Review

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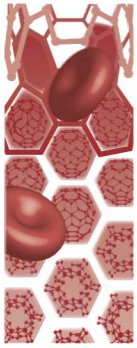

Immunotherapy for cancer: effects of iron oxide nanoparticles on polarization of tumor-associated macrophages

Camila Sales Nascimento[1], Érica Alessandra Rocha Alves[1], Celso Pinto de Melo[2], Rodrigo Corrêa-Oliveira[1] & Carlos Eduardo Calzavara-Silva*,[1]

[1]Grupo de Pesquisa em Imunologia Celular e Molecular, Instituto René Rachou – Fiocruz Minas, Av. Augusto de Lima, 1715 – Barro Preto – Belo Horizonte–MG, 30190-002, Brazil

[2]Grupo de Polímeros Não-Convencionais, Departamento de Física, Universidade Federal de Pernambuco, Av. Prof. Moraes Rego, 1235 – Cidade Universitária, Recife-PE, 50670-901, Brazil

*Author for correspondence: Tel.: +55 313 349 7711; carlos.calzavara@fiocruz.br

Cancer immunotherapy is the most promising trend in oncology, focusing on helping or activating the patient's immune system to identify and fight against cancer. In the last decade, interest in metabolic reprogramming of tumor-associated macrophages from M2-like phenotype (promoting tumor progression) to M1-like phenotypes (suppressing tumor growth) as a therapeutic strategy against cancer has increased considerably. Iron metabolism has been standing out as a target for the reprogramming of tumor-associated macrophages to M1-like phenotype with therapeutic purposes against cancer. Due to the importance of the iron levels in macrophage polarization states, iron oxide nanoparticles can be used to change the activation state of tumor-associated macrophages for a tumor suppressor phenotype and as an anti-tumor strategy.

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Cancer is a disease characterized by the uncontrolled growth and spread of abnormal cells [1]. This pathology is considered a major global health problem, and its burden continues to grow globally, exerting tremendous physical, emotional and financial pressure on individuals, families, communities and health systems [2]. In addition, the increasing incidence of cancer and its mortality rate reveal the importance of developing research focused on new therapeutic approaches [3].

Currently, surgery, radiotherapy and chemotherapy are the three main therapeutic tools. These treatments contribute to the recovery of many patients, but they are also associated with risks of injury or toxicity to normal tissues [4]. Despite constant improvements in cancer treatment, current therapeutics are not always effective in preventing the progression of the disease and the high mortality rate among cancer patients, as well as severe side effects during anti-neoplastic treatment, thus reinforcing the importance of developing more effective and safer therapies [4].

Cancer immunotherapy is the most promising trend in oncology. This alternative treatment is focused on modulating the patient's immune system to identify and fight against cancer. Different cells of the immune system have been investigated as potential targets in cancer immunotherapy [5]. More recently, tumor-associated macrophages (TAMs) have aroused great interest as a therapeutic target because these cells are one of the most abundant leukocytes in the tumor microenvironment and may influence both tumor progression and anti-tumor response [6]. In this field of investigation, different research approaches have sought to develop strategies to reprogram TAMs to exert anti-tumor action [7].

Among all strategies investigated in cancer immunotherapy, the metabolic reprogramming of TAMs is considered a promising method. In these cells, the metabolism is not only a system of energy production but also a source of

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several intermediates with relevant biological functions. In this context, intracellular iron balance is one important element engaged in metabolic reprogramming of TAMs. Emerging evidence supports that alteration in the iron metabolic profile of macrophages changes their activation state and biological functions [8].

As iron levels regulate macrophage polarization states, different methods for manipulating the functions of these cells through modification of the intracellular iron balance have been investigated. One of these strategies is treatment with iron oxide nanoparticles to modulate the activation state of TAMs [9,10]. These nanoparticles are physically and chemically stable, biocompatible and environmentally safe, presenting unique characteristics for clinical applications [11]. Owing to their ability to accumulate preferentially in macrophages and considering that intracellular iron balance is crucial to regulate the expression of proteins correlated to macrophage polarization states, iron oxide nanoparticles are considered a potential tool for cancer treatment [12]. Recent studies have described that treatment with iron oxide nanoparticles is able to reduce the growth of tumor cells through the reprogramming/reeducation of TAMs into a tumor suppressor phenotype [13–15].

This review initially discusses TAMs and their phenotypic characteristics, as well as their interaction with the tumor microenvironment and the main reprogramming pathways. Then, the discussion focuses on iron metabolism as a therapeutic strategy for reprogramming TAMs and the use of iron oxide nanoparticles as an anti-tumor strategy.

TAMs

The composition of the tumor microenvironment is a critical factor for establishing more aggressive forms of cancer. This microenvironment is composed not only by the tumor cells but also by a variety of leukocytes, fibroblasts, the extracellular matrix and various bioactive molecules derived from both tumor and nontumor cells [16]. This complex cellular and molecular network that constitutes the tumor microenvironment is recognized as a critical element for modulating tumor growth and invasiveness [17]. In the tumor microenvironment, several proteins are involved in cell growth, migration and production, leading to apoptosis, proteolysis of the membrane and cell signaling [17]. Due to the close association between the composition of the tumor microenvironment and tumor aggressiveness, there is a growing interest in the tumor microenvironment as a therapeutic target.

Inside the tumor microenvironment, interactions between tumor cells and other cells are bidirectional, resulting in the remodeling of the tumor microenvironment and conditions favorable to mutual growth and support [18]. As for the immune cells, it is known that immunosuppressive cells such as Tregs and TAMs with an anti-inflammatory phenotype are responsible for accelerating tumor growth and aggressiveness [19]. TAMs have attracted great interest in recent years as a therapeutic target because they represent a significant part of the cellular component of the tumor microenvironment.

Macrophages are cells with high plasticity that constantly change their functional state in response to environmental stimuli. In the tumor microenvironment, the polarization of macrophages is regulated by multiple cytokines, chemokines, growth factors and other signals derived from tumor and stromal cells [20]. The activation state of macrophages has historically been simplified to a binary categorization: M1 and M2 macrophages (Figure 1). According to Mantovani *et al.*, the M1 nomenclature should be used for the macrophage phenotypes stimulated by IFN-γ and bacterial products and the nomenclature M2 should be used to refer to macrophages stimulated by IL-4 and/or IL-13 [21]. Moreover, the nomenclature M1-like is used to include the activation states that lead to anti-tumor and cytotoxicity responses and M2-like to cover a range of diverse phenotypes that share functional states related to the promotion of tumors [21]. Although this is useful for generic classification, macrophages exhibit a wide spectrum of *in vivo* activation states [20].

In tumor tissues, the pattern of macrophage activation depends on the surrounding microenvironment, which explains the heterogeneity between macrophage populations in the tumor area. M1 macrophages (classically activated) stimulate the development of T helper type 1 (Th1) immune response and produce proinflammatory cytokines such as TNF-α, IL-1β, IL-12, reactive oxygen species (ROS) and reactive nitrogen species (RNS) [22]. These molecules produced by M1 macrophages are highly toxic and therefore crucial for the defense of the host and the destruction of tumor cells [21]. In contrast, M2 macrophages – also referred to as 'alternatively activated' – are phenotypically characterized by the expression of specific markers. These macrophages mainly express scavenger receptors (CD204 and CD163) and mannose receptor-1 (CD206), stimulate the development of T helper type 2 (Th2) immune response and produce large amounts of IL-10, TGF-β and chemokines [21,23]. The M2 macrophages are inefficient antigen-presenting cells that suppress Th1 responses, contribute to attenuating inflammation and promote wound healing, angiogenesis, tissue remodeling and, consequently, tumor progression [24].

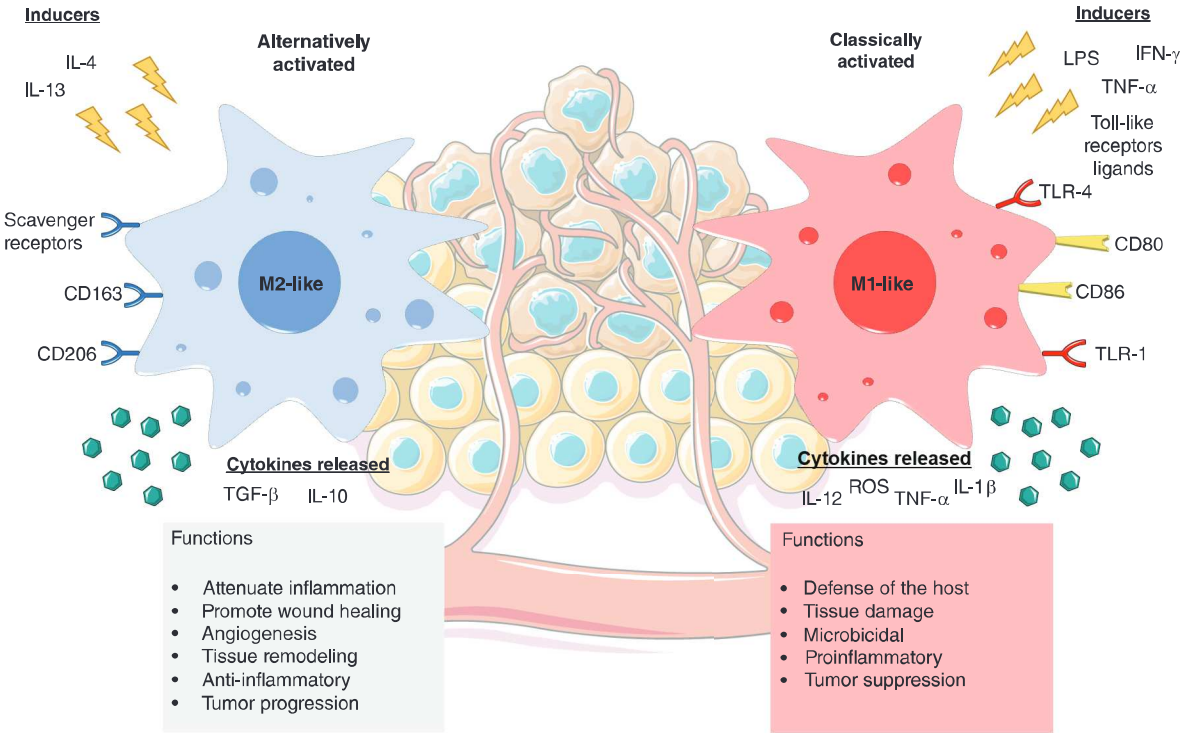

Figure 1. Binary categorization of proinflammatory (M1-like) and anti-inflammatory (M2-like) macrophages in the tumor microenvironment.
LPS: Lipopolysaccharide; ROS: Reactive oxygen species.

The origin of the infiltrated macrophages present in the tumor microenvironment was initially believed to be exclusively from blood monocytes derived from bone marrow hematopoietic stem cells [25]. However, recent studies have shown that most TAMs originate from resident macrophages from the yolk sac that are deposited in the tissues during embryonic development and persist into adulthood due to local proliferation [26]. Some studies have also suggested a double origin; in mouse models with brain and pancreatic cancer, TAMs were derived from either blood monocytes or embryonic macrophages recruited and/or activated by various signals from the tumor microenvironment such as hypoxia and products of cellular damage [23,27,28]. In addition, it is known that several factors produced by malignant tumor cells are chemotactic for macrophages. The most studied factors are MCP-1, M-CSF, G-CSF, and VEGF [29]. In breast tumors, MCP-1 is expressed in neoplastic cells and TAMs, and its high expression has been correlated with low survival of patients [30]. M-CSF and G-CSF are produced by a variety of tumor types and are chemotactic for macrophages, as they are stimulators for many cell types involved in immune responses, being used in the past to elicit immune response against tumor cells [31]. VEGF is a potent angiogenic cytokine produced by neoplastic cells and is an important macrophage chemotactic factor [31]. The increase in VEGF expression in breast cancers was correlated with increased content of macrophages in the tumor microenvironment [18].

Thus, as M2-like macrophages are abundantly present in almost all solid tumors and contribute significantly to cancer progression, they are considered an interesting target for treating solid tumors through their reprogramming from anti-inflammatory M2-like to proinflammatory M1-like phenotype.

The role of iron as a tool for metabolic reprogramming of macrophages

Different approaches have been proposed to modulate TAMs for cancer treatment. In general, the main approaches are focused on TAMs depletion, recruitment inhibition of circulating monocytes, M2-like phenotype blocking, enhanced activation of M1-like macrophages and TAMs reprogramming to M1-like macrophages [32,33].

As toll-like receptor agonists induce M1-like phenotype, they have been investigated as a promising tool to reprogram TAMs to anti-tumor phenotype. In a study conducted by Rodell *et al.*, it was found that β-cyclodextrin nanoparticles loaded with Resiquimod (R848), an agonist of TLR7 and TLR8, induce the nuclear translocation of

nuclear NF-κB in macrophages from BALB/c mice (J774.A), leading to the production of the proinflammatory proteins TNF-α and IL-12 and tumor destruction [34]. Likewise, heat-treated *Mycobacterium indicus pranii* has been investigated in combination with an agonist anti-GITR monoclonal antibody DTA-1. The GITR acts as a co-stimulatory molecule in the activation of cytotoxic T cells, stimulating the production of IFN-γ and IL-2. The combination of heat-treated *Mycobacterium indicus pranii* and DTA-1 promoted the polarization of TAMs to a tumor suppressor phenotype and reduced the frequency of intratumor regulatory T cells in a mouse model with advanced-stage melanoma by activation of TLR4 [35].

Another strategy investigated to promote TAMs reprogramming is the use of antibodies. The effectiveness of the combination of CD40 agonist and CSF-1R blocking also was investigated [36]. Considerable changes were observed in the populations of macrophages and monocytes after treatment with anti-CD40/anti-CSF-1R. The combination resulted in an increase in M1-like macrophages and a reduction in M2-like macrophages [36]. In addition, the treatment promoted profound changes in the composition of immune infiltrate, causing a decrease in immunosuppressive cells and changing the tumor microenvironment to a more inflammatory profile. This combined approach also increased the maturation and differentiation of proinflammatory macrophages and dendritic cells and stimulated the response of effector T cells [36].

Macrophage receptor with collagenous structure (MARCO) was another molecule targeted in antibody therapy against cancer. This surface receptor is a pattern recognition receptor of the Class A scavenger receptor family that was identified as a gene overexpressed in immunosuppressive TAMs and linked to poor prognosis of human cancer [37]. In this approach, MARCO blocking caused inhibition of tumor growth and metastasis through TAMs reprogramming to a proinflammatory phenotype, increasing the anti-tumor immune response against melanoma and colon carcinoma [37]. PD-1 blocking was also investigated and an *in vivo* study in murine model showed that treatment with antibody against PD-1 promoted increased phagocytosis by macrophages, reducing tumor growth and prolonging survival in mice with colon cancer [38].

A third alternative that has been investigated to reprogram TAMs is the use of cytokines. It has been observed that IL-12 treatment in mice with Lewis lung carcinoma induced tumor regression. Analysis of macrophages infiltrated in tumors revealed that treatment with IL-12 induced, both *in vivo* and *in vitro*, a rapid reduction in the production of tumor-promoting cytokines by macrophages (IL-10, MCP-1 and TGF-β) and a concomitant increase in the production of proinflammatory and pro-immunogenic cytokines (TNF-α, IL-15 and IL-18) [39]. Similar changes in the polarization of TAMs to a tumor suppressor phenotype were induced by IL-12 in TAMs isolated from lung containing metastases [39]. It has also been observed that nanoparticles of copolymers of poly (β-aminoester) loaded with IL-12 (IL-12⊂P1) promoted the release of IL-12 in the tumor microenvironment and re-educated TAMs to a proinflammatory phenotype [40].

The cytokine IFN-γ was also able to reverse the immunosuppressive properties of TAMs. In a study with TAMs isolated from ovarian cancer, IFN-γ was the most potent cytokine for the polarization of these macrophages to the M1-like phenotype, inducing low production of IL-10 and high production of IL-12 by macrophages [41]. A polyelectrolyte multilayer (PEM) film complex composed of chitosan (Ch), γ-glutamic acid (γ-PGA), and IFN-γ was also used as a drug-delivery system to modulate the macrophage phenotype. This complex was able to reduce the invasion of gastric cancer cells *in vitro* from four- to two-times, which was followed by a decrease in the production of IL-10 and the pro-invasive role of TAMs [42].

In the last decade, interest in immunometabolism – the interaction between the immune response and metabolic systems – has increased considerably, with many studies focusing on the reprogramming of macrophages through the modification of cell metabolism to treat diseases [8]. In fact, the metabolic system not only produces energy but is also an important source of several intermediates with relevant biological functions in innate immunity and inflammatory response [8]. Emerging evidence suggests that immunometabolism is an important factor in determining the functional phenotype that characterizes different populations of macrophages. Iron metabolism has been recognized as an important component of macrophage plasticity and relevant metabolic characteristics in the differentiation between different classes of macrophages [43].

Given the association of iron accumulation with the function of M1 macrophages, several studies have focused on the hypothesis that an increase of intracellular iron in macrophages can induce reprogramming and stimulate the anti-tumor immune response. Studies show that short-term iron overload is associated with increased TNF-α production [44]. Iron chelators have also been shown to promote M1-like phenotype. Application of the intracellular iron chelator disulfide prochelator $(TC3\text{-}S)_2$ shifted the macrophage phenotype from iron release toward sequestration (M1), as determined by the iron-gene profile [45]. Iron chelated melanin-like nanoparticle (Fe@PDA-PEG)

treatment showed that M1 macrophages repolarized from TAMs displayed a significant increase of iNOS/IL-10 ratio in macrophage mRNA level, which indicates the capability of repolarizing M2-like macrophages into M1-like phenotype [46]. Furthermore, experiments established that TAMs exposed to hemolytic red blood cells were converted into proinflammatory macrophages capable of directly killing tumor cells [13]. Au/Fe_3O_4 composite nanoparticles with spherical core–shell morphologies induced a significant proinflammatory cytokine expression and secretion, indicating a response to the proinflammatory state of RAW 264.7 macrophages [47].

In the context of nanoparticles, the use of iron oxide nanoparticles to induce metabolic reprogramming of macrophages has been growing in recent years, being considered an extremely promising therapeutic strategy not only due to its ability to modulate the activation state of macrophages but also due to its low toxicity and biological compatibility [48]. To contextualize iron metabolism as a therapeutic approach to reprogramming macrophages, in the next sections the authors detail the role of macrophages in iron homeostasis, iron metabolism and macrophage polarization states and the use of iron oxide nanoparticles (IONPs) as a tool for reprogramming macrophages as an anti-tumor strategy.

Iron metabolism

Iron is an essential component for mammals, playing versatile roles in many basic cellular processes such as ATP production in mitochondria, oxygen transport by hemoglobin, DNA synthesis, energetic metabolism, cellular respiration and mechanisms of cell growth and death [49].

In biological systems, iron can be found in two main types of oxidation states, ferrous ion (Fe^{2+}) and ferric ion (Fe^{3+}), which are capable of stably interconverting between the two ionic forms of iron via oxidation or reduction reactions [49]. Iron is an element essential for the function of proteins known as metalloproteins, which are responsible for oxygen transport (hemoglobin), oxygen storage (myoglobin), energy production (cytochrome-c), cell metabolism (amino acid oxidases, fatty acid desaturases), detoxification (cytochrome P450, catalase) and host defense (myeloperoxidases, nitric oxide synthases, NADPH oxidases) [49,50].

Although iron plays important roles in the human body, its quantity must remain in balance, since an excess can cause cellular toxicity and lead to oxidative stress. This toxicity is related to iron's ability to donate or receive electrons and generate ROS by the reactions of Fenton (1) and Haber-Weiss (2) [48].

$$(1)\ Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH. + OH^-$$

$$(2)\ Fe^{3+}\ H_2O_2 \rightarrow Fe^{2+} + HOO. + H$$

$$= O^{2-} + H_2O_2 \rightarrow OH. + OH - + O_2$$

Therefore, iron levels in the body are strictly controlled by complex mechanisms to preserve physiologically tolerable iron levels to serve as a critical catalytic component of many proteins and enzymes and, simultaneously, avoid cytotoxicity induced by iron accumulation [51]. A variety of mechanisms are responsible for adjusting iron concentrations on systemic and cellular levels. In response to the erythrocyte iron demand, an orchestrated interaction between iron-processing cells, including tissue macrophages, hepatocytes, erythrocytes and duodenal epithelial cells, controls and maintains iron homeostasis [50].

Most of the body's iron requirements are supplied by the digestion of senescent or short-lived erythrocytes, mediated by macrophages [52]. Recycling iron is mainly performed by red pulp macrophages (RPMs) that are localized in the splenic red pulp. These cells phagocytose senescent erythrocytes and release iron after catabolism of hemoglobin [53]. Around 20 to 21 mg of iron is recycled from hemoglobin and is sufficient to maintain the daily iron requirement for erythropoiesis [53].

Macrophages & systemic iron homeostasis

Iron metabolism is regulated by a variety of mechanisms that are important to adjust iron concentrations on systemic and cellular levels. In response to iron and red blood cell (RBC) demand, an orchestrated interplay between iron-processing cells is responsible for maintenance of iron homeostasis [49]. Macrophages are important regulators of iron metabolism by controlling cellular iron import and export. Macrophages can absorb iron or molecules containing iron through receptors such as TfR1; CD71, LRP1; CD91 and CD163 [51]. These receptors connect to iron bound to plasma proteins responsible for iron transport, known as transferrin (Tf), heme-hemopexin

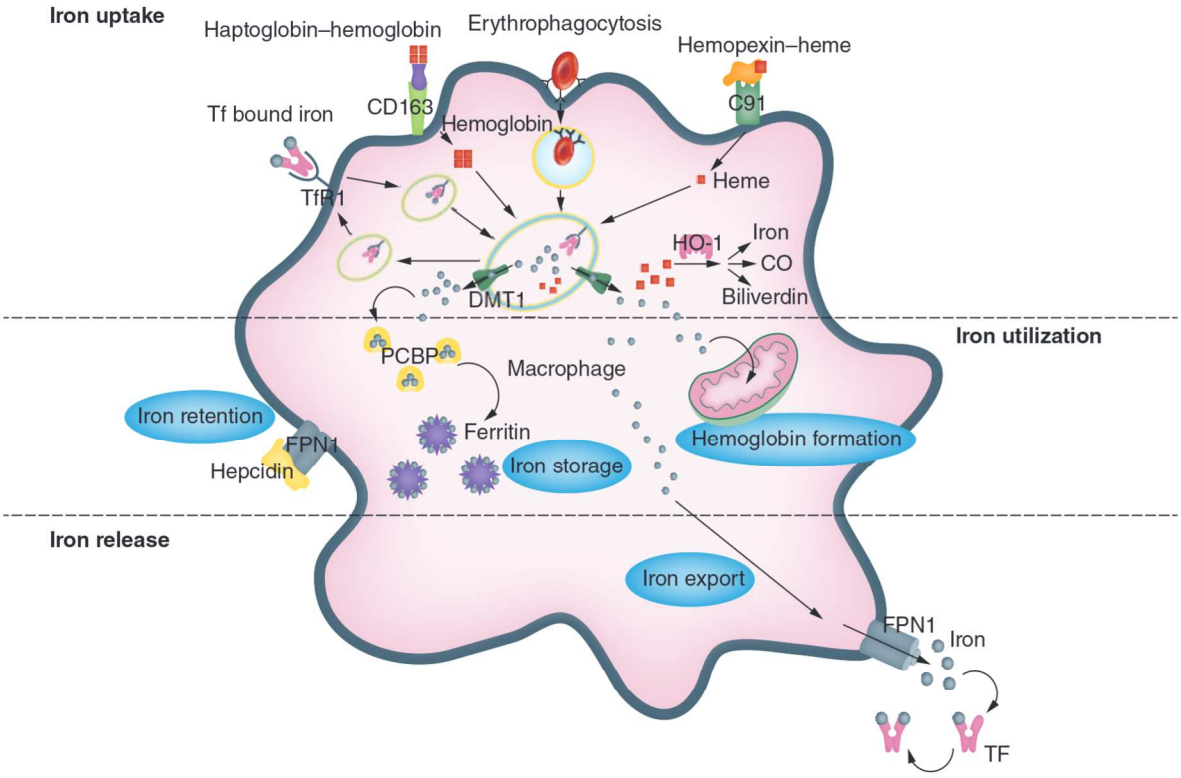

Figure 2. Iron homeostasis in macrophages. The main iron source for macrophages is the phagocytosis of senescent erythrocytes, but macrophages can acquire iron from different sources through different receptors. The molecule heme-hemopexin is scavenged by the CD91 receptor, hemoglobin-haptoglobin is scavenged by the CD163 receptor and transferrin-bound iron can be internalized by TfR1. Whether phagocytosed in erythrocytes or endocytosed by scavenging, the heme moieties are degraded by HO-1 in CO, biliverdin and free iron (Fe^{2+}), which is exported to the macrophage cytoplasm by DMT1. TF-bound iron (Fe^{3+}) is internalized, followed by Steap3 reduction of Fe^{3+} to Fe^{2+} and DMT1-mediated transport across the endosomal membrane. The Fe^{2+} obtained can be delivered to ferritin for storage by PCBP or delivered to FPN1 for cell export. The rate of iron export is controlled by extracellular hepcidin through its ability to induce the endocytosis and proteolysis of ferroportin. For the export of iron, the CLP oxidizes Fe^{2+} to Fe^{3+} and thus allows the loading of iron in the TF protein. Iron-loaded transferrin (Fe^{3+}) transports iron to cells where it will be reused and internalized by specific receptors. Cytoplasmic iron can be subsequently transported to the mitochondria, where iron is inserted into the protoporphyrin molecule, forming heme. Subsequently, the heme is added to the globin chains, forming hemoglobin.
CO: Carbon monoxide; Fe^{2+}: Ferrous iron; Fe^{3+}: Ferric iron.

(Hx-heme) and hemoglobin-haptoglobin (Hb-Hp), respectively [54]. In addition, macrophages also take up iron via phagocytosis of erythrocytes and other cells (Figure 2) [51,55].

During the recycling process of erythrocytes, macrophages recognize phosphatidylserine in the surface of senescent erythrocytes and internalize them using the CD36 receptor [56]. After engulfment, the erythrocytes are subjected to the action of ROS and hydrolytic enzymes in the phagolysosome that promotes the release of heme in the vacuolar fluid [57]. The released HO-1 from macrophages catalyzes the degradation of heme in carbon monoxide, biliverdin and Fe^{2+} [57]. Subsequently, the free Fe^{2+} iron is exported from the phagolysosome to the cytoplasm by DMT-1 and by the Nramp1 [58]. The Fe^{2+} obtained can be delivered to ferritin for storage by cytoplasmic iron chaperones proteins known as the PCBP or delivered to FPN1 for cell export [56,59].

The iron export rate is controlled by extracellular hepcidin. The binding of hepcidin to FPN1 leads to internalization and lysosomal degradation of ferroportin and decreased iron release from macrophages and, consequently, the retention of iron into the macrophages [60]. This mechanism allows the reduction of circulating iron that would be available to extracellular pathogens [60].

For the export of iron, the ceruloplasmin ferroxidase oxidizes Fe^{2+} to Fe^{3+} and thus allows the loading of iron in the transferrin protein [59]. The binding of iron to TF allows its solubilization and attenuates its reactivity, ensuring safe transport and release to cells [59]. Iron-loaded transferrin (Fe^{3+}) will transport iron to the sites where

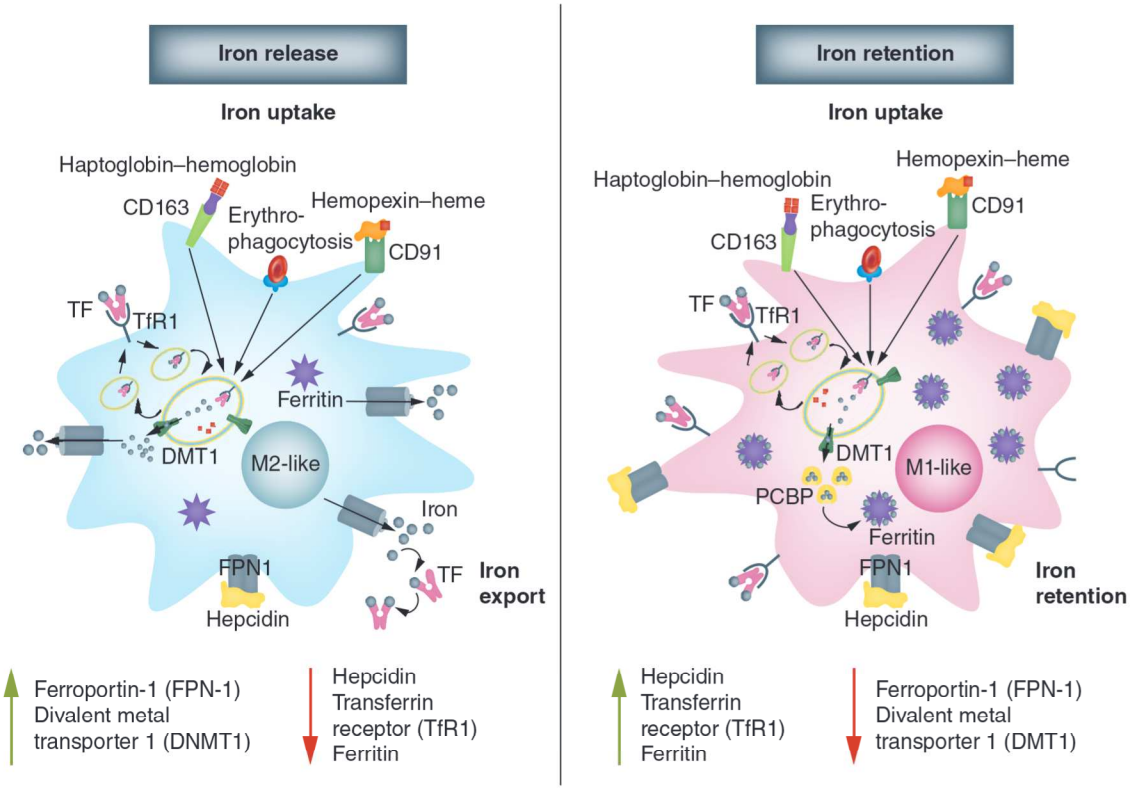

Figure 3. Macrophage phenotype and iron homeostasis. The M2-like phenotype has high expression levels of ferroportin-1 and DMT-1 and low levels of the hepcidin, TF and ferritin protein, which favors the iron export phenotype. M1 macrophages exhibit the iron storage phenotype, promoting intracellular iron retention through the upregulation of ferritin, hepcidin and the TF receptor.

it will be reused, predominantly in the bone marrow, where iron will participate in the hemoglobinization of new erythrocytes [61]. The internalization of iron is initiated by the binding of specific receptors, TfR-1 and TfR-2, which are expressed in all cells capable of cell division, especially erythrocytes [61]. After internalization, the iron present in acidified endosomes is released by transferrin receptors and reduced to Fe^{2+} by STEAP3 [62]. Subsequently, the Fe^{2+} is exported to the cytoplasm by DMT-1 [62]. Cytoplasmic iron can be subsequently transported to the mitochondria, where iron is inserted into the protoporphyrin molecule, forming heme [59]. The heme leaves the mitochondria and is added to the globin chains, forming hemoglobin [59].

Iron metabolism & macrophage polarization states

Macrophages play an essential role in iron homeostasis and immune defense. Reprogramming of intracellular metabolism in response to microenvironmental signals is required for M1/M2 macrophage polarization and function [63]. The acquisition of a specific macrophage phenotype determines the expression of iron-regulated genes and determines the accumulation and traffic of cellular iron [8]. Recent studies have shown that more than 60% of genes related to iron metabolism, including differential expression of iron uptake, storage and release, occurs in the two final stages of macrophage polarization (Figure 3) [64].

M1 macrophages exhibit the iron storage phenotype with high levels of ferritin, transferrin, and hepcidin; low levels of FPN1; CD163; and HO-1 – which favors intracellular iron sequestration and storage of phenotype improving antimicrobial effector functions through the increased expression of TNF-α (proinflammatory) and suppressed expression of IL-10 cytokine (anti-inflammatory) [65,66].

On the other hand, M2 macrophages exhibit the iron export phenotype, with increased expression of CD163, HO-1-mediated catabolism and FPN1 and decreased expression of ferritin [8]. The ferroportin-mediated iron release can significantly contribute to the role of M2 macrophages in various pathophysiological conditions, such as tissue repair and tumor growth [8]. In the context of the tumor microenvironment, M2-like TAMs are known

as 'iron releasers' and support the growth and establishment of the tumor, whereas M1-like TAMs are considered 'iron retainers' and are responsible for limiting tumor progression [66].

In general, the direction of macrophage polarization can be stimulated by iron deficiency, supplementation or iron overload. The polarization pathways of macrophages by iron are complex and diverse, and the specific mechanisms still need to be explored. However, studies show that iron supplementation can shape macrophage polarization to the M1-like phenotype through cell signaling pathways, epigenetic regulation/modification and cell metabolism [67]. Macrophages isolated from iron-deficient rats have shown that iron deprivation can suppress NF-κB activation to block the polarization of type M1-like macrophages [68]. Iron loading contributes to the polarization of M1-like macrophages by inducing inflammation and increasing glycolysis in bone marrow–derived macrophages (BMDMs) [69]. Iron can modulate macrophage polarization to the M1-like phenotype by increasing *miR-214* expression and reducing *miR-29a* expression [70].

In relation to the influence of different oxidation states of iron on the reprogramming of macrophages, the effect of Fe^{2+} and/or Fe^{3+}on regulating proinflammatory macrophages is not consistent. Most studies demonstrate that both oxidation states of iron in different valence states can alter the polarization state of macrophages to M1-like phenotype. Iron supplementation with ferumoxytol ($Fe_3H_2O_4$), (Fe^{2+}), ferric citrate (Fe^{3+}) and ferrous citrate (Fe^{2+}) were able to promote the expression of M1 markers (e.g., iNOS, TNF-α) [15,44]. In contrast, studies have also shown that both irons oxidate states can promote M2-like macrophage polarization. Ferric ammonium citrate (Fe^{3+}) and $FeSO_4$ (Fe^{2+}) enhance THP-1 cells polarizing into the M2-like phenotype [71,72]. Therefore, more studies should be conducted to elucidate the type of factor involved in the regulation of the final polarization of macrophages by iron. Such different findings may be related to the features such as the oxidation states of iron (Fe^{2+} and/or Fe^{3+}); the proportion of oxidation states of iron in nanoparticles; different models *in vitro* and *in vivo*, processing time and others. Subsequently, more studies must be realized to understand the effect of different oxidation states of iron on the polarization of macrophages.

Considering that iron-loaded macrophages generally exhibit the proinflammatory M1-like phenotype, which are cells with anti-cancer properties, alteration of the macrophage phenotype through the modulation of iron metabolism has been considered an interesting target for anti-tumor therapeutic strategy [65,73]. In lung cancer, for example, it was demonstrated that iron-loaded macrophages presented increased production of ROS and TNF-α and directly killed tumor cells, suggesting that iron supplementation for TAMs may be a therapeutic strategy for cancer treatment [13]. Therefore, new approaches are being investigated for manipulating iron homeostasis and metabolically reprogramming macrophages in the tumor microenvironment [65]. These studies provide new insights into the role of intracellular iron regulation in the function of macrophages and open a new avenue for the development of advanced iron-based anti-cancer technologies [65]. In this context, several studies have shown promising results using IONPs as a tool for the reprogramming of macrophags and cancer treatment.

Reprogramming of TAMs by iron oxide nanoparticles

Considering the complex molecular and functional machinery driven by iron in macrophages and the influence of intracellular iron on macrophage polarization, IONPs have the potential as an immunotherapeutic agent for reprogramming macrophages. Currently, several studies show promising results in the use of nanoparticles as a tool for the treatment of cancer.

Iron oxide nanoparticles

Nanoparticles are a broad class of materials that includes particulate substances with at least one dimension less than 100 nm in size. They are comprised of the nucleus (central portion) and shell layer, which can be functionalized with a variety of small molecules, metal ions, surfactants and polymers. Due to such exceptional characteristics, these materials have aroused great interest among researchers of multidisciplinary areas [74].

Bearing in mind the different applications to nanotechnology, metallic nanoparticles, specifically IONPs, have been extensively investigated for biomedical applications due to their excellent electrical and magnetic properties, biocompatibility and good physical and chemical stability [11]. In addition, they are one of the few nanomaterials that can be injected into the human body and incorporated by natural metabolic pathways [75].

The use of IONPs presents some advantages relative to other nanoparticles, such as ease of synthesis, magnetism, low toxicity, biocompatibility, high adsorption capacity and low cost [76–82]. The main iron oxides applied to the production of nanoparticles are magnetite (Fe_3O_4), maghemite (γ-Fe_2O_3) and hematite (α-Fe_2O_3) [83]. Applying

changes in temperature and atmospheric control, it is possible to obtain iron oxides in different phases, and currently magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) are the most used in biomedical applications [83].

In general, IONPs typically have two structural configurations: a magnetic particle core coated with a biocompatible polymer or porous polymer biocompatible with IONPs present within the pores [84]. The coating acts to protect the magnetic particle from the surrounding environment, preventing oxidation and providing stability. In addition, it can be functionalized by binding carboxyl, biotin, avidin, carbodiimide groups and other molecules that act as binding points for coupling cytotoxic drugs, antibodies and others to increase targeting performance [84]. Another important advantage is that IONPs have low toxic effects on human cells, showing low or no toxicity at doses up to 100 μg/ml [85].

In terms of products approved for use in humans, nanomaterials such as polymers and liposomes dominate the list of nanomedicines approved by the US FDA. However, considering inorganic nanomaterials, only IONPs have been approved by the FDA as a drug to treat iron deficiency in patients with chronic renal failure or patients who do not tolerate oral iron supplementation [12,86]. Of those available on the market and approved by the FDA, Feraheme® (ferumoxytol) (AMAG Pharmaceuticals, Inc., MA, USA) is a formulation composed of IONPs coated with a semi-synthetic carbohydrate. However, its use is limited due to the high cost involved in the treatment [87]. Thus, the development of new formulations based on more accessible IONPs that allows the exploration of new applications is presented as a very attractive field of study. Due to the propensity of macrophages to uptake nanoparticles, one of the new approaches that has been tested is the use of IONPs for reprograming macrophages in different cancers.

Reprogramming of TAMs by IONPs as an anti-tumor strategy

Due to the multiple effects of iron on macrophage phenotypes, it has been observed that IONPs have strong effects on the reprogramming of macrophages in the tumor microenvironment and they have been widely explored in preclinical and clinical studies for TAMs reprogramming and the treatment of several types of solid tumors [12]. Different from other scientific research articles that considered nanoparticles only as vehicles for anti-tumor drugs, these studies suggest that the modulation of TAMs phenotype functions by IONPs may be an important tool in cancer treatment [88]. Here, the authors have gathered the current studies and summarized the main effects of nanoparticles with an iron core on the re-education of TAMs and on the treatment of different solid tumors (Table 1) [13,15,89–94].

Studies involving the polarization of macrophages by IONPs for cancer treatment show that they can induce a phenotypic shift of M2-like macrophages to M1-like macrophages *in vitro* and *in vivo* [13,15,91,94]. The anti-tumor effect is attributed to the accumulation of intracellular iron in macrophages, promoting the transcriptional reprogramming of macrophage phenotypes (Figure 4).

The roles of iron nanoparticles in regulating proinflammatory macrophages are not always consistent. Until now, the molecular basis of IONPs and macrophage interactions has remained unclear due to the diversity of the chemical composition and physical properties of nanoparticles (size, charge and morphology) and to a variety of models *in vitro* and *in vivo* [95]. A summary of molecular mechanisms for IONP-mediated macrophage reprogramming is described below (Figure 5).

The process starts with the interaction between IONPs and macrophage surface receptors, when reprogramming can be initiated. In general, the proinflammatory potential of IONPs is mediated by pattern recognition receptors expressed on innate immune cells, including TLRs, complement receptors, scavenger receptors, and FCγ receptors, which are involved in downstream MAPK/mTOR cascades and transcription factors – STATs , NF-κB, and IRFs, which regulate immune activation and inflammatory response [96]. Studies show that in the STAT family and c-Fos/c-Jun complex, MAPK pathways are involved in the reprogramming of macrophages [97,98]. It has also been found that high intracellular iron concentration can activate NF-κB and induce proinflammatory pathways initiating the reprogramming of macrophages to a proinflammatory phenotype associated with TNF-α expression and other proinflammatory cytokines [93]. In addition, IONPs modulate IRF-5 signaling pathway to improve the polarization of anti-tumor M1-like macrophages [99]. Accordingly, most IONPs are positive regulators of autophagy in macrophages [96]. Scavenger receptors participate directly in macrophage polarization and IONPs uptake; IONPs internalization is significantly reduced by scavenger receptor ligands and dextran sulfate [97]. Mainly, IONPs uptake and inflammatory response are promoted by TLR4 activation [100]. Activation of TLR4 by IONPs also results in the production of ROS and NO, which are mediators of the proinflammatory response [96].

Table 1. Current studies on the effects of iron oxide nanoparticles on reprogramming tumor-associated macrophages as an anti-tumor strategy.

Iron oxide nanoparticle	Macrophage type (*in vitro*)	Described effects	Ref.
Ferumoxytol	RAW264.7 and BDMB from MMTV-PyMT mice	*In vitro*: Ferumoxytol increased caspase-3 expression in cancer cells and production of ROS; upregulated TNF-α and CD86 markers. *In vivo*: Ferumoxytol induced suppression of tumor growth; increased presence of M1 macrophages in the tumor tissues; increased presence of $CD80^+$ cells; inhibit metastases in the liver and lungs.	[14]
CLIO	BDMB from C57BL/6N and $Slc40a1^{C326S/C326S}$ mice	*In vitro*: CLIO nanoparticles induced the decrease of CD206 expression in macrophages and reduction of tumor cell viability. *In vivo*: CLIO nanoparticles induced suppression of tumor growth and increase CD86 expression.	[15]
Ferumoxytol/ferumoxytol functionalized or combined with the TLR3 agonist PIC	RAW 264.7; bone BDMB from C57BL/6 mice	*In vitro*: Ferumoxytol combined or functionalized with PIC induced the retardation of B16F10 cells growth; upregulated TNF-α and iNOS expression; increased NO secretion. *In vivo*: Ferumoxytol combined or functionalized with PIC promoted the melanoma regression; reduced number of lung tumor colonies; upregulated *iNOS* and *TNF-α* expression.	[70]
IO-LPMONs and IO-LPMON-OVA nanoparticles	RAW264.7; BDMB from C57BL/6 mice	*In vitro*: IO-LPMONs increased the number of apoptotic tumor cells and upregulated M1-related iNOS, TNF-α and CD86 markers. In vivo: IO-LPMONs decreased tumor volume and increased CD80+ cells. In addition, treatment with IO-LPMON-OVA combined with IO-LPMONs promoted T cell activation and macrophage polarization to M1-like phenotype with complete prevention of tumor establishment.	[71]
Fe_3O_4-OVA	RAW264.7; BDMB from C57BL/6 mice	*In vitro:* Fe_3O_4-OVA activated macrophages to M1 phenotype with release of TNF-α. *In vivo*: Fe_3O_4-OVA inhibited tumor growth and metastasis; prevented tumor formation with no damage to mice.	[72]
HIONs	RAW 264.7	*In vitro*: HIONs caused a pro-apoptotic effect against cancer cells by increasing the expression of caspase-3 with an efficient generation of ROS and proinflammatory cytokines by macrophages.*In vivo*: HIONs effectively inhibited tumor growth and increased TNF-α production and TNF-α and iNOS expression in macrophages.	[73]
CD206-Fe_3O_4-PLGA nanoparticles	RAW 264.7	*In vitro*: CD206-Fe_3O_4-PLGA significantly promoted the increase of TNF-α, IL-1β and iNOS expression and increased ROS production. *In vivo*: CD206-Fe_3O_4-PLGA inhibited tumor growth and decreased expression of CD86 in TAMs.	[74]
$_{LP}Fe_3O_4$ NPs	RAW 264.7; BDMB from BALB/c mice	*In vitro*: $_{LP}Fe_3O_4$ NPs increased tumor cell death, TNF-α and NO production and expression of CD16/32 M1 markers. *In vivo*: The treatment with $_{LP}Fe_3O_4$ NPs caused effective inhibition of the tumor growth and enhanced apoptotic characteristics in the tumor.	[75]

BDMB: Bone marrow–derived macrophage; CLIO; Fe_3O_4 nanoparticles coated by dextran; HION: Fe_3O_4 nanoparticles functionalized with hyaluronic acid; IO-LPMON: Fe_3O_4-embedded large-pore mesoporous organosilica nanoparticle; LP: L-arginine and poly(acrylic acid); $_{LP}Fe_3O_4$ NP: Fe_3O_4 nanoparticles loaded with l-arginine and sealed with poly(acrylic acid); NO: Nitric oxide; NP: Nanoparticle; PIC: Polyinosinic-polycytidylic acid; PLGA: Poly(lactic-co-glycolic) acid; ROS: Reactive species oxygen.

After uptake, IONPs are degraded into lysosomes by enzymes, and during this process they can be involved in the multifactorial activation of different signaling pathways. The effect of IONPs on transcriptional reprogramming of macrophages has been presented by several groups. In general, these studies show that IONPs can induce a variety of transcription factors related to the expression of iron metabolism-related proteins [99]. It is important to note that the molecular mechanisms involved in the reprogramming of macrophages are still not well understood and more studies to understand this phenomenon must be carried out. At present, it is known that these effects may be related to ROS production by Fenton reaction, TLR4 activation and cytokine production.

The biological effects of nanoparticles are widely influenced by their physicochemical properties, such as chemical composition, shape, size and surface charge. These properties affect several aspects of the interaction between nanoparticles and the mammal organism, including absorption and degradation by the reticuloendothelial system, internalization of target cell, and *in vivo* biodistribution and binding to proteins in body fluids and matrix structures [101,102]. It has been demonstrated that the size, shape and surface charge of nanoparticles significantly modify their ability to modulate the inflammatory response of TAMs. In an interesting *in vitro* study, Zhang *et al.* (2020) evaluated whether IONPs with different charges have distinct effects on macrophage polarization. It was observed that IONPs with positive (S^+) and negative (S-) charges exhibit higher cellular uptake and polarization effects on macrophages [103]. However, S-IONPs showed higher cellular toxicity than S^+IONPs [103]. In addition, when IONPs with neutral charge (SN) were evaluated, lower uptake and cell toxicity were observed as well as

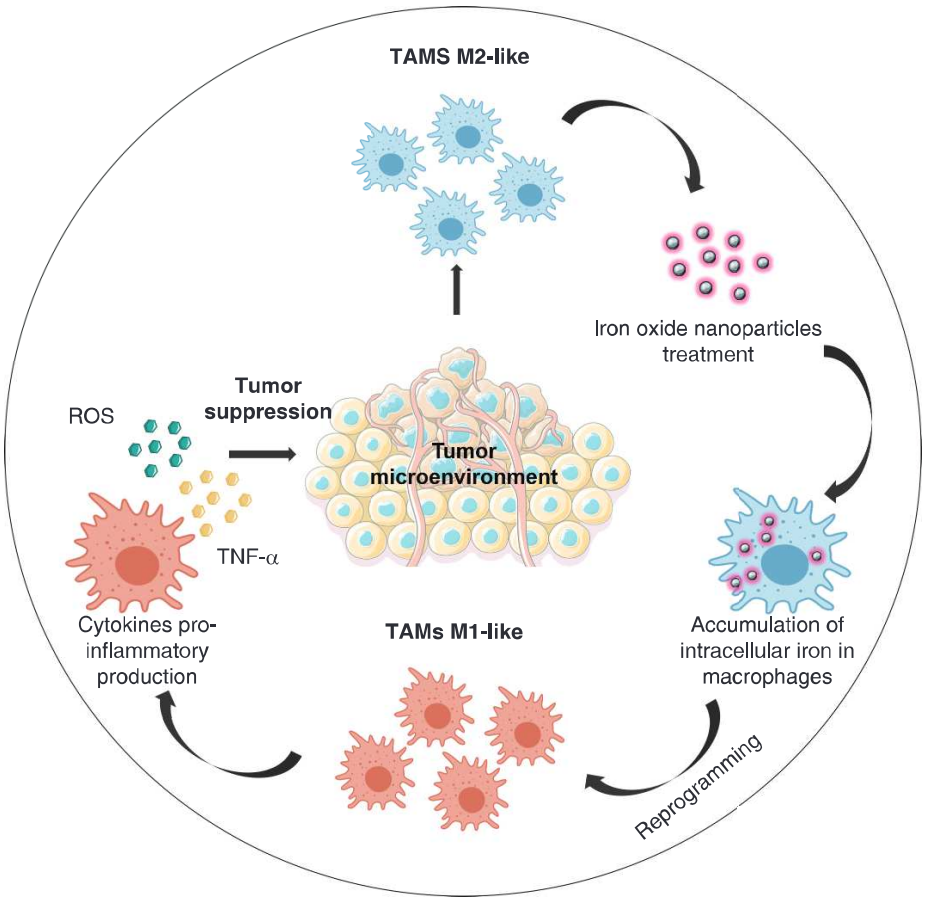

Figure 4. Reprogramming of tumor-associated macrophages by iron oxide nanoparticles as an anti-tumor therapeutic strategy. The anti-tumor effect is attributed to the accumulation of intracellular iron in M2-like macrophages present in the tumor microenvironment, promoting the reprogramming of macrophages to the proinflammatory M1-like phenotype. These M1-like macrophages produce high levels of TNF-α and ROS with significant tumor suppression.
IONP: Iron oxide nanoparticle; ROS: Reactive oxygen species; TAM: Tumor-associated macrophage.

their inefficiency in inducing macrophage polarization [103]. The importance of IONPs shape in the activation of macrophages was also analyzed by Liu *et al.* In their work, they synthesized four types of morphologically distinct IONPs with similar composition and surface charge, creating an ideal system for evaluating the effect of nanoparticle morphology on biological systems [101]. Their results demonstrated that morphology is a critical factor for IONP-induced IL-1β release by macrophages, and IONPs with octopod morphology exhibited significantly greater activity than cube and sphere IONPs [101]. Thus, taken together, these data suggest that the formulation and design of IONPs are characteristics that must be considered for the development of IONPs with applications for the reprogramming of macrophages and cancer treatment.

Another possibility that has been pointed out is the use of IONPs as immunopotentiators in combination with other immunotherapies. In this context, IONPs act as modulators of macrophage phenotype and as nanocarriers to deliver tumor antigens and/or adjuvants for cancer immunotherapy. This strategy could promote the simultaneous activation of T cells and the reprogramming of macrophages to promote tumor suppression, thus improving the efficacy of treatment [89,91]. In addition, IONPs conjugated or manipulated with auxiliary molecules such as antibodies, cytokines, miRNAs or TLR agonists may further amplify the immunomodulatory anti-tumor properties of macrophages [44]. Furthermore, IONPs could protect molecules from degradation and facilitate their immunostimulant properties for anti-tumor immunotherapy [44]. Therefore, the untapped potential of IONPs as immunotherapeutic agents for the activation of macrophages and/or their use as vehicles for other immunotherapies may represent a new, efficient strategy for cancer treatment.

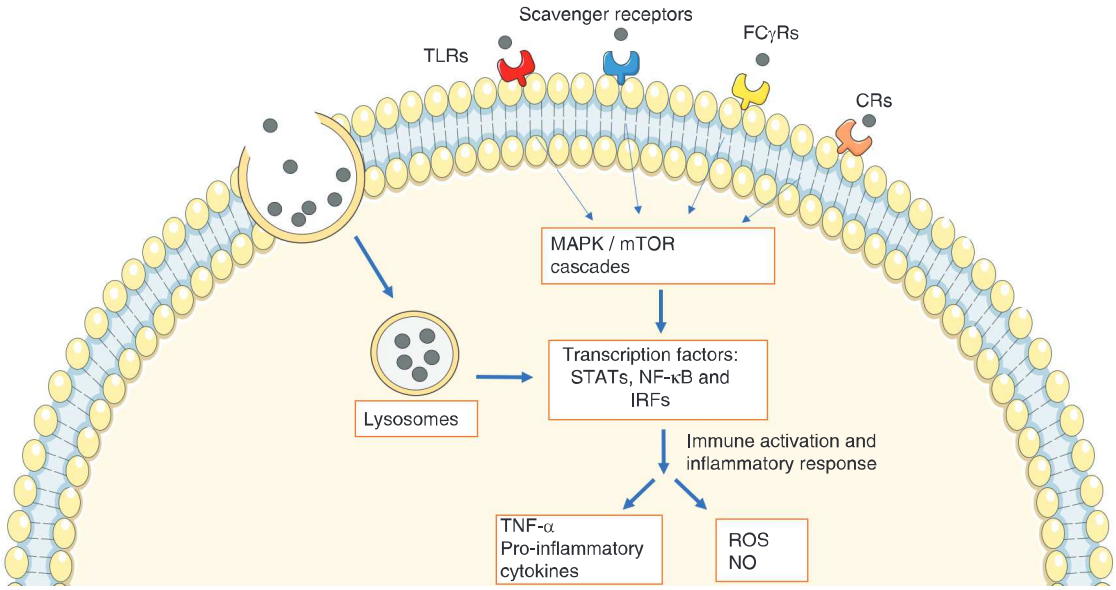

Figure 5. Molecular mechanisms of activation of tumor-associated macrophages to M1-like phenotype by iron oxide nanoparticles. The reprograming of macrophage to the M1-like phenotype by IONPs started with the interaction of nanoparticles with macrophage surface receptors, including TLRs, CRs, scavenger receptors and FCγRs. The binding of IONPs with receptors can promote the downstream MAPK/mTOR cascades and activation of STATs, NF-κB and IRF transcription factors, which regulates immune activation and inflammatory response. In addition, scavenger receptors and TLRs also participate in IONPs uptake. In lysosomes, IONPs are degraded by enzymes and during this process they can be involved in the activation of STATs, NF-κB and IRF transcription factors and transcription factors that regulate iron metabolism–related proteins. The activation results in expression/production of TNF-α, proinflammatory cytokines, ROS and NO, which are mediators of the proinflammatory response.
IONP: Iron oxide nanoparticle; NO: Nitric oxide; ROS: Reactive oxygen species.

Limitations & challenges in clinical application

IONP-based immunotherapy for cancer provides new opportunities to overcome the limitations associated with traditional cancer treatment. The ability of IONPs in reprogramming macrophages makes them a promising candidate for cancer treatment. Their efficiency in the accumulation of IONPs at a tumor site can be improved by increasing extravasation by the enhanced permeation and retention (EPR) effect [104]. However, the development of studies based on the reprogramming of macrophages using IONPs remains largely empirical. The lack of standardization of preclinical studies and variability of experimental conditions pose a barrier for human trials and contribute to slow development of the field. Many fundamental issues need to be established before the results can be transferred from bench to bedside. Each IONP formulation is unique, and several challenges still need to be overcome to achieve their clinical translation. Such challenges to be faced before entering human trials include the physico-chemical properties of IONPs, the preclinical aspects of safety, the administration route, the timing of delivery, pharmacokinetics and biodistribution (Figure 6) [105]. In addition, the challenges of the large-scale manufacturing of IONP systems need to be addressed before successful translation to clinical settings can occur. Comprehending the relationship between the physicochemical properties of IONPs and their behavior in living systems is the absolute requirement for realizing their translational potential [106]. Thus, significant efforts should continue to be made to explore the capacity of IONPs to reprogram macrophages to the M1-like phenotype.

Conclusion

In the tumor microenvironment, different subpopulations of TAMs can influence tumorigenesis. Whereas TAMs polarized for the M2-like phenotype promote cancer progression, TAMs polarized for the M1-like phenotype can reduce tumor growth by selectively killing cancer cells in the tumor microenvironment.

Studies have shown that therapeutic strategies that promote polarization of the M2-like to M1-like phenotype can contribute to cancer control. Among the most promising strategies, the modulation of iron metabolism is an important target for reprogramming TAMs to M1-like tumor suppressor phenotype due to its close relationship with the polarization states of macrophages.

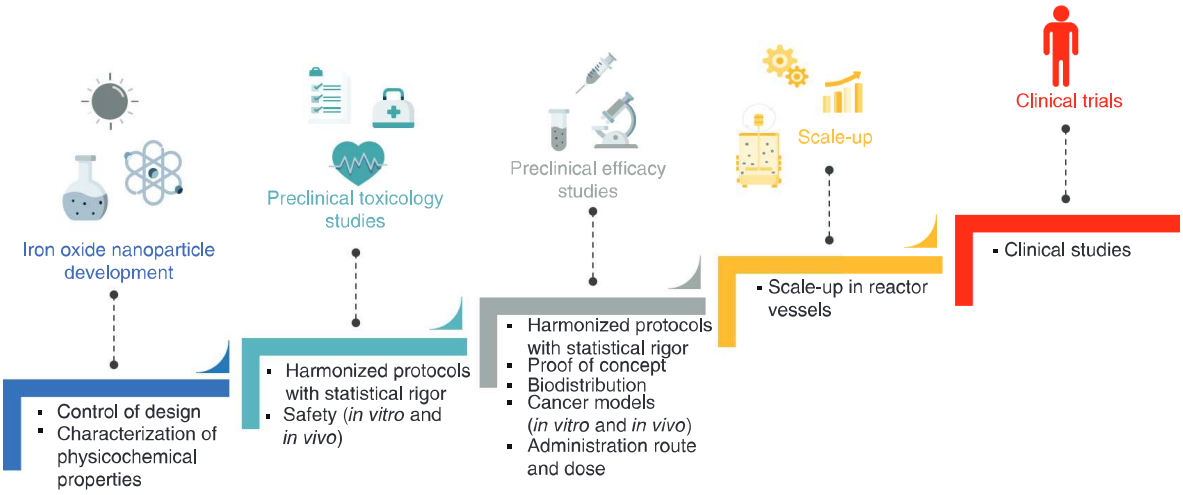

Figure 6. Challenges in the clinical application of iron oxide nanoparticles. Several challenges need to be overcome in the clinical translation of iron oxide nanoparticles (IONPs). Current studies should focus on harmonized protocols with statistical rigor in the physico-chemical characterization of IONPs, preclinical aspects of safety and efficacy, administration route, biodistribution and scale-up manufacturing of IONPs before successful translation to clinical settings.

Due to the importance of iron levels in macrophage polarization states, IONPs can be used to change the activation state of TAMs. The use of IONPs as a treatment for cancer has demonstrated great potential for the modulation of macrophages that acquire features of the proinflammatory M1-like phenotype and their ability to suppress tumor growth. In addition, these nanoparticles can be used as nano-immunopotentiators, acting as a vehicle for other immunotherapies and improving inflammatory response. The feasibility of this potential anti-tumor strategy is due to its biocompatibility, good physical and chemical stability, preferential accumulation in macrophages and ability to affect the activation state of macrophages to a tumor suppressor phenotype through the alteration of intracellular iron levels.

Future perspective

New research should consider IONPs as an instrument for reprogramming macrophages and cancer therapy, as they have had positive and potent results in recent studies. However, the development of TAM-reprogramming therapies based on IONPs is still facing great challenges and new studies should be conducted to clarify the mechanisms that drive the reprogramming of macrophages, how this effect can improve the effect of reprogramming and how to acquire a sufficient and long-lasting anti-tumor response. Research related to the development of immunotherapies for the treatment of cancer with a focus on the use of IONPs should consider the physical and chemical characteristics of the particles as a potential to maximize their therapeutic efficacy.

Author contributions

All authors conceived the review idea and designed the review. CS Nascimento, EA Rocha Alves and CE Calzavara-Silva also collected the references and wrote the manuscript.

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Executive summary

Background

- Cancer is a major public health problem and the increasing incidence of cancer and its mortality rate reveal the importance of developing new therapeutic approaches.
- The treatment of cancer is still a challenge due to various factors such as the increase in radio/chemoresistance and the occurrence of residual tumors.
- Cancer immunotherapy is the most promising trend in oncology, focusing on helping or activating the patient's immune system to identify and fight cancer.
- The metabolic reprogramming of tumor-associated macrophages (TAMs) from the M2 phenotype (promoting tumor progression) to the M1-like phenotype (suppressing tumor growth) it is a new therapeutic strategy against solid tumors.
- Iron oxide nanoparticles are able to reduce the growth of tumor cells through the reprogramming/re-education of TAMs into a tumor suppressor phenotype.

TAMs

- TAMs have aroused great interest as a therapeutic target because they are abundantly present in almost all solid tumors and contribute significantly to cancer progression.
- The TAMs M2-like phenotype promotes tumor progression and the TAMs M1-like phenotype suppresses tumor growth.
- Due to the high plasticity of macrophages, these cells can be a key to treating solid tumors through their reprogramming to the proinflammatory M1-like phenotype.

The role of iron as a tool for metabolic reprogramming of macrophages

- Macrophages are important regulators of iron metabolism by controlling cellular iron import and export.
- Exposure to iron to regulate the expression of proteins correlates to macrophage M1 and M2 polarization states.

The reprogramming of tumor-associated macrophages by iron oxide nanoparticles as an anti-tumor strategy

- Studies involving the polarization of macrophages by iron oxide nanoparticles (IONPs) show that they can induce a phenotypic shift of M2-like macrophages to M1-like macrophages *in vitro* and *in vivo*, leading to tumor cell death.
- High intracellular iron concentration in macrophages can activate several signaling pathways, which are responsible for initiating the reprogramming of macrophages to a proinflammatory phenotype associated with the expression and production of proinflammatory cytokines and reactive oxygen species.
- The biological effects of nanoparticles are widely dictated by their physico-chemical properties.
- IONPs can also be used as immunopotentiators in combination with other immunotherapies.

Limitations & challenges in the clinical application

- The ability of IONPs to reprogram macrophages makes them a promising candidate for cancer treatment.
- The lack of standardization of preclinical studies and variability of experimental conditions are a barrier for human trials and contribute to the slow development of the field.
- Each IONP formulation is unique, and several challenges such as the physico-chemical properties of IONPs, the preclinical aspects of safety, the administration route, the timing of delivery, pharmacokinetics, and biodistribution need to be overcome in order to achieve their clinical translation.
- Significant efforts should continue to be made to explore the capacity of IONPs to reprogram macrophages to the M1-like phenotype.

Future perspective

- New studies should be conducted to clarify the mechanisms that drive the reprogramming macrophages by iron oxide nanoparticles.
- Studies focused on the use of iron oxide nanoparticles for the treatment of cancer should consider the physical and chemical characteristics of the particles as a potential to maximize their therapeutic efficacy.

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