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Implications de l'hème oxygénase-1 myéloïde dans l'échappement à la réponse antitumorale, développement d'un modèle préclinique

Thèse présentée par Emmanuelle ALALUF
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Institut d'immunologie médicale

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I am Emmanuelle Alaluf, I was born on 25th of January, 1987.

I went to Beth-Aviv and then Ganenou schools in Brussels and followed in parallel intense ballet dance training.

I studied medicine at the ULB (Université Libre de Bruxelles) from 2005 to 2012.

Next, I worked as resident in internal medicine till 2015 in ULB hospitals.

Then, convinced that clinical work and medical research are complementary and synergetic approaches, I performed a PhD in immuno-oncology at Institute for Medical Immunology, ULB, from October 2015 to March 2019. I have focused my work on tumor-associated macrophages and the impact of myeloid heme oxygenase-1 on tumor immune escape mechanisms in the mouse.

In 2019, I came back to the clinical work to pursue a specialization in medical oncology at Jules Bordet Institute (Brussels) and Sourasky Ichilov Hospital (Tel Aviv) to become a medical oncologist in 2021.

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ABBREVIATIONS

Akt or PKB	Protein kinase B
AP-1	Activator protein-1
APCs	Antigen-presenting cells
Arg-1	Arginase-1
ATP	Adenosine triphosphate
Bach1	BTB and CNC homolog 1
CAFs	Caner-associated fibroblasts
CD206	Mannose receptor
C/EBPb	CCAAT/enhancer binding protein b
CO	Carbon monoxide
CoPPIX	Cobalt protoporphyrin IX
COX	Cyclooxygenase
CBP	CREB-binding protein
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CTLs	Cytotoxic T lymphocytes
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
EGF	Epidermal growth factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Hb	Hemoglobin
HMGB1	High-mobility group box 1
HO	Heme oxygenase
HIF-1α	Hypoxia-inducible factor 1-a
IDO	Indoleamine 2,3-dioxygenase
IL(-)	Interleukin(-)
iNOS	Inducible nitric oxide synthase
IRAK-M	Interleukin-1R-associated kinase-M

IRF	Interferon regulatory factor
JAK2/STAT3	Janus kinase 2/signal transducer and activator of transcription 3
Keap1	Kelch-like ECH-associated protein 1
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage-colony stimulating factor
MMP	Matrix metalloproteinase
MDSCs	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex class
NF-κB	Nuclear factor-κB
NK (cells)	Natural killer (cells)
NO	Nitric oxide
PAMPs	Pathogen-associated molecular patterns
PD-1	Programmed cell death protein 1
PI3K	Phosphatidylinositol 3-kinase
PPARg	Peroxisome proliferator-activated receptor g
PRRs	Pattern recognition receptors
RAGE	Advanced glycation end products
RNS	Reactive nitrogen species
SHIP	Src homology 2 domain-containing inositol-5-phosphatase
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
ROS	Reactive oxygen species
TAMs	Tumor-associated macrophages
TANs	Tumor-associated neutrophils
TCR	T-cell receptor
TILs	Tumor infiltrating lymphocytes
TLR	Toll-like receptor
TME	Tumor microenvironment
TGF-β	Transforming growth factor- β
TNFα	Tumor Necrosis Factor α

Tollip	Toll-interacting protein
Treg (cells)	Regulatory T (cells)
VEGF	Vascular endothelial growth factor

ABSTRACT

Immunotherapy has revolutionized the treatment of certain cancers by facilitating the antitumor immune response and represents today one of the mainstays of cancer therapy. However, only a subset of patients responds to immunotherapy, which can also lead to serious complications.

The tumor microenvironment is composed of multiple and complex cellular and molecular interactions providing to cancer cells not only a supportive framework but promoting also many steps of immunosuppression and tumor progression. To date, the mechanisms that drive the acquisition of these immunosuppressive features are still poorly defined. Tumor-associated macrophages can be highly represented in the tumor microenvironment where they are shaped and become key players in the innate and adaptive immune escape of the tumor cells.

Heme oxygenase-1 is the rate-limiting enzyme that catabolizes heme into three major biologically active byproducts which display cytoprotective, antioxidant and immunomodulatory effects. We hypothesized that tumor-associated macrophages might suppress anti-tumor T-cell response through heme oxygenase-1 induction in the tumor microenvironment and macrophage polarization.

We showed that heme oxygenase-1 is highly expressed in tumor-associated macrophages. By using a subcutaneous EG7-OVA lymphoma model on genetically engineered mice with a conditional deletion of heme oxygenase-1 in macrophages, our data show that myeloid-restricted heme oxygenase-1 deficiency improves the effect of a therapeutic antitumor immunization by enhancing tumor-infiltrating antitumor CD8+ T-cell proliferation and cytotoxicity and represses tumor growth. Our data suggest a major role of myeloid heme oxygenase-1 in the differentiation and the phenotypic, functional, transcriptional and epigenetic reprograming of tumor-associated macrophages.

Myeloid HO-1 inhibition might be considered as a new myeloid HO-1-mediated immune checkpoint blockade. Targeting myeloid compartment could reprogram the tumor microenvironment and synergize with other cancer therapies.

RESUME

L'immunothérapie a révolutionné le traitement de certains cancers en facilitant la réponse immunitaire anti-tumorale, et représente aujourd'hui l'un des piliers du traitement contre le cancer. Cependant, seule une minorité de patients répond à l'immunothérapie, qui peut également mener à de sérieuses complications.

Le microenvironnement tumoral est composé d'interactions cellulaires et moléculaires multiples et complexes, fournissant aux cellules tumorales non seulement une structure de soutien mais favorisant également de nombreux aspects d'immunosuppression et de progression tumorale. Aujourd'hui, les mécanismes régulant l'acquisition de cette immunosuppression sont encore peu connus. Les macrophages tumoraux peuvent être abondants dans le microenvironnement tumoral où ils sont modulés et deviennent des acteurs clés de l'échappement des cellules tumorales à l'immunité innée et adaptative.

L'hème oxygenase-1 est l'enzyme limitante qui catabolise l'hème en trois produits de dégradation majeurs biologiquement actifs, qui possèdent des effets cytoprotecteurs, antioxydants et immunomodulateurs. Nous avons émis l'hypothèse que les macrophages tumoraux pouvaient supprimer la réponse anti-tumorale lymphocytaire T via l'induction de l'hème oxygenase-1 dans le microenvironnement tumoral et la polarisation des macrophages.

Nous avons montré que l'hème oxygenase-1 est fortement exprimée dans les macrophages tumoraux. En utilisant un modèle lymphomateux d'EG7-OVA sous-cutané sur des souris génétiquement modifiées possédant une délétion de l'hème oxygénase-1 restreinte aux macrophages, nos données montrent que la déficience en hème oxygénase-1 myéloïde améliore l'effet d'une vaccination thérapeutique anti-tumorale en augmentant la prolifération et la cytotoxicité des lymphocytes T CD8+ anti-tumoraux qui infiltrent la tumeur, et réprime la croissance tumorale. Nos données suggèrent un rôle majeur de l'hème oxygénase-1 myéloïde dans la différenciation ainsi que dans la reprogrammation phénotypique, fonctionnelle, transcriptionnelle et épigénétique des macrophages tumoraux.

L'inhibition de l'hème oxygénase-1 myéloïde pourrait être considérée comme un nouvel inhibiteur de checkpoint immunitaire. Cibler le compartiment myéloïde pourrait reprogrammer le microenvironement tumoral et entrer en synergie avec d'autres traitements contre le cancer.

INTRODUCTION

1. History of cancer immunotherapy

The first cases of immunotherapy have been developed in 1891 by Dr.William Coley, who used bacterial toxins and hypothesized that it would stimulate phagocytes and promote tumor cell killing. There were some successes over the next decades but not well reproducible (except regarding a live weakend BCG bacterium for bladder cancer, which is still used today). In 1957, Drs.Macfarlane Burnet and Lewis Thomas proposed the concept of immunosurveillance against pathogens and abnormal cells, in spite of the lack of reliable data. Progress has been made during the next decades in radio- and chemotherapy. In 1970, Drs.Georges Kohler and Cesar Milstein develop the production of mouse monoclonal antibodies, giving rise to major tools for cancer therapy such as rituximab, imatinib, bevacizumab or trastuzumab. In 2010, Sipuleucel-T therapeutic vaccine for castration-resistant prostate cancer started to extend moderately patient overall survival, and other different therapeutic cancer vaccines are currently under development or in clinical trials. From 2011, first immune checkpoint inhibitors such as ipilimumab (anti-CTLA-4), pembrolizumab and nivolumab (anti-PD1) and avelumab, atezolizumab and durvalumab (anti-PD-L1) were approved by the FDA for Hodgkin's lymphoma, advanced melanoma, non-small-cell lung cancer, metastatic renal cell carcinoma, head and neck cancer and advanced urothelial cancer. These molecules brought emerging encouraging results but only for a minority of cancer patients with previously incurable diseases. In 2017, adoptive cell transfer emerged with particularly thrilling results for B cell acute lymphoblastic leukemia. However, cancer immunotherapy has provided the desired clinical efficacy only to a small fraction of patients. Today, hundreds of combined immunotherapy trials take place for various indications (1). Growing evidence has shown that a major barrier to efficient immunotherapy is the tumor microenvironment (TME).

2. A tumor-promoting tumor microenvironment

Cancer cells are endowed with fundamental capabilities, such as a chronic and often uncontrolled cell proliferation associated with the evasion of growth suppressors, resistance to cell death, replicative immortality, angiogenesis, invasion, dissemination, adapted energy metabolism and antitumor immune escape (2). The TME is a complex dynamic network of extracellular matrix components, growth factors, cytokines, chemokines, tumor cells and neighboring stromal cells including fibroblast cells, endothelial cells, epithelial cells and immune cells. These components interact with each other and exploit the immune system to evade immune attack, promote tumor progression and resistance to immunotherapies. The TME alters the trafficking, metabolism and function of T cells. It induces also T-cell apoptosis within the TME (for example through the upregulation of co-inhibitory molecules such as PD-L1, B7-H4, Fas ligand, TRAIL, RANTES, RCAS1 and Galectin-1). These tumor regulatory pathways impair T-cell mediated clearance of tumor cells (3).

Cross-communication between tumor cells and the TME is mediated by secreted cytokines. Interleukin (IL)-4, a well-known Th2 cytokine, is produced by activated tumor-infiltrated Th2-polarized $CD4^+$ T helper cells and by tumor cells, and is suggested to be an indicator of tumor aggressiveness. **IL-4** induces on the one hand T-cell anergy and loss of T cell-mediated cytotoxicity, and on the other hand tumor-promoting and anti-inflammatory tumor-associated macrophage (TAM) polarization. In addition, IL-4 is involved in tumor progression, metastasis, and has anti-apoptotic properties on cancer stem cells. IL-4 could also promote cancer cell proliferation. **IL-1β** is a potent pro-inflammatory cytokine and is abundant in the TME. IL-1β influences tumor growth and invasion and is correlated with poor cancer patient outcome. Its role in carcinogenesis could be attributed to mutagenesis induction through ROS and NOS production. **IL-6**, also elevated in the TME, is mainly produced by TAMs. IL-6 contributes to the proliferation, dissemination, VEGF production and resistance of many tumor cells. IL-6 is also a key regulator of cancer stem cell self-renew (4). **IL-10** is known for its anti-inflammatory activity and suppressive properties on antitumor immune response. However, mouse models lacking IL-10 or IL-10 receptor show inflammatory bowel disease and cancer. IL-10 has been reported to stimulate antitumor immunity. Mouse tumor models have shown IL-10-mediated rejection of tumors, through both its anti-inflammatory properties and its stimulation of antitumor-specific $CD8^+$ T-cell

cytotoxicity. For example, mice genetically engineered to express IL-10 in myeloid cells have been reported to reject transplantable tumors through a mechanism depending on $CD8^+$ T cells. On the other hand, IL-10 -/- mice were found to be susceptible to chemically induced skin cancers (5). The transforming growth factor-β (**TGF-β**) plays a role as both tumor suppressor and tumor promoter depending on the microenvironmental context. It seems to initiate growth arrest early during tumor development, but in late-stage tumors it promotes substantial changes in the tumor stroma, angiogenesis, epithelial-to-mesenchymal transition and cellular migration and invasion. It also decreases tumor cell recognition and clearing by the antitumor immune system, recruits regulatory T (Treg) cells and myeloid-derived suppressor cells (MDSCs) into the TME, drives the phenotypical conversion of naive to Treg cells, inhibits the functional maturation of natural killer (NK) cells, inhibits the activation of antigen-presenting cells (APCs), induces M2-like macrophage polarization and suppresses T-cell proliferation and Th1 $CD4^+$ T-cell phenotype. TGF-β induces cancer-associated fibroblast (CAFs) differentiation which results in collagen deposition and extracellular matrix remodeling. This ultimately promotes tumor progression in a TGF-β-dependent manner, although there is also controversial evidence showing a tumor-suppressive role of TGF-β. An example among others is the suppression of TGF-β signaling in fibroblasts that increases inflammatory cell infiltration and tumor progression (6).

The **extracellular matrix** displays a dysregulated architecture in solid tumors, and provides not only a structural framework for connective tissues but also affects T cells and favors tumor progression (7).

Tumor cells produce several types of vascular endothelial growth factors (**VEGF**) and other growth factors that enhance the formation of new tumor-associated blood and lymphatic vessels which support tumor outgrowth, metastatic spreading of tumor cells and immune evasion (8). The blood vessels are irregularly organized, leak, display suboptimal blood flow, resulting in hypoxia and further VEGF production, promoting tumor growth.

Tumor **endothelial cells** display abnormal morphologies and phenotypes and actively affect tumor cells to promote tumor metastasis and progression. These cells are proangiogenic and secrete matrix metalloproteinases to migrate into the tumor during angiogenesis. They do not undergo senescence unlike normal endothelial cells, they contain cytogenetic abnormalities, show resistance to antiangiogenic and chemotherapeutic drugs and suppress T-cell recruitment, adhesion

and activity (9). In addition, pericytes have also been suggested to contribute to the regulation of innate and adaptive immunity (10).

Red blood cells and hemoglobin from tumor bleeding promote tumor cell proliferation and induce chemoresistance. This is associated with endogenous danger signals, an inflammatory signature, an increased tumor neoangiogenesis and a recruitment of anti-inflammatory macrophages (11).

In most studies, **hypoxia** also contributes to tumor immune escape (12). For example, neoangiogenesis is induced by hypoxia within the TME via the production of VEGF. This decreases the presentation of tumor-associated antigens to T cells and increases the accumulation of MDSCs in the TME and secondary lymphoid organs. Furthermore, hypoxia-inducible factor 1-α (HIF-1α), induced by hypoxia, modulates TAM phenotype and T-cell proliferation and cytotoxicity. Under hypoxic conditions, anti-inflammatory tumor-derived cytokines are secreted such as IL-10 and TGF-β. Furthermore, hypoxia reduces energy sources and increases extracellular levels of adenosine in the TME, which binds to its receptors on immune cells and further contributes to the establishment of an immunosuppressive environment (13).

In addition, rapidly proliferating malignant cells generate large amounts of **lactate** to the extracellular microenvironment, resulting from aerobic glycolysis. This lactate contributes to acidosis, stimulates angiogenesis, acts as cancer cell metabolic fuel, and exerts deleterious effect on tumor-infiltrating immune cells. For example, lactate impairs cytotoxic T-cell function, inhibits the differentiation from monocytes to dendritic cells (DCs), inactivates DC cytokine production, and induces VEFG and arginase-1 (Arg-1) expression in TAMs, contributing thereby to tumor evasion (14).

Dying cells release adenosine triphosphate (ATP) which is metabolized to adenosine that ultimately suppresses tumor-infiltrating T-cell function (13).

Lastly, immunosuppression in the TME is orchestrated by a variety of **stromal myeloid and lymphoid cells** that will be detailed further below.

3. Impact of the immune infiltrate on cancer outcome

A meta-analysis of gene expression profiles from 18 000 tumors from cancer patients across 39 different malignancies largely established associations between diverse tumor-associated leukocyte subsets and overall survival and highlights the impact of tumor immune heterogeneity on the clinical outcome of cancer patients (15). The correlation between the level of immune cell infiltration of the TME and the clinical outcome has been shown in many different cancer types. In contrast with tumor-infiltrating myeloid cells and specific subtypes of lymphoid cells, tumor-infiltrating $CD8^+$ T cells and Th1 $CD4^+$ T cells are often strongly associated with a better clinical outcome and is a predictor of disease-free survival and overall survival in addition to the widely used TNM classification (16). The adaptive immune system found in the TME plays indeed a major role in immune surveillance with specialized T-cell subpopulations playing specialized effector functions (17). This concept is exploited for immunotherapy purposes. In addition, T cell-mediated cytotoxic response releases new tumor-associated antigens. This allows a new extension of the antitumor response and is called immunotherapy-induced antigen spread (18).

4. Cancer immunoediting

Three types of tumor antigens with high tumoral specificity can elicit a tumor-specific immune response. They are viral antigens, antigens resulting from a mutated gene-coding sequence from ubiquitously expressed genes, and antigens encoded by cancer-germline genes that are normally silent in almost all normal adult tissues and shared among distinct tumors. Among antigens of low tumoral specificity, there are differentiation antigens and antigens derived from proteins but that are overexpressed in tumors (19). Most tumor cells express antigens that can be recognized by T cells, potentially allowing an efficient antitumor immune response, although they are often not eradicated. Indeed, immune rejection of established tumors are rarely seen, and adoptive cell transfer in mouse and human tumors show often initial tumor regression followed by inefficient control of tumor development. In fact, immune system plays a dual role in cancer: suppressing tumor growth and promoting tumor progression by developing an immunosuppressive TME, selecting for tumor cells that will be able to survive in an immunocompetent host. Cancer immunosurveillance concept is evolving towards a new cancer immunoediting concept consisting

in three phases, the elimination, the equilibrium and the escape phases. The tumor **elimination phase** of the antitumor immune response rests on host effector molecules such as both type I and type II interferons, perforin, granzyme, Fas/FasL, TRAIL, NKG2D, an intact lymphocyte compartment of the adaptive immunity, and cells of the innate immunity such as NK cells and macrophages. The molecular mechanisms underlying the **equilibrium phase** are poorly understood but would involve a balance between positive and negative immunoregulation maintaining tumor cells in a state of immune-mediated dormancy with arrest of cancer progression. During the **escape phase**, immunologically sculpted tumors grow. This involves tumors with loss of antigenicity (by lack or mutation of immunogenic tumor antigens or by downregulation of Major Histocompatibility Complex (MHC) and costimulatory molecules) and immunogenicity (by a multitude of negative immunoregulatory mechanisms) (20), ultimately leading to a defective recruitment of antitumor T cells to the tumor site, to T-cell functional impairment and to T-cell suppression within the TME (21) (22). Tumor cells escape antitumor immunity and cancer progresses in spite of antigen-specific immune response.

5. Tumor-infiltrating immune cell dysfunction

As described above, the immune system is a critical regulator of tumor progression. It surveys actively for tumor cells and can eliminate them, but tumor cells evolve to avoid this elimination. Tumor cells orchestrate active sites of "immune privilege" within the TME. Indeed, while it is not clearly established in the periphery, the antitumor immune response within the TME is defective. Tumors can be divided in so-called hot tumors which are T-cell inflamed and cold tumors which are T-cell noninflamed. The latter can be subdivided into immune-excluded tumors where T cells are attracted to the periphery of the tumor but fail to penetrate and immunologically ignorant tumors where it seems to be no recruitment of T cells at all (23). T-cell intrinsic factors (such as the immune checkpoints) and extrinsic functional inhibition through different tumor-derived cell types and factors (cell surface proteins, cytokines, chemokines, high reactive soluble oxygen and nitrogen species, gangliosides, toxic metabolites, amino acid metabolism) vary according to tumor type and tumor stage (24). Overall, the impact of tumor-infiltrating effector T cells can be heavily modulated by the coinfiltration of regulatory tumor-infiltrating lymphoid and myeloid cells that inhibit antitumor immune response using numerous mechanisms.

5.1. Tumor-infiltrating CD8+ T cells

Priming of naïve CD8+ T cells occurs mostly in the tumor-draining lymph nodes after T-cell receptor (TCR) - antigenic peptide - MHC I interaction and costimulation by APCs, leading to differentiation into effector cytotoxic T lymphocytes (CTLs). **CTLs are highly cytotoxic and play a crucial role in killing tumor cells** upon recognition, by TCR, of tumor-specific antigenic peptides bound to MHC I molecules on the surface of target cells, associated with the CD3 and CD8 molecules and the interaction of adhesion molecules (25) (26). This is followed by a specific cytolysis of the target cell which is mediated by exocytosis of cytotoxic granules including perforin, granulysin and granzymes and through cytokine secretion such as IFNɤ and TNFα (27) (28). After antigen-bearing cell apoptosis, effector cells undergo apoptosis, but a small subset of antigen-experienced CD8+ T cells persists as memory CD8+ T cells that can readily differentiate into effector T cells upon a secondary antigen challenge. Central memory cells acquire less rapidly an effector function but have a high proliferative potential.

Tumor-infiltrating lymphocytes (TILs) **often display multiple states of CD8+ T-cell dysfunction** in mouse tumor models and cancer patients (29). The TCR is exposed to a persistent antigen or to epitopes presented in large amounts, leading to a state of **exhausted CD8+ T cells**, which is a gradual state of T-cell dysfunction. This is characterized by an affected transcriptional state which impairs the optimal control of tumor development (30), although a specific transcription factor has not been identified yet. Exhausted T cells display a poor effector function and can compromise memory T-cell function. This involves the upregulation of inhibitory receptors such as PD-1, CTLA-4, TIM-3, LAG-3, CD160, BTLA, TIGIT, 2B4 and express high amounts of Blimp-1. Immunoregulatory cytokines such as IL-10 and TGF-β also influence T-cell exhaustion. Tumor-specific T cells become dysfunctional already at early malignant stage and are initially therapeutically reversible but evolve later into an irreversible state. Immunoregulatory cell types such as Treg cells, but also CD8+ regulatory T cells, alternatively activated macrophages and altered APCs are known to suppress effector functions in the TME. They may also be directly involved in T-cell exhaustion but that is poorly described to date (31). Immunosuppressive mechanisms in the TME and APCs during CD8+ T-cell priming drive TILs to an anergic phenotype, making them unable to control the tumor. **CD8+ T-cell anergy** takes place in the early

stages of tumorigenesis. Anergy involves specific genes, but others could be shared between exhausted and anergic T cells (30). Another $CD8^+$ T-cell dysfunctional subtype described is the heterogeneous population consisting in both senescent and/or regulatory $CD8^+$ T cells, defined by the loss of CD28 expression. Tissue-resident memory $CD8^+$ T cells accumulate in human cancer where their retention may be important in effector antitumor CTL response and may be associated with a favorable prognosis. However, they exhibit also often a dysfunctional phenotype in tumors (32).

5.2. Tumor-infiltrating $CD4^+$ T cells

Because $CD8^+$ CTLs are able to target and directly kill tumor cells expressing MHC class I molecules, they have long been considered as the main effector cells required for tumor control and eradication. Moreover, effector CTLs do not require $CD4^+$ T cells or co-stimulation for tumor cell lysis. However, many other cells of the immune system play an important role. The TME consists in a complex network of T-cell subsets. $CD4^+$ T cells show high plasticity depending on developmental and environmental cues and display a broad range of lineages in terms of cytokine production and effector functions, contributing to the antitumoral or protumoral activity (33).

$CD4^+$ T-cell population improves the antitumoral effects of tumor-reactive $CD8^+$ T cells and optimize the antitumor immune response. After a required tumor antigen recognition on APCs during priming in tumor draining-lymph nodes, $CD4^+$ T cells can differentiate into Th1 cells and migrate to the tumor site where they contribute directly and indirectly to the eradication of MHC II^+ and MHC II^- tumor cells, respectively (34). Cancer cells can indeed present intracellular tumor antigens on MHC II by several nonclassical antigen-processing pathways which are directly recognized by tumor antigen-specific $CD4^+$ T cells (35). $CD4^+$ Th1 cells have the capacity to directly kill MHC II^+ tumor cells through perforine and granzyme B, TRAIL receptor and Fas/Fas ligand pathways. However, the majority of tumor cells do not express MHC II. **Th1 $CD4^+$ T-cell subpopulation plays an indirect role in antitumor immunity against tumor cells**. For example, Th1 cells produce proinflammatory cytokines such as IFNɤ, TNFα and IL-2, displaying by this way antiproliferative and pro-apoptotic effects on tumor cells. The production of IFNɤ from Th1 cells induces the expression of MHC molecules on tumor cells. Th1 $CD4^+$ T cells activate

macrophage-mediated killing of tumor cells and indiscriminate surrounding cells (36), and activate also APCs, providing antigen presentation and costimulatory signals to effector $CD8^+$ T cells through the secretion of cytokines such as IFNɤ. In addition, Th1 $CD4^+$ T cells promote $CD8^+$ T-cell infiltration into the tumor through the secretion of chemokines and recruit inflammatory cells such as macrophages, granulocytes and NK cells. They also help CTLs in the expression of cytotoxic effector molecules and downregulation of inhibitory receptors. However, as $CD8^+$ T cells, $CD4^+$ T cells are also subject to tolerance if the tumor antigen-secreting tumor cells are not efficiently eliminated within a short timeframe. Th2 cells in cancer are not well described compared to Th1 cells. Th2 $CD4^+$ T cells are not directly cytotoxic but mediate their effector function by releasing cytokines that activate other immune cells such as mast cells, eosinophils, B cells and macrophages. Besides extracellular parasite immunity and allergic inflammatory response, **Th2 cells are generally considered to be detrimental in cancer immunity by producing Th2-associated cytokines** such as IL-4, IL-5 and IL-13 at the tumor site. However, Th2 cells have been reported to exert a long-lasting tumor antigen-specific immunity with a capacity of tumor eradication which does not require B cells, NK cells, or $CD8^+$ T cells, through massive infiltration of M2-like macrophages producing arginase (in close contact with tumor cells) along with a more modest recruitment of eosinophils (37). Th17 cells, involved in antimicrobial and autoimmunity, produce proinflammatory cytokines including IL-17A, IL-17F and IL-22. These cells have a long lifespan and a high ability to self-renew, in contrast to Th1 cells. $CD4^+$ T cell-derived IL-17 cytokines seem to have a dual function in cancer. They display antitumor functions and prevent tumor progression by recruiting TILs, promoting NK cell and CTL activity. On the other hand, they exert an oncogenic function by preventing tumor cell apoptosis and increasing tumor angiogenesis, invasion and metastasis. It mediates also the recruitment of myeloid cells and Treg cells into the TME (38). Follicular helper Tfh cells contribute to immunity against tumor growth. They promote B cell differentiation into antibody-secreting cells and play an important role in immune cell recruitment to the tumor and in the formation of intratumoral tertiary lymphoid structure germinal centers associated with a good prognosis in cancer patients (39). They are suggested to be central players in long-term protection against tumor growth. Although their functions and underlying mechanisms remain elusive, the emerging IL-9-producing Th9 T-cell subset has pro-inflammatory function in a broad spectrum of autoimmune and allergic disease. In tumor models, it may exert potent antitumor properties in solid tumors by activating

both innate and adaptive immune response by an IL-9 dependent manner, although a tumor-promoting role for Th9 cells has also been suggested (40).

5.3. Tumor-infiltrating regulatory T cells

Another major component involved in the immunosuppression of the TME are Treg cells. Indeed, Treg cells exert protumoral effects by using cell-contact dependent and independent mechanisms to suppress effector T cells within the TME (41). They are often found at elevated densities in tumor lesions, suppress the activation and differentiation of $CD4^+$ and $CD8^+$ T cells and are often predictive of a poor clinical outcome in cancer (42). Treg cells, defined as $CD4^+$ T cells with a high expression of CD25 (IL-2 receptor α-chain) and the transcription factor Foxp3 when they are activated and highly suppressive cells, play a central role in the maintenance of self-tolerance but a detrimental role in antitumor immune response. They exhibit their suppressive activity by **inhibiting the maturation of APCs in an antigen-specific manner**, by a **high consumption (and low production) of IL-2** from the extracellular milieu and by the secretion of **anti-inflammatory cytokines** such as IL-10 and TGF-β.

5.4. Tumor-infiltrating dendritic cells

DCs are critical modulators of the antitumor immune response using a sophisticated presentation of peptides to the adaptive immune system to **initiate a long-lasting and antigen-specific response**. Indeed, immature DCs patrol in peripheral tissues searching for endocytosis of invading pathogens and dying cells for example. After DC stimulation by a variety of proinflammatory signals (pathogen-associated molecular patterns (PAMPs), danger-associated molecular patterns (DAMPs), inflammatory mediators, CD40 ligand, …), DCs are triggered to mature, migrate to lymphoid organs and upregulate efficiently peptide-MHC complexes, costimulatory molecules, immunostimulatory cytokine production and chemokines necessary for the recruitment of the effector immune cells. Proteins are partially degraded in the endosomal-lysosomal system, and in the cytosol and endoplasmic reticulum, to stably load antigenic peptides onto MHC I or MHC II molecules and transport them to the cell surface. Then, the recognition by antigen-specific naive

or memory T cells and costimulation (interaction between CD80, CD86 and CD28) takes place. This is followed by activation and expansion of antigen-specific T cells and T-cell differentiation into effector lymphocytes. DCs optimize their functions notably by producing high amounts of proinflammatory cytokines such as IL-12 to enhance T-cell stimulation, but these cells can also display different maturation pathways and differently polarize T-cell response. MHC II pathway is mainly at play for the presentation of antigens derived from extracellular sources, where MHC I pathway is associated with the presentation of endogenous antigens. DCs are also peculiarly capable of "cross presentation" where antigens captured from the extracellular space can be loaded onto MHC I and presented to $CD8^+$ T cells (43). Some tumor-infiltrating DC populations have been shown to be strong CTL stimulators and correlate with clinical outcome across several mouse tumor models and human cancers (44). In cancer, however, DCs generally fail to induce an efficient antitumor effector T-cell response and to control tumor growth. Indeed, DC phenotype and function are modulated by tumor cells and tumor-associated cells and factors in the TME. Tumor-derived factors such as IL-6, M-CSF, IL-10, TGF-β, VEGF have been shown to negatively regulate DC function, inhibiting DC differentiation, maturation, activation and migration. This induces a tolerogenic phenotype with a **decreased ability to present antigens and to prime T cells**. This induces also **anergic cytotoxic $CD8^+$ T cells**, an **inability to produce IL-12**, a **skewing towards a Th2 response** and the **induction of Treg cells**. The **inhibitory molecules** such as PD-L1, PD-L2, Tim3, LAG3 also contribute to the negative regulation of DC function in the TME and the tumor-draining lymph nodes (45). Tumor-altered DC function by tumor-derived factors is also accompagnied by an **alteration of the differentiation program** of DC precursors, promoting the accumulation of immunosuppressive myeloid cells such as immature MDSCs and TAMs (46).

5.5. Myeloid-derived suppressor cells

Cancer is associated with an atypical expansion of myeloid cells that are immature. These myeloid cells fail to differentiate and to behave as efficient APCs and macrophages. This includes the inability to provide adequate levels of MHC/antigen presentation, co-stimulation molecules and pro-inflammatory cytokines (47). MDSCs are myeloid cells at different stages of maturation, known for their potent ability to suppress various types of immune responses, including antitumor immune response (48). They are also known to play an important role in the

remodeling of the TME, tumor angiogenesis, tumor dissemination and drug resistance. In mouse, these cells are defined as $CD11b^{+}Ly6G^{+}Ly6C^{lo}$ **granulocytic PMN-MDSCs** and $CD11b^{+}Ly6G^{-}Ly6C^{+}$ **monocytic M-MDSCs**, phenotypically and morphologically similar to neutrophils and monocytes, respectively. In human, MDSCs are defined with common myeloid markers such as CD11b and CD33 but lack markers of mature cells and HLA-DR. MDSCs are not present in the steady state of healthy individuals but accumulate in chronic pathological conditions such as cancer where they create a tolerogenic environment, mainly by blocking activation and proliferation of T cells, notably through Arg-1, indoleamine 2,3-dioxygenase (IDO), inducible nitric oxide synthase (iNOS), TGF-β, IL-10 and cyclooxygenase 2. Monocytic MDSCs suppress T-cell response both in an antigen-specific and nonspecific manner, using NO and cytokines, while granulocytic MDSCs mainly use reactive oxygen species (ROS) to suppress antitumor immune response in an antigen-specific manner (MDSC, Gabrilovich). In the TME, M-MDSCs are more prevalent than PMN-MDSCs and more suppressive. **Most M-MDSCs rapidly differentiate into mature immunosuppressive TAMs within the TME** (49) (50) (51). It is not clear whether they constitute a transitory step to mature TAMs or whether they are a terminal cell type.

5.6. Tumor-associated macrophages

As briefly summarized above, the TME is composed of multiple and complex cellular interactions providing not only a supportive framework but also a malignant potential, where immune cells can be highly represented. TAMs are among the most abundant of these immune cells and influence many steps of tumorigenesis and tumor progression, including genetic alterations and instability, regulation of senescence, angiogenesis and lymphangiogenesis, remodeling of the extracellular matrix, tumor survival, invasion and dissemination. TAMs are also widely recognized as **key modulators of the antitumor immune response**. This will be detailed further below.

5.7. Other tumor-infiltrating cells

Tumor-associated neutrophils (TANs) are phagocytic cells that migrate rapidly to a site of injury where they release cytokines and toxic molecules to elicit an acute inflammatory response and

eliminate pathogens. TANs play a complex role in cancer. They have been reported to exert ROS-mediated tumor cell apoptosis. They can also function directly or indirectly as antigen-presenting cells and promote antitumor activity of T cells. These neutrophils generate extracellular traps that are networks of extracellular fibers typically released from neutrophils to bind pathogens but recently found to inhibit the proliferation and the growth of tumor cells. However, these cells can have divergent antitumor or protumor functions, acting on the tumor cells and on the TME. Neutrophil phenotypes could be modulated by local cues from the microenvironment. In tumor-bearing mouse models, a TGF-β-rich environment induces a **"N2" tumor-promoting phenotype**, contrasting with the inhibition of TGF-β or the presence of IFNβ. It has been established that TANs play a role in the initiation, development and progression of tumor. They contribute to the establishment of an immune tolerance within the TME. They are indeed major producers of ROS, which cause oxidative stress in T cells, and of Arg-1, which metabolizes arginine that is required for T-cell CD3ζ chain expression. They also express direct immunosuppressive molecules such as PD-L1. Once in the TME, TANs produce cytokines, chemokines and proteases that regulate tumor cell proliferation, angiogenesis and metastasis. They accumulate in the blood of cancer patients and are associated with a poor clinical outcome in several tumor types, although few studies have been carried out on TANs in human with controversial data (52)(53).

Innate effector cells including natural killer (**NK**) cells are a heterogeneous and plastic cell population lacking genetically rearranged antigen receptors, known to kill virally infected and abnormal cells. Many studies demonstrated their **cytotoxic properties against tumor cells** through inhibitory receptors such as KIRs for example. They are strong producers of cytokines such as IFNɤ which promotes Th1 cell polarization and DC activation and chemokines which recruit DCs into solid tumors (54). However, the exact role of these tumor-infiltrating cells in tumor immunity and cancer prognosis is unclear. A reduction of NK cell cytotoxicity often occurs during malignant transformation and some subpopulations of innate effector cells can have a regulatory rather than a cytotoxic function in the TME (55).

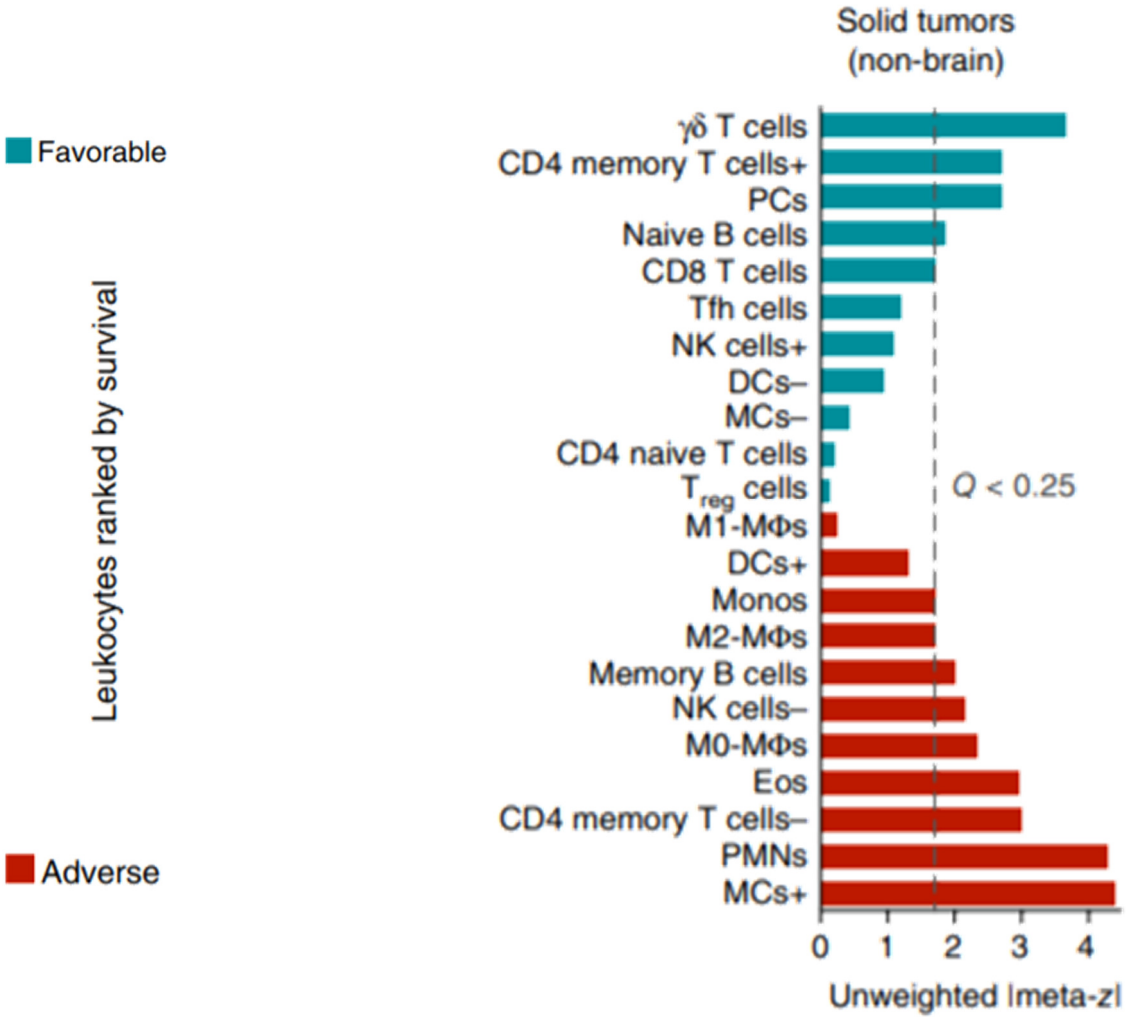
ɤδ**T cells** have shown a strong antitumor immune response (by direct cytotoxic activity and by the production of huge amounts of pro-inflammatory cytokines). These cells also stimulate B cells, αβT cells, NK cells, macrophages and DC maturation and can even display characteristics of APC allowing αβT cell activation. However, ɤδT cells are often impaired by immunosuppressive signals

from the TME showing a dual, either anti-tumoral or protumoral role, by differentiating into different functional subsets upon polarizing microenvironment (56).

B cells can also modulate the immune response in both solid and hematologic cancer. In several tumor models, regulatory B-cell subset produces IL-10 and TGF-β and suppress T cells and NK cells through immunosuppressive ligands such as PD-L1, or induce the generation of Treg cells, promoting tumor growth. Regulatory B cells have also a tumor-promoting effect on tumor-infiltrating myeloid cell education. Regulatory B cells are upregulated in cancer patients with solid tumors and are associated with a more aggressive disease (57).

Cancer-associated fibroblasts (CAFs) constitute another important component of the tumor stroma where they are a major source of TGF-β in many tumor types. They produce the cytokines and chemokines required to attract and retain immunosuppressive cells into the TME, contribute to the extracellular matrix-mediated T-cell trapping and actively polarize macrophages toward a tumor-promoting phenotype (58).

Although it has been considered to play a role in the eradication of tumor cells, a growing body of evidence indicates that the complement activities can play a tumor-promoting and immunoregulatory role in several tumor types. For example, proteins of the complement have been reported to promote the recruitment and polarization of MDSCs, TAMs, Th2 $CD4^+$ T cells and the induction of Treg cells. In addition, the activation of products of the complements have been shown to upregulate the expression of molecules such as PD-L1, IL-10, TGF-β or Arg-1 in the TME (59).



Gentles, Nat Med, 2015

Main cellular features and immune dysfunctions in the tumor microenvironment

	Mainly antitumoral activity	Mainly protumoral activity
$CD8^+$ T cells	Cytotoxicity	Exhaustion, anergy
NK cells	Cytotoxicity Th1-polarization DC activation	Reduced cytotoxic function
$CD4^+$ T cells	Th1 (indirect role)	Th2 (indirect role)
DCs	Initiation of a long-lasting and antigen-specific response	Treg-cell induction CTL anergy Th2-response induction Low IL-12 secretion Decreased APC capacity MDSC/TAM differentiation
TANs	N1-like neutrophils	N2-like neutrophils
MDSCs		M-MDSCs PMN-MDSCs
TAMs	M1-like macrophages	M2-like macrophages
Treg cells		T-cell suppression and inhibition of APC maturation in an antigen-specific manner High consumption/low production of IL-2 Anti-inflammatory cytokines
CAFs		Matrix remodeling T-cell trapping M2 polarization
Endothelial cells		Angiogenesis Matrix remodeling
Cancer cells		Uncontrolled cell proliferation Immortality Angiogenesis Invasion and dissemination Immune escape

6. Tumor-associated macrophages

Cancer leads to an expanded and profoundly altered myelopoiesis. Indeed, tumor-derived factors (such as M-CSF, CCL2) expand myeloid precursors and induce their recruitment into the TME. Once in the TME, monocytes rapidly differentiate into TAMs (60). Fully differentiated TAMs display self-renewal capability, although circulating monocytic progenitor migration to the TME is required in the long term. This monocyte recruitment can originate from the bone marrow but also from tumor-induced extramedullary hematopoiesis within the spleen (61). In turn, these altered tumor-reprogrammed myeloid cells drive the neoplastic process by immune-dependent (both direct and indirect effects) and independent mechanisms.

6.1. Protumoral role of TAMs

Clinical studies and experimental mouse models demonstrate that TAMs are educated by the TME and generally adopt a protumoral role, in contrast to their tumoricidal role after in vitro activation (62). Macrophage-derived factors released in the TME have a broad impact on multiple aspects of tumor growth and progression.

Through the release of inflammatory mediators like reactive oxygen species (ROS) and reactive nitrogen species (RNS), tumor necrosis factor α (TNFα), IL-1β and IL-6, TAMs are believed to **provoke DNA damages promoting neoplastic transformation** (62). Furthermore, TAMs release Epidermal Growth Factor (EGF) that **activates cancer stem cell specific pathways** including Signal Transducer and Activator of Transcription (STAT) 3 as well as drug resistance. In turn, cancer stem cells could be able to promote TAM conversion (63).

TAM express also the SIRPα marker on their surface, which recognizes a CD47 marker (**"don't eat me" signal**) on tumor cells. Blocking the CD47-SIRPα axis could promote the phagocytosis of tumor cells by TAMs (64).

Through a paracrine loop, EGF released by TAMs interact with M-CSF released by tumor cells, promoting **tumor cell intravasation and dissemination**, while **tumor cell invasion** is promoted by TAMs through the degradation of extracellular matrix proteins through MMP2 and MMP9 (65) (66).

Consistent with their role in maintaining tissue homeostasis, TAMs have an impact on **neoangiogenesis**, **dissolution and remodeling of the interstitial matrix**, contributing to tumor protection and progression. Indeed, TAMs stimulate the acquisition of a vasculature within the TME often designated as "angiogenic switch" through the production of pro-angiogenic factors like TGF-β, VEGF, platelet-derived growth factor (PDGF), matrix metalloproteinases (MMP) and angiogenic chemokines. Notably, Tie-2-expressing monocytes and macrophages have been demonstrated to have a strong angiogenic activity. The matrix remodeling is promoted by enzymes secreted by TAMs such as MMP, plasmin, urokinase-type plasminogen activator and the uPA receptor, regulating matrix digestion and composition. TAMs also increase the secretion of VEGF-C, inducing lymphangiogenesis which has been shown to facilitate tumor cell dissemination to lymph nodes and metastasis (67).

All these protumoral properties are often further reinforced after cancer treatments, making TAMs a **regulator of tumor response to chemotherapy and radiotherapy** (68). Indeed, although some radio-chemotherapies result in alarm signals which trigger an adaptive immune response against tumor cells (some treatments induce immunogenic cell death or non-specific immune stimulation that sensitize tumor cells to killing by cytotoxic immune cells), they can also limit their therapeutic effects. For example, conventional cytotoxic drugs induce tumor cells to produce macrophage recruitment factors, and chemotherapy associated with blockade of macrophage recruitment could increase neoplastic tissue damages and be a new therapeutic approach (69). Likewise, **TAMs can be recruited and polarized** during radiotherapy treatment, inducing tissue repair. This limits the efficacy of chemotherapy and radiotherapy and promotes early tumor recurrence. TAM depletion in combination with radiotherapy inhibits more efficiently tumor growth (70) (71). Tumor regression has also been observed in patients with surgically incurable pancreatic ductal adenocarcinoma through stimulation of the CD40 receptor by an agonist antibody, associated with gemcitabine chemotherapy (72).

6.2. Immunosuppressive role of TAMs

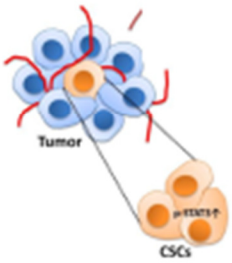
TAMs have been associated with **poor clinical prognosis** in the vast majority of cancers and represent a major obstacle for cancer immunotherapy (73) (74).

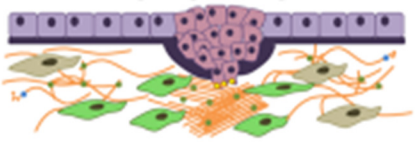
TAMs regulate various aspects of the TME such as angiogenesis and the composition of the extracellular matrix that **indirectly regulate antitumor T-cell activity** (75). For example, TAMs promote fibrosis within the stroma of the TME through the expression of MMP and the activation of TGF-β, and could by this way contribute to shield tumor islets from T-cell infiltration. In addition, TAMs, through the production of TGF-β and IL-10, induce Treg cells within the TME. TAMs could also regulate T-cell recruitment through the regulation of vascular adhesion molecules such as vascular cell adhesion molecule-1. Nitration of chemokines such as CCL2 or CCL5 might also be involved in the suppression of T-cell recruitment, without impacting monocyte recruitment. Although TAMs are phagocytic cells, they compete with efficient APCs since they don't express CCR7 and are unable to migrate into the draining lymph nodes to initiate an antitumor T-cell response (76).

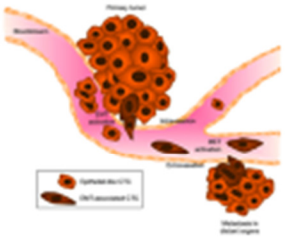
TAMs display also multiple direct immunosuppressive effects. They inhibit TCR signaling by expressing the ligands for the inhibitory receptor programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) which are **PD-L1**, **PD-L2** and **B7-1 (CD80)**, **B7-2 (CD86)**, respectively. These immune checkpoint ligands are abundant in the TME and play a crucial role in T-cell suppression in multiple animal models and human cancers (77). Although tumor murine models show conflicting results, another immune checkpoint ligand in human TAMs is B7-H4, which is involved in T-cell suppression and whose receptor is currently unknown. PD-L1 and PD-L2 (specific to APCs) are regulated in TAMs and MDSCs, for example by hypoxia as a consequence of HIF-1α signaling, which induces T-cell suppression (78). B7-1 and B7-2 are the ligands for the T-cell costimulatory CD28, but display higher affinity with CTLA-4. TAMs express also non classical HLA-E (that inhibit NK cells) and HLA-G (that inhibit T-cell function) molecules. TAMs are an important source of cytokines such as **TGF-β** and **IL-10** that directly inhibit T-cell function, and chemokines such as CCL5, CCL20 and CCL22 that **recruit Treg cells** (79). TAMs deplete L-arginine from the extracellular milieu via the secretion of Arg-1 enzyme, the signature of many M2 macrophages and TAMs. **Arg-1** metabolizes L-arginine to urea and L-ornithine and therefore plays an important role in the suppression of T-cell proliferation and activity in the TME (by inhibiting the re-expression of the CD3ζ chain after TCR stimulation and CD3 internalization) (80). In addition, arginase-derived ornithine is important for the synthesis of (proline-derived) collagen, and may by this way play a role in tissue remodeling processes. It is worth noting that in spite of mouse models, Arg-1 from human macrophages has not shown a T-

cell suppressive activity. Another enzyme secreted by TAMs is the inducible nitric oxide synthase (**iNOS**). This enzyme generates nitric oxide (NO) which endows macrophages with cytotoxic antimicrobial and antitumor properties (81). NO provokes also the nitration of TCR and MHC molecules and therefore prevents their interaction (through a direct effect of NO on T cells and through a secondary production of peroxynitrites). However, iNOS expression by macrophages in vivo seems rather to promote a T-cell response. **IDO** is expressed in vivo in tumor-infiltrating myeloid cells and blocks the activation of specific effector T cells at the tumor site by depleting tryptophan locally. It is induced by many inflammatory cytokines or activation markers such as IFNɤ or CD69+ on T cells (82).

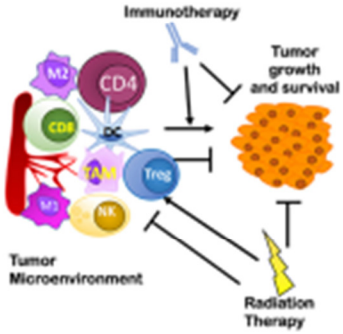
However, the specific inhibitory impact and dominant mechanisms of TAMs in vivo are still to be determined, and a better understanding of the molecular mechanisms of TAM-mediated immunosuppression will likely help improve therapeutic approaches of immunotherapy.

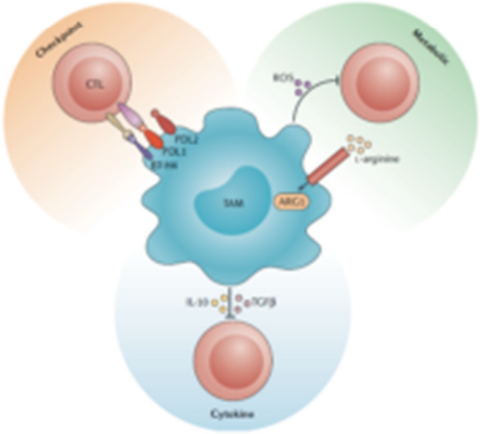


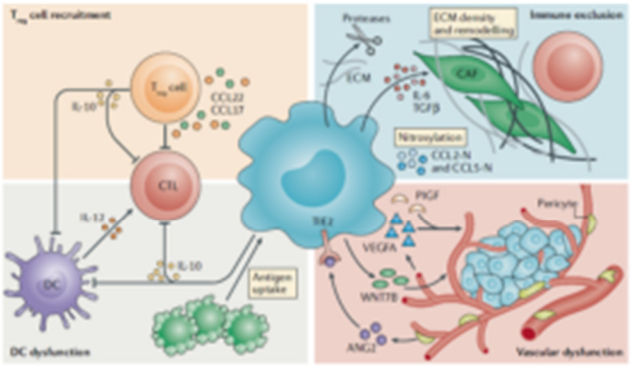




Multiple protumoral and immunosuppressive effects of TAMs







Darragh, Front Immunol, 2019

DeNardo and Ruffel, Nature Rev Imm, 2019

Features of TAMs

Protumoral properties	Immunosuppressive properties
Cancer cell stemness	PD-L1 - PD-1
DNA damages	PD-L2 - PD-1
SIRP-α	CD80 - CTLA-4
Tumor cell intravasation and dissemination	CD86 - CTLA-4
Tumor cell invasion	Arg-1
Matrix remodeling	IDO
Neoangiogenesis	TGF-β
Lymphoangiogenesis	IL-10
Poor clinical prognosis	Treg induction
Resistance to therapy	Poor APC function
	Nitration of chemokines
	Fibrosis-mediated T-cell trapping

6.3. TAM characterization

Phenotypic definition has been challenging because of macrophage heterogeneity within each tumor and within each tumor type, with multiple distinct subpopulations with overlapping features and distinct and dynamic activation states. In mouse, general surface markers of macrophages in tumor-models are often CD11b, Ly6C, MHC II, F4/80, CD64 and the absence of Ly6G. In human, cell surface markers include CD11b, LPS co-receptor (CD14), HLA-DR and the FcγRIII receptor (CD16). However, these markers are still in their infancy. Macrophages can be phenotyped with four complementary approaches: cell surface markers, expression of transcription factors, production of cytokines and specific enzymes related to their function. These features are described further elsewhere below (83).

6.4. Macrophage heterogeneity, ontogeny and microenvironmental influences

Macrophage phenotypic heterogeneity would come from their **high capacity of plasticity** following a complex spatio-temporal dynamics, adapting to the stage of any pathological context through local microenvironment cues. The analysis of monocytes and different tissue-resident macrophages in the mouse show that, despite their common lineage, they can be distinguished by very different gene expression profiles across cell types. The adoptive transfer of fully differentiated macrophages to different tissues is sufficient to reprogram the macrophages. This highlights the inherent plasticity of these cells which are educated by microenvironmental specificities and drive tissue-specific macrophage function (84).

Macrophages undergo a **phenotypic switch during the course of tumor progression**, predisposing tissue to tumor initiation by the release of factors that promote neoplastic transformation, followed by a tumor-promoting and immunosuppressive phenotype. The **location of TAMs** could also influence macrophage phenotype. For example, TAMs do not show the same phenotype within tumor islet or tumor stroma, or in poorly vascularized tumor areas. In addition, evidence exists for mixed phenotype (85). Furthermore, tissue-resident macrophages and TAMs show minimal similarities in gene expression profiles. The analysis between different cancer types indicates also that, as resident macrophages from different healthy tissues exhibit a distinct

transcriptional profile, cancers activate **cancer tissue-specific transcriptional profiles** in TAMs (86).

Macrophage heterogeneity could also be partially derived from **different specific lineages**. Indeed, until recently, TAMs were described as originating exclusively from circulating monocyte precursors from adult hematopoiesis and differentiating upon tissue infiltration. However, recent studies indicated embryonic-derived populations of resident macrophages in mouse cancer models (yolk sac, fetal liver) that self-maintain throughout adulthood independently of bone marrow contribution (87) and proliferate and accumulate with tumor expansion. The association of TAM origin with specific TAM functional profile is nevertheless not clear across the few different models and in particular regarding their immunosuppressive functions so far (88).

6.5. Macrophage polarization

Macrophages are programmed to respond locally to specific needs. This is mediated by locally produced signals that activate the corresponding functional polarization programs through dedicated transcription factors (89). Unlike the process of cell differentiation, which is a stable and irreversible transition from progenitor cells, the polarization process is a **stable and reversible program** induced on demand. Once the functional demand is met, the signals decrease and result in a reversal of the polarization to the initial state, ready for other functional demands. Nevertheless, when a signal reporting on functional demand is constitutively present in a given tissue, the corresponding polarization program may become fixed as a differentiation program (90). Macrophages can therefore exert **dual influences** by either antagonizing the cytotoxic activity of immune cells or by improving the immune response. Functional polarization of fully differentiated macrophages is controlled by the reversible induction of tissue-specific signals under physiological and pathological conditions, although the molecular mechanisms remain largely unknown. Mirroring the paradigm of Th1/Th2 $CD4^+$ T helper lymphocytes, macrophages can be classified into **two polarized states following an M1/M2 classification based on in vitro data**. However, this is an oversimplified classification and macrophages exhibit **in vivo a more complex continuum of functional states between end-stages of macrophage polarization**. I put, in the Figure A, the TAMs in the middle of a suggested view of this continuum of different states. In line

with their various essential functions, macrophages are important contributors to various pathological conditions depending on their different functions. An M1/M2 imbalance is seen in cancer and play a pathogenic role. Remarkably, in many publications, in vivo M1 is widely considered synonymous with in vitro classically activated, while in vivo M2 is considered synonymous with in vitro alternatively activated state. However, although there are some overlaps, many discrepancies exist. In vivo M1/M2 markers remain to be clarified (91).

6.6. M1 phenotype

Fully polarized "classically activated" or "M1" macrophages play critical role in innate host defense. They contribute to the elimination of pathogens, infected and cancer cells but can also damage contiguous tissues (92). They are commonly described as **potent antigen-presenting cells** with a pronounced **proinflammatory profile** and the capacity to **kill tumor cells and inhibit tumor growth**.

M1 macrophages are part of a polarized Th1 response and as such, they are key effector cells induced in response to granulocyte-macrophage colony stimulating factor (**GM-CSF**), to NK- and **Th1-derived cytokines**, to pro-inflammatory mediators including **IFNs**, and to various pathogen-associated molecules such as TLR agonists. This activates a cellular reprogramming of the macrophage. TLRs are the most well-known pattern recognition receptors. These receptors, mostly on dendritic cells and macrophages, are key sensors for the detection of various "danger-associated" highly conserved molecules expressed by pathogens (**PAMPs**) and endogenous damage-associated molecular patterns (**DAMPs**) derived from injured tissues. For example, the most studied TLR is the TLR-4, involving two distinct adaptors, MyD88 and TRIF, that mediate the signaling downstream of it. MyD88 induces a cascade of kinase activation including IRAK4, TRAF6 leading ultimately to the activation of transcription factors such as nuclear factor-κB (**NF-κB)** (p65 and p50) and Activator protein-1 (**AP-1)**. Canonical NF-κB signaling involves p65/p50 NF-κB heterodimer that translocates into the nucleus to bind to the relevant gene promoters after that its inhibitor protein IκB is phosphorylated and undergoes ubiquitin-mediated degradation, resulting in the transcription of proinflammatory genes. This includes for example genes coding for IL-1β, TNFα, IL-6, IL-12b, COX2 and iNOS (93). TRIF, also activated in the TLR-3 signaling pathway, activates the transcription factor **IRF3**, inducing IFNβ secretion binding to type I

interferon receptor (IFNAR) and **STAT1** activation, further resulting in the transcription of proinflammatory genes. It should be added that Myd88 adaptor is also involved in several other signaling pathways such as those mediating the stimulation by IL-1 or CD40 ligand for example. M1 macrophages express elevated **MHC II** and **costimulatory molecules** (CD80 and CD86) that lead to an **efficient antigen presentation, T-cell priming and the recruitment of a Th1 response**. M1 macrophages produce **RNS (such as NO), ROS, COX-2 enzyme** and large amounts of pro-inflammatory cytokines such as **TNFα, IL-1β, IL-6, IL-12, IL-23 and type I IFN**. They produce **low expression of IL-10**. In addition, they promote the recruitment of Th1 cells through the expression of chemokines such as **CXCL9 and CXCL10**. Furthermore, they possess various classes of Fcɤ receptors, allowing efficient **antibody-dependent cellular phagocytosis** leading to the eradication of tumor cells by intracellular digestion in lysosomes (94). The NO-producing enzyme **iNOS** is considered as a hallmark cytotoxic enzyme used by M1 macrophages to kill pathogens and tumor cells. Nonetheless, NO may exhibit a dual activity. For example, although macrophages are the main producers of NO in the TME, iNOS exerts immunoregulatory effects when produced by other immune cells such as MDSCs. The underlying mechanisms in vivo and the prognostic significance of iNOS expression in the TME is still unclear but it could depend on NO concentration. High concentrations of NO and ROS from macrophages stimulate apoptosis of tumor cells in addition to direct cell damages through free radical oxidation reactions (95). M1 macrophages rely mainly on glycolysis and the pentose phosphate pathway to meet their ATP requirements, where HIF-1α could play a key role, also under normoxic conditions (96).

6.7. M2 phenotype

In contrast, various forms of non-classically activated macrophages, called alternatively activated M2 macrophages, observed in healing-type circumstances, respond to cytokines typically secreted by **Th2 cells** such as **IL-4 and IL-13**. The M2-polarization is also induced by **IL-10**, immune complexes, glucocorticoid, **TGF-β** and growth factors produced by the tumor cells such as **M-CSF**. IL-4 can also be secreted by tumor cells. M2 macrophages possess strong pro-tumoral properties such as tissue-remodeling, angiogenesis, lymphangiogenesis and metastasis. They suppress antitumor immunity and promote the proliferation of contiguous cells (97) (98). M2 macrophages **highly express IL-10** and produce **low levels of IL-12**. They express chemokines

such as CCL17, CCL22 and CCL24 whose corresponding receptors CCR4 and CCR3 are present on Treg cells and Th2 cells, eosinophils and basophils, resulting in an **amplification of a polarized Th2 response**. M2 macrophages show more **phagocytic activity** but **lose their antigen-presenting capabilities**.

M2 macrophages display typical M2 markers. For example, **Arg-1** is an enzyme that depletes L-arginine from the extracellular milieu and therefore plays an important role in the suppression of T-cell proliferation and activity (by inhibiting the re-expression of the CD3ζ chain after TCR stimulation and CD3 internalization) (80). Arg-1 is considered as a signature of M2 macrophages and TAMs. Furthermore, Arg-1 seems to be crucial for wound healing as opposed to NO-producing macrophages that are at play during the initial phase of antibacterial inflammation (99). The **CD163** is also seen as an M2 marker. It is a specific scavenger receptor for extracellular hemoglobin-haptoglobin complex or free hemoglobin, and results in receptor-mediated endocytosis and clearance of hemoglobin associated with heme oxygenase-1 induction (100) that will be further described below. The mannose receptor **CD206** is involved in the initiation of endocytosis, phagosome-lysosome formation and autophagy. It is considered as an M2 marker and is associated with a decreased intratumoral immunity (101). Its activation has however recently been proposed to be involved in the shift from an immunosuppressive state to a proinflammatory, phagocyting phenotype and to improved innate and adaptive antitumor immune response (102). Alternatively activated macrophages show also high expression of chitinase-3-like protein (Chi3l3 or Ym1), Found in Inflammatory Zone 1 or Resistin-Like Molecule a (Fizz1 or RELMα), Dectin1, macrophage galactose C-type lectin (MGL1 and MGL2) and scavenger receptors. **Ym1** is considered as a M2 marker in the mouse, but its role in macrophages is poorly understood. It is a lectin that binds glycosaminoglycans and chitin and that is synthesized by macrophages during parasitic and fungal infections, allergy or eosinophilic meningoencephalitis. It could exert an anti-inflammatory effect by compete with leukocyte for extracellular matrix binding. The role of **FIZZ1** is unknown, but it is upregulated by helminth infection and in allergic airway inflammation in the mouse, IL-4, IL-13 and suppressed by IFNɤ. Dectin 1 is a lectin-like innate immune receptor that binds beta-glucans present on fungal cell wall and initiates fungal phagocytosis by macrophages. Tumor cells can express glycans that could be recognized by Dectin-1 (103). Scavenger receptors contribute to inflammation resolution by clearing surrounding tissues of oxidized molecules and cellular debris. The pattern recognition scavenger receptor MARCO could

be involved in a subset of suppressive macrophages in the secondary lymphoid organs and in the TME, promoting tumor growth and metastasis (104). In contrast to M1 macrophages, M2 macrophages rely mainly on oxidative phosphorylation and fatty acid uptake and oxidation (96).

In fact, at least three different subsets of M2 macrophages could be described, M2a, M2b, and M2c. M2a macrophage subset is induced by IL-4/IL-13 cytokines that act through IL-4Rα. These M2a macrophages upregulate the expression of the CD206 and MHC II molecules and exhibit a stimulated endocytosis and antigen presentation. Their master regulators are STAT6, IRF4, KLF4, PPARɤ and JMJD3. M2b phenotype, whose master regulator is ERK, is induced by immune complexes, in combination with either IL-1β or TLR4-signaling inducers, underlining the collaborative interplay between M1 and M2b macrophages for an appropriate inflammatory response. Their phenotype could explain a type of macrophages sharing common features between pro-inflammatory and anti-inflammatory macrophages. TGF-β and IL-10 have different effects on macrophage gene expression and induce M2c macrophage subset polarization associated with characteristic cytokine phenotype of IL-10^{hi}, IL-12^{lo}, IL-23^{lo} and TGF-β^{+}, the expression of the transcription factor STAT3, the downregulation of MHC II molecules. They promote scavenging, **tissue repair** and **tumor progression**. Without external stimuli, M2 macrophages have stable phenotypes and maintain their state. The repolarization from M2 to M1 in specific microenvironments seems to show higher inertia demonstrated by only 30% of the pro-inflammatory genes expression at the activated level. Hybrid macrophage states of these five canonical phenotypes (M0, M1, M2a, M2b, M2c) could exist although in vivo models are lacking. Various combinations of environmental-dependent defined stimuli could give rise to a continuous multitude of M2-like functional macrophage phenotypes yet sharing some properties in common (105).

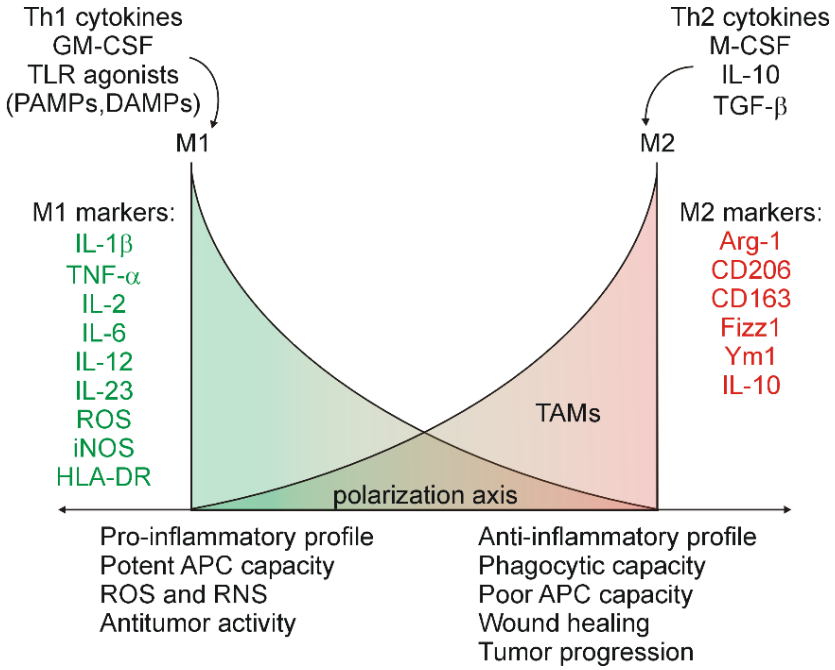


Figure A

Macrophage polarization and TAM phenotype suggestion

6.8. Endotoxin tolerance

Macrophage tolerance is a long-recognized property of macrophages resulting in an altered response to repeated or chronic inflammatory stimuli probably to limit potential tissue damages from prolonged release of pro-inflammatory cytokines. Rather than simply unresponsiveness, it represents a distinct hybrid transcriptional response induced by different TLR ligands which are pro-inflammatory in nature but with the activation of a large number of anti-inflammatory and pro-resolution genes. The mechanisms involved in the refractory state of macrophages during endotoxin tolerance are not completely understood but involve notably the downregulation of genes coding for inflammatory cytokines and chemokines and the upregulation of genes coding for anti-inflammatory cytokines, scavenging receptors and negative regulators (such as Tollip, SOCS1, SHIP-1, A20 and IRAK-M) to negatively regulate the signaling pathway downstream of TLR-4 (106). Functionally, macrophages exhibit increased phagocytic and wound healing activities but with an impaired antigen presentation capacity and reduced expression of co-stimulatory and MHC II molecules.

6.9. TAM phenotype

Although the macrophages within the TME can share some features of both M1 and M2 profile, including **mixed coexisting phenotypes** (107), with a complex dynamics, it is suggested that in the majority of cases tumor-infiltrating myeloid cells are educated by the tumor cells to be preferentially skewed away from M1-like phenotype to express **a protumoral M2-like phenotype**. In cancer patients, these tumor-infiltrating myeloid cells are generally related to a poor clinical outcome in many solid tumor types (74) (108). TAMs are also known to be associated with resistance to cancer therapy such as chemotherapy, irradiation, angiogenic inhibitors, and represent a major obstacle for efficient immunotherapy (68).

However, the oversimplified M1/M2 macrophage classification overlooks the **continuous spectrum of activation state of macrophages in vivo** (109) which act in a **timely and spatially appropriate manner**. Functionally distinct phenotypes of TAMs arise within different tumor

compartments (110). For example, at the site of invasion of cancer cells at early stage of tumor development, invasive macrophages would have an important role in the transition of preinvasive malignant lesions to early invasive carcinoma. TAM seem to display specific function in close proximity to cancer nests. In the stroma, where tumor cells are generally sparse, the components of the extracellular matrix and the non-malignant cells could regulate macrophage phenotype and function. The perivascular macrophages often express high levels of Tie-2 (the receptor for angiopoietin) besides other M2 markers and play a role in angiogenesis and metastasis. Specific TAM subpopulations are found in the poorly vascularized **hypoxic or necrotic areas** of the TME. They upregulate HIF transcription factors to adapt to low oxygen availability, shaping macrophage phenotype to promote immune escape and tumor progression (111). On the other hand, distinct TAM subpopulations can be found during tumor initiation, progression and metastasis and represent dynamic changes in tumor-promoting subpopulations of macrophages (112). For example, macrophages are suggested to be key cells in cancers that are induced by inflammation. Acute inflammation dominated by macrophages, can be followed by a persistent unresolved lower inflammation or a chronic infection, which can be designated as "**smoldering" inflammation**. This creates a tumor-promoting microenvironment known to cause several cancers. Indeed, myeloid cells produce a mutagenic environment through ROS and RNS, in association with infiltrating myeloid cell-released cytokines and growth factors that amplify these mutagenic influences, leading to genetic instability. The benign-to-malignant transition is also promoted by the macrophage-mediated **angiogenic switch** (highly enriched in transcripts coding for angiogenic molecules). The TME can also phenocopy the cytokine milieu and extracellular matrix of a wound healing process, where the macrophage phenotype can be deviated to one promoting **tissue repair** through the production of angiogenic factors, TGF-β and tissue remodeling enzymes such as MMPs. These macrophages could be particularly involved in tumor progression, intravasation of tumor cells into the blood stream and metastatic spread (113) (114). Other TAMs are the **metastatic-associated macrophages** found in the primary tumor and at the metastatic site. They influence processes such as cancer cell adhesion to the vasculature and colonization of secondary tumor sites. This could be mediated by primary tumor-derived factors and the recruitment of macrophages into pre-metastatic niches (115). Hemorrhage-associated macrophages are induced by ingestion of **hemoglobin**-haptoglobin complexes and are suggested to drive an anti-inflammatory phenotype (116). Oxidative tissue-associated macrophages have been proposed to

play an important role in redox status control (117). These last two phenotypes are found in atherosclerosis lesions but might contribute to the "homeostasis" of the TME.

In my opinion, TAM polarization axis could therefore be seen as a multiple variable axis in a multi-dimensional view depending on multiples suggested variables, in contrast to the binary described M1-M2 axis (Figure B).

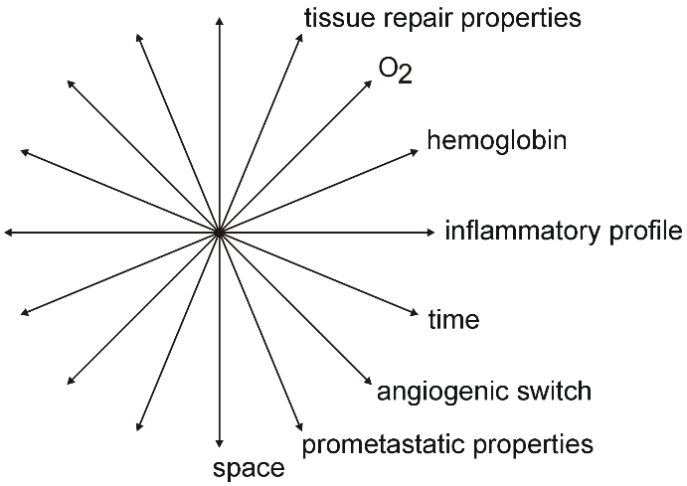


Figure B

Multiple variable axis

6.10. Modulators of TAM polarization

Macrophages, characterized by a high heterogeneity and plasticity, can exert opposed functions in response to different microenvironmental signals (118) (119). In cancer patients, tumor-infiltrating myeloid cells are generally associated with poor survival, although contradictory data do exist (120). These contradictory data might be explained by the fact that macrophages could act as powerful stimulators of the inflammatory response or as strong anti-inflammatory cells with various tumor-promoting properties. Indeed, M2-like macrophages are associated with worse outcomes than M1-like macrophages in various cancers (121) (15). In fact, macrophages can be considered as sentinel cells for tissue damage and specific needs by monitoring tissue microenvironment through a broad array of sensing molecules such as scavenger receptors, pattern

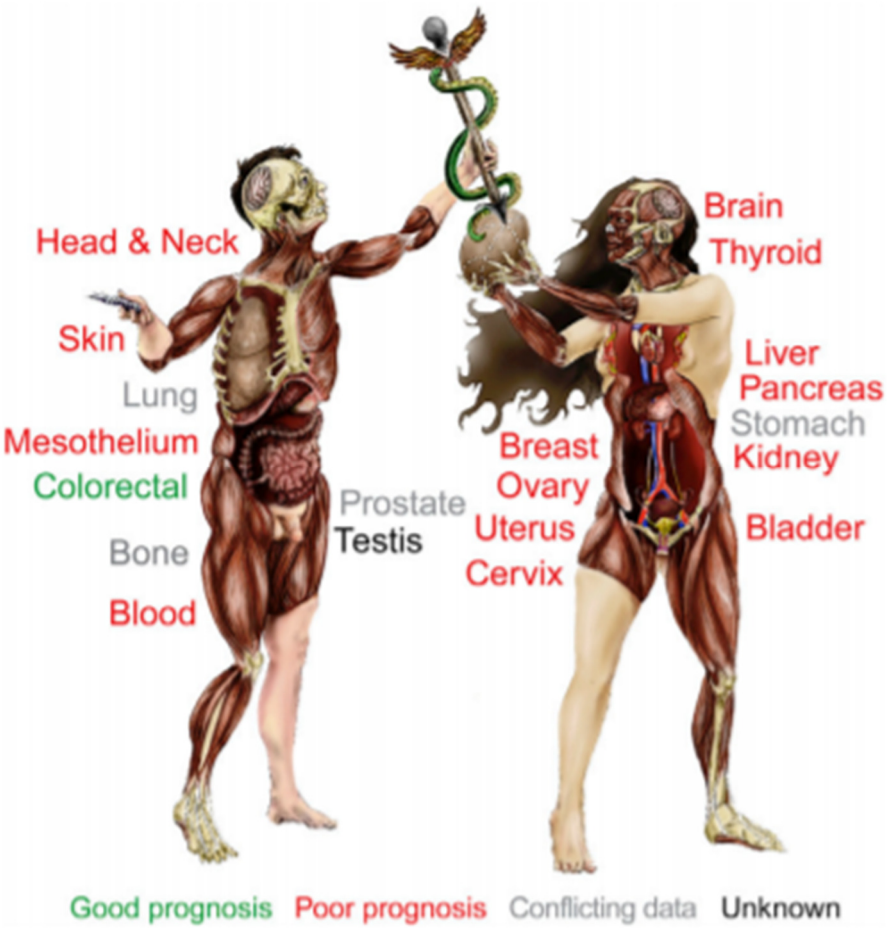
recognition receptors and cytokine receptors. This reprograms the macrophages and alerts the immune system to adapt their functions depending on the microenvironment needs.

Interplay of signaling molecules and transcription factors can reverse macrophage polarization. Although the molecular mechanisms governing macrophage polarization remain incompletely defined, it is known that the modulators of macrophage polarization include the canonical IRF/STAT signaling pathway. **IRF4**, **STAT3** and **STAT6** promote the expression of typical M2-associated genes, while **NF-κB** p65/p50 heterodimer (a key transcription factor for the expression of pro-inflammatory genes), **IRF3**, **IRF5**, **STAT1** and STAT5 are involved in the induction of M1-associated genes. Generally, there is a reciprocal regulation of M1 and M2 genes by the same transcription factors. The SOCS family regulates STAT-mediated activation of macrophages (122). The importance of the **MAPK** pathway, described further below, has also been suggested in TAM polarization and tumor progression by several studies, in particular the p38 protein (123). Macrophages can be driven into an M2-like polarization by various M2 stimuli. As detailed above, Th2 cytokines polarize macrophages. TAMs induce Treg cells but a reciprocal regulation is also described since Treg cells have been shown to profoundly alter macrophage function (124). Cytokines from the TME such as M-CSF (125) and chemokines such as CCL2 have been reported to promote M2-polarization of macrophages (126), with the existence of an amplification loop for their recruitment and polarization as CCL2 is produced by tumor cells but also by TAMs themselves. Once entered into the TME, CCR2 is downregulated, as a mechanism to trap recruited macrophages within the TME. **IL-10** biding to its receptor IL-10R results in **STAT3** activation, with subsequent reduction of pro-inflammatory cytokine expression (92). The reason why STAT3 activation by IL-6 or IL-10 results in opposing functions is not clear, but could be due to a transient (activating signal) or prolonged (becoming an inhibitory signal) STAT3 activation state, respectively (127). TGF-β-driven M2 polarization is mediated by SMAD2/3 and PI3K/Akt signaling pathways (128). **IL-4** and **IL-13** can promote an M2-like polarization of TAMs through **STAT6** activation, which further triggers IL-4 secretion in the TME (129). STAT6 can interact with other transcription factors such as IRF4, PPARϒ or KLF4. CREB-C/EBPβ axis is also important for Arg-1 expression in macrophages in response to TLR ligands (130). **Hypoxia**, which is a major characteristic of solid tumors because of their poorly organized vascular structures and which generally increase along with tumor progression, has been suggested to be a crucial factor for driving the phenotype of TAMs. Indeed, myeloid HIF-1α deficiency in a progressive murine

model of breast cancer slows tumor progression and blocks hypoxia-induced T-cell suppression, associated with increased tumor apoptosis. The suppression of T-cell proliferation by macrophages in vitro increased with lower oxygen tensions in a manner dependent on macrophage expression of HIF-1α (131). Besides hypoxia, **tumor-derived lactic acid**, a by-product of tumoral aerobic glycolysis, plays also an important role in driving TAM polarization and subsequent tumor growth and is mediated by **HIF-1α** (132). A specific population of phagocytic macrophages with receptors for apoptotic cells is associated with genes that limit the TLR-mediated inflammatory response to **apoptotic cell-derived nucleic acids** (133). The **Tyro3-Axl-MerTK receptors** inhibit M1-polarization and skew macrophages towards a pro-tumor M2-like phenotype through their shared ligands Gas6 and Protein S and the induction of **SOCS1**. They promote also apoptotic cell clearance which further amplifies macrophage polarization (134). This supports tissue homeostasis that could otherwise be disrupted by apoptotic cells in pathological contexts. High-Mobility Group Box1 protein (**HMGB1**) released by dying tumor cells can promote M2-like macrophage accumulation, associated with IL-10 production, through the receptor for advanced glycation end products (RAGE) signaling and could have an important role in tumor progression (135). In addition, although **RAGE** activation promotes the production of pro-inflammatory molecules through danger signals from the TME, it is paradoxically also expressed on M2 macrophages where it could be involved in protumoral activities through a negative feedback affecting downstream pathways leading to similarities with classical LPS tolerance (136). **Arginine metabolism** can direct macrophages into two opposing pathways and be seen as a key M1/M2 macrophage modulator. Indeed, M1 macrophages express iNOS that metabolizes arginine to NO and citrulline, while M2 macrophages express Arg-1 that metabolizes arginine to urea and ornithine. Arginine is therefore a precursor for mainly two important distinct enzymatic pathways in macrophages, Arg-1 enzyme limiting arginine availability for iNOS activity (and subsequent NO synthesis) and vice versa (137). In most cases, mutually exclusive expression of iNOS or Arg-1 occurs in macrophages (138).

Suggested molecular determinants of TAM polarization

"M1-like" polarization	**"M2-like" polarization**
iNOS	Arg-1
NF-κB p65/p50	NF-κB p50/p50
IFN (type I or II) - STAT1	IL-4/IL-13 - STAT6
IL-6 - STAT3	IL-10 - STAT3
IRF3/IRF5	IRF4
	SOCS1
	HIF-1α



Ruffell et Coussens, Cancer Cell, 2015

7. Heme oxygenase-1

Heme is crucially involved in transport (hemoglobin) and storage (myoglobin) of oxygen, and has other critical functions in other hemoproteins such as those involved in electron transfer of the respiratory chain, drug metabolism, oxygenase and peroxidase reactions. Heme is tightly regulated under physiological conditions and its enclosed iron is recycled by tissue-specific macrophages to prevent the cytotoxic effects of free iron atom (mainly an excess of ROS production, with lipid peroxidation, protein cross-linking and DNA damages).

Heme oxygenase (HO) plays an important role in iron recycling and erythropoiesis. It is the first and rate-limiting enzyme that catabolizes heme into three major biologically active byproducts: CO, ferrous iron, and biliverdin-IXa, the last one being converted to bilirubin-IXa by biliverdin reductase (Figure C). By this way, HO in macrophages carries out the clearance of hemoglobin (mainly bound to haptoglobin) and excess free heme (bound to hemopexin) while allowing beneficial cytoprotective effects through its heme degradation by-products (139). The constitutive HO-2 isoform is highly expressed in many tissues such as the spleen, the testes, the brain, the liver, the gut, the kidney and the cardiovascular system, and does not respond to transcriptional activation by environmental cues. By contrast, heme oxygenase-1 (HO-1), the inducible form of HO, is considered as an essential sensor of cellular stress and inflammation and a regulator of tissue homeostasis (140). Indeed, HO-1 is highly expressed in the spleen and other tissues that degrade senescent red blood cells (such as specialized reticuloendothelial cells of the liver and the bone marrow). In most other tissues not directly involved in erythrocyte or hemoglobin metabolism, **HO-1 is ubiquitously expressed at low levels under basal conditions but is highly up-regulated by a wide diversity of stimuli** that can cause cellular stress, regardless of cell type or inducing compound, and leads to various cytoprotective effects detailed below.

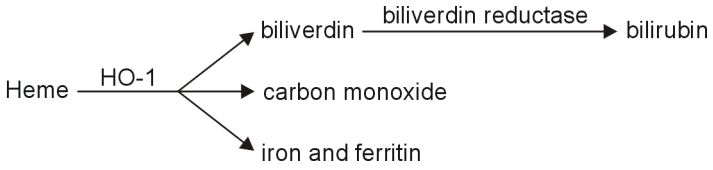


Figure C

HO-1 degradation byproducts

7.1. HO-1 inducers

The inducers of HO-1 are many and various. They include for example its natural substrate, **heme**, but also chemical and physical stimuli such as **hypoxia/**hyperoxia (through HIF-1α), environmental or industrial polluants, heavy metal salts, solar ultraviolet radiation. The cytoprotective role of HO-1 is not limited to stress from exogenous origin but is induced also in response to systemic stress caused by inflammation or infection for example. This includes the hepatic acute phase reaction, **inflammatory cytokines**, oxidative cellular stress (involving the generation of **ROS and RNS**) and pathogens (141). HO-1 could be seen as a marker of proinflammatory state with regulatory anti-inflammatory properties. The release of **PAMPs and DAMPs** occurs during ongoing bacterial infection. It is reported for example that **LPS** injections induce HO-1 expression in myeloid cells (142). The release of DAMPs can also occur during various clinical events in the absence of pathogens, generally known as "sterile inflammation" in tissue injury. Remarkably, extracellular heme can act as a DAMP, released during tissue damages and acting as a "danger molecule" by activating Pattern Recognition Receptors (PRRs) and inducing stress response-associated genes and a strong induction of HO-1. Indeed, TLR4 has been shown to have various non-pathogen-associated ligands. PAMPs and DAMPs, including ROS and heme, also activate the intracellular PRR Nod-like receptors that activate the inflammasome protein multiplexes (143).

7.2. Regulation of HO-1 transcription

The gene coding for HO-1, *Hmox1*, is found in most living organisms. The regulation of gene transcription is carried out by multiple complex mechanisms depending on a multiplicity of physiological and pathophysiological conditions. The various HO-1 inducers activate signaling pathways, such as the **mitogen-activated protein kinase (MAPK) pathway** (including p38, ERK and JNK). The phosphatidylinositol 3-kinase (PI3K), tyrosine kinases and protein kinases A, B, C, and G can also be activated, although it has been poorly studied. These pathways ultimately activate transcription factors such as **AP-1**, **Nrf2** and **NF-κB** leading to HO-1 transcription (144).

Among its multiple transcriptional factor binding sites, the binding of Nuclear factor erythroid 2-related factor 2 (**Nrf2**) to the antioxidant response element motifs is the most important that regulates *Hmox1* gene transcription. Nrf2 is anchored in the cytoplasm by Kelch-like ECH-associated protein 1 (**Keap1**), which under basal conditions prevents its nuclear translocation. When exposed to stimuli, Nrf2 dissociates from Keap1, migrates to the nucleus where it can exert its transcriptional activity that requires small Maf proteins. BTB and CNC homolog 1 (**Bach1**) is a transcriptional repressor of *Hmox1* by binding with small Maf proteins at the Maf recognition element on the *Hmox1* promoter. When heme binds ton Bach1, the latter is exported from the nucleus and degraded, allowing Nrf2 to induce the transcription of *Hmox1* and other iron-regulatory genes such as ferritin and ferroportin.

HO-1 expression depends also on IL-10 signaling and vice versa. IL-10 binds to its receptor complex that activates the phosphorylation of **STAT3** resulting in the translocation to the nucleus and the binding to the promoters of various genes. STAT3 and PI3K pathways are required for the IL-10-mediated HO-1 induction. In turn, HO-1 and CO modulate IL-10 production in monocytes and macrophages. This **positive feedback loop between HO-1 and IL-10** could amplify the anti-inflammatory effects of HO-1 in myeloid cells (145).

Although Nrf2 activation by HO-1 inducers is known to be mediated by the activation of protein phosphorylation-dependent signaling cascades, the link between redox changes and the regulation of protein phosphorylations and dephosphorylations remain poorly understood (141).

HO-1 inducers:
Hemoglobin, heme
PAPMs, DAMPs
ROS, RNS
Cytokines
Hypoxia

Transcriptional regulators:
Nrf2
IL-10 - STAT3 axis
NF-κB
AP-1
HIF-1α

7.3. Cytoprotective and protumoral role of HO-1

The crucial **tissue-protective properties** of HO-1 is supported by a vast array of evidences in a variety of animal and human disorders. For example, HO-1 has beneficial effects on acute kidney injury by regulating cell cycle, autophagic response and inflammatory response. In addition, oxidative stress induces HO-1 and its by-products, further improving cytoprotection in acute kidney disease models (146). In the digestive system, HO-1 plays a critical role in the resolution of inflammation in diseases such as inflammatory bowel diseases, radiation enteritis or necrotizing enterocolitis (147). HO-1 has also shown to be protective in ischemia/reperfusion injury in various models (148). HO-1-derived CO is involved in multiple anti-inflammatory and pro-resolving activities resulting in protective effects observed in mouse models of sepsis, without decreasing circulating inflammatory cells or their accumulation at the site of injury, for example by enhancing bacterial clearance through increased phagocytosis (149). The immune modulatory activity of HO-1 has a protective effect on semi-allogeneic fetus tolerance and fetal development during pregnancy, as well as in organ allograft tolerance, through the blockade of dendritic cell maturation and an increased number of Treg cells for example. This is associated with a shift towards a Th2 protective profile in the uterine cytokine milieu and with anti-apoptotic molecules in the fetal tissue (150). The properties of HO-1 play a protective role in auto-immunity. For example, HO-1 has been proved to be neuroprotective in experimental models of multiple sclerosis, and HO-1 expression is reduced in peripheral blood mononuclear cells of multiple sclerosis patients during exacerbations of the disease. HO-1 induction by CoPP in a diabetes mouse model attenuates β-cell destruction, prevents DC infiltration into the pancreas and inhibits the development of diabetes. A reduced expression of HO-1 in monocytes has also been observed in patients with systemic lupus erythematosus. It seems to be conflicting results on the role of HO-1 in rheumatoid arthritis though (151). In preclinical models of ischemic stroke, HO-1 reduces infarct volume and attenuates neurological symptoms (152).

In human, the length of a guanine-thymidine (GT)n-repeat polymorphism in the promoter region of *Hmox1* gene determines the level of HO-1 activity and inducibility. A short homozygous polymorphism leads to a higher expression of HO-1 than a long homozygous polymorphism. HO-1 expression is associated with decreased risk of coronary heart disease (153), decreased susceptibility to auto-immune diseases such as systemic lupus erythematous and rheumatoid

arthritis (151), and reduced susceptibility to chronic obstructive pulmonary disease (154). Genetic variation in *Hmox1* length has also been observed to be associated with decreased episodes of acute chest syndrome in patients with sickle cell disease (155). In addition, a shorter (GT)n microsatellite polymorphism has been correlated with a longer life (156). Cases of HO-1 deficiency have been reported in human. These patients exhibit severe hemolytic anemia, iron accumulation and a pro-inflammatory phenotype (157) (158), but further precise clinical manifestations of HO-1 deficiency are lacking to date.

The association between HO-1 polymorphism and the risk of cancer is not clear (159). However, associations have been demonstrated between HO-1 expression, **advanced stage and shorter survival in cancer patients**, for example for gallbladder cancer (160), bladder cancer (161), renal cell carcinoma (162) and non-small cell lung cancer (163). Studies suggested HO-1 and circulating levels of CO as diagnostic and prognostic marker (164), although opposite data have been observed, showing for example a lower rate of lymph node invasion and a better survival for colorectal cancer patient with colonic HO-1 expression in cancer cells and macrophages (165).

The mechanisms of cytoprotection of HO-1 are incompletely understood, but may be explained at least in part by the removal of the pro-oxidant heme (which contributes to the formation of oxidative radicals resulting in oxidative injury) and by the enzymatic production of its biologically active byproducts which possess anti-inflammatory and antioxidant properties. In addition, these molecules **modulate cell proliferation and apoptosis positively or negatively, in a manner that seems to contribute dynamically to homeostasis** (166). For example, in contrast to its suppressor effect on T cells, CO inhibits apoptosis in many non-hematopoietic cells, allowing tissue protection (167) (168). HO-1 expression is associated with **resistance to cancer therapy** in high-risk neuroblastoma cell lines (169), pancreatic cancer cells (170), ovarian carcinoma cells (171) and chronic myeloid leukemia from patients resistant to imatinib (172) (173). Its blockade leads to increased tumor sensitivity to chemo-and radiotherapy and growth inhibition. In contrast, there was no effect on apoptosis on normal cells (172) (173). The protective effect of HO-1 in imatinib treated cells involves also its nuclear translocation (174). HO-1 and CO are involved in **DNA repair** and improved cell survival in vitro and in vivo (175). HO-1 and CO contribute to tumor **angiogenesis and vasodilatation**. HO-1 expression is induced by VEGF and enhances the proliferation of endothelial cells. In turn, HO-1 and its activators induce VEGF expression. In

addition, HO-1 is a mediator of NO-induced VEGF production in various cells. The proangiogenic properties of HO-1 are due to all 3 by-products of HO-1 activity and to CO in particular, whose vasodilatory and antiapoptotic effects potentiate angiogenesis process (176). Furthermore, HO-1 has been reported to increase the proliferation and viability of tumor cells and **metastasis** in murine tumor models (177). However, the beneficial role of HO-1 in blocking tumor progression and invasion has also been reported in human breast cancer cells (178). HO-1 has plethora of inducers and is involved in many different pathways through various different mechanisms. Its role in malignancy is far from being completely understood.

7.4. Antioxidant role of HO-1

HO-1 and its byproducts play a key role in cellular and tissue homeostasis notably through its antioxidant properties. HO-1 **degrades the pro-oxidant heme** but generates labile ferrous iron, which is toxic because of its capacity to catalyze the generation of ROS via Fenton chemistry, eventually leading to oxidative tissue damages (causing lipid peroxidation, DNA and protein oxidative damages, eventually inducing ferroptosis). **Labile iron is however quickly sequestered by ferritin** (which is co-induced with HO-1 induction), which converts ferrous iron ($Fe2^{+}$) into ferric iron ($Fe3^{+}$). By binding iron, ferritin acts as an antioxidant since it limits the generation of ROS. Furthermore, iron is oxidized from the ferrous to the ferric state, further enhancing the antioxidant effects of HO-1 (179). **Bilirubin is a potent antioxidant that scavenges ROS** (180). It is itself oxidized to biliverdin, the latter being reduced and recycled by biliverdin reductase into bilirubin. The risk for cancer mortality has been shown to decrease with increased concentrations of serum bilirubin and could be related to its endogenous antioxidant properties (181). Although CO is not an antioxidant molecule per se, it shows indirectly antioxidant properties, since **CO-induced ROS in macrophages likely leads to the induction of antioxidant and cytoprotective enzymes**. ROS have been shown to both activate and repress NF-κB signaling. Oxidative stress activates NF-κB in the early phase, but inhibits it in the late phase (182). Conversely, inflammatory stress increases the production of ROS and RNS that contribute to cytotoxicity (183). However, **NF-κB** possesses antiapoptotic and antioxidant protective activities and is coordinated with **Nrf2** to resolve inflammation and maintain redox homeostasis. For example, Nrf2 decreases oxidative and nitrosative stress, which restrains NF-κB (a redox-sensitive transcription factor) activation and

cytokine production (184). Likewise, the induction of ferritin controls ROS accumulation following exposure to TNFα (185) (Figure D).

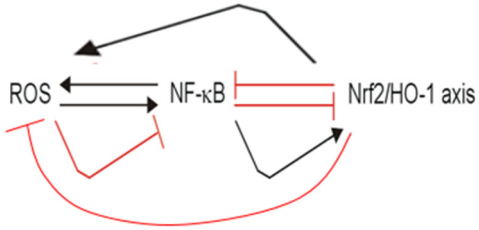


Figure D

Crosstalk between inflammation and redox homeostasis

HO-1 acts as a cytoprotective agent against oxidative injury in healthy tissues and **prevents ROS-mediated malignant transformation**. Paradoxically, this cytoprotective enzyme is frequently upregulated in many tumors compared to surrounding healthy tissues and several studies highlight it could sustain cancer progression and correlate with poor prognosis in tumor-bearing mice and in cancer patients (186) (187) (188) (172). Indeed, the metabolic aberrations of tumor cells lead to high levels of ROS, balanced by an upregulated antioxidant activity (189). **Due to its strong antioxidant and antiapoptotic properties, HO-1 provides a strong survival advantage to tumor cells** (190). HO-1 expression is further upregulated in response to cancer therapy, suggesting that HO-1 could be a **mechanism of resistance to therapy** (191). HO-1 inhibition can enhance sensitivity of cancer cells to chemotherapeutic agents with potential synergistic effect, as it has been shown in gemcitabine-induced cytotoxicity in a model of cholangiocarcinoma, which was associated with massive formation of ROS (192). This could be explained by the fact that normal cells display lower basal levels of ROS and are therefore less dependent on antioxidant repairing enzyme. Most of the evidences suggest that HO-1 has a cytoprotective role against oxidative attacks, although the association between HO-1 and ferroptosis (a non-programmed cell death induced by overloading of free iron) has provided some contradictory results. In fact, it has been suggested that a moderate level of HO-1 expression exerts a cytoprotective effect by neutralizing ROS, but that an overexpression of HO-1 could become cytotoxic because of an excessive increase of labile ferrous iron and ROS overload (193).

7.5. Immunomodulatory mechanisms of HO-1

Despite convincing data demonstrating the immunomodulatory function of HO-1, the underlying mechanisms are incompletely understood. To study its pleiotropic effects, HO-1 can first be analyzed through the direct immunomodulatory effects of its byproducts, described hereunder.

7.5.1. HO-1

There are several **mechanisms of competition between Nrf2/HO-1 axis and NF-κB signaling pathway**. For example, the p65 subunit of NF-κB and Nrf2 compete for their transcriptional co-activator CREB-binding protein (CBP) (184). This could explain that HO-1 induction by Nrf2 binding to CPB decreases the expression of NF-κB target genes such as iNOS. In addition, Keap1 can become more available in the cytoplasm after the dissociation and nuclear translocation of Nrf2 that leads to HO-1 transcription upon cellular stress. Keap1 may then downregulate TNFα-stimulated NF-κB signaling by inhibiting IKKβ phosphorylation (194). In turn, p65, the canonical NF-κB subunit, may promote the nuclear translocation of Keap1 to repress Nrf2 activity (195). Moreover, HO-1 has been shown to ameliorate liver ischemia/reperfusion injury by inhibiting TLR4-triggered Myd88- and TRIF-dependent signaling pathways. This is associated with the downregulation of IRAK-1, TRAF6 and TBK1, and the upregulation of negative regulators of TLR signaling such as Toll-interacting protein (Tollip), suppressor of cytokine signaling (SOCS)-1, interleukin-1R-associated kinase-M (IRAK-M) and Src homology 2 domain-containing inositol-5-phosphatase (SHIP)-1. This leads to the inhibition of NF-κB and IRF3 signaling pathways, and is mediated by a diminished kinase phosphorylation of IκB-α, NF-κB, p38 and IRF3 (196). Other results suggested that the activation of Nrf2/HO-1 pathway in RAW 264.7 macrophages protects against LPS-induced inflammatory and oxidative response by inhibiting NF-κB translocation, NF-κB and AP-1-DNA binding, MAPK and PI3K/Akt (PKB) pathways (197) (198) (199).

Some immunoregulatory properties of HO-1 can be mediated by **IL-10**, while myeloid HO-1 has also been identified as an induced downstream effector of IL-10 (200).

7.5.2. CO

Although CO can be toxic or even lethal, CO at low concentrations mediates potent anti-inflammatory effects in in vitro and in vivo models (Figure E). Indeed, it **inhibits LPS-induced pro-inflammatory cytokine production** from monocytes/macrophages (IL-1β, TNFα, MIP-1β) and **increases LPS-induced IL-10** through the MAPK pathway (201). The anti-inflammatory effect of CO in monocytes/macrophages is also suggested to be mediated by CO-induced low intensity oxidative burst followed by PPARɤ activation (202). In vitro, CO **decreases iNOS expression and impairs NO and TNFα production in LPS-stimulated macrophages** (203). Exogenous and endogenous CO displays also a **suppressive effect on T-cell proliferation and IL-2 secretion**, possibly due to the inhibition of ERK phosphorylation in T cells (204). Moreover, upon TLR ligand stimulation, HO-1 and CO may **traffic to the caveolae and reduce inflammatory signals** by promoting the interaction between TLR4 and caveolin-1 (which inhibits TLR4 association with MyD88 and TRIF) (205) and by inhibiting the recruitment of TLR4 to lipid rafts (206). This results in a negative regulatory feedback of the inflammatory signal in macrophages. In addition, **HO-1 and its enzymatic byproducts can modulate the activity of its transcription factors**. For example, CO decreases JNK phosphorylation in macrophages and AP-1 inflammatory activity with subsequent decreased expression of pro-inflammatory cytokines such as IL-1β and IL-6 (207). HO-1 and CO also negatively regulate NF-κB inflammatory activity in different in vitro and in vivo models (208). On the opposite, nuclear HO-1 could promote its own transcription by interacting and stabilizing its transcription factor Nrf2, further promoting an anti-inflammatory response (209). Taken together, this could represent a protective mechanism of negative regulatory feedback against the deleterious consequences of an exacerbated inflammation. HO-1 and CO may also limit the inflammatory response by downregulating the activation of the NLRP3 inflammasome and the subsequent secretion of IL-1β and IL-18 in macrophages (210).

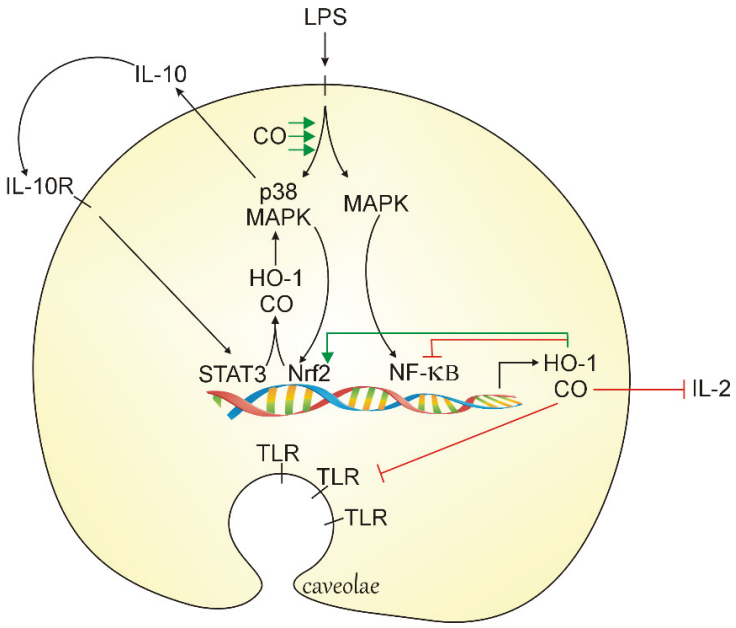


Figure E

Immunomodulatory effects of CO

7.5.3. Bilirubin

Bilirubin, independently of its antioxidant activity, is also a powerful immunomodulatory agent (Figure F). Bilirubin has for example been reported to suppress an experimental autoimmune encephalomyelitis. It suppressed Ag-specific and polyclonal T-cell response through multiple mechanisms, including the inhibition of TCR signaling, the suppression of NF-κB activity and the downregulation of MHC II and costimulatory molecules on macrophages and DCs. Bilirubin can also induce direct T-cell apoptosis when high concentrations are at play (211).

Moreover, biliverdin reductase could be required for heme transport into the nucleus to further promote HO-1 expression and its byproducts (212).

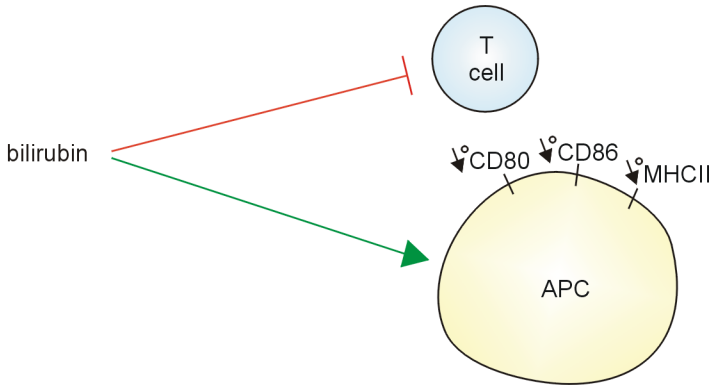


Figure F

Immunomodulatory effects of bilirubin

7.5.4. Labile iron

Labile iron released by heme catabolism through HO-1 is mainly sequestered by ferritin or exported through ferroportin and bound by transferrin to be recycled. Iron and iron regulatory proteins have been suggested to modulate the proliferation and effector function of the innate and adaptive immune cells, as well as the growth of pathogens and tumor cells (Figure G). It could therefore be involved in various diseases such as infectious diseases or cancer. Iron-loaded macrophages display a pro-inflammatory phenotype that are capable of directly killing tumor cells through ROS production (213). In contrast, **M2 macrophages** have shown a higher expression of genes involved in **iron metabolism** such as heme uptake, heme catabolism by HO-1 and ferrous iron export (214). Moreover, perivascular macrophages catabolizing heme are particularly able to release iron to tumor cells, reinforcing the iron supply to demanding tumor cells (215). In an experimental mouse model, iron deprivation fails to provoke auto-immune encephalomyelitis, suggesting an impairment in T-cell generation and activity independently of the reduced oxidative damages of the central nervous system (216).

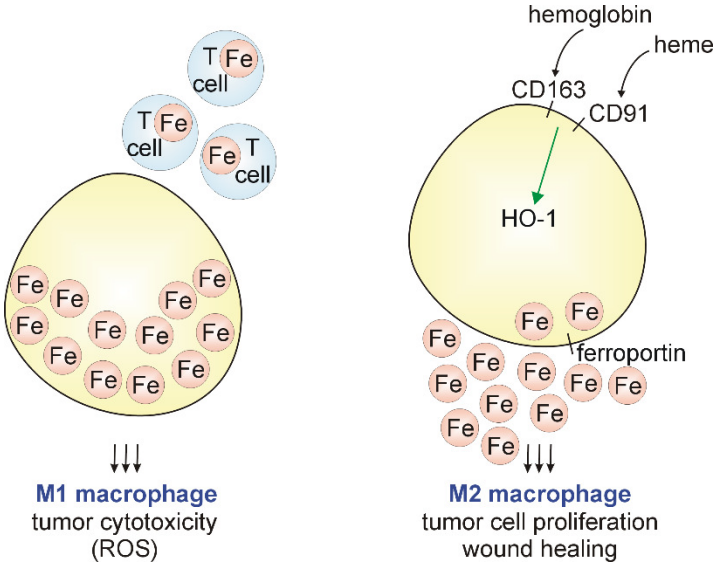


Figure G

Immunomodulatory effects of iron

7.5.5. Nrf2

Nrf2 reduces the levels of proinflammatory mediators such as iNOS in LPS-stimulated peritoneal macrophages (217). Scratched microglial cells from Nrf2 KO mice exhibit a more aggressive inflammation with enhanced NF-κB activity and enhanced expression of pro-inflammatory cytokines (218). In another mouse model, Nrf2 determines the survival of mice in an experimental model of septic shock through a regulation of the innate immune response. Indeed, severe lung inflammation occurs in the Nrf2 KO mice, associated with greater NF-κB and IRF3 expression in fibroblasts in a Myd88-dependent (TLR4 agonist) and -independent (TLR3 agonist) manner (219).

The pleiotropic effects of HO-1

Cytoprotective/protumoral	Antioxidant	Immunomodulatory
DNA repair	Heme degradation	HO-1 ↔ Nrf2
Antiapoptotic effect	Ferritin-iron	Nrf2/HO-1 axis ⊩—⊩ NF-κB
Resistance to cancer therapy	Bilirubin	HO-1 ↔ IL-10
Angiogenesis	CO-induced ROS : → NF-κB (early phase) —⊩ NF-κB (late phase) ==> Nrf2, HO-1	CO-mediated anti-inflammatory state of LPS-stimulated myeloid cells
Metastasis	NF-κB → Nrf2, HO-1	CO → T-cell suppression
	Prevention of ROS-mediated malignant transformation, but cancer progression	CO-mediated inhibition of TLR4 signaling (caveolae)
	Resistance to cancer therapy	Bilirubin?
		Iron metabolism ↔ M2 macrophages?
		Nrf2-mediated anti-inflammatory effect

7.6. HO-1 localizations

HO-1 localizes to the smooth endoplasmic reticulum, the inner mitochondrial membrane, the plasma membrane (caveolae). HO-1 is also found in the nucleus in an enzymatically inactive form.

HO-1 is bound to the **endoplasmic reticulum** and faces the cytosol, facilitating iron recycling from heme breakdown products generated in the cytosol (220). HO-1 trafficking to other subcellular locations in response to cellular stress or after erythrophagocytosis is not clear. **Mitochondrial translocation of HO-1** has been shown to be cytoprotective in a model of NSAID-induced gastric injury by preventing NSAID-induced mitochondrial dysfunction and oxidative stress (221). **At the plasma membrane**, Caveolin-1 physically interacts with HO-1 and modulates HO-1 activity (222). **Nuclear HO-1** is enzymatically inactive. Although its function is not clear, it could interact with its transcription factor Nrf2 and be involved in an adaptive reprogramming of the cell (209). The effects of HO-1 go indeed beyond its only enzymatic activity. In many tissues there is no available substrate, and the enzymatically inactive HO-1 protein has been reported to keep cytoprotective effects by migrating to different cell compartments and binding to a variety of other proteins (resulting for example in the regulation of gene expression via activation of transcription factors) and thereby modifying cellular functions. The role of nuclear HO-1 may be to promote the transcription of antioxidant proteins and to improve DNA repair (223). The nuclear translocation of HO-1 has also been shown to promote tumor cell proliferation independently of HO-1 enzymatic activity (224). The presence of HO-1 protein, but not HO-2, has also been reported in **extracellular compartments** and biological fluids (such as plasma, cerebrospinal fluid, human milk) where it may play additional roles in oxidative stress conditions, but its mechanisms of secretion and biological importance are unknown (225).

7.7. Cellular source of HO-1 and its immunosuppressive impact

7.7.1. HO-1-expressing regulatory T cells

Several studies have suggested that HO-1 is involved in Treg-mediated immunosuppression. Indeed, Foxp3 induces HO-1 production (226) and HO-1 induces IL-10, TGF-β and Foxp3

expression (227) (228). HO-1 induction has been shown to enhance Treg activity in patients with acute coronary syndrome by promoting LAP and GARP expression on activated T cells (229). However, other more recent studies have concluded that HO-1 induction does not result in Foxp3 expression or immunoregulatory function in $CD4^+$ $CD25^-$ T cells, and HO-1 inhibition in Treg cells does not affect their immunosuppressive activity (230).

7.7.2. HO-1-expressing B cells

HO-1 can be induced in B cells by its transcription factor Nrf2, and in particular in malignant B cells where its induction is 10-50-fold higher (231). Its expression has been suggested to be involved in the dampening of the humoral immune response to factor VIII administration in a mouse model of hemophilia (232). However, the data on HO-1-expressing B cells are scarce.

7.7.3. HO-1-expressing endothelial cells

HO-1 in endothelial cells inhibits the ability of proinflammatory cytokines to induce endothelial cell activation, leukocyte adhesion, activation and transmigration. The likely underlying mechanism could be the inhibition of NF-κB activation (233). HO-1-expressing human endothelial cells display a better survival under oxidative stress, a better proliferation rate in response to VEGF and a lower production of inflammatory mediators (234).

7.7.4. HO-1-expressing tumor cells

HO-1 is upregulated in quickly proliferating cells such as tumor cells where it plays a cytoprotective role, namely, among others, by scavenging ROS, exerting antiapoptotic function and contributing to resistance to chemo- and radiotherapy. HO-1 is elevated in various human cancers and is generally associated with tumor progression, angiogenesis and dissemination (160) (161) (162) (163). Mechanisms of cell division driven by HO-1 are unclear, but could involve the downregulation of negative regulators of the cell cycle such as p21, as well as the upregulation of

EGF. However, an antiproliferative effect of HO-1 has been shown for example on breast cancer cells (235). Various cancer therapies exert an antitumor activity through the production of ROS resulting in oxidative stress from an imbalanced intracellular reduction-oxidation status with diminishing antioxidant enzymes. In contrast, excessive activation of HO-1 could also increase tumor cell death in some cancers, namely through labile iron accumulation. In addition, ROS further promote HO-1 expression. These contradictory data could be explained by a differential role of HO-1 depending on the degree of ROS production (193). HO-1 might also be involved in the induction of the differentiation of cancer cells, through the regulation of the p38-MAPK pathway, as it has been reported for other cell types. However, this hypothesis is mostly speculative to date.

7.7.5. HO-1 expression by the mononuclear phagocytic system and its involvement in the regulation of the inflammatory response

The mononuclear phagocytic system is the first immunological responder to tissue injury. Its high expression of HO-1 appears to be of critical importance in the inflammatory response and injury outcome in many different organ systems. HO-1 expression in macrophages and dendritic cells seems to be closely correlated to their differentiation and functions including surface receptors, cytokine production, maturation and polarization (236).

7.7.5.1.HO-1-expressing antigen-presenting cells

HO-1 expression is found in immature dendritic cells in animal and human, and decreases during DC maturation. In vitro, HO-1 and CO treatment **blocks TLR3 and TLR4-induced DC maturation**, **antigen presentation and pro-inflammatory cytokine production while preserving IL-10 production**, and results in the **inhibition of alloreactive T-cell proliferation** (237). In a transgenic mouse model of induced auto-immune diabetes (through an adoptive T-cell transfer associated with an immunization with matured and loaded DCs), HO-1 and CO treatment of DCs **prevents DC immunogenicity** and the induction of a diabetes (238). Notably, HO-1 inhibits LPS-induced NF-κB activation of DCs (239). HO-1 and CO induction can reverse the

paralysis of mice in an experimental autoimmune encephalomyelitis, and is associated with the downregulation of MHC II molecules by APCs and the inhibition of T-cell proliferation and effector function (240). Likewise, HO-1 expression has been found upregulated in inflammatory dermatoses. Its induction limits the differentiation, maturation and antigen-presenting capacity of dendritic cells, shifts their cytokine profile to an anti-inflammatory phenotype and limits T cell-dependent cutaneous inflammation in a psoriatic skin model (241). In vitro, while HO-1 expression in Treg doesn't seem to play a significant role in their suppressive function, the absence of HO-1 in APC abolishes **Treg suppressive activity** and restores effector T-cell proliferation (242).

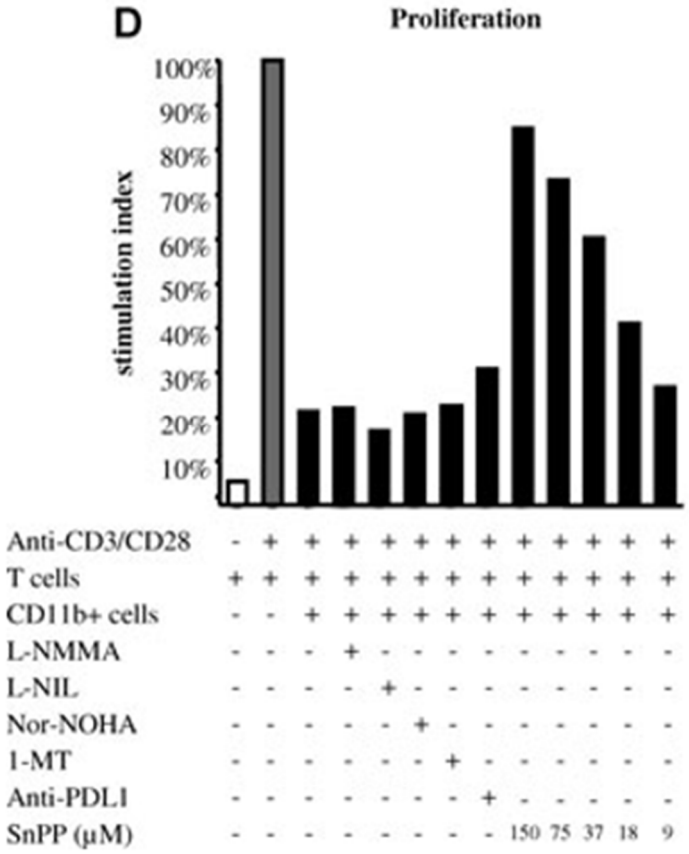
7.7.5.2.HO-1-expressing macrophages

In vitro, **M2 macrophages** have shown a strikingly higher expression of genes involved in iron metabolism such as **heme uptake**, **heme catabolism by HO-1** and **ferrous iron export**. These macrophages show an increased LPS-triggered IL-10 production after HO-1 induction (214). Proinflammatory M1 macrophages show a shift in iron metabolism that favors intracellular iron sequestration and reduced iron excretion. This could contribute to the defense strategy of M1 macrophages against extracellular pathogens (microbicidal effect). Pathophysiological implications have been suggested for iron trafficking and metabolism in the regulation of macrophage function. For example, alternatively activated macrophage activity could play a role in **homeostasis**, **tissue repair** and **tumor growth** (243) (244). Indeed, M2 macrophages only slightly increase their production of ferritin that is usually associated with the release of ferrous iron by HO-1, but increase ferroportin expression and actively export non-heme-associated iron (bound by transferrin) that might be used for a variety of purposes, according to their iron-recycling role. Increased extracellular iron availability could promote fibroblast growth and extracellular matrix deposition (depending on iron-dependent enzymes) during wound healing processes, by contrast with damaged adjacent fibroblasts resulting from M1 macrophage activity. Ferroportin-mediated iron release has been shown to affect the capacity of conditioned media to sustain cell growth. The conditioned media from M2 macrophages promote tumor cell line proliferation more efficiently than those of M1 macrophages. Remarkably, the effect is blunted by iron chelation (245). HO-1-expressing myeloid cells may contribute to tumor growth by preventing labile heme

cytotoxicity (pro-oxidant and proinflammatory effects) and supplying iron to the actively proliferating malignant cells inside the TME (246) (213) (247).

Iron metabolism in macrophages has also been suggested to modulate the immune response. For example, a deficiency in the iron exporter ferroportin in mouse macrophages results in elevated levels of proinflammatory cytokines. This effect is dependent on the iron content of the macrophages since reducing iron levels with an iron chelator inhibits LPS-induced proinflammatory cytokine production (248). In addition, M2 macrophages do not seem to activate T cells in condition of iron deficiency, in contrast with M1 macrophages (249). Moreover, HO-1 and CO might inactivate inflammatory hemoproteins such as iNOS, COX or NADPH oxidase (250). In vivo, several lines of evidence suggest that HO-1 induction in macrophages can drive a shift toward **phenotypically and functionally anti-inflammatory macrophages**, with a suggested control of the adaptive immune response, although the molecular mechanisms remain to be established. For example, in a mouse model of liver ischemia-reperfusion injury, myeloid-specific HO-1 deletion led to increased hepatocellular damages associated with pro-inflammatory markers of macrophages, suggesting that myeloid HO-1 favors an M2-macrophage phenotype and tissue homeostasis (251). Helicobacter pylori activates HO-1 expression in macrophages to favor its own survival by inducing a switch towards a regulatory phenotype associated with a dampening of the anti-bacterial immune response. HO-1 deficiency in Helicobacter pylori-infected mice exacerbates gastritis and reduces bacterial colonization. Ex vivo, Helicobacter Pylori infected-macrophages from HO-1-deficient mice exhibit a pro-inflammatory phenotype (252). In a diabetic mouse model, $CD206^{+}$ M2-like macrophages expressing HO-1 were required to protect against diabetes-induced gastroparesis (253). Myeloid-specific HO-1 deficiency shows, in a mouse model of experimental autoimmune encephalomyelitis, the activation of antigen-presenting cells and an enhanced myelin-specific T-cell activity associated with an increased disease activity (254). In addition, myeloid HO-1 modulates IFNβ production. Conditional myeloid HO-1 deletion has been shown to play a major role in innate immunity, resulting in improved clearance and survival to Listeria monocytogenes infection (254). In a pancreatic allograft model, the induction of HO-1 in donor macrophages prevents pancreatitis and improves allograft function and survival. This is associated with a decrease of proinflammatory cytokine production such as TNFα, IL-2, IFNɤ, and an increase of IL-10 (255). Myeloid HO-1 was also shown to limit the inflammatory response associated with damages after myocardial infarction (256).

Furthermore, we previously showed that HO-1 can play a key role in the **immunosuppressive function** of myeloid cells. Indeed, a prolonged skin allograft survival was observed after a transfer of myeloid cells coming from mice previously treated with repetitive LPS injections. These myeloid cells inhibit polyclonal T-cell proliferation and cytokine production in vitro through an HO-1-dependent mechanism (257). Moreover, our laboratory showed the key role of myeloid HO-1 from the donor in an experimental mouse model of allogeneic stem cell transplantation. Indeed, there was 100% of mortality from graft versus host disease when the donor was HO-1 deficient in the myeloid compartment and only 25% in the control group (to be published). The mortality was dependent on $CD4^+$ and $CD8^+$ T-cell alloreactivity. In patients with allogeneic hematopoietic stem cell transplantation, the donor with a long poly(GT)n polymorphism in the HO-1 gene promoter, associated with a low HO-1 expression, was at higher risk to develop severe graft versus host disease (to be published).



De Wilde, Am Journ of Transpl, 2009

Iron metabolism and M2-like macrophages

Heme and hemoglobin uptake	→	Prevents labile heme cytotoxicity
Iron efflux and extracellular iron availability	→	Wound healing (fibrobasts, …)
	→	Tumor cell proliferation
	→	Angiogenesis
Macrophage iron deficiency	→	Anti-inflammatory state of macrophages
Heme catabolism	→	Hemoprotein inactivation (iNOS, COX-2 …)
HO-1 expression	→	Anti-inflammatory state of macrophages
	→	T-cell suppression
	→	Mechanisms?

AIMS OF THE WORK

Previous studies support the tumor-promoting role of HO-1 and its association with bad prognosis and resistance to cancer therapy. Molecular mechanisms of macrophage polarization and in vivo dominant suppressive mechanisms of TAMs are poorly understood. Based on our previous data suggesting an immunomodulatory impact of myeloid HO-1, we hypothesized that TAMs might suppress anti-tumor T cells through HO-1 induction and macrophage polarization in the TME. Therefore, this work aimed at investigating the role of myeloid HO-1 in the antitumor immune response, by:

1. Studying the impact of myeloid HO-1 on tumor growth and on the antitumor T-cell response in an immunogenic tumor-bearing mouse model, mainly through antitumor immunization model and T-cell transfer model as it is already used in clinics. A chemotherapy model will also be tested to generalize our observations with clinically useful tools.
2. Characterizing HO-1-expressing myeloid cells in tumor-bearing mice by analyzing lymphoid organs and tumors and by identifying HO-1-expressing myeloid cells and tumor-infiltrating myeloid cells.
3. Studying the role of myeloid HO-1 on TAM phenotype (such as MHC and costimulatory molecules) and function (such as macrophage activation state, cytokine production, co-cultured T-cell proliferation) in vitro through bone marrow-derived macrophages and in vivo.
4. Deciphering the molecular mechanisms used by myeloid HO-1 that could contribute to tumor immune escape through flow cytometry, RNA sequencing and ATAC sequencing.

METHODS

We used genetically engineered C57BL/6 mice with a Cre-loxP system (where a Cre recombinase is expressed by the LysM promoter and excises *Hmox1* gene (coding for HO-1 protein) flanked by two loxP sites) to generate conditional deletion of *Hmox1* gene in macrophages ($LysMCre^{+/-}$ $Hmox1^{fl/fl}$ with $Hmox1^{fl/fl}$ used as littermate control mice). This is a very helpful and powerful genetic technology for conditional deletion, although the Cre-mediated deletion is limited in term of efficiency and specificity (258).

We used EG7-OVA tumor cell line which is a C57BL/6 (H-2 b) mouse EL4 lymphoma cell line transfected with the plasmid pAc-neo-OVA (genes coding for complete chicken ovalbumin (OVA) and neomycin resistance (G418)). These tumor cells synthesize and secrete OVA constitutively, giving rise to H-2 Kb restricted cytotoxic lymphocytes specific for the OVA 258-276 peptide.

This syngeneic tumor model is widely used for preclinical models in cancer immunotherapy and allowed us to easily assess the tumor growth and the antitumor immune response with experimental reproductibility. Nevertheless, it should be noted that this method lacks the genomic and microenvironmental heterogeneity compared to other more physiologically relevant tumor models such as carcinogen-induced models. Another detrimental aspect of this model is the fact that the tumor cells don't undergo the pathophysiological steps of tumor development in interaction with stromal and immune cells of the TME, such as it is observed in genetically engineered models. In addition, the EG7-OVA subcutaneous transplantation is not an orthotopic one and is therefore not representative of a tumor growth in its original organ (259).

The methods are detailed further in the article below.

RESULTS

Heme oxygenase-1 orchestrates the immunosuppressive program of tumor-associated macrophages

Emmanuelle Alaluf[1], Benoît Vokaer[1], Aurélie Detavernier[1], Abdulkader Azouz[1], Marion Splittgerber[1], Alice Carrette[1], Louis Boon[2], Frédérick Libert[3], Miguel Soares[4], Alain Le Moine[1*] and Stanislas Goriely[1*]

Affiliations :

[1] Université Libre de Bruxelles, Institute for Medical Immunology, Gosselies, Belgium

[2] Bioceros B.V., Utrecht, the Netherlands

[3] Université Libre de Bruxelles, BRIGHTcore ULB-VUB and Institute of Interdisciplinary Research in Human and Molecular Biology (IRIBHM), Brussels, Belgium

[4] Instituto Gulbenkian de Ciência, Oeiras, Portugal.

* These authors jointly supervised this work

Lead contact:

S. GORIELY, Institute for Medical Immunology, 8 rue Adrienne Bolland, B-6041 Charleroi-Gosselies, Belgium; Phone: 32-2-650-9588; Fax: 32-2-650-9563; E-mail: stgoriel@ulb.ac.be

a. Abstract

Tumor-Associated Macrophages (TAMs) contribute to the maintenance of a strong immunosuppressive environment, supporting tumor progression and resistance to treatment. To date, the mechanisms that drive acquisition of these immunosuppressive features are still poorly defined. Heme oxygenase-1 (HO-1) is the rate-limiting enzyme that catabolizes free heme. It displays important cytoprotective, anti-inflammatory and antioxidant properties. A growing body of evidence suggests that HO-1 may also promote tumor development. Herein, we show that HO1 is highly expressed in monocytic cells in the tumor microenvironment (TME) once they differentiate into TAMs. Deletion of HO-1 in the myeloid compartment enhances the beneficial effects of a therapeutic antitumor vaccine by restoring CD8 T-cell proliferation and cytotoxicity. We further show that induction of HO-1 plays a major role on monocyte education by tumor cells by modulating their transcriptional and epigenetic programs. These results identify HO-1 as a valuable therapeutic target to reprogram the TME and synergize with current cancer therapies to facilitate antitumoral response.

b. Introduction

Immunotherapy represents a paradigm shift in the treatment of cancer. In the past few years, approaches such as immune checkpoint inhibition or adoptive transfer of engineered T cells have produced durable responses and long-term survival of many patients for whom previous therapeutic options were ineffective[1–9]. These clinical successes demonstrate the essential role of the cancer-immunity interface in tumor progression and therapy. However, only a subset of patients respond to immunotherapies, and some of them acquire resistance to the treatment. The cellular and molecular determinants of responsiveness versus resistance to immunotherapy are incompletely understood[10,11]. This may be partly due to the current focus of therapies on the T-cell compartment only, with little attention to the side of the antigen presenting cell. It is likely that a better understanding of how tumors shape their microenvironment, and alter myeloid cell functions, would reveal novel principles of cancer immunotherapy and enable innovative clinical applications, benefiting patients refractory to current immunotherapies[12,13].

In this context, tumor-associated macrophages (TAMs) generally play a deleterious role by supporting multiple aspects of tumor progression[14]. They were shown to contribute to the resistance of tumors to biologic therapies, chemotherapies and radiotherapies through direct trophic support to cancer cells. For example, TAMs may promote angiogenesis, the maintenance of cancer stem cells and metastatic processes[15]. Macrophage infiltration may also interfere with immunotherapy, hampering efforts to reactivate $CD8^+$ T cells by targeting immune checkpoints. Indeed, they express inhibitory receptors (such as PD-L1 or B7-H4) and represent an important source of cytokines (such as IL-10 or TGF-β), chemokines and enzymes (such as arginase 1, inducible nitric oxide synthase or indoleamine 2,3-dioxygenase) that contribute to the maintenance of a strong immunosuppressive environment by inhibiting effector T cells while inducing regulatory T cell recruitment and expansion[16,17].

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme that catalyzes free heme into three major biologically active byproducts: carbon monoxide, ferrous iron and biliverdin (converted to bilirubin). In numerous pathological contexts, HO-1 displays important cytoprotective, antiinflammatory, antioxidant and anti-apoptotic properties[18–26]. In the context of alloreactivity, we previously demonstrated that HO-1 contributed to the immunosuppressive properties of

myeloid cells[27]. A growing body of evidence suggests that HO-1 also promotes tumor development. It is expressed in a wide variety of cancers and is generally associated with poor prognosis[28–30]. In preclinical models, the administration of HO-1 pharmacological inhibitors displays anti-tumoral effects[31,32] and improves the response to chemotherapy[33]. This effect is at least partially mediated by $CD8^+$ T cells but the underlying mechanisms of immunomodulation by HO-1 remain unclear[34]. Furthermore, expression of HO-1 by TAMs was recently shown to promote transendothelial migration and metastatic spread[35].

Herein, we investigated the role of HO-1 in TAMs. We show that deletion of HO-1 in the myeloid compartment enhances the beneficial effects of a therapeutic antitumor vaccine by restoring T-cell proliferation and cytotoxicity in the tumor microenvironment (TME). We further show that induction of HO-1 plays a major role on monocyte education by tumor cells by modulating their transcriptional and epigenetic programs. Taken together, these results identify HO-1 as a valuable target to reprogram TAMs and improve current strategies of immunotherapy.

c. Results

1. Monocytic cells express HO-1 upon differentiation into macrophages in the tumor microenvironment

In order to evaluate the expression of HO-1 in the myeloid compartment during tumor development, we implanted thymoma cells (EG7-OVA) intradermally in C57BL/6 mice as this model was shown to strongly promote the expansion of myeloid suppressive cells[36]. We first assessed HO-1 expression in the TME by immunofluorescence staining (Fig 1A). We observed HO-1 staining in a portion of $CD11b^+$ and $F4/80^+$ infiltrating cells. Of note, HO-1 was found to be present in the cytoplasm and/or the nucleus of these cells. This is consistent with previous reports indicating that HO-1 can migrate to the nucleus and modulate transcriptional activity independently of its enzymatic activity[37–39]. To further define the cellular sources of HO-1, we evaluated its expression by flow cytometry. $Ly6C^{hi}$ monocytes that are recruited in the TME gradually differentiate into $Ly6C^{lo}$MHC II^+ TAMs[40]. The proportion of $Ly6G^{hi}$ granulocytes or $LyC6^{hi}$ monocytes (MHC II^- or MHC II^+) expressing HO-1 was low (Fig 1B). In sharp contrast, it was expressed by a significant proportion of $Ly6C^{lo}$MHC II^+ TAMs, suggesting that it is part of the program induced in monocytic cells upon exposure to tumor-derived factors. Expression of HO-1 by TAMs was associated with strong expression of classical phenotypic markers of macrophage differentiation such as F4/80, CD64, CD206 and CD163 (Fig 1C). As compared to $HO\text{-}1^-$ TAMs, MHC II expression was found to be decreased. Tumors also influenced myelopoiesis and induced the accumulation of immature $CD11b^+Gr1^+$ myeloid cells in the bone marrow and the spleen (Fig 1D). Expression of HO-1 in immature $CD11b^+Gr1^+$ myeloid cells from naïve and tumor-bearing mice was found to be comparable (Fig 1E). These results indicate that HO-1 expression is specifically induced upon differentiation of monocytic cells in the TME.

FIGURE 1

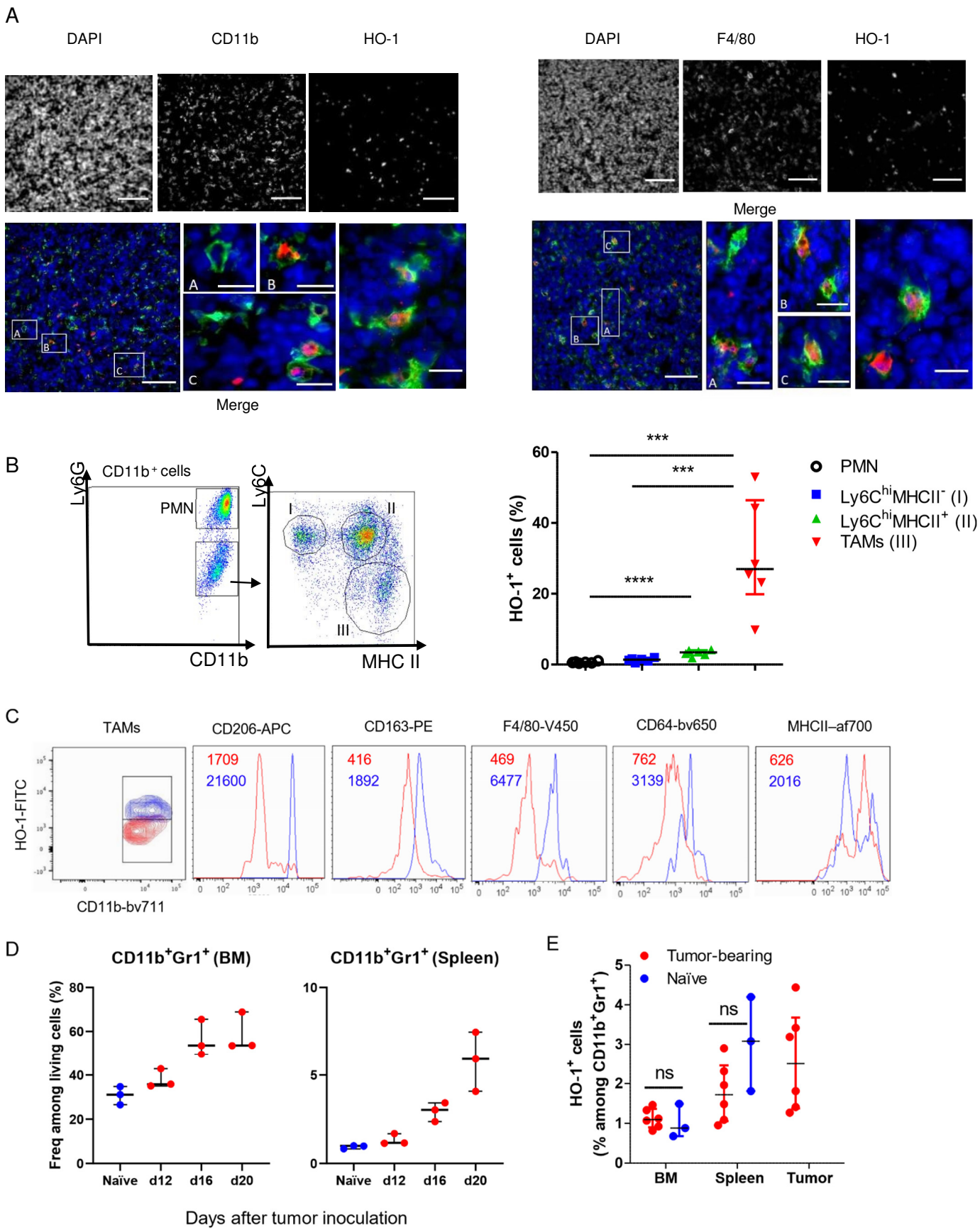

A
DAPI
CD11b
HO-1
DAPI
F4/80
HO-1
Merge
Merge
B
CD11b⁺ cells
Ly6G
Ly6C
PMN
I
II
III
CD11b
MHC II
***
***
****
HO-1⁺ cells (%)
PMN
Ly6C^hiMHCII^- (I)
Ly6C^hiMHCII^+ (II)
TAMs (III)
C
TAMs
CD206-APC
CD163-PE
F4/80-V450
CD64-bv650
MHCII–af700
HO-1-FITC
CD11b-bv711
1709
21600
416
1892
469
6477
762
3139
626
2016
D
CD11b⁺Gr1⁺ (BM)
CD11b⁺Gr1⁺ (Spleen)
Freq among living cells (%)
Naïve
d12
d16
d20
Days after tumor inoculation
E
Tumor-bearing
Naïve
HO-1⁺ cells (% among CD11b⁺Gr1⁺)
ns
ns
BM
Spleen
Tumor

Figure 1.

HO-1 expression is specifically induced by monocytic cells upon differentiation into macrophages in the tumor microenvironment

A) HO-1 staining (in red) combined with DAPI co-staining showing nuclei (in blue) are visualized in tumor slices by immunofluorescence in $CD11b^+$ and $F4/80^+$ myeloid cells (in green) in an EG7OVA tumor 21 days after tumor inoculation in a wild type mouse. Scale bar = 5 µm. B) Flow cytometry plots pregated on live $CD11b^+$ cells indicate 12 days after tumor inoculation the proportion of HO-1 producing cells among different tumor-infiltrating myeloid cell subtypes : the $CD11b^{hi}Ly6G^+$ neutrophils (PMN), the $CD11b^{hi}Ly6G^-Ly6C^{hi}$MHC II^- monocytes (I), the $CD11b^{hi}Ly6G^-Ly6C^{hi}$MHC II^+ cells (II) and the $CD11b^{hi}Ly6G^-Ly6C^{lo}$MHC II^+ TAMs (III). C) Histograms indicate by median fluorescence intensity (MFI) the expression of the specified markers in $HO\text{-}1^+$ (blue) versus $HO\text{-}1^-$ (red) TAMs. D) Representative flow cytometry plots of the accumulation of immature myeloid cells compatible with myeloid-derived suppressor cell (MDSC) phenotype ($CD11b^{hi}Ly6C^+Ly6G^-$and $CD11b^{hi}Ly6C^{int}Ly6G^+$ summarized as $CD11b^+Gr1^+$cells) in the bone marrow (BM) and spleen from tumor-bearing wild type mice. E) HO-1 expression measured by flow cytometry among $CD11b^+Gr1^+$ cells from bone marrow, spleen and EG7-OVA tumor from tumor-bearing wild type mice, compared to tumor-free wild type mice (naïve).

2. Myeloid HO-1 promotes tumor growth by an immunosuppressive mechanism

To determine the impact of HO-1 expression by TAMs on tumor growth, we invalidated *Hmox1* in myeloid cells (*LysMCre*$^{+/wt}$ *Hmox1*$^{fl/fl}$: *Hmox1*$^{\Delta M}$ mice). We evaluated the growth of intradermally implanted EG7-OVA tumors at regular intervals. Tumor growth in *Hmox1*$^{\Delta M}$ mice was found to be comparable to *Hmox1*$^{fl/fl}$ littermates (Fig 2A). Next, we repeated these experiments and induced antitumoral T cell response by immunizing the mice against OVA at day 7 and day 14 post-implantation. As adjuvant, we used poly(I:C) as this dsRNA analogue was shown to promote CD8 T cell responses in pre-clinical tumor immunotherapy settings[41]. As expected, using this regiment, we observed a delay in tumor growth in *Hmox1*$^{fl/fl}$ mice. However, in most cases, mice had to be sacrificed at latter time-points because of tumor escape. In contrast, we observed complete tumor regression upon therapeutic immunization in a majority of *Hmox1*$^{\Delta M}$ mice. This antitumoral effect was abrogated upon depletion of CD8 T cells by antibody treatment (Fig 2B). To further evaluate the antigen-specific nature of this enhanced antitumoral response, we implanted EG7-OVA tumor cells on one flank and parental EL4 cells on the other flank of the same animal. Upon immunization and transfer of OVA-specific CD8 T cells (OT-1), progression of EG7-OVA tumors was reduced in *Hmox1*$^{\Delta M}$ as compared to *Hmox1*$^{fl/fl}$ mice (Fig 2C). However, in the same animals, growth of EL4 tumors was not restrained in the *Hmox1*$^{\Delta M}$ group. Taken together, this set of experiments shows that myeloid specific inactivation of HO-1 potentiates antigen-specific antitumoral CD8 T cell responses in the context of therapeutic immunization.

FIGURE 2

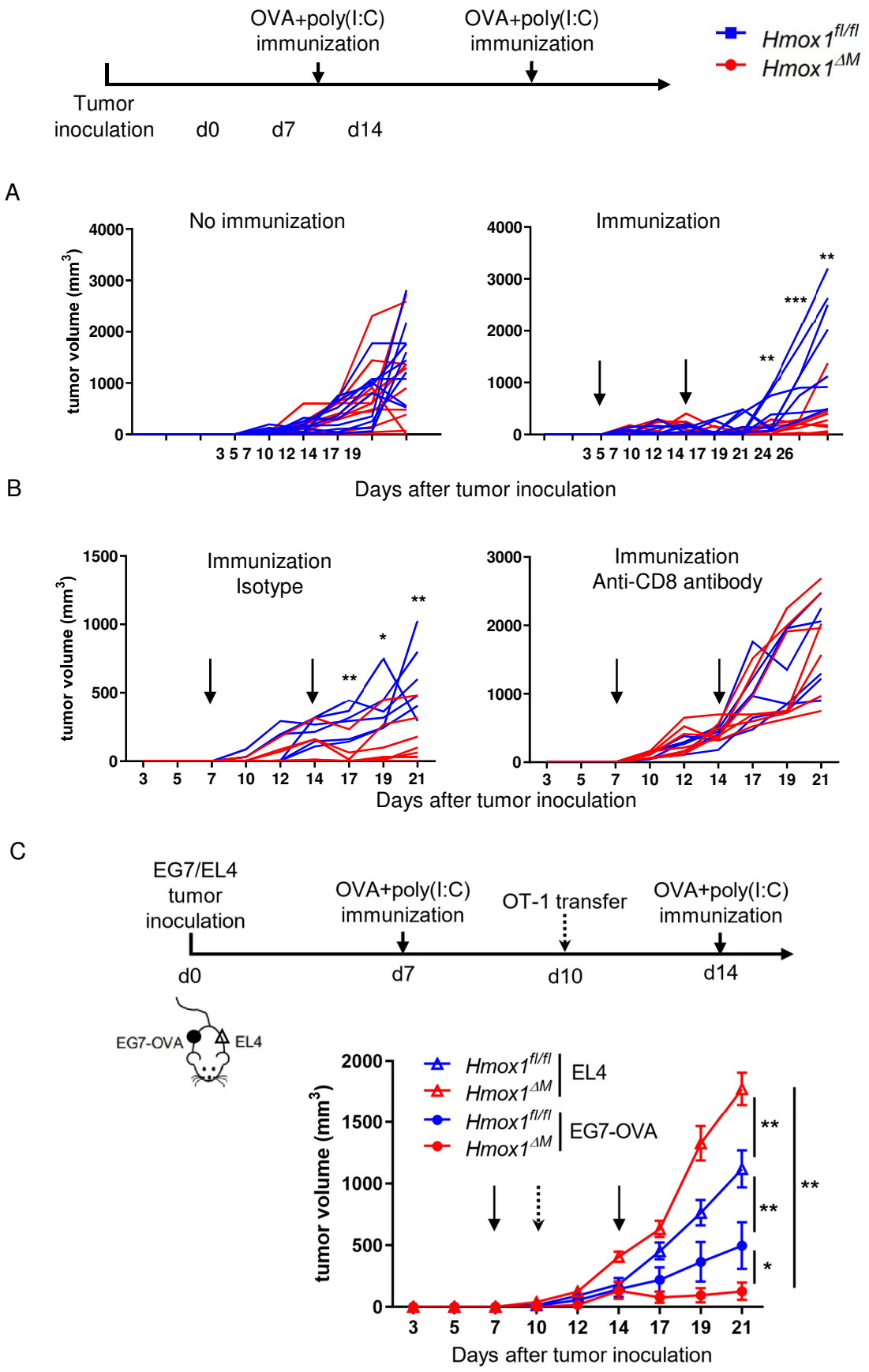

OVA+poly(I:C) immunization
OVA+poly(I:C) immunization
Hmox1fl/fl
Hmox1ΔM
Tumor inoculation
d0
d7
d14
A
No immunization
Immunization
tumor volume (mm3)
Days after tumor inoculation
B
Immunization Isotype
Immunization Anti-CD8 antibody
tumor volume (mm3)
Days after tumor inoculation
C
EG7/EL4 tumor inoculation
OVA+poly(I:C) immunization
OT-1 transfer
OVA+poly(I:C) immunization
d0
d7
d10
d14
EG7-OVA
EL4
Hmox1fl/fl
Hmox1ΔM
EL4
Hmox1fl/fl
Hmox1ΔM
EG7-OVA
tumor volume (mm3)
Days after tumor inoculation

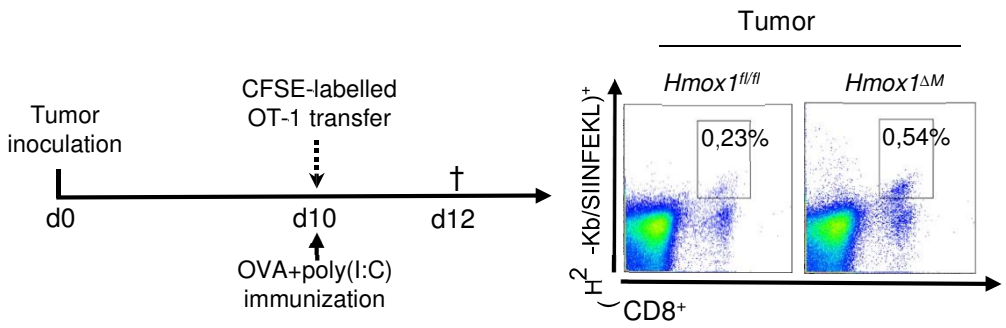
Figure 2. Myeloid HO-1 promotes tumor growth by an immunosuppressive mechanism

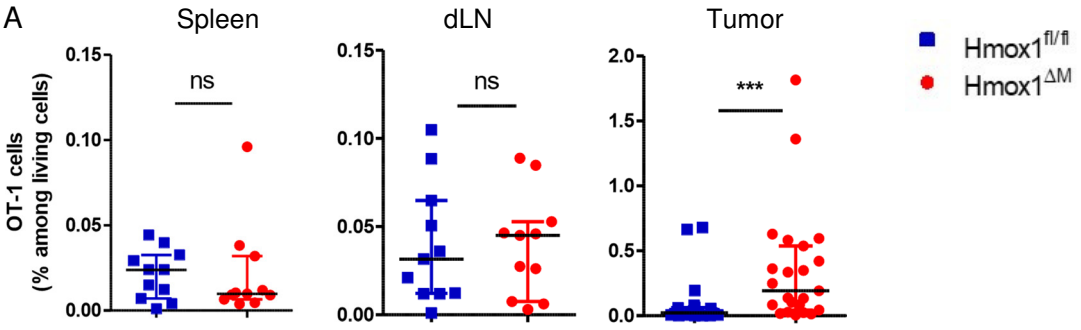
EG7-OVA tumor cells were inoculated intradermally at day 0 on the right flank of *Hmox1*$^{\Delta M}$ *mice* (n = 11). Their tumor volumes were compared to *Hmox1*$^{fl/fl}$ littermates (n = 10) at regular intervals following implantation. A) There was no significant differences between the groups of tumors. However, a blockade of tumor growth was observed in Hmox1$^{\Delta M}$ mice (n = 11) compared to *Hmox1*$^{fl/fl}$ littermates (n = 8) after therapeutic immunization with subcutaneous injection of ovalbumin protein (10 µg/mouse) and poly(I:C) (50 µg/mouse) 7 days after tumor inoculation and reboost 7 days later on the right flank of the animals. B) Intraperitoneal administration of isotype control or CD8$^+$ T cell-depleting monoclonal antibody (clone YTS169) 1x/week (500 µg/mouse). C) Bilateral tumor model, where EG7-OVA tumor cells were inoculated on the right flank and EL4 cells on the left flank from *Hmox1*$^{\Delta M}$ mice (n = 8) and *Hmox1*$^{fl/fl}$ mice (n = 10) which were therapeutically immunized such as described above and adoptively transferred with OT-1 cells (10^6 cells/mouse) at day 10. Data are representative of three independent experiments.

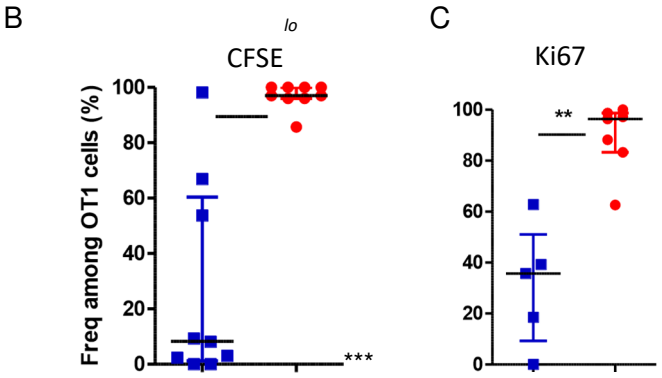
3. Myeloid HO-1 controls antitumor T-cell proliferation and cytotoxicity in the tumor microenvironment

In order to further assess the effect of myeloid HO-1 on antigen-specific T cell responses, we performed adoptive transfer of CFSE-labelled OT-1 cells 10 days after EG7-OVA implantation. Mice were immunized concomitantly. Two days after, we assessed their frequency. While the proportions of OT-1 cells in the spleen or the draining lymph nodes were comparable in both groups, it was strongly increased within the tumors of *Hmox1*$^{\Delta M}$ mice as compared to *Hmox1*$^{fl/fl}$ controls (Fig 3A). This was accompanied by high proliferation rate, assessed by CFSE dilution (Fig 3B) and Ki67 staining (Fig 3C). In order to evaluate their functionality, we analyzed Granzyme B and IFNɤ expression following *ex vivo* stimulation of tumor-infiltrating lymphocytes with OVA SIINFEKL peptide. We observed increased expression of these cytotoxic mediators in OT-1 cells transferred in tumor-bearing *Hmox1*$^{\Delta M}$ mice (Fig 3D and Fig 3E). This was accompanied by higher T-bet levels (Fig 3F). In contrast, expression of Eomes was similar in both groups (Fig 3G). Taken together, these data indicate that HO-1 expression by TAMs leads to strong immunosuppressive activity in the TME that limits antigen-specific CD8 T-cell effector function against tumor cells.

FIGURE 3


Tumor
Tumor inoculation
CFSE-labelled OT-1 transfer
d0
d10
d12
†
OVA+poly(I:C) immunization
Hmox1fl/fl
Hmox1ΔM
0,23%
0,54%
H2-Kb/SIINFEKL)+
CD8+


A
Spleen
dLN
Tumor
OT-1 cells (% among living cells)
ns
ns
***
Hmox1fl/fl
Hmox1ΔM


B
CFSElo
Freq among OT1 cells (%)
***
C
Ki67
**

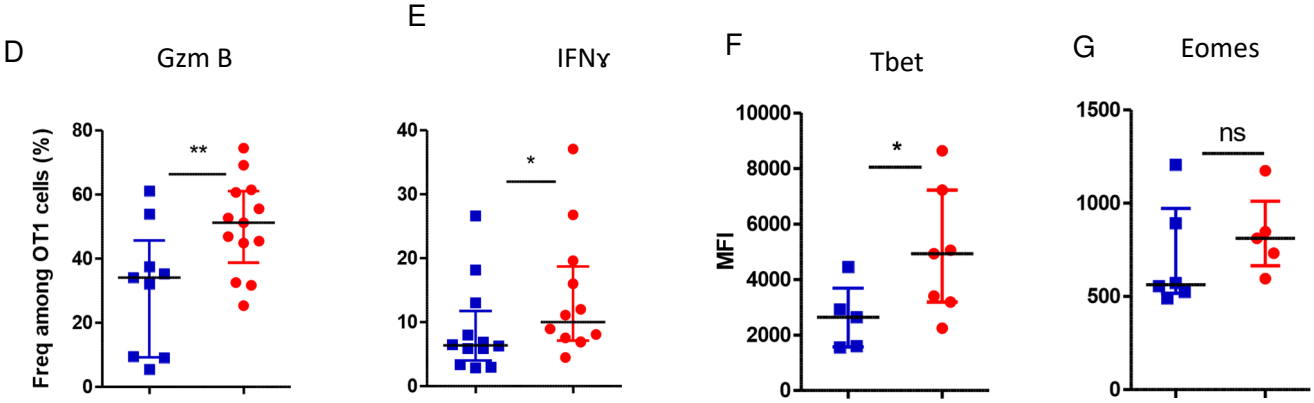

D
Gzm B
Freq among OT1 cells (%)
**
E
IFNγ
*
F
Tbet
MFI
*
G
Eomes
ns

Figure 3. Myeloid HO-1 controls antitumor T-cell proliferation and cytotoxicity in the tumor microenvironment

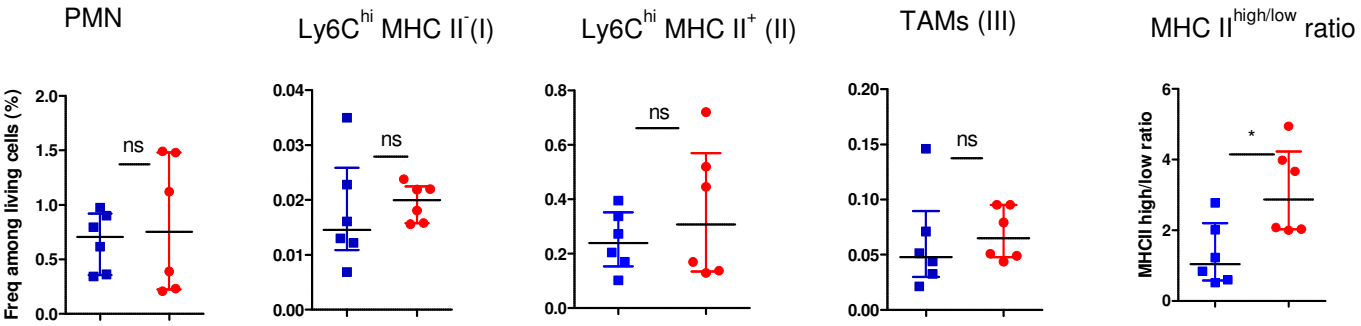
An intravenous adoptive transfer of OT-1 cells ($2x10^6$ cells/mouse) has been performed 10 days after tumor inoculation. This was followed by an immunization with subcutaneous injection of ovalbumin protein (50 µg/mouse) and poly(I:C) (50 µg/mouse) one hour later on the right flank of the animals. Two days later, EG7-OVA tumors were enzymatically and mechanistically digested and analyzed by flow cytometry. A) Proportions of OT-1 cells in the spleen, the draining lymph nodes (dLN) (axillary and inguinal lymph nodes on the right side) and the tumor of *Hmox1*$^{\Delta M}$ mice compared to *Hmox1*$^{fl/fl}$ littermates. OT-1 cells were labeled with CFSE before intravenous adoptive transfer ($2x10^6$ cells/mouse). This was followed by an immunization of the mice as described above. B) Tumor-infiltrating OT-1 cell proliferation assessed by CFSE dilution and C) Ki67 expression among OT-1 cells. D) Granzyme B (GzmB) was analyzed by intracytoplasmic staining in tumor-infiltrating OT-1 cells. E) Production of IFNɣ and MFI of F) T-bet and G) Eomes were assessed by *ex vivo* stimulation overnight with OVA SIINFEKL peptide (and brefeldine A added 2 hours later). Data are pooled from three/four experiments.

4. HO-1 drives transcriptional and epigenomic programs of TAMs

In order to define the role of HO-1 in myeloid cells, we examined the proportions of Ly6G^{hi} granulocytes, Ly6C^{hi} monocytes and Ly6C^{lo}MHC II^{+} TAMs in tumors of *Hmox1*$^{\Delta M}$ and *Hmox1*$^{fl/fl}$ mice 12 days after implantation. There were no significant changes in proportions between these two groups. However, we observed a higher proportion of MHC II^{hi} cells in TAMs from *Hmox1*$^{\Delta M}$ mice (Fig 4A). In addition, myeloid HO-1 deletion led to increased iNOS and decreased Arg-1 expression in TAMs (Fig 4B). Taken together, this suggests that their differentiation status could be impacted by HO-1, resulting in the antitumor effect observed associated with the restoration of tumor-infiltrating T-cell proliferation. Next, we performed RNA-seq experiment on CD11b^{hi}CD64^{+}Ly6C^{lo}MHC II^{+} TAMs from *Hmox1*$^{\Delta M}$ and *Hmox1*$^{fl/fl}$ mice. We confirmed the lack of a full-length RNA of *Hmox1* gene upon LysM-driven expression of the Cre recombinase in these cells (Fig 4C) and identified more than 1000 differentially expressed genes (594 down and 539 up-regulated genes in HO-1-deficient cells as compared to their controls, FDR < 0.05, FC > 2) (Fig 4D). We performed geneset enrichment analysis (GSEA) in order to evaluate the expression of genes that were shown to be upregulated in TAMs as compared to splenic monocytic cells (Fig 4E). We observed a global decrease in the expression of these genes in HO-1deficient cells, indicating that the core molecular signature induced by the TME is dysregulated in absence of HO-1. The profile observed in HO-deficient TAMs did not follow a simple M1/M2 dichotomy as both M1 and M2 signatures were found to be significantly affected. Importantly, multiple genes encoding molecules that participate to the immunosuppressive features of TAMs such as Arg1, iNOS, IL-10, PDL-1 or PDL-2 were downregulated in HO-1-deficient cells (Fig 4F). Expression of IL-4-dependent genes were also globally decreased in this group. Furthermore, classical pro-inflammatory M1 genes such as IL-27 or IL12 were also affected (Fig 4F).

FIGURE 4

A


PMN
Ly6C^hi MHC II^- (I)
Ly6C^hi MHC II^+ (II)
TAMs (III)
MHC II^high/low ratio
Freq among living cells (%)
MHCII high/low ratio
ns
ns
ns
ns
*

B

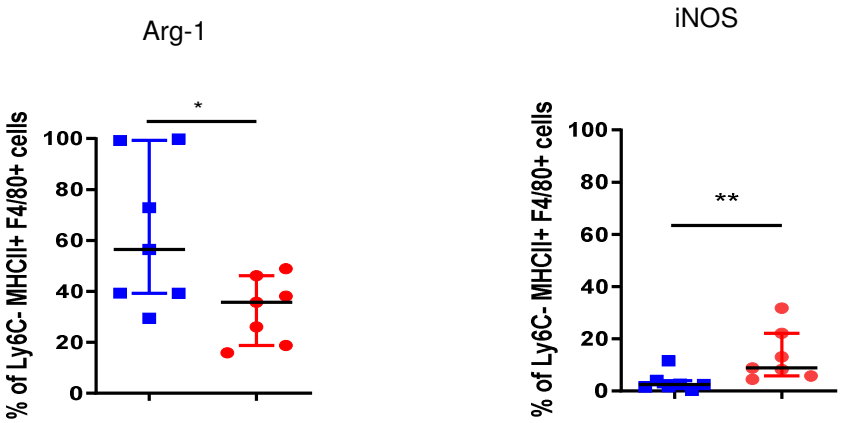
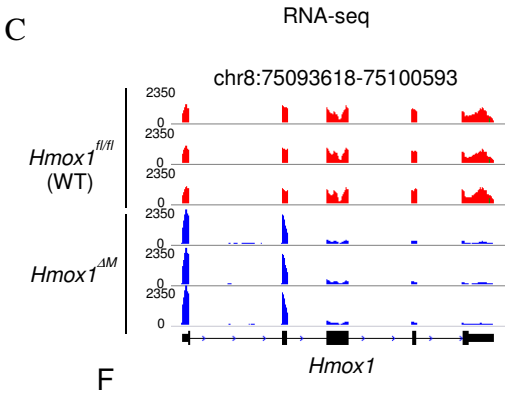
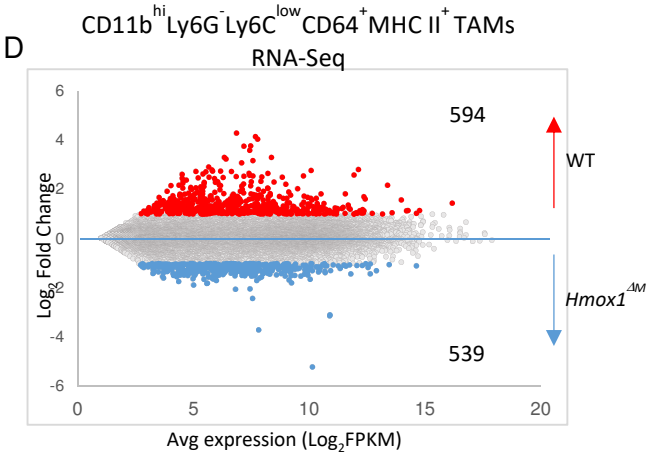
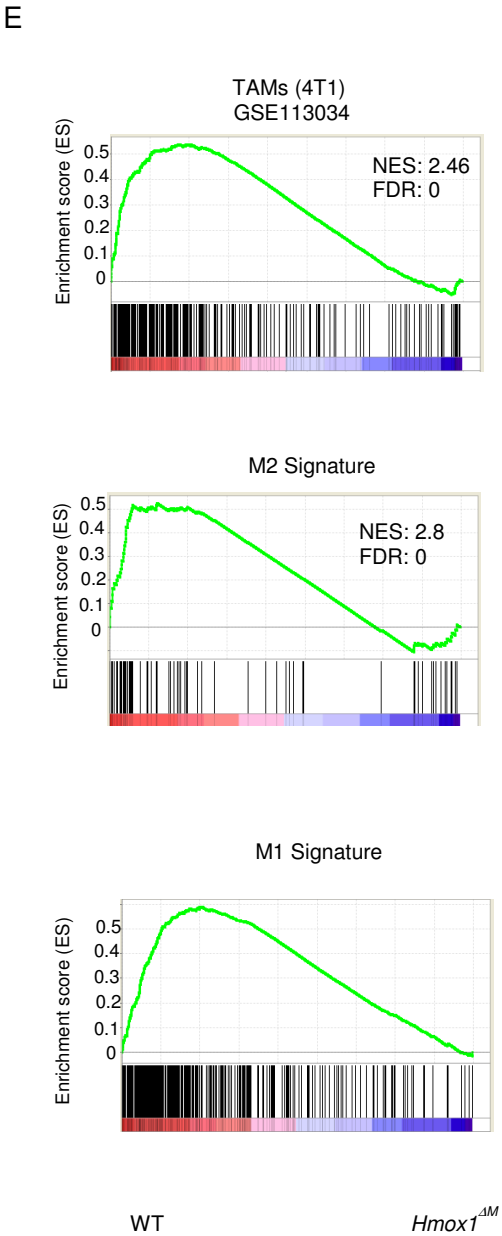

Arg-1
iNOS
% of Ly6C- MHCII+ F4/80+ cells
% of Ly6C- MHCII+ F4/80+ cells
*
**

FIGURE 4


C
RNA-seq
chr8:75093618-75100593
Hmox1fl/fl (WT)
Hmox1ΔM
2350
0
Hmox1


D
CD11bhi Ly6G- Ly6Clow CD64+ MHC II+ TAMs
RNA-Seq
594
539
WT
Hmox1ΔM
Log2 Fold Change
Avg expression (Log2FPKM)


E
TAMs (4T1)
GSE113034
NES: 2.46
FDR: 0
M2 Signature
NES: 2.8
FDR: 0
M1 Signature
Enrichment score (ES)
WT
Hmox1ΔM

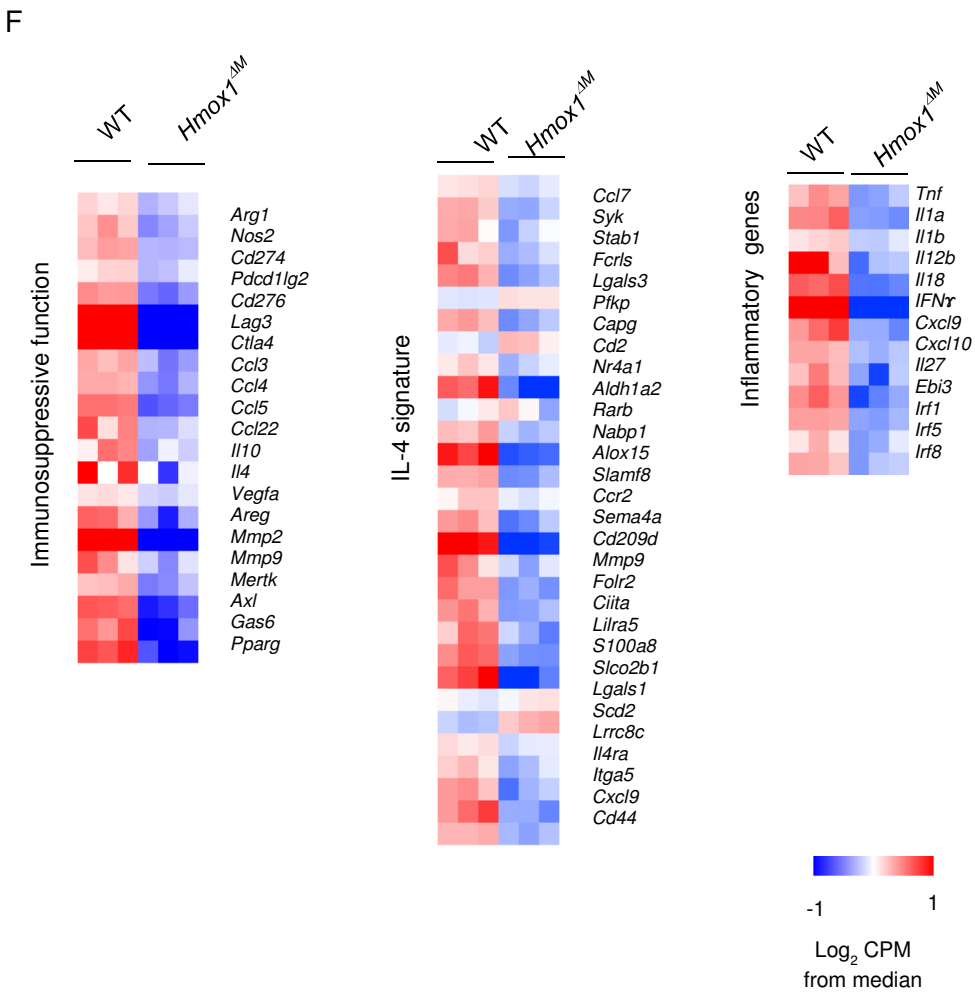

F
WT
Hmox1ΔM
Immunosuppressive function
Arg1
Nos2
Cd274
Pdcd1lg2
Cd276
Lag3
Ctla4
Ccl3
Ccl4
Ccl5
Ccl22
Il10
Il4
Vegfa
Areg
Mmp2
Mmp9
Mertk
Axl
Gas6
Pparg
IL-4 signature
Ccl7
Syk
Stab1
Fcrls
Lgals3
Pfkp
Capg
Cd2
Nr4a1
Aldh1a2
Rarb
Nabp1
Alox15
Slamf8
Ccr2
Sema4a
Cd209d
Mmp9
Folr2
Ciita
Lilra5
S100a8
Slco2b1
Lgals1
Scd2
Lrrc8c
Il4ra
Itga5
Cxcl9
Cd44
Inflammatory genes
Tnf
Il1a
Il1b
Il12b
Il18
IFNγ
Cxcl9
Cxcl10
Il27
Ebi3
Irf1
Irf5
Irf8
-1
1
Log2 CPM from median

Figure 4. HO-1 drives the transcriptional program of TAMs

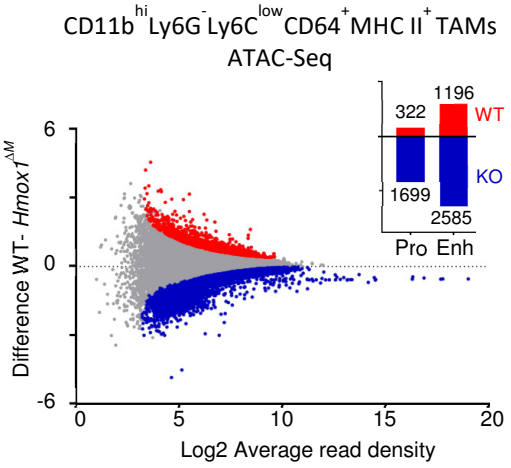
A) Flow cytometry data showing the frequency of different tumor-infiltrating myeloid cell subtypes : the CD11b^{hi}Ly6G^{+} neutrophils (PMN), the CD11b^{hi}Ly6G^{-}Ly6C^{hi}MHC II^{-} monocytes (I), the CD11b^{hi}Ly6G^{-}Ly6C^{hi}MHC II^{+} cells (II) and the CD11b^{hi}Ly6G^{-}Ly6C^{lo}MHC II^{+} TAMs (III) among living cells. The ratio between MHC II^{high} and MHC II^{low} TAMs is also shown. B) Production of Arg-1 and iNOS by CD11b^{hi}Ly6G^{-}Ly6C^{lo}CD64^{+}MHC II^{+} TAMs assessed by flow cytometry intracellular staining, in *Hmox1*$^{\Delta M}$ mice and *Hmox1*$^{fl/fl}$ littermates, at day 17 (sc immunization with OVA 50µg/mouse and poly(I:C) 50µg/mouse at day 12) C) MA plot showing differentially expressed genes in WT (red) and *Hmox1*$^{\Delta M}$ (blue) CD11b^{hi}Ly6GLy6C^{lo}CD64^{+}MHC II^{+} TAMs with the indicated number of genes. D) Integrative Genomics Viewer (IGV) tracks showing read coverage for RNA expression of *Hmox1* gene in WT (red) and *Hmox1*$^{\Delta M}$ (blue). Gene position is indicated at the top of the panel. E) Gene set enrichment analysis (GSEA) plots using our RNA-seq as a data set and the indicated publicly available gene sets. Normalized Enrichment Score (NES) and False Discovery Rate (FDR) are shown. F) Gene expression heatmap from RNA-seq data showing the log2 Count Per Million (CPM) of selected pathways.

To further determine underlying molecular processes at play, we analyzed epigenomic landscapes of these cells by ATAC-Seq approaches. This technique allows us to map open chromatin regions throughout the genome[42]. We observed extensive modifications in HO-1 deficient cells *Hmox1*$^{\Delta M}$. 1518 and 4284 regions were found to be significantly more or less accessible in controls, respectively (Fig 5A). Most of the differentially accessible peaks were located in enhancers rather than in promoters. We used Binding and Expression Target Analysis (BETA) package[43] to predict the activating or repressive function of these differentially accessible regions. Regulatory regions that were more/less accessible were clearly associated with genes that were up or down-regulated in WT or HO-1 deficient TAMs, respectively (Fig. 5B). This observation indicates that the impact of HO-1 on their transcriptional profile has a strong epigenetic component. For example, we observed decreased accessibility in regulatory elements associated with the genes that encode the immune checkpoint molecules PD-L1, PD-L2 or MERTK (Fig 5C)[44], in line with their reduced expression in absence of HO-1. Similarly, we identified regions that were less accessible within the locus of *Mmp2* that together with other matrix metalloproteinases favors neovascularisation and tumor dissemination[45]. Next, we performed gene-ontology analysis using GREAT[46] (genomic regions enrichment of annotations tool). The most relevant pathways were associated with regions that were less accessible in HO-1 deficient cells (Fig 5D). As expected, many of these were involved in the regulation of inflammatory response or cytokine signaling. In line with the cytoprotective functions of HO-1[47], we also observed signatures for wound healing and cell redox homeostasis pathways. Importantly, several different metabolic processes were also identified along with other important tumor-related pathways such as signaling by EGFR, VEGF and TGF-βR (Fig 5D). These observations suggest important and widespread functional impact of HO-1 on the epigenetic programming of TAMs. We then scanned for binding motifs at the center of ATAC peaks located in these differentially accessible regions using Ciiider algorithm[48]. We observed strong enrichment for consensus binding motifs characteristic of basic region/leucine zipper (bZIP) and Zinc fingers families of transcription factors in WT and HO-1 deficient cells respectively (Fig 5E). Among these bZIP factors, we noted motifs for CCAAT-enhancer-binding proteins (C/EBPs), key factors involved in myeloid cell differentiation and for Fos- and Jun-related factors. Of note, the consensus antioxidant response elements (AREs) bound by NRF2 was significantly enriched in WT cells. This is of particular interest as it represents the main transcriptional pathway responsible for induction of

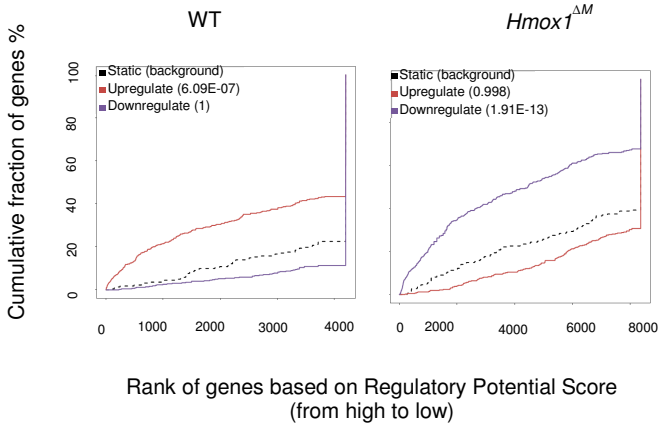
Hmox1 in response to oxidative stress[49]. Conversely, nuclear HO-1 was shown to interact with NRF2 and to promote its transcriptional activity[37]. Taken together, these data indicate that HO-1 sets a major transcriptional and epigenetic reprogramming of monocytic cells once they enter the TME.

FIGURE 5

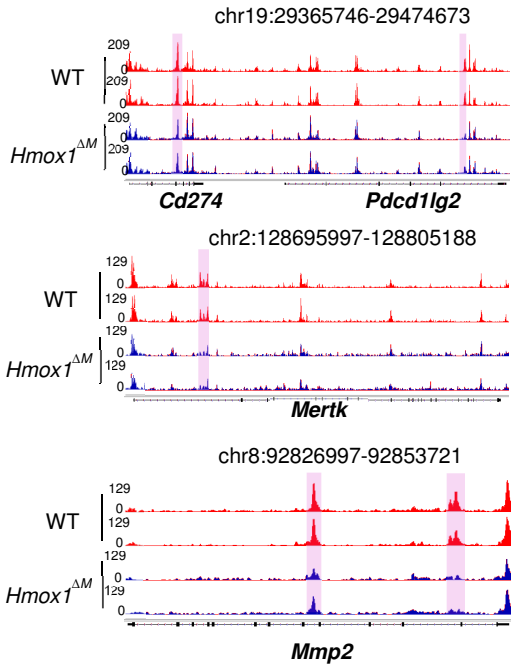
A



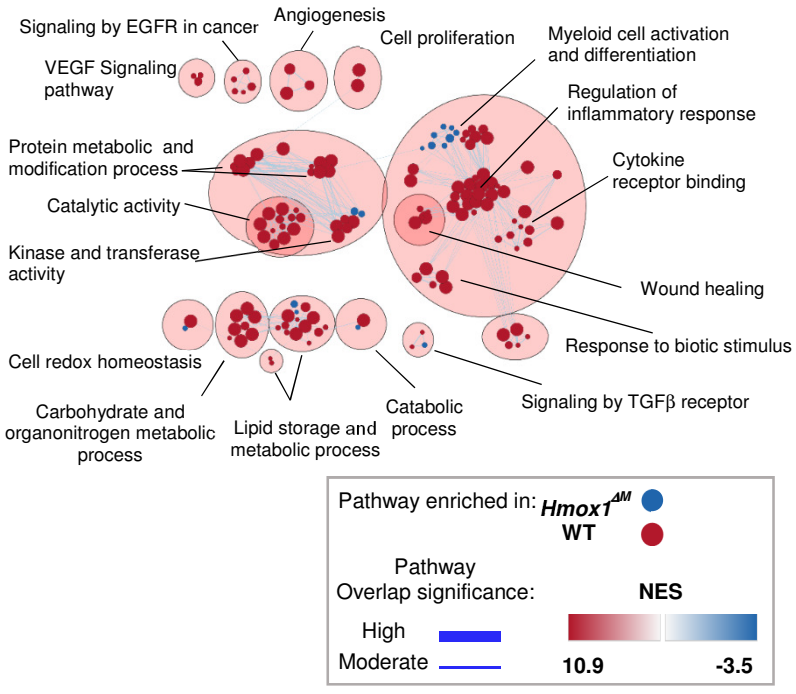
B



C



D



E

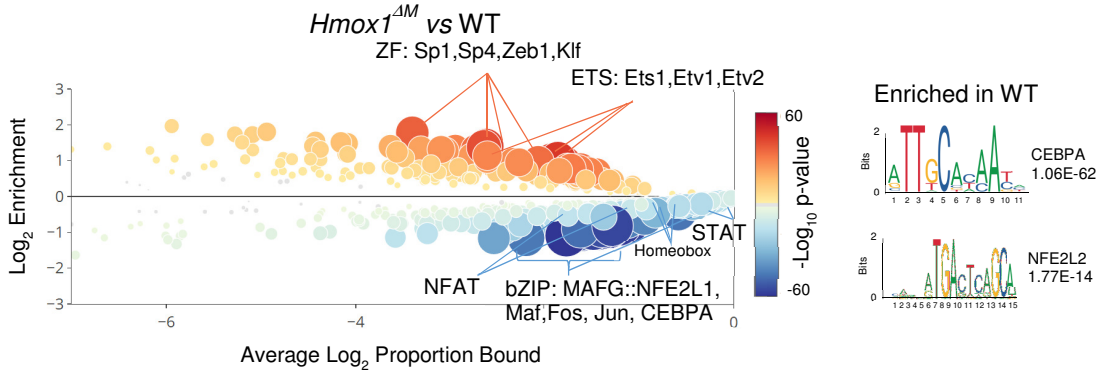


Figure 5. HO-1 drives the epigenomic program of TAMs

A) MA plot showing Log2 Average read density of differentially open regions in WT (red) and *Hmox1*$^{\Delta M}$ (blue) CD11b^{hi}Ly6G^{-}Ly6C^{lo}CD64^{+}MHC II^{+} TAMs. Histograms indicate the number of opening (red) or closing (blue) regions in WT compared to *Hmox1*$^{\Delta M}$ cells at promoters (Pro) and enhancers (Enh). B) Cumulative distribution plot generated by BETA algorithm showing the predicted activating/repressive functions of differentially open regions in CD11b^{hi}Ly6G^{-} Ly6C^{low}CD64^{+}MHC II^{+} TAMs with the indicated P-values determined by the Kolmogorov–Smirnov test. C) Representative ATAC-seq tracks showing enhancers highlighted in purple at the loci of Cd274, Pdcd1lg2, Mertk and Mmp2. Position of each loci in the genome is indicated at the top of each track. D) Gene sets enrichment network displays clusters of redundant pathways overrepresented in WT (red) and *Hmox1*$^{\Delta M}$ (blue) TAMs, respectively. Nodes represent gene sets and edges represent mutual overlap. Overlap significance is indicated by edge's thickness. NodesColor denseness indicates normalized enrichment score (NES). E) CiiiDER analysis for putative transcription factors motifs from differentially open regions of TAMs *Hmox1*$^{\Delta M}$ and WT. Transcription factors are colored according to their gene coverage P-value and whether they are over- (red) or under- (blue) represented. The size of each point is also proportional to log10 Pvalue. Consensus sequence of CEBPA and NFE2L2 transcription factors are shown with their respective P-value.

d. Discussion

Tissues macrophages are highly heterogeneous and plastic, and acquire specific functions in respond to their environmental cues. In the TME, they integrate multiple signals that reshape their enhancer landscape and as a consequence their transcriptional and functional programs[16]. Here we show that HO-1 is induced in monocytic cells that infiltrate the tumor bed upon differentiation into TAMs. Several signals could contribute to this observation. For example, tissue hypoxia and accumulation of lactic acid, key metabolic features of the TME[50,51], are known to induce HO-1 through the activation of HIF1α[52]. Intratumor hemorrhage, commonly encountered in cancer might lead to extravasation of hemoglobin[53], the physiological inducer of HO-1. In addition, cytokines such as IL-6, produced by cancer-associated fibroblasts[54], endothelial cells[55] or TAMs themselves[40] was recently shown to be a potent inducer of HO-1 as part of a "wound healing" signature[35].

We demonstrate that myeloid-restricted HO-1 ablation strongly improves the response towards therapeutic immunization by enhancing antitumoral $CD8^+$ T-cell proliferation and cytotoxicity. In line with this observation, enzymatic inhibition of HO-1 by metalloporphyrins was previously shown to promote tumor regression or complement conventional cancer therapies and facilitate cytotoxic antitumor immune response[31–34,56]. Several lines of evidence in different pathological conditions suggested that HO-1 induction in macrophages plays a critical role in controlling the adaptive immune response by influencing their polarization[57–63]. Our data indicate that HO-1 ablation has a major impact on the transcriptional and epigenetic profiles of TAMs. We suggest that this could be independent of its enzymatic activity as multiple pieces of evidence support a role for HO-1 as transcriptional modulator. After exposure to hypoxia, HO-1 translocates to the nucleus in a cleaved and enzymatically inactive form where it directly interacts with transcription factors such Nrf2 or JunD and modulates their activity[37–39]. We propose that similar processes could be at play in TAMs and account for their acquisition of an immunosuppressive program. Further understanding the underlying mechanisms will be important to develop adequate pharmacological approaches.

Collectively, our data indicate that HO-1 in tumor-infiltrating monocytic cells represents a molecular switch that promotes their immunosuppressive functions. It could therefore represent a

valuable target to reprogram the TME and potentially synergize with the current therapeutic approaches focused on the T cell compartment.

e. Material and Methods

Mice.

Hmox1$^{\Delta M}$ mice were generated at the Institue For Medical Immunology by crossing *Hmox1*$^{fl/fl}$ mice (in which *Hmox1* allele was flanked by *loxP* sites) with *LysMCre*$^{+/wt}$ mice, both of which had been backcrossed onto the C57BL/6 background for more than 10 generations (Instituto Gulbenkian de Ciência, Oeiras, Portugal). The *LysMCre* transgene causes a specific deletion of *Hmox1* gene in macrophages. *Hmox1*$^{fl/fl}$ mice (*Hmox1*$^{fl/fl}$ *LysMCre*$^{wt/wt}$) were used as controls for *Hmox1*$^{\Delta M}$ mice and were littermates. MHC class I-restricted OVA-specific TCR-transgenic OT-1 mice with a Rag1-/- background were obtained from the Jackson Lab. All experimental and control mice were eight- to twelve-week-old animals and were of the same sex for each experiment. All experiments were conducted in agreement with and approved by the local committee for Animal Welfare (Comission d'éthique du Biopole ULB Charleroi, Université Libre de Bruxelles).

Tumor cell line.

The EL-4 lymphoma cell line was obtained from American Type Culture Collection (ATCC, TIB39). The EG7-OVA tumor cell line is derived from the tumor cell line EL4 (ATCC CRL-2113) by transfection with a plasmid carrying the chicken ovalbumin (OVA) and neomycin phosphotransferase - G418 resistance - genes. The cells were maintained at 37°C and 5% CO2 in RPMI 1640 medium (Lonza) supplemented with 10% fetal calf serum (FCS), 0,1 mM nonessential amino acids, 100 U/ml of penicillin and 100 U/ml of streptomycin (all reagents from Lonza, which is hereunder referred as complete medium). The EG7-OVA tumor cells were cultured in a complete medium supplemented with 1 mg/ml of G418 sulfate (Geneticin Selective Antibiotic, ThermoFisher) once a week. OVA peptide expression on MHCI molecules of EG7OVA tumor cells was regularly verified by flow cytometry.

Tumor inoculation and therapeutic immunizations.

Tumors were initiated by intradermally injection of $2,5\times10^5$ tumor cells (in 100 µl of sterile PBS) per mouse into the right flank at day 0. When indicated, at day 7, 10 or 14 after EG7-OVA tumor inoculation, mice were injected subcutaneously with 10 or 50 µg of ovalbumin protein (grade VI, A2512, Sigma-Aldrich) associated with 50 µg of poly(I:C) (Poly(I:C) (HMW) VacciGrade, InvivoGen) in 100 µl of sterile PBS per mouse on the right flank. Prior to the immunization, mice were anesthetized intraperitoneally with weight-adjusted amounts of ketamine (1%, 100 µg/g, Nimatek)-xylazine (2%, 10 µg/g, Bayer) solution. In other experiments, the mice were injected intraperitoneally with 0,5 mg of depleting monoclonal antibodies in 200 µl of sterile PBS per mouse for $CD8\alpha^+$ T cell depletion (YTS169, University of Cambridge), 1 day before tumor inoculation and then once a week. The control mice were injected with IgG2b isotype control antibodies. Peripheral blood samples were collected once a week after depletion and analyzed by flow cytometry to confirm the depletion.

Tumor monitoring.

Mice were monitored every other day for tumor growth by using fine calipers. Tumor volume

(mm^3) is described as $(AxB^2)/2$, where A and B represent tumor length and width, respectively. Mice were sacrificed for tumor analysis when specified or when the total volume of the tumor reached 3000 mm^3.

Tissue digestion protocol.

Tumors were dissected, finely chopped and perfused with a digestion solution containing DNAse

I 1 mg/ml (Grade II, Sigma-Aldrich, 10104159001) 20 µl and collagenase I and II 2,5 mg/ml (Liberase TL Research Grade, Roche) 20 µl in 5 ml of RPMI 1640 (Lonza) each and were incubated 30 minutes at 37°C. Five ml of RPMI FCS 5% with ethylenediaminetetraacetic acid 2 mM (EDTA, Sigma) was added to each sample and tumor pieces were mashed and filtered, twice. Cell suspensions from spleens were obtained by homogenizing individual spleens to release

splenocytes in 5 ml of RPMI 1640 with 10% FCS each. The red blood cells were lysed briefly in 800 µl of ACK lysis buffer. Lymph node cell suspensions were prepared by dissecting (inguinal, deep and superficial axillary lymph nodes on the right flank) and grinding the tissue in RPMI 1640 with 10% FCS. To obtain bone marrow cell suspensions, femur and tibia from the mice were dissected, rinsed in ethanol, transferred to RPMI 1640 with 10% FCS. Bone ends were cut with sterile sharp scissors and the contents of the bone marrows were flushed with the medium. Bone marrow cells were diluted by vigorous pipetting. All the cell suspensions from individual organs were filtered through a 40 µm cell strainer, centrifuged and resuspended in RPMI 1640 with 10% FCS.

OT-1 T-cell isolation and adoptive transfer.

Cell suspensions from the lymph nodes of the MHC class I-restricted OVA-specific TCRtransgenic OT-1 mice (OT-1 cells) (8 weeks to 4 months old) were harvested (see tissue digestion protocol) and adoptively transferred by tail vein injection (2×10^6 cells per mouse) on specified day after tumor inoculation. When specified, OT-1 cells were labeled before intravenous injection with 2 µM carboxyfluorescein diacetate succinimidyl ester (CFSE) (CellTrace CFSE Cell Proliferation Kit, Invitrogen) by incubating them for 20 minutes at 37°C according to manufacturer's instructions. This was followed by an immunization with OVA protein (50 µg) and poly(I:C) (50 µg) one hour later. Cell division accompagnied by CFSE dilution was analyzed by flow cytometry two days later (detected in the FITC channel).

Flow cytometry.

Cell suspensions were washed, resuspended and incubated (for 30 minutes at 37°C in the dark) in 50 µl of PBS with 10% FCS and containing an antibody mix with a Fc-blocking reagent (rat antimouse CD16/CD32, BD, clone 2.4G2, dilution 1/200). EDTA 2 mM was added to the tumor cell suspensions. The cell surface staining was performed using monoclonal antibodies against the following molecules (clone, company) : BV510-conjugated rat anti-mouse CD90.2 (53-2.1, BD), af700-conjugated rat anti-mouse CD4 (RM 4-5, eBioscience), pacific blue-conjugated rat

antimouse CD8α (53-6.7, BD), FITC-conjugated hamster anti-mouse TCRβ (H57-597, BD), PEconjugated rat anti-mouse CD163 (TNKUPJ, eBioscience), APC-conjugated rat anti-mouse CD206 (CO68C2, BioLegend), pacific blue-conjugated rat anti-mouse F4/80 (BM8, eBioscience), bv650-conjugated mouse anti-mouse CD64 (X54-5/7.1, BD), AF647-conjugated mouse antimouse CD64 (X54-5/7.1, BD), BV711-conjugated rat anti-mouse CD11b (M1/70, BD), AF700conjugated rat anti-mouse CD11b (M1/70, BD), BV605-conjugated rat anti-mouse Ly6G (1A8, BioLegend), PE-conjugated rat anti-mouse Ly6G (1A8, BD), PerCP/Cy5.5-conjugated rat antimouse Ly6C (HK1.4, BioLegend), AF700-conjugated MHC II (I-A/I-E) (M5/114.15.2, eBioscience), AF700-conjugated rat anti-mouse IFNɤ (XMG1.2, BD), APC-conjugated rat antimouse Granzyme B (NGZB, eBioscience), APC-conjugated mouse anti-mouse Tbet (4B10, BioLegend), PE-conjugated anti-mouse Eomes (Dan11mag, eBioscience), BV605-conjugated rat anti-mouse Ki67 (16A8, BioLegend), APC-conjugated mouse anti-mouse OVA 257-264 (SIINFEKL) peptide bound to H-2Kb (25-D1.16, eBioscience). Cells were stained to exclude dead cells (LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit, 1/1000 APC-Cy7 ThermoFisher). Intracytoplasmic staining was performed using the Intracellular Fixation/Permeabilization Buffer Set (eBioscience) according to manufacturer's instructions. HO-1 intracytoplasmic staining was performed through primary unconjugated mouse anti-mouse HO-1 antibody (ab13248, Abcam) (ab172730, Abcam, for isotype control) and then a secondary FITC-conjugated rat anti-mouse IgG1 (A85-1, BD). When indicated, cells were harvested after density gradient preparation (Lymphoprep) and stimulated *ex vivo* overnight at 37°C with OVA 257-264 SIINFEKL peptide, which consists of 17 15-mer peptides selected for H2-Kb epitope content (1 µg/ml, Polypeptides Laboratories, Strasbourg, France), in complete medium, in the presence of recombinant human IL-2 (10 ng/ml, R&D) and brefeldine A (5 µg/ml, BD Biosciences) added 2 hours later. IFNɤ production was then assessed by $CD8^+$ T-cell intracytoplasmic staining. To assess OVA-specific $CD8^+$ T-cell response, PE-labelled MHC class I SIINFEKL specific pentamers (ProImmune) were used. Granzyme B intracytoplasmic staining was used in $pentamer^+$ $CD8^+$ T cells. Samples were acquired on a BD LSRFortessa flow cytometer. Analyses were performed using FlowJo software (Flowjo LLC).

Immunofluorescence.

For imaging, mice were sacrificed 21 days after tumor inoculation. Tumors were dissected, placed in a solution of OCT (Optimum Cutting Temperature compound) embedding media (Tissue-Tek O.C.T. Compound, Sakura Finetek). OCT-embedded samples were frozen and sectioned on the cryostat microtome (5 µm thickness) and then fixed in methanol. Slides were incubated overnight with a primary antibody mix (FITC-conjugated rat anti-mouse CD11b, clone M1/70, dilution of 1/100, BD; FITC-conjugated rat anti-mouse F4/80, clone BM8, dilution of 1/200, eBioscience; Texas Red-conjugated rabbit anti-mouse HO-1, clone ADI-SPA-895, dilution of 1/100, Enzo) in the dark at 4°C, then washed and incubated for 3h with a secondary antibody mix (FITCconjugated donkey anti-rat IgG, clone A21208, dilution of 1/200, Invitrogen; Texas-r-Redconjugated goat anti-rabbit IgG, clone A11006, dilution of 1/150, Invitrogen). The mix was composed of PBS with 0,1% of Tween 20 (Sigma-Aldrich) and 5% of bovine serum albumin. Nuclear staining was visualized with Diamidino-2-Phenylindole (DAPI) (dilution of 1/10000). Isotype controls (1/100) were used instead of primary antibodies to assess the nonspecific background. Images were captured with a Zeiss Axio Observer Z.1 microscope and analyzed with Zen Pro and ImageJ softwares.

TAM cell sorting.

$CD11b^+$ cells were first purified from tumor cell suspensions using positive magnetic selection with a cell isolation kit (Miltenyi). $Live/dead^-$ $CD11b^+$ $Ly6G^-$ $Ly6C^-$ MHC II^+ $CD64^+$ TAMs were sorted using a BD FACS Aria III cell sorter (100 000 cells from $Hmox1^{\Delta M}$ mice and from wild type mice, in triplicates) after surface staining with monoclonal antibody mix (see the section "flow cytometry").

RNA-sequencing

TAMs were isolated by FACS in RLT buffer and flash frozen. RNA extraction was performed using RNeasy kit (Qiagen) and sample quality was tested on a 2100 Bioanalyzer (Agilent). Libraries were prepared using Ovation® SoLo RNA-Seq system (NuGEN Technologies) and underwent paired-end sequencing (25×10^6 paired-end reads/sample, Novaseq 6000 platform)

performed by BRIGHTcore ULB-VUB, Belgium (http://www.brightcore.be). Adapters were removed with Trimmomatic-0.36 (with the following parameters: Truseq3-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36 HEADCROP:4) Reads were then mapped to the reference genome mm10 by using STAR_2.5.3 software with default parameters. We then sorted the reads from the alignment according to chromosome positions and indexed the resulting BAM-files. Read counts in the alignment BAM-files that overlap with the gene features were obtained using HTSeq-0.9.1 with "--nonunique all" option (if the read pair aligns to more than one location in the reference genome, it is counted in all features to which it was assigned and scored multiple times). Genes with no raw read count greater or equal to 20 in at least 1 sample were filtered out with an R script, and raw read counts were normalized and a differential expression analysis was performed with DESeq2 by applying an adjusted p-value <0.05 and an absolute log2-ratio larger than 1.

ATAC-seq

Assay for transposase accessible chromatin (ATAC) followed by sequencing was performed as following: 20 000 sorted TAMs were collected in 1mL of PBS + 3% FBS at 4°C. Cells were centrifuged, then cell pellets were resuspended in 50 µL of lysis buffer (Tris HCl 10 mM, NaCl 10 mM, MgCl2 3 mM, Igepal 0,1%) and centrifuged (500 g) for 25 minutes at 4°C. Supernatant was discarded and nuclei were resuspended in 50 µL of reaction buffer (Tn5 transposase 2,5 µL, TD buffer 22,5 µL and 25 µL H_2O – Nextera DNA sample preparation kit, Illumina). The reaction was performed for 30 minutes at 37°C. DNA was purified using the MinElute purification kit (QIAGEN). Purified DNA was amplified and indexed by PCR using NEBNext High-Fidelity 2× PCR Master Mix (New England Biolabs) with 10-12 cycles. Amplified libraries were purified using MinElute PCR Purification Kit (Qiagen), followed by a double AMPURE XP purification (0,5:1 and 1.2:1 ratios) and quality controlled using a Bioanalyzer High-Sensitivity DNA Analysis kit (Agilent). Paired-end sequencing was performed on NovaSeq platforms (Illumina). Adapters in obtained reads were removed with Trimmomatic 0.36 with the following parameters : Nextera1.fa:1:25:6 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36. Pairedend reads were mapped to mouse genome mm10 with Bowtie2[64,65] using the following

parameters for paired-end reads –X 2000 –fr –very-sensitive –no-discordant –no-mixed –non-deterministic. Reads from the alignment were sorted and indexed according to chromosomes. Reads located within the blacklist of the ENCODE project 46 were then removed. Duplicate reads were removed with MarkDuplicates tools (Picard suite). Peaks were called with MACS2[66] using the following parameters: -f BAMPE -g mm -q 0.05 --nomodel --call-summits -B –SPMR. Regions obtained by MACS2 were subjected to differential analysis using DESeq2 provided by SeqMonk 1.43.0 (Mapped Sequence Analysis Tool, Babraham Bioinformatics, http://www.bioinformatics.babraham.ac.uk/projects/seqmonk/). First, we created an atlas containing all obtained peaks for all the populations using bedtools[67] with a minimum overlapping of 1 bp. We used DESeq2[68] with a p-adjusted cutoff of 0.05. Resulted peaks were separated into two categories: peaks located in promoters (located within 2kb around TSS) and peaks located in enhancers (not located in the defined promoters' regions). For downstream visualisation, a scaling factor was calculated using deepTools package[69] to normalise peak intensity to FRiP (fraction of reads in peaks) and generate bigwig files. For Gene ontology analysis, we introduced BED files from differential ATAC-seq peaks to GREAT tool with default parameters[46]. For motif analysis, Ciiider algorithm was used to perform motif enrichment in the differentially accessible regions. We used BETA package with default parameters to integrate ATAC-seq (differentially accessible regions) and RNA-seq (transcriptome) data and evaluate the regulatory potential of chromatin accessibility to promote/repress genes expression.

Statistical analysis

All data points were included with median and interquartile range. A two-tailed non parametric Mann-Whitney U test was used to compare 2 data sets and a Wilcoxon matched-pairs signed rank test when the different tumors from the same animal were compared. Differences were considered statistically significant as followed : p-values less than 0,05 were flagged with *, less than 0,01 with **, less than 0,001 with *** and less than 0,0001 with ****. NS means not statistically significant. All graphs and statistical analysis were performed using GraphPad Prism 6 software.

Data Availability

RNA-Seq and ATAC-Seq data that support the findings reported in this study have been deposited in the GEO Repository (in process).

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Author Contributions

EA conducted most of the experiments. BV, AD, AA and AC contributed to some experiments. AA, MS and FL performed bioinformatics analysis. EA and BV analyzed the data and prepared the figures. MS and LB provided critical reagents. AL and SG supervised the work and wrote the manuscript. All authors were involved in critically revising the manuscript for important intellectual content. All authors had full access to the data and approved the manuscript before it was submitted by the corresponding author.

Competing Interests

The authors declare no competing interests.

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DISCUSSION AND PERSPECTIVES

Although their functions and underlying mechanisms are incompletely understood, macrophages are being rediscovered as major immunological regulators of cancer disease. Notably, as detailed above, they are key players in the innate and adaptive immune response against tumor cells. We hypothesized that the anti-tumor immune response might be modulated by myeloid HO-1. Consistent with our hypothesis, **our data suggest a major role of myeloid HO-1 in the control of antitumor immunity and tumor growth by modulating the differentiation and the phenotypic, functional, transcriptional and epigenetic program of TAMs**.

Myeloid HO-1 and tumor progression

Myeloid HO-1 has already been suggested to be associated with tumor progression. In mouse, myeloid-specific deletion of HO-1 suppresses tumor growth and progression in prostate intraepithelial neoplasia but not in PC3 xenograft model (260). The dynamic infiltration of the myeloid cells into the tumor is different in these tumor models and might explain the different effects observed. In vitro co-culture of prostate cancer cell line with HO-1-expressing macrophages suppress tumor growth, while macrophages lacking HO-1 fail to alter tumor growth (260). This could be explained by the fact that the antitumor effect exerted by HO-1-deficient macrophages in vivo could be mediated by TILs. Another study showed, in rat models of prostate cancer, that HO-1^+ macrophages were increased mainly at the tumor border, in the surrounding tumor-bearing prostate lobe, where it was related to tumor size and aggressiveness, and in metastatic tumors. In human prostate cancer samples, HO-1^+ macrophages correlated with high-grade and metastatic tumors (261). Myeloid HO-1 has been shown to promote tumor colonization at metastatic sites in mouse (262). TAMs co-expressing HO-1 and fibroblast activation protein-α have been reported to display a similar phenotype to that observed during an innate wound healing response. This involves genes such as IL-1β and IL-6 that are conserved in human wound transcriptome data sets, expressed in multiple human cancers, and associated with poor prognosis. Furthermore, these HO-1^+ TAMs could promote tumor cell transendothelial migration and metastatic dissemination (114). Lastly, HO-1 in TAMs has been associated with breast cancer

tumor growth and its inhibition suppresses cancer growth in vitro and in vivo (263). In human, an old report supported a correlation between HO-1-expressing macrophages and angiogenesis in human gliomas (264). HO-1 was also specifically expressed in tumor-infiltrating macrophages of melanomas and were correlated with depth of tumors (265). HO-1-expressing macrophages were also associated with lymph node metastasis and shorter disease-free survival in patients with advanced colorectal cancer (266). On the other hand, M2 macrophages expressing HO-1 have been reported as protective cells associated with benign tissue and which could prevent malignant transformation (267).

HO-1 is often upregulated in cancer and is associated with disease progression and poor prognosis in many tumors in mouse and human. This led consequently to the attempt for anticancer strategies to target HO-1 not only to stimulate the innate and adaptive antitumor immune response, but also to block the other cytoprotective and antioxidant properties of this molecule. **HO-1 inhibitors** can be categorized into two classes of inhibitors. The metalloporphyrins and the imidazole-dioxolane compounds. Metalloporphyrins (such as ZnPPIX or SnPPIX) are non-selective competitive inhibitors of the enzyme activity, based on their structural similarity to heme (therefore, they can paradoxically also induce HO-1 transcription and expression, although to varying degrees. For example, CoPPIX is a potent inducer of HO-1 activity in vivo). These molecules generally display antitumor effects in vitro and in vivo (water-soluble pegylated ZnPP and encapsulated ZnPP in micelles of amphiphilic copoly (styrene-maleic-acid)) with no apparent side effects and leaving normal cells untouched (268). Some of them accumulate preferentially in solid-tumor tissue. The imidazole-dioxolane compounds, such as azalanstat, which are highly specific for HO-1 inhibition but have been mainly tested in vitro up to now (269), have shown in vivo first enthusiastic results in hormone-refractory prostate cancer (270).

HO-1 inhibition

Although some studies reported contradictory effects of HO-1 inhibition in cancer growth and cancer cell migration (271) (272) (273), the administration of **HO-1 inhibitors in vivo has shown a control of tumor growth** in many experiments. For example, HO-1 inhibitor ZnPP inhibits tumor angiogenesis and growth in a xenograft model of human colorectal carcinoma (274). A

water-soluble derivate of ZnPP, PEG-ZnPP, which has a longer circulating time in blood, accumulates in solid tumor tissue and suppresses mouse sarcoma growth and was associated with tumor cell apoptosis in vivo, and oxidative stress of tumor cells in vitro. There were no side effects assessed by blood tests, neither on liver and spleen histological examination (275). In an orthotopic model of pancreatic ductal adenocarcinoma, the HO-1 inhibitor SnPP decreases metastasis and improves the efficacy of gemcitabine chemotherapy. This was explained by enhanced tumor cell apoptosis (276). In addition, HO-1 is upregulated in tumor cells and is involved in cellular resistance to cancer therapy in human tumor cell lines. The inhibition of HO-1 in vivo has been reported in several models to sensitize the cancer to radiotherapy and chemotherapy with decreased tumor growth, lymph node involvement and metastasis (270) (170). The byproducts of HO-1 seem to be involved in HO-1-mediated resistance to cancer therapy, and the specific chelation of iron revealed also an increased susceptibility to cancer therapy in vivo (277). In a Lewis lung carcinoma model, TAMs that express fibroblast activation protein-α are the major tumoral source of HO-1. Their depletion, or the administration of an HO-1 inhibitor, showed an arrest of tumor growth that was suggested to be, at least partially, mediated by the immune response (278), although the underlying mechanisms of immunomodulation by HO-1 were not further investigated.

HO-1 inhibitors have been assessed in randomized trials to prevent bilirubin-mediated neurological dysfunction in preterm newborns suffering from neonatal hyperbilirubinemia. It ameliorated significantly the bilirubin concentration in a dose-dependent manner, without significant side effects (279).

Myeloid-targeting strategies for cancer therapy

Macrophage-targeting strategies have been studied and yielded mitigated benefits. For example, targeting the receptor CSF-1R in pre-clinical tumor models shows encouraging results but leads to an enhanced recruitment of monocytes to the tumor site (280) (281) so that continuous CSF-1R inhibition would be needed to keep a therapeutic effect. Blockade of the CCL2/CCR2 axis in experimental cancer models suggests it is an attractive target, although disappointing results have been observed in clinical settings as monotherapy to date (282). However, macrophages are mainly targeted as a whole population, without taking functional macrophage properties into

consideration. In this line, targeting a specific M2-like subpopulation of TAMs has recently been reported to promote T cell-mediated tumor regression in mouse (283) (284).

Based on the current literature and on our data, **the modulation of TAM polarization by myeloid HO-1 inhibition** could represent a therapeutic approach to promote an efficient antitumor immunity. Specifically targeted delivery of drugs to TAMs could be achieved by nanoparticles that accumulate preferentially in these cells (285). Various molecules regulating TAM polarization start to emerge in preclinical models as a potential therapeutic target to reprogram TAMs into antitumor macrophages to activate the antitumor immune response (286). A challenge is still to prevent the induction of a systemic inflammation and strategies should be adopted therefore to selectively reprogram TAM or the TME (287). In this line, HO-1 might be an interesting target since HO-1 seems to be highly induced in TAMs.

My various mechanistic hypotheses

In our EG7-OVA tumor-bearing mouse model, myeloid-restricted HO-1 deficiency improves the effect of a therapeutic antitumor immunization by enhancing tumor-infiltrating antitumor $CD8^+$ T-cell proliferation and cytotoxicity and represses tumor growth. We observed similar results with antitumor $CD4^+$ T cells (data not shown). The impact of myeloid HO-1 on tumor growth is not observed without any immunization but appears when the mice are immunized with ovalbumin protein and poly(I:C) adjuvant. This could be explained by **different hypotheses,** which are not mutually exclusive (Figure H):

1) A "myeloid HO-1-mediated immune checkpoint" blockade

We have described hereinabove that TLR signaling pathways can be associated with HO-1-mediated mechanisms of negative regulation. TLR-mediated reprogramming of macrophages plays an important role in the regulation of the tumor-infiltrating innate and adaptive immune cells (288). Myeloid HO-1 deficiency could therefore act as a "myeloid HO-1-mediated immune checkpoint" blockade. This could potentiate the inflammatory response of TAMs and the subsequent restoration of the antitumor T-cell response. In line with this hypothesis, myeloid HO-

1 inhibition has recently been favorably compared to an immune checkpoint inhibitor by blocking tumor induced immunosuppression in an immune-stimulating chemotherapy model (289).

2) A shift of macrophage polarization

More generally, poly(I:C) could lead to expanded and mature myeloid cells (290), where myeloid HO-1 deficiency could promote the conversion from M2- to M1-like macrophages. This shift of macrophage polarization could be mediated by the activation of key transcription factors detailed further below. A shift of macrophage polarization is suggested by several features observed in our experiments:

- A **better antigen-presenting capacity** of ovalbumin peptide to antitumor T cells by macrophages. Indeed, we observed in a bilateral tumor growth model (EG7-OVA tumor on the right flank and EL4 tumor on the left flank of the mice) that the EL4 tumor growth was dramatically increased compared to the EG7-OVA tumor growth in the same mice which were deficient for myeloid HO-1. The inoculated tumor cells were the only difference in this experimental setting, the EL4 tumor cells lacking the OVA peptide on their tumor cell surface compared to the EG7-OVA tumor cells.

- iNOS and Arg-1 enzymes are competitive enzymes since they use the same extracellular substrate arginine, and are considered as typical M1- and M2-associated enzymes, respectively (137). In line with this M1/M2 macrophage polarization hypothesis, we observed an improved myeloid **iNOS/Arg-1 ratio** after immunization of the myeloid-restricted HO-1-deficient mice.

- Bone marrow-derived macrophages were cultured and stimulated with LPS and poly(I:C) in vitro. This led to an **increased proinflammatory profile** in the absence of myeloid HO-1, and an increased co-cultured T-cell proliferation rate (data not shown).

3) TAMs as poor antigen-presenting cells

Myeloid HO-1 has been suggested to play an important role in the phagocytic activity of macrophages, improving bacterial and apoptotic cell clearance (149). Although TAMs are phagocytic cells, they compete with efficient APCs since they are poor antigen-presenting cells and are unable to migrate into the draining lymph nodes to initiate an antitumor T-cell response (76). Myeloid HO-1 inhibition associated with antitumor immunization may therefore improve APC activity and subsequent antitumor T-cell response.

4) Macrophage-independent factors

It could be considered that myeloid HO-1 inhibition has only marginal effects on macrophages. The immunization could reveal a stronger antitumor effect through an amplification of the antitumor T-cell response mainly through macrophage-independent factors such as DC activation. The immunization could also amplify the myeloid cell infiltrate and its impact. However, the impact of HO-1 deficiency in TAMs seems rather broad in terms of cell reprogramming.

5) Myeloid HO-1-mediated control of Treg cell suppressive function

Another hypothesis could be that HO-1-expressing macrophages could act indirectly by promoting Treg cell activity. A similar hypothesis has been suggested in vitro with higher proportion of Treg cells but abolished suppressive activity when co-cultured with HO-1-deficient APCs (242). However, although we observed in vivo an increased proportion of tumor-infiltrating Treg cells (data not shown), we did not assess Treg function in our experiments.

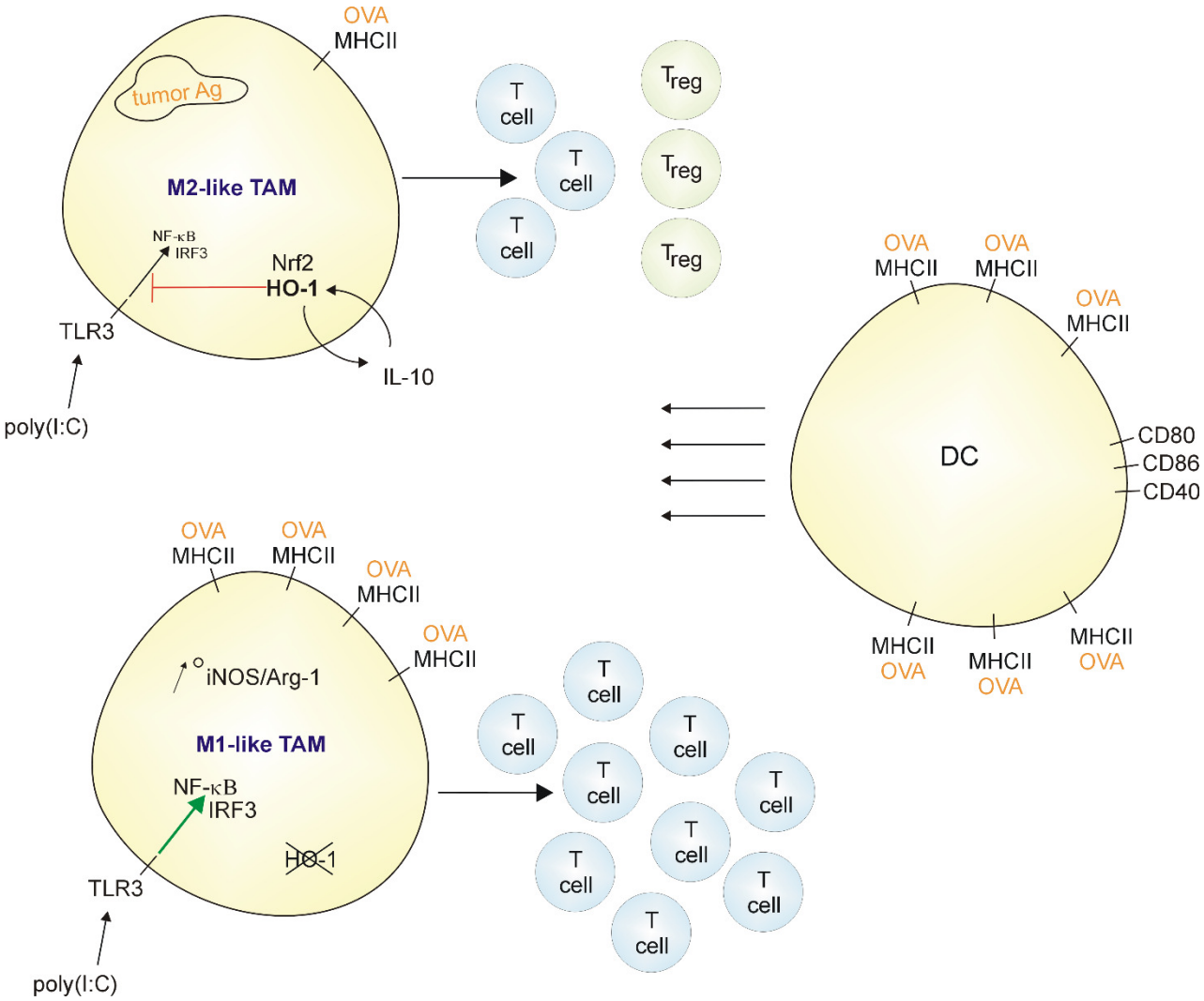

Figure H

Various mechanistic hypotheses for the enhancement of the antitumor T-cell response in the absence of HO-1 in TAMs

Myeloid HO-1 inhibition may therefore be considered for TLR-based cancer therapy. Although our experiments were mainly performed with poly(I:C) adjuvant, other settings should be assessed. For example, we observed an inhibition of tumor growth in the absence of myeloid HO-1 in a preliminary experiment with cyclophosphamide.

6) Endotoxin desensitization in the tumor microenvironment

Another important hypothesis could be the involvement of myeloid HO-1 in an endotoxin tolerance-like process in TAMs, resulting from persistent and aberrant endogenous TLR stimulations. This could contribute to tumor progression which could be reversed by myeloid HO-1 inhibition (Figure I):

- While TLRs mediate immune surveillance, several reports suggest that **TLR expression in the TME correlates with immune dysfunction** (291) (292) (293). Although they are not clearly defined, the TME releases endogenous TLR ligands that display a dual nature by being involved in cancer-associated inflammation (294), tissue repair (295), immunosuppression (296), tumor progression (297) and metastasis (298). Diverse molecules, designated as alarmins or DAMPs, may act as endogenous TLR ligands (299), such as HMGB1 protein for example, a DNA-binding nuclear protein released from damaged cells (300). As an example, LPS has been shown to be inefficient to trigger IL-12 production by TAMs compared to peritoneal elicited macrophages from naïve and tumor-bearing mice. This was associated with an alteration in the molecular mechanisms leading to NF-κB activation (301). In human tumor biopsies, most macrophages show a gene signature of LPS unresponsiveness which correlates with death from many different cancer types (302).

  We previously suggested that **HO-1 plays a crucial role in the immunosuppressive function of myeloid cells previously treated with in vivo repetitive LPS injections** (257). HO-1 has also been shown to downregulate the TLR4-triggered Myd88- and TRIF-dependent signaling pathways (resulting in the inhibition of NF-κB and IRF3) and to upregulate the implicated negative regulators in a model of liver ischemia/reperfusion injury (196).

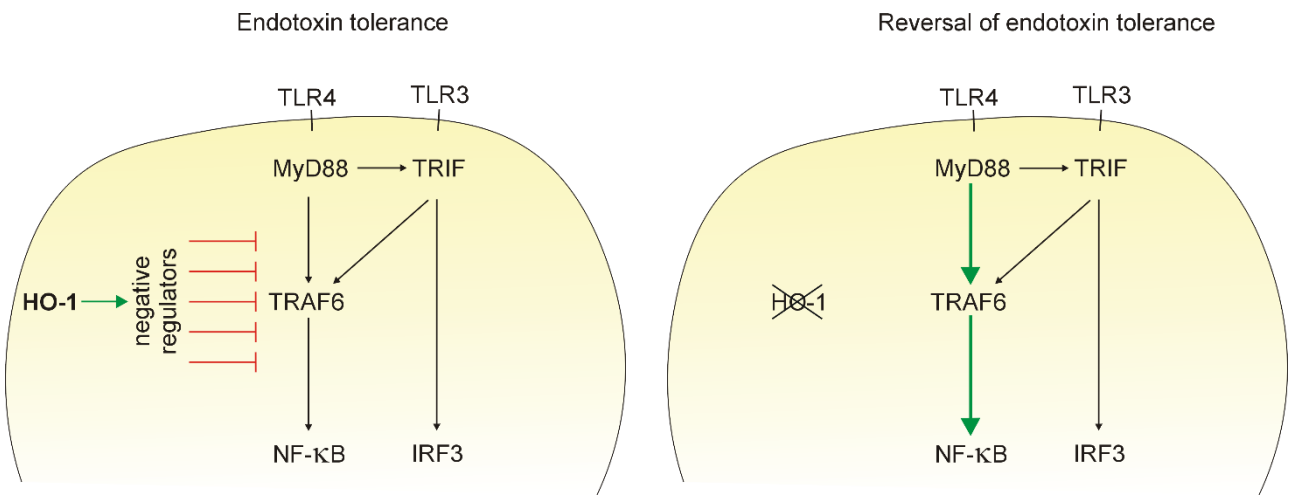

Figure I

Endotoxin tolerance-like process mediated by HO-1 in TAMs

- Many diseases display dynamic changes in macrophage state, with activated M1 macrophages involved in initiating and sustaining inflammation while M2 macrophages are involved in resolution or smoldering chronic inflammation. The tumor stroma shares similar properties with wound healing except that the response in the TME is a persistent process that never heals completely (303). TAMs did not show in our results a preferential enrichment for M2-associated genes, suggesting that TAM transcriptional profile is much more complex than the binary M1/M2 signature. It may be possible that a **gradual dynamic conversion of myeloid cell states under numerous spatio-temporal signals from the TME** plays a role in tumor progression (Figure J). Although the signaling pathways leading to endotoxin tolerance and M2 macrophage polarization have been suggested to be different (304), their gene response are similar, including reduced pro-inflammatory gene expression such as NF-κB cascade and enhanced phagocytic and wound healing activities (305). NF-κB could determine the balance between the protumoral and antitumoral activity of macrophages by acting as a "rheostat" that regulates the inflammatory response of macrophages (306) (307), endowed with a genetic program of negative feedback loop essential for resolution of inflammation (308). Inflammatory triggers of NF-κB activation, such as

the stimulation of IL-1 receptor or TLR for example, have been suggested to be important to maintain the immunosuppressive phenotype of TAMs (309), while NF-κB has been reported to play opposite roles in the inflammatory response depending on its stage (310).

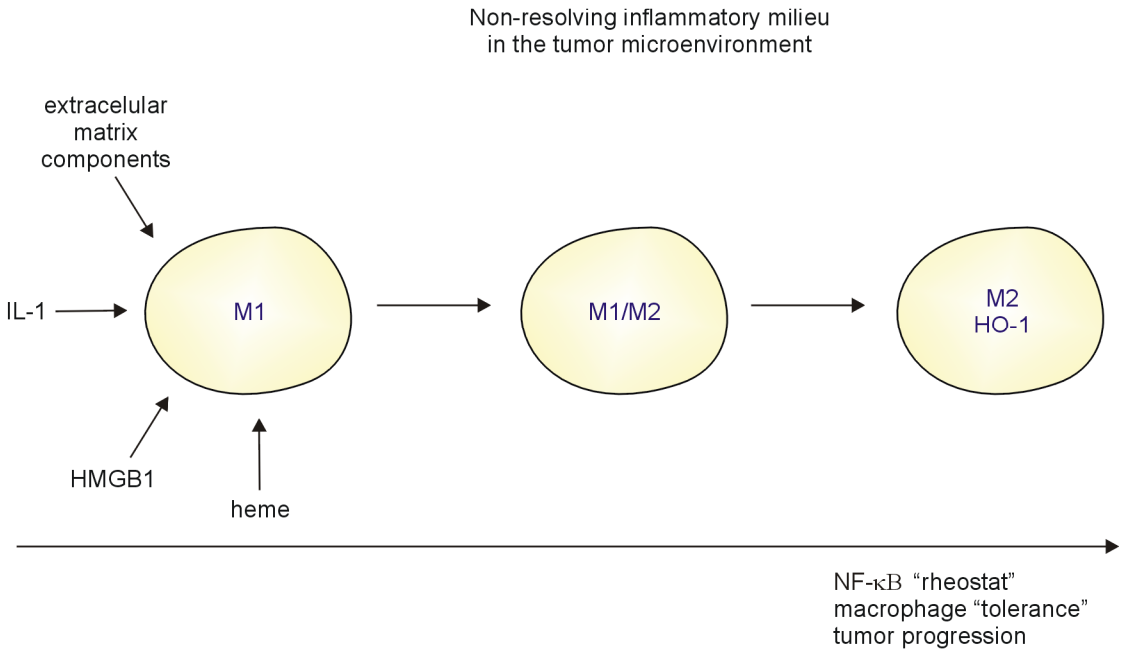

Figure J

Gradual dynamic conversion of myeloid cell states under persistent and aberrant signals from the TME

TLR agonists for cancer therapy are now used in preclinical and clinical trials. There is a rationale for targeting TLR with activating ligands for cancer immunotherapy, since TLR agonists are potent stimulators of the innate immunity and inducers of a long-lasting adaptive immunity. However, clinical applications using TLR agonists as monotherapy showed limited results to date. Further experiments should be performed to better understand the mechanisms of TLR dysfunctions in tumor-infiltrating myeloid cells. In addition, other experiments should be performed with more physiologically relevant tumor models. Indeed, the implanted EG7-OVA tumor model that we used does not undergo the physiopathological steps of tumor development in interaction with the tumor-infiltrating myeloid cells of the TME.

7) HO-1 degradation byproducts

The various effects of HO-1 degradation byproducts could also explain the beneficial impact of myeloid HO-1 inhibition on tumor progression. HO-1 enzymatic expression in the TME could directly impair the inflammatory state of macrophages and subsequent T-cell proliferation, as detailed in the introduction above. HO-1 byproducts could also impact other tumor-infiltrating immune cells. For example, CO could prevent DC immunogenicity, as it has already been observed in a mouse model of diabetes for example (238).

8) A role for myeloid HO-1 in TAM differentiation

Our RNAseq results show a major genomic deletion in TAMs from the myeloid HO-1 KO condition, suggesting that myeloid HO-1 induction in the TME plays an important role in the differentiation of TAMs, which are mainly immunosuppressive and protumoral macrophages. Our ATACseq results further support a strong epigenetic involvement of HO-1 in TAM transcriptional activity. In our flow cytometry data, monocyte-derived TAMs highly express HO-1 compared to other tumor-infiltrating myeloid cells and extratumoral sites, further supporting the important role of HO-1 in macrophage differentiation. Myeloid HO-1 has already been suggested to be a critical molecule for myeloid progenitor cell differentiation into a functional subset of macrophages (311). In addition, it has been suggested a role for Nrf2 in **myeloid cell survival** and function, which attenuates oxidative stress in a context of highly toxic ROS content (312). Remarkably, the transcriptome of M-CSF-mediated differentiated macrophages and M2-polarized macrophages seem to be close, in contrast to that of M1-polarized macrophages (313) (91). This could suggest that M-CSF-driven **differentiation of macrophages leads per se to the acquisition of M2 properties given its homeostatic growth factor properties, while M1 properties of macrophages could be induced upon specific inflammatory context** (Figure K). In line with this hypothesis, it has been shown for example that Kupffer cell differentiation plays a critical role in homeostasis in a model of liver ischemia reperfusion injury. This tissue-resident macrophage differentiation is dependent on HO-1 expression whose deletion results in a pro-inflammatory

monocytic phenotype (314). In addition, hypoxia in the TME has been suggested to convert immature myeloid cells into differentiated immune suppressive TAMs (111).

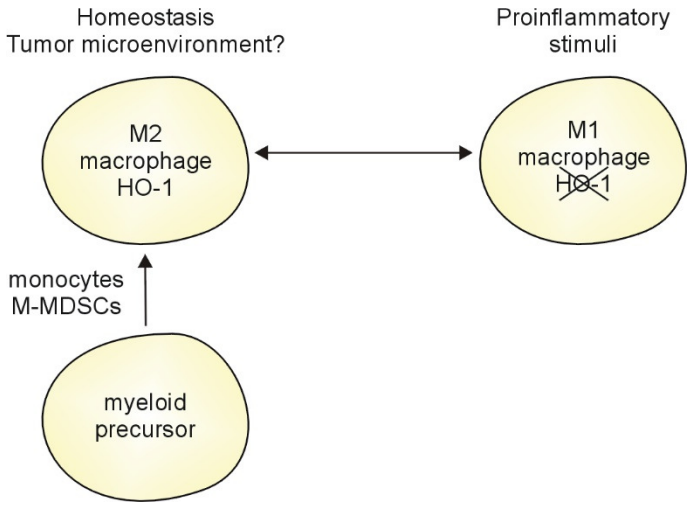

Figure K

M2 differentiation of macrophages per se

Activation state modulation by the inflammatory context

These last two hypotheses do not explain why myeloid HO-1 inhibition has no impact on tumor growth in the absence of immunization. We could speculate that a broad inhibition of macrophage differentiation, including M1 and M2-like macrophages, may lead to unclear final benefits on tumor progression. It would be interesting to study macrophage differentiation in the TME and its M1/M2 properties, with a view to exploiting the effector function of fully differentiated macrophages.

Potential HO-1 inducers in TAMs

The impact of myeloid HO-1 inhibition was highlighted by the observation that the antitumor T-cell response was not enhanced in the secondary lymphoid organs (data not shown) where myeloid HO-1 is not much expressed compared to the TME. **Various HO-1 inducers could be at play within the TME** (Figure L). This is the case for the transcription factor HIF-1α, which is induced

by hypoxia and lactic acid that are both major characteristics of solid tumors. In addition, these molecules have been shown to be involved in macrophage polarization (132). The upregulation of myeloid HIF-1α has also been suggested as one of the mechanisms suppressing the antitumor response (111). Another potent inducer of HO-1 is IL-10. Myeloid HO-1 has been identified as an induced downstream effector of IL-10 (200) which is a major tumor-derived cytokine. It involves the phosphorylation of p38 from MAPK pathway and STAT3 signaling pathway. Concurrently, IL-10 has been implicated in the regulation of macrophage phenotype (315). IL-6 in the TME has been also shown to be an important inducer of HO-1 in TAMs (114), probably by activating the Janus kinase 2/Signal Transducer and Activator of Transcription 3 (JAK2/STAT3) pathway, like IL-10 does. The importance of HO-1 in macrophages is underscored by their expression of specific receptors that uptake heme-containing substrates to deliver it to HO-1. Extracellular hemoglobin is scavenged by haptoglobin and captured by macrophages through the CD163 (high affinity). CD163 can also bind to free hemoglobin when haptoglobin is depleted (low affinity). This results in receptor-mediated endocytosis, clearance of hemoglobin, and induction of HO-1 expression (100). Like the CD163 for haptoglobin-hemoglobin complexes, hemopexin-heme complexes are delivered to HO-1 in macrophages after CD91 receptor-mediated endocytosis. HO-1 transcription is highly increased by heme uptake and is also induced by hemopexin-heme complexes (316). In cancer, neovascularization often occurs, promoting intratumor bleeding where hemoglobin is released. Tissue injury, associated with lysed erythrocytes and intracellular organelles (nucleus or mitochondria), can release other heme-containing proteins. These pathological processes are common manifestations in cancer patients. Hemoglobin from intratumor bleeding triggers an inflammatory response. However, it has recently been suggested that the removal of the proinflammatory hemoglobin by macrophages leads to their recruitment and to a M2-like macrophage phenotypical switch in the TME, associated with a localized anti-inflammatory response, tissue repair, tumor cell proliferation and tumor growth (11). Although the underlying mechanisms are not well understood, CD163 contribute to this process and is therefore considered as a typical M2 cell surface marker (100). Besides, extracellular labile heme has been proposed to trigger, through TLR-4 stimulation, an adaptive and protective response that attenuate the pathogenic outcome of diseases and promote tissue damage repair, although the underlying mechanisms are unclear (317). Moreover, perivascular macrophages catabolizing heme are

particularly able to release iron to tumor cells, reinforcing their tumor-promoting function through iron supply to demanding tumor cells (215).

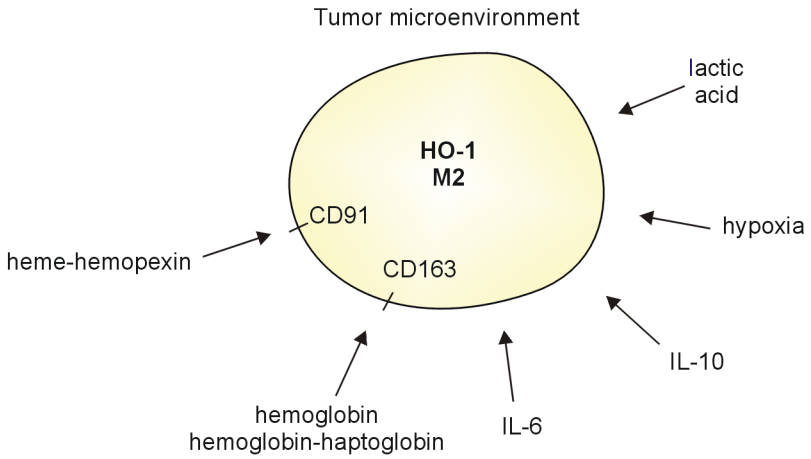

Figure L

Potential HO-1 inducers in TAMs

In our experiments, we observed that IL-10 and/or IL-6 inhibition in wild type tumor-bearing mice displayed similar tumor growth and HO-1 expression in TAMs compared to isotype control injection (data not shown). This could be explained by the numerous redundant inducers of HO-1, and suggests that myeloid HO-1 should be targeted and not its various inducers, as it is a central downstream regulatory molecule.

It is tempting to speculate that HO-1 drives M2-macrophage polarization through its numerous inducers within the TME. HO-1 is not a transcription factor and does not possess DNA binding motifs, a direct transcriptional activity is therefore unlikely. However, the broad effect of myeloid HO-1 could be mediated by transcription factors involved in macrophage polarization, as described above.

A myeloid transcriptional modulation by HO-1

- For example, nuclear HO-1 could amplify its own transcription by the nuclear stabilization of its transcription factor **Nrf2** (209) (Figure M). Furthermore, Nrf2 activation has been shown to negatively regulate oxidative stress, but also a subset of M1-associated genes in macrophages independently of the redox state. Indeed, Nrf2 activation disrupts the recruitment of RNA polymerase II to loci such as those of IL-1β and IL-6 (320) and has been suggested to be a key regulator for two cytoprotective pathways, the anti-inflammatory and the antioxidative ones.

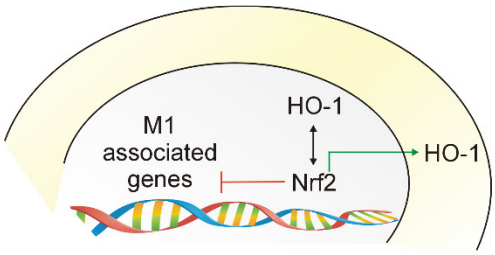

Figure M

Nrf2 stabilization by HO-1

- IRF transcription factors play key role in macrophage differentiation and polarization. HO-1 could bind to **IRF3 and/or IRF4** and modulate their nuclear translocation and activity, allowing macrophage reprogramming (Figure N). In line with this hypothesis, myeloid HO-1 has been reported to form a complex with IRF3 in models of Listeria monocytogenes infection and autoimmune encephalomyelitis (254). This HO-1-IRF3 complex was essential for IRF3 activation and subsequent gene expression in response to TLR3/TLR4 stimulation. A similar transcriptional factor complex could be at play with IRF4 and contribute to TAM polarization.

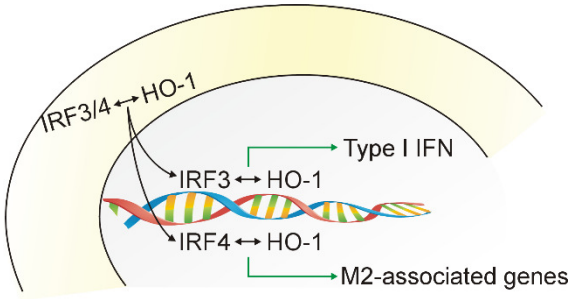

Figure N

HO-1 – IRF complex

- Another hypothesis to explain the impact of HO-1 on the reprogramming of macrophages is the involvement of **STAT3** (Figure O). STAT3 plays an important role in myeloid cell differentiation and M2 polarization and is associated with tumor progression (321). STAT3 is known to induce HO-1 transcription, IL-10 production, and IL-10-induced STAT3 activation (145). On the other hand, the activation of STAT3 by HO-1 has been shown to regulate innate immune response and confer cytoprotection in a mouse model of liver ischemia/reperfusion injury by reducing TLR4/NF-κB-mediated inflammation (322). To drive its broad effect on macrophage reprogramming, HO-1 could directly interact with STAT3 in the nucleus. Alternatively, HO-1 has been shown to interfere with STAT3 signaling by inducing its cytoplasmic retention (323), while STAT3 could increase the nuclear retention of NF-κB through formation of nuclear complexes (324). In addition, since STAT3 can interact with **STAT6**, they could synergize to promote M2-like TAM differentiation and activity. In parallel, STAT3 can antagonize IFN-induced STAT1 activity that promote M1 macrophage activity (321).

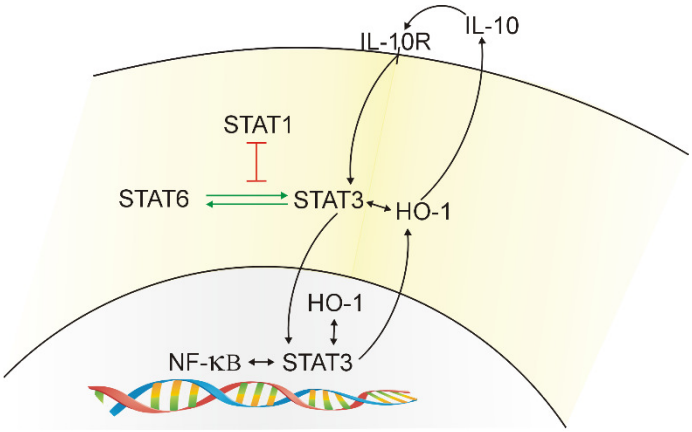

Figure O

HO-1 and STAT3 interactions

However, in our tumor-bearing mouse model, myeloid HO-1 deficiency alone was not sufficient to inhibit tumor growth. Moreover, in our in vitro experimental data, bone marrow-derived macrophages deficient for HO-1 did not reduce Arg-1 levels when stimulated with IL-4, IL-10, IL-6, lactate, dimethylfumarate or tumor-conditioned media (data not shown). Instead, as described above, myeloid HO-1 seems to act mainly when TLR stimulation is at play. It should be noted that in these in vitro experiments, bone marrow-derived macrophages deficient for HO-1 could have stayed in an immature state of myeloid cells, compatible for example with MDSCs, and were perhaps not representative of tumor-infiltrating macrophages. Other experiments aiming at understating the nuclear mechanisms of HO-1 and its precise effects in cancer are needed.

A potential synergy with cancer therapies

The TME plays a crucial role in cancer treatment outcome. Indeed, as described above, the TME not only provides a supportive framework but also an active protumoral and immunosuppressive activity. Moreover, these properties are often further reinforced after cancer treatments, for example through TAM recruitment. **Targeting myeloid compartment could synergize with immunotherapy or other conventional cancer therapies**. Since immunotherapy

is limited by the same constraints as the other effector immune cells in the immunosuppressive TME, an efficient antitumor immune response might require the complementary approach targeting both T-cell and myeloid-cell compartment (325). Several clinical trials are currently in progress in this optic (326). In addition, mirroring T-cell transfer, an adoptive transfer of ex-vivo modified HO-1-deficient macrophages could be an interesting strategy, as it has been reported to be protective in a model of liver ischemia/reperfusion injury with myeloid HO-1 induction (327).

PD-L1 expression on tumor biopsy does not seem to be an adequate biomarker to predict which patients would benefit from immune checkpoint blockade. New potential predictive biomarkers are emerging. They could involve, for example, the analysis of peripheral T-cell populations (and particularly T-cell receptor gene sequences or reactivity to neoantigens, although technically complex), TILs, mutational burden and intratumor neoantigen heterogeneity, immune gene signatures and assessment of tumor and immune cell phenotypes by multiplex immunohistochemistry (328). **HO-1 might be considered as a biomarker** of macrophage functional state and therefore a potential predictor of prognosis and response to cancer therapy.

CONCLUSIONS

Cancer-educated TAMs impede T cells from infiltrating the TME and from exerting their cytotoxic function within the TME. Our data suggest a major impact of HO-1-expressing TAMs on the control of the antitumor immunity and tumor growth. Indeed, myeloid HO-1 seems to be crucial for monocytic cell differentiation into TAMs and for the phenotypic, functional, transcriptional and epigenetic plasticity of TAMs. Myeloid HO-1 inhibition improves the beneficial effects of a therapeutic antitumor vaccine through a switch of TAM inflammatory state associated with the restoration of tumor-infiltrating lymphocyte proliferation and cytotoxicity and inhibition of tumor growth. As such, myeloid HO-1 inhibition might be considered as a new myeloid HO-1-mediated immune checkpoint blockade to reprogram the TME and facilitate the antitumor immune response.

Immunotherapy benefits only a minority of cancer patients, and there can be serious immuno-mediated toxic side effects which can lead to life-threatening complications. Many strategies are currently tested in preclinical and clinical settings with various efficacy, including immune checkpoint inhibitors and adoptive T-cell transfer. Notably, many combination strategies are ongoing. A better understanding of the multiple immunoregulatory mechanisms at play within the TME, including the myeloid compartment, is essential. In this context, we should keep into account the fact that the TME plays a crucial role in tumor immune escape mechanisms and cancer outcome. Targeting only the T-cell compartment is unlikely to be enough to overcome tumor progression. Targeting the myeloid compartment, such as TAM polarization, could be a valuable complementary approach to facilitate the antitumor immune response and ultimately improve the clinical outcome of cancer patients.

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