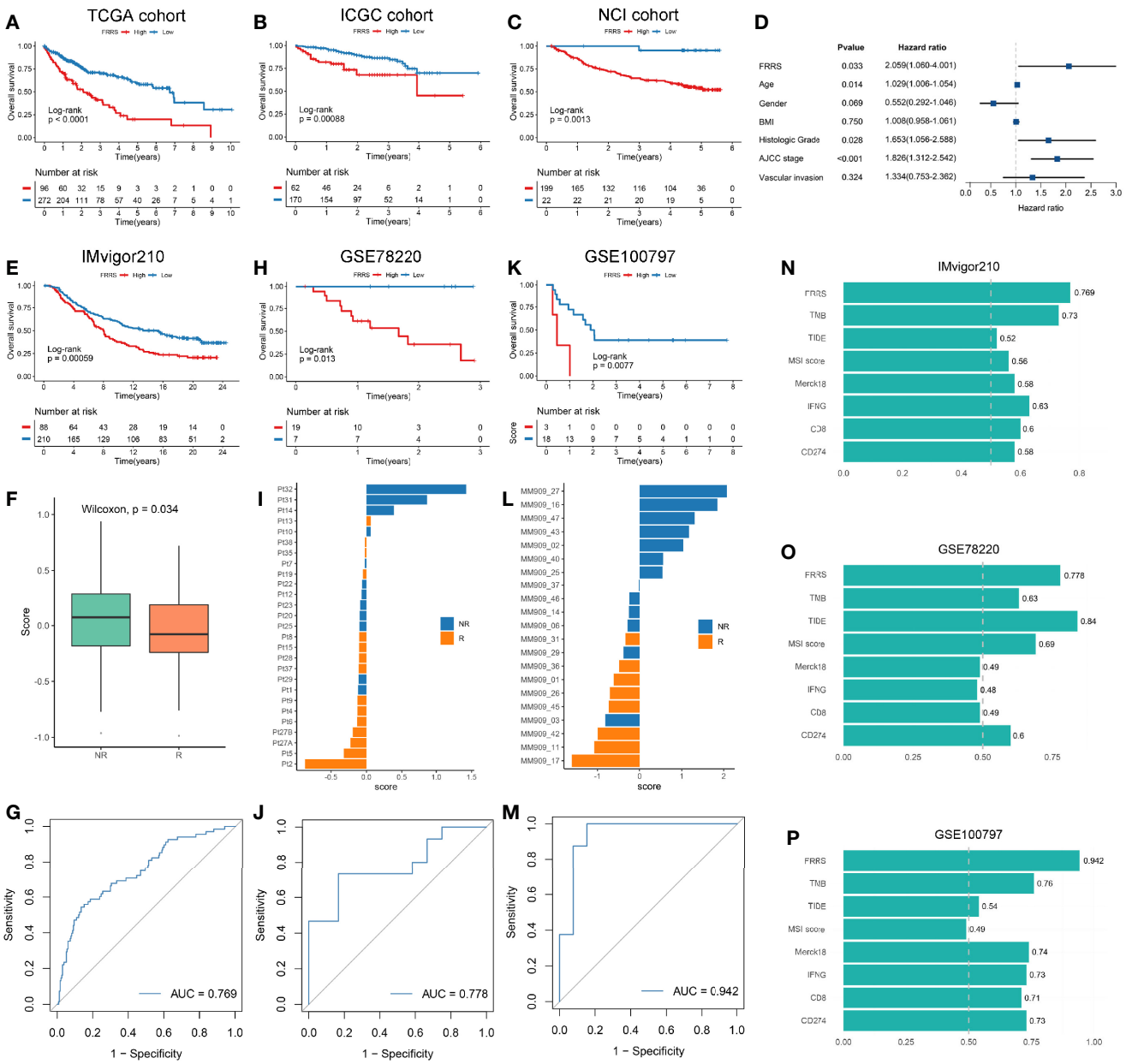

FIGURE 7 | **(A–C)** Kaplan-Meier survival analysis of high FRRS and low FRRS group in TCGA **(A)**, ICGC **(B)**, and NCI **(C)** cohorts. **(D)** FRRS and clinical factors were combined for multivariate Cox regression analysis. **(E–G)** Kaplan-Meier survival analysis of high FRRS and low FRRS groups **(E)**, the distribution of FRRS between response and nonresponse groups **(F)**, and ROC curve of the FRRS signature for predicting immunotherapy response **(G)** in IMvigor210 cohort. **(H–J)** Kaplan-Meier survival analysis of high FRRS and low FRRS groups **(H)**, the distribution of FRRS between response and nonresponse groups **(I)**, and ROC curve of the FRRS signature for predicting immunotherapy response **(J)** in GSE78220 cohort. **(K–M)** Kaplan-Meier survival analysis of high FRRS and low FRRS groups **(K)**, the distribution of FRRS between response and nonresponse groups **(L)**, and ROC curve of the FRRS signature for predicting immunotherapy response **(M)** in GSE100797 cohort. **(N–P)** AUC values of FRRS and seven other biomarkers for predicting the immunotherapy response in IMvigor210 **(N)**, GSE78220 **(O)**, and GSE100797 **(P)** cohorts.

In addition, C1 was more prone to occur in the patients with clinical characteristics such as younger, female, advanced stage, higher grade, vascular invasion relative to C2. Further predictions for sorafenib displayed the drug sensitivity of C2 was higher than C1, which might be due to the overexpression of FRGs that could be targeted by sorafenib in C2 (38). Conversely, C1 displayed superior response to immunotherapy. These results might facilitate personalized treatment for patients with HCC.

We then explored the specific immune escape mechanisms of the two subtypes. The TME of C1 accumulated more immunosuppressive cells and inhibitory cytokines, and its overexpressed ICPs could evade immune recognition and clearance after activation. C2 had a lower

TABLE 1 | Summary of FRGs expression, TME cells infiltration, biological and clinical characteristics, immune escape mechanisms, and genome-driven events for the two ferroptosis subtypes.

Subtype	Cluster 1	Cluster 2
FRGs expression	lower	higher
TME cells infiltration	higher	lower
Biological characteristics	inflammation	metabolism
Dominant clinical characteristics		
Prognosis	worse	better
Age	younger	older
Gender	female	male
Stage	more advanced	less advanced
Grade	senior	junior
Vascular invasion	macro or microvascular	none
Sensitivity to sorafenib	lower	higher
Sensitivity to immunotherapy	higher	lower
Extrinsic immune escape mechanism		
All TME cells	higher	lower
Innate immune cells	higher	lower
Adaptive immune cells	higher	lower
Immunosuppressive cells	higher	lower
Fibroblasts	higher	lower
Intrinsic immune escape mechanism		
MHC expression	higher	lower
APS score	higher	lower
ICPs expression	higher	lower
Immunogenicity	higher	lower
TMB	had no significant difference	
Neoantigen load		
MSI status		
CTA score	higher	lower
CNV load	higher	lower
TCR/BCR diversity	higher	lower
Cluster-specific genomic variation landscape		
Mutations	BAP1	ARID1A, ACVR2A, and CDKN2A
Copy number amplifications	8q24.21 (MYC);1p11.2 (NOTCH2)	6p21.1(VEGFA);17q25.1 (CDK3)
Copy number deletions	10q23.1(GRID1)	3q28 (TP63);13q13.3 (BRCA2, CDK8); 22q13.33 (MAPK11)
DNA methylation		
GML	higher	lower
ESGs	ACOX2; SCD	WIPF3; LAMA3

abundance of immune killer cells, which might arise from its inferior immunogenicity and antigen presentation capacity. These results provided critical references for immunotherapy of HCC. In addition, we also observed that CNV and DNA methylation might play a master role in regulating immunoregulatory factors compared to mutations, which points out the directions for the development of ICIs.

Next, in order to depict the molecular characteristics of the two subtypes, we separately investigated the distinct genome alterations of the two subtypes. As a particular SMG of C1, BAP1 has been certified to block cystine uptake by inhibiting the expression of SLC7A11, leading to lipid peroxidation and ferroptosis, thereby inhibiting tumor progression (50). However, the mutation of BAP1 deprived the above ability, which might partially explain its poor prognosis to some extent. Consistent with the immune escape mechanism, the amplification of oncogene MYC was widespread in C1, which could further inhibit immune surveillance by increasing the expression of CD47 and PD-L1 (51). Topper and colleagues had demonstrated that depletion of MYC could reversed immune evasion in mouse, which in turn achieved the purpose of treating non-small cell lung cancer, corresponding clinical trial is still ongoing (52). In addition, an intervention study indicated that higher methylation levels of SCD1 were related to weight loss in subject, which was consistent with the lower BMI of C1 (53). The unique SMGs of C2 such as ARID1A, ACVR2A, and CDKN2A were closely associated with chromatin remodeling, which could inhibit the ferroptosis process by altering lipid metabolic genes (54, 55). This suggested that we can target chromatin remodeling to develop drugs for C2. Notably, C2 is more sensitive to the multi-kinase inhibitor sorafenib, which might be attributed to its significant copy number alterations in cell cycle-related kinases such as CDK3, CDK8, and MAPK11 (56). Overall, the specific genomic variation landscape of the two subtypes not only might lead to the formation of heterogeneous ferroptosis subtypes, but also partially contributed to the underlying mechanism of their sensitivity to different drugs. In addition, these results also point the directions for drug development and clinical treatment of HCC patients.

Finally, we developed and validated a prognosis signature termed FRRS in three independent cohorts. The high FRRS predominantly associated with poor prognosis. FRRS demonstrated a favorable performance in predicting the prognosis, and was an independent prognosis factor in HCC. Taking into account the close link between FRRS and TME cells, we further explored the potential significance in predicting immunotherapy response and it turned out FRRS also achieved a high accuracy. In addition, the accuracy of FRRS was superior to seven prevalent indicators including TMB, TIDE, MSI score, Merck18, IFGN, CD8, and CD274 in predicting immunotherapy response, which hinted FRRS was a promising marker for selecting patients who might be sensitive to immunotherapy.

Nevertheless, the study also had several limitations. First, owing to the lack of data, our study only considered the interpatient heterogeneity and did not take into account the intratumoral heterogeneity. Second, although we had applied some algorithms to assess the two subtypes in predicting the sensitivity of sorafenib and immunotherapy, prospective cohort studies and clinical data are still need.

In summary, our work identified and validated two heterogeneous ferroptosis subtypes. The two subtypes also exhibited heterogeneity in functional status, immune escape mechanisms, genome-driven events, and clinical outcomes. In addition, we developed a scoring system termed FRRS, which was a reliable prognosis and immunotherapy signature. These

results facilitated the understood of ferroptosis as well as clinical management and precise therapy of HCC.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

XH and YS designed the research. ZL and LW performed data acquisition and data analysis. LL and TL assisted with data acquisition and data analysis. ZL, LW, and DJ wrote the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We thank the researchers who provided sequencing data as well as the workers who developed the R package.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.619242/full#supplementary-material

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Conflict of Interest: 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.

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frontiers in Immunology

REVIEW
published: 11 March 2021
doi: 10.3389/fimmu.2021.635899

Flipside of the Coin: Iron Deficiency and Colorectal Cancer

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Iron deficiency, with or without anemia, is the most frequent hematological manifestation in individuals with cancer, and is especially common in patients with colorectal cancer. Iron is a vital micronutrient that plays an essential role in many biological functions, in the context of which it has been found to be intimately linked to cancer biology. To date, however, whereas a large number of studies have comprehensively investigated and reviewed the effects of excess iron on cancer initiation and progression, potential interrelations of iron deficiency with cancer have been largely neglected and are not well-defined. Emerging evidence indicates that reduced iron intake and low systemic iron levels are associated with the pathogenesis of colorectal cancer, suggesting that optimal iron intake must be carefully balanced to avoid both iron deficiency and iron excess. Since iron is vital in the maintenance of immunological functions, insufficient iron availability may enhance oncogenicity by impairing immunosurveillance for neoplastic changes and potentially altering the tumor immune microenvironment. Data from clinical studies support these concepts, showing that iron deficiency is associated with inferior outcomes and reduced response to therapy in patients with colorectal cancer. Here, we elucidate cancer-related effects of iron deficiency, examine preclinical and clinical evidence of its role in tumorigenesis, cancer progression and treatment response. and highlight the importance of adequate iron supplementation to limit these outcomes.

Keywords: iron deficiency, iron deficiency anemia, colorectal cancer, immune host defense, intravenous iron therapy

OPEN ACCESS

Edited by:
Paola Zacchi,
University of Trieste, Italy

Reviewed by:
Tiziana Schioppa,
University of Brescia, Italy
Tariq Iqbal,
Queen Elizabeth Hospital Birmingham, United Kingdom

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

Received: *30 November 2020*
Accepted: *18 February 2021*
Published: *11 March 2021*

Citation:
Aksan A, Farrag K, Aksan S, Schroeder O and Stein J (2021) Flipside of the Coin: Iron Deficiency and Colorectal Cancer. Front. Immunol. 12:635899. doi: 10.3389/fimmu.2021.635899

INTRODUCTION

Colorectal cancer (CRC) is the third most deadly and fourth most diagnosed cancer worldwide, and its incidence is steadily rising in developing nations (1). Both genetic characteristics and environmental factors play a role in intestinal carcinogenesis (2, 3). Alongside other well-established contributors, iron has recently emerged as a possible culprit in colorectal carcinogenicity (4). Published data support the hypothesis that excess oral iron intake is associated with an increased risk of CRC (5–8).

Iron is a vital micronutrient that has an essential role in many biological functions, in the context of which it has been found to be intimately linked to cancer biology (4, 9, 10). The trace element is required for energy production and intermediary metabolic actions as a catalyzer for REDOX-mediating enzymes. Proteins may bind directly to iron or contain iron in the form of heme or iron–sulfur clusters (11). Iron generates oxygen free radicals, which may in turn cause

iron-induced apoptosis or ferroptosis. Furthermore, these iron-oxygen complexes are complicit in promoting mutagenicity and malignant transformation. Having undergone transformation, malignant cells require large quantities of iron in order to proliferate. Iron is also an important mediator of immune functions, including tumor surveillance carried out by the immune cells (9). Cytokine production in macrophages, a key aspect of host defense, is regulated by their iron content (11). Ideal iron intake must therefore be carefully balanced between iron deficiency and iron excess, since both can have potentially crucial clinical consequences with regard to cancer development. To date, however, although a large number of studies have comprehensively investigated and reviewed the role of excess iron in cancer initiation and progression (5, 9, 10, 12–14), potentially tumorigenic effects of iron deficiency have been largely neglected and are not yet well defined (4). This certainly deserves more research, since iron deficiency occurs particularly frequently in patients with CRC, both at the time of diagnosis and throughout the duration of disease (15–17).

Just as the effects of excess iron intake can potentially influence both the etiology and prognosis of CRC, so too can the physiological effects of iron deficiency (18–20). The risk of CRC has been found to be significantly elevated among patients with iron deficiency anemia (IDA) (15, 16, 21). Moreover, iron deficiency is evidentially associated with shorter survival times in patients with cancer (19). These findings are not surprising, since iron deficiency can limit hematopoiesis, a prerequisite for immune cell production, and iron is necessary for the correct functioning of the immune cells (22, 23). Thus, in cancer patients, iron deficiency can result in a diminished immune response and, consequentially, an impaired treatment response, a poor prognosis and reduced overall survival (18–20). In this review, we investigate the flipside of the coin regarding the role of iron in cancer, addressing consequences of iron deficiency on immune functions key to tumor development and progression, particularly in CRC, and elucidating current options for iron therapy to limit these outcomes.

DEFINITION OF IRON DEFICIENCY

Iron deficiency, with or without anemia, is the most frequent hematological manifestation in individuals with cancer, occurring in over 40% of patients. In patients with CRC, the reported rate is even higher, at around 60% (17, 24, 25). Two forms of iron deficiency can be observed in patients with cancer: absolute iron deficiency (AID) and functional iron deficiency (FID).

Whereas AID is characterized by depleted iron stores and inadequate iron supply, in FID, iron stores are adequate, but there is insufficient iron supply for erythropoiesis and other iron-dependent pathways (26, 27). The main cause of FID in cancer is the release of cancer-associated pro-inflammatory cytokines such as interleukin (IL)-6, IL-1, TNF-α, and IFN-γ. These cytokines upregulate hepcidin synthesis, thus reducing the quantity of iron released into the circulation (27–29). FID may also develop due to chemo- and/or radiotherapy-induced myelosuppression or increased erythropoiesis under therapy with erythropoiesis-stimulating agents (ESAs) (27, 29). Chronic kidney disease, a frequent comorbidity in cancer patients, can cause FID by reducing erythropoiesis and increasing levels of hepcidin (30, 31). FID is one of the major contributors to anemia of chronic disease (ACD), in this context also known as anemia of cancer or cancer-related anemia (29, 32).

In AID, on the other hand, iron stores are genuinely depleted. Nutritional deficiencies (e.g., malabsorption, tumor-induced anorexia, malnutrition) and especially manifest or occult blood loss, which are not uncommon in CRC, contribute to AID (26, 27, 29).

Figure 1 presents an overview of the consequences of iron deficiency and anemia in patients with cancer.

Clinical Insight: Diagnosing Iron Deficiency in Patients With Cancer

Differentiation between AID and FID is essential, since the specific etiology of iron deficiency in patients with cancer is an important determinant of the treatment approach (26, 27, 33, 34).

Analysis of iron supply in the bone marrow with Perls' Prussian blue staining is the "gold standard" for diagnosis of iron deficiency (35). However, this technique is costly, highly invasive and non-automated, as a result of which it is largely impracticable in routine practice.

In healthy individuals, serum ferritin (SF) is recognized as a marker of iron stores, while other parameters, such as transferrin saturation (TSAT), mean corpuscular volume (MCV), percentage of hypochromic erythrocytes (%HYPO), Hb content of reticulocytes (CHr), soluble transferrin (sTfR), red blood cells (RBCs) and zinc protoporphyrin (ZnPP) reflect the amount of biologically available iron (26, 27). However, most of these parameters are altered in patients with cancer. Therefore, the differentiation of types of iron deficiency in this setting poses multiple challenges (27).

Iron deficiency is defined as transferrin saturation (TSAT) $<$ 20%, and can be further characterized as AID (SF $<$ 100 ng/mL) or FID (SF $>$ 100 ng/mL) (27, 33, 34). Since SF behaves as an acute-phase protein, due to the presence of cancer-related inflammation, its cut-off point is higher in patients with cancer than in persons without inflammatory disease (cut-off for SF in the latter is 30 ng/mL) (34). In addition to the more established markers TSAT and SF, ZnPP could represent a valuable addition to differential diagnostics, since it has been found to be increased in AID (34, 36, 37). While levels of soluble transferrin receptor

Abbreviations: AID, absolute iron deficiency; CAT, catalase; CHr, hemoglobin content of reticulocytes; CRC, colorectal cancer; EMT, epithelial to mesenchymal transition; ESA, erythropoiesis-stimulating agent; Fe-S cluster, iron-sulfur cluster; FID, functional iron deficiency; GSH-Px, glutathione peroxidase; Hb, hemoglobin; HIF, hypoxia-inducible factor; IDA, iron deficiency anemia; IFN, interferon; IL, interleukin; JHDM, Jumonji-C (JmjC)-domain-containing histone demethylase; MCV, mean corpuscular volume; MiRNA, microRNA; NF, nuclear factor; NK, natural killer; MPO, myeloperoxidase; RBC, red blood cell; REDOX, oxidation-reduction; SF, serum ferritin; SOD, superoxide dismutase; TNF, tumor necrosis factor; TSAT, transferrin saturation; ZnPP, zinc protoporphyrin; sTfR, soluble transferrin receptor; UIBC, unsaturated iron binding capacity; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau; %HYPO, percentage of hypochromic erythrocytes.

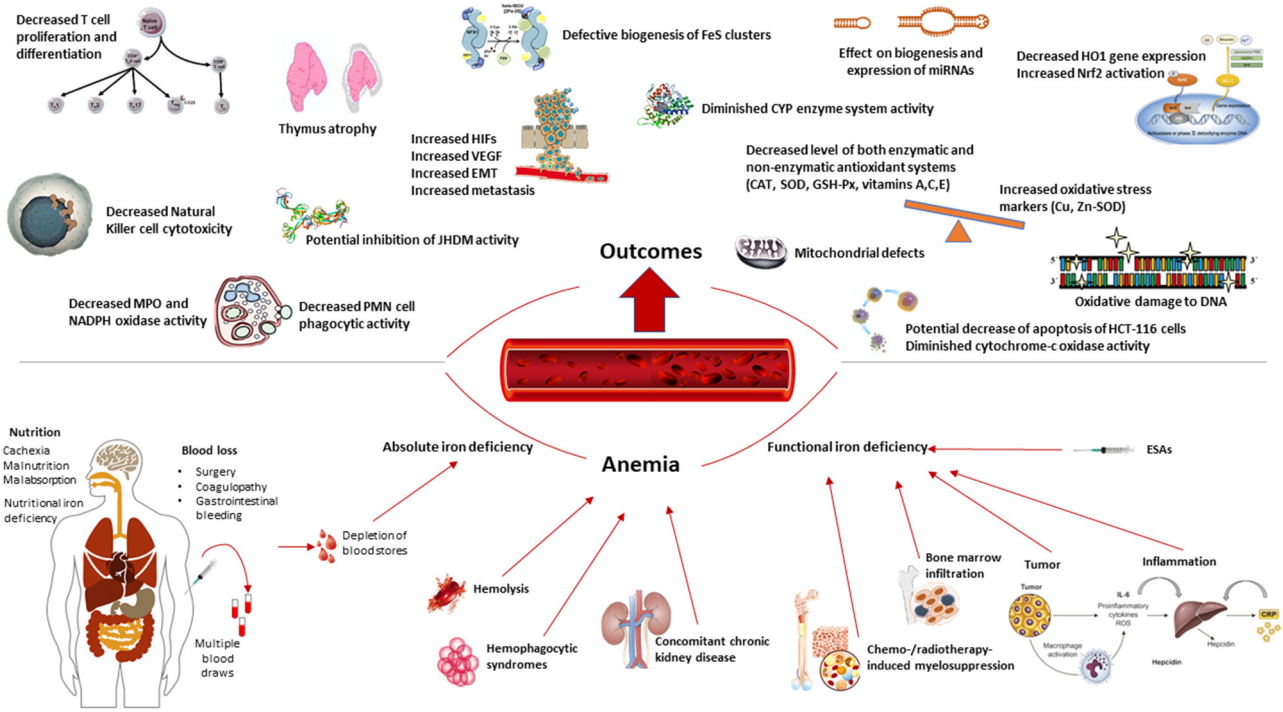

FIGURE 1 | Overview of the consequences of iron deficiency and anemia in patients with cancer.

(sTfR) have also been reported to be increased in AID and reduced in FID (27, 32), its levels may decrease following chemotherapy and increase after ESA treatment. Therefore, sTfR and markers related to sTfR, such as sTfR/log ferritin index, are less suitable as markers in an oncological setting (32, 34, 37). Other markers of iron deficiency, including CHr, %HYPO, MCV, and RBCs, fail to discriminate between AID and FID (34).

Measurement of circulating hepcidin could offer additional utility, not only in assessing iron status, but also in predicting response to iron therapy (38, 39). As yet, however, there is neither a validated clinical cut-off for hepcidin nor a simple standard test that would allow it to be fully used in clinical practice (27, 38).

IMPACTS OF IRON DEFICIENCY ON CANCER

Iron has anti-inflammatory and antioxidant properties and is vitally involved in functions of the immune system (4, 20, 40). It also plays an indispensable role in many other essential physiological processes, such as cell proliferation and differentiation, the maintenance of intestinal health, DNA synthesis and repair, and the metabolic breakdown of drugs and toxins (41–43). Iron homeostasis (23, 44, 45) and the role of iron in the initiation, progression and therapy of cancer have already been comprehensively reviewed in numerous publications (9, 10, 12, 13, 46). In this section, we specifically focus on the impacts of iron deficiency on CRC, from basic science to clinical outcomes (**Figure 1**).

Iron Deficiency and Cancer Epigenetics

Epigenetic mechanisms have emerged as major actors that play diverse and important roles in the initiation and progression of cancer (47–49). While the role of iron in epigenetics has been described, the underlying mechanisms have not yet been thoroughly elucidated. Iron is essential for iron–sulfur (Fe-S) cluster synthesis in every cell of the body (50) and it is known that the key enzymes of DNA duplication, repair, and epigenetics have Fe-S clusters as prosthetic groups (50–54). Iron deficiency causes defective biogenesis of the Fe-S clusters, inducing DNA replication stress and genome instability, both of which are indications of malignant transformation (20, 54).

Jumonji-C (JmjC)-domain-containing histone demethylases (JHDMs) affect gene expression by demethylating lysine residues of histone tails, the most common sites of post-translational changes. Genetic alterations in JHDMs have been reported in various human cancers (55–57). Consequently, JHDMs are believed to be involved in oncogenesis (55). JHDMs are iron-dependent enzymes, having iron as a cofactor (51, 57). Therefore, iron deficiency might inhibit the activity of JHDMs, with possible oncologically relevant effects. Furthermore, hypoxia, a common feature of iron deficiency, has also been found to result in a loss of JHDM activity and probably contribute to changes in chemokine expression (56). The role of JHDMs can be two-sided, depending

on the cancer type. Overall, therefore, it is important to maintain optimal iron levels (55).

The role of microRNAs (miRNAs), members of the noncoding RNA family, in the initiation, progression, metastasis and invasive activity of tumors has been characterized over the past decade. miRNAs are evolutionarily conserved, endogenous, single-stranded small RNAs of 18–22 nucleotides in length, that are encoded by eukaryotic genomic DNA. Aberrant expression of miRNAs may modify the normal expression of various genes including oncogenes and tumor-suppressor genes (47, 58). Ultimately, dysregulation of miRNA expression and related biological processes leads to poor outcomes in terms of cancer progression and development, and also to poorer therapeutic response (58–60). In addition, ∼50% of miRNAs are located at genomic cancer-associated regions of loss of heterozygosity or loss of amplification and at fragile sites within chromosomes, underlining the important role of miRNAs in tumorigenesis (61).

Iron deficiency is suspected to affect miRNA biogenesis and expression and alter miRNA-mediated gene regulation networks by causing defective heme biosynthesis and degradation, hypoxia and increased ROS (62–66). Thus, iron deficiency can also increase the risk of tumorigenesis and lead to poor cancer prognosis and poor therapeutic outcomes by negatively influencing the gene regulation system of miRNAs (67).

Hypoxia, a common feature of iron deficiency, has been demonstrated to play a major part in tumor progression and treatment resistance in mice by corrupting the von Hippel-Lindau (VHL) gene, the master regulator of hypoxia-inducible factor (HIF) and thus a tumor suppressor (68). In iron-deficient, immunodeficient mouse xenograft models, the Notch signaling pathway was shown to be disrupted and expression of the transcription factor Snail elevated (69). Snail has numerous effects relevant to tumor growth, metastasis and treatment resistance: Its increased expression promotes cell motility and invasiveness by altering epithelial-mesenchymal transition (by repressing epithelial and enhancing mesenchymal markers). Furthermore, Snail endows stem cell-like characteristics on tumor cells, thus increasing therapy resistance (70).

Iron deficiency, through hypoxia, has been associated with enhanced expression of BCL2L1, the protein-coding gene that inhibits mitochondria-mediated cell death. Furthermore, iron deficiency has been shown to inhibit expression not only of CTSZ, the gene for the cysteine protease cathepsin Z, which has been associated with malignancy and inflammation, but also of CASP5, the gene for the cysteine peptide Caspase 5, which is involved in cellular apoptosis (71).

Iron deficiency is therefore associated with a variety of epigenetic changes and epigenetic mechanisms that are likely associated with oncogenesis. However, their role in cancer development and progression remains to be fully elucidated.

Iron Deficiency and Pro-oxidant and Antioxidant Activities

It has been suggested that iron deficiency might cause an imbalance of the pro- and anti-oxidant systems (REDOX) (20). When iron is lacking, the level of both enzymatic and non-enzymatic antioxidant systems, such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and vitamins A, C, and E have been found to be decreased (6, 72–74). On the other hand, oxidative stress markers like Cu and Zn-SOD are increased (20, 75). These changes lead to an increased generation of reactive oxygen species (ROS), accompanied by a decrease in the body's total antioxidant capacity (74, 76–78). While ROS display varying reactivities toward different targets, they share the ability to damage cells by oxidizing proteins, lipids and DNA. This potential of ROS to cause cell damage and DNA mutation suggests that it may be directly or indirectly associated with tumor cell development, metastasis, tumor aggressiveness and treatment resistance as a reflection of accumulated ROS damage over time (20, 79, 80).

It has been demonstrated that by increasing oxidative stress, iron deficiency can cause damage to the mitochondria, corrupting mitochondrial DNA (81). Mitochondria are organelles of the cell that are primarily responsible for oxidative phosphorylation, the production of intracellular energy from oxygen and nutrients, as well as heme synthesis (82) and assembly of eukaryotic iron-sulfur (Fe-S) protein clusters (83). Mitochondria are also responsible for autoreproduction. Disruption of mitochondrial functions can therefore impair the integrity of the nuclear genome (84).

Hemoproteins are conjugated proteins with a variety of structures and functions that contain a non-protein component or prosthetic group called heme (or a derivative thereof). Increased ROS due to oxidative stress may induce the hemoproteins to discharge these heme groups, resulting in circulating free heme that can trigger additional production of free radicals. There are a number of mechanisms that can counteract pro-oxidant effects of free heme, such as rapid induction of heme oxygenase-1 gene (HMOX1) transcription and heme oxygenase-1 (HO-1) isoenzyme protein expression, which generates rapid catabolism of free heme in order to limit resultant cell damage (85, 86). As well as being involved in cellular homeostasis, HO-1 plays an important part in preventing oxidative tissue damage and mediating intracellular inflammatory mechanisms, apoptosis and cell proliferation (85). Lai et al. (87) reported that without adequate iron, HCT-116 human colon adenocarcinoma cells were unable to express the HO-1 gene completely, in response to toxicity. Since iron is essential for HO-1 gene expression, iron deficiency might lead to decreased cytoprotection through HO-1 expression (20).

Heme is an integral part of the CYP (intestinal cytochrome P450) antioxidant enzyme system (88–90). Iron deficiency has been shown to diminish CYP system activity in intestinal cells. Both in a xenograft murine model and in CRC cells, CYP2S1 gene depletion was identified to promote colorectal carcinogenesis (91–93). Thus, the effects of iron deficiency on heme synthesis can interfere with the CYP system, posing a risk factor for CRC.

In vitro studies in human brain cells have shown iron deficiency to result in significant reduction of the heme-containing electron transport protein (cytochrome-c oxidase/complex IV) (94). This has been shown to cause

impairment of the heme metabolism, an increase in oxidative stress, and mitochondrial dysfunction (94). All of these are characteristic indications of cancer (20, 95).

The transcription factor Nrf2 (nuclear factor-E2-related factor-2) functions as a cellular sensor for oxidative stress. The genetic transcription of phase-II proteins via Nrf2 activation probably represents the most important signaling pathway for the body's immune response to oxidative stress and toxins. Nrf2 thus plays an essential role in cell protection. Iron deficiency has been found to activate autophagy and Nrf2 signaling for oxidative stress (96). Nrf2 activation has been implicated in cancer and is associated with a poor outcome and reduced survival in tumor types such as non-small cell lung cancer (97, 98). It has been proposed that constitutive activation of Nrf2 may encourage oncogenesis (99, 100) through actions promoting angiogenesis, metabolic reprogramming, chronic proliferation, and resistance to cell death (101, 102). Therefore, iron deficiency may promote oncogenesis by activating autography and Nrf2 signaling for oxidative stress.

Iron Deficiency, Immune Response, and Cell Function

The interplay of iron homeostasis with cellular immune responses is complex and context dependent. Impairment of cellular immunity and antimicrobial activities of immune cells due to iron deficiency may create a microenvironment unconducive to the immunosurveillance mechanisms of the immune system that should identify and eliminate potential for malignant transformation. Furthermore, within the modified tumor microenvironment, immune cells may themselves exert a pro-tumorigenic response (4, 14, 20, 85).

The nuclear factor (NF-κB) and hypoxia-inducible factors (HIFs) are transcription factors that are critical to immune system regulation (103). The physiology of tumor cells allows them to grow and multiply rapidly and avoid apoptosis. Also characteristic of these cells are their capacities to ignore growth-inhibitory signals, to instigate angiogenesis, tissue invasion and metastasis, and to replicate infinitely. Almost all of the genes involved in the mediation of these processes are regulated by NF-κB transcription (104). Low levels of intracellular iron evidentially reduce phosphorylation of Re1A, a subunit of the NF-κB family of genes, and impair prolyl hydroxylation of HIFs (71, 105). Iron deficiency *per se* and iron deficiency-induced hypoxia can trigger the activation of HIFs, which are known to mediate cancer progression by upregulating target genes associated with angiogenesis and the metabolic reprogramming of tumor cells (106, 107), thus causing resistance to chemo- and radiotherapies (108, 109). HIF-1α plays a key role in the growth, progression and metastasis of solid tumors (110, 111). Iron deficiency has been found to promote HIF-1 transcription and inhibit HIF-2 transcription, thus corrupting the synergistic signaling pathways between the HIFs and NF-κB (71). Consequently, iron deficiency may weaken the immune response, increasing both the risk of oncogenesis and the probability of a poor prognosis and resistance to therapy when malignancy occurs.

Cellular iron depletion induced by the iron chelator desferoxamine mesylate (DFO) has been shown to increase HIF-1α (112). The transcription factor HIF-1α mediates expression of vascular endothelial growth factor (VEGF), a potent inducer of malignant angiogenesis and metastasis. Thus, iron deficiency has been reported to have important effects on HIF-1α stabilization, VEGF formation, angiogenesis and tumor progression in breast cancer, in both *in vitro* and *in vivo* studies (68, 113). Jacobsen et al. (114) found increased VEGF levels to be associated with a poor outcome in human renal cell carcinoma. Moreover, in one of these models, iron supplementation was found to significantly decrease VEGF levels in hypoxia, indicating a role for iron in counteracting HIF-1α stabilization and thus, possibly, in preventing angiogenesis (113).

Myeloperoxidase (MPO) and NADPH oxidase are enzymes that play a key role in interferon-γ (IFN-γ) induction by monocytes, and in microbial killing and phagocytosis by means of ROS production in neutrophils. These enzymes are iron dependent (115–118): Their catalytic activity is suppressed when iron deficiency is present, causing phagocytosis to be impaired. As a result, susceptibility to infections and tumor development may be increased (20, 118).

Natural killer (NK) cells are cytotoxic effector lymphocytes that perform unique functions including immunosurveillance and anti-tumor actions within the innate immune system (119). Hypoxia, which is characteristic of the iron deficient state, has been shown to inhibit the expression of vital activating NK-cell receptors and NK-cell ligands on tumor cell membranes (120, 121). Iron deficiency therefore disrupts the cytotoxic and specifically anti-tumor activities of NK cells and is conducive to oncogenesis and tumor growth.

Lymphocytes, comprising natural killer cells, T cells and B cells, are the major cellular constituents of cell mediated immunity. Cytotoxic T cells have several functions, one of which is the lysis of tumor cells. Iron deficiency has been shown to inhibit T cell proliferation and secretion of the potent anti-tumor cytokine IFN-γ (122). In murine models, iron deficiency was found to lead to atrophy of the thymus gland and the reduced excretion of CD28 thymocytes and spleen cells, causing impairment to lymphocytic motility and functions (123, 124). In addition, protein kinase-C translocation from cytosol to the plasma membrane, vitally necessary for T cell migration and immunological synapse, is reduced in the iron deficient state (125, 126). Furthermore, iron deficiency inhibits overall the expression of various diversely acting cytokines from cells of the immune system (127–129). Cell mediated immunity is therefore impaired due to iron deficiency, paving the way for cancer development and growth.

It has been demonstrated that intracellular iron plays a key role in apoptosis of HCT-116 (human cancer) cells (130). Furthermore, cytochrome-c oxidase activity, a significant marker of apoptosis resistance, is evidentially diminished in the presence of iron deficiency (131, 132). Therefore, the cancer-related effects of iron deficiency may influence not only tumor development and progression, but also apoptosis and treatment response.

EVIDENCE FROM HUMAN CLINICAL STUDIES OF IRON DEFICIENCY ANEMIA IN RELATION TO COLORECTAL CANCER

The abundant biological and immunological evidence describing important cancer-related effects of iron deficiency has direct implications for human health. Clinical and epidemiological studies have focused on various aspects of the relationship between iron deficiency and CRC, from etiology to progression and metastasis, therapeutic response and long-term outcomes.

Studies of patients with CRC found a significant association with low transferrin saturation in a cohort of Californian males (133) and with low serum ferritin in a case-control nested study of New York females (134). In another cohort study, men and postmenopausal women with iron deficiency without anemia had a five-fold and those with IDA a 31-fold increased risk of developing gastrointestinal cancer in comparison to individuals with normal hemoglobin (Hb) and TSAT levels (15).

In a large cohort of 965 men and women aged 50–75 years, Bird et al. (135) found a U-shaped relation between iron intake and colorectal polyps, with those consuming high (>27.3 mg/day) or low (<11.6 mg/day) quantities of iron more likely to develop colorectal polyps, a precursor lesion to CRC. In line with this, Cross et al. (136) showed that CRC risk was inversely associated with serum ferritin levels and positively associated with serum unsaturated iron binding capacity (UIBC). Moreover, serum iron and TSAT were found to have an inverse association with the risk of colon cancer, specifically (136). In a recent study by Hamarneh et al. (137) assessing risk factors for CRC following a positive fecal immunochemical test, IDA was reported as a significant risk factor for CRC [OR 7.93, 95% Cl (2.90–21.69), $p < 0.001$] independent of age. While the above findings suggest that iron deficiency could contribute to the pathogenesis of CRC, just as excessive iron intake does, the mechanisms are not yet fully understood. However, as presented above, preclinical research points to a role of iron deficiency in blunting the immune response, allowing tumor cell invasion under diminished immunosurveillance or switching to a pro-tumorigenic immune cell function in the tumor microenvironment (4, 9, 22, 23).

Not only may iron deficiency substantially influence oncogenesis, but it has also been found to influence oncological outcomes in patients with CRC. Zhen et al. (138) investigated long term effects of iron deficiency on the outcomes of 644 patients (19–83 years) with TNM stage II CRC and found IDA to be an independent predictor of long-term outcome in patients with T3N0M0 stage colon cancer. Patients with IDA had inferior outcomes and presented with worse tumor staging and lower disease-free survival than non-anemic patients (138). These findings suggest that IDA can influence CRC prognosis and outcomes, presumably by inhibiting immune system mechanisms that limit tumor growth, hindering responsiveness to treatments such as chemotherapy or surgery, and restricting the immune system's response to circulating tumor cells that can develop into distant metastasis (4, 9, 139). Lorenzi et al. (140) found that patients with both high and low serum ferritin levels who underwent curative or palliative surgery had shorter survival after a follow up period of at least 5 years in comparison to those with normal levels. Another study by An et al. (141) showed that patients with preoperative anemia treated with combined FOLFOX-based adjuvant chemotherapy had a worse prognosis than those without anemia. Additionally, a systematic review of 60 studies identified a 65% overall elevated mortality risk among cancer patients with anemia in comparison with those without anemia (19).

Overall, therefore, the evidence from epidemiological and clinical research corroborates data from preclinical studies, suggesting that iron deficiency, like iron surplus, might have a considerable negative influence with regard to oncogenesis, tumor progression and individual outcomes. Iron deficiency, with or without anemia, is associated with a poor prognosis, worse tumor staging, lower disease-free survival rates and a poorer response to oncological therapies in patients with CRC.

ON A THERAPEUTIC KNIFE-EDGE: IRON REPLACEMENT THERAPY IN PATIENTS WITH COLORECTAL CANCER AND IRON DEFICIENCY/ANEMIA

There are currently three main treatment approaches for iron deficiency in the context of CRC; blood transfusions (RBC transfusions), erythropoiesis-stimulating agents (ESAs) and iron supplementation (26, 34). Since both RBC transfusions and ESAs are, like iron deficiency/anemia, independently associated with an increased risk of CRC recurrence and mortality (142–144), the use of iron substitution therapy to reverse anemia has gained more attention. In principle, iron can be replaced either orally or intravenously.

Oral Iron

Oral substitution of iron has long been favored due to its simplicity and low costs, and as a result of lingering safety concerns due to adverse events associated with early intravenous iron compounds. However, its suitability in cancer patients is generally limited by concurrent inflammation, gastrointestinal discomfort and polypharmacy. Furthermore, oral iron has not been associated with consistent clinical or hematological improvement in patients with cancer (82, 145–147). On the contrary; it has been found to be ineffective in individuals with cancer and especially CRC, since intestinal iron absorption is greatly reduced in these patients (nearly 95% of the iron being excreted) (33). Furthermore, the increased availability of iron in the gut due to reduced intestinal iron absorption may support the proliferation of pathogenic gut bacteria conducive to tumor progression in preference to protective passenger bacteria that are more likely to hinder disease progression (148). As for the very small quantity of iron absorbed, most remains trapped within the enterocytes, where it is largely blocked by inflammatory cytokines and thus cannot be metabolized (33, 149). Overall,

therefore, oral iron is unsuitable for iron replacement in patients with CRC.

Intravenous Iron

Intravenous (IV) iron can overcome the absorptive inflammatory blockade of iron, since iron is directly captured by the macrophages (33). There is growing evidence to support benefits of IV iron therapy (without additional ESAs) in patients with cancer (150–160) and IV iron has been shown to optimize preoperative hemoglobin levels specifically in patients with CRC (158–163). On the other hand, in the extended IVICA trial, a randomized study including 116 patients with anemia and colorectal cancer treated preoperatively with oral or IV iron, no significant difference was found for 5-year overall survival or disease-free survival (164). There are some concerns about the possible role of iron overload in cancer, including promotion of tumor growth, enhanced oxidative stress and poor disease progression (165–167). Wilson et al. (168) suggest that "iron therapy may worsen colorectal tumor prognosis by supporting colorectal tumor growth and increasing the metastatic potential." However, there is no direct evidence from experimental studies to substantiate this hypothesis and the clinical applicability of such experimental data in patients with cancer is limited, since they are based on high iron doses, differing routes of injection and a variety of iron formulations that are not typically used in clinical settings (27, 169). Furthermore, iron overload is rare in patients with cancer (34).

In rodent models of CRC induced by inflammatory or carcinogenic agents, whereas elevated oral iron intake was shown to increase the incidence of tumors, systemic (IV) iron supplementation did not have the same effect (170, 171). This suggests that increased luminal iron, but not systemic iron levels, increase colorectal carcinogenesis in inflammatory models of CRC (172, 173). Radulescu et al., who showed in a rodent model that luminal iron cooperates with Apc (*adenomatous polyposis coli gene*) loss to promote intestinal tumorigenesis, propose that in patients with CRC, a combination of colonic luminal iron chelation and concurrent systemic iron replacement therapy would both resolve anemia and at the same time diminish the carcinogenic pool of residual iron within the colon (174).

Evidence from prospective clinical trials describing outcomes of IV iron therapy (alone or in combination with ESAs) in an oncological population are relatively scarce but their results are in line with the findings of rodent model studies. Short-term studies are reassuring, having not shown increased tumor progression in patients treated with IV iron and ESAs (34). One prospective randomized controlled trial evaluating treatment with IV iron and ESAs in patients with cancer (175), with a median follow-up period of 1.4 years, failed to find any negative effects on long-term outcomes or survival. A retrospective cohort study of patients who underwent surgery for CRC, with an extended follow-up period (median 3.9 years), confirmed that overall and disease-free survival did not significantly differ in subjects treated with IV iron (in this case, ferric carboxymaltose at a dose of 1,000–2,000 mg) as compared with a matched group not receiving IV iron (176). A comprehensive review of iron dextran use by Gilreath et al. concluded that there was no clinical evidence to support an elevated risk of cancer growth due to iron overload (167).

Regarding the risk of infections, no alarming signs have emerged in patients with cancer treated with IV iron. Nevertheless, given the role of iron in immune response and microbial proliferation (177), current guidelines prudently advise that IV iron should not be administered to patients who have, or are suspected to have, active infections (34).

No increase in cardiovascular morbidity has been observed in connection with IV iron therapy (82, 145, 178–180). However, it is recommended to avoid concomitant administration of IV iron and cardiotoxic chemotherapy: IV iron should be administered either before or after application of chemotherapy, or at the end of the chemotherapy treatment cycle (34).

CONCLUSION

In contrast to the large amount of research already dedicated to the effects of excess iron as a probable (co-)trigger and driver of oncogenesis, the role of iron deficiency has been largely neglected and—on the evidence of the reviewed preclinical and clinical data—possibly underestimated. In particular, iron is vital for optimal functioning of the immune system, playing major roles in a multitude of different immune processes and pathways. Iron deficiency influences crucial mechanisms such as immune surveillance, gene regulation and cell apoptosis, all of which are key to host defense against malignant transformation and tumor growth. Clinical studies in patients with cancer and iron deficiency/anemia suggest that that unlike oral iron, IV iron therapy (with/without ESAs) improves overall outcomes without increasing risk of infection or cardiovascular morbidity. Excess (uningested/residual) oral iron can cause oncogenic effects in the intestinal tract and is thus generally unsuitable for patients with CRC (although its use may occasionally be justified, employing "defensive" dosing strategies). In general, IV iron does not appear to have this potential for local exacerbation, as confirmed by rodent studies. Iron overload is rarely seen in patients with cancer and there is no clinical evidence that IV iron negatively affects tumor progression. Nevertheless, in view of the abounding evidence of effects of iron overload on tumor growth, we suggest that IV iron should be cautiously supplemented with the goal of avoiding anemia and maintaining iron stores. Additional research is needed to confirm the appropriateness of IV iron replacement in patients with cancer, to explore the feasibility of concurrent luminal iron chelation, to determine target levels for iron store maintenance, and to shed further light on the effects of chronic iron deficiency on iron-dependent mechanisms in the context of the tumor microenvironment.

AUTHOR CONTRIBUTIONS

AA performed the initial literature search. All authors participated in the data analysis, preparation of the manuscript draft, reviewed the manuscript for important intellectual content and approved the final version for submission.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance of Janet Collins (Interdisziplinäres Crohn Colitis Centrum Rhein-Main, Frankfurt am Main, Germany) in editing and proofreading the manuscript.

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Conflict of Interest: AA: Congress expenses, consultancy and lecturing fees: Vifor Pharma and Immundiagnostik AG. KF: Speakers' honoraria: Immundiagnostik AG. OS: Congress expenses: Abbvie, Falk, Janssen, Takeda; Lecturing fees: Abbvie, Falk, Janssen, Takeda, Norgine, Pfizer. JS: Consulting: Pharmacosmos, Vifor. Abbvie, Bristol Myers Squibb, Dr Schär, Falk, Ferring, Fresenius Kabi, Immundiagnostik, Janssen, Medice, MSD, Pfizer, Shire, Takeda, Thermofisher. Board member: Pharmacosmos, Vifor, Abbvie, Bristol Myers Squibb, Dr Schär, Ferring, Fresenius Kabi, Immundiagnostik, Janssen, MSD, NPS, Takeda, Shield. Lecturing: Vifor.

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frontiers in Oncology

HYPOTHESIS AND THEORY
published: 25 March 2021
doi: 10.3389/fonc.2021.627223

TAM-ing the CIA—Tumor-Associated Macrophages and Their Potential Role in Unintended Side Effects of Therapeutics for Cancer-Induced Anemia

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OPEN ACCESS

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

Received: *08 November 2020*
Accepted: *01 March 2021*
Published: *25 March 2021*

Citation:
Weiler S and Nairz M (2021) TAM-ing the CIA—Tumor-Associated Macrophages and Their Potential Role in Unintended Side Effects of Therapeutics for Cancer-Induced Anemia. Front. Oncol. 11:627223. doi: 10.3389/fonc.2021.627223

Cancer-induced anemia (CIA) is a common consequence of neoplasia and has a multifactorial pathophysiology. The immune response and tumor treatment, both intended to primarily target malignant cells, also affect erythropoiesis in the bone marrow. In parallel, immune activation inevitably induces the iron-regulatory hormone hepcidin to direct iron fluxes away from erythroid progenitors and into compartments of the mononuclear phagocyte system. Moreover, many inflammatory mediators inhibit the synthesis of erythropoietin, which is essential for stimulation and differentiation of erythroid progenitor cells to mature cells ready for release into the blood stream. These pathophysiological hallmarks of CIA imply that the bone marrow is not only deprived of iron as nutrient but also of erythropoietin as central growth factor for erythropoiesis. Tumor-associated macrophages (TAM) are present in the tumor microenvironment and display altered immune and iron phenotypes. On the one hand, their functions are altered by adjacent tumor cells so that they promote rather than inhibit the growth of malignant cells. As consequences, TAM may deliver iron to tumor cells and produce reduced amounts of cytotoxic mediators. Furthermore, their ability to stimulate adaptive anti-tumor immune responses is severely compromised. On the other hand, TAM are potential off-targets of therapeutic interventions against CIA. Red blood cell transfusions, intravenous iron preparations, erythropoiesis-stimulating agents and novel treatment options for CIA may interfere with TAM function and thus exhibit secondary effects on the underlying malignancy. In this Hypothesis and Theory, we summarize the pathophysiological hallmarks, clinical implications and treatment strategies for CIA. Focusing on TAM, we speculate on the potential intended and unintended effects that therapeutic options for CIA may have on the innate immune response and, consequently, on the course of the underlying malignancy.

Keywords: cancer-induced anemia (CIA), tumor-associated macrophage (TAM), iron, hepcidin, ferroportin, BMP - Smad signaling pathway, IL-6 (interleukin 6)

CANCER-INDUCED ANEMIA IS A FREQUENT CONSEQUENCE OF MALIGNANCY

Cancer-induced anemia (CIA) occurs in roughly one to two thirds of patients with solid tumors and complicates the course of malignancy (1–5). Its incidence is highly dependent on patient-related factors, on the entity and stage of the underlying disease and on therapeutic interventions. Specifically, the frequency and degree of anemia is higher in metastatic cancers, in aggressive hematologic malignancies and following treatment with high-dose chemotherapy, multi-targeted tyrosine kinase inhibitors and chimeric antigen receptor T cells (6–9). Therefore, CIA forms a spectrum which can broadly be categorized into three principal etiologies: First, CIA present before the initiation of anti-tumor therapy is typical of advanced disease stages with infiltration and replacement of the bone marrow or when the primary neoplasia results in substantial bleeding such as in colorectal or genitourinary malignancies. Second, indirect effects of products of neoplasms can lead to hemophagocytosis, autoantibody induced hemolysis or cytokine inhibition of erythropoiesis. Third, CIA with initial presentation only after the onset of anti-neoplastic treatment is one of the most common side effects of chemotherapy, yet also occurs as sign of progressive disease (10). According to the common terminology criteria for adverse events by the World Health Organization and National Cancer Institute, anemia is categorized into 5 grades from mild (Hemoglobin (Hb) 10 g/dL – lower limit of normal), to moderate (Hb 8.0 – 9.9 g/dL), severe (Hb <8 g/dL) and life-threatening with urgent interventions indicated (grade 4) or even death (grade 5) (11).

CANCER-INDUCED ANEMIA HAS DISTINCT PATHOPHYSIOLOGICAL HALLMARKS

The pathophysiology of CIA is complex and involves several contributing mechanisms. First, the immune response against malignant cells inevitably induces the iron-regulatory hormone hepcidin, which then directs iron fluxes away from the erythron and into compartments of the mononuclear phagocyte system (MPS) (**Figure 1**). In addition, certain inflammatory mediators, many of them cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, transforming growth factor (TGF)-ß and IL-10, stimulate iron uptake into the MPS, induce iron storage in the form of ferritin (FT) and/or block iron recycling (12–15). Together, these effects of inflammatory mediators result in a functional iron deficiency, and erythropoietic cells are cut off their iron supply by macrophages. Presumably, this iron-storing macrophage phenotype deprives infectious agents as well as malignant cells from circulating iron sources. However, in the tumor microenvironment (TME), tumor-associated macrophages (TAM) may lose their ability to store iron because they are re-programmed by neoplastic cells to resume iron export. The mechanisms that result in metabolic reprogramming of TAM are incompletely understood but may involve transcriptional regulations and epigenetic changes (16).

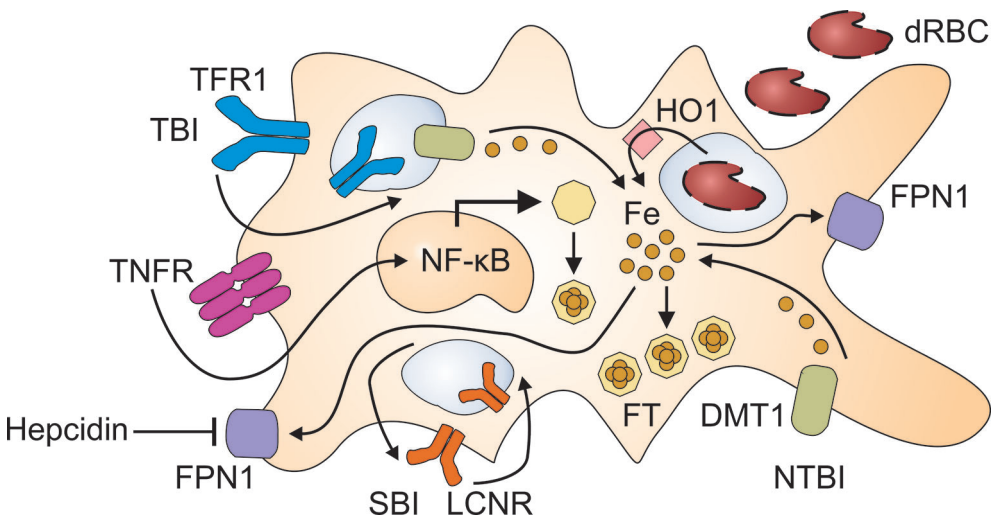

FIGURE 1 | Pathways for the retention of iron in macrophages. Immune activation results in the production of inflammatory cytokines such as tumor necrosis factor (TNF) which damage red blood cells (dRBC) and activate nuclear factor (NF)-κB. The uptake of dRBC delivers large amounts of heme to macrophages which recycle heme-derived iron after its release by heme oxygenase (HO)-1. Moreover, inflammatory cytokines stimulate the uptake of transferrin-bound iron (TBI) *via* transferrin receptor (TFR)-1 and of non-transferrin-bound iron (NTBI) *via* divalent metal transporter (DMT)-1. TBI incorporated *via* TFR1 undergoes reduction to its ferrous form in the endosome and subsequent transfer to the cytoplasm through DMT1. The lipocalin-2 receptor (LCNR) can mediate both the uptake and the release of siderophore-bound iron (SBI). Inflammatory cytokines and hepcidin reduce ferroportin (FPN)-1 mediated iron export, which further contributes to iron retention in macrophages. To avoid elevated iron levels in the cytoplasm, labile iron is incorporated into ferritin (FT) which is upregulated both by iron and NF-κB.

Second, inflammatory mediators such as TNF and hydrogen peroxide inhibit the production of erythropoietin (EPO) in renal peritubular fibroblasts (**Figure 2**) (17, 18). Again, this mechanism aims at reducing the oxygen supply to tumor cells. However, these cells may switch to anaerobic glycolysis and induce tumor neovascularization by starting to generate angiogenetic factors. However, macrophages, endothelial cells or other cell types in the TME are also able to secrete angiogenetic mediators (19–21). Therefore, hypoxia in the TME is also a potential driving force for disease progression and metastasis (22). Although EPO levels are elevated in patients with CIA, this elevation remains insufficient for the degree of anemia (23–25).

Third, the immune response, intended to primarily target malignant cells, also impairs erythropoiesis in the bone marrow (**Figure 2**) due to inhibition of and damage to erythroid progenitors (EP) and hematopoietic stem cells (26–28).

Fourth, in advanced solid tumors, proliferating cancer cells may deprive EP of cobalamin and folate, infiltrate the bone marrow, displace normal hematopoiesis and destroy its niches similar to what is typical of hematologic malignancies. Without cancer treatment, all these mechanisms interact in a vicious circle to aggravate the severity of CIA. However, when cancer treatment is initiated, the toxic effects of chemotherapeutics or radiation therapy on the bone marrow and on other organs such as the kidney may contribute to anemia, too. Moreover, cellular iron retention counteracts the ability of hypoxia inducible factors (HIF) to stimulate EPO production, a mechanisms which is relevant to peritubular fibroblasts (29).

In summary, many of the major pathways that contribute to anemia in cancer patients interact with each other, and specific treatment for CIA needs to be taken into consideration to break this circle of mechanisms causing and aggravating conditions of anemia (30).

INFLAMMATION AND CANCER DYSREGULATE IRON METABOLISM

Iron metabolism and the immune response are interlinked (31). Iron is an essential nutrient to almost all microbes (32, 33). One important role of the acute phase response (APR) is the

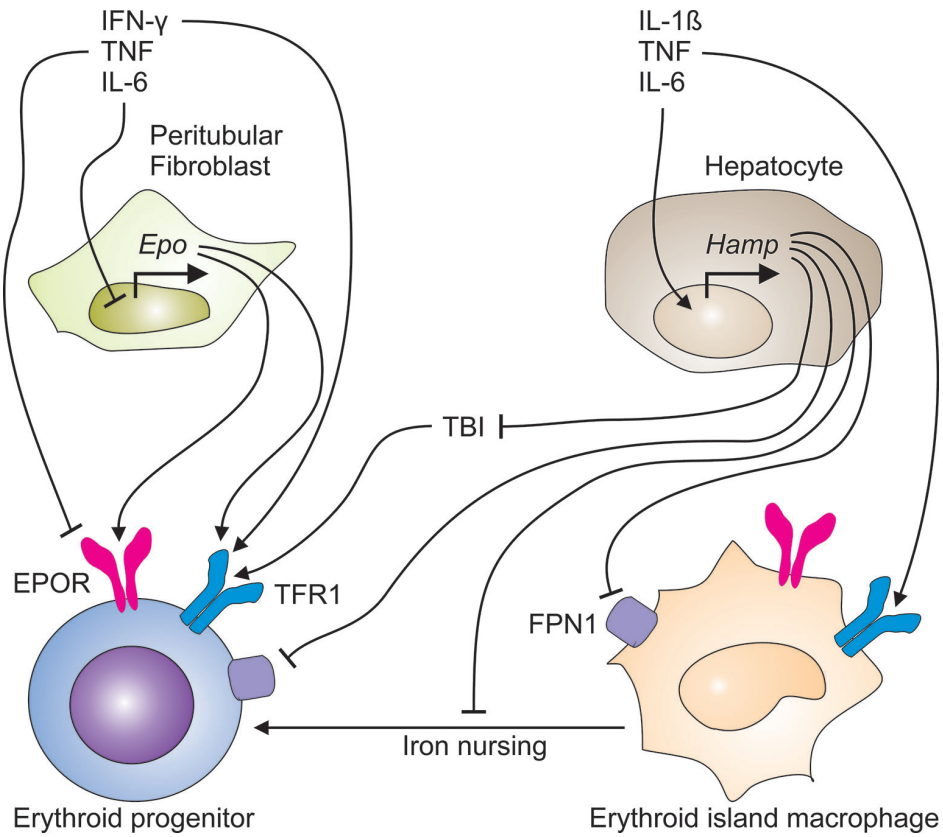

FIGURE 2 | Immune-mediated pathways contribute to the development of cancer-induced anemia. The immune response to cancer cells generates a plethora of pro-inflammatory cytokines. TNF and other T_H1 cytokines inhibit both, the production of erythropoietin (EPO) in peritubular fibroblasts in the kidney and the expression of the EPO receptor (EPOR) on erythroid progenitor cells in the bone marrow. In parallel, TNF, IL-1ß and IL-6 induce the transcriptional expression of the *hepcidin antimicrobial peptide* (*Hamp*) gene in hepatocytes. Liver-derived hepcidin acts on ferroportin (FPN)-1 and blocks its iron export function, thus reducing the transfer of iron to the circulation and the amount of transferrin-bound iron (TBI), consequently. In addition, hepcidin may undermine the transport of iron from erythroid island macrophages to erythroid progenitors, thus impairing their proliferation and hemoglobin synthesis.

reduction of iron levels in extracellular compartments (i.e. the serum) and iron storage in the intracellular compartment (i.e. in FT). As for many other immune functions, this important role is fulfilled by monocytes, macrophages and other cellular players of the mononuclear phagocyte system (MPS). Macrophages possess a broad spectrum of pattern recognition and scavenger receptors for the sensing and uptake of potentially harmful macromolecules and the recognition of malignant cells. These receptors are linked to intracellular signaling cascades which converge at the level of key inflammatory transcription factors including the nuclear factor (NF)-κB (**Figure 1**). Once activated, NF-κB orchestrates the transcriptional responses of macrophages and other immune-competent cell types to *trans*-activate inflammatory gene products such as TNF, IL-6 and IL-22 which also play an important role in the cancer development and aggravation of CIA (34). These pro-inflammatory cytokines, especially the APR-initiator IL-6, target the liver and stimulate hepatocytes to increase hepcidin output (35–37). In inflammatory conditions such as in patients with malignancies, hepcidin expression is induced by the concerted interaction of two pathways involving IL-6, Janus kinases (JAK) and signal transducers and activators of transcription (STAT) or activin B and activation of the SMAD (for sisters of mothers against decapentaplegic)-1/5/8 signaling pathway (38–42). On the contrary, patients treated with an anti-TNF antibody or an anti-IL-6 antibodies exhibit reduced levels of inflammatory markers such as IL-6, hepcidin, and/or C-reactive protein, correlating with improvement in anemia related to autoimmune inflammatory conditions (43).

THE ACUTE PHASE RESPONSE DRIVES IRON RETENTION IN MACROPHAGES

The immune response to pathogen associated molecular patterns (PAMP) such as lipopolysaccharide and to danger associated molecular patterns (DAMP), present in the TME, such as free heme, adenosine, IL-1α, high-mobility group box-1 (HMGB1) and S100 proteins are similar. The recognition of either PAMP or DAMP results in the activation of NF-κB, p53, mitogen-activated (MAP) kinases and inflammasomes (44). As a consequence, the hypoferremia of the APR also limits the availability of iron for malignant cells. Yet, prolonged hypoferremia may be regarded as a maladaptation to persistent immune stimulation in the setting of chronic infections, autoimmune or neoplastic diseases.

T cells are major mediators of the immune response against malignant cells. Specifically, major histo-compatibility (MHC) class I molecules present on tumor cells present neoantigens on their surfaces. These neoantigens are detected by cytotoxic T cells and elicit the secretion of granzyme B, perforins and of cytokines such as TNF and IL-6. Both cytokines induce hepcidin, a hormone with a unique mode of action: hepcidin binds to its receptor ferroportin (FPN)-1, an iron export channel, and blocks its transport function. Hepcidin thus mediates a negative feedback loop because it reduces iron recycling by macrophages and iron absorption by enterocytes when serum and tissue iron levels are elevated. Therefore, excess hepcidin deactivates iron transport to the blood stream resulting in a reduction of serum iron concentrations during ongoing iron consumption by transferrin receptor (TFR)-1 expressing cells in the face of reduced resupply (**Figure 2**).

To further support the iron withdrawal from malignant cells, IL-6, IL-10 and other pathways induce the iron storage protein FT (45, 46). FT is composed of 24 subunits of heavy and light chains which assemble in variable proportions to form a shell-shaped heteromultimer. Serum FT is primarily produced and secreted by macrophages (47). Therefore, the levels of serum FT reflect body iron stores and the state of immune activation. In other words, in the presence of cancer cells or other immune stimuli, serum FT does not accurately predict the amount of iron stored in macrophages and other cell types such as hepatocytes. Consequently, in these scenarios, normal serum FT does not rule out the depletion of iron stores that characterizes absolute iron deficiency. *Vice versa*, increased serum iron (hyperferritinemia) can indicate either parenchymal iron overload or immune activation with subsequent uptake and storage of iron in macrophages. In clinical settings, it is fundamental to distinguish the different etiologies, as treatment is directed toward the underlying morbidity and does not automatically result in iron reduction approaches.

IRON HAS PLEIOTROPIC EFFECTS ON THE IMMUNE RESPONSE

The adaptation of iron metabolism during the APR may have evolved to deal with acute stressors such as bacterial infections. However, when inflammatory stimuli persist, as is the case in neoplasms that cannot be fully resected, this immune-mediated storage of iron in the MPS may be of disadvantage for the affected individual for at least two reasons. First, iron storage in the MPS is the basis for a functional iron deficiency in the erythron. Second, while iron promotes the non-enzymatic generation of reactive oxygen species (ROS), it has negative effects on many other immune effector pathways. Macrophage iron overload, commonly resulting from chronic hemolysis and/or repetitive red blood cell (RBC) transfusions, impairs their effector functions which are promoted by TNF and interferon (IFN)-γ. These key cytokines are produced by cytotoxic T cells, T helper type 1 (T_H1) cells and natural killer (NK) cells. Specifically, an increased macrophage iron content results in impaired production of nitric oxide (NO). This is a consequence of iron inhibiting the abilities of HIF-1 and of NF-IL6 to *trans*-activate the *NOS2* (for NO synthase-2) gene (**Figure 3**) (48, 49). Similarly, iron impairs MHC class II expression in macrophages (50). The transcriptional mechanisms are unknown yet. Therefore, iron may inhibit most T_H cell responses. In a similar fashion, surplus iron can also be directly toxic to T cells and inhibit their proliferation or induce ferroptosis (Tymoszuk et al., 2020). The latter is a specific form of cell death that is dependent on iron, ROS and lipid peroxides. Ferroptosis is mediated by inactivation of the lipid repair enzyme glutathione peroxidase 4 (GPX4) (51). In macrophages, the induction of ferroptosis results in degradation of FT. This process is known as

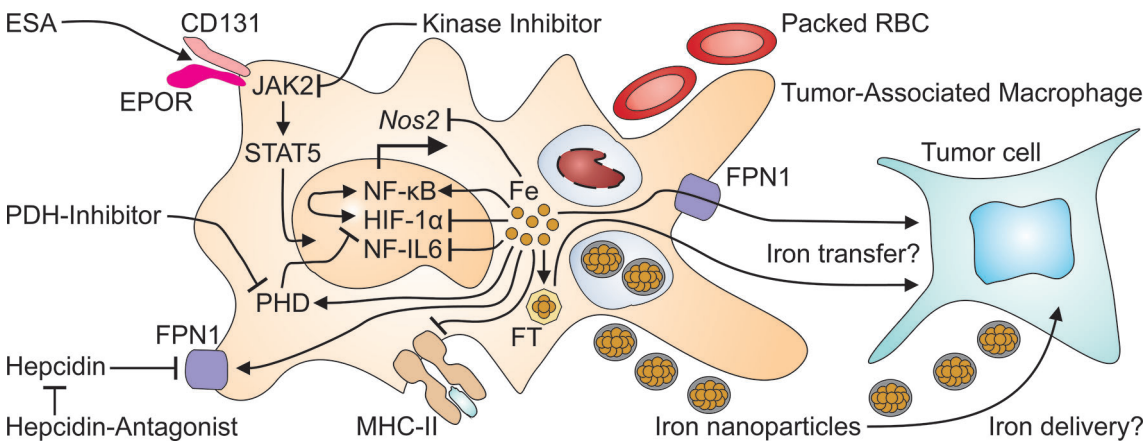

FIGURE 3 | Many therapeutics for cancer-induced anemia may exert off-target effects on tumor-associated macrophages. Erythropoiesis-stimulating agents (ESA) activate the EPOR/CD131 heterodimer to initiate signaling *via* Janus kinase (JAK)-2 and signal transducer and activator of transcription (STAT)-5. On the other hand, kinase inhibitors, prescribed to treat cancer or to reduce hepcidin production, may interfere with JAK2 signaling. Packed red blood cells (RBC) are taken up by macrophages including TAM at the end of their life span and increase intramacrophage iron levels. Intravenous iron preparations are nanoparticles which are taken up by TAM by phagocytosis and increase the cellular iron content. Surplus intracellular iron in turn, can inhibit the transcriptional expression of the *nitric oxide synthase* (*Nos*)*-2* and of *major histocompatibility* (*MHC*) *class II* unless it is exported through FPN1 or stored in ferritin (FT). Presumably, either form of iron may be transferred from TAM to adjacent tumor cells. This may also be relevant for the application of hepcidin antagonists which prevent the action of hepcidin on FPN1, thus restoring macrophage iron export. Intracellular iron also has profound effects on TAM themselves because iron stimulates the binding activity of nuclear factor (NF)-κB, while reducing the activation of NF-IL6 and of hypoxia-inducible factor (HIF)-1α. The latter effect is also relevant to treatment with prolyl hydroxylase domain (PHD) inhibitors which impair the degradation of HIF-1α, much like iron deficiency which stabilizes HIF-1α's active form.

ferritinophagy. Another effect of ferroptosis is the release of iron by nuclear receptor coactivator (NCOA)-4-mediated autophagy. As ferroptosis is an increasingly recognized mechanism of action of chemotherapeutics, the pathway provides another possible mechanism of interaction between iron metabolism, anti-tumor immunity and cancer biology (52–54).

In conclusion, iron has multiple, predominately negative effects on the immune response.

SOME MACROPHAGE POPULATIONS PROFESSIONALLY HANDLE IRON

Macrophages are dispersed throughout the human body to assume organ- and tissue-specific forms and functions. Several macrophage populations exist for example in the spleen. Of major importance for the maintenance of body iron homeostasis are SpiC- and FPN1-expressing iron-recycling red pulp macrophages (RPM) (55, 56). Under steady-state conditions, RBC have a normal life span of 120 days. Finally, they display altered molecular surface patterns and begin to lose 'don't eat me' signals. Moreover, RBC start to flip phosphatidylserine to their outer surface (57–59). RPM respond to these alterations and take up these RBC marked as 'aged' by a process known as erythrophagocytosis. In brief, engulfed RBC are degraded and their heme-iron is recycled to the systemic circulation by FPN1-mediated iron export (**Figure 3**). Alternatively, a novel study suggests that RBC may undergo physiologic hemolysis in the spleen and that their remnants are taken up by RPM for rapid turn-over (60). In situations when this capacity of RPM to take up damaged RBC (dRBC) and to recycle iron is overwhelmed, such as in massive hemolysis, Kupffer cells (KC) in the liver take over RBC uptake and degradation as a back-up mechanism. In addition, the liver-derived chemokines CCL2 and CCL5 stimulate the bone marrow to release new monocytes (61). These will then also participate in RBC uptake and iron recycling as a support to RPM and KC. Apart of these iron homeostatic functions, KC may play a role in tumor progression, too. *Per se* the liver is one of the organs most commonly affected by metastasis. Therefore, resident KC may be considered as TAM analogues for metastatic cells that have reached the liver. In this setting, iron-recycling by KC may facilitate the growth of liver metastasis. Similarly, in hepatocellular carcinoma, KC may be re-programmed to supply cancer cells with iron, resulting in adverse outcome (62, 63).

In summary, KC are a paradigm for macrophage populations that recycle iron and deliver this nutrient to cancer cells.

TAM ARE MACROPHAGES IN THE TME

TAM are located in the TME and thus have a strategic position in immunity against malignant cells. However, tumor cells enter a cross-talk with TAM by producing soluble mediators and metabolites with which they can manipulate anti-tumor immune responses. Iron is a decisive factor in this interaction: The immune-mediated uptake of Hb by and the sequestration of iron in TAM both aim at withholding this nutrient from malignant cells to counteract disease progression (64, 65). *Vice versa*, tumor cells seek at undermining these mechanisms and

may promote the release of iron from TAM (66). FPN1-mediated export of ionic iron and the secretion of iron-laden FT or lipocalin-2 may be the most relevant pathways by which re-programmed TAM supply neoplastic cells with iron (67, 68). In addition, malignant cells themselves produce lipocalin-2 and its receptor for the uptake of siderophore-bound iron (SBI) which supports cancer cell growth (69, 70). Moreover, tumor cells can express ionic iron importers such as divalent metal transporter (DMT)-1 and solute carrier family 39 member 14 (SLC39A14; also known as ZIP14) as well as receptors for FT and transferrin-bound iron (TBI) (71–73).

By producing the vascular endothelial growth factor (VEGF), TAM may also be implicated in tumor neovascularization which is triggered by tissue hypoxia, influenced by cardiovascular function and CIA, respectively (74). Hypoxia is sensed by oxygen sensitive prolyl hydroxylases (PHD) which stabilize HIF, a heterodimeric transcription factor composed of α and ß subunits. Under normoxic conditions, PHD continuously hydroxylate HIF-1α at two specific proline residues. This enzymatic process is modified by concentrations of ferrous iron and 2-oxoglutarate and tags HIF-1α for proteosomal degradation following binding by the von Hippel Lindau (VHL) E3 ubiquitin ligase complex. During cellular hypoxia, which commonly occurs in the TME, HIF-1α is stabilized thus enabling *trans*-activation of HIF target genes such as VEGF. VEGF also constitutes a relevant target for monoclonal antibodies, which are used as add-ons in combination with chemotherapeutics to counteract disease progression. The cross-talk between TAM and cancer cells is bidirectional, though. On the one hand, cells release lactate in breast cancer in order to stimulate TAM to transfer a HIF-1α stabilizing RNA into malignant cells. This impairs the action of chemotherapy (75).

On the other hand, PDH inhibitors, which are of potential use to treat CIA, may exert effects on TAM: PDH3 and HIF-1α play central roles in the activation of macrophages and in their interaction with T cells (76, 77). In addition, the central transcription factors HIF-1α and NF-κB are linked and put the PDH-HIF pathway right at the interface between the sensing of reduced oxygen and iron levels and immune effector functions (78, 79). Despite the key role of HIF-1α in TAM however (**Figure 3**), it is hard to predict whether these immunologic effects or potential pharmacologic intervention will favor or impair the progression of the underlying malignancy. In line, we currently lack a comprehensive understanding, how compounds for the treatment of CIA may affect the pleiotropic functions of TAM. In addition, many studies on the cross-regulation of iron metabolism and macrophage function address the functions of inflammatory cytokines and infectious agents but still need to be carefully translated into cancer models and, subsequently, cancer patients.

TAM AS POTENTIAL TARGETS OF CIA-DIRECTED THERAPIES

Macrophages form the central executive part of the MPS and are equipped with a plethora of pattern recognition and scavenger receptors. In order to fulfill their immune and homeostatic functions in the human body, macrophages need to respond to endocrine, paracrine and metabolic signals. The corresponding receptors present on macrophages include – but are not limited to – bone morphogenic protein receptor (BMPR)-I and -RII, hemojuvelin (HJV), transferrin receptor (TFR)-2, FPN1, IL6R, TNFRI, EPOR and CD (for cluster of differentiation)-131. Therefore, many therapeutic options for CIA are predicted to affect macrophage functions in general and TAM functions, specifically (**Figure 3**).

INTRAVENOUS IRON PREPARATIONS TARGET MYELOID CELLS

Intravenous iron preparations can be administered to cancer patients for two indications. First, patients with CIA and relevant absolute iron deficiency may benefit from intravenous iron supplementation as long as the underlying malignancy is under therapeutic control as well. Second, superparamagnetic iron oxide nanoparticles (SPION) are used for diagnostic purposes in imaging studies. Both classes of intravenous iron preparations are nanoparticles which primarily target classical monocytes and macrophages. Concretely, classical monocytes and macrophages take up iron-containing nanoparticles *via* their set of scavenger receptors and degrade them in phagolysosomes (80, 81). As a consequence, these cells accumulate their total and cytoplasmatic iron content before they transfer iron to the circulation (82). However, it is increasingly recognized that iron-containing nanoparticles exert immune-modulation on macrophages (83). For example, the clinically used intravenous iron preparations iron sucrose and sodium ferric gluconate impair the adhesion and phagocytosis of monocytes, while ferric carboxymaltose and iron isomaltoside lack these effects (84). However, also the latter two compounds affect the MPS. This is also evident from the fact that monocytes and macrophages take up iron carboxymaltose and deliver these iron-nanoparticles to tumors. There, they inhibit monocyte chemoattractant protein (MCP)-1 and to a lesser degree NO production (85). In contrast, the uptake of iron sucrose by circulating monocytes results in activation of NF-κB and in enhanced production of TNF, IL-6 and IL-8 (86, 87).

The uptake of iron nanoparticles by monocytes and macrophages is also relevant for the use of SPION as contrast enhancers for magnetic resonance imaging. SPION coated with dextran are phagocytozed by human monocytes, in which they activate mitogen-activated protein (MAP) kinases and stimulate TNF and IL-1ß production (88). Other SPION preparations however, counteract toll-like receptor (TLR)-4 signaling and NF-κB activation, thus reducing TNF, IL-1ß and IL-6 production (89). Further results obtained with monocyte-derived dendritic cells suggest that SPION impair antigen processing and T_H cell stimulation which may have important implications for anti-tumor immunity (90). Furthermore, efforts have been made to selectively label distinct macrophage phenotypes with SPION, which may be relevant for cancer patients because TAM represent distinct macrophage phenotypes (91).

In the face of these heterogenous data, it is currently difficult or even impossible to predict the clinical implications of the use of iron nanoparticles from their specific effects on TAM function. Therefore, more preclinical studies and following clinical data are desirable to better understand the effects of intravenous iron preparations on CIA and cancer.

BLOCKING IL-6 OR BMP-6 TO INHIBIT HEPCIDIN IN CIA

The inhibition of hepcidin is an attractive treatment strategy for CIA patients, too. Hepcidin antagonism can be achieved *via* multiple approaches such as inhibitors of hepcidin expression, HJV inhibitors, hepcidin-binding oligoribonucleotides, neutralizing antibodies to hepcidin or antibodies which block its interaction with FPN1 (92, 93).

Tocilizumab, a monoclonal antibody approved for several indications such as rheumatoid arthritis, giant cell vasculitis or cytokine release syndrome, targets the IL-6R and thus one of the major pathways to induce the transcription of the *HAMP* (for hepcidin-antimicrobial peptide) gene (37, 94, 95). Therefore, tocolizumab ameliorates disease activity and lowers circulating hepcidin levels in rheumatologic diseases (96, 97). The latter effect improves ACD, presumably by a direct effect on hepatocytes (98). Nevertheless, one might speculate that blockade of IL-6R may also modulated the function of macrophages including TAM (99). For example, in macrophage activation syndromes, tocilizumab results in lower serum FT levels (100). The ligand IL-6 is also involved in the cross-talk between TAM and tumor cells and promotes the survival of tumor cells in hypoxic conditions and the differentiation of cancer stem cells (101–104). Whether or not this is relevant for the clinical application of tocilizumab in cancer patients remains to be addressed in further clinical trials. However, a plethora of studies has implicated chronic inflammation in general and IL-6, specifically, in tumor initiation and progression (105, 106). Therefore, IL-6R blockade may impair the progression and metastasis of some forms of cancer (107). Yet, these data do not exclude the possibility that tocilizumab facilitates malignant transformation in other malignant diseases (108). Thus, only clinical trials in cancer patients will give relevant answers as to whether or not tocilizumab is a safe and efficient therapy for CIA.

Other potential targets for the treatment of CIA are bone morphogenetic proteins (BMP) and their receptors. As members of the TGF-ß superfamily of cytokines, BMP-2 and BMP-6, are specifically involved in the maintenance of iron homeostasis, yet also immune regulation and cancer progression (41, 109). BMP-6 is secreted by endothelial cells in the liver to inform adjacent hepatocytes about elevated serum iron levels and replenished iron stores (110). Accordingly, BMP-6 is a major inducer of *HAMP* transcription and a potential mediator of CIA (111–113). Given the key role of BMP-6 for the induction of hepcidin, it is not surprising that neutralizing antibodies have been used to block hepcidin induction in preclinical and clinical models of ACD (114–116). As many cell types including normal and neoplastic epithelial cells as well as macrophages possess BMPR-I and -II, BMP-6 can affect both tumor cells and TAM.

Several studies have implicated BMP-6 in the linkage of immune regulation and cancer progression. In a mouse model of malignant melanoma for example, the absence of BMP-6 resulted in a substantial delay in tumor onset and progression by a mechanism depending on mast cells (117). In non-small cell lung cancer however, reduced BMP-6 expression was associated with reduced overall survival and BMP-6 inhibited the proliferation of lung cancer cells (118). Similarly, BMP-6 inhibited the growth of breast cancer cells induced by estrogens (119). A high expression of BMP-6 was associated with higher immune cell infiltration and better survival in estrogen receptor-positive breast cancer in a cohort study (120). In prostate cancer, BMP-6 produced by neoplastic cells acts on adjacent macrophages and activates NF-κB and SMAD1 signaling to increase IL-1α and IL-6 secretion. IL-1α, in turn, stimulates endothelial cells and promotes tumor angiogenesis (121). In parallel, IL-6 acts on BMP-6 secreting malignant cells and enhances the expression of the androgen receptor, a major determinant of tumor growth and treatment response in prostate cancer (122). In renal cell carcinoma (RCC), BMP-6 mediates a crosstalk between tumor cells and TAM, too. BMP-6 instructs TAM to assume an anti-inflammatory phenotype with increased IL-10 production. The expression of BMP-6 in RCC cell lines compared to that in a nonmalignant renal cell line correlated with RCC cell line proliferation Increased IL-10 levels predicted poor prognosis of RCC in human subjects (123, 124).

Given the pleiotropic effects of BMP-6 in the crosstalk between immune and neoplastic cells in the TME, it will be important to assess the effects of BMP-6 neutralizing antibodies on the clinical course of the underlying malignancies. In light of the available data, it is tempting to speculate that the gene signatures in biopsies from primary lesions and the role played by BMP-6 in a given tumor entity may enable us to predict whether BMP-6 blockade exerts stimulatory or inhibitory effects on tumor growth in an individual patient.

THE HEPCIDIN-FPN1 AXIS ITSELF IS A PHARMACOLOGIC TARGET

Located downstream of IL-6 and BMP-6, the hepcidin-FPN1 axis itself is an attractive target for the therapy of CIA and other forms of ACD (125, 126). However, both FPN1 and its ligand hepcidin are potential regulators of tumor growth and the immune response directed against it. Loss-of-function mutations in *SLC40A1*, the gene encoding for FPN1, are in discussion to produce a molecule that does not traffic appropriately to the cell surface or that has limited ability to export iron (127–129). Excess accumulation of iron in macrophages is the result with accompanying high serum FT levels. In gain-of-function mutations of *SLC40A1*, the binding site of hepcidin is altered, resulting in a hepcidin-resistant protein and in iron overload (130–132). In TAM in RCC, FPN1 expression is elevated, especially in high grade lesions.

Importantly, high FPN1 levels predict poor overall survival because iron export by TAM supports the proliferation and migration of RCC cells (133). Similarly, in breast cancer biopsies taken from the primary lesion and axillary lymph node metastases, TAM exhibit high FPN1 expression and cancer cells display high TFR1 levels, suggesting that TAM supply tumor cells with iron (134). Importantly, the influence that FPN1 levels on TAM exert on disease outcome, extend to other relevant malignancies including hepatocellular carcinoma (135). This may be a general observation for cancers with predominant infiltration by monocytes and macrophages. However, this effect may also partly be attributable to the role of FPN1 on macrophages in the control of their cellular iron status and immune response. Specifically, macrophages lacking FPN1 are impaired in their function and secrete higher amounts of TNF and IL-6, possibly because intracellular iron can stimulate the translation of these cytokines (136–138). On the other hand, over-expression of FPN1 in macrophages enhances their NO output because low intramacrophage iron levels promote the transcription of the *NOS2* gene (139, 140). FPN1 inhibitors are being evaluated in clinical trials for the treatment of thalassemia (141). However, to date, no data exist on the use of allosteric FPN1 modulators for ACD or CIA.

In contrast, humanized monoclonal antibodies which display a high affinity toward hepcidin and lead to its premature degradation have been developed. One of these, LY2787106, was shown to be tolerated well during its phase one clinical trial, demonstrating a significant increase in serum iron levels (142). Short hairpin RNA (shRNA) which target hepcidin were demonstrated to cause a reduction in hepcidin production and alleviate anemia when used in conjunction with erythropoiesis stimulating agents (ESA) because they may inhibit hepcidin more robustly than anti-hepcidin antibodies (143). Similarly, aptamers, an emerging class of synthetic, structured oligonucleotide therapeutics, can inhibit *HAMP* expression with high affinity and specificity, thus increasing iron availability for erythropoiesis in a preclinical ACD model (144, 145).

In conclusion, several approaches targeting the hepcidin-FPN1 axis may be effective in CIA because they have the potential to improve anemia in preclinical models of ACD (93, 146, 147). Not only does hepcidin target macrophages to limit iron recycling, it is also produced by macrophages themselves, possibly to autoregulate their iron homeostasis (148–150). Therefore, it is feasible to assume that hepcidin-targeting therapies will affect the immune functions of macrophage populations including TAM, raising safety concerns.

THE HEPCIDIN-FPN1 INTERACTION IN MALIGNANCIES

The functional importance of the hepcidin-FPN1 interaction is not limited to TAM, though. In cancer cells, up-regulation of iron uptake pathways such as DMT1 or TFR1 and down-regulation of FPN1 keep cellular iron levels high for metabolism and proliferation (151–153). Also, low FPN1 expression in malignant cells has been linked to the proliferation of malignant myeloma (154, 155). It therefore comes as a surprise that low FPN1 levels are associated with improved prognosis in acute myeloid leukemia (156). However, this observation has been linked to increased sensitivity to chemotherapy, and it is currently being investigated whether iron-induced cytotoxicity or ferroptosis are contributing mechanisms (157).

As for solid tumors, the most conclusive data are available for breast cancer (134). In this tumor entity, several mechanisms cooperate to reduce FPN1 expression on tumor cells including epigenetic modifications in the *FPN1* promoter region and down-regulation of FPN1 protein by hepcidin which is secreted by cancer cells and adjacent fibroblasts (158–160). Of relevance, expression levels of hepcidin and FPN1 govern disease outcome in breast cancer and decreased *SCL40A1* gene expression is an independent predictor of reduced metastasis-free and disease-specific survival (161).

In conclusion, there is overwhelming evidence that the hepcidin-FPN1 axis regulates both, the function of TAM and the growth of cancer cells. While antagonizing the hepcidin-FPN1 interaction may ameliorate CIA because one of the key molecular mechanisms of functional iron deficiency is undermined, there may be relevant further consequences for the underlying disease (**Figure 4**). Blocking the hepcidin-FPN1 interaction on TAM will reduce their iron content and may promote NO production, MHC class II expression and alter cytokine production, consecutively increasing their anti-tumor activities. In contrast, FPN1 mediated iron export from TAM may increase the availability of iron not only for EP, as intended, but also for cancer cells. On the other hand, the neutralization of hepcidin's effects on FPN1 present on cancer cells may inhibit their proliferation yet impair ROS-mediated effects of chemotherapeutics. Therefore, any compound that targets the hepcidin-FPN1 axis exerts both systemic and local effects and needs to be thoroughly tested in preclinical cancer models and clinical cancer trials to carefully balance the benefit-risk ratio of such products before approval for clinical use.

DIAGNOSIS OF CIA

Individual Approach for Cancer Patients

CIA is a subtype of ACD and may be viewed as spectrum by itself that ranges from mild and asymptomatic forms in individuals with well-controlled residual malignancy to severe forms that, if left uncorrected, will limit patients' life expectancy just as does the underlying neoplasia (162). Anemia in cancer patients can be classified as CIA when the anemia has hyporegenerative features as indicated by a reduced reticulocyte count or reticulocyte production index (163). Often, the underlying cancer is advanced or not in remission or patients are under chemotherapy (164). In many cases of CIA however, substantial hemolysis, evident from haptoglobin consumption, contributes to anemia as do other contributing factors such as

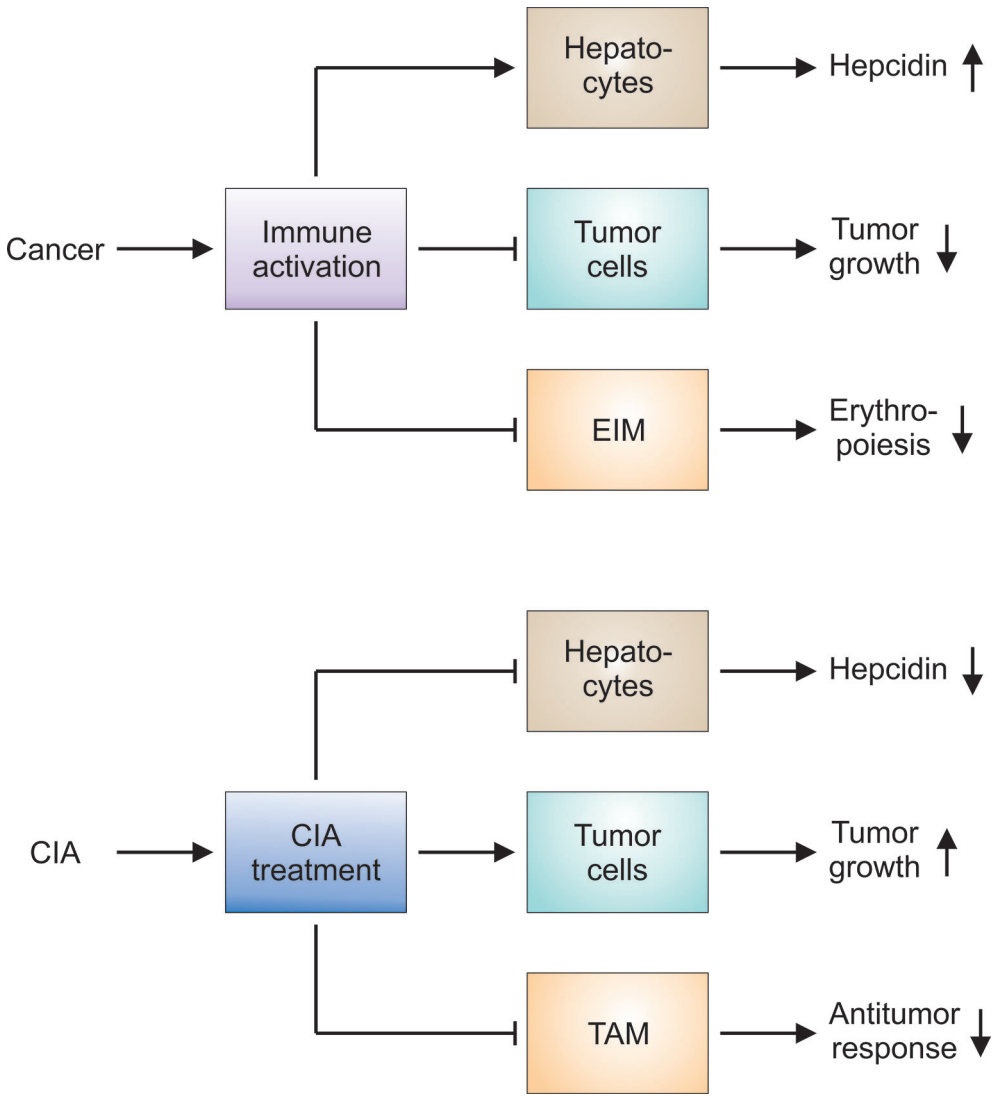

FIGURE 4 | Hypotheses on the pathophysiology of CIA and the effects of CIA treatment on the underlying tumor. Upper panel: Cancer results in prolonged immune activation, especially when the disease is advanced or progressive. The immune response directed against malignant cells impairs tumor growth. At the same time, proinflammatory mediators stimulate hepatocytes to produce hepcidin: Hepcidin in turn counteracts iron export by erythroid island macrophages (EIM). Other immune mechanisms exert similar effects on EIM and aggravate the functional iron deficiency observed in cancer patients. Thus, several mechanisms contribute to the development of cancer-induced anemia (CIA). Lower panel: CIA and the underlying functional iron deficiency can be considered maladaptations to prolonged immune activation. CIA treatment is intended to counteract these immune-mediated pathways and to promote erythropoiesis. Many of these therapeutic approaches reduce hepcidin production or block its negative action on ferroportin-mediated iron export. However, CIA treatment may exert unintended side effects on tumor cells and on tumor-associated macrophages (TAM). The latter may be attributable to the fact that EIM cannot be selectively targeted and other macrophage populations such as TAM are inhibited by CIA treatment, too. Therefore, all therapeutic interventions for CIA have to be thoroughly tested in preclinical models and clinical trials to check for unintended effects on tumor growth and the antitumor response.

EPO and vitamin deficiencies. Given this multifactorial etiology of CIA, cancer patients often require an individual approach in the diagnosis and treatment.

CIA and Laboratory Markers

CIA is often normocytic or microcytic but occasionally macrocytic. Because of the fact that serum FT is elevated as part of the APR, a higher cut-off of 100 rather than 30 ng/ml for the assessment of body iron stores in cancer patients may be necessary (165). In cancer patients, serum FT < 100 ng/ml and TSAT < 20% suggest absolute iron deficiency but the Hb content of reticulocytes may be a more reliable parameter (166). Similarly, the soluble TFR is less affected by inflammation and may thus be a reliable indicator of absolute iron deficiency, even in the setting of cancer (167). This may be attributable to the idea that the Hb content of reticulocytes is a marker of the functional ID whereas the FT index (soluble TFR/log FT) primarily reflects the iron availability for erythropoiesis (168, 169). Therefore, the

FT index facilitates the differential diagnosis between iron deficiency and ACD (170). An sTFR-FT index <1 suggests ACD, whereas, an sTFR-FT index >2 suggests ACD accompanied by absolute iron deficiency anemia. The use of the FT index for daily clinical routine is limited by the lack of internationally standardized assays, though (171).

BMP-6 and Hepcidin Elevation in CIA

BMP-6 and hepcidin levels are elevated in CIA which may aid in the diagnosis (112, 113). Yet it remains unclear whether serum concentrations of these mediators predict the response to currently available treatments such as ESA and to therapies specifically targeting them (172). Also, hepcidin levels may be regulated by cancer-induced inflammation through IL-6 dependent and independent pathways (167, 172, 173).

In conclusion, both patient's history as well as classical and novel laboratory parameters enable the accurate classification of CIA. Given the increasing number of parameters available, we expect that improved algorithms for the differential diagnosis of CIA and the prediction of treatment responses will become available in the near future.

TREATMENT INDICATIONS AND OPTIONS FOR CIA

Aim of CIA Treatment

All inner organs depend on a sufficient supply with oxygen from its iron-containing carrier Hb. CIA thus impairs organ functions and activities of daily living in patients with neoplastic disorders. In addition, CIA may negatively affect the survival of cancer patients (174, 175). Therefore, a correction of the reduced Hb levels is warranted although the desired target levels remain incompletely studied and thus still under debate.

Different Treatment Options in CIA

The therapeutic options to correct the CIA have increased in recent years (**Table 1**). Apart of RBC transfusions and intravenous iron preparations, ESA have long been used to treat CIA. Recently, hepcidin antagonists such as monoclonal antibodies, short interfering RNA (siRNA), shRNA, aptamers, TFR2 inhibitors as well as pharmacologic inhibitors of HIF prolyl hydroxylases, e.g. vadadustat, roxadustat and daproustat, have emerged as novel therapeutic concepts for CIA. RBC transfusions and ESA are described more in detail as cornerstones of CIA treatment in the further sections.

RED BLOOD CELL TRANSFUSIONS

RBC transfusions have long been used to correct anemia, including CIA. The direct replacement of RBC and rise in Hb levels promptly ameliorates oxygen supply to vital organs including the central nervous system and myocardium and thus promotes quality of life and exercise capacity. Yet, this strategy can also worsen the underlying malignancy because oxygen delivery to neoplastic cells will increase, too. Furthermore, RBC transfusions may also modulate the immune response in recipients because packed RBC contain up to 0.8% of hemolyzed cells (176). Therefore, during transfusion, substantial amounts of free Hb, heme and iron as well as microvesicles and membrane fragments can enter the circulation. To avoid tissue damage and inflammation, strategically located macrophage populations such as RPM in the spleen and KC in the liver will neutralize these compounds by CD163, CD91 and other scavenger receptors. CD163 recognizes both free and haptoglobin-bound Hb and eliminates it by receptor-mediated endocytosis. CD91, on the other hand, binds heme-hemopexin complexes. Subsequently, intracellular heme is degraded by heme oxygenase-1 (HO1) and detoxified to bilirubin. When HO1's enzymatic capacity is overwhelmed, and heme starts to accumulate within cells, free heme activates the NLRP3 inflammasome (177). Similarly, in the extracellular space, macrophages sense free heme as DAMP and initiate the APR (178). Therefore, by capturing free Hb and free heme, the CD163 and CD91 pathways protect from pro-oxidative tissue damage at the systemic level. In the TME, however, their functions may be different because CD163 is a marker of TAM and may enhance the delivery of iron to the tumor (179).

In clinical practice, the major advantage of RBC transfusions is the rapid improvement of Hb levels, which must be balanced against the risks for immune-mediated adverse reactions and transmissible infections.

TABLE 1 | Selected approved and experimental treatment options for CIA.

Compound/group	Target/mechanism	Pharmacological/clinical effect
Intravenous iron preparations	Deliver iron-containing nanoparticles to macrophages	Correction of absolute iron deficiency
Tocilizumab	Blocks the IL-6 receptor	Suppresses IL-6 induced immune pathways including hepcidin production
LY2787106	Neutralizes circulating hepcidin	Blocks hepcidin and restores iron transfer to the circulation *via* FPN1
KY1070	Neutralizes circulating BMP-6	Reduces hepcidin transcription and restores iron transfer to the circulation *via* FPN1
Momelotinib	Off-target inhibition of BMPR kinase activin A receptor, type I	Reduces hepcidin transcription and restores iron transfer to the circulation *via* FPN1
TFR2 inhibitors	Inhibit TFR2 on erythroid cells	Improve EPO sensitivity
ESA	Stimulate the EPOR on erythroid progenitor cells	Restore erythropoiesis
HIF prolyl hydroxylase inhibitors	Stabilize HIF	Restore endogenous EPO production
RBC	Transfusion of allogenic RBC	Delivery of RBC as oxygen carriers

ERYTHROPOIESIS-STIMULATING AGENTS FOR CIA TREATMENT

EPO Functions and Signaling in CIA

In general, ESA are derivatives of the endogenous hormone EPO. EPO itself has a dual function in human biology: On the one hand, it is the key growth factor for EP in the bone marrow. There, EPO activates its homodimeric receptor to promote the differentiation of multipotent hematopoietic progenitors along the erythroid lineage and inhibit the apoptotic elimination of surplus cells (**Figure 2**) (180). On the other hand, EPO exerts functions of an anti-inflammatory and tissue-protective cytokine throughout the body. These latter functions are mediated by a distinct molecular form of its receptor, a heteroreceptor of EPOR and CD131 (181). This extraerythropoietic receptor is also expressed by immune cells including T cells and macrophages (**Figure 3**). EPO's effects on the immune system may be either pro- or anti-inflammatory dependent on the type of EPO-responsive cells, the context of the tissue microenvironment or the entity of underlying disease. In CIA, ESA raise Hb levels and reduce the frequency of RBC transfusion in patients.

Cell-, Organ- and Malignancy-Specificities of EPO

In the liver, EPO enhances the phagocytotic capacity of KC and the production of CCL2. This chemokine, in turn, promotes the recruitment of monocytes from the bone marrow to the liver, but it is also implicated in tumor metastasis (182–184). Also, in lung epithelial cells, EPOR expression is higher in malignant cells than in normal cell types (185). This suggests that unintended side effects of EPO are more likely to occur in CIA than in other forms of ACD. In malignant myeloma for instance, EPO stimulates IFN-γ production and counteracts disease progression. In bone marrow derived macrophages in contrast, EPO promotes the secretion of angiogenetic factors which may drive myeloma progression. This function may be especially relevant in the setting of multiple myeloma because in these patients, bone marrow macrophages overexpress EPOR (186). On the other hand, EPO may induce apoptosis in myeloma cells (187). This may be relevant for erythroid island macrophages, on which EPOR is highly expressed and may be important for their nursing function and for the delivery of iron to adjacent EP (188).

As EPO and ESA can act on both, TAM and cancer cells, these compounds may impact on the malignant disease underlying CIA. ESA in the presence of EPOR may promote angiogenesis, tumor growth, tumor cell survival, or resistance to treatment. However, it is impossible to predict the net effect of ESA therapy in a given tumor entity. In gastroesophageal cancer for example, ESA therapy initiated at a similar Hb cut-off of 11 g/dl, tended to have improved clinical outcome, implying that in this context, ESA is a valuable adjunct therapy (189). In breast cancer patients with CIA however, treatment with ESA may not affect overall survival yet increase the risk of venous thromboembolic events (190, 191).

Benefit/Risk Considerations of ESA in CIA

Previous meta-analysis have suggested that ESA may be efficient and safe for the treatment of CIA (192). However, more recent work has led to opposite conclusions, questioning the safety of ESA and raising concerns about the increased risk for thromboembolic events and deaths in CIA patients receiving these compounds: A systematic review and meta-analysis of 6,769 cancer patients in 35 clinical trials exhibited an increased risk of thromboembolic events with recombinant human erythropoietin compared with controls (relative risk (RR), 1.67; 95% confidence interval (CI), 1.35 to 2.06) (193). A meta-analysis of patient-level data from 53 controlled trials in cancer patients who received chemotherapy, radiation therapy, chemoradiotherapy, or no therapy with epoetin therapy (n=13,933) reported a consistently significantly increased risk of thromboembolic events (194). The absolute event rates ranged from 0 to 30.8% (pooled 5.8%) in the treatment arms and from 0 to 14.5% (pooled 3.2%) in the control arms. Besides, other off-target effects of ESA might contribute to mechanisms of tumor regulation, such as cell activation and neovascularization. Based on the results on thromboembolic safety and mortality, ESA are not recommended for the treatment of anemia that is unrelated to chemotherapy in patients with malignancy. Potential exceptions of use of ESA are in patients with lower risk myelodysplastic syndromes to avoid RBC transfusions and the use in patients with concomitant renal failure. The main advantage of ESA is the decreased need for RBC transfusions.

To date, it remains unknown whether and how ESA affect tumor growth or their control by the immune system. From a clinical standpoint, in the absence of conclusive evidence, a personalized approach is mandatory in order to balance the potential benefits of ESA treatment, taking into account each patient's individual circumstances and preferences, against the increased risk of thromboembolic events and death.

In summary, we need further preclinical and clinical research to characterize the cellular mechanisms and molecular pathways by which ESA affect thrombogenesis and tumor growth in patients with CIA (195).

CONCLUSIONS

As for other forms of ACD, treating the underlying disease is the preferred therapeutic approach to patients with CIA. In patients with progressive disease however, it may be required to treat CIA *per se* in order to positively influence the cancer patient's quality of life, physical performance and life expectancy.

Nowadays, physicians have an increasing armamentarium at hand to treat CIA. In patients, in whom the underlying malignancy is in full remission and in regular follow-up, treatment of CIA may be relatively safe and the erythropoietic bone marrow is likely to benefit from ESA, iron supplementation or hepcidin antagonism. In progressive cancers however, there may be an increased risk that CIA-directed interventions in fact provide malignant cells with growth promoting nutrients and/or signals. Therefore, a combination therapy that stimulates erythropoiesis with ESA on the one hand and provides EP with iron by medications targeting the IL-6R, BMP-6, hepcidin or FPN1 may be the preferred approach because in combination,

these compounds may preferentially supply EP with signals and nutrients for proliferation.

In the future, we expect to have improved mathematical models and IT supported algorithms to diagnose and subclassify CIA, select multimodal therapies and predict treatment responses. We need to conduct appropriately powered randomized controlled trials in order to evaluate the benefits and risks of therapeutic interventions for CIA. Further objectives are trials with end points that focus on overall survival, disease free survival, progression free survival, exercise capacity, infection rate and quality of life. Before complementing these clinical studies, ongoing work in preclinical cancer models aims to gain further mechanistic insight in the effects that CIA-directed therapies exert both locally, on TAM and other tumor infiltrating leukocyte populations, as well as systemically (**Figure 4**).

Therefore, both preclinical and clinical investigations are inevitable to ameliorate – or TAM-e – CIA with acceptable risks of medicine.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SW has written the manuscript. MN has written the manuscript and drawn the figures. All authors contributed to the article and approved the submitted version.

FUNDING

Work in MN's laboratory is supported by grants from the Austrian Research Fund (FWF stand-alone project P 33062) and the Tyrolean Research Fund (TWF).

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Conflict of Interest: 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.

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frontiers in Immunology

ORIGINAL RESEARCH
published: 01 April 2021
doi: 10.3389/fimmu.2021.612144

Hepcidin Upregulation in Lung Cancer: A Potential Therapeutic Target Associated With Immune Infiltration

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Lung cancer has the highest death rate among cancers globally. Hepcidin is a fascinating regulator of iron metabolism; however, the prognostic value of hepcidin and its correlation with immune cell infiltration in lung cancer remain unclear. Here, we comprehensively clarified the prognostic value and potential function of hepcidin in lung cancer. Hepcidin expression was significantly increased in lung cancer. High hepcidin expression was associated with sex, age, metastasis, and pathological stage and significantly predicted an unfavorable prognosis in lung cancer patients. Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Set Enrichment Analysis (GSEA) results suggested that hepcidin is involved in the immune response. Furthermore, hepcidin expression was positively correlated with the infiltration levels of immune cells and the expression of diverse immune cell marker sets. Importantly, hepcidin may affect prognosis partially by regulating immune infiltration in lung cancer patients. Hepcidin may serve as a candidate prognostic biomarker for determining prognosis associated with immune infiltration in lung cancer.

Keywords: hepcidin, lung cancer, prognostic biomarker, immune infiltration, iron

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Edited by:
Stefania Recalcati, University of Milan, Italy

Reviewed by:
Michaela Semeraro, Assistance Publique Hopitaux De Paris, France
Hui Chen, University of Technology Sydney, Australia

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Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *30 September 2020*
Accepted: *17 March 2021*
Published: *01 April 2021*

Citation:
Fan Y, Liu B, Chen F, Song Z, Han B, Meng Y, Hou J, Cao P, Chang Y and Tan K (2021) Hepcidin Upregulation in Lung Cancer: A Potential Therapeutic Target Associated With Immune Infiltration. Front. Immunol. 12:612144. doi: 10.3389/fimmu.2021.612144

INTRODUCTION

Lung cancer is the leading cause of cancer-related death worldwide. Approximately 2.1 million new cases of lung cancer were diagnosed, and 1.8 million deaths were predicted in 2018 according to Global Cancer Statistics (1). Based on histological features, non-small-cell lung cancer (NSCLC) accounts for 80-85% of lung cancers and mainly includes lung squamous cell carcinoma (LUSC), lung adenocarcinoma (LUAD), and large-cell carcinoma (LCC) (2). Most NSCLC patients are diagnosed at late stages due to the absence of early typical clinical symptoms and effective diagnostic methods (3). Despite improvements in surgery and targeted therapeutic drugs, these current treatments still fail to yield desirable survival in lung cancer patients (2, 3). Therefore, there is a pressing need to explore novel prognostic predictors and therapeutic targets for lung cancer (4).

Iron is the most abundant trace element and plays critical roles in multiple cellular functions (5). In recent years, iron metabolism has attracted great attention as a mechanism in tumorigenesis (6–8).

Among the regulators of iron homeostasis, hepcidin is thought to play an important role (9, 10). Hepcidin is a small (25-amino acid) antimicrobial regulator that prevents iron absorption by enterocytes, iron release from macrophages, and iron transport across the placenta (9, 10). The role of hepcidin is shown to be related to its regulation of the iron transporter ferroportin (FPN1). FPN1 is an important mediator of iron metabolism and is the only known iron exporter in mammals that transfers intracellular iron to the extracellular environment (5). Hepcidin can bind to FPN1 on the cell surface and cause internalization and ubiquitin-dependent degradation of FPN1, which increases intracellular iron levels (11, 12). When hepcidin expression is chronically increased, persistent hypoferremia can result in the development of iron-restricted anemia (13). In contrast, chronic hepcidin deficiency leads to excessive iron absorption, increased levels of nontransferrin-bound iron in circulation, and the development of hyperferremia-related diseases, such as hemochromatosis (13). Consistently, transgenic mice overexpressing hepcidin exhibit iron-deficient anemia, whereas hepcidin-deficient mice show iron overload in many organs (14, 15). Because of its critical role in mediating iron homeostasis and the pathogenesis of iron disorders, hepcidin has emerged as a promising drug target.

Hepcidin is a pivotal peptide hormone that exhibits bactericidal and fungicidal properties *in vitro* (16). It is prominently produced in the liver, released into plasma and excreted in urine (17). The expression of hepcidin is mainly regulated by iron excess, hypoxia, and inflammatory stimuli (16–18). Hepcidin synthesis is significantly induced by infection and inflammation. The upregulation of hepcidin by inflammation is regulated, at least in part, by the inflammatory cytokine interleukin-6 (IL-6), a major mediator of the acute phase response in hepatocytes (19, 20). IL-6 treatment promoted the expression of hepcidin in isolated hepatocytes and hepatoma cell lines through the Jak/signal transducer and activator of transcription (STAT) pathway (19, 20). Hepcidin expression did not increase in mice lacking IL-6 when treated with LPS. Administration of IL-6 to mice and human volunteers increased hepcidin production and led to hypoferremia (21). Importantly, bone morphogenic protein (BMP)/sma and mothers against the decapentaplegic homologue (SMAD) pathway contribute to the maximal induction of hepcidin by inflammation (19, 20). Moreover, IL-1 also upregulated hepcidin mRNA expression in mouse primary hepatocytes from both wild-type and IL-6 knockout mice, indicating that IL-1 may play an IL-6-independent role in the upregulation of hepcidin by inflammation (22). Therefore, cross talk may exist between different pathways regulating hepcidin expression. Because inflammation is often accompanied by tumorigenesis and hepcidin is closely related to inflammation, the relationship between hepcidin, inflammation and tumors remains to be further explored.

A growing body of studies has revealed that dysregulation of iron homeostasis is one of the metabolic hallmarks of cancer cells, illustrating that iron is required for tumor development, progression and metastasis (6–8). Consistent with this notion, previous studies have demonstrated that hepcidin expression is upregulated in several types of cancer, including breast cancer, renal cell carcinoma (RCC), pancreatic cancer, prostate cancer, and colorectal cancer (23). In breast cancer patients, hepcidin expression in both serum and cancer tissues is significantly upregulated compared with that in normal individuals (24, 25). Hepcidin exhibits diagnostic value in both breast cancer and breast cancer with bone metastasis (26). Furthermore, increased levels of hepcidin are also involved in the development of the malignant phenotype of breast cancer cells and resistance to doxorubicin (27). Hepcidin mRNA expression is higher in RCC patients with metastasis than in those without metastasis (28). High hepcidin expression is significantly correlated with poor survival in RCC patients (29). In pancreatic cancer, high expression of hepcidin is significantly associated with a poor prognosis in patients (30). Moreover, hepcidin expression is also associated with the pathological stage and vascular invasion of pancreatic cancer (30). The synthesis and secretion of hepcidin are also markedly increased in prostate cancer cells and tissues (31, 32). In addition, hepcidin expression is increased in colorectal cancer tissues compared to matched normal tissues and is related to advanced T stage (T3 and T4) (33). Therefore, hepcidin can serve as an independent risk factor and prognostic biomarker of different types of cancer (34–38). However, the role of hepcidin in lung cancer metastasis and its association with immune cell infiltration in lung cancer are less well understood.

Given the close relationship between iron homeostasis and tumorigenesis, limited evidence has illustrated the function and clinical significance of hepcidin in lung cancer pathogenesis and prognosis. The present study aims to integrate multiple bioinformatics approaches to investigate whether hepcidin is involved in lung cancer metastasis and immune infiltration and to explore its molecular regulation. We found that hepcidin expression was significantly upregulated in lung cancer tissues compared with nontumor tissues. Moreover, hepcidin expression was increased in tumor stages and correlated with axillary lymph node metastasis. High expression of hepcidin was negatively correlated with the prognosis of lung cancer patients. In addition, there was a significant relationship between the expression of hepcidin and the infiltration levels of B cells, CD4+ T cells, CD8+ T cells, macrophages, neutrophils, and dendritic cells in lung cancer. Importantly, hepcidin seemed to affect the prognosis of lung cancer patients partially through immune cell infiltration. These observations emphasize a noticeable role of hepcidin in carcinogenesis and indicate that hepcidin may play an important role in the regulation of immune cell infiltration in lung cancer.

MATERIALS AND METHODS

Oncomine

Oncomine (www.oncomine.org) is a gene chip-based database for facilitating data mining of the transcriptional expression of genes in various cancers. The mRNA level of hepcidin in lung cancer was

examined using Oncomine. The P-value was set as 0.05, the fold-change was set as 1.5, and the gene rank was set as all.

UALCAN

UALCAN (http://ualcan.path.uab.edu/) is a web-based tool that provides in-depth analyses of transcriptome data from The Cancer Genome Atlas (TCGA) and MET500 data. UALCAN was used to investigate hepcidin expression and the association between hepcidin and various clinicopathological parameters (sex, cancer stages, nodal metastasis status, age, race and TP53 mutation status) of lung cancer.

Gene Expression Profiling Interactive Analysis (GEPIA)

GEPIA (http://gepia.cancer-pku.cn/index.html) is a user-friendly web portal for gene expression analysis based on TCGA and GTEx data. In the current study, expression analysis of hepcidin was evaluated using TCGA-LUAD and TCGA-LUSC datasets. In the module "Expression DIY" of GEPIA, the expression of hepcidin between LUAD/LUSC and normal adjacent lung tissue samples was investigated with the option of matching TCGA normal and GTEx data and log2 (TPM+1) for log-scale. Additionally, the relationships between hepcidin and PD-1, PD-L1 and CTLA-4 were determined using Spearman's correlation coefficient in "correlation analysis".

cBioPortal

The cBioPortal for Cancer Genomics contains a large-scale cancer genomics dataset and has functions such as visualization, download, and analysis. We chose three lung cancer datasets with 2197 cases for further analysis by using cBioPortal. The genomic alteration types and alteration frequency of hepcidin in lung cancer were analyzed through the "OncoPrint" module and "Cancer Types Summary" module. The OS and disease-free survival (DFS) of hepcidin were analyzed through the "Comparison/Survival" module in cBioPortal.

Gene Ontology (GO) Term and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analysis and Gene Set Enrichment Analysis (GSEA)

GO and KEGG analyses were applied to explore the biological functions of hepcidin in lung cancer. GO analysis is a powerful bioinformatics tool to determine the biological processes (BPs), cellular components (CCs) and molecular functions (MFs) related to hepcidin. GSEA was used to investigate the potential mechanisms of hepcidin. GO, KEGG and GSEA were performed by the R package ClusterProfiler.

Tumor Immune Estimation Resource (TIMER)

TIMER (https://cistrome.shinyapps.io/timer/), an interactive web portal, could perform comprehensive analysis on the infiltration levels of different immune cells. In the present study, hepcidin expression in multiple types of cancer was evaluated through the "Diff Exp" module. The correlation of hepcidin and immune cell infiltration in LUAD and LUSC was analyzed in TIMER. The "Gene" module can investigate the relationship between hepcidin expression and immune cell infiltration levels (B cells, CD8+ T cells, CD4+ T cells, neutrophils, macrophages, and dendritic cells) using the TCGA database. TIMER was also applied to investigate the relationship between hepcidin expression and different gene marker sets of immune cells by using the "Correlation" module. The correlations of hepcidin expression with immune infiltration were evaluated by purity-correlated partial Spearman's correlation and statistical significance.

Immune Cell Infiltration With the CIBERSORT Algorithm

CIBERSORT (https://cibersort.stanford.edu/), an established computational resource, was applied to characterize the immune cell composition based on a validated leukocyte gene signature matrix containing 547 genes and 22 human immune cell subpopulations. Our current analysis gauged the proportions of tumor-infiltrating immune cells in lung cancer through CIBERSORT and examined the correlations between hepcidin expression and the immune cell subpopulation. A p-value <0.05 was set as the criterion to select lymphocytes possibly affected by hepcidin expression.

Kaplan-Meier Plotter Database Analysis

We used KM Plotter (http://kmplot.com), an online database that contains gene expression data and survival information of 3452 clinical lung cancer patients, to analyze the prognostic value of hepcidin in lung cancer. The patient samples were separated into two groups by median expression (high expression and low expression) to analyze the overall survival (OS), progression-free survival (PFS) and postprogression survival (PPS) with hazard ratios (HRs) with 95% confidence intervals (95% CIs) and log-rank p-values.

PrognoScan Database Analysis

The correlation between hepcidin expression and survival in lung cancer was also analyzed by the PrognoScan database (http://www.abren.net/PrognoScan/). The relationships between hepcidin expression and patient prognosis, such as OS and relapse-free survival (RFS), across a large collection of publicly available cancer microarray datasets can be investigated by using PrognoScan. To select the datasets to be included in this study, the screening parameters were set as follows: "Cancer Type" as lung cancer, "Subtype" as "adenocarcinoma" and "squamous cell carcinoma". HR with 95% CIs was calculated. The threshold was adjusted to a Cox P-value <0.05.

Analysis of Hepcidin-Interacting Genes and Proteins

The GeneMANIA database (http://www.genemania.org) was applied to construct the hepcidin interaction network. The STRING online database (https://string-db.org/) was applied to construct a protein-protein interaction (PPI) network of hepcidin.

Cell Culture, RNA Isolation and Real-Time PCR

The human lung epithelial cell line BEAS-2B and NSCLC cell lines HCC827 and A549 were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cells were incubated in an incubator with 5% CO^2 at 37°C. Real-time PCR was conducted to evaluate gene expression. Total RNA was extracted from fresh renal tissues or cells using a TRIzol-based method as previously described (39, 40). Real-time PCR was performed in triplicate using samples derived from three independent experiments. Primers for hepcidin (forward, 5'- CTGACCAGTGGCTCTGTTTTCC-3', reverse, 5'- AAGTGGGTGTCTCGCCTCCTTC-3') and S18 (forward, 5'-GTTCCGACCATAAACGATGCC-3', reverse, 5'-TGGTGGTGCCCTTCCGTCAAT-3') were used for qPCR.

Immunohistochemistry (IHC) Staining

This study was approved by the Institutional Research Ethics Committee of HanDan Central Hospital. Written informed consent was obtained from the participants. Ten formalin-fixed, paraffin-embedded lung cancer tissues and normal lung tissues were used for IHC staining. Briefly, 4-μm sections of tissues were mounted on glass microscope slides, deparaffinized in xylene, and then rehydrated in sequentially increasing dilutions of alcohol. Antigen retrieval was performed at a high temperature using a water bath. The sections were cooled and rinsed, and endogenous peroxidases were quenched using 3% hydrogen peroxide. Then, the sections were washed three times with PBS, incubated with calf serum to block nonspecific antigens for 10 min, incubated with anti-hepcidin polyclonal primary antibody (1:200, ab30760, Abcam, Cambridge, MA, USA) overnight at 4°C, washed with PBS three times, and then incubated with secondary antibody for 30-40 min at room temperature (RT). Dried sections were observed with an optical microscope. The IHC staining results were analyzed and scored by two pathologists who were blinded to the sources of the clinical samples. A semiquantitative integration method was used to analyze the intensity of staining.

Statistical Analysis

The results generated in Oncomine are displayed with P-values, fold changes, and ranks. The results of Kaplan-Meier plots, PrognoScan, and GEPIA are displayed with HR and P or Cox P-values from a log-rank test. The correlation of gene expression was evaluated by Spearman's correlation and statistical significance. The heat map of the correlations between hepcidin and iron metabolism-related genes was generated by the R software package pheatmap with Spearman's correlation. P-values < 0.05 were considered statistically significant.

RESULTS

Hepcidin Expression Is Increased in Lung Cancer Patients

The mRNA expression of hepcidin in human cancers was first analyzed using the Tumor Immune Estimation Resource (TIMER) online database. Higher expression of hepcidin was observed in breast invasive carcinoma (BRCA), colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal papillary cell carcinoma (KIRP), LUAD, LUSC and stomach adenocarcinoma (STAD) compared with the corresponding normal tissues (**Figure 1A**). Consistently, we also found that higher mRNA of hepcidin was expressed in LUAD and LUSC tissues than in normal lung tissues in the gene expression profiling interactive analysis (GEPIA) and UALCAN databases (**Figures 1B, C**). The expression of hepcidin mRNA was further examined using the Oncomine database (**Supplementary Figure 1**). We found that hepcidin expression was higher in LUAD, LUSC and LCC tissues from 5 different cohorts (**Supplementary Figure 1**). In addition, hepcidin expression in LUAD and LUSC samples and adjacent normal tissues was analyzed using data directly obtained from The Cancer Genome Atlas (TCGA). Hepcidin expression was significantly elevated in LUAD and LUSC tissues (**Figure 1D**). Furthermore, a marked increase in hepcidin expression in LUAD and LUSC was observed in 58 and 50 paired tumor samples compared with adjacent normal samples, respectively (**Figure 1E**). These findings illustrate that hepcidin expression is upregulated in lung cancer and indicate that hepcidin may play an important regulatory role in lung cancer progression.

The protein expression of hepcidin was further investigated in lung cancer by IHC staining, and we found that the hepcidin protein level was obviously increased in lung cancer tissues compared with normal lung tissues (**Figures 2A, B**). Moreover, we found that hepcidin mRNA expression was significantly upregulated in two NSCLC cell lines (A549 and HCC827) compared to that in a nonmalignant lung epithelial cell line (BEAS-2B) (**Figure 2C**).

Hepcidin Expression and Clinical Parameters of Lung Cancer Patients

By using the UALCAN online tool, we then investigated hepcidin expression among groups of patients according to different clinical parameters. According to sex, hepcidin expression was significantly upregulated in lung cancer samples from both males and females compared to the corresponding normal controls (**Figure 3A**). Regarding tumor stage, a significant increase in hepcidin expression was observed in LUAD patients in stages 1, 2, 3 and 4 and in LUSC patients in stages 1 and 3 (**Figure 3B**). Based on cancer stage, hepcidin expression was higher in patients with LUAD classified as N0, N1 or N2 and in patients with LUSC classified as N0 (**Figure 3C**). Upregulation of hepcidin expression was observed in both TP53-mutant and TP53 wild-type lung cancer patients compared to normal controls (**Supplementary Figure 2A**). In terms of age, the hepcidin level was significantly elevated in the lung cancer tissues of patients from different age groups (21-40 years, 41-60 years, 61-80 years and 81-100 years in LUAD; 61-80 years in LUSC) (**Supplementary Figure 2B**). In addition, hepcidin expression was dramatically increased in Caucasian lung cancer patients (**Supplementary Figure 2C**). These results suggest that there is a

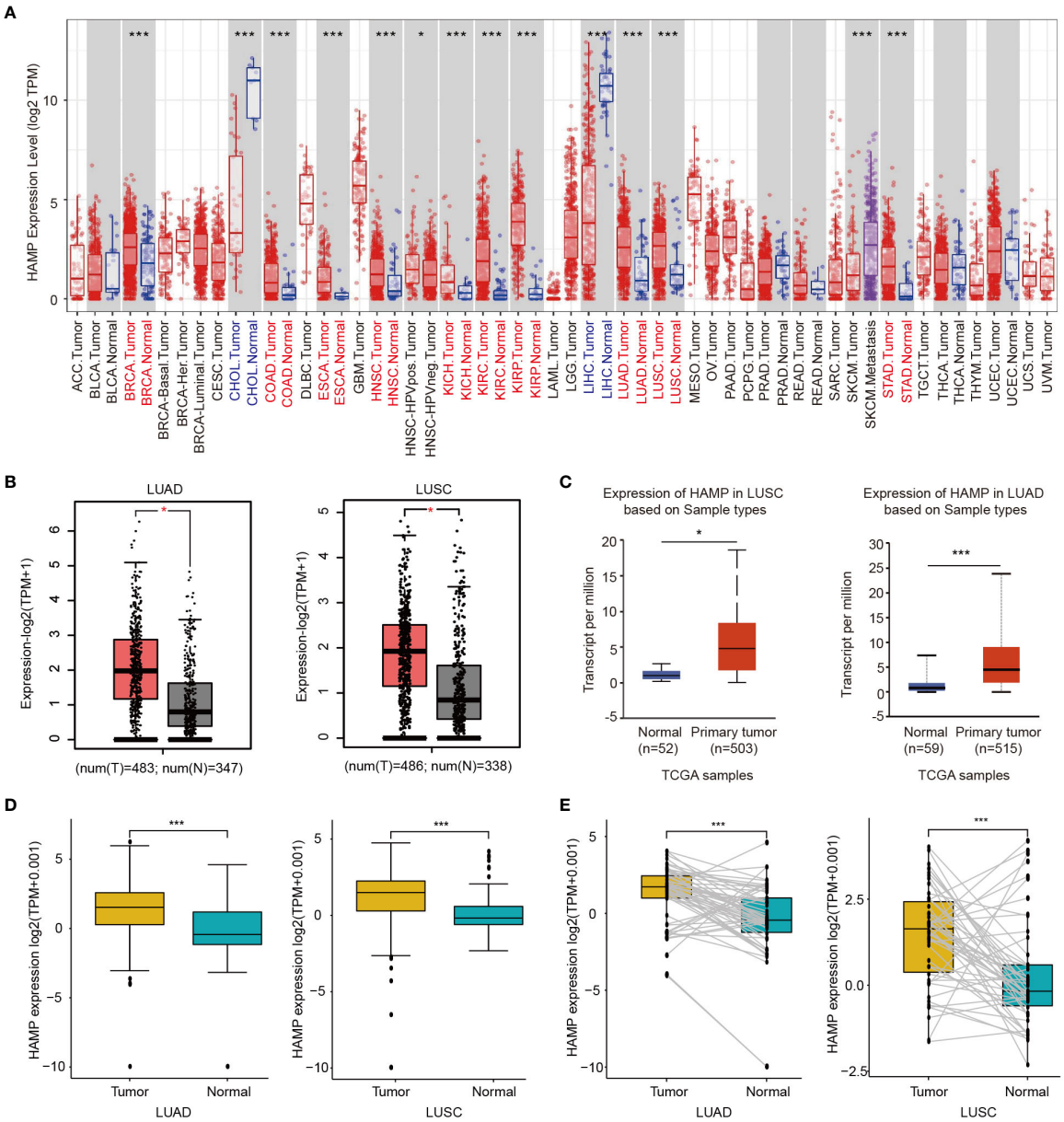

FIGURE 1 | Expression of hepcidin in lung cancer. **(A)** Hepcidin expression in different types of cancer was investigated with the TIMER database. **(B)** Increased or decreased expression of hepcidin in lung cancer compared to normal tissues in the GEPIA database. **(C)** Hepcidin expression in lung cancer was examined by using the UALCAN database. **(D)** Analysis of hepcidin expression in lung cancer and adjacent normal tissues in the TCGA database. **(E)** TCGA database and statistical analyses of hepcidin expression in 58 pairs of LUAD tissues and adjacent normal tissues and 50 pairs of LUSC tissues and adjacent normal tissues, respectively. *p < 0.05, ***p < 0.001.

close correlation between hepcidin expression and tumor progression and metastasis.

Increased Hepcidin Expression Correlates With Poor Prognosis in Lung Cancer Patients

Since the hepcidin expression level is intimately related to lung cancer progression and metastasis, we then examined the prognostic value of the hepcidin gene. Lung cancer patients with higher expression of the hepcidin gene exhibited poor overall survival (OS) and progression-free survival (PFS) but not postprogression survival (PPS) according to the Kaplan-Meier plotter database (**Figure 4A**). Moreover, the PrognoScan database demonstrated that elevated expression of hepcidin was significantly associated with poor OS and RFS in the GSE31210 and GSE4573 cohorts (**Figure 4B**). These results indicate that hepcidin is significantly associated with the prognosis of lung cancer patients.

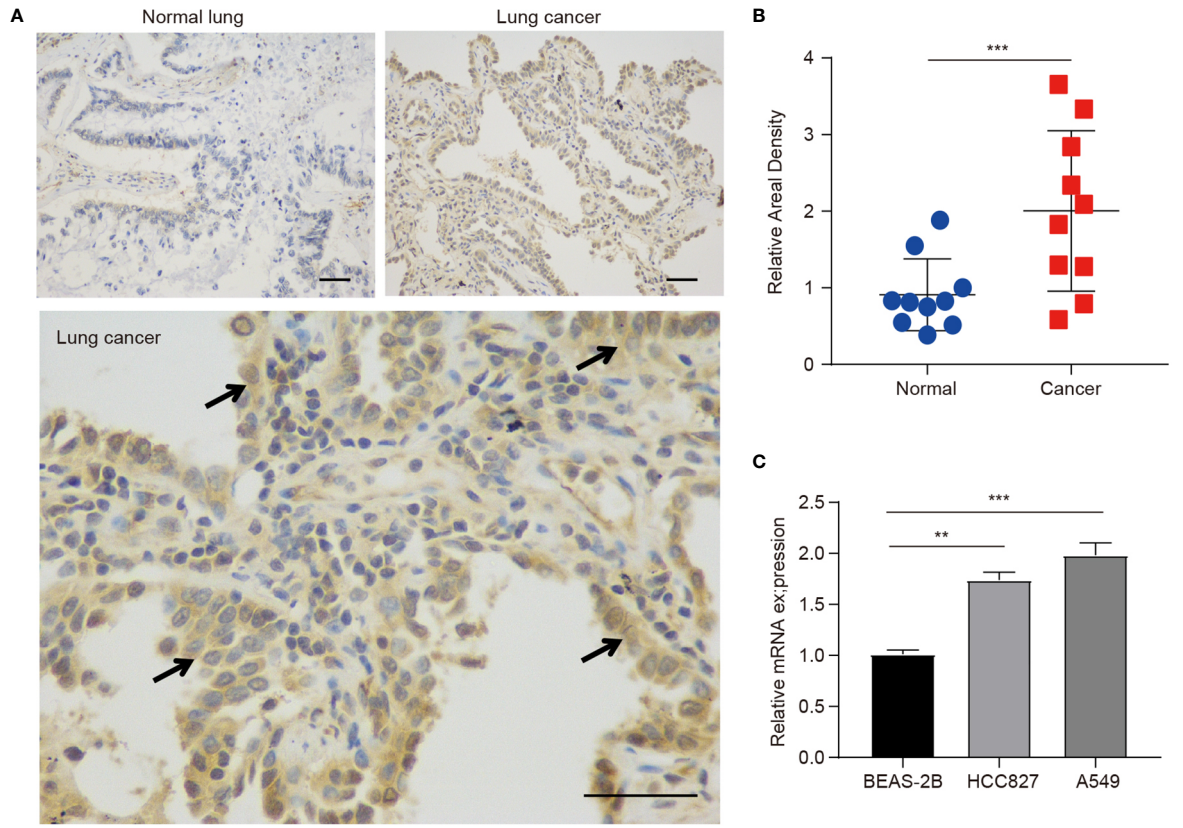

FIGURE 2 | Protein expression of hepcidin in lung cancer patients. **(A)** Immunohistochemical staining of hepcidin was performed in lung cancer and normal lung tissues. Representative images are shown. Scare bars, 50 μM. **(B)** The staining was quantified, as shown. The dot plot depicts the means and standard deviation of 10 images of lung cancer patient tissues and normal lung tissues. **(C)** Hepcidin expression in three different cell lines was examined by real-time PCR. The mean ± s.d. is shown. Statistical significance was determined using one-way ANOVA with the post hoc Tukey test. $^{**}p < 0.01$, $^{***}p < 0.001$.

Validation of the Prognostic Value of Hepcidin Based on Various Clinicopathological Features

To better understand the prognostic value and potential mechanism of hepcidin expression in lung cancer, we explored the association between hepcidin mRNA expression and clinical characteristics using the Kaplan-Meier database. Interestingly, hepcidin upregulation was correlated with poor OS and poor PFS in LUAD patients but not in LUSC patients (**Figure 4C**). High hepcidin expression was significantly correlated with poor OS and PFS in male and female lung cancer patients (**Figure 4C**). Regarding different tumor stages, high hepcidin expression was associated with poor OS and poor PFS only in stage 1 lung cancer patients (**Figure 4C**). A significant correlation between hepcidin expression and poor OS was observed in American Joint Committee on Cancer (AJCC) stage T-1 lung cancer patients (**Figure 3C**). Moreover, we found a significant association between hepcidin expression and unfavorable OS in both smoking and nonsmoking lung cancer patients (**Figure 4C**). In addition, high hepcidin expression was significantly associated with poor OS and PFS in lung cancer patients with negative surgical margins (**Figure 4C**). In contrast, upregulated hepcidin levels corresponded with better OS and PFS in patients with chemotherapy (**Figure 4C**). These results imply that hepcidin mRNA expression possesses prognostic value in lung cancer.

Identification of Hepcidin-Interacting Genes and Proteins and Genetic Alterations

We constructed the gene-gene interaction network for hepcidin and the altered neighboring genes by using GeneMania. The results showed that the 20 most frequently altered genes were closely correlated with hepcidin, including SLC40A1, CEBPB, and STAT1 (**Figure 5A**). Functional analysis suggested that these genes were significantly associated with the acute inflammatory response (**Figure 5A**). A protein-protein interaction (PPI) network of hepcidin was generated using the STRING database (**Figure 5B**). There were 43 edges and 11 nodes, including SLC40A1, TFR2 and HFE (**Figure 5B**). In addition, the correlations between hepcidin and iron metabolism-related genes were investigated based on TCGA database. Hepcidin was positively and significantly correlated with CP, FTH1, FTL, SLC40A1 and TFRC but negatively correlated with TFR2 IREB2 in LUAD (**Figure 5C**). Moreover, hepcidin was positively and significantly correlated with

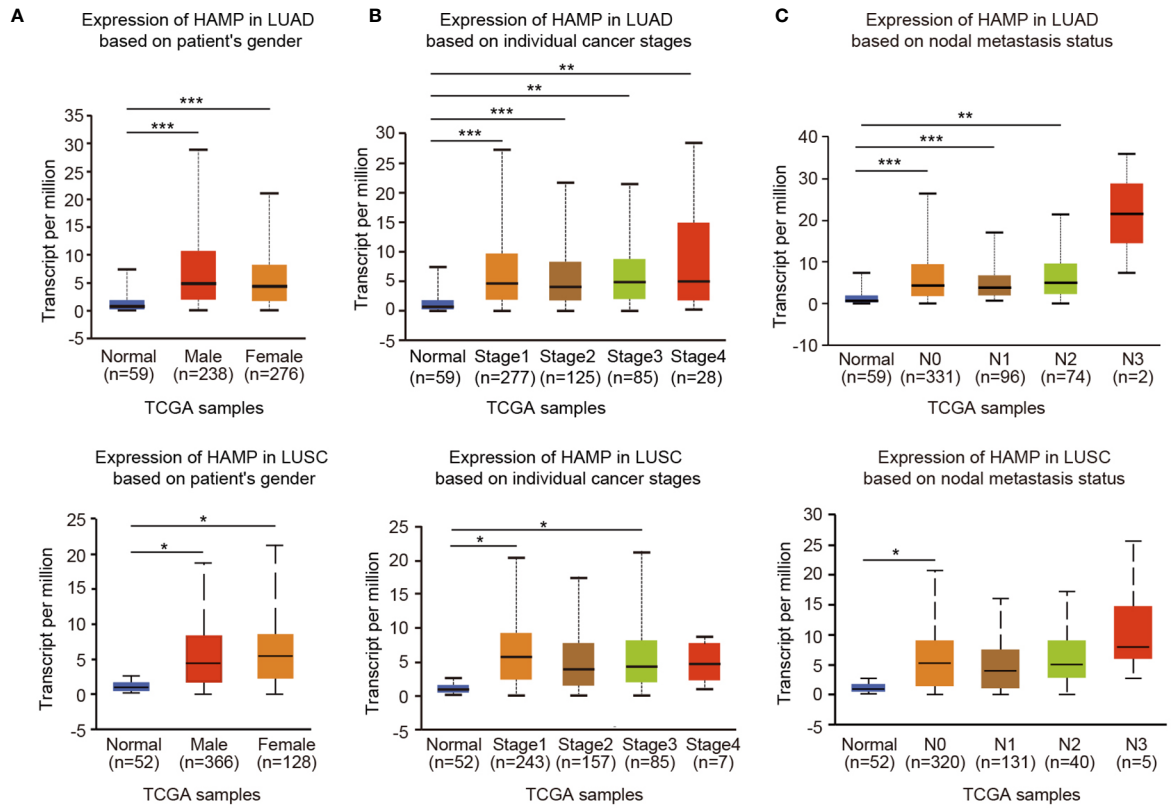

FIGURE 3 | Box plots evaluating hepcidin expression among different groups of patients based on clinical parameters using the UALCAN database. Analysis is shown for sex **(A)**, cancer stage **(B)**, and metastasis **(C)**. N0: no regional lymph node metastasis; N1: metastases in 1 to 3 axillary lymph nodes; N2: metastases in 4 to 9 axillary lymph nodes; N3: metastases in 10 or more axillary lymph nodes. *p < 0.05, **p < 0.01, ***p < 0.001.

ACO1, CP, FTL and SLC40A1 but negatively correlated with IREB2, TFR2 and TFRC in LUSC (**Figure 5D**).

The alteration frequency of hepcidin in lung cancer was analyzed using cBioPortal. A total of 2197 patients from three datasets of lung cancer were analyzed (NSCLC, TCGA, 2016; LUSC, TCGA, pancancer altas; LUAD, TCGA, pancancer altas). Genetic variations in hepcidin showed incidence rates of 5.59%, 5.34%, and 3.18% in these three datasets, respectively (**Supplementary Figures 3A, B**). Amplification was the most common type (**Supplementary Figures 3A, B**). However, the results of Kaplan–Meier plotter and log-rank test indicated that there was no statistically significant difference between OS and PFS and lung cancer patients with or without alterations of hepcidin (**Supplementary Figure 3C**).

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis of Hepcidin and Its Coexpressed Genes in TCGA Lung Cancer

Data mining from TCGA database was used to identify genes positively or negatively coexpressed with hepcidin. The top 50 genes that were positively and negatively correlated with hepcidin in LUAD and LUSC are shown (**Figures 6A, B**; **Figures S4A, B**). Then, a total of 300 genes positively related to hepcidin were used for KEGG and GO enrichment analyses to explore the hepcidin-related pathways and biological functions. The top 20 significant terms of BP, MF and CC enrichment analysis are presented (**Figure 6**; **Supplementary Figures 4C–F**). Notably, in terms of BP, hepcidin was enriched in immune response-related processes, such as neutrophil activation, T cell activation, leukocyte proliferation and migration, and positive regulation of cytokine production in LUAD; the enriched processes in LUSC were T cell activation, regulation of lymphocyte activation, immune response-activating cell surface receptor signaling pathway, lymphocyte differentiation, leukocyte proliferation, etc. (**Figures 6C, E**).

In addition, the top 20 KEGG pathways for hepcidin and its-correlated genes are shown in **Figures 6D, F**. Among these pathways, many immune-related pathways were highly associated with hepcidin, including cytokine-cytokine receptor interaction, chemokine signaling pathway, B cell receptor signaling pathway, Th17 cell differentiation, natural killer cell-mediated cytotoxicity, Th1 and Th2 cell differentiation and intestinal immune network for IgA production in LUAD; and chemokine signaling pathway, Th17 cell differentiation, Th1 and Th2 cell differentiation, B cell receptor signaling pathway, antigen processing and presentation, and intestinal immune network for IgA production in LUSC (**Figures 6D, F**).

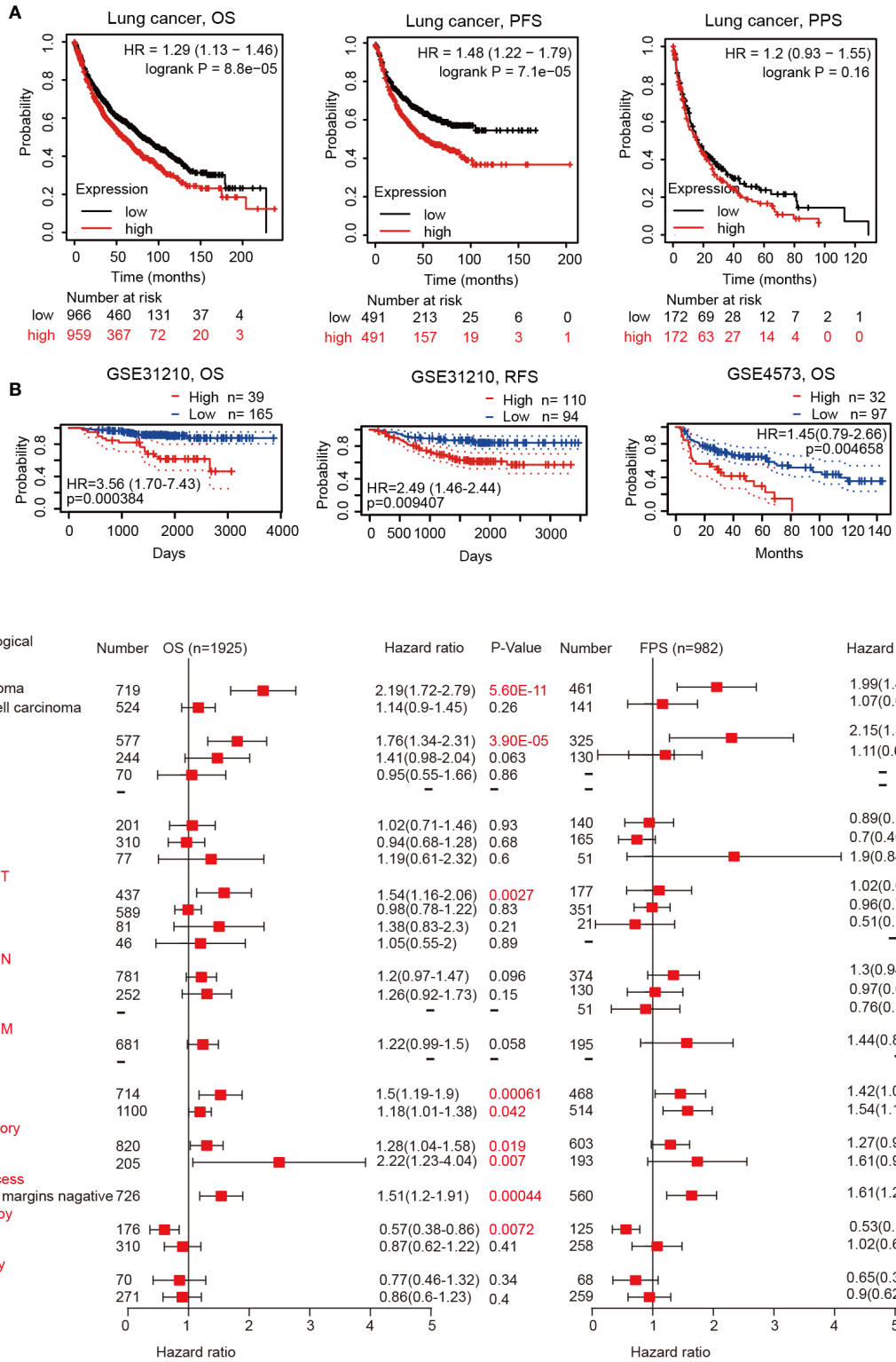

FIGURE 4 | Survival curve evaluating the prognostic value of hepcidin. **(A)** Survival curves using the Kaplan-Meier plotter are shown for OS, PFS and PPS. **(B)** Survival curves using the PrognoScan database are shown for OS and RFS. **(C)** A forest plot shows the correlation between hepcidin expression and clinicopathological parameters in LUAD and LUSC patients.

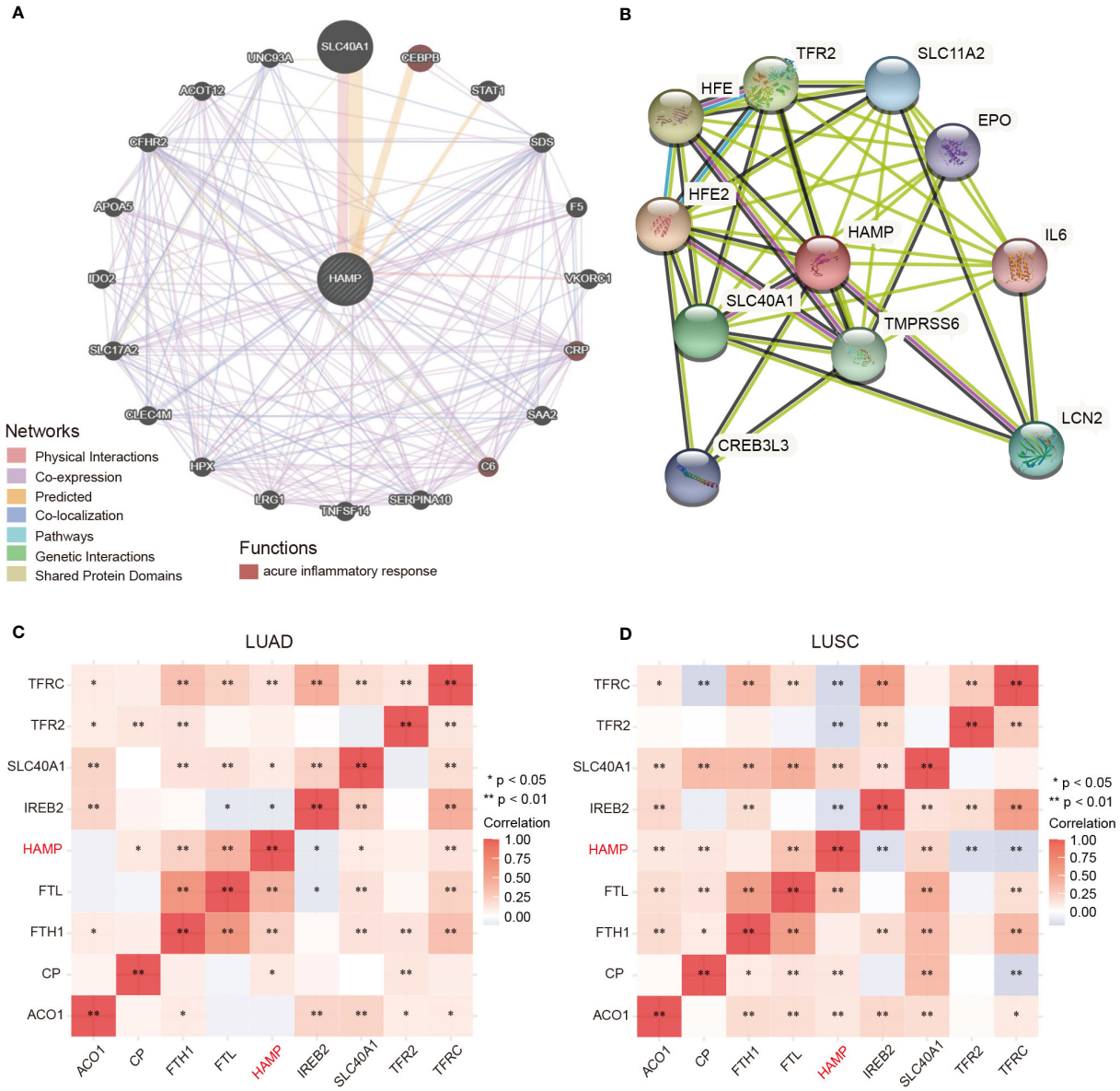

FIGURE 5 | **(A)** The gene-gene interaction network of hepcidin was constructed using GeneMania. **(B)** The PPI network of hepcidin was generated using STRING. **(C, D)** A heat map shows the correlations between hepcidin and iron metabolism-related genes in LUAD and LUSC, respectively. *p < 0.05, **p < 0.01.

Gene Set Enrichment Analysis (GSEA) Identified Hepcidin-Related Signaling Pathways

To further explore the molecular mechanisms affected by hepcidin in lung cancer, GSEA was conducted. Among the GO terms, the top 20 signaling pathways influenced by hepcidin were enriched mainly in immune-related activities, including adaptive immune response, immune effector process, activation of immune response, cytokine production, activation of innate immune response, and regulation of cytokine-mediated signaling pathway in LUAD; and adaptive immune response, leukocyte mediated immunity, cell activation involved in immune response, cytokine production, myeloid cell activation involved in immune response and neutrophil activation involved in immune response in LUSC (**Supplementary Figures 5A, B**). Similarly, among the KEGG terms, GSEA revealed multiple immune functional gene sets that were enriched in lung cancer, including those related to viral protein interactions with cytokine and cytokine receptors, natural killer cell-mediated cytotoxicity, cytokine-cytokine receptor interaction and chemokine signaling pathways (**Supplementary Figures 5C, D**). These results strongly imply that hepcidin is involved in the regulation of the immune response in lung cancer.

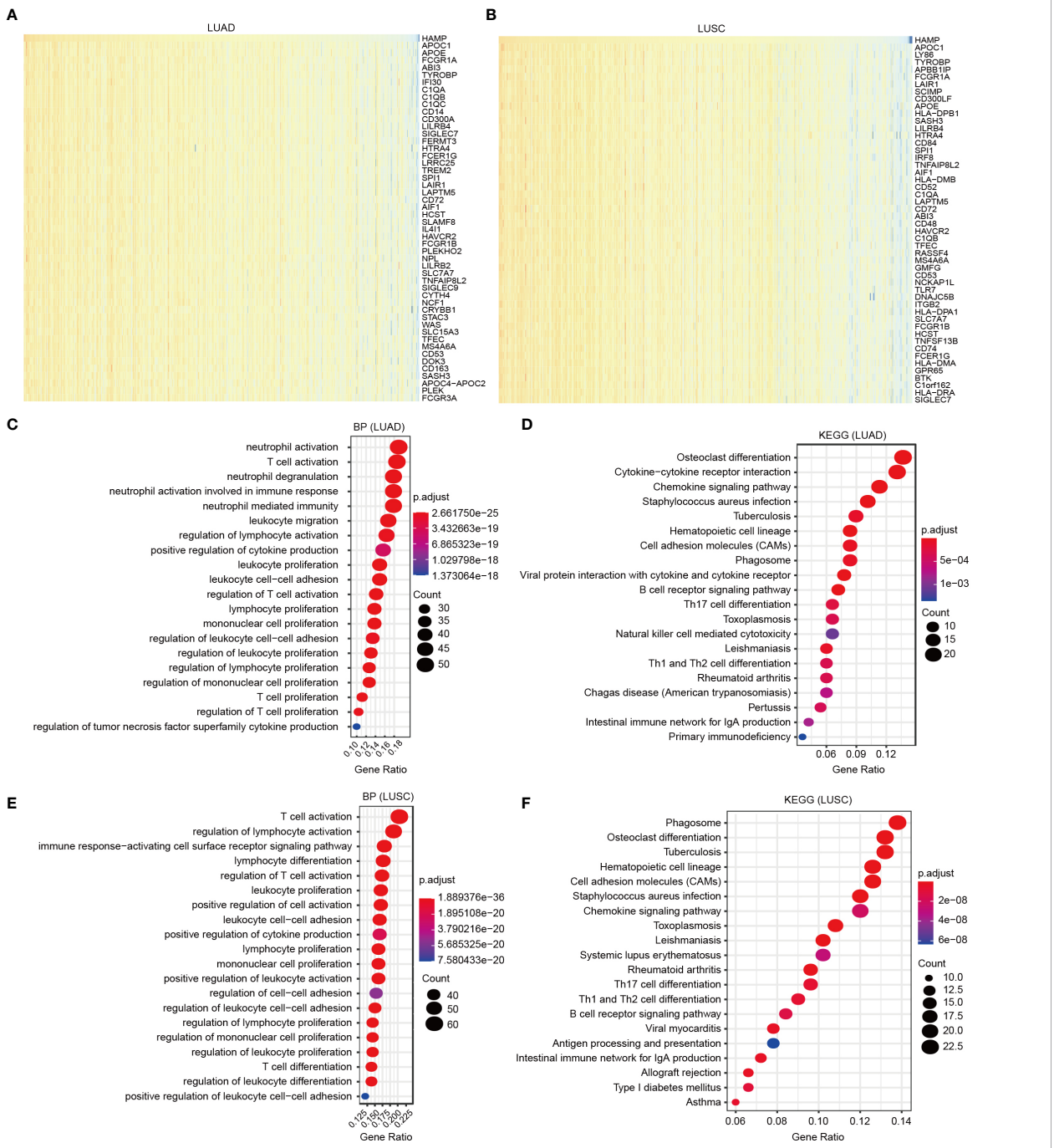

FIGURE 6 | GO and KEGG enrichment analysis for hepcidin. **(A)** Heat maps showing the top 50 genes positively correlated with hepcidin in LUAD. **(B)** Heat maps showing the top 50 genes positively correlated with hepcidin in LUSC. **(C)** Top 20 enrichment terms in BP categories in LUAD. **(D)** Top 20 enrichment terms in BP categories in LUSC. **(E)** Top 20 KEGG enrichment pathways in LUAD. **(F)** Top 20 KEGG enrichment pathways in LUSC.

Correlation Analysis Between Hepcidin Expression and Infiltrating Immune Cells

We analyzed the correlation between hepcidin expression and six types of infiltrating immune cells, including B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells. The results showed that hepcidin expression levels had a significant positive correlation with the infiltration of B cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells and no significant correlations with CD8+ T cells in LUAD (**Figure 7A**). Moreover, hepcidin expression was positively and significantly associated

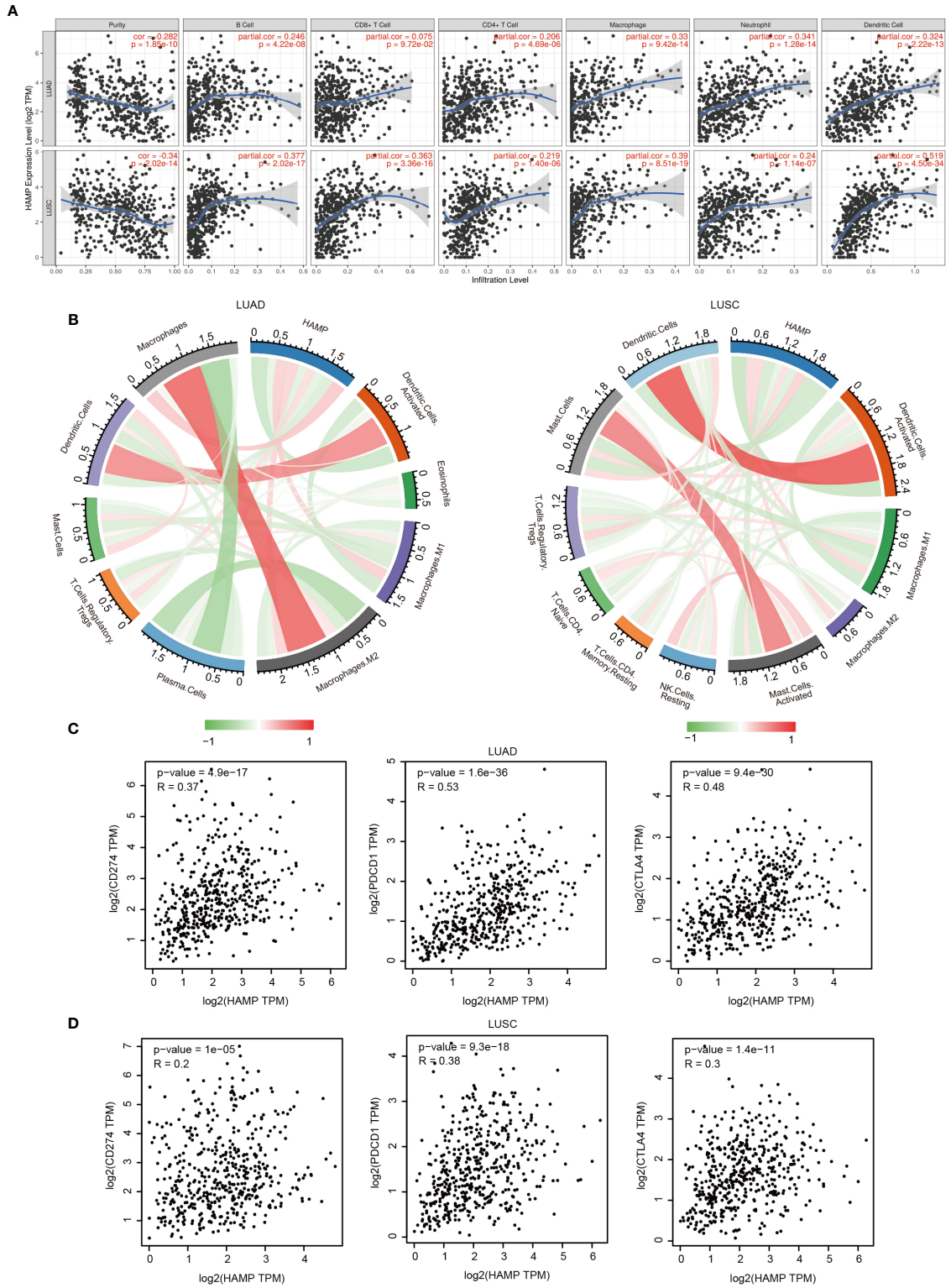

FIGURE 7 | Correlation of hepcidin expression with immune infiltration level. **(A)** Hepcidin is significantly associated with tumor purity and is positively correlated with the infiltration of different immune cells using the TIMER database. **(B)** Hepcidin expression has a significant correlation with the infiltration of immune cells in lung cancer using the CIBERSORT algorithm. **(C, D)** Scatterplots of the correlations between hepcidin expression and PD-1, PD-L1 and CTLA-4 in LUAD and LUSC using the GEPIA database.

with infiltration of all six types of immune cells in LUSC (**Figure 7A**).

To further assess the effect of hepcidin on the tumor microenvironment (TME), we estimated the correlation between hepcidin and immune infiltration using the established computational resource CIBERSORT. Notably, hepcidin was positively correlated with the infiltration levels of macrophages, M1 macrophages, M2 macrophages and regulatory Treg cells but negatively correlated with the infiltration levels of dendritic cells, activated dendritic cells, mast cells, resting mast cells, monocytes, plasma cells, lymphocytes, and eosinophils in LUAD (**Figure 7B**; **Supplementary Figure 6**). Moreover, hepcidin was positively correlated with the infiltration levels of CD4 memory T cells, gamma delta T cells, regulatory Treg cells, activated memory CD4 T cells, macrophages, M1 macrophages and M2 macrophages but negatively correlated with the infiltration levels of naïve CD4 T cells, mast cells, activated mast cells, dendritic cells, activated dendritic cells, eosinophils, and M0 macrophages in LUSC (**Figure 7B**; **Supplementary Figure 7**).

Correlation Between Hepcidin Expression and Various Immune Markers

To deepen our understanding of hepcidin crosstalk with the immune response, we validated the correlations between hepcidin expression and diverse immune signatures in both LUAD and LUSC using the TIMER database. The genes listed in **Table 1** were used to characterize immune cells, including B cells, T cells, CD8+ T cells, monocytes, tumor-associating macrophages (TAMs), M1 macrophages, M2 macrophages, neutrophils, natural killer (NK) cells and dendritic cells. Tumor purity is an important aspect affecting the dissection of immune infiltration in clinical cancer biopsies. After adjusting for tumor purity, hepcidin expression was significantly associated with most immune markers in divergent types of immune cells in LUSC and LUAD (**Table 1**).

TABLE 1 | Correlation analysis between hepcidin and gene markers of immune cells in TIMER.

Description	Gene markers	LUAD				LUSC			
		None		Purity		None		Purity	
		Cor	p	Cor	p	Cor	p	Cor	p
B cell	CD19	0.285	***	0.191	***	0.463	***	0.36	***
	CD79A	0.25	***	0.156	***	0.457	***	0.354	***
T cell (general)	CD3D	0.358	***	0.27	***	0.58	***	0.508	***
	CD3E	0.354	***	0.262	***	0.586	***	0.517	***
	CD2	0.394	***	0.313	***	0.61	***	0.55	***
CD8+ T cell	CD8A	0.337	***	0.247	***	0.517	***	0.463	***
	CD8B	0.318	***	0.249	***	0.4	***	0.376	***
Monocyte	CD86	0.482	***	0.421	***	0.574	***	0.501	***
	CSF1R	0.442	***	0.383	***	0.576	***	0.499	***
TAM	CCL2	0.358	***	0.295	***	0.436	***	0.366	***
	CD68	0.468	***	0.405	***	0.492	***	0.424	***
	IL10	0.467	***	0.392	***	0.46	***	0.393	***
M1	IRF5	0.32	***	0.257	***	0.213	***	0.19	***
	PTGS2	-0.153	***	-0.171	***	0.182	***	0.265	**
	NOS2	0.201	***	0.152	***	0.068	0.129	0.096	*
M2	CD163	0.416	***	0.346	***	0.553	***	0.486	***
	VSIG4	0.444	***	0.385	***	0.59	***	0.534	***
	MS4A4A	0.476	***	0.413	***	0.633	***	0.579	***
Neutrophils	CEACAM8	-0.05	0.260	-0.073	0.105	0.084	0.0588	0.074	0.108
	ITGAM	0.348	***	0.283	***	0.481	***	0.398	***
	CCR7	0.274	***	0.164	***	0.496	***	0.413	***
Natural killer cell	KIR2DL1	0.177	***	0.148	***	0.201	***	0.163	***
	KIR2DL3	0.217	***	0.161	***	0.273	***	0.227	***
	KIR2DL4	0.265	***	0.214	***	0.232	***	0.169	***
	KIR3DL1	0.218	***	0.185	***	0.335	***	0.289	***
	KIR3DL2	0.206	***	0.15	***	0.327	***	0.286	***
	KIR3DL3	0.12	**	0.114	*	0.119	**	0.118	**
	KIR2DS4	0.208	***	0.17	***	0.24	***	0.216	***
Dendritic cell	HLA-DPB1	0.319	***	0.241	***	0.682	***	0.627	***
	HLADQB1	0.29	***	0.219	***	0.526	***	0.453	***
	HLA-DRA	0.315	***	0.235	***	0.649	***	0.588	***
	HLA-DPA1	0.294	***	0.216	***	0.657	***	0.597	***
	CD1C	0.085	0.0551	0.015	0.735	0.423	***	0.298	***
	NRP1	-0.045	0.308	-0.074	0.0986	0.261	***	0.158	***
	ITGAX	0.474	***	0.413	***	0.557	***	0.477	***

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

We also examined the correlation between hepcidin expression and various functional T cells, including Th1, Th1-like, Th2, Treg, resting Tregs, effector Tregs, effector T cells, naïve T cells, effector memory T cells, resistant memory T cells, and exhausted T cells (**Table 2**). By using the TIMER database, we found that the hepcidin expression level was significantly correlated with 33 of 38 T cell markers in LUAD and with 32 of 38 T cell markers in LUSC after adjusting for tumor purity (**Table 2**).

We further investigated the interrelationship between hepcidin expression and famous T cell checkpoints, such as PD-1, PD-L1 and CTLA-4, in the GEPIA database. Hepcidin expression was significantly correlated with the expression of PD-1, PD-L1 and CTLA-4 in LUAD and LUSC (**Figures 7C, D**). These findings further support that hepcidin expression is significantly related to immune infiltration and suggest that hepcidin plays an important role in immune escape in the lung cancer microenvironment.

Prognostic Analysis of Hepcidin Expression Based on Immune Cells in LUSC Patients

Since hepcidin expression is significantly correlated with immune infiltration and poor prognosis in LUSC, we investigated whether hepcidin expression affects the prognosis of LUSC because of immune infiltration. We performed prognosis analyses based on the expression levels of hepcidin in LUSC in related immune cell subgroups. As shown in **Figures 8A, B**, LUSC patients with high expression of hepcidin and decreased infiltration of B cells, CD4+ memory T cells,

TABLE 2 | Correlation analysis between hepcidin and gene markers of different types of T cells in TIMER.

Description	Gene markers	LUAD				LUSC			
		None		Purity		None		Purity	
		Cor	p	Cor	p	Cor	p	Cor	p
Th1	TBX21	0.336	***	0.243	***	0.53	***	0.46	***
	STAT4	0.24	***	0.136	**	0.469	***	0.38	***
	STAT1	0.272	***	0.196	***	0.241	***	0.194	***
	TNF	0.215	***	0.126	**	0.17	***	0.049	0.285
	IFNG	0.352	***	0.284	***	0.389	***	0.346	***
Th1-like	HAVCR2	0.546	***	0.495	***	0.681	***	0.63	***
	IFNG	0.352	***	0.284	***	0.389	***	0.346	***
	CXCR3	0.383	***	0.301	***	0.615	***	0.559	***
	BHLHE40	-0.007	0.879	-0.029	0.514	-0.005	0.920	-0.076	0.0954
	CD4	0.426	***	0.355	***	0.637	***	0.577	***
Th2	STAT6	-0.085	0.0535	-0.097	*	-0.071	0.112	-0.075	0.101
	STAT5A	0.316	***	0.236	***	0.404	***	0.314	***
Treg	FOXP3	0.298	***	0.208	***	0.49	***	0.403	***
	CCR8	0.209	***	0.117	**	0.444	***	0.365	***
	TGFB1	0.165	***	0.089	*	0.055	0.221	-0.044	0.333
Resting Treg	FOXP3	0.298	***	0.208	***	0.49	***	0.403	***
	IL2RA	0.308	***	0.23	***	0.505	***	0.431	***
Effector Treg T-cell	FOXP3	0.298	***	0.208	***	0.49	***	0.403	***
	CCR8	0.209	***	0.117	**	0.444	***	0.365	***
	TNFRSF9	0.318	***	0.23	***	0.431	***	0.344	***
Effector T-cell	CX3CR1	0.239	***	0.2	***	0.481	***	0.402	***
	FGFBP2	0.184	***	0.145	**	-0.017	0.702	0.025	0.579
	FCGR3A	0.517	***	0.461	***	0.604	***	0.545	***
Naïve T-cell	CCR7	0.274	***	0.164	***	0.496	***	0.413	***
	SELL	0.3	***	0.205	***	0.508	***	0.422	***
Effector memory T-cell	DUSP4	0.072	0.101	0.068	0.131	0.294	***	0.232	***
	GZMK	0.328	***	0.242	***	0.582	***	0.518	***
	GZMA	0.409	***	0.341	***	0.461	***	0.395	***
Resident memory T-cell	CD69	0.24	***	0.14	**	0.5	***	0.413	***
	CXCR6	0.371	***	0.287	***	0.587	***	0.531	***
	MYADM	0.118	**	0.042	0.350	0.299	***	0.215	***
General memory T-cell	CCR7	0.274	***	0.164	***	0.496	***	0.413	***
	SELL	0.3	***	0.205	***	0.508	***	0.422	***
	IL7R	0.211	***	0.106	0.0181	0.331	***	0.215	***
Exhausted T-cell	HAVCR2	0.546	***	0.495	***	0.681	***	0.63	***
	LAG3	0.368	***	0.305	***	0.417	***	0.359	***
	CXCL13	0.259	***	0.16	***	0.361	***	0.263	***
	LAYN	0.17	***	0.077	0.0886	-0.002	0.971	-0.005	0.919

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

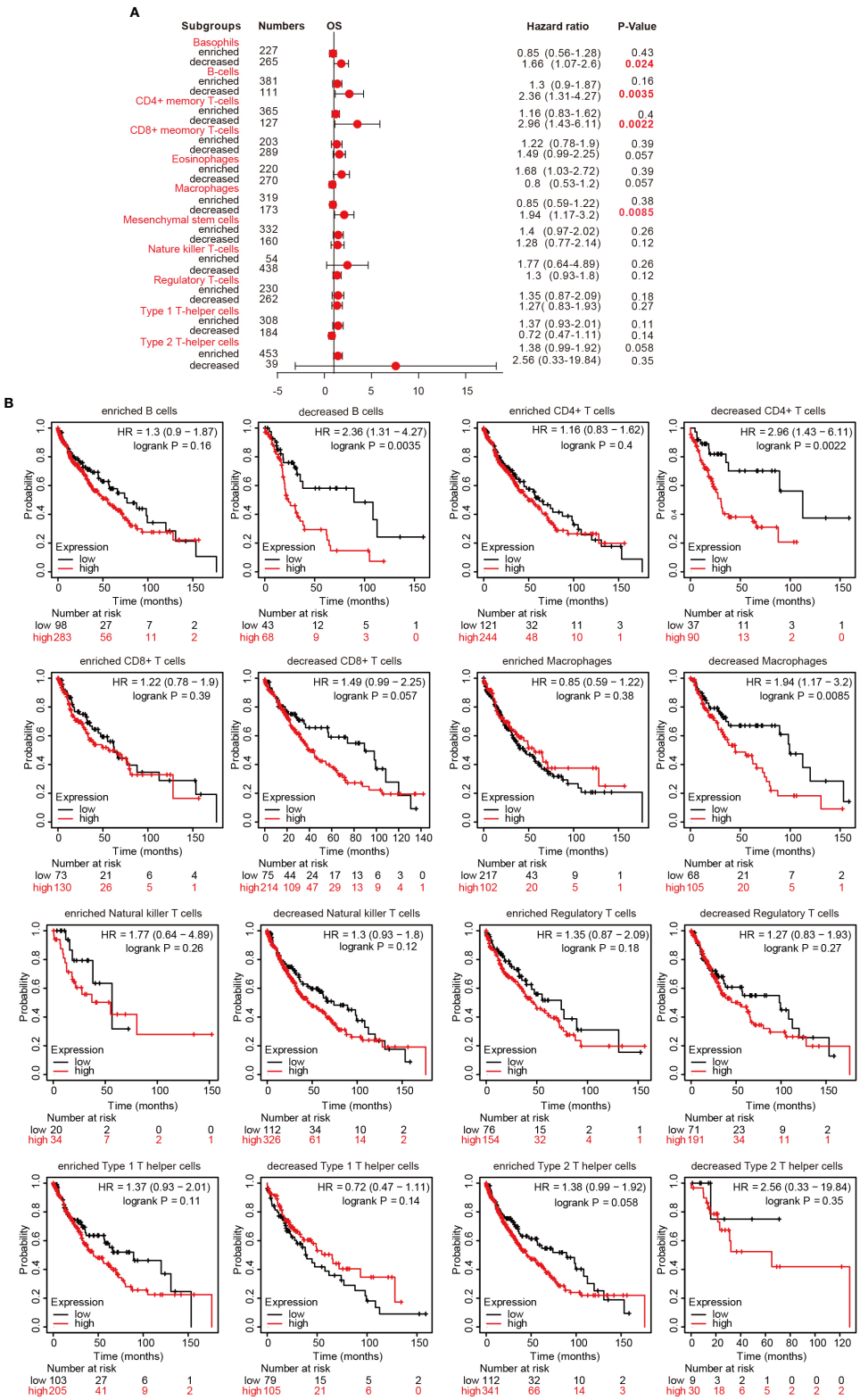

FIGURE 8 | Kaplan-Meier survival curves according to high and low expression of hepcidin in immune cell subgroups in lung cancer. **(A)** A forest plot shows the prognostic value of hepcidin expression according to different immune cell subgroups in LUSC patients. **(B)** Correlations between hepcidin expression and OS in different immune cell subgroups in LUSC patients were estimated by Kaplan-Meier plotter.

macrophages and basophils had a poor prognosis. However, there was no significant correlation between hepcidin expression and the prognosis of LUSC in the group with different levels of CD8+, NK, Treg, Th1 and Th2 cell infiltration (**Figures 8A, B**). These results indicate that hepcidin may affect the prognosis of LUSC patients in part due to immune infiltration.

DISCUSSION

Among malignancies, lung cancer has the highest morbidity rates and is the leading cause of cancer-related death in both males and females worldwide (1). Despite advances in early diagnosis and targeted and immune therapies, lung cancer is often diagnosed at an advanced stage and has a poor prognosis (1–3). Thus, it is important to explore mechanisms that result in the incidence of lung cancer metastasis and identify useful prognostic biomarkers of lung cancer. In the present study, we showed that the expression of hepcidin in lung cancer was higher than that in normal lung tissue by means of bioinformatics analysis of the TIMER, Oncomine, UALCAN and TCGA public databases (**Figure 1**). These findings were consistent with a previous report and suggested that hepcidin may act as an oncogene by promoting the development and progression of lung cancer (34). Subsequently, the clinical prognostic significance of hepcidin in lung cancer patients was investigated. High expression of hepcidin was significantly correlated with sex, age, clinical stage, histological grade and metastasis in lung cancer patients (**Figure 3**). According to the patient samples in the cBioPortal database, approximately 5% of lung cancer patients possess genetic alterations in hepcidin (**Supplementary Figure 3**). We also unearthed the fact that most of the alterations of hepcidin are gene amplifications in lung cancer patients. Furthermore, Kaplan-Meier survival analyses indicated that lung cancer patients with high hepcidin expression exhibited a markedly worse survival rate than those with low expression (**Figure 4**). These results substantiated that hepcidin may be an independent prognostic biomarker in lung cancer and may facilitate the development of targeted precision oncology.

As the most common subtype of NSCLC, accumulating evidence has demonstrated that LUAD and LUSC differ from each other in their biopathology, molecular, clinical characteristics and therapeutic effect (41). For example, the subtypes of LUSC include primitive, classical, secretory and basal (42). Three distinct subtypes of LUAD were introduced in 2014, including proximal inflammatory (PI), proximal proliferative (PP), and terminal respiratory unit (TRU) (43). LUAD usually arises from the distal airway, while LUSC is associated with more proximal airways (44). LUSC is generally more strongly associated with smoking and inflammatory diseases than LUAD (44). In general, LUAD grows more slowly and has smaller lumps than its contemporaneous counterpart LUSC but tends to metastasize at an early stage (45). The most commonly mutated genes in LUAD include oncogenes (KRAS and EGFR) and tumor suppressor genes (TP53, KEAP1, STK11 and NF1) (46). The frequency of EGFR-activating mutations varies greatly by region and ethnicity. In contrast, the commonly mutated gene in LUSC is TP53, which is observed in more than 80% of the samples. Recurrent mutations in NFE2L2, KEAP1, CDKN2A, FBXW7, BAI3, GRM8, MUC16, RUNX1T1, STK11 and ERBB4 have been reported in LUSC (46). Moreover, many studies have investigated the differences in the mRNA and circRNA expression profiles and methylation patterns of LUAD and LUSC. These findings provide more insights into the molecular mechanism of LUSC and LUAD. Consistent with these observations in LUAD and LUSC, we also found that there were some differences in our analysis results between LUAD and LUSC. For instance, upregulated hepcidin expression only significantly correlated with poor OS and PFS in LUAD but not in LUSC (**Figure 4**). Moreover, there were few overlapping enrichment terms in GO and KEGG analyses between LUAD and LUSC. Nevertheless, we still found that hepcidin was closely associated with immune response-related pathways in both LUAD and LUSC (**Figure 6** and **Supplementary Figure 5**).

Human hepcidin is highly expressed in hepatocytes. In addition to the liver, hepcidin is also synthesized in a number of other organs and tissues, such as the brain, heart, kidney, spleen, pancreas, stomach and adipose tissue (47). The function of this extrahepatic hepcidin remains unclear, but one hypothesis is that it is associated with local iron homeostasis. A growing number of studies have suggested that increased serum hepcidin accompanies multiple cancers, including breast cancer, prostate cancer, renal cell carcinoma and myeloma (23). Furthermore, recent studies have revealed that hepcidin can be produced by cancer cells. For example, hepcidin expression was observed in normal breast cells and was significantly increased in breast cancer cells (25, 48). Suppression of hepcidin synthesis by heparin, a potent inhibitor of liver-derived hepcidin production, induced significant inhibition of tumor growth due to diminished intracellular iron retention (48). These results imply that circulating hepcidin secreted from the liver exerts a robust effect on tumor growth by mediating ferroportin-regulated iron export in cancer cells. More importantly, knockdown of tumor hepcidin expression also caused robust inhibition of tumor growth of MDA-MB-231 cells (48). Hepcidin was also highly expressed in prostate cancer cells compared with normal prostate epithelial cells (32, 49). Hepcidin synthesis in prostate cancer cells is regulated by Wnt- and SOSTDC1-associated pathways (32, 49). Inhibition of hepcidin obviously suppressed prostate cancer cell survival. Hepcidin could be detected in pancreatic cancer and gastric cancer tissues by IHC staining (30, 50). Strongly stained hepcidin patients showed a worse OS than weakly stained hepcidin patients with pancreatic cancer (30). Furthermore, hepcidin expression was significantly increased in thyroid cancer cells, especially K1 and 8505C cells, compared with normal cells (51). Mechanistically, SOSTDC1 silencing by E4BP4 and G9a complex-mediated promoter hypermethylation promoted hepcidin secretion in thyroid cancer (51). In addition, knockout of *hepcidin* led to a marked reduction in the development of cancer in a mouse lung cancer

model (52). A previous study demonstrated that hepcidin expression in doxorubicin-resistant MCF-7 cells was increased compared with that in doxorubicin-sensitive MCF-7 cells (27). Moreover, the development of resistance to doxorubicin in Walker-256 carcinosarcoma *in vivo* was accompanied by an increase in hepcidin expression (27). However, the underlying mechanism between hepcidin and chemoresistance is still unclear. A possible explanation is the upregulation of IL-6 expression and consequent upregulation of hepcidin associated with inflammatory conditions typically observed in many patients with metastatic cancer. Increased IL-6 concentrations have been demonstrated to be closely associated with chemoresistance. In esophageal squamous cell carcinoma, IL-6 derived from cancer-associated fibroblasts plays the most important role in chemoresistance by upregulating the expression of C-X-C motif chemokine receptor 7 (CXCR7) *via* the STAT3/nuclear factor-κB (NF-kB) pathway (53). IL-6 contributes to chemoresistance in MDA-MB-231 cells by upregulating HIF-1α through the activation of STAT3 (54). In addition, IL-6 also enhanced the chemoresistance of ovarian cancer cells against cisplatin through the IL-6/STAT3/HIF-1α loop *in vitro* and *in vivo* (55). Hepcidin has been considered a particularly attractive target, and agents that inhibit hepcidin are under active investigation as potential therapies for cancer treatment. Here, we found that hepcidin expression was upregulated in lung cancer tissues compared with normal lung tissues (**Figure 2A**). The expression of hepcidin in A549 and HCC827 cells was also higher than that in normal lung cells (**Figure 2C**). These findings suggest that lung cancer may synthesize functional hepcidin to promote its proliferation. However, the ways and methods to reduce the expression of hepcidin still need to be further explored. Excessive reduction of systemic hepcidin can lead to iron deposition, which is another risk factor for tumor development and progression. Over the last decade, there has been increasing interest in developing pulmonary drug delivery systems suitable for lung cancer therapy (56, 57). A number of nanocarrier systems, including nanoparticles, liposomes, micelles and polymers, have been developed to selectively deliver various anticancer molecules and drugs at the tumor site. Nanocarrier systems have potential advantages, such as improved drug solubility, prolonged systemic circulation, controlled release and targeted drug delivery (56, 57). Moreover, topical delivery of hepcidin-targeted drugs to the lung *via* inhalation is also deemed to be an effective approach for the treatment of lung cancer (58).

Hepcidin is upregulated in response to iron overload (9, 10). Hepcidin is also an acute-phase reactant induced by inflammatory stimuli. A previous study reported that the induction of hepcidin can be triggered by IL-6, which plays an important role in the regulation of inflammation and the immune response (20). However, to our knowledge, the relationship between hepcidin and immune cell infiltration in lung cancer has not been investigated. In the present study, GO and KEGG pathway enrichment analyses of hepcidin and its related genes revealed that hepcidin is involved in numerous pathways, especially the immune system in lung cancer (**Figure 6**). This finding was consistent with the literature and GSEA results we presented in this study, solidifying the association between hepcidin and the immune response (**Supplementary Figure 5**). Here, we first report that high hepcidin expression in lung cancer is correlated with the increased infiltration of B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells (**Figure 7**). Moreover, a significant association between hepcidin and various immune cell marker sets was observed in lung cancer (**Table 1** and **Table 2**). Hepcidin expression was also positively correlated with PD-1 and CTLA-4 expression (**Figure 7**). More importantly, hepcidin influences the survival time of lung cancer patients partially through immune cell infiltration (**Figure 8**). These findings indicate that hepcidin could be a novel immune-related therapeutic target in lung cancer. However, the precise role of hepcidin in the tumor-immune microenvironment still needs further in-depth exploration.

The present study improves our understanding of the relationship between hepcidin and lung cancer, but some limitations still exist. First, although we investigated the correlation between hepcidin and immune infiltration in LUAD and LUSC patients, there is a lack of interpretation of the immune analysis according to the different subgroups. Second, we observed that hepcidin was strongly expressed in lung cancer cells by IHC analysis. However, the molecular mechanisms and roles of hepcidin in tumor growth, metastasis and immune infiltration and escape need to be explored in further studies. Third, most of the analyses were performed based on mRNA levels of hepcidin in the present study. A deeper analysis, based on protein levels, would make the data more convincing. Fourth, we did not investigate the diagnostic and prognostic value of hepcidin in small cell lung cancer (SCLC) and large cell lung cancer (LCLC) in this study. Overall, our results indicate that hepcidin could serve as a potential novel prognostic biomarker for lung cancer. Moreover, we explored the underlying evidence indicating that hepcidin regulates immune cell infiltration in the TME in lung cancer patients. Therefore, these findings are potentially valuable in advancing our current understanding of not only the role of hepcidin but also its translational use in lung cancer prognosis and immunotherapy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Institutional Research Ethics Committee of

HanDan Central Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Study concept and design: YF and KT. Acquisition of data: KT, YF, ZS, BL, and FC. Analysis and interpretation of data: KT, ZS, BH, YM, JH, and FC. Statistical analysis: PC, KT, YF, and BH. Drafting of the manuscript: KT. Critical revision and final approval of the manuscript: KT and YC. Obtained funding: YF and KT. Study supervision: KT. All authors contributed to the article and approved the submitted version.

FUNDING

This work was partially supported by the China Postdoctoral Science Foundation (2017M621099), the Key Projects of Hebei Normal University (L2018Z07), the Graduate Student Innovation Funding of Hebei Normal University (CXZZSS2021064) and the One Hundred Person Project of Hebei Province (E2016100019).

ACKNOWLEDGMENTS

We would like to thank the staff of the Sheng Xin Xiao Ke Tang WeChat platform for their help in data analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.612144/full#supplementary-material

Supplementary Figure 1 | Box plots comparing hepcidin expression in normal individuals and lung cancer patients obtained from the Oncomine database.

Supplementary Figure 2 | Box plots evaluating hepcidin expression among different groups of patients based on clinical parameters using the UALCAN database. Analysis is shown for TP53 mutation status **(A)**, age **(B)**, and race **(C)**. *p < 0.5, **p < 0.01, ***p < 0.001.

Supplementary Figure 3 | Alteration frequency of hepcidin. **(A)** Summary of alterations in hepcidin. **(B)** OncoPrint visual summary of alterations in a query of hepcidin from cBioPortal. **(C)** Kaplan-Meier plots comparing OS and PFS in cases with or without hepcidin gene alterations.

Supplementary Figure 4 | GO analysis for hepcidin. **(A)** A heat map showing the top 50 genes negatively correlated with hepcidin in LUAD. **(B)** A heat map showing the top 50 genes negatively correlated with hepcidin in LUSC. **(C)** Top 20 enrichment terms in MF categories in LUAD. **(D)** Top 20 enrichment terms in MF categories in LUSC. **(E)** Top 20 enrichment terms in CC categories in LUAD. **(F)** Top 20 enrichment terms in CC categories in LUSC.

Supplementary Figure 5 | Enrichment plots from GSEA. **(A, B)** A merged plot showing the pathways associated with hepcidin expression in LUAD and LUSC based on GO analyses. **(C, D)** A merged plot showing the pathways associated with hepcidin expression in LUAD and LUSC based on KEGG analyses.

Supplementary Figure 6 | Scatterplots of correlations between hepcidin expression and infiltration levels of immune cells in LUAD.

Supplementary Figure 7 | Scatterplots of correlations between hepcidin expression and infiltration levels of immune cells in LUSC.

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

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frontiers in Immunology

REVIEW
published: 27 April 2021
doi: 10.3389/fimmu.2021.614294

The Iron Curtain: Macrophages at the Interface of Systemic and Microenvironmental Iron Metabolism and Immune Response in Cancer

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Macrophages fulfill central functions in systemic iron metabolism and immune response. Infiltration and polarization of macrophages in the tumor microenvironment is associated with differential cancer prognosis. Distinct metabolic iron and immune phenotypes in tumor associated macrophages have been observed in most cancers. While this prompts the hypothesis that macroenvironmental manifestations of dysfunctional iron metabolism have direct associations with microenvironmental tumor immune response, these functional connections are still emerging. We review our current understanding of the role of macrophages in systemic and microenvironmental immune response and iron metabolism and discuss these functions in the context of cancer and immunometabolic precision therapy approaches. Accumulation of tumor associated macrophages with distinct iron pathologies at the invasive tumor front suggests an "Iron Curtain" presenting as an innate functional interface between systemic and microenvironmental iron metabolism and immune response that can be harnessed therapeutically to further our goal of treating and eliminating cancer.

Keywords: cancer systems, immunotherapy, iron metabolism, macrophage polarization, tumor microenvironment

OPEN ACCESS

Edited by:
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University of Milan, Italy

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

Received: *05 October 2020*
Accepted: *29 March 2021*
Published: *27 April 2021*

Citation:
DeRosa A and Leftin A (2021) The Iron Curtain: Macrophages at the Interface of Systemic and Microenvironmental Iron Metabolism and Immune Response in Cancer. Front. Immunol. 12:614294. doi: 10.3389/fimmu.2021.614294

INTRODUCTION

Defining patient prognosis, potential precision therapeutic avenues, and ultimately survival outcomes on the basis of metabolism is complicated by the need to integrate macroenvironmental and microenvironmental processes and multi-cellular metabolic systems interactions. Systemic metabolism establishes a unique profile of metabolites in the tumor microenvironment (TME), but distribution of these metabolites in the TME and their characterization is complicated by the multi-cellular dynamic composition of the tumor that introduces spatial heterogeneity of the metabolite distribution due to inter-cellular competition for metabolites that can promote tumor growth and hinder effective anti-tumor responses (1–5). This intersection of metabolism and cellular function in the TME is increasingly recognized as being of critical importance in cancer immune response. Metabolic gradients in the TME and systemic

changes in metabolism alter immune cell activity and notably plays a prominent role in mediating immunotherapeutic responses (6, 7).

Of the various cells involved in cancer, macrophages play a central role in systemic and microenvironmental metabolism that has prominent effects on immune response in cancer. Tumor associated macrophages (TAMs) are implicated in all stages of cancer from tumorigenesis, to metastasis outgrowth, and therapy response as they plastically change their immune response according to local and systemic cues (8, 9). Metabolically, in non-malignant diseases and homeostatic contexts, macrophages exhibit a unique metabolic trait throughout the body in diverse tissue microenvironments in that macrophages can shift the fate of immune response in a manner dependent upon their central function in iron recycling (10–12). Investigators are increasingly focused on similar connections between cancer, TAM immune response, and iron metabolism. Thus, here we review our current understanding of macrophages in metabolic iron handling and immunologic response in cancer. To contextualize the role of TAMs in iron handling we review macrophage's dual roles in iron handling and immune response both systemically in organs throughout the body, and in various tumors. Further, we detail how macrophages are central to the axis of immune system and iron metabolism in cancer therapy and demonstrate how harnessing either their iron level or immune response jointly effects the other enhancing our ability to treat cancer. These new insights will support new opportunities for therapeutic interventions at the multi-systems level.

REGULATION OF IRON METABOLISM BY MACROPHAGES

Molecular Mechanisms of Iron Handling

Macrophages are involved in controlling iron import, export, and storage. These functions are regulated post-transcriptionally. mRNA-binding iron regulatory proteins 1 and 2 (IRP1 and IRP2) mediate cellular iron uptake, transport, storage and utilization in macrophages and hepatocytes (13). IRPs bind their target transcripts to regulate protein transcription and iron regulatory elements in response to iron levels in the body (14). In iron deficient conditions, IRPs will bind with high affinity to the iron regulatory element (IRE) in heavy and light chain ferritin mRNA and inhibit their translation to prevent storage of iron. In replete iron cells, IRP binding to IRE is reduced to allow for degradation of TfR1 mRNA and translation of ferritin mRNA to support cellular iron storage (15).

Macrophages take-up different forms of iron. Transferrin bound, and non-transferrin bound free iron (NTBI) enter the macrophage *via* specific cell surface receptors (16). The transferrin receptor (TfR) sits on the macrophage cell surface and recognizes transferrin-bound iron, or holo-transferrin, which becomes endocytosed upon binding (16–18). Similarly, receptors for lactoferrin (LFN), a member of the transferrin family that binds iron and has numerous functions (19), are present on many immune cells, including macrophages (20). NTBI can be transported by ZIP14, a ZIP family member of metal ion transporters, where it is upregulated on human primary macrophages under inflammatory conditions (21). NTBI can also be taken up by the divalent metal transporter 1 (DMT-1), also known as SLC11A2 which is associated with duodenal cytochrome B on the surface of the macrophage. In the endosome at low pH, iron is released if bound to transferrin, then reduced and stabilized by the endosomal reductase six-transmembrane epithelial antigen of the prostate 3 (STEAP3), from ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) and transported through DMT-1 into the cytosol for ferritin storage, metabolic cofactor processes, or export through ferroportin (FPN), the cellular iron exporter (16).

Macrophages recycle heme iron from phagocytosis of senescent erythrocytes. Senescent red blood cells (RBCs) present cell surface markers to be recognized by macrophage scavenger receptors for phagocytosis. CD91 and CD163 are two scavenger receptors expressed at high levels on the surface of the macrophage. CD91 binds hemopexin-bound heme iron while CD163 bind both free iron and haptoglobin-hemoglobin complexed iron (22). RBCs are engulfed and digested by macrophages *via* erythrophagocytosis. RBCs phagocytized by the macrophage will be degraded, iron will be released from heme in the phagolysosome by heme oxygenase 1 (HO-1), transported to the cytosol of the macrophage, and processed for storage or recycling (11). NTBI can be exported through FPN out of the cell, supported by the ferroxidase ceruloplasmin, and loaded into transferrin to be transported to other target cells (16).

Macrophages store iron in ferritin. Ferritin is normally found within cells but can also be found in plasma. Ferritin is an iron storage protein complex consisting of 24 molecules of light (FTL) and/or heavy (FTH) chains. Extracellular ferritin can bind cell surface ferritin receptors, mainly including heavy chain H-ferritin receptor T cell immunoglobin and mucin domain-2 and light chain L-ferritin receptor scavenger receptor member 5, mediate the uptake of ferritin-bound iron (16). After uptake, NTBI is freed from ferritin protein and processed further by the cell for export.

Macrophages in Systemic Iron Metabolism

Iron is regulated systemically by specific organs including the liver, spleen, and bone marrow. Tissue-specific macrophage populations are present within these organs as distinct phenotypic subsets (23). These resident macrophages have broad roles in removing debris, such as senescent and apoptotic cells, they help in development by promoting angiogenesis and bone break-down, and in regulation of metabolism (23). They also aid in controlling iron homeostasis at the local and systemic levels (24). Here we review the molecular mechanisms by which macrophages regulate iron, and present examples of macrophages in systemic contexts where they perform these iron recycling roles to integrate our forthcoming observations of TAM iron handling and immune response within the larger context of macrophage iron recycling systems of the body such as drawn schamtically in **Figure 1**.

The liver is a major center for iron regulation. Hepatic hepcidin production regulates systemic iron export by promoting the

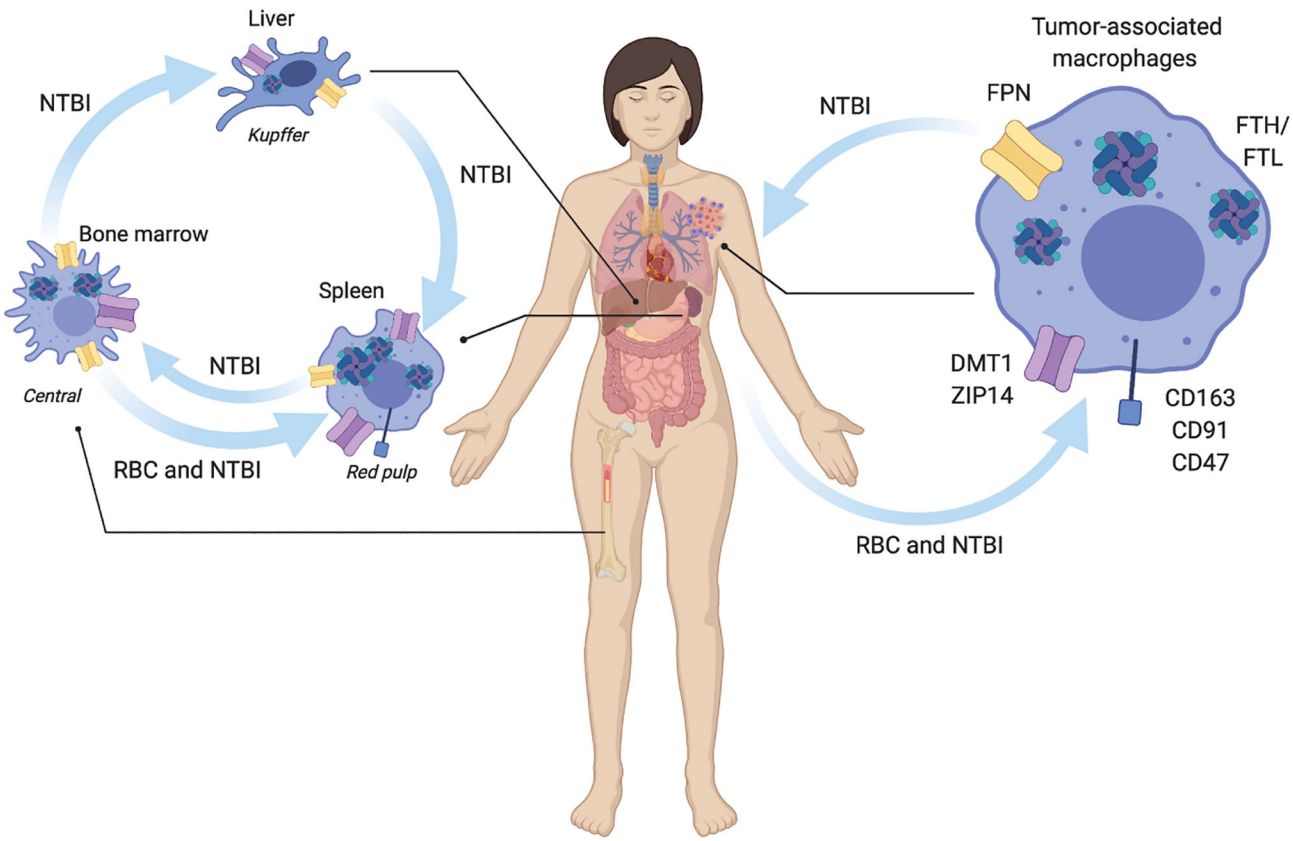

FIGURE 1 | Macrophage regulation of systemic iron metabolism. Macrophages are central regulators of iron metabolism systemically throughout the body where they regulate largely unidirectional flux of non-transferrin bound iron NTBI, and red blood cell (RBC) erythrophagocytosis. Macrophages across the body and in tumors share similar uptake, storage and release mechanisms. NTBI iron is taken up by ZIP14, and DMT1. RBC are recognized for phagocytosis *via* CD163, CD91, and CD47 receptors. NTBI is stored as ferritin heavy chain (FTH) and light chain (FTL) complexes. NTBI is released by ferroportin (FPN). In liver Kupffer cells integrate inflammatory cues with NTBI iron recycling to regulate iron homeostasis. Spleen red-pulp macrophages respond to systemic metabolic iron needs by phagocytosis of senescent red blood cells (RBC) and export of recycled NTBI. Bone marrow central macrophages process NTBI received from the periphery to support heme synthesis during erythropoiesis. In the tumor, tumor associated macrophages similarly recycle NTBI and RBC.

internalization of the iron exporter FPN on macrophages and other cells, lowering circulating NTBI concentrations (24). This leads to a decrease in the concentration of systemic iron and the accumulation of iron within iron-handling macrophages such as Kupffer cells in the liver. Liver Kupffer cells take up and store NTBI from senescent erythrocytes and release it in response to systemic need. In instances of chronic inflammation over-accumulation of liver iron leads to less iron circulation throughout the body and decreased RBCs production (25, 26) in anemia of chronic disease (27, 28).

The red-pulp macrophages of the spleen filter the blood of senescent erythrocytes acting as a quality control mechanism to regulate circulating RBCs and plays important roles during inflammation by serving as a depot of immune cells. Splenic macrophages rapidly clear senescent RBCs from the blood that do not express the CD47 "don't eat me" cell surface signaling molecule and recycle the heme iron they contain. The extracted NTBI is trafficked within red pulp macrophages to be stored in ferritin, utilized by the mitochondria, or exported to other organs, such as the bone marrow.

In the bone marrow, erythroid island macrophages express high levels of iron regulation protein machinery including TfR, HO-1, and FPN to support RBC heme production (29). Osteoclasts are viewed as bone marrow resident macrophages, but non-osteoclast macrophages exist as well (30). Due to their bone repair function, non-osteoclast resident bone macrophages can be examined to determine their specific function as iron-handling regulatory cells. Iron release was shown to be necessary for osteoclastogenesis and general skeletal homeostasis and a population of resident bone marrow macrophages in mice. Thus, iron metabolism is implicated in osteoclasts, bone macrophages that drive bone reabsorption and bone healing.

Lastly, most cells and tissues participate in regulation of iron metabolism, as iron is critical for their function and is potentially harmful if accumulation or depletion is left unchecked. For example, systemic iron metabolism is tightly regulated by the kidney to properly carry out cellular functions such as erythropoiesis *via* erythropoietin, hypoxia signaling, mitochondrial respiration and DNA synthesis, while avoiding

toxicity from free iron (31). Oxidative metabolism associated with renal iron overload is associated with renal cell carcinoma development (32). Ferroptosis, an iron-dependent form of cell death is also identified and associated with renal ischemia-reperfusion injury (33). Cells and tissue with high mitochondrial respiration needs, such as skeletal muscle myocytes, require iron for respiration and myoglobin production. This is due to the role of iron as cofactor for many of the respiratory chain proteins. Macrophages present in muscle express higher levels of haptoglobin, HO-1, CD163, and ferritin, suggesting they sequester myoglobin iron when released from damaged monocytes in response to acute injury and dysregulation of iron homeostasis in muscle can lead to myopathies under iron deficiency or aberrant oxidative stress which contributes to muscular atrophy. After injury, skeletal muscle macrophages upregulate FPN to release iron and contribute to myofiber regeneration, indicating that iron is necessary for muscle healing (24).

MACROPHAGE POLARIZATION AND IRON METABOLISM

As we have shown above, macrophages play a central role in systemic iron metabolism. Cells of the innate immune system more famously play essential roles in inflammation and systemic host defense (34, 35). In response to local damage, detection of pathogens, or stimulation with lipopolysaccharide in the laboratory, macrophages become activated and polarize towards an "M1" like phenotype (35). These classically activated, pro-inflammatory M1 macrophages are characterized by high levels of pro-inflammatory cytokines, such as interferon-γ (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin 1 (IL-1), interleukin 6 (IL-6), produce inducible nitric oxide synthase (iNOS) and high reactive oxygen species (ROS) to promote bactericidal and anti-tumor activity (36). Stimulation with a variety of other cytokines and signaling molecules, including IL-4, IL-10, IL-21, and transforming growth factor – β (TGF-β) triggers a shift in macrophage polarization to an "M2" like phenotype (37). This subset of alternatively activated macrophages function in response to tissue damage and aid in repair, matrix remodeling, angiogenesis, and tumor promotion (38). M2 macrophages contribute to inflammation resolution by initiating wound repair. They produce angiogenesis mediators such as TGF-β, vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) (39).

The iron handling function of macrophages is coupled with shifts in their polarization over the course of their immune response. Macrophages are found polarized in the M1 state and engaged in iron sequestration as part of the acute inflammatory signaling response to bacterial and fungal infection (40, 41). The M1 iron retaining macrophages store ferritin iron and reduce import and export to prevent pathogens and non-self-cells from utilizing iron to proliferate (16). Along with inflammation markers, M1 macrophages are characterized by low expression levels FPN, CD163, and HO-1 while expressing high levels of ferritin to support this iron retention phenotype. With respect to iron metabolism, M2 macrophages demonstrate more of an iron release phenotype at sub-acute stages of immune resolution. Unlike M1, M2 macrophages have high expression levels of FPN, CD163, HO-1, and low expression levels of ferritin, contributing to an iron donating phenotype.

IRON METABOLISM AND MACROPHAGES IN CANCER

Cancer related inflammation is characterized by a polarized distribution of macrophages at the site of the tumor. Macrophages are essential for promotion of cancer during both early and late stages of tumorigenesis. Pro-inflammatory M1 macrophages help counteract tumor growth by eliciting acute immune responses or direct killing *via* phagocytosis, while alternatively activated M2-like macrophages promote immune suppression, angiogenesis and tissue remodeling functions that sustain cancer growth in the sub-acute phase of the immune response (38). Within the TME, the presence of TAMs with a higher M2 to M1 ratio is linked to worse clinical outcomes, including poor survival rate, increased metastasis, and evasion of immune response (32, 42–46). Given these functional consequences of macrophage infiltration in cancer, and their critical role in iron metabolism in other organs of the body in the absence of malignancy, it stands to reason that tumor macrophages also are central to iron metabolism. Indeed, several studies have related immunological response and polarization with macrophage iron handling and cancer iron metabolism. The picture that emerges is one in which a macrophage "Iron Curtain" is established by the TME to directs iron flux and immune response towards tumor growth as shown in **Figure 2**. Here we review recent studies that have focused on the intersection of iron metabolism and macrophage polarization to generalize the cellular, and metabolic traits linking iron and immune response across cancers.

Breast Cancer

Dysregulation of iron metabolism in breast cancer is evident by changes of protein gene expression and accompanies polarization of macrophages towards pro-tumor states. In a normal breast, unique populations of iron-handling M2 macrophages serve to regulate iron levels within the adipose-rich tissue to maintain normal adipogenesis and control peroxidative stress (24). In both murine and human breast tumor tissue, iron accumulation in TAMs is observed, with higher levels of TAM iron being associated with M1 polarization and less invasive cancer, while M2 polarization and reduced iron was observed in invasive breast cancer (47). Correlation of dysfunctional iron metabolism with breast malignancy is supported by differential expression of the high iron FE gene (HFE) gene variants in patients. Patients with major HFE variants have an increased risk of developing breast cancer (48). Iron associated proteins such as hepcidin, FPN, TfR1, and ferritin are highly expressed in breast tissue macrophages and

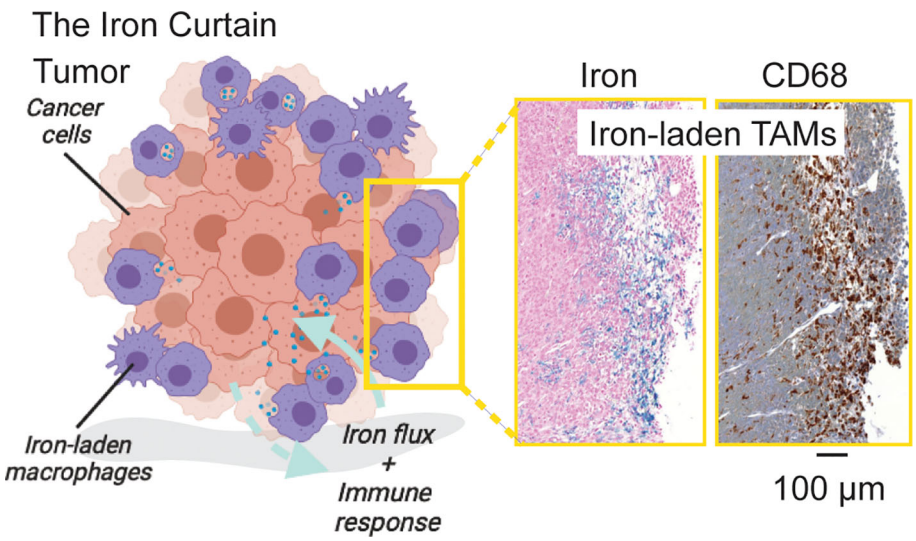

FIGURE 2 | The Iron Curtain. Iron-laden macrophages occupy a unique cellular niche in the tumor where they act as an interfacial boundary mediating systemic and microenvironment metabolic flux and immune response. Prussian Blue iron histochemistry which is specific for ferric iron deposits from endogenous hemosiderin (shown here) or iron nanoparticle contrast agent (not shown) in such macrophages beside pan-macrophage CD68 immunohistochemical staining reveals a distinct spatial pathology of such iron-laden macrophages suggestive of an **"**Iron Curtain**"** where colonies of TAMs exhibiting similar iron accumulation phenotypes form physical borders in the TME.

lymphocytes in patients with the HFE variant gene, suggesting that increases in hepcidin and TfR1 favor tumor growth and lead to more aggressive forms of cancer (48). Findings by Pinnix et al. reveal a substantial reduction in FPN in breast cancer cells, where FPN abundance correlates with metabolically available iron. In this case, high levels of FPN and low hepcidin expression demonstrates a favorable cohort of breast cancer patients with an increased survival rate (49). Macrophages associated with breast cancer express high levels of ferritin light chain that promotes the M2 macrophage phenotype and fosters a pro-tumor environment in breast cancer by secreting ferritin iron into the stroma (43, 45). TAMs in more aggressive forms of breast cancer secrete lipocalin 2 (Lcn2), a small molecule that increases iron concentration and the iron labile pool of cancer cells within the TME to promote growth and resistance to chemotherapy (50, 51). The upregulation of Lcn2 significantly increases the iron concentration at metastatic tumor stages. while Targeting Lcn2 iron secretion for inhibition could starve cancer cells of iron, being a potential therapy to reduce tumor growth.

Central Nervous System (CNS) Cancer

Microglia are the resident macrophages of the CNS that provide immune surveillance and play central roles in iron metabolism. Microglial polarization response to inflammation or wound healing cues accompanies shifts in iron regulatory proteins such as TfR, FPN, ferritin and others that signals the accumulation or release of iron, respectively (24). In brain cancers microglia and blood-derived macrophages have a variety of functional differences in tumor immune response, including iron metabolism. The genes that regulate iron uptake (CD163 and TfR1), metabolism, storage (ferritin light and heavy chain, NCOA4), and catabolism (HO-1) are more highly expressed in bone marrow derived macrophages, revealing that bone marrow derived macrophages sustain an iron-recycling metabolism. These bone marrow derived macrophages are known to infiltrate glioma and glioblastoma where their association with M2 immunosuppressive functions increases towards the middle of the tumor (44, 52). Pathological studies of brain tumors such as brain metastasis from breast cancer show that TAM populations associated with the growing tumor edge have higher iron levels than the center consistent with an M1 to M2 polarization gradient (53). Interestingly, recent studies of leptomeningeal metastasis have shown that similar to breast cancer cells, these cancer cells utilize Lcn2 to obtain iron from macrophages in the CNS space which promotes tumor growth (54). Future studies will shed light on whether this cooption of metabolic function occurring in the CSF is generalizable to other cancers and how macrophages mediate this iron transfer in accord with their polarization state.

Lung Cancer

Alveolar macrophages have been recently implicated in iron trafficking and may exhibit some independence from the hepcidin/FPN axis (24), but macrophage polarization, as well as TfR, ferritin, and FPN expression within the lung predicts iron-recycling activity similar to other localized macrophages (55). Human lung adenocarcinoma and mouse models of Lewis lung carcinoma (LLC) with elevated levels of M2-like TAMs have poorer clinical outcomes, such as increased tumor growth, metastasis, and reduced survival compared with M1-like TAM infiltration (46). In lung adenocarcinoma and LLC, iron and heme can repolarize TAMs from the M2 to M1 cytotoxic

phenotype, leading to direct tumor killing and reduced tumor growth. TAMs loaded with iron have low expression of FPN and are CD163, CD86, and HO-1 positive which are expected to prevent supplying iron to the tumor, thereby inhibiting growth. Patients with lung adenocarcinoma that accumulate iron show more M1 TAMs along with improved survival (42, 46). TAM exposure to heme or iron promotes an anti-cancer immune response by repolarizing TAMs to harness their direct tumor killing ability. Increasing the amount of iron loaded TAMs can be used as a potential therapy to help counteract tumor growth and increase patient survival.

Kidney Cancer

In renal cell carcinoma (RCC), analysis of iron metabolism genes, FPN, ferritin light and heavy chains, IRP2, and TfR1, revealed that these genes are all highly expressed in RCC, similar to other cancers, and TfR1 expression is used as a biomarker of RCC and is associated with worse survival outcomes (56). Iron levels are elevated in RCC as well as genes responsible for iron handling, where this cancer depends on iron for escape of apoptosis and cell cycle arrest. The role of iron in kidney cancer was also linked to the von Hippel Lindau (VHL)/hypoxia inducible factor-α (HIF-α) axis, which is a major regulator of iron metabolism which is dysregulated in RCC. Iron dependency introduced by VHL inactivation reveals an interplay between VHL/HIF-α dysregulation and iron metabolism in RCC. TAMs were shown to have an M2 iron release phenotype with an increase of FPN receptor expression, promoting growth of RCC. To further confirm that iron promotes tumor growth, extracellular fluid from tumor tissue was applied to renal tumor cells, showing that proliferation along with metastasis was enhanced (32). These studies further identified that pathological iron accumulation occurs in TAMs compared with normal iron levels in kidney macrophages. It is intriguing to suppose that TAMs in kidney cancer contribute to tumor proliferation and dissemination by sustaining an iron release phenotype *via* upregulation of FPN and M2 polarization.

Prostate Cancer

Metabolic iron feedback between prostate cancer cells and macrophages provides a putative connection between macrophage infiltration and tumor iron dysfunction observed in prostate cancer. Prostate cancer cells are highly dependent on iron for their proliferation (57). In this iron addicted state, they exhibit a low FPN high TfR phenotype and synthesize hepcidin to induce neighboring tissue iron retention to support their cellular program. Approximately 80% of prostate cancer patients exhibit anemia of chronic disease (ACI), that paradoxically, is associated with iron accumulation in macrophages occurring *via* hepcidin signaling (58). While studies thus far have not definitively linked hepcidin signaling with macrophage polarization in prostate cancer, supporting the idea that prostate cancer cells induce non-heme macrophage iron in tumors clinical studies have found elevated non-heme ferritin tissue iron is associated with malignant tumors compared with benign (59, 60). Iron loading specifically in macrophages has also been observed in prostate cancer. Studies in mouse models show accumulation of macrophage iron in tumors and in systemic iron handling macrophage populations is related to tumor growth and extent of macrophage infiltration in the tumors (61). Generalizing these combined findings prostate cancer TAMs at the invasive margin of the tumor are associated with M1 polarization and high iron levels, where more invasive TAM found deeper in the tumor were primarily M2 polarized and have less iron stores.

Hematological Malignancy

In addition to infiltrating macrophages of solid tumors, it is of interest to consider changes in iron metabolism that are brought about by malignancy involving myeloid cells themselves. Myelodysplastic syndromes constitute a diverse group of hematopoietic stem cell disorders resulting from ineffective hematopoiesis that can lead to acute myeloid leukemia (AML) (62). AML is a malignant hematologic disorder within the bone marrow, blood, and other tissues containing cells of the hematopoietic system (63). Cytotoxic chemotherapy and ineffective hematopoiesis contribute to iron accumulation in these patients and serum ferritin levels have been correlated with an increased risk of relapse (64). Clinical studies have shown that elevated levels of serum ferritin were associated with poor prognosis in patients with hematological malignancies (65). However, serum ferritin concentration is controversial in determining a prognosis in patients with AML because chronic blood transfusion commonly seen in patients with myelodysplastic syndromes and AML that improve anemia and increase the quality of life, also exacerbates iron loading confounding the prognostic value of serum ferritin (63).

IMMUNE RESPONSE AND IRON METABOLISM IN CANCER THERAPY

The above studies provide evidence supporting the central role of iron in tumor growth that is regulated in the microenvironment by macrophages. However, it remains unknown whether changes in iron metabolism stimulate changes in immune status and response, or whether changes in immune status effect macrophage iron handling. Further, it is still unknown whether these metabolic and immunologic responses arise from microenvironmental cues, or if systemic changes in iron metabolism and immune response dictate the iron handling and immune response of TAMs locally. We can derive some insight into these mechanisms by examining therapeutics targeting either iron metabolism or immunity and evaluate their reciprocal effects. Tumor response to drugs such as iron-depleting chelators or iron accumulating nanoparticles can be evaluated to investigate the role of macrophages in transmitting systemic metabolic cues to the tumor-immune microenvironment. Similarly, tumor metabolism modulates immunotherapy response, and given the previously mentioned disruptions in immune cell signaling likely has effects on macrophage iron metabolism both systemically and in the tumor microenvironment such as shown schematically in **Figure 3**.

Here we review several therapy studies that highlight the reciprocity between iron metabolism and immune response and place macrophages at the intersection of immune-metabolic processes as they relate to cancer therapy efficacy.

Tumor Immune Response in Iron Chelation Therapy

Iron chelators, such as clinically used deferoxamine (DFO), deferiprone (DFP) and investigational chelators such as EC1 tropolone, have been shown to inhibit cancer cell growth *via* a variety of mechanisms, involving inhibition of iron-dependent processes *via* their role as enzymatic co-factors, catalysts for reactive species generation, and others (66, 67). Here we review several iron-chelation therapy studies in which immune response of macrophages is induced.

Iron chelators have been shown to alter iron metabolism, macrophage polarization, and immune signaling. Supporting these effects in cancer, DFO administration decreases iron availability from gastric cancer tissue slice cultures, reduces viability of cancer cells, and leads to high iron efflux by decreasing ferritin expression in the TME and TAMs (43). DFO also has effects on immune signaling cytokine factors in cancer. To characterize the relationship between TNF-α and iron metabolism during inflammation, the regulatory interactions between metabolism, cellular differentiation, and TNF-α release was investigated in the human monocyte cell line THP-1 with DFO (68). DFO decreased TNF-α expression and when added to phorbol-12-myristate-13-acetate (PMA) stimulated cells DFO rapidly inhibited TNF-α release. Addition of iron salts to PMA-differentiating cells increased TNF-α mRNA expression and protein release, supporting that iron may mediate the pro-inflammatory response. In other studies of DFO, the role of iron and ferritin have been investigated according to their role modulating MHC-I expression and natural killer cell signaling. Macrophages, critical innate immune cells and iron regulators, express MHC-I and II proteins to present antigens to other lymphocytes such as natural killer cells. When given the iron chelator DFO, MHC-I cell surface expression decreases together with degradation of ferritin and ferritin heavy chain shRNA (69). Additionally, in

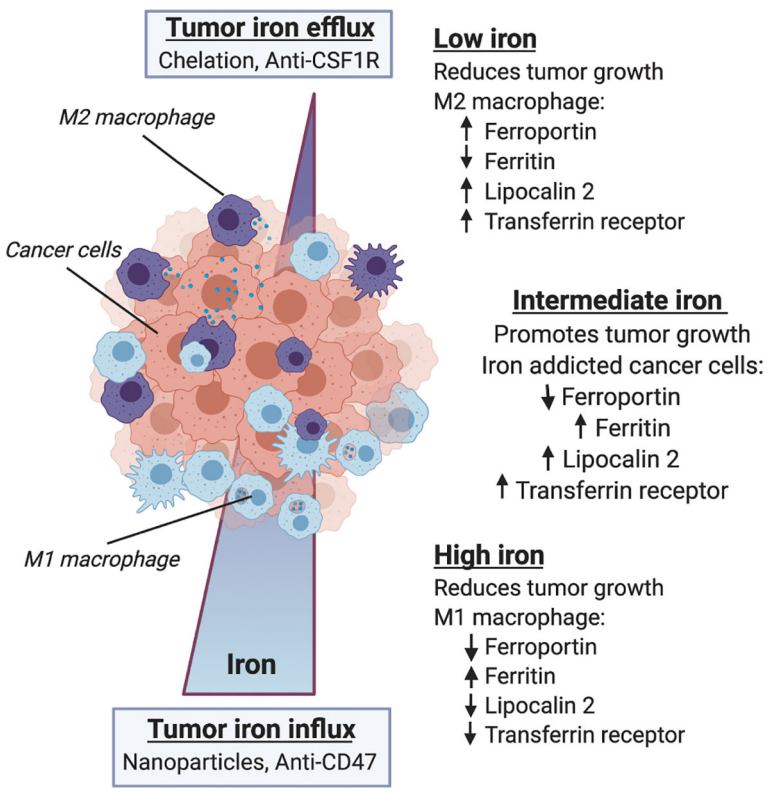

FIGURE 3 | Macrophage iron metabolism and immune response in the tumor microenvironment. Within the microenvironment macrophage phenotype is influenced by iron metabolism, and both iron and immune status are correlated with tumor growth and therapy response. Along a gradient of tumor iron concentration established according to systemic metabolic background and mode of therapy, macrophages can adopt various polarization states spanning a continuum between M1 anti-tumor/proinflammatory activation and M2 pro-tumor wound-healing states. Low tumor iron is associated with reduced tumor growth and favors M2-like macrophage polarization with increased expression of ferroportin, lipocalin 2 and transferrin receptor, and reduced ferritin content. High tumor iron is similarly associated with reduced tumor growth and favors M1-like macrophage polarization with lower expression of ferroportin, lipocalin 2 and transferrin receptor, and increases in ferritin iron storage. Between these two extremes the heterogeneous distribution of macrophage polarization states supports an intermediate iron regime where iron-addicted cancer cells expressing low levels of ferroportin, and high levels of ferritin, lipocalin 2 and transferrin receptor co-opt macrophage's innate role in iron handling to support malignancy.

mouse models of ferritin accumulation, expression of MHC-I cell surface receptors was increased, and DFO reduced ferritin levels and MHC-I. This supports a role for iron chelators in ferritin regulation of iron metabolism in parallel to their inhibitory effects on inflammatory immune signaling factors such as TNF-α and MHC-I.

DFP is another FDA approved small molecule iron chelator prescribed in cases of iron overload. The mechanism of action of deferiprone is similar to DFO, but the two agents different in that DFO chelates extracellular iron while DFP is an intracellular iron chelator. This allows DFP to mobilize cellular iron which is likely related to its effects on cancer cell proliferation. The efficacy of DFP has also been evaluated in the context of macrophage iron accumulation. Macrophages sequester iron as hemosiderin, ferritin protein aggregates, and are known as hemosiderin laden macrophages (HLM) to prevent depletion of iron and maintain levels of cytotoxic free iron. This ability of macrophages to store and metabolize iron puts them in a position to be used as putative iron reservoirs that can be exploited by tumor cells to promote their growth. In vivo treatment of Myc-CaP and TRAMP-C2 mice models of prostate cancer with DFP led to a significant anti-tumor response that was directly proportional to the amount of iron found in tumor, tumor associated macrophages and peripheral macrophages of the liver and spleen as detected by immunopathology and MRI (61). Importantly, these studies enabled the direct *in vivo* observation of the "Iron curtain" which defined a new prognostic biomarker of macrophage iron handling associated with their spatial infiltration and cancer therapy response.

Supporting the role of macrophages in providing iron to support tumor growth, the iron chelator, EC1 was investigated. EC1 is a thiosemicarbazone chelator with a tridentate binding unit that ensures high affinity iron binding. The role of macrophage secreted iron was examined in renal cell carcinoma cell lines and patient samples of tumor progression by applying this novel chelation approach (32). These authors found that iron regulating genes were significantly upregulated in tumors when compared to healthy tissue with tumor cells retaining iron and TAMs exhibited an iron releasing M2-like phenotype. Iron concentration increased in macrophage extracellular fluids which when added to tumors stimulated tumor growth. Macrophage derived iron had pro-tumor functions but was seen to be blocked once EC1 chelator was administered. The addition of EC1 reversed the effect of macrophage conditioned media on cancer cell proliferation and reduced the effect of iron supplementation on tumor cell proliferation and migration. This study shows that the labile iron pool in the TME is regulated by tumor macrophages which drives cancer, and that this interaction can be disrupted by small molecule iron chelators to reduce tumor growth.

Tumor Immune Response to Iron

The above studies highlight the ability of systemically administered iron chelators to interfere with the iron-regulating functions of macrophages, which has complementary effects on microenvironmental immune response and tumor growth. As counterpoint to these studies, we can consider effects of iron accumulation rather than depletion *via* chelation and evaluate how macrophages handle increases in systemic iron concentrations in the tumor microenvironment.

Given the association of iron accumulation with M1-type macrophage function, many investigators have proposed the hypothesis that increasing iron in macrophages as cancer therapy can induce this effect and thereby stimulate anti-tumor immune response, including cytokine formation. Short-term iron overload has been associated with production of TNF-α and long-term iron overload leads to inactivation of macrophages, reflected by decreased TNF-α, IL-6, IL-12, MHC-II, ICAM1, and iNOS expression (70, 71). This leads to induction of anti-inflammatory pathways and impaired control in numerous infectious diseases (72). This also has an impact on the effects of iron chelators, which have been shown to promote the M1-like macrophage phenotype (73, 74). Iron loading may also result in de-activation of macrophages *via* induction of HO-1, resulting in tolerance developing, where the presence of tumor cells is tolerated (75).

Iron nanoparticle injection has been shown to induce M1 polarization, triggering apoptosis of cancer cells *via* an autocrine feedback loop that maintains TNF-α and nitric oxide within the TME to continuously inhibit tumor growth, reduce cell migration, and inhibit pulmonary and hepatic metastasis (76). These nanoparticles are also commonly used as TAM imaging contrast agents in MRI preclinically and clinically as shown in **Figure 4**. Such nanoparticles are able to polarize RAW264.7 macrophages to an M1-like phenotype characterized by elevated expression levels of TNF-α, INOS, CD11b, and CD80. ROS is also enhanced in tumor cells by iron nanoparticles that triggers caspase 9 expression and apoptosis (77). Additionally, nanoparticles alone and in combination with other therapeutic intervention such as photothermal therapy promote tumor associated antigen release and recruitment of T-helper and T-effector cells at the tumor site through repolarization of M2 TAMs to the M1 phenotype (78). This indicates that due to the dependence on both cancer cells and macrophages on iron, these nanoparticles have pleiotropic effects on immune response in tumors that can be exploited to induce transient anti-tumor responses involving oxidative stress.

Iron Metabolism and Macrophage-Targeted Immunotherapy

In the same light that we consider iron chelation as promoting iron release and depletion from the tumor, here we begin with discussion of immunotherapies that are reported to have similar cellular iron reducing effects. The colony stimulating factor 1 receptor (CSF1R) is a key regulator of monocyte function that drives the recruitment of macrophages to the TME and promotes their differentiation to pro-tumorigenic TAMs (79–81). Preclinically, inhibition of CSF1R using monoclonal antibodies (mAbs) or small molecule drugs, such as BLZ945 and PLX3397, have been used to treat malignancies including breast, ovarian, brain, pancreatic and other cancers where they decrease TAM accumulation and promote tumor growth inhibition (47, 82–84). The overexpression of CSF1R has been associated with poor prognosis in many cancers and accumulated evidence has made

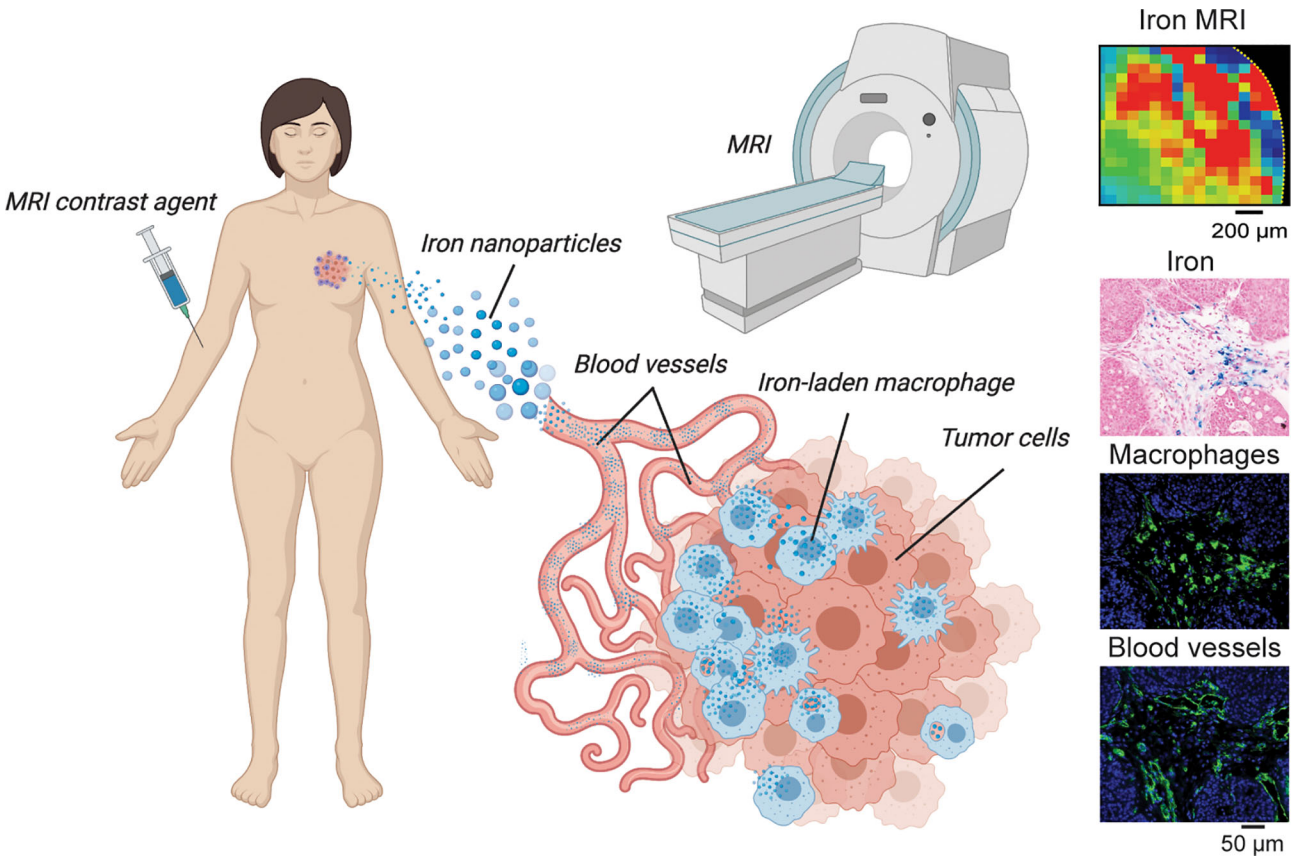

FIGURE 4 | TAM iron imaging in cancer. In many studies focusing on iron-laden population of macrophages magnetic resonance imaging (MRI) is used to localize and monitor these cells during tumor growth and immunometabolic therapy response. Here, iron nanoparticle contrast agents are injected intravenously and subsequently are delivered to the tumor where TAM phagocytosis occurs. Quantitative iron MRI provides *in vivo* quantitative detection of iron containing macrophages in animal models and patients. Cytological imaging confirms associations between iron deposits within macrophage accumulation and vascular infiltration.

it clear that combination of CSF1R immunotherapy with other standard-of-care often improves therapeutic response which is currently of clinical interest (85, 86).

On the other side of the coin, we can also consider potential effects of immunotherapies on inducing cellular iron accumulation in the tumor. For example, CD47 expression is an independent poor prognostic marker and serves as the ligand for signal-regulatory protein alpha (SIRPα), present on macrophages and other phagocytic cells that inhibits phagocytosis when activated. Monoclonal antibodies against CD47 (CD47 mAb) inhibit the interaction between SIRPα and CD47 effectively blocking the "don't eat me" signal to activate TAMs and promote macrophage phagocytosis of, for example, of malignant osteosarcoma cells (87), and self-renewing leukemia stem cells (LSC) that promote AML (88). To prevent tumor dissemination, CD47 mAb may be administered systemically or locally upon surgical resection to eliminate circulating tumor cells (89). Given the role of macrophages in recycling iron *via* cellular phagocytosis, anti-CD47 immunotherapy likely effects tumor iron metabolism. This has been investigated by Daldrup-Link and coworkers where combination of doxorubicin and CD47 mAb significantly inhibited tumor growth and improved survival in a manner proportional to the increase in iron metabolism that was detected by increases in TAM iron using histology and MRI (90). Outside of the tumor microenvironment, one notable effect on iron metabolism that occurs with anti-CD47 therapy is the onset of systemic anemia which likely has contributions from over-accumulation of iron in tissue macrophages that can lead to reductions of the systemic availability of iron for RBC heme synthesis (91–93). This effect would suggest that anti-CSF1R also effects systemic iron metabolism whereby reduced macrophage iron accumulation vis a vis cellular macrophage depletion would increase peripheral iron availability, but this mechanism has yet to be proven.

Immune Checkpoint Blockade and Iron Metabolism

The ligand for programmed cell death protein 1 (PD-L1) is frequently overexpressed on tumor cells enabling their escape from immune surveillance. Monoclonal antibodies blocking PD-L1/PD-1, so called immune checkpoint blockade inhibitors (ICB), have been clinically shown to have efficacy in patients with

a variety of cancers by activating T lymphocytes (94, 95). While iron metabolism *per se* has not been an area of focus in adaptive immunotherapy to date, research on T lymphocyte biology have noted that iron metabolism plays an important role in T cell migration and activation throughout the body (25, 96, 97). Specific involvement of macrophage iron metabolism in instances of ICB nonetheless can be speculated from recent studies. For example, along with T cells, PD-1 blockade rescues macrophage and dendritic cell function in the TME, activating the immune cells against the tumor. It was shown that TAM PD-1 expression is negatively correlated with phagocytotic potency against tumor cells, but blockage of PD-1/PD-L1 *in vivo* increased macrophage phagocytosis, reduced tumor growth, and increased survival (98). Considering the effects that anti-CD47 has on macrophage iron accumulation, these similar observations during ICB suggest a corresponding effect on macrophage iron accumulation. Additionally, anemia has been reported as a correctable but significant effect in a clinical ICB trials that further suggests a parallel between systemic iron metabolism in ICB and the metabolic status observed during direct targeting of macrophage by other immunotherapies (99, 100).

An area of additional relevance relating effect of ICB with iron metabolism comes from new developments in the field of cell death, specifically in the context of ferroptosis. Ferroptosis is a novel cell death mechanism that proceeds *via* iron catalyzed peroxidation of polyunsaturated lipids and its' regulation by factor, such as system Xc^- and the glutathione peroxidase 4 enzyme (GPX4) which maintains cellular redox homeostasis (101). Sensitivity of cancer to this cell death mechanism is attributed to a variety of factors such as tissue iron, lipid composition, and the expression of redox regulating proteins (102–104). Thus, macrophages likely represent a central cellular player in the mechanism as they mediate iron storage and release in the tissue microenvironment. Indeed, the iron accumulating properties of M1 macrophages have been implicated in driving ferroptosis by harboring higher concentrations of iron that under appropriate conditions brought about by iron challenge or ferroptosis-targeted agents that block reactive species scavengers, can increase levels of lipid peroxides and sustain ferroptotic cell death (105–108). In ICB cancer immunotherapy, tumor growth inhibition caused by the drug is associated with increased lipid peroxidation and can be further amplified by ferroptosis-targeted drugs and significantly inhibited by iron chelators (109). While this suggests that modulation of iron metabolism is a clear avenue for altering immunotherapy efficacy, the field of ferroptosis must still address some outstanding questions regarding tolerance of ferroptosis-targeted agents, and more fundamentally, how to reconcile the association of increased iron and lipid peroxidation in driving ferroptosis with the same associations of these metabolic factors in also increasing oxidative stress and causing peroxidative DNA damage that promotes carcinogenesis (76, 110, 111). The balance between ferroptotic cell death and peroxidative carcinogenesis in the context of tumor susceptibility to iron chelators is of special significance regarding efficacy of susceptibility to ferroptosis-targeted drugs and their combinations with ICB in the context of iron metabolism and immune response. These observations support further investigation into macrophage iron handling as a critical factor in ICB response that can potentially be harnessed by tapping into ferroptosis pathways to improve this mode of cancer therapy.

Iron Metabolism and Adoptive Cell Therapy

Adoptive cell therapy seeks to modulate the immune response by engineering immune cells from the patient and reintroducing them to reset the patient's immune system (112). Currently, the most pursued adoptive cell therapy involves chimeric antigen receptor (CAR) T cells that are engineered to express receptor binding motifs tethered to T cell activating constructs that when re-introduced to a patient bind and eliminate specific malignancies such as leukemias and lymphomas, and increasingly, solid tumor cancers. One major drawback of these therapies has been patient toxicity due to the rapid and amplified immune response these agents induce. This so-called cytokine release syndrome, or storm, that results from these infusions and their target interactions is part of the acute phase response (113). One of the signatures of the acute phase response linking adoptive cell therapy to iron metabolism is elevated serum ferritin (114, 115). Definitive connections between this metabolic iron response in cytokine storm and macrophages have not been drawn in CAR-T cell therapy. However, investigators have shown that macrophages are central mediators of the cytokine storm and that inhibiting their signaling, for example by targeting IL-6 or granulocyte-macrophage colony stimulating factor (GM-CSF), can alleviate these over-responses while maintaining therapeutic efficacy (116, 117). Further, iron homeostasis can affect migration, function, and differentiation of T lymphocytes, therefore not only does iron metabolism effect TAMs, but also T cells and other tumor-infiltrating lymphocytes are directly and/or indirectly affected. Iron has been shown to trigger CD4+ T cell differentiation and alter CD8+ T cell expansion. Immunosuppressive effects of iron on T cells have been described in individuals with hereditary or transfusion mediated iron overload, where these patients have altered T cell numbers and function (118). In tumor infiltrating lymphocytes, iron may impair the proliferation, differentiation or maturation by generating mitochondrial ROS, resulting in cell death. Pursuit of this role of macrophages and lymphocytes in mediating ferritin iron efflux is likely to be an important correlate of adoptive cell therapy and ensuing cellular responses.

CONCLUDING REMARKS

Macrophage infiltration in cancer is associated with poor patient outcomes and therapy resistance. There is an incomplete understanding of the balance between macrophage polarization and functional phenotype related to these effects. Dysfunctional primary metabolism is a hallmark of cancer cells that is now accepted as contributing to the pro- versus anti-tumor response

decisions of macrophages. As we review above, it is evident that iron metabolism plays a major role in the cellular plasticity of tumor macrophages and their involvement in cancer. Here we considered two aspects of macrophage function to advance our understanding of this interaction space. The first is the role of macrophages as central regulators of systemic iron metabolism. The second is their contribution to the immune landscape of the tumor microenvironment. At the intersection of these two critical roles, we focused on a metabolically distinct cellular population of iron-containing macrophages. We find that unique populations of macrophages throughout the body and in tumors perform similar iron handling functions where macrophage iron accumulation and release in the tissue is synchronized with the phase of systemic and microenvironmental immune responses. In tumors specifically, sites of inflammation are indicated by M1 polarized tumor-associated macrophages (TAM) which accumulate iron, accumulation while release of iron to cancer cells by M2 polarized macrophages supports the iron-addicted metabolic program of the cancer cells within the TME.

To fully establish the role of macrophage iron metabolism in cancer, however, more research is needed to answer pressing questions such as: How spatial heterogeneity in tumor microenvironment influences the molecular networks that allow iron exchange between cancer cells and macrophages? How iron flux in macrophages changes their communication with other immune cell and stromal components? How systemic metabolic dynamics of iron alters macrophage plasticity longitudinally over the many complex steps of tumorigenesis, cancer progression, metastasis, and therapeutic response? In the future we will tackle these multiscale phenomena from a bench-to-beside approach, making use of translational advances in cytology, bioinformatics, and imaging to reveal new ways to therapeutically harness these targets and translate these insights to the clinic. Our insights here support these further investigations into the crosstalk between iron metabolism and immune response and strongly warrants further development of anti-cancer therapies that target this immunometabolic axis.

AUTHOR CONTRIBUTIONS

AD and AL conducted literature searches, prepared figures, and wrote the article. All authors contributed to the article and approved the submitted version.

FUNDING

We gratefully acknowledge funding from the Carol M. Baldwin Breast Cancer Research Fund, Stony Brook School of Medicine Departments of Radiology and Pharmacological Sciences, and the Bahl Center for Metabolomics and Imaging at the Stony Brook Cancer Center.

ACKNOWLEDGMENTS

We would like to thank Dr. Jason Koutcher of Memorial Sloan Kettering Cancer Center for critical reading of this review.

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Conflict of Interest: 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.

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frontiers in Oncology

ORIGINAL RESEARCH
published: 19 May 2021
doi: 10.3389/fonc.2021.638856

The Ferroxidase Hephaestin in Lung Cancer: Pathological Significance and Prognostic Value

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Hephaestin (HEPH) belongs to a group of exocytoplasmic ferroxidases which contribute to cellular iron homeostasis by favouring its export. Down-regulation of HEPH expression, possibly by stimulating cell proliferation due to an increase in iron availability, has shown to correlate with poor survival in breast cancer. The lung is particularly sensitive to iron-induced oxidative stress, given the high oxygen tension present, however, HEPH distribution in lung cancer and its influence on prognosis have not been investigated yet. In this study we explored the prognostic value of HEPH and its expression pattern in the most prevalent histotypes of lung cancers, namely lung adenocarcinoma and lung squamous cell carcinoma. *In silico* analyses, based on UALCAN, Gene Expression Profiling Interactive Analysis (GEPIA) and Kaplan–Meier plotter bioinformatics, revealed a significant correlation between higher levels of HEPH expression and favorable prognosis, in both cancer histotypes. Moreover, TIMER web platform showed a statistically significant association between HEPH expression and cell elements belonging to the tumor microenvironment identified as endothelial cells and a subpopulation of cancer-associated fibroblasts, further confirmed by double immunohistochemical labeling with cell type specific markers. Taken together, these data shed a light on the complex mechanisms of local iron handling lung cancer can exploit to support tumorigenesis.

Keywords: lung cancer, hephaestin, iron, immunohistochemistry, bioinformatics

OPEN ACCESS

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

Reviewed by:
Gregory Jon Anderson, The University of Queensland, Australia
Manfred Nairz, Innsbruck Medical University, Austria

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

Received: *07 December 2020*
Accepted: *26 April 2021*
Published: *19 May 2021*

Citation:
Zacchi P, Belmonte B, Mangogna A, Morello G, Scola L, Martorana A and Borelli V (2021) The Ferroxidase Hephaestin in Lung Cancer: Pathological Significance and Prognostic Value. Front. Oncol. 11:638856. doi: 10.3389/fonc.2021.638856

INTRODUCTION

Lung cancer represents the most frequent malignant neoplasm in most countries and the leading cause of death worldwide in both sexes (1). The incidence of lung cancer is low in people aged below 40 years but it dramatically increases up to ages 60–65 years in most populations. The most common subtype of lung cancer is non-small cell lung cancer (NSCLC; 85%), the most prevalent form being lung adenocarcinoma (LUAD), followed by lung squamous cell carcinoma (LUSC) and large cell carcinoma (2). Smoking status is certainly the most important causative link in lung cancer development even though air pollution represents another paramount source of risk factor (3).

Airborne Particulate matter (PM), in particular the small size components (PM_{10}, $PM_{2.5}$ and ultrafine particles-UFP), which include combustion products, soot, exhaust emission from vehicles and industrial processes, have attracted attention mainly for two reasons: firstly, due to their small size, these particles remain suspended in the air for quite a long time, thus increasing the chance of being inhaled; secondly, these particles are vehicles for chemical compounds, in particular transition metals, since iron is present in significant concentration (4). Iron is also found in cigarette smoke, the strongest causative link to pulmonary pathology (5, 6), and in asbestos fibers, which are the most frequent cause of occupational cancer (7).

Iron toxicity derives from its high redox cycling reactivity which can drive the production of free radical species (ROS) known to promote many aspects of tumor development and progression (8). The lung is extremely sensitive to metal-induced oxidative stress due to its unique role in the massive transfer of oxygen into the bloodstream (9). Therefore, as a protective strategy to prevent ROS generation, lung epithelial cells have developed a tight control on iron import, storage and export in order to keep intracellular iron concentration low, while sustaining the metabolic demand (10). Efficient iron uptake and intracellular sequestration can limit its toxicity, but if iron import exceeds the long-term storage capacity of the cell, as it occurs in iron overload conditions, the chances it may mobilize increase, resulting in oxidative stress and cell damage. Iron export mechanisms are therefore necessary to prevent excessive intracellular accumulation, as may occur when exogenous iron supplies increase as a result of airborne pollutants inhalation. The only known non-heme iron export pathway relies on the activity of the transmembrane ferrous iron transporter Ferroportin 1 (FPN1), also known as solute carrier family 40 member 1 (SLC40A1) (11), in conjunction with members of the multicopper ferroxidases family, which are required to oxidize ferrous iron to its ferric form (12). Only three multi-copper oxidases have been identified so far, namely ceruloplasmin (CP), hephaestin (HEPH) and zyklopen (ZP) (13–15). These ferroxidases promote iron transport in different tissues: HEPH is mostly expressed in the small intestine (14) but it is also present in other tissues (16); CP is mainly found as a soluble serum protein, but it is also membrane-bound *via* a glycosylphosphatidylinositol (GPI)-anchor in astrocytes and kidney (17, 18); ZP has been proposed to be involved in placental iron transport, but this has not yet been verified (19). In enterocytes FPN1, functionally associated with HEPH, allows the translocation of iron across the basolateral membrane and its release into the bloodstream (20). In the lung, instead, FPN1 is mainly expressed in the apical membrane of the airway epithelium (21) where it is believed to promote iron release into the airways or the lumen of the alveoli to meet the need for detoxification. This egress pathway has been shown to be compromised in various types of cancers (22). In particular FPN1 mRNA expression levels appeared significantly down-regulated in lung tumor, as compared to matched healthy tissue, a condition that is likely to guarantee an increase in the intracellular labile iron pool necessary for all metabolic processes involved in cell proliferation (23).

The role played by HEPH in iron metabolism in lung is still poorly characterized, and so is its possible contribution to lung carcinogenesis and growth. We recently identified a single-nucleotide polymorphism within *HEPH* gene, leading to a missense variation of this multicopper ferroxidase, which confers protection against asbestos-dependent malignant pleural mesothelioma and lung carcinoma in exposed subjects (24, 25). Moreover, in breast cancer HEPH expression has been shown to be down-regulated by the histone methyltransferase G9a, leading to changes in iron homeostasis that burst cancer growth (26).

In the current study, we examined the expression and prognostic value of HEPH expression in LUAD and LUSC patients in databases such as UALCAN, GEPIA and Kaplan–Meier plotter. Moreover, we investigated the correlation of HEPH expression with tumor-infiltrating immune and non-immune cells that characterize the tumor microenvironment, *via* Tumor Immune Estimation Resource (TIMER). Finally, we assessed the distribution of endogenous HEPH in lung cancer tissues. Taken together, these data further support the key role played by iron dysregulation in the tumor microenvironment of lung malignancies. In this context HEPH expression, if further confirmed by retrospective studies on a broader cohort of patients, could serve as a potential prognostic marker in lung cancer pathogenesis.

MATERIALS AND METHODS

Gene Expression and Survival Analysis

Our analysis focused on the prognostic value of the *HEPH* gene in lung adenocarcinoma (LUAD) and in lung squamous cell carcinoma (LUSC). The expression level of the gene in different carcinomas was analyzed using UALCAN (http://ualcan.path.uab.edu) and GEPIA (http://gepia.cancer-pku.cn). Those tools estimate the effect of gene expression level on the patient survival, as well as being web resources for analyzing cancer transcriptome data (27, 28). We compared the differences in mRNA level between cancers and normal tissue, using genomics data from "The Cancer Genome Atlas" (TCGA lung). The prognostic significance of *HEPH* mRNA expression and survival in LUAD and LUSC were analyzed by Kaplan–Meier plotter (https://kmplot.com/analysis). The Kaplan–Meier plotter uses genomic data from the Gene Expression Omnibus and the European Genome-phenome Archive to generate survival probability plots and to perform survival analysis. The same analysis was performed for the following cell-type specific genes: *ACTA2* (α-SMA), a marker of vascular muscular cells and pericytes (29); fibroblasts activation protein (FAP), platelet-derived growth factor receptor-α/β (*PDGFRA/B*), biological markers for CAFs (30); *PECAM1* (*CD31*) and von Willebrand Factor (*vWF*) markers for endothelial cells (31, 32). The hazard ratio with 95% confidence intervals and log-rank *p*-value were also computed.

Protein Expression Analysis

The expression of HEPH proteins between cancer and normal tissue were analyzed using UALCAN, which provides a protein

expression analysis option using data from the Clinical Proteomic Tumor Analysis Consortium (CPTAC) Confirmatory/Discovery dataset (33). The *CPTAC* dataset relies on the RPPA platform, which involves micro-blots of protein lysates from multiple samples of tissues on a single array, with each sample represented by at least one spot. Each array is incubated with one specific antibody, in order to detect the relative expression of the corresponding protein across many samples simultaneously. Protein levels are quantitated by mass spectrometry-based proteomics analysis. Unfortunately, at the time of writing, the UALCAN tool only provided data for the LUAD histotype.

TIMER Database Analysis

TIMER is a comprehensive resource for systematic analysis of immune infiltrates across diverse cancer types (www.cistrome.shinyapps.io/timer/) (34). TIMER applies a statistical method to infer the abundance of tumor-infiltrating immune cells (TIICs) from gene expression profiles using data from the TCGA dataset (35). We analyzed *HEPH* expression in lung cancers, and the correlation between its expression and the abundance of immune infiltrates, including B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, cancer associated fibroblasts and endothelial cells *via* gene modules. These gene markers are referenced in prior studies. Gene expression levels against tumor purity are also displayed (36, 37). Tumor purity is defined as the proportion of cancer cells present in the tumor tissue, and reflects the characteristics of tumor microenvironment. Low tumor purity is associated with a consistent recruitment of diverse kinds of tumor-infiltrating immune cells as well as stromal cells (fibroblasts, endothelial cells and pericytes). The computational algorithms of TIMER take "tumor purity" into account when analyzing a specific gene expression profile. Thus, a gene characterized by a negative association with the tumor purity parameter is expected to be expressed in cells of the microenvironment, while a gene that shows a positive correlation is expected to be mostly expressed by cancer cells. The correlation module generated the expression scatter plots between several genes and defined genes of TIICs in chosen carcinomas, together with the Spearman's correlation and the estimated statistical significance. Several genes were used for the x-axis, and the related marker genes were represented on the y-axis as genes of TIICs. The gene expression level was displayed with log2 RSEM.

Statistical Analysis

Survival curves were generated by the Kaplan–Meier plotter (38). All results are displayed with *p*-values from a log-rank test. *p*-values <0.05 were considered significant. In TIMER, the correlation of gene expression was evaluated by Spearman's correlation and statistical significance, and the strength of the correlation was determined using the following guide for the absolute value: 0.00–0.19 "very weak," 0.20–0.39 "weak," 0.40–0.59 "moderate," 0.60–0.79 "strong," 0.80–1.0 "very strong".

Immunohistochemistry Analysis on Tumor Tissues

All lung cancer tissue samples for this study were collected according to the Helsinki Declaration and the study was approved by the University of Palermo Ethical Review Board (approval number 09/2018). A specific informed consent was not required at the time of tissue sample collection for immunohistochemical analysis of archival tissue sections, since the patients were not identified and genetic analysis was not carried out. Surgically removed malignant tissue samples, together with the adjacent non-tumor tissue, were selected for immunohistochemical analysis for HEPH expression. Invasive malignant neoplasia specimens included the two most represented histotypes including LUAD and LUSC. Tissue sections were obtained from at least ten different patients for each histotype. The study was approved by the Institutional review board of the University of Palermo (09/2018).

Immunohistochemistry was carried out on FFPE human tissue sections. Briefly, 4 micron-thick sections were cut from paraffin blocks, dried, de-waxed and rehydrated. The antigen unmasking technique was performed using Target Retrieval Solutions, pH = 6 EDTA-based buffer in thermostatic bath at 98°C for 30 min. After the sections were brought at room temperature, neutralization of endogenous peroxidase with 3% H_2O_2 and protein blocking by a specific protein block, were performed. For HEPH immunostaining, sections were probed with mouse monoclonal anti-human HEPH (dilution 1:100, pH 6, Clone sc-365365 Santa Cruz Biotecnology) overnight at 4°C. Antibody–Antigen recognition was detected using Novolink Polymer Detection Systems (Novocastra Leica Biosystems, Newcastle), and high sensitivity AEC (3-Amino9-Ethylcarbazole) as chromogen. Slides were counterstained with Harris Hematoxylin (Novocastra, Ltd).

For double-labeling experiments, sections were additionally probed with rabbit polyclonal anti-human CD31 (dilution 1:50, pH 9, ab28364 Abcam), rabbit monoclonal anti-human PDGFRβ (dilution 1:250, pH 6, clone Y92, ab32570 Abcam) and rabbit polyclonal anti-Ferroportin (1:1,000, pH 6, PA5-64232, Invitrogen) and anti-Myeloperoxidase antibody (1:50, pH 6, ab9535 Abcam). Staining was carried out *via* Novolink Polymer Detection Systems (Novocastra, Leica Biosystems) and DAB (3,3′ -Diaminobenzidine; Dako, Denmark) substrate-chromogen. All the sections were analyzed under Zeiss Axio Scope A1 optical microscope (Zeiss, Germany) and microphotographs were acquired using an Axiocam 503 Color digital camera with the ZEN2 imaging software (Zeiss Germany).

RESULTS

The mRNA Expression Levels of HEPH in Different Types of Human Cancers

Ferroxidase HEPH has recently been shown to play a role in breast tumor cell growth; in particular its decreased expression has been significantly correlated with poor survival in affected patients (26). In order to expand the analysis to other cancer types, we examined HEPH expression using UALCAN to analyse TCGA RNA-sequencing and patients' clinical data from 33 different cancer types, including several metastatic tumors (34). This analysis revealed that a significant down-regulation of HEPH mRNA expression levels is found in several other malignancies such

as BLCA (bladder urothelial carcinoma), BRCA (breast invasive carcinoma), COAD (colon adenocarcinoma), KICH (kidney chromophobe), KIRP (kidney renal clear cell carcinoma), LIHC (liver hepatocellular carcinoma), LUAD (lung adenocarcinoma), LUSC (lung squamous adenocarcinoma), PRAD (prostate adenocarcinoma), READ (rectum adenocarcinoma), and UCEC (uterine corpus endometrial carcinoma) compared to the corresponding normal tissues (**Figure 1A**).

Given our interest in better understanding the role iron dysregulation may exert in lung cancer development and prognosis, we evaluated HEPH mRNA expression levels in the most prevalent histological types, LUAD and LUSC, as compared to normal tissue, utilizing the GEPIA database. Consistent with the previous analysis, a significant decrease in HEPH mRNA expression was found in LUAD and LUSC compared to healthy controls (**Figure 1B**). This reduction was confirmed at protein level only for the LUAD histotype based on the UALCAN dataset (**Figure 1C**), since correspondent proteomic data for LUSC are still not available.

To investigate the correlation between HEPH expression and patient outcome we employed the Kaplan–Meier overall survival curves to establish and compare the survival differences between patients with high and low expression of the ferroxidase (grouped according "Auto select best cutoff") (**Figure 1D**). In both the LUAD and LUSC datasets, the high expression group had a significantly longer overall survival than the low expression group, thus indicating that higher HEPH expression correlates with better prognosis.

HEPH Expression Is Correlated Mostly With Non-Immune Infiltration

It is well established that cancer cells are characterized by an iron-seeking phenotype, which is fundamental to support the enhanced metabolic demand characteristic of actively proliferating cells (39). The increased request in iron supply is met not only by up-regulating iron import pathways while down-regulating storage and export routes, but also by altering how other cell types of the tumor microenvironment, including immune cells, endothelial cells, pericytes and fibroblasts, metabolize iron (40, 41). We therefore investigated the correlations of HEPH expression and immune and non-immune infiltration levels, using the TIMER web resource (34). In particular, we assessed B cells, CD4+ T cells, CD8+ T cells, macrophages and dendritic cells, as immune infiltrates, while cancer associated fibroblasts and endothelial cells were analysed as infiltrating non-immune cell types. The results showed that, in both types of lung cancer, HEPH expression had a significant negative correlation with tumor purity, the parameter that identifies the proportion of cancer cells present in the tumor tissue (**Figure 2**). In addition, HEPH expression showed a very weak correlation with all infiltrating immune elements tested (**Table 1**), while a strong positive correlation was found only with cancer associated fibroblasts (CAFs) and endothelial cells (ECs) (**Figure 2**).

CAFs are the most abundant cells in solid cancer. They can be derived from several sources including activation of resident fibroblasts (42), epithelial-mesenchymal transition of epithelial cells (43), endothelial–mesenchymal transition of resident endothelial cells (44). Compared to normal fibroblasts they are characterized by enhanced proliferative and migratory features, and they are also more metabolically active. Tumor endothelial cells are the cells lining the tumor-associated blood vessels that provide nutrition and oxygen to the tumor, contributing to its growth and development. They also constitute one of the main sources of cancer-associated fibroblasts (CAFs).

To further characterize the relationship between HEPH and these infiltrating cells in lung malignancies, we explore the correlation between HEPH and a list of marker sets known to be widely used to identified CAFs and ECs, using the TIMER Gene Correlation module. In particular, we used α-SMA (ACTA2, also marker for vascular muscular cells and pericytes), fibroblasts activation protein (FAP, also expressed in a subset of CD45+ immune cells), and platelet-derived growth factor receptor-α/β (PDGFRA/B) as biological markers for CAFs (**Figure 3A**); PECAM1 (CD31) and von Willebrand Factor (vWF) as markers for endothelial cells (**Figure 3B**). After adjusting the correlation by tumor purity, HEPH expression level was significantly correlated with all tested marker sets (**Figure 3**).

Interestingly, we also found that the mRNA expression level of all these marker genes, with the only exception of FAP, were significantly down-regulated in both lung malignancies, as compared to paired normal tissues, based on GEPIA datasets (**Figure 4A**). Moreover, Kaplan–Meier analysis indicated that high expression of ACTA2 and PDGFRA, as well as PECAM1 and vWF, was associated with better overall survival, as it is for HEPH expression (**Figure 4B**).

Distribution of HEPH in Clinical LUAD and LUSC Specimens

Based on the results obtained from the TIMER database analysis, we set out to better understand the distribution of HEPH in a series of specimens of LUAD and LUSC upon ferroxidase immunohistochemical labeling. Immunolocalization on normal lung specimes showed that HEPH was expressed by several cell types (**Figures 5A, C**): the epithelial cells of the alveoli, mainly type II pneumocytes, identified based on their round-shape morphology (**Figure 5B**, arrow-head); the epithelial cells of the bronchiole together with the smooth muscle fibers surrounding the bronchiolar epithelium (**Figure 5B**, black arrow); the endothelial cells of the micro vessels (**Figures 5C, D**). HEPH was mainly observed in the cytoplasm.

HEPH distribution in cancer tissues, appeared to be quite different in the two malignancies. Cancer cells in most of the analyzed LUAD specimens were totally lacking HEPH (**Figure 5E**), even though a few clumps of neoplastic cells surrounded by stroma, the so-called tumor nests (**Figure 5F**), positive to HEPH labeling, could be observed. In LUSC, instead, approximately 30% of cancer cells, identifiable by their characteristic large polygonal shape, expressed the ferroxidase to variable extent (**Figures 5G, H**). Cancer cells HEPH staining was mainly cytosolic but, in some cases, also it was also clearly detected at the cell membrane (**Supplemental Figure 1A**). In both malignancies, HEPH expression was quite intense on the vascular endothelium in the peri-tumoral tissues (**Figures 6A, B**,

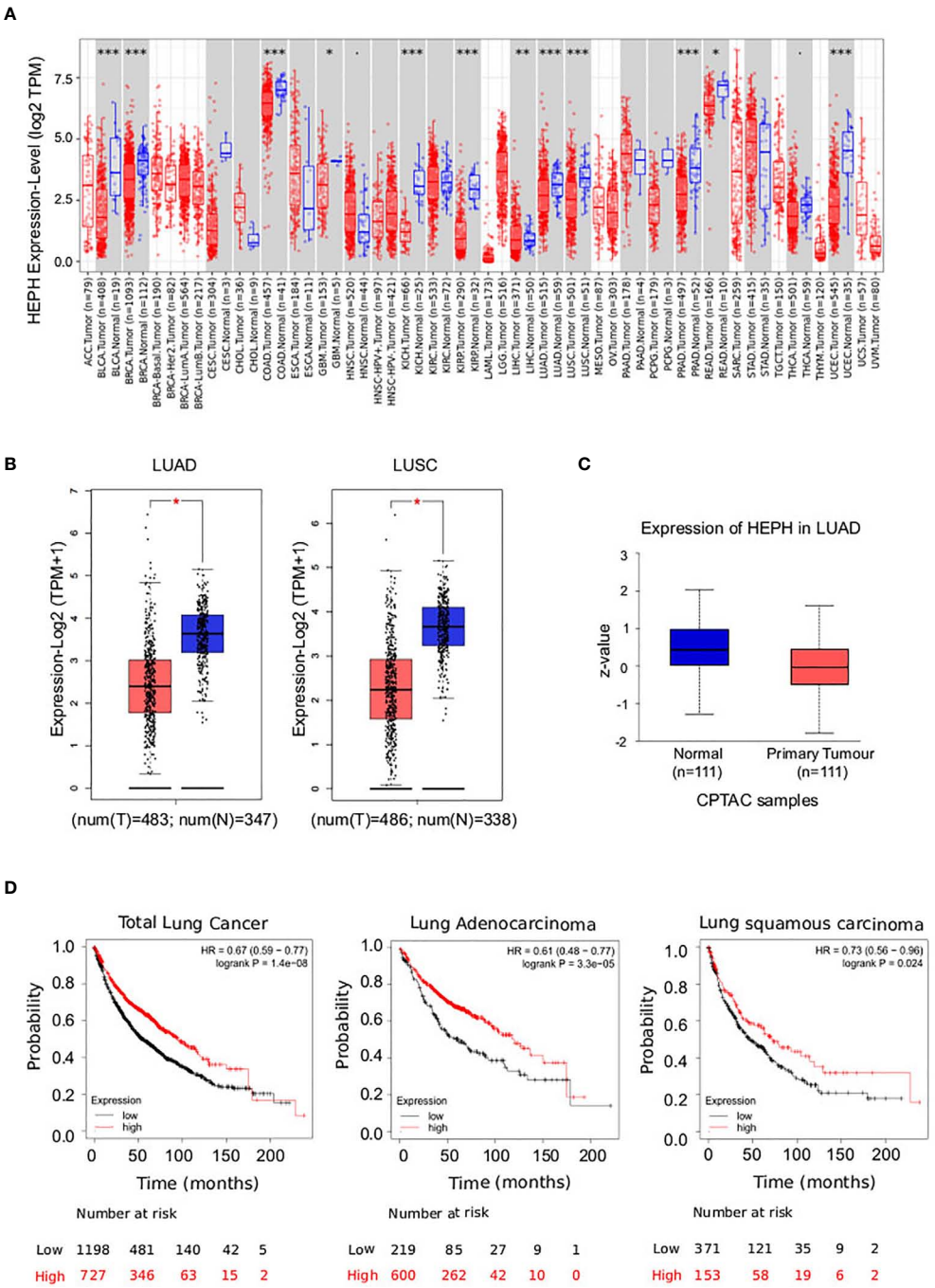

FIGURE 1 | Pathological significance of HEPH expression in different types of human cancer, and in-depth evaluation in LUAD and LUSC. **(A)** Human HEPH expression levels in different tumor types from TCGA database were determined by TIMER (*P < 0.05, **P < 0.01, ***P < 0.001). **(B)** HEPH mRNA expression comparisons between normal (blue) and tumor tissues (red) obtained from the GEPIA web tool. **(C)** HEPH protein expression comparison between normal and tumor tissues obtained from the UALCAN web tool (Wilcoxon test). P-value <0.05 was used to assess differences. **(D)** Survival analyses of HEPH by Kaplan–Meier estimator with log-rank test obtained from the Kaplan–Meier plotter web tool. Survival differences are compared between patients with high (red) and low (black) HEPH expression (grouped according to Auto select best cut-off). H, high expression; L, low expression.

LUAD and LUSC panels **C**, **D**), as identified by PECAM1 (also known as CD-31)/HEPH double-labeling immunoreactivities (**Figures 6I, J**, LUAD panels and LUSC panels **K, L**).

Moreover, the tumor masses in LUSC and LUAD were also characterized by the presence of HEPH positive mesenchymal cells exhibiting spindle-shaped morphology, reminiscent of

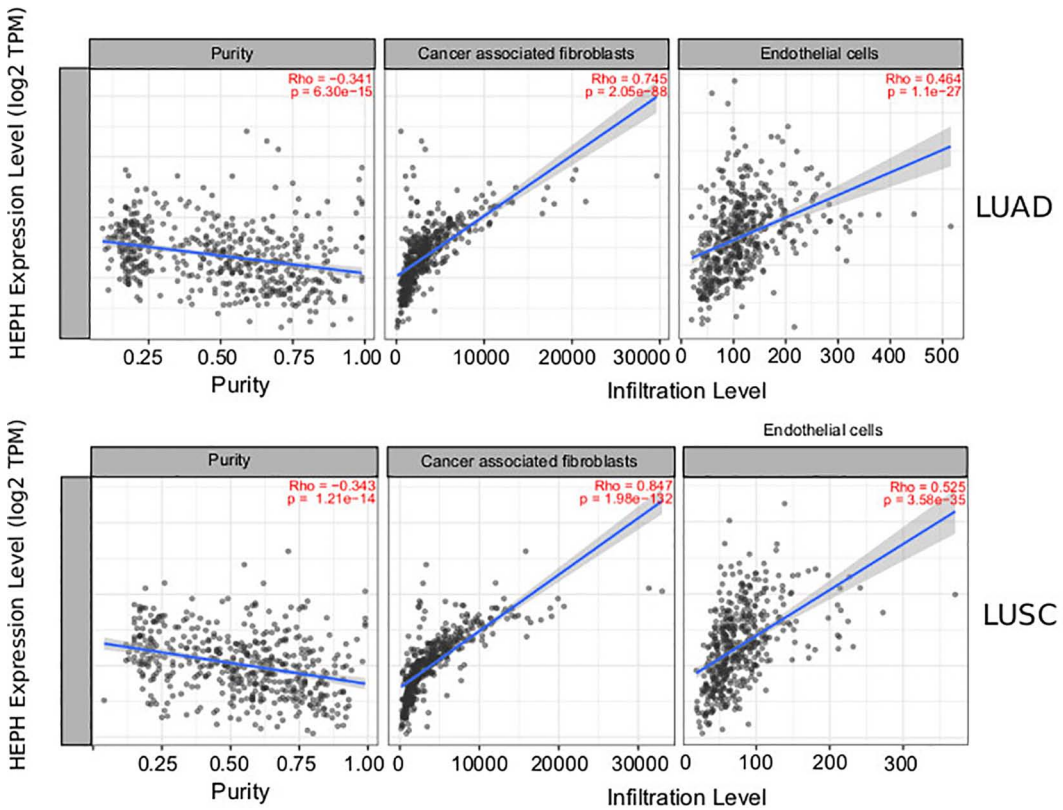

FIGURE 2 | Correlation of HEPH expression with infiltration level of non-immune cells in LUAD and LUSC. HEPH expression is significantly negatively correlated to tumor purity and has significant positive correlations with the infiltrating levels of cancer-associated fibroblasts and endothelial cells.

TABLE 1 | Correlation analysis between HEPH expression and immune infiltration level of the indicated immune cells.

	LUAD		LUSC	
	Rho	*p*	*Rho*	*p*
Purity	−0.341	6.3e−15	−0.343	1.21e−14
CD8+ T cell	0.184	3.9e−05	0.273	1.38e−09
CD4+ T cell	0.167	2.03e−04	0.233	2.55e−07
Macrophages	0.406	5.7e−21	0.206	5.89e−06
Neutrophis	0.285	1.03e−10	0.278	6.15e−10

The "Purity Adjustment" option was applied to all analyses performed.

fibroblastic stromal component (**Figures 6E, F**, LUAD and LUSC panels **G, H**). In double-labeling experiments only few of these stromal cellular elements co-labeled with PDGFRβ, a recognized marker for CAFs (**Figures 6M, N**, LUAD and LUSC panels **O, P**).

Regarding immune infiltrates, in both histotypes we observed the presence of some HEPH expressing monocyte/macrophages, identified by their spherical appearance and their positivity for the marker CD14, a glycolipid-anchored membrane glycoprotein expressed on cells of the myelomonocyte lineage (**Figure 7**, see arrows). On the contrary, neutrophils, identified by their small round shape, the presence of a clearly identifiable multi-lobed nucleus and by their positivity for the marker MPO, were only occasionally found positive for HEPH staining, in both LUAD and LUSC specimens (**Supplemental Figure 1B**).

Overall, the immune-labeling experiments, in concordance with the bioinformatics analysis, confirm the hypothesis that HEPH is expressed mostly by endothelial cells and stromal elements infiltrating the tumor microenvironment.

DISCUSSION

Lung cancer still represents the leading cause of cancer-related deaths both in men and in women, especially in developed countries (45, 46). Lung adenocarcinoma is the most common histologic subtype, its incidence having risen dramatically, surpassing in fact that of squamous cell carcinoma, due to the increased incidence of lung cancer in women (47). Despite advances in diagnosis and treatments, the overall 5-year survival rate remains dismal, especially when lung cancer is diagnosed at advanced stages (48). Therefore, a better understanding of the molecular mechanisms underlying lung carcinogenesis could contribute to the development of novel strategies for prevention and therapy. Cigarette smoking represents the main risk factor for lung cancer, however,

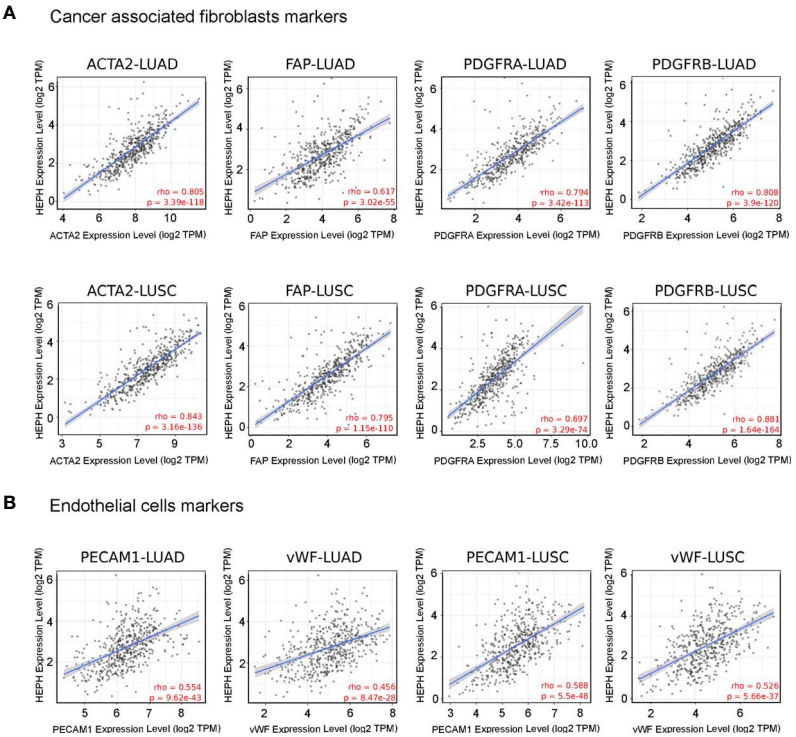

FIGURE 3 | HEPH expression positively correlated with markers of cancer-associated fibroblasts **(A)** and endothelial cells **(B)** in both LUAD and LUSC. Scatterplots of correlations between HEPH and gene markers include ACTA2, FAP, PDGFRA, PDGFRB for cancer-associated fibroblasts and PECAM1 (CD31) and vWF for endothelial cells.

inhalation of iron-rich air pollution particles (49) as well as asbestos fibers, even if with a lower risk factor (50), are also accountable for the increased incidence of this type of malignancies. Air pollution and tobacco smoking have been shown to impact on lung iron metabolism, increasing iron supply in a tissue that is physiologically exposed to oxidative stress. In the present study, supported by bioinformatic evidence, we identified HEPH, a protein involved in exporting iron out of the cell, as a promising predictor of clinical prognosis in lung cancer.

HEPH is a multi-copper oxidase whose function has been better characterized for the small intestine, where it is required for iron egress from the enterocyte into the circulatory system (14, 51). HEPH has been shown to act in concert with Ferroportin (FPN1) (52), the only known mammalian iron exporter for non-heme iron, the mRNA down-regulation of which has been detected in several cancers, usually correlated to poor prognosis (23). In the healthy lung, FPN1 is facing the lumen of the alveoli and this localization has been associated to a role in iron detoxification (21). Indeed, environmental iron reaching the lung epithelium can initially be buffered by the activity of antioxidant molecules such as ascorbic acid, reduced glutathione, and mucin. Once loaded on the transferrin and lactoferrin herein present, it can undergo transferrin receptor 1 (TfR1) and lactoferrin receptor (LfR) internalization by epithelial alveolar cells (53) and alveolar macrophages, and be stored safely, bound to ferritin (54). Under conditions of iron overload, excess pulmonary iron can be released into the lumen of the alveoli *via* FPN1 permease, and possibly oxidized by GPI-anchored or soluble ceruloplasmin, a ferroxidase homologous to HEPH (55).

In the context of cancer, the observed reduction in FPN1 is expected to increase the concentration of the intracellular iron pool, a condition required to sustain the high metabolic demand of actively proliferating cells. Based on bioinformatic evidence, also HEPH mRNA expression levels are downregulated in several malignancies, including lung cancer and, similarly to FPN1, such down-regulation correlates with poor prognosis. Interestingly, HEPH/FPN1 double-labeling experiments showed that both ferroxidase and its functionally-coupled iron permease were both poorly expressed in most of the cancer cells of the analysed LUAD and LUSC specimens, while their expression was still maintained in nesting arrangement of cancer cells having a characteristic epithelial differentiation (**Supplemental Figure 2**). Tumor cell differentiation status is a very important aspect; it is scored and evaluated for clinical diagnosis since it correlates with tumor aggressiveness and worse prognosis (56). The fact that a higher expression of HEPH/FPN1 partners is detected in still well-differentiated cancer cell nests may prove their ability to correctly handle iron. By conferring a better prognosis, this feature could make HEPH expression a relevant prognostic marker to predict a patient's clinical course

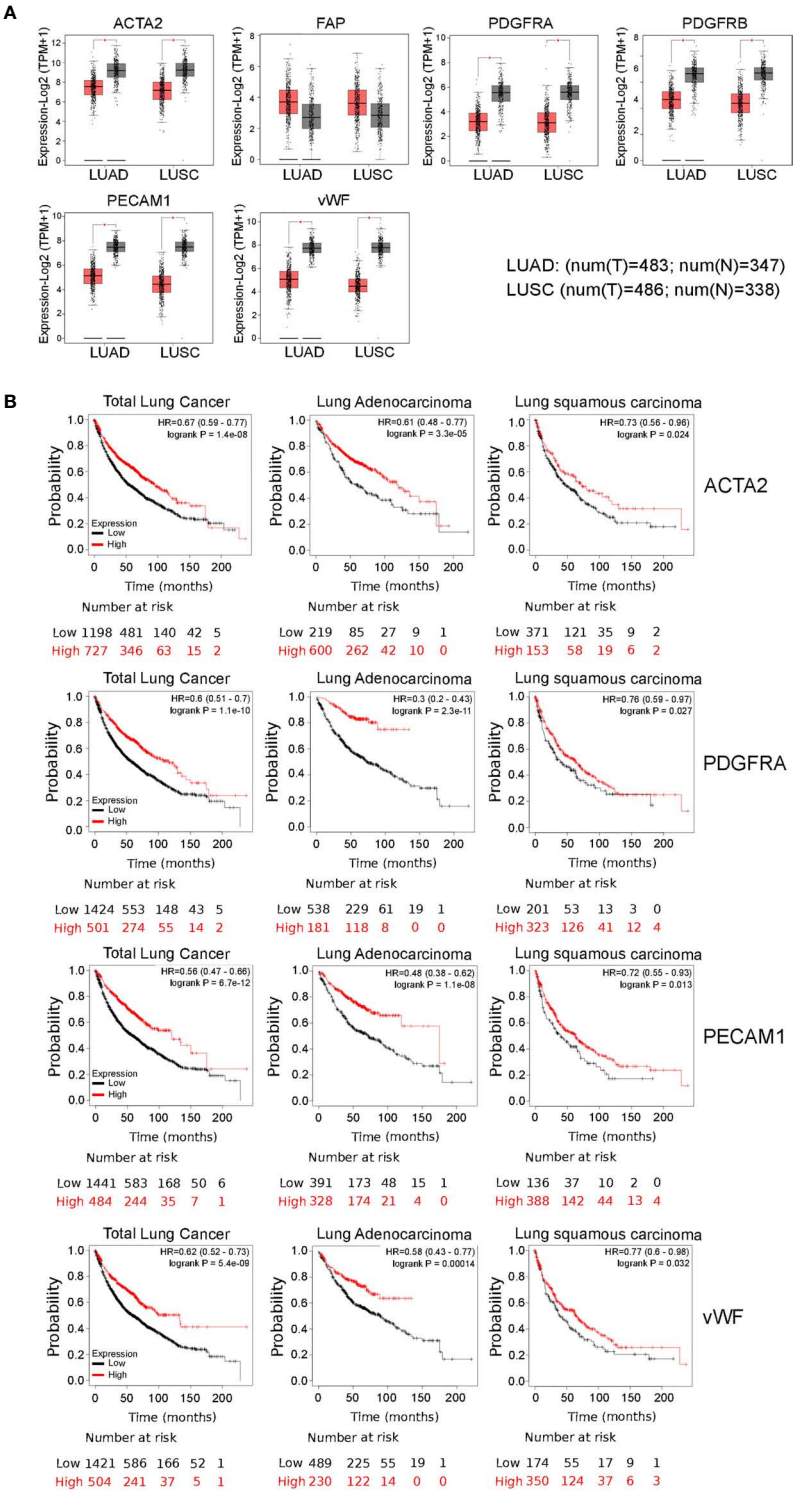

FIGURE 4 | Comparisons of the mRNA expression levels of cancer-associated fibfroblasts and endothelial cells markers between normal (grey) and tumor tissues (red) **(A)**. Overall survival curve of each cancer-associated fibroblasts and endothelial marker shown to correlate with HEPH expression and produced by Kaplan–Meier website resource **(B)**. OS differences are compared between patients with high and low HEPH expression (grouped according to Auto select best cut-off). H, high expression; L, low expression.

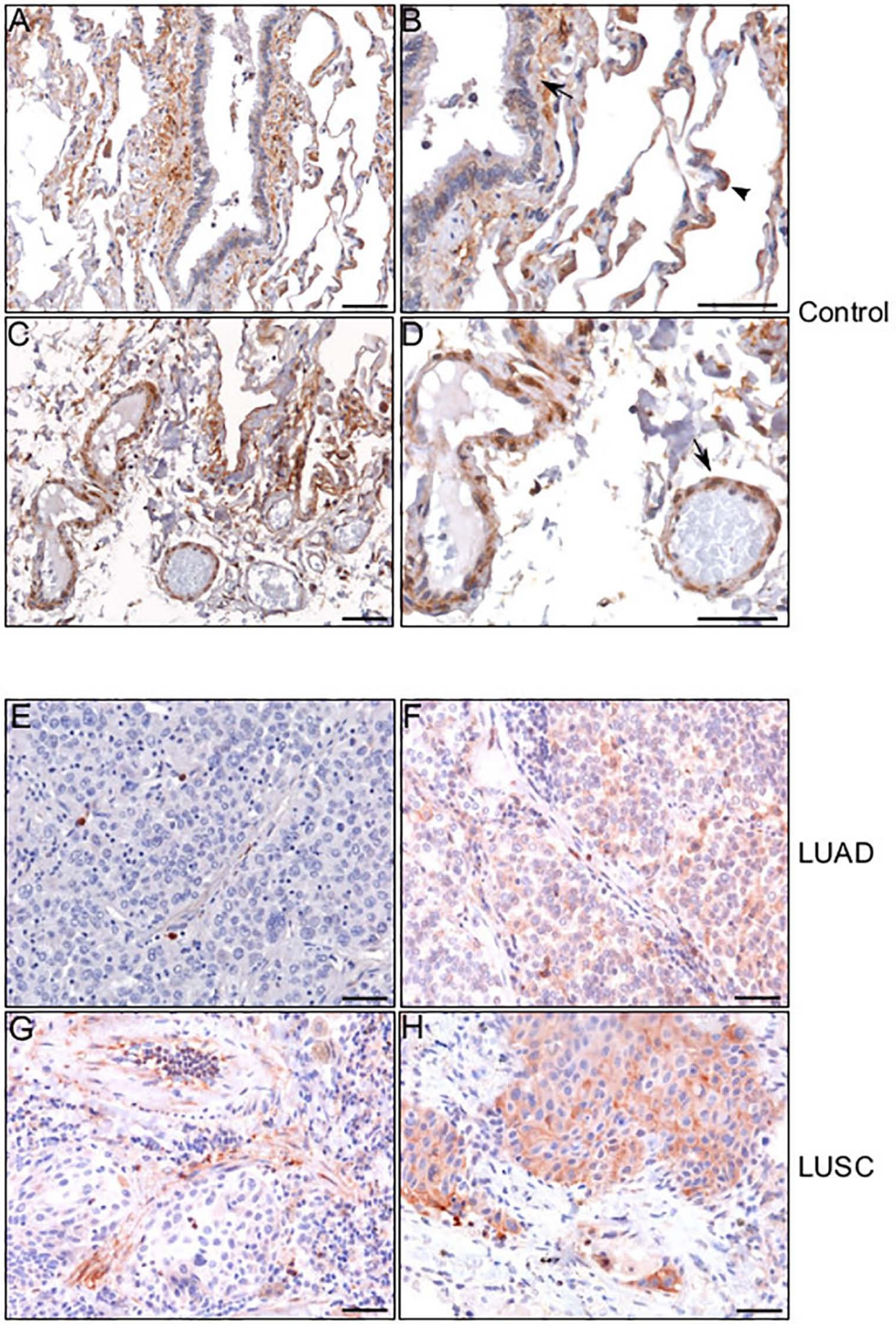

FIGURE 5 | HEPH distribution in control non-tumor lung and in LUAD and LUSC specimens. Representative microphotographs relative to HEPH distribution in non-tumor lung **(A–D)**. In panel **(B)** an HEPH expressing type II pneumocyte is indicated by arrow-head while a black arrow points to smooth muscle fibers and bronchiolar epithelium. In panel **(D)** endothelial cells are indicated by an arrow. HEPH distribution by cancer cells in the context of the two histotypes. Panels **(E)** (LUAD) and **(G)** (LUSC) show the tumoral areas in which HEPH is poorly or not expressed. Panels **(F)** (LUAD) and **(H)** (LUSC) correspond to cancer nests expressing HEPH. Polymer detection system with AEC (red) chromogen; scale bars, 50 μm.

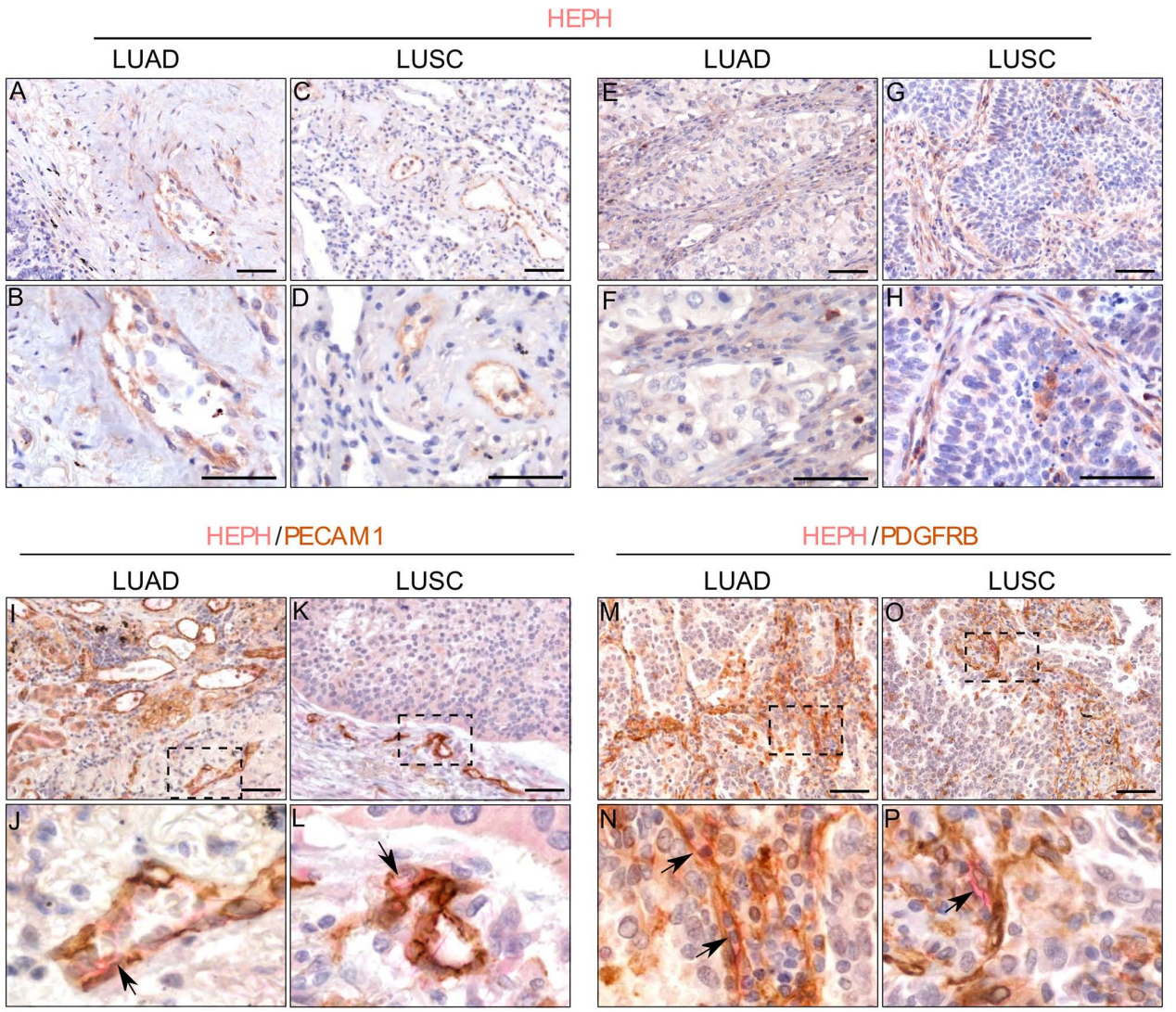

FIGURE 6 | HEPH expression by endothelial and stromal cells in LUAD and LUSC specimens. Endothelial cells identified morphologically in single HEPH immunostaining (panels **(A, B)** for LUAD and **(C, D)** for LUSC), and by way of the strong expression of PECAM1 (CD31) upon double-labeling (panels **(I, J)** for LUAD and **(L, M)** for LUSC). Black arrows indicate HEPH/PECAM1 colocalization. Fibroblasts identified based on their spindle-shaped morphology in single HEPH immunostaining [panels **(E, F)** for LUAD and **(G, H)** for LUSC], and by the expression of PDGFR-β upon double-labeling. Back arrows indicate HEPH/PDGFR-β colocalization [panels **(M, N)** for LUAD and **(O, P)** for LUSC]. Panels **(J, M, N, P)** represent higher magnifications of the corresponding dashed area indicated on the corresponding upper panel. Polymer detection system with AEC (red) chromogen for HEPH and DAB (3,3′-Diaminobenzidine) chromogen for PECAM1; scale bars, 50 μm.

Our study has also shown that HEPH is expressed by the endothelial cells of the lung vasculature in the peri-tumoral tissue of both histotypes. To our knowledge, this peculiar HEPH distribution is only seen in the capillaries of the central nervous system, where brain microvascular endothelial cells, in association with astrocytes and pericytes, exert a tight control on iron entry into the brain (57). In this context, the ferroxidase has been shown to localize on the endothelium abluminal side, where it is presumed to convert ferrous iron, released in the extracellular space by endothelial FPN1, into ferric iron, thus limiting the oxidative damage. HEPH/FPN1 double labeling experiments demonstrated a partial co-localization of the two markers on endothelial cells belonging to the blood vessels situated close to the cancerous mass (**Supplemental Figures 3A**, panels **A, B**). Taken together, these data would support the notion that iron flux in the lung could operate similarly to what has been described for the brain, with endothelial-localized HEPH assisting FPN1 in shipping nutritional ferrous iron into the interstitial space, making it available for resident cell uptake.

TIMER bioinformatics identified a strong positive correlation between HEPH expression and cancer associated fibroblasts (CAFs). These cells are the most dominant cellular component

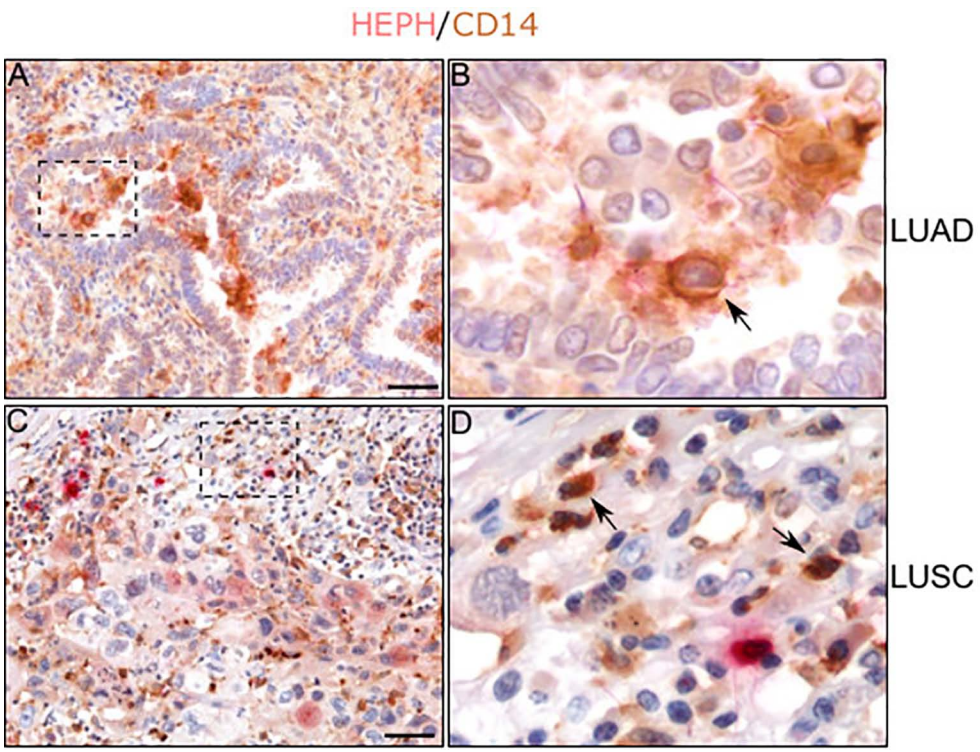

FIGURE 7 | HEPH distribution in tumor-associated macrophages. Representative microphotographs relative to HEPH expression in monocytes/macrophages recognized by CD14 immunoreactivity in LUAD **(A, B)** and LUSC **(C, D)**. Black arrows indicate HEPH/CD14 colocalization. Polymer detection system with AEC (red) chromogen for HEPH and DAB (3,3′ -Diaminobenzidine) chromogen for CD14; scale bars, 50 μm.

in the tumor stroma,. They not only provide physical support to tumor cells but also play key role in promoting or hampering tumorigenesis in a context-dependent manner. CAFs are tremendously heterogeneous in phenotype, function and prognostic significance (58, 59) and can originate from resident fibroblasts, bone marrow-derived progenitor cells or epithelial/endothelial cells that have undergone epithelial to mesenchymal transition (60). Through HEPH immune-labeling of LUAD and LUSC specimens, we clearly identified HEPH-expressing cells, characterized by the typical elongated spindle-shaped morphology of fibroblasts, enveloping some tumor nests in both LUAD and LUSC cancer histotypes. A subpopulation of these cellular elements also co-labeled with PDGFRβ, a key regulator of mesenchymal cell activity in the tumor microenvironment (61), while most of them were negative for α-smooth muscle actin (α-SMA) expression (data not shown). Heterogeneity was further underlined by the observed variable degree on Ferroportin/HEPH co-labeling (**Supplemental Figures 3A**, panels **C, D**), thus increasing the complexity of the scenario. It is interesting to note that a recent study identified at least seven diverse subpopulations of fibroblasts in lung cancer, varying in abundance between cancer subtypes, and shown to accumulate in spatially distinct niches, possibly associated to achieve functional synergy (62). Our results introduce an additional layer of complexity by highlighting the multifaced and interconnected ways in which each cell type tailors iron handling to fulfil its own needs. An aspect, this, that requires further investigations.

Finally, our study underscored the presence of CD14 positive monocyte/macrophages, expressing HEPH to different extents, in all LUAD and LUSC specimens analyzed. Tumor associated macrophages (TAMs) are found in most malignancies, where they facilitate angiogenesis, remodelling of the extracellular matrix, tumor cell invasion and migration while suppressing immune-response (63). TAMs are characterized by an iron-release phenotype achieved by lowering the expression of the iron storage protein ferritin, while increasing the expression of the only iron exporter FPN1 (64). Based on double labeling experiments, we observed that HEPH expressing TAMs were mostly colocalizing with FPN1 immuno-reactivity (**Supplemental Figure 3B**), thus supporting their possible role as iron suppliers for tumor cells.

In conclusion, our results further underline the complex, and still poorly understood, association that exists between iron metabolism and the cancerogenic mechanisms operating in different organ landscapes. Bioinformatic analysis based on mRNA expression dataset, indicates HEPH as a potential novel prognostic biomarker for lung cancer pathologies. Up-regulation of HEPH in LUAD and LUSC correlates with a better outcome since, in association with ferroportin activity, it's expected to

avoid the increase of the intracellular concentration of free iron, known to promote cell proliferation. The novelty of our study lays in having shown that HEPH, together with FPN1, resides mainly on stromal cellular elements, in particular endothelial cells and fibroblasts, key players in the tumorigenic process. Despite the limitations of our immunohistochemical characterization of HEPH distribution in LUAD and LUSC histotypes, which requires further validation on a broader cohort of patients, the current findings illustrate how complex, multifaced and still poorly understood, is the contribution of iron handling in the pathogenesis of lung cancer. In fact, only upon gaining a full understanding of the functional cross-talk that occurs between the different cell types, HEPH-expressing cells and cancer cells, will it be possible to envisage a clinical use for HEPH as a prognostic marker, exploiting it as new therapeutic target to fight these devastating diseases.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Palermo Ethical Review Board (approval number 09/2018). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Conception and design: PZ, AM, and VB. Development of methodology: PZ and AlM. Acquisition of data: BB, LS, GM, and AnM. Analysis and interpretation of data (e.g., statistical analysis, biostatistics, and computational analysis): AlM, PZ, and VB. Writing, review, and/or revision of the manuscript: PZ, AlM, and VB. Study supervision: VB. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the Italian League for the Fight Against Cancer (LILT), Gorizia section (Bando di Ricerca sanitaria 2017-programma 5 per mille anno 2015).

ACKNOWLEDGMENTS

We thank Prof. Giuliano Zabucchi and Dr. Alessandra Knowles for critical review of the manuscript, and Dr. Armando Gentile and Dr. Angela D'Amato for their technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.638856/full#supplementary-material

Supplementary Figure 1 | (A) HEPH is clearly detected at the cell membrane is some neoplastic nests **(**panel **A)**. A higher magnification of the dashed area is reported on panel B. **(B)** HEPH is not expressed by neutrophils. Representative microphotographs relative to lack of HEPH expression in neutrophils recognized by MPO immunoreactivity (indicated by arrows) in LUAD **(A, B)** and LUSC **(C, D)**. Polymer detection system with AEC (red) chromogen for HEPH an d DAB (3,3′ -Diaminobenzidine) chromogen for CD14; scale bars, 50μm.

Supplementary Figure 2 | HEPH co-expresses with FPN1 in some cancer nests. Representative microphotographs relative to HEPH/FPN1 co-expression in two cases of LUAD **(A, B)** and LUSC **(C, D)**. Polymer detection system with AEC (red) chromogen for HEPH an d DAB (3,3′ -Diaminobenzidine) chromogen for CD14; scale bars, 50μm.

Supplementary Figure 3 | HEPH/FPN1 are partially co-expressed on endothelial cells and stromal fibbroblasts, as well as on macrophages. **(A)** Representative microphotographs relative to HEPH/FPN1 co-expression on endothelial cells **(A, B)** and fibroblasts **(C, D)** (see black arrows). **(B)** Representative microphotographs relative to HEPH/FPN1 co-expression on macrophages in LUAD **(A, B)** and LUSC **(C, D)** (see black arrows). Polymer detection system with AEC (red) chromogen for HEPH an d DAB (3,3′ -Diaminobenzidine) chromogen for CD14; scale bars, 50μm.

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Conflict of Interest: 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.

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