Immunosuppressive Mechanisms of Tumor-Associated Macrophages: Bioinformatic Analyses and Targeting

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ABSTRACT: In recent years, the treatment of various cancers with immunotherapeutic strategies has revolutionized the classical treatments with chemotherapy or radiation. Such immunotherapeutic strategies are effective only in a subset of cancer patients who were unresponsive to conventional therapies and are not generalized to all cancer types. Several mechanisms have been reported that underlie the failure of the natural anti-tumor immunity or the administered immunotherapeutic agents in the treatment of cancer. Among these mechanisms is the pivotal role played by the immunosuppressive tumor microenvironment (TME). The TME is complex and consists of the stroma, blood vessels, and several cell types that have a direct relationship with the tumor as well as the tumor-relationship with the TME. Among the immunosuppressive cells in the TME are the tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), T regulatory cells (Tregs), and cancer-associated fibroblasts (CAFs). These cells altogether inhibit both innate and the adaptive anti-tumor immune responses. Noteworthy, TAMs represent > 50 % of all the infiltrating cells in the TME and their frequencies correlate with poor prognoses in many cancers. The depletion or inactivation of TAMs has been reported to restore, in large part, the anti-tumor immune response in several cancers. In this review, we discuss (i) the interrelationship between TAMs and cancer stem cells, (ii) the various mechanisms by which TAMs suppress the immune response [e.g., expression of inhibitory receptors and ligands, secretion of immunosuppressive cytokines, secretion of chemokines, secretion of arginase 1, secretion of IDO1 and expression of the triggering receptor expressed on myeloid cells (TREM)], and (iii) targeting TAMs for immunotherapy (e.g., depletion of TAMs, killing of TAMs, inhibition of TAM recruitment, reprogramming of TAMs, targeting Toll-like receptors, inhibition of PI3K gamma, HDAC inhibitors, and inhibition of specific miRNA activities, and targeting TREM and exosomes). In addition, we present bioinformatic analyses that demonstrated that (i) TAM infiltration into many cancers correlated with poor survival (ii) the TAM infiltration was associated with the clinical stages of the cancer and (iii) there is a strong correlation between the TAM infiltrates and various immunosuppressive gene products. Although many clinical studies are underway to inhibit the immunosuppressive functions of TAMs through a variety of mechanisms, by either targeting TAMs alone or in combination with other therapeutics, we present various perspectives that need to be considered for the successful translational application of TAMs targeting alone or in combination with other therapies in the clinic.

KEY WORDS: cancer, TAMs, immunosuppression, targeting TAMs

ABBREVIATIONS: ARG1, arginase-1; **CAR**, chimeric antigen receptor; **CCL2**, chemokine (C-C motif) ligand 2; **CCR2**, C-C motif chemokine receptor 2; **CD40**, cluster of differentiation 40; **CSC**, cancer stem cell; **CSF-1**, colony stimulating factor 1; **CSF-1R**, colony stimulating factor 1 receptor; **CTLA-4**, cytotoxic T-lymphocyte-associated protein 4; **HDAC**, histone deacetylase; **IDO1**, indoleamine 2,3-dioxygenase; **IFN-γ**, interferon-gamma; **mAb**, monoclonal antibody; **M1**, classically activated macrophages; **M2**, alternatively activated macrophages; **MARCO**, macrophage receptor with collagenous structure; **MDSC**, myeloid-derived suppressor cell; **NK**, natural killer; **PD-1**, programmed cell death protein 1; **PD-L1**, programmed death-ligand 1; **PI3K-γ**, phosphoinositide 3-kinase gamma; **SIRP-α**, signal-regulatory protein alpha; **TAM**, tumor-associated macrophage; **TIMER**, tumor immune estimation resource; **TLR**, Toll-like receptor; **TME**, tumor microenvironment; **TREM-1**, triggering receptor expressed on myeloid cells 1

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I. INTRODUCTION

We have witnessed this last decade new milestones in immunotherapeutic strategies against various cancers, particularly those unresponsive and refractory to conventional therapies. These immunotherapeutic strategies include the wide spectrum of U.S. Food and Drug Administration (FDA)–approved monoclonal anticancer antibodies and various T cell-mediated cytotoxic anti-tumor activities. These latter include the genetic engineered T cells with specific T cell receptors, the development of chimeric antigen receptor (CAR)-T cells and the development of checkpoint inhibitors targeting CTLA-4 and both PD-1 receptors and PDL-1/2 ligands.[1] Although these new therapies resulted in significant clinical responses, nevertheless, they were consistent with only a subset of the patients. The unresponsive patients had tumors that were either intrinsically resistant and/or the tumor microenvironment (TME) was immunosuppressive.[2]

Clearly, the immunosuppressive TME is the result of many factors and infiltrating cells such as the regulatory T cells (Tregs), tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and cancer-associated fibroblasts (CAFs). Interestingly, the TME has over 50% of the cells belonging to TAMs and those frequencies correlated with worse prognoses. TAMs affect the immune cells and MDCs and express chemokines (CCL5, CCL22, and CCL20) and cytokines [interleukin-10 (IL-10) and transforming growth factor beta (TGF-β)] that recruit and activate Treg, cells and participate in immune suppression.[3]

Due to the immunosuppressive dominant role of TAMs in inhibiting T-cell mediated anti-tumor cytotoxicity as well as regulating the expression of checkpoint inhibitory receptors on T cells and corresponding ligands on the tumor cells and on MDSCs, specific targeting TAMs may reverse the immunosuppression and restore the T-cell-mediated cytotoxic activity, both directly and in the presence of checkpoint inhibitors.[4]

In this review, we will briefly examine the immunosuppressive roles of TAMs on anti-tumor T cell-mediated immune response and tumor regression, bioinformatic analyses on the association of TAMs with immunosuppressive factors in human cancers and how targeting TAMs may restore immunity and tumor regression.

II. TAMS VERSUS CANCER STEM CELLS (CSCS)

CSCs constitute a very small subpopulation of cancer cells that have distinct phenotypic and molecular properties that endow them with the ability for self-renewal and multilineage differentiation and are highly resistant to cytotoxic therapies. They are in large part responsible for tumor relapses.[5,6] Hence, novel immunotherapeutic intervening strategies might be effective provided the delineation of the various resistant mechanisms.[7]

The specific identification of CSCs has been a challenge, but some phenotypic markers were found to be associated with CSCs such as $CD34^{-}$ $CD38^{-}$.[8,9] These markers cannot distinguish between stem cells and CSCs. Other markers were also reported such as the ATP binding cassette, CD133 on the membrane or ALDH1 in the cytoplasm. Likewise, a recent biomarker was reported, namely, the expression of $EPCAM^{+}$ (CD44) on epithelial cells.[10]

CSCs escape immune mechanisms via down regulation of MHC I expression that is essential for recognition of T cells and APCs.[11,12] Also, the antigen-processing machinery is defective in CSCs and, hence, results in poor immunogenicity.[13]

CSCs exist amidst numerous cell types including TAMs. TAMs primarily role is to provide for the CSCs functions. For instance, the TAMs provide necessary signals to promote CSC survival, self-renewal, migration, and their maintenance. In return, the CSCs also help in promoting the TAMs that enhance the CSCs tumorigenicity. Thus, significant cross-talks are established between the TAMs and the CSCs.[14] Huang et al. (2020) reported, in prostate cancer models, that TAMs secrete CCL5 that promotes the migration, invasion, the EMT of prostate cancer cells as well as the self-renewal of prostate CSCs.[15] The CCL5 effect is mediated by the activation of the β-catenin/STAT3 signaling pathway. They also found that the expression of CCL5 correlated significantly with high Gleason grade, poor

prognosis, metastasis and enhanced activity of the CSCs. In a recent review by Aramini et al. (2021) they discussed the various interactions between TAMs and CSCs in various tumors.[16] For instance, in liver cancer the TAMs secrete IL-6 that promotes the expression of the CSC marker, $CD44^+$ and leading to tumor development. The IL-6 release, together with CCL5 and IL-8, has been linked to the β-catenin/Wnt pathway, leading to the spread of CSCs.

III. IMMUNOSUPPRESSIVE CELLS IN THE TME

In the TME, there exists a variety of both mature differentiated and immature myeloid cells which consist of monocytes, macrophages, neutrophils and MDSCs and which play a major role in tumor growth and metastasis.[17–19] Proangiogenic factors and pro-inflammatory cytokines derived from hypoxia and the acidic environment promote the infiltration of myeloid cells and their activation.[20,21]These myeloid cells infiltration regulate both the T cell trafficking as well as regulating their activity and exhaustion.[22,23] Therefore, targeting or eliminating these myeloid derived immunosuppressor cells may render the natural immune T cell responses against tumors more effective.[24,25] For instance, depleting TAMs with clodronate liposomes resulted in significant improvement of T cell trafficking.[26]

Poor T cell traffic is also the result of reduced chemokines in the TME and corresponding receptors on cytotoxic T cells.[27] Disruption of the immunosuppressive chemokine/cytokine network can restore T cell traffic and sensitize tumors to cytotoxic immunotherapy.[28] The M2-like macrophages represent the major player in the inadequate CD8 T cell trafficking.[27]

Peripheral blood monocytes are recruited into the TME via the CCL2/CCR2 chemokine pathway and transdifferentiate into M2-like macrophages under the influence of the CSF1/CSF1R pathway.[28] Depleting M2 macrophages by inhabiting either the CSF1/CSF1R or the CCL2/CCR2 overcome T cell exhaustion within the tumor.[29,30]

TAMs are also engaged in metabolic pathways that deplete essential elements (e.g. tryptophan) and also produce immunosuppressive metabolites like Indo-kynurenines.[31]

Lymphocyte exhaustion is a hallmark of CD4 and CD8 T cells in the TME as a result of their inability to function and their failure to produce effector cytokines [TNF, interferon-gamma (IFN-γ), and IL-10] and inability to kill tumor cells.[28,32] The exhausted cells express a variety of checkpoint inhibitory receptors such as PD-1, LAG-3 TIM3, CTLA-4, BTLA, and TIGIT.[33] Ligands for immune checkpoint receptors are expressed on M2 macrophages and promote immune evasion.

IV. TAMS VERSUS IMMUNITY

TAMs have been reported to limit the efficacy of immunotherapy. Several FDA-approved monoclonal antibodies (mAbs) directed against checkpoint inhibitory receptors and ligands have been used in cancer therapies with significant clinical responses in a subset of patients in certain cancers but not all cancers (e.g., pancreas, colorectal, and ovarian cancers).[27]

Macrophages exhibit plasticity by acquiring the proper phenotype to respond depending on the stimulus. This plasticity is referred to as polarization and is based on gene expression, surface molecules and metabolites that can switch from M1 inflammatory cells to M2 anti-inflammatory. M1 can be activated by Th1 cytokines such as IFN-γ, TNF-α, or by LPS and secrete high levels of pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-12, IL-23) through the NADPH oxidase system and consequent ROS production exert anti-microbial and anti-tumoral activities. M2 macrophages are induced by Th2 cytokines (IL-4, IL-13, IL-10) and glucocorticoids. They produce anti-inflammatory cytokines such as IL-10, TGF-β. They have potent phagocytic activity and promote tissue repair and wound healing and pro-angiogenic activity.[34,35]

TAMs represent the major infiltrating immune cells in the TME. TAMs play a major role in immunosuppression by inhibiting TH1 cells and activating Th2 cells.[36,37] TAMs inhibit CD8 T cells proliferation and activation.[38,39] The M1/M2 macrophage ratio score along with the tumor mutational burden and $CD8^+$ scores were all predictors to ICIs.[40]

V. MECHANISMS OF IMMUNE SUPPRESSION BY TAMS

Several mechanisms have been reported to describe the multiple means by which TAMs lead to immune suppression. Some of these mechanisms are briefly discussed below.

A. Expression of Inhibitory Receptors Including HLA-E and HLA-G

These inhibit the activities of natural killer (NK) and T cells via the interaction of these receptors with CD94 and LIT-2, respectively.[41] HLA-E and HLA-G (non-classical) membrane bound or soluble forms can inhibit the activation of NK cells and a subset of activated T cells upon the ligation of HLA-E to the killer cell immunoglobulin like receptor CD94 (also known as NKG2)[42] or the inhibitory leukocyte immunoglobulin-like receptor LIT-2, where it binds to HLA-G molecules on antigen-presenting cells and macrophages.[43] Both transduce a negative signal that inhibits stimulation of the NK or T cell mediated immune response.

Under physiological conditions, there is a strong correlation between HLA-E and HLA-G as they are involved in inducing anergy of activated immune NK cells and T cells. Hence, they both establish an immunosuppressive environment in human tumors, and thus, tumor escape from the immune system.

For example, breast cancer patients who displayed loss of HLA-I molecules with either HLA-G or HLA-E expression correlated with worse overall and event-free survival even though these patients have activated NK cells.[44] In addition, in patients with colon cancer, both HLA-E and HLA-G co-expression correlated with metastasis and with a worse event-free and overall survival.[45]

B. Expression of the Inhibitory PD-1, PD-L1/2, and B7-1/2 Ligands

TAMs also express T cell checkpoint inhibitors (PD-1 and CTLA-4) and ligands PDL-1/2 and B7-1/2 that directly inhibit T cell functions.[46,47] PD-1 receptor is highly expressed by activated T cells, B cells, and natural killer cells. The well-known ligands of PD-1 are PD-L1 (or B7-H1) and PD-L2 (or B7-DC) are expressed in TAMs and can be induced by inflammatory cytokines on tumors, immune cells, and various tissues. After ligand binding, PD-1 inhibits kinase signaling pathways involved in T-cell activation; thus, this process prevents overstimulation of immune response. PD-L1 also binds the CD80 receptor, which is another negative regulator of T-lymphocyte activation. PD-1 primarily inhibits T-cell activity in the effector phase within tissues and tumors, whereas CTLA-4 regulates immune responses early in T-cell activation.

The activation of T cells requires more than one signal in addition to the TCR binding to the corresponding MHC-peptide complex. TCR binding to the MHC provides specificity to T-cell activation, but further costimulatory signals are required. For instance, the binding of B7-1 (CD80) or B7-2 (CD86) molecules on the APC and macrophages with CD28 molecules on the T cell leads to signaling within the T cell. Sufficient levels of CD28:B7-1/2 binding lead to proliferation of T cells, increased T-cell survival, and differentiation through the production of growth cytokines such as IL-2, increased energy metabolism, and upregulation of cell survival genes. CTLA-4 is a CD28 homolog with much higher binding affinity for B77; however, unlike CD28, binding of CTLA-4 to B7 does not produce a stimulatory signal. As such, this competitive binding can prevent the costimulatory signal normally provided by CD28:B7 binding.[48]

Interestingly, patients whose TAMs express PD-L1 responded favorably to anti-PD-L1 antibody.[49] The expression of PD-1 on TAMs inhibited phagocytosis of the tumor cells and blocking of the PD-1–PD-L1 axis restored the macrophage phagocytic activity.[47] For example, in a mouse model of a tumor resistant to anti-PD1 antibody, the combination treatment with anti-CD40 resulted in a synergistic anti-tumor response.[50] In a murine model for melanoma, the treatment with the combination of anti-PD-1 and anti-CSF1R resulted in tumor regression.[51]

C. Secretion of Immunosuppressive Cytokines and TGF-β

TAMs also secrete several cytokines such as IL-10 and TGF-β that contribute to the maintenance of a strong immune suppressive microenvironment by inhibiting CD4 (Th1 and Th2) and CD8 T cells and by inducing T-regulatory cells expansion.

TAMs interfere with T cell activation via interaction with inhibitory immune checkpoints and inhibiting T cell recruitment.[52,53] TGF-β excludes CD8 from the tumor parenchyma and their delocalization in peri-tumoral stroma.[54]

D. Secretion of Chemokines

TAM-mediated release several chemokines such as CCL2, CCL3, CCL4, CCL5, and CCL20 further contributing to the recruitment of regulatory T cells in the TME and participate in the suppression of CD8 T cells.[43,55]

E. Secretion of Arginase 1

TAMs also directly inhibit T cell cytotoxicity by depletion of L-arginine (L-Arg), essential for the re-expression of the CD3 zeta chain in the TCR after antigen engagement on T cells, by the release of arginase I that metabolizes L-Arg to urea and L-ornithine.[56,57]

Arginase production by macrophages not only leads to the inhibition of anti-tumor response via L-Arg degradation, but also increases the proliferation of tumor cells, which is associated with the production of L-ornithine and then a polyamine-putrescine that promotes tumor cells proliferation. Moreover, L-Arg depletion in the TME attenuates NO production and reduces its cytotoxic effects on tumor cells.[58,59]

F. Depletion of Tryptophan by IDO1

Similarly, depletion of tryptophan or production of tryptophan metabolites by indoleamine 2,3-dioxygenase (IDO) expressed by macrophages can inhibit cytotoxic T cells. The mechanism of IDO1-elicited immunosuppression is not fully understood; however, increased IDO1 and Kyn levels are known to inhibit natural killer (NK) cell function, prevent the activation of effector T cells.[60] stimulate the activation of Treg, cells[61] and promote the expansion and activation of MDSCs.[62]

Lack of any single essential amino acids restricts T-cells activation and proliferation and this phenomenon is not specific to L-Arg. Depletion of L-histidine, L-leucine, L-lysine, L-phenylalanine, L-threonine, and L-valine inhibited the proliferation of T-cells to a similar extent as L-Arg depletion. Of importance, however, only arginases as well as IDO that hydrolyzes L-tryptophan are substantially increased in cancer.[63,64]

G. Expression of TREM

The triggering receptor expressed on myeloid cells (TREM) is a 30-kDa glycoprotein expressed on monocytes and macrophages. It is a type 1 membrane receptor that is also secreted. TREM-1 interacts with DAP12 to stimulate neutrophil and monocyte mediated inflammatory responses through the triggering and release of pro-inflammatory cytokines and chemokines.[65]

Six *TREM* genes in the mouse (*TREM* 1-6) and 4 *TREM* genes in humans (*TREM* 1-4) have been identified and the human genes are clustered on human chromosome 6p21. TREM-1 (CD354) is the first identified and best-characterized family member and an important regulator of myeloid cell immune response.[66]

TREM-1 is overexpressed in macrophages and M1-M2 polarization.[65] The TREM-1 ligand is not known and it is an area of controversy. The cell signaling mediated by TREM-1 has been recently reviewed.[66,67] *TREM-1* expression on macrophages is an independent predictor of tumor progression.[66,68] The immunosuppressive proprieties of TAMs described in this section are summarized in Fig. 1.

VI. TARGETING TAMS FOR IMMUNOTHERAPY

Tumors expressing neoantigens have the ability to elicit an anti-tumor immune response, both antibody-mediated and T-cell-mediated. The T-cell mediated response is the result of the interactions of the

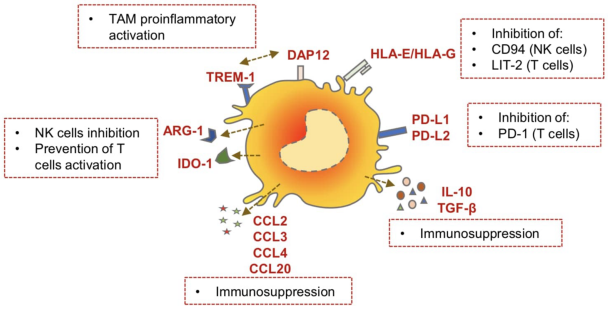

FIG. 1: Immunosuppressive properties of TAMs. A schematic diagram represents all the multiple pathways by which the TAMs mediate their tumor immune suppression, which include: (1) HLA-E- and HLA-G-mediated response, (2) PD-L1 and PD-L2 checkpoint response, (3) secretion of relevant immunosuppressive cytokines and chemokines, (4) secretion of enzymes (ARG-1 and IDO-1), and (5) TREM-1/DAP12-mediated signaling.

T cell with the MHC loaded with the tumor peptide and the additional signaling via the CD28 signaling on the T cells via the co-stimulatory molecules (CD80 and CD86) expressed on the APCs.

However, this activation process is complex as it is affected by various immunosuppressive factors. For instance, the solid tumors are infiltrated with immunosuppressive cells (Tregs, TAMs, MDSCs, and CAFs) and endothelial cells,[69] as well the tumor is regulated by immunosuppressive cytokines such as IL-10 and TGF-β. An important development was the discovery of immune checkpoint blocking receptors, such as CTLA-4 and PD-1/2 whose main functions were to block the induction and the effector functions of T cells, respectively. These have led to the development of FDA-approved checkpoint inhibitors allowing a restoration of the antitumor immune response.[70]

The TME is complex with diverse populations of non-tumor stromal cells that impact tumor immune evasion, response to immunotherapy, and patient survival.[71] Several reports have indicated that TAMs are directly involved in immune resistance.[29] Therefore, several strategies were devised to target TAMs to inhibit TAMs immunosuppressive activities, namely, depletion of TAMs, inhibition of TAM recruitment, reprogramming of TAMs, targeting TREM-1, targeting the CD47/SIRPapj=ha axis, and targeting exosomes. Such TAM-targeting strategies have been extensively reviewed elsewhere.[72–76] Some strategies are briefly described below and are summarized in Fig. 2.

A. Depletion of TAMs

Because TAMs are dependent on CSF1R signaling for survival and proliferation, antibodies directed against CSF1 or CSF1R have been developed as well as small chemical molecules targeting CSF1R have been synthesized such as PLX3397 or pexidartinib.[74,75] A humanized mAb targeting CSF1R, emactuzumab, in animal models reduced the number of TAMs in the TME and increased the ratio of CD8/CD8 T lymphocytes.[77] In patients with advanced solid tumors and treated with a combination of emactuzumab and paclitaxel resulted in a safety profile although with no significant improvement of outcomes.[78] Other clinical trials using other anti-CSF1R mAbs alone or in combination are being investigated.[74]

Depletion of TAMs with the Plexicon small molecule PLX3397 was tested in patients with different cancers showed clinical responses.[79] In murine pancreatic cancer, the combination of PLX3397 with either anti-CTLA4 or anti PD-1 checkpoint inhibitors ameliorated the anti-tumor immune response and

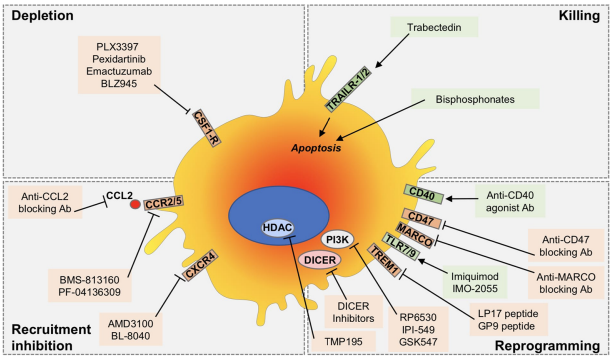

FIG. 2: Various means of targeting TAMs. A summary of the main strategies pursued to target TAMs are schematically represented: (1) depletion of TAMs, (2) killing of TAMs (through drug-mediated apoptosis induction), (3) inhibition of TAM recruitment at the tumor site, and (4) reprogramming of TAMs (from the pro-inflammatory to the anti-inflammatory phenotype).

the regression of established pancreatic tumors.[80] Several clinical trials are currently being investigated regarding the efficacy of treatment with the inhibitors of the CSF-1/CSF1R axis.[74]

In an *in vivo* study in mice, the infiltration of T cells increased following treatment with a tyrosine kinase inhibitor, pexidartinib, that targets CSF1, and depleted TAMs. This resulted in T cell migration and anti-tumor effect. In a mouse tumor model of pancreatic cancer, macrophages were depleted by the use of an inhibitor against CSF-1R, PLX3397, and the findings revealed that CD8 T cells from the periphery migrated and infiltrated into the tumor cells though with minimal effect on tumor growth. However, the combination treatment with anti-PD-1 mAb enhanced the CD8 T cells interaction with the tumor cells and resulted in significant inhibition of tumor growth.[81,82] The combination of anti-PD1 or anti-CTLA-4 antibodies with the CSF1R inhibitor, PLX3397, resulted in a significant improvement if anti-tumor immunity in established pancreatic cancer models.[80]

B. Killing TAMs

Another approach to deplete and kill TAMs is via the use of bisphosphonates. These agents have been reported to have a both direct effect on the tumor cells and the TME.[83] A member of the family of non-nitrogen bisphosphonates, clodronate, was used in liposomes and induces apoptosis in macrophages.[84] In tumor bearing mice, administration of the liposomes inhibited tumor growth and depleted TAMs.[85] Another bisphosphonate, zoledronic acid, inhibited tumor growth in a mouse model of breast carcinoma and reduced the number of TAMs and their repolarization status.[86,87]

A registered anti-neoplastic drug, trabectedin, can target tumor cells and also deletes circulating monocytes and TAMs through a TRAIL-dependent pathway of apoptosis.[88] Monocytes and TAMs express the functional TRAIL receptors, TRAIL R1, and TRAIL R2 and are susceptible to the cytotoxic effect of trabectedin. Its anti-tumor effect was shown in preclinical animal cancer models.[89–91]

Macrophages and TAMs express the scavenger receptor, CD163, which promotes pro-tumorigenic activities[92] and, targeting CD163 with an antibody, results in depletion of TAMs and consequently anti-tumor activity.[92,93]

C. Inhibition of TAM Recruitment

The recruitment of monocytes into the TME as TAMs may be targeted to prevent the recruitment and prevent TAM-mediated immunosuppression. The recruitment of circulating monocytes depends on various chemokines signaling and therefore targeting these chemokines will prevent the TAM recruitment.[94] CCL2 is synthesized by tumor cells and is a potent chemoattractant for monocytes. The CCL2/CCR2 signaling is involved in the regulation of circulating monocytes and their infiltration into the TME and their inhibition showed an anti-tumor activity.[95] Hence, for example the use of neutralizing antibodies to CCL2 inhibited the recruitment of circulating monocytes and significantly reducing the number of TAMs concomitantly with an increase of CD8 T and NK cells.[96,97] The therapeutic effect of BMS-813160 in clinical studies was induced by examining a small molecule inhibitor of CCR2/5, in combination with nivolumab and/or the tumor vaccine GVAX in several solid tumors.

Carlumab is a human antibody that binds CCL2 and its administration in a prostate cancer model reduced tumor growth and the infiltration of TAMs. In a phase II clinical study treatment with carlumab in patients with refractory prostate cancer did not show a therapeutic effect.[98]

In a phase Ib randomized trial in 47 patents, the CCR2 small molecule inhibitor PF-04136309 was tested in combination with FOLFIRINOX chemotherapy and the findings showed that unlike treatment with FOLFIRINOX alone, the combination resulted in 16/33 patients to have an overall response and 32 patients had local tumor control.[99]

Hypoxia was also reported to be involved in the recruitment to TAMs via hypoxia-induced upregulation of stromal cell-derived factor 1 alpha (SDF-1α/CXCL12).[100] Treatment with the inhibitor SDF1 alpha receptor (CXCR4) using the antagonist AMD 3100 reversed immunosuppression and enhanced anti-PD-1 treatment in a sorafenib resistant HCC model.[101] Therefore, CXCR4 antagonists, such

as AMD3100 and BL-8040, should be justifiably considered in the future design of clinical trials for immunotherapies.

D. Reprogramming of TAMs

Several approaches have been used to reprogram M2 macrophages into anti-tumor M1 macrophages. Reprogramming is feasible due to the plasticity of the macrophages. These include the restoration of phagocytic activity by targeting the CD-47-SIRP-α axis using antibodies directed against either, targeting the Toll-like receptors, inhibition of PI3Kgamma, anti-CD40 mAbs, HDAC inhibitors, anti-MARCO Ab, inhibitors for CSF1R or CCR2 and RNA delivery.

1. Anti-CD40 Antibodies

Macrophages and DCs express on their surface the CD-40 receptor, a member of the TNF receptor superfamily. Its interaction with its ligand expressed on T cells upregulates the expression of MHC molecules and the secretion of pro-inflammatory cytokines that promote T cell activation.[102] The administration of anti-CD40 agonists led to the anti-tumor effect and reprogramming TAMs towards the M1 phenotype.[103,104]

2. Inhibition of CSF1R or CCR2

Inhibition of CSF1R or CCR2 resulted in inhibition of tumor growth. This finding was the result of reprogramming M2 into M1. Pyonteck et al. reported that the CSF1R inhibitor BLZ945 enhanced survival in tumor bearing mice and was due in part of TAM reprogramming to M1.[105]

3. Anti-MARCO Antibody

The macrophage receptor with collagenous structure (MARCO) belongs to the class A scavenger receptor family and is a pattern recognition receptor and primarily expressed on macrophages.[106] MARCO is overexposed in breast cancer and metastatic melanoma patients and its neutralization by antibodies inhibited tumor growth and metastasis and enhancement of anti-CTLA- 4 antibody.[107]

4. Targeting Toll-Like Receptors

Toll-like receptors are pattern recognition receptors in innate immunity and their interactions with corresponding ligands stimulate macrophages. Hence, targeting TAMs by TLRs agonists can reprogram TAMs into anti-tumor effector cells.[108] TLRs located in the endosomal compartment (TLR3, 7, 8, 9) exhibit a higher capacity, compared to extremal TLRs, to trigger a better anti-tumor response[74]. The TLR7 agonist imiquinod is the only FDA-approved for topical administration in squamous and basal cell carcinoma.[109–112] Two TLR7 agonists (imiquinod and 852A0) and one TLR9 ligand (IMO-2055) are being tested clinically.[38]

An agonist of TLR7/8, Resquimod R848, was shown to reprogram TAMs and triggers a strong anti-tumor response.[113,114] MEDI19197, a different formulation of R848 and less toxic was used. Beta-cyclodextrin-NPs loaded with R848 and was targeted for TAMs *in vivo*. The findings showed that this treatment resulted in the production of pro-inflammatory cytokines by TAMs and its combination with PD-1 antibodies restored the anti-tumor activity in cancers resistant to anti-PD 1antibody.[115]

5. Inhibition of PI3K-γ

PI3K-γ in TAMs regulate their immunosuppressive activity.[116] The selective inhibition of PI3K-γ resulted in CD8 T cells recruitment, pro-inflammatory cytokine secretion and inhibition of tumor growth. The PI3K-delta/gamma inhibitor, RP6530, was able to reprogram the TAMs into an M1 phenotype and inhibited angiogenesis and inhibited tumor growth in a Hodgkin's lymphoma tumor xenograft.[117]

A PI3K-γ inhibitor, IPI-549, reprograms M2 into an M1 phenotype and its administration *in vivo* upregulated PD-1 and CTLA4 expressions on CD8 T cells and the combination treatment with IPI-549 and either anti-PD1 or anti-CTLA4 Abs in ICI resistant tumors resulted in significant tumor delay and remission in a number of mice.[118]

Although IFN-γ has been reported to amplify anti-tumor immunity, it also can hinder it. The secretion of IFN-γ by TAMs induces the expression of PD-L1 on tumor cells through the JAK/STAT3 AND

PI3K/AKT pathways leading to inhibition of CD8 T cells and tumor progression.[119] Also, TAMs secrete IL-10 that upregulates PD-L1 on tumor cells.[120] Tumor cells also secrete phosphoprotein 1 (SPP1) that induces PD-L1 expression on TAMs.[119] Therefore, there is a vicious circle between the TAMs and tumor cells in the upregulation of PD-L1 on both TAMs and the tumor cells and thereby enhancing immunosuppression via the inhibition of anti-tumor CD8 T cytotoxic cells expressing PD-1.

The receptor-interacting serine/threonine protein kinase 1 (RIPK1) is upregulated in TAMs in PDAC and targeting TAMs with the inhibitor GSK547 repolarizes TAMs towards a pro-inflammatory phenotype and increases the infiltration of CTLs and tumor suppression.[121] In addition, there was a synergistic activity with the combination of the inhibitor and anti-PD1 antibody.

6. HDAC Inhibitors

HDAC inhibitors are enzymes that remove the acetyl groups on histones during epigenetic regulation or gene expression.[122] They reported that the administration of the class IIA HDAC inhibitor, TMP195, resulted in the recruitment and the reprogramming of TAM-CD40^{+} into anti-tumor effector cells and resulting in tumor eradication. In addition, the combination of TMP195 with chemotherapy or checkpoint inhibitor anti PD-1 augmented the anti-tumor response.

The specific inhibitor of class IIA HDAC, TMP195, modifies the epigenomic profile of monocytes and macrophages and promotes a pro-inflammatory phenotype.[123] In a model of breast cancer, the administration of TMP195 resulted in infiltration of myeloid cells into the tumor where they differentiated into anti-tumoral macrophages. In addition, they enhanced the treatment with chemotherapeutic drugs or anti-PD-1 antibodies.[122]

7. Inhibition of miRNA Activity

The RNAse-III enzyme DICER inhibition in macrophages affected TAM reprogramming and was associated with inhibition of tumor growth an infiltration of anti-tumor immune cells.[124]

8. Targeting TREM-1

TREM-1 targeting represents a new modality to inhibit M2-mediated chronic inflammation associated with tumor development.[65] The TREM-1 peptide LP17 antagonist was reported to have a therapeutic effect in a mouse model of colon cancer by reducing production of pro-inflammatory mediators by intestinal macrophages.[125] The GP9 inhibitory peptide was tested in two human NSCLC xenograft models suppressed tumor growth.[126] GF9 also attenuated the resistance to PD-L1 blockade and improved the therapeutic efficacy.[127]

Studies by Zhou et al. reported that targeting TREM-1 with LP17 inhibited tumor growth in a murine model of colon carcinogenesis.[125] Similar findings were reported in human NSCLC xenografts and human pancreatic cancer xenografts by the administration of the GP-9 peptide inhibitor.[126,128–130] In an orthotopic HCC-bearing models, GP9 abrogated the TREM-1 TAM mediated immunosuppression and attenuated resistance to PD-1 blockade.[127]

9. Targeting the CD47-SIRP-α Axis

Tumor cells express CD47 that interacts with the signal regulatory protein 1 alpha (SIRP-α) present on the surface of phagocytic cells and inhibits phagocytosis. Blocking CD47 restores phagocytosis and killing of tumor cells by macrophages. Repolarization of TAMs was achieved by the combination of anti-CD47 and anti-CSF1R antibodies and was accompanied by anti-tumor effect.[128,129] Several clinical trials using a combination of anti-CD47mAbs or CD47-Fc fusion proteins with anti-PD1 antibodies in the treatment of different tumor types.[74]

10. Targeting Exosomes

Exosomes derived from TAMs promote cellular migration and invasion. It has been reported that TAM-derived exosomes exhibit high expression levels of miR-21-5-p and miR-155-5p, which significantly promote cellular migration and invasion of colorectal cancer cell. Besides, exosomes from macrophages also contain a high level of Wnt, which is considered to provide critical signaling in

EMT induction and mesenchymal-phenotype maintenance. In addition to metastasis, TAM-derived exosomes also contribute to extracellular matrix remodeling. It has been shown that MMP-12 and MMP-13, as well as cathepsin B, D, K, L, S, and Z, are all upregulated in TAM-derived exosomes, suggesting their positive roles in extracellular remodeling. Notably, exosomes released from TAMs also contain miRNAs that lead to Treg/Th17 cell imbalance in ovarian cancer, resulting in the generation of an immune-suppressive TME and thus promoting cancer progression and metastasis. Altogether, targeting TAM-derived exosomal signaling represents a novel and promising approach for cancer treatment.[131]

VII. BIOINFORMATIC ANALYSES

A. TAMs in Human Cancers

During the last years, several molecular and clinical-pathological data of tumors have been collected.[132] The huge amount of bioinformatics data generated has led researchers worldwide to develop tools and software able to fasten the analysis of such data thus favoring the identification of specific characteristics of each tumor as well as novel diagnostic biomarkers or therapeutic targets.[133,134]

Among the biggest tumor bioinformatics data repository there is the Cancer Genome Atlas database which collects omics data obtained for 33 different tumors useful to identify transcriptomics, proteomics, epigenomics and clinical features of tumors.[135] Through the integration of gene expression, protein expression and clinical data it has been possible to evaluate the interaction of the immune system with different tumors thus highlighting the role of the different immune cells in cancer development and progression.[136]

To fasten the analysis of the interaction existing between tumor cells and immune cells, Li and colleagues have developed a web resource, Tumor Immune Estimation Resource (TIMER), for the analysis of the abundance of immune cell infiltrates as well as to establish the correlation between specific immune cell types and the expression of selected genes.[137] The software uses a well-validated statistical method that takes into account the immune infiltrates' abundances estimated by multiple immune deconvolution methods across diverse cancer types correlating these infiltrates with immunological, clinical and genomic features.[137]

TIMER analysis revealed how macrophage infiltrates are generally increased in tumors samples. Statistical increment of macrophages was also observed in bladder cancer (BLCA), breast cancer (BRCA) with higher increment in luminal B breast cancer (BRCA-LumB), kidney chromophobe cancer (KICH), low-grade glioma (LGG), liver hepatocellular carcinoma (LIHC), malignant mesothelioma (MESO), primary skin cutaneous melanoma (SKCM-Primary) and stomach adenocarcinoma (STAD) (Fig. 3).

For all these tumors, except for KICH, the increment of macrophage infiltrates is associated with a lower cumulative overall survival (Fig. 3). Therefore, macrophage infiltrates are associated with a worse prognosis in different tumors.

B. TAMs in Human Cancers and Clinical Stages

By performing the same analysis and considering tumor stages as a key clinical feature, TIMER analysis revealed how the increment of macrophage infiltrates is associated with an increased risk of BLCA, BRCA, BRCA-LumB, LGG, LIHC, MESO and STAD advanced stages (Fig. 4). As for the previous analysis, the increment of macrophage infiltrates is associated with lower cumulative survival of patients affected by these tumors (Fig. 4).

C. Correlation of TAM Infiltrates and Immunosuppressive Factors

To further unveil the role of TAMs in human cancers, the correlations between macrophage infiltrates and the expression levels of immunosuppressant genes were also established.

Therefore, using TIMER it is possible to evaluate the statistical variation of macrophage infiltrates abundance between normal and tumor tissues as well as to establish the prognostic significance of this infiltration in terms of cumulative overall

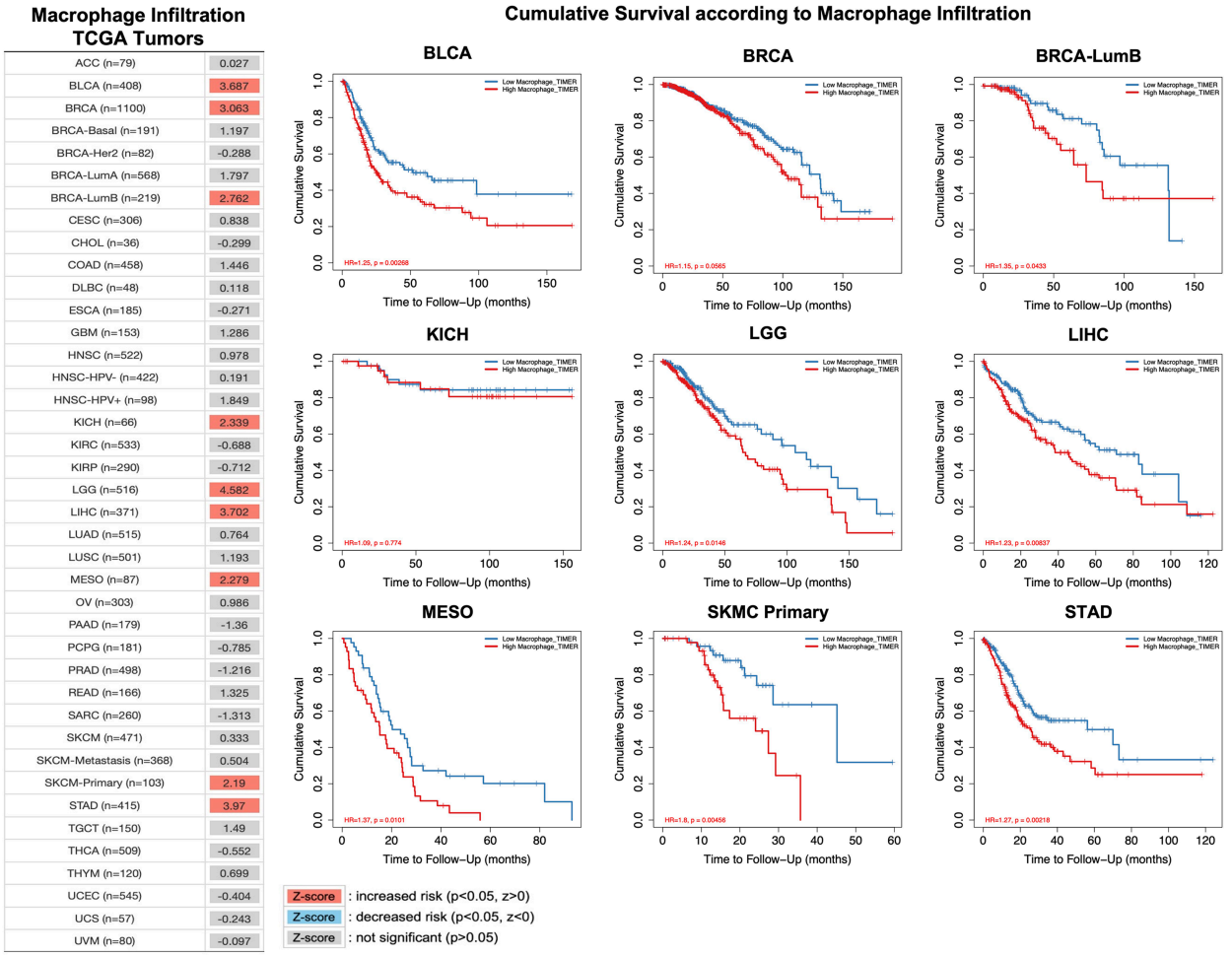

FIG. 3: Macrophage infiltrate abundance using the TIMER multivariable Cox proportional hazard model in different TCGA tumors. *Z*-scores indicate the normalized coefficient of the macrophage infiltrates across multiple tumors. Each cell of heatmap corresponds to an independent cox model. Kaplan-Meier curves show the clinical relevance of macrophage infiltrates dividing tumor samples into low and high levels of infiltration. The hazard ratio and *P* value for the Cox model were considered significant when $Z > 0$, $Z < 0$, and $P < 0.05$. Log-rank *P* value for the Kaplan-Meier curve was considered significant when $P < 0.05$.

survival. In addition, TIMER can correlate the levels of macrophage infiltrates with the expression of immunosuppressant genes like PDCD1 (PD-1), CD274 (PD-L1), PDCD1LG2 (PD-L2), CTLA4, CD80 (B7-1), and CD86 (B7-2).

Overall, macrophage infiltrates are mainly positively correlated with the expression levels of immunosuppressant genes like PDCD1 (PD-1), CD274 (PD-L1), PDCD1LG2 (PD-L2), CTLA4, CD80 (B7-1), and CD86 (B7-2). In particular, macrophage infiltrates are positively correlated with the expression levels of CD86 in almost all of the TCGA tumors (moderate correlation). Similarly, macrophage infiltrates are associated with the over-expression of CD86 in 28 out of 40 TCGA tumor categories (mild and moderate positive correlation) (Fig. 5). As regards the PD-1/PD-L1/2 axis, macrophages play a more heterogeneous role in immunosuppressant genes' regulation. Similar to what observed for the CTLA-4/CD80-CD86 axis, macrophage infiltrates are positively correlated with PD-1, PD-L1, and PD-L2 expression (mild and moderate correlation)

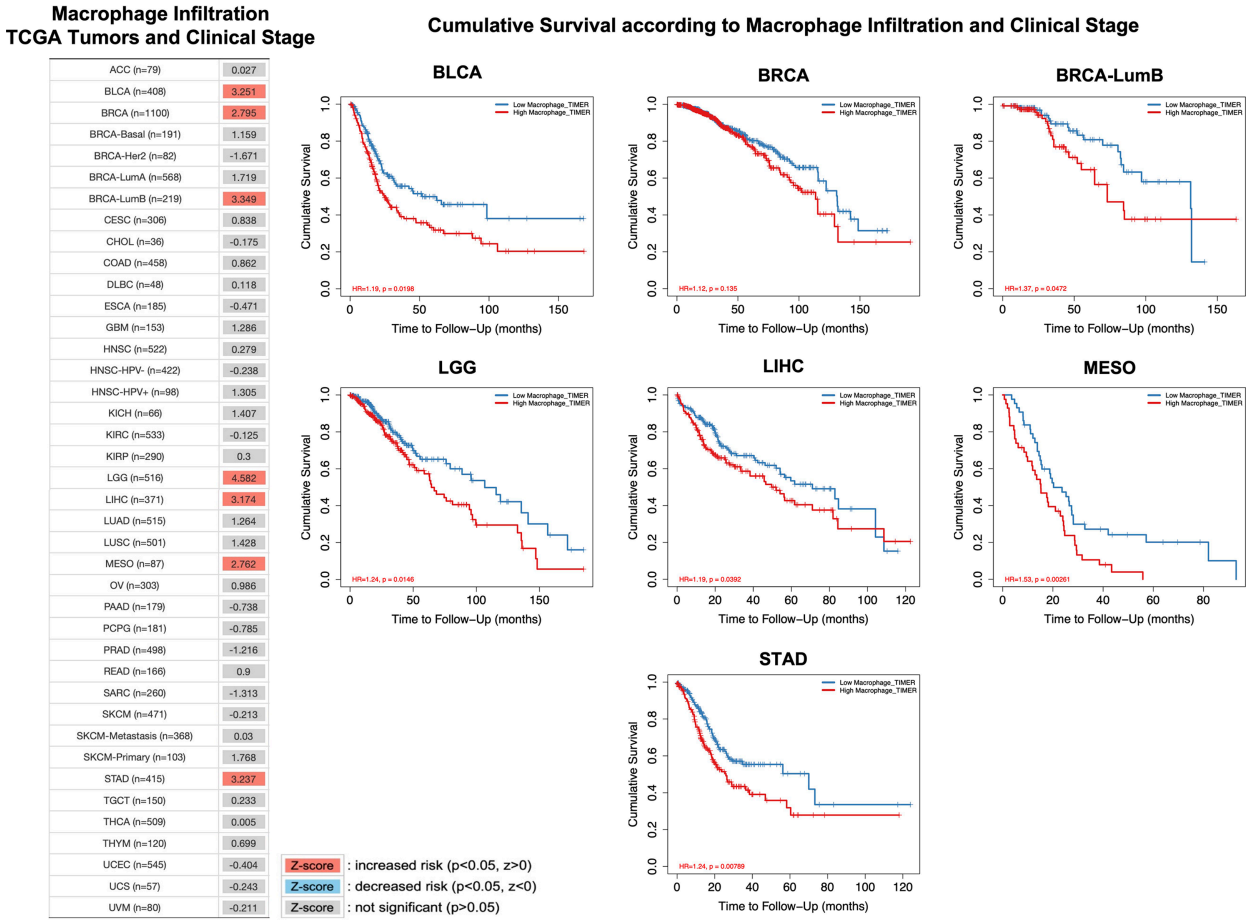

FIG. 4: Clinical relevance of macrophage infiltrates using the TIMER multivariable Cox proportional hazard model considering tumor stages. Z-scores indicate the normalized coefficient of the macrophage infiltrates across multiple tumors considering the tumor stages. Each cell of the heatmap corresponds to an independent Cox model. Kaplan-Meier curves show the clinical relevance of macrophage infiltrates dividing tumor samples into low and high levels of infiltration considering the tumor stages. The hazard ratio and p-value for Cox model were considered significant when $Z > 0$, $Z < 0$, and $P < 0.05$. Log-rank P value for the Kaplan-Meier curve was considered significant when $P < 0.05$.

except for BRCA tumors where a wild-moderate negative correlation between macrophage infiltrates and PD-1 gene expression has been observed (Fig. 5).

Taking into account both PD-1/PD-L1/2 and CTLA-4/CD80-CD86 axes, macrophage infiltrates in adrenocortical carcinoma (ACC), colon adenocarcinoma (COAD), glioblastoma multiforme (GBM), HPV-negative head and neck cancer (HNSC-HPV-), kidney renal papillary cell carcinoma (KIRP), LGG, LIHC, pancreatic adenocarcinoma (PAAD), pheochromocytoma and paraganglioma (PCPG), rectum adenocarcinoma (READ) and STAD were positively correlated with the expression levels of all the relevant immunosuppressant genes (PD-1, PD-L1, PD-L2, CTLA4, CD80 and CD86). Of these tumors, GBM and LIHC showed the highest positive correlation levels (Fig. 5).

VIII. PERSPECTIVES AND CONCLUSIONS

We have briefly discussed the complex role TAMs play in the TME and how they regulate the cell-mediated anti-tumor immune response. Clearly, immunotherapy has become a major therapeutic approach in the treatment of many cancers and particularly those

TCGA Tumors	PDCD1 (PD-1)	CD274 (PD-L1)	PDCD1LG2 (PD-L2)	CTLA4 (CTLA-4)	CD80 (B7-1)	CD86 (B7-2)
ACC (n=79)	0.278	0.279	0.477	0.318	0.442	0.437
BLCA (n=408)	-0.089	-0.079	0.08	-0.134	0.013	0.162
BRCA (n=1100)	-0.278	0.095	0.187	-0.13	0.222	0.267
BRCA-Basal (n=191)	-0.323	-0.094	-0.005	-0.25	0.059	0.106
BRCA-Her2 (n=82)	-0.292	0.113	0.325	-0.169	0.444	0.364
BRCA-LumA (n=568)	-0.199	0.192	0.331	-0.022	0.347	0.361
BRCA-LumB (n=219)	-0.292	0.141	0.287	-0.043	0.334	0.362
CESC (n=306)	0.028	-0.096	-0.022	-0.019	0.071	0.138
CHOL (n=36)	0.11	0.39	0.335	0.142	0.219	0.408
COAD (n=458)	0.19	0.278	0.496	0.239	0.359	0.543
DLBC (n=48)	-0.175	-0.321	-0.431	-0.253	-0.131	-0.049
ESCA (n=185)	0.131	0.065	0.387	0.214	0.37	0.466
GBM (n=153)	0.425	0.216	0.334	0.432	0.381	0.671
HNSC (n=522)	0.062	0.135	0.294	0.121	0.296	0.384
HNSC-HPV- (n=422)	0.165	0.174	0.31	0.195	0.33	0.414
HNSC-HPV+ (n=98)	-0.24	-0.043	0.209	-0.124	0.179	0.33
KICH (n=66)	0.2	0.293	0.517	0.27	0.245	0.415
KIRC (n=533)	-0.228	0.171	0.362	-0.068	0.24	0.406
KIRP (n=290)	0.223	0.244	0.529	0.159	0.473	0.732
LGG (n=516)	0.235	0.151	0.282	0.162	0.165	0.345
LIHC (n=371)	0.286	0.355	0.386	0.303	0.5	0.593
LUAD (n=515)	-0.006	0.225	0.338	0.002	0.311	0.46
LUSC (n=501)	0.054	0.07	0.1	-0.03	0.106	0.207
MESO (n=87)	-0.05	0.136	0.246	0.021	0.19	0.434
OV (n=303)	0.076	0.191	0.239	0.042	0.238	0.301
PAAD (n=179)	0.211	0.477	0.54	0.252	0.278	0.523
PCPG (n=181)	0.363	0.403	0.473	0.379	0.295	0.552
PRAD (n=498)	-0.022	0.215	0.232	-0.084	0.078	0.243
READ (n=166)	0.282	0.229	0.468	0.207	0.326	0.512
SARC (n=260)	-0.073	-0.194	-0.03	0.024	0.132	0.452
SKCM (n=471)	-0.07	-0.036	0.072	-0.067	0.104	0.2
SKCM-Metastasis (n=368)	-0.145	-0.084	-0.005	-0.132	0.004	0.119
SKCM-Primary (n=103)	-0.024	0.02	0.163	0	0.226	0.319
STAD (n=415)	0.104	0.166	0.545	0.12	0.359	0.532
TGCT (n=150)	0.014	0.416	0.397	0.127	0.293	0.34
THCA (n=509)	0.03	0.131	-0.033	-0.17	-0.061	0.003
THYM (n=120)	-0.408	0.191	-0.197	-0.182	-0.003	0.191
UCEC (n=545)	0.315	0	0.351	0.346	0.308	0.621
UCS (n=57)	-0.044	0.401	0.577	0.047	0.342	0.605
UVM (n=80)	0.15	0.26	0.269	0.14	-0.001	0.148

Spearman's ρ : positive correlation (p<0.05, ρ>0)
Spearman's ρ : negative correlation (p<0.05, ρ<0)
Spearman's ρ : not significant (p>0.05)

FIG. 5: Correlation analysis between macrophage infiltrates and expression levels of immunosuppressant genes in diverse cancer types. Correlation levels are expressed as purity-adjusted Spearman's rho values. When a particular cell on the heatmap is clicked, a scatter plot will pop out to present the relationship between infiltrate estimation value and gene expression. Data were considered significant when $P < 0.05$.

that were refractory to conventional treatments. In addition, immunotherapy is a targeted therapy that is not manifested by various side and toxic effects observed with chemotherapy or radiation. For solid tumors, the TME dictates the efficacy of the anti-tumor response. Several mechanisms have been identified that underlie the immunosuppressive nature of the TME due to its complex constituents of cellular and liquid mediators. These constitute the infiltrating cells (macrophages and TAMs, MDSCs, CAFs, Treg, CD4 and CD8 T cells, NK cells, Dcs, Ecs, etc.) and a variety of chemokines, cytokines and other molecules that establish a network whose end result is to maintain tumor growth and survival and by inhibiting the anti-tumor response.

A major cellular constituent in the TME was found to be TAMs that represent an average of > 50% of the cellular infiltrates. These TAMs regulate tumor growth and inhibit T-cell mediated anti-tumor response and their elimination or reprogramming has been shown to restore immunity and tumor regression in many experimental cancer models.

The bioinformatic analyzes presented herein are in line with other scientific findings demonstrating how macrophage infiltrates are associated with a lower response to immune checkpoint inhibitors. Recently, Mehta and colleagues have widely described the immunosuppressant roles of TAMs in breast cancer highlighting how macrophages can prompt breast cancer progression through the promotion of tumorigenesis, the facilitation of angiogenesis, the promotion of metastasis, the inhibition of antigen presentation and the suppression of T cell function.[138] Similarly, DeNardo and Ruffell have elegantly described the direct and indirect mechanisms of T cell regulation in different tumors including prostate cancer, pancreatic cancer, hepatocellular carcinoma, ovarian cancer and melanoma.[55] These observations, together with several studies already reported in the literature, suggest that precise characterization of macrophage infiltrates within the tumor bulk could give important prognostic information for the administration of ICIs and the development of tailored anti-cancer treatments.

Several approaches have been devised to inhibit TAMs in the TME that include their depletion, reprogramming them to the anti-tumor M1 phenotype

and such approaches were shown to be effective in restoring the anti-tumor response and inhibition of tumor growth and metastasis in experimental cancer models. Of interest are the findings that targeting TAMs resulted in the therapeutic response to checkpoint inhibitors in cancer resistant to such inhibitors. However, while the preclinical models were significant, clinical applications in cancer patients are still at an early stage in clinical trials.

There are many questions regarding targeting TAMs and immunity that need to be considered for translational applications in cancer patients. A few of these are discussed below.

- The TAM population is not homogeneous and is distinctly different in different tumors and there are various subsets of TAMs that may function differently in the TME of individual cancers. Also, the subsets of TAMs in primary tumors may be different in metastases. More research is needed to sort out these differences and design more specific targeting agents for different cancers.
- An important area of investigation is the role played by TAMs in CSCs and whether such TAMs are distinct from those in the TME and need to develop novel therapeutic strategies.
- TAMs are not the only immunosuppressive cells in the TME and other immunosuppressive cells may be the dominant ones and targeting TAMs may not be effective by itself and may require targeting other immunosuppressive cells. This will depend on the tumor studied and the stage of the intervention.
- It must also be considered that targeting TAMs may not be a good approach in some immunotherapeutic strategies such as vaccination.
- It is not clear what is the optimal therapeutic approach due to the heterogeneity of TAMs and their role in the tumor is dependent on the environmental conditions.
- It has been shown that checkpoint inhibitors such as anti-PD-1 antibodies not only acts on the CD8 T cell but also TAMs express PD-1 to maintain an immunosuppressive M2 phenotype and is a useful target and reprograms the TAMs into the M1 phenotype. However, TAMs also express other inhibitory receptors (LAG-3, TIGIT) and it is not clear how these receptors will influence the response to anti-PD-1 treatments.
- Although experimentally targeting TAMs as a monotherapy was effective in several tumor models, combination treatments were more effective. Therefore, it is important to determine which combination is appropriate for certain cancers and make the correct choices.
- Although we are recently considering individual medicine, the challenge remains as how to determine the therapeutic approach for particular individual patients.
- Some patients may be refractory to TAMs targeting therapies due, for example, to gene mutations. For instance, certain single nucleotide polymorphisms in the CSF-21R decreases the efficacy of emactuzumab.
- There are also basic research questions that need to be examined such as the molecular mechanisms of TAM development, the key factor responsible for the phenotypic changes of TAMs in the TME, and the molecular regulation of the expression of checkpoint inhibitory receptors and ligands.

We feel very optimistic in the forthcoming advances in targeting TAMs and other immunosuppressive cells in the TME and their use in combination with other therapies in leading to new advances in the treatment of unresponsive tumors and significant prolongation of survival and minimal side effects.

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