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Review

Tumor-induced perturbations of cytokines and immune cell networks ☆

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ABSTRACT

Until recently, the intrinsically high level of cross-talk between immune cells, the complexity of immune cell development, and the pleiotropic nature of cytokine signaling have hampered progress in understanding the mechanisms of immunosuppression by which tumor cells circumvent native and adaptive immune responses. One technology that has helped to shed light on this complex signaling network is the cytokine antibody array, which facilitates simultaneous screening of dozens to hundreds of secreted signal proteins in complex biological samples. The combined applications of traditional methods of molecular and cell biology with the high-content, high-throughput screening capabilities of cytokine antibody arrays and other multiplexed immunoassays have revealed a complex mechanism that involves multiple cytokine signals contributed not just by tumor cells but by stromal cells and a wide spectrum of immune cell types. This review will summarize the interactions among cancerous and immune cell types, as well as the key cytokine signals that are required for tumors to survive immunoediting in a dormant state or to grow and spread by escaping it. Additionally, it will present examples of how probing secreted cell–cell signal networks in the tumor microenvironment (TME) with cytokine screens have contributed to our current understanding of these processes and discuss the implications of this understanding to antitumor therapies.

Contents

Abbreviations: APC(s), antigen presentation cell(s); ARG1, arginase-1; Breg(s), regulatory B (cells); CAF(s), cancer-associated fibroblast(s); CD, cluster of differentiation protein; CD40L, cluster of differentiation protein 40 ligand; C/EBP, CAAT/enhancer binding protein; CCL, C–C motif (chemokine) ligand family member; CCR, C–C motif (chemokine) receptor family member; COX, cyclooxygenase; CTL(s), cytotoxic T lymphocyte(s); CTLA-4, cytotoxic T lymphocyte-associated protein-4; CXCL, C-X-C motif (chemokine) ligand family member; CXCR, C-X-C motif (chemokine) receptor family member; CSF, colony stimulating factor; DC(s), dendritic cell(s); ECM, extracellular matrix; EGF, epidermal growth factor; ELR+, contains amino-acid sequence "glutamate–leucine–arginine"; ENA-78, neutrophil activating peptide 78 (CXCL5); FasL, Fas ligand (TNFSF6); G-CSF, granulocyte colony stimulating factor (CSF3); GM-CSF, granulocyte-macrophage colony stimulating factor (CSF2); Gr-1, granulocytic myeloid marker protein; GRO, growth related oncogene α (KC/CXCL1), β (MIP-2/CXCL2) and/or γ (CXCL3); HIF-1α, hypoxia-induced factor-1α; HNSCC, head and neck squamous cell carcinoma; IDO, indolamine 2,3-deoxygenase; IFN, interferon; IgA, immunoglobulin A; IGF-1, insulin-like growth factor 1; IκB, inhibitor of kappa B; IL, Interleukin; IP-10, IFN-γ-induced protein 10 (CXCL10); IRF, interferon regulatory factor; LGALS, galectin (lecithin, galactoside-binding, soluble protein); KC, keratinocyte chemoattractant (mouse CXCL1); M1, type 1 macrophage; M2, type 2 macrophage; M-CSF, macrophage colony stimulating factor (CSF1); MCP-1, monocyte chemoattractant protein-1 (CCL2); MDSC(s), myeloid-derived suppressor cell(s); MHC, major histocompatibility complex; MIG, monokine induced by IFN-γ protein (CXCL9); MMP, matrix metalloproteinase; MPO, myeloperoxidase; MyD88, myeloid differentiation primary response gene 88; N1, type 1 neutrophil; N2, type 2 neutrophil; NF-κB, nuclear factor kappa B; NK(s), natural killer (cells); NK-T(s), natural killer T (cells); NO, nitric oxide; NOS2, nitric oxide synthase 2; PD-1, programmed death receptor 1; PD-L1, programmed death receptor 1 receptor; PGE_2, prostaglandin E_2; RNOS, reactive nitrogen oxide species; ROS, reactive oxygen species; SDF-1, stromal cell-derived factor 1 (CXCL12); SMAD, homolog of *Drosophila* "mothers against decapentaplegic" protein; TAAs, tumor-associated antigens; TCR, T-cell receptor; TGF, transforming growth factor; TGFBR2, type II TGF-β receptor protein; Th0, immature T helper cell precursor; Th1, type 1 T-helper cell; Th2, type 2 T-helper cell; Th17, type 17 T-helper cell; TIMP, tissue inhibitor of metalloproteinases; TME, tumor microenvironment; TNF, tumor necrosis factor; TNFRSF, TNF receptor superfamily member; TNFSF, TNF superfamily member (ligand); Treg(s), regulatory T (cells); TAM(s), tumor-associated macrophage(s); TAN(s), tumor-associated neutrophil(s); VEGF, vascular endothelial growth factor

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1. Introduction

Tumors are not homogenous population of cancerous cells; instead, tumors are heterogeneous groups of cells from diverse origins, such as stem cells, stromal cells, endothelial cells and a wide range of immune cells [1]. These heterogeneous populations secrete multiple signals that, individually and collectively, may promote or hinder any or all of the hallmarks of cancer required for tumor growth, development, and progression, as well as the enabling characteristics of genomic instability and tumor-promoting inflammation [2]. The immune system is constantly patrolling the body for foreign invaders and aberrant cells to destroy, a process commonly referred to as immunosurveillance [3]. Thus, to remain viable and to continue to grow and thrive, tumors must not just blunt the antitumor immune response in its microenvironment, the growing tumor must maintain other hallmarks of cancer, such as avoiding apoptosis and maintaining self-sufficiency of growth signals, inflammation, and angiogenesis [4].

Tipping the balance of immunosurveillance from tumor elimination to tumor promotion appears to be a complex process that spans multiple signal pathways that can be influenced by cytokine expression from tumor cells [5], immune cells [6], and other non-cancerous cell types, such as epithelial cells or cancer-associated fibroblasts (CAFs) [7], in the surrounding tissue. Thus, net cytokine signal inputs from multiple cell types in the tumor microenvironment (TME) can tip the overall balance in immunoediting from tumor-promoting to tumor-suppressing, or vice versa. As we described in a previous review, cytokine biology is quite complex [8]. How, then, does one go about decoding such a complex network of multiple signals (some of which may be additive, antagonistic, or synergistic) that may originate from multiple potential sources and pleiotropic effects on the heterogeneous cell types in the TME?

A proteomic technique that has shown great promise in detecting key cytokines in cancers and in decoding tumor-related cytokine signal networks is the multiplexed immunoassay [9–13]. The two major types of multiplexed immunoassays are antibody arrays (wherein capture antibodies are printed on a planar solid support, such as membranes, glass slides, or microtiter plates) and bead-based assays, in which capture antibodies are attached to fluorescently-tagged beads. The capabilities, challenges, advantages, and disadvantages associated with these various multiplexed immunoassay technologies—compared with one another and with more traditional approaches to proteomics and genomics—have been well documented in the literature [8,13–17] and are beyond the scope of this review.

However, the salient aspects of these methods that make them useful are the sensitivity and specificity of immunoassays and the ability to detect multiple proteins as once. Low-density bead-based assays and antibody arrays may detect a dozen or so targets simultaneously, but high-density (high-content) antibody arrays can potentially screen for hundreds of proteins in as little as 20 μl of sample. Thus, targeted screens for known inflammatory, angiogenic, apoptotic, or growth factors can help identify key biomarkers related to cancer hallmarks from among many potential candidates. High-density screens that allow for detection of cytokines with diverse functions may reveal crucial but unexpected expression of key factors by one or more cell types. Moreover, the heterogeneous nature of tumors lends itself naturally to analysis of the collective secretion profiles within tumor lysates or to dissect secretion profiles of single cell types (cancerous or non-cancerous) or co-cultures in vitro to discover which cells are contributing these critical signals and which cells are receiving them. After key factors are identified by these cytokine screens, more traditional methods of cell biology, molecular biology, and genetics can be used to complement these results, confirming and further dissecting and defining the complex network of secreted cell–cell signals and intracellular signaling pathways that contribute to the biological process being studied. Thus, antibody arrays are extremely powerful tools for the identification of cancer-specific biomarkers either secreted by cells in the local microenvironment or by the cancer cells themselves.

The balance of this review will summarize much of what is known of the cellular and secreted signaling networks that constitute the native immune response to tumors and those that allow tumors to escape immunoediting, as well as presenting some selected examples of how multiplexed immunoassays, in conjunction with standard techniques of molecular and cellular biology, have helped to shed light on these complex processes. Unanswered and remaining questions will be identified and implications of the mechanisms of antitumor immune response and tumor-induced immunosuppression on strategies for antitumor therapies will be discussed.

2. Native antitumor immune response

2.1. Coley's observation: shared mechanisms of anti-bacterial and antitumor immunity

The idea that the immune system could scan the body for microbial pathogens and aberrant cancer cells and subsequently employ innate and adaptive immune responses to eliminate them is not new; this theory was conceived more than 100 years ago by Paul Erlich [18]. In the 1890s, physician William B. Coley had observed that tumors shrank in some cancer patients with incident bacterial infections, and many of these patients appeared to be completely cured [19]. However, it was not immediately apparent whether the mechanisms of immunity used to fight pathogens would be the same as for tumor clearance.

More recently, Agrawal, et al., used a xenograft model in mice to test Coley's observation and found a molecular connection between bacterial infection and shrinking tumors [20]. In their study, mice bearing subcutaneous tumor xenografts (colorectal or renal cell carcinoma) were injected with spores of *Clostridium novyi-NT*, an obligate anaerobe. This initiated *C. novyi-NT* infection exclusively within tumors, and hemorrhagic necrosis of tumors began within 24 h. After the bacterial infection resolved, tumors returned in 66% of mice, but the other 34% remained tumor free for at least 60 days. Similar results were obtained using tumor-bearing rabbits. Moreover, this effect was long-lasting, as 80% of mice and 100% of rabbits cured of tumors by *C. novyi-NT* inoculation resisted further tumorigenic challenges for months after the initial treatment. Cytokine antibody array analysis, screening for expression of 32 different proteins at once, revealed that *C. novyi-NT* infection in tumor-bearing mice increased serum levels of several inflammatory markers, including IL-6, MIP-2 (GRO-β)/CXCL2, G-CSF/CSF3, TIMP-1, and KC (GRO-α)/CXCL1. These cytokines also attracted a massive influx of pro-inflammatory cells, largely neutrophils, followed within a few days by monocyte and lymphocyte

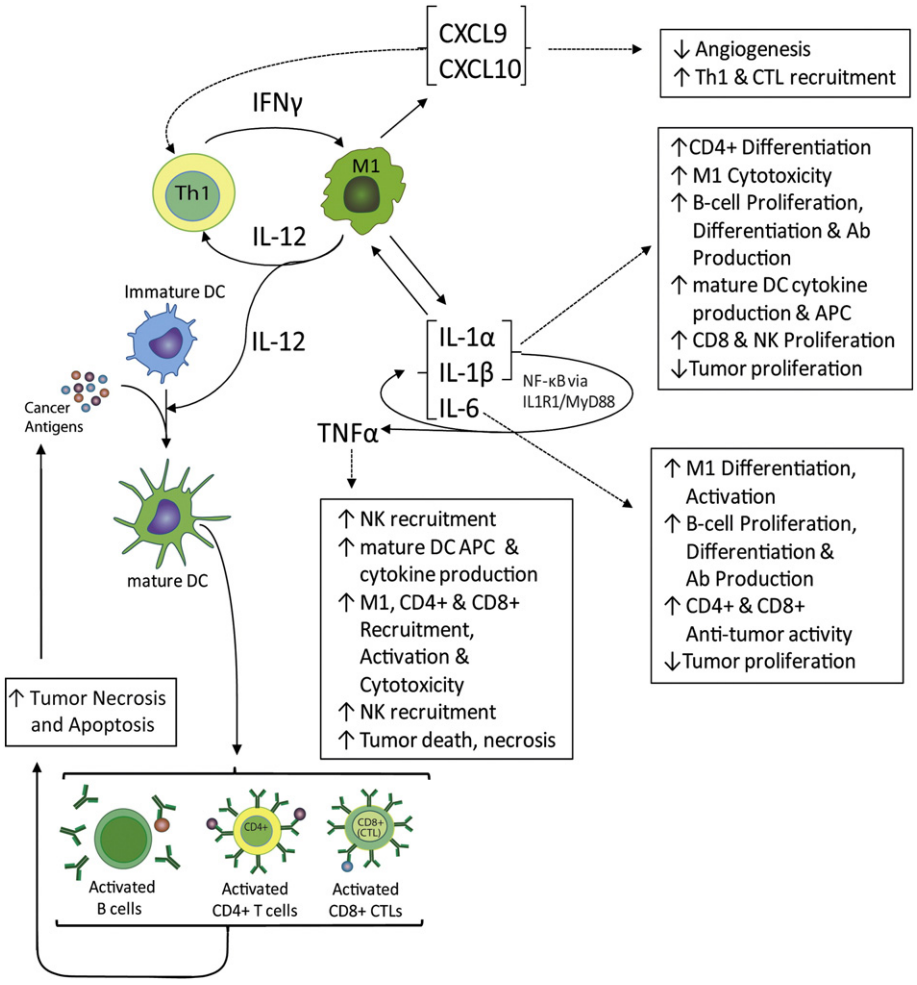

Fig. 1. A model of tumor-suppressing inflammation. Th1 lymphocytes and M1 macrophages are the primary sources of pro-inflammatory cytokines that also promote cancer immunosurveillance and cytotoxicity. Their interactions are mutually reinforcing: Secretion IFN-γ by Th1 cells results in the recruitment of M1 macrophages and maintenance of M1 phenotype, while IL-12 produced by M1 macrophages recruits, activates and maintains a Th1 phenotype. Secretion of MIG/CXCL9 and IP-10/CXCL10 also promotes the recruitment of Th1 cells and CTLs and inhibits angiogenesis. IL-1α, IL-1β and IL-6 form an autocrine feedback loop by stimulation of myeloid differentiation primary response gene 88 (MyD88)-mediated activation of NF-κB signaling. TNF-α, also released by the activation of NF-κB signaling, which activates APC functions of DCs and the recruitment and cytotoxic activation of M1 macrophages, effector CD4+ T cells, and CD8+ cells, as well as the recruitment of NK cells.

infiltration. These, in turn, initiated a cell-mediated tumor immune response, primarily by cytotoxic T cells, characterized by the expression of cluster of differentiation protein 8 ($CD8^+$) on their surface, that inhibited tumor growth even when transplanted to uninfected tumor-bearing mice.

2.2. A model of native antitumor inflammation

A wide range of "antitumor" immune cells are known to support the clearance of tumor cells, including T helper type 1 (Th1) cells, cytotoxic $CD8^+$ T lymphocytes (CTLs), natural killer (NK) cells, natural killer T (NK-T) cells, type 1 macrophages (M1), type 1 neutrophils (N1), eosinophils, and mature dendritic cells (DCs) [21–24]. However, some cancers appear to benefit from increased numbers of Th1 cells, and paradoxical roles for CTLs and NK-T cells in cancer have also been reported [21,22,25,26]. A model of the immunostimulatory effects of Th1 and M1 cells resulting in antitumor inflammation was recently published by Haabeth et al., in which they depicted results of their studies of the cytokine profiles of infiltrating immune cells in a collagen gel matrix [21,27]. The cytokines trapped in the gel were measured by cytokine multiplexed bead assays, and the immune cell types were characterized by cell surface antigens using flow cytometry or immunohistochemistry (IHC). Using this approach, they discovered that tumor-suppressive inflammation in the early immune response is regulated by a relatively small number of cytokines (summarized in Fig. 1).

Th1 and M1 cells were the predominant T helper and macrophage phenotypes in the TME of the collagen matrix. Production of IL-1α and IL-1β from M1 cells enhanced B cell proliferation and antibody production, increased antigen-presentation cell (APC) capabilities of DCs, synergized with IL-2 to stimulate proliferation of cytotoxic cells and the proliferation and differentiation of $CD4^+$ effector T cells, and increased the cytotoxicity of M1 cells by an autocrine (positive feedback) loop via stimulation of MyD88-mediated activation of NF-κB signaling. IL-6 also stimulated B cell proliferation, differentiation, and antibody production, increased the antitumor activity of $CD4^+$ and $CD8^+$ T cells, and promoted the differentiation and activation of M1 cells by an autocrine loop. MIG/CXCL9 and IP-10/CXCL10 increased the recruitment of Th1 and CTLs to the gel matrix and inhibited angiogenesis. TNF-α, also up-regulated by the activation of NF-κB signaling, was important for the activation of APC functions of DCs and the recruitment and cytotoxic activation of M1 macrophages, effector CD4 + T cells, and CD8 + cells, as well as the recruitment of NK cells. When stimulated by IFN-γ and TNF-α, Th1 cells are efficient at arresting cancer progression [25,28]. Additionally, Th1 and M1 cells mutually reinforced one another, with Th1-produced IFN-γ driving the differentiation and activation of the M1 phenotype while M1-produced IL-12 reinforced the Th1 phenotype. This model clearly illustrates the importance of Th1 and M1 cells in antitumor immunity, which has been well established in the literature [21,25]. Importantly, levels of M1 macrophages and Th1 lymphocytes in the TME have been positively correlated with prognosis and survival times in many cancers [29–32].

While it is clear that induction of a vigorous inflammatory response is one of the primary mechanisms of native immune responses to tumors, the inability to resolve chronic inflammation is widely considered one of the primary causes of carcinogenesis. About 20% of cancers are linked to inflammation associated with chronic infections [33]. In stomach cancer, for example, the most established risk factor is chronic gastric infection by *Helicobacter pylori* [34]. Chronic inflammation in the TME is a major contributor to angiogenesis, invasion and metastasis [23,35]. In addition to enhancing the proliferation of mutated cells, inflammatory microenvironments can also increase mutation rates. Activated inflammatory cells serve as sources of reactive oxygen species (ROS) and reactive nitrogen oxide species (RNOS) that are capable of inducing DNA damage and genomic instability [23,26]. The clinical significance of this was recently confirmed in a study that reported lower rates of both somatic genomic anomalies and progression to esophageal adenocarcinoma in patients with Barrett's esophagus who routinely took non-steroidal inflammatory drugs (NSAIDs) [23,36].

2.3. Immunosurveillance & immunoediting: elimination, equilibrium and escape

A second mechanism of antitumor immunity is known as immunosurveillance: the identification and elimination of tumor cells by the immune system. This was first proposed in the 1950s by Sir Macfarlane Burnet and Lewis Thomas (reviewed in [3]). Results of early testing of the immunosurveillance concept in mouse models, primarily in immunocompromised mice, such as nude mice, were either inconclusive or contradictory to its central predictions, and by the late 1970s the concept was all but abandoned. By the late 1990s, however, following the discovery of cytotoxic cells, such as NK cells [37], the elucidation of the critical role of antigen presentation by DCs [38] and the development of severe combined immune-deficient (SCID) mice [39], studies of mechanisms of antitumor immunity that were dependent on IFN-γ [40], perforin [41,42], and T-cell receptor (TCR) interactions with antigens bound to the surfaces of APCs by MHC proteins [43] presented strong evidence for cancer immunosurveillance due to both humoral and adaptive immune responses. The prominent role of immunosurveillance in the natural defenses opposing tumorigenesis was illustrated by significant increases seen in spontaneous tumor incidence in immunodeficient mice strains, such as SCID mice, which collectively represent disruptions in more than two dozen immune-related genes [44,45].

Activation of both $CD8^+$ cytotoxic T lymphocytes (CTLs), including αβ T cells, γδ T cells, NK cells, NK-T cells, and effector ($CD4^+$) T cells by antigen presentation is critical for tumor elimination via immunosurveillance [25]. Moreover, infiltration of CTLs and effector T cells in the tumor microenvironment is a critical factor in prognosis for many cancers [29,30,32]. Two separate signals are required to activate the adaptive immune response in naive T cells (see Fig. 2). The first is generated by TCR on naive T cells binding to an antigen presented by an MHC expressed on the surface of another cell. Antigen presentation by mature DCs (Fig. 2A) is considered to be critical for potent antitumor immune responses by $CD4^+$ T cells. CD4 on $CD4^+$ T cells bind to class II MHC (MHC-II), which can be expressed on any cell type, but primarily by cells specialized for APC (macrophages, B cells, and DCs). TCRs and CD8 on CTLs bind to class I MHC molecules (MHC-I), which are expressed on the surface of all mammalian cells except red blood cells (Fig. 2B). The second signal is provided by interaction of CD28 protein on the T cell with B7-1 (CD80) or B7-2 (CD86) on the surface of the APC. Failure of the T cell to receive the second signal generally results in T cell anergy (inactivation). This can also be accomplished by interaction of the CD28 ligands (CD80 and/or CD86) with a co-inhibitory molecule, CTLA-4; this is part of the natural fail–safe mechanism to limit overactive immune responses that could lead to an auto-immune disease. As we shall see in Sections 7.1 and 7.2, CTLA-4 is a target for immunotherapy that is generating much interest because it is almost exclusively expressed on T cells, including immunosuppressive regulatory T cells (Tregs) [46,47].

Effective antitumor immune responses lead to the targeting and elimination of tumor cells and, ultimately, shrinkage of the tumor. However, immunosurveillance can create selective pressure for tumor cells that may suppress certain cell surface markers or otherwise gain the capability to persist in equilibrium state (dormancy) or to escape antitumor immune responses entirely, leading to tumor progression. Collectively, the processes of immunosurveillance and its three possible outcomes—elimination, equilibrium and escape—are referred to as immunoediting (see Fig. 3) [3,48]. Both innate and adaptive immunity appear to be involved in elimination, and inhibition of both innate and adaptive immunity is important for the ultimate success of tumors achieving escape, but it is the adaptive immune system that is primarily responsible for maintaining occult tumors in equilibrium [45].

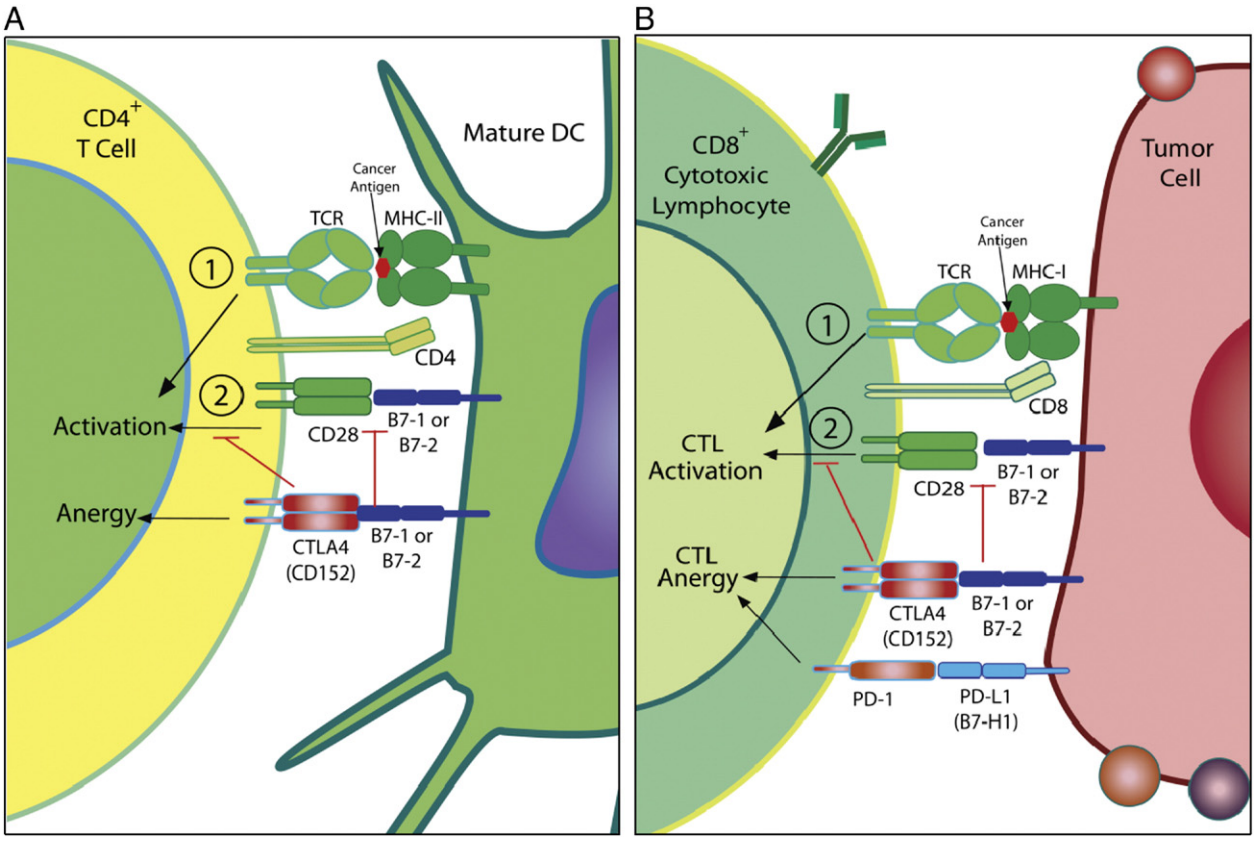

Fig. 2. Antigen presentation and T cell anergy. T lymphocytes become activated via a two-signal process in which 1) T-cell receptor must bind to an antigen presented on a major histocompatibility complex (MHC) protein expressed on the cell surface of another cell, and 2) CD28 must concurrently bind with its ligands, B7-1 or B7-2. Without this second signal, T cell anergy (inactivation) will result, and the adaptive immune response is inhibited. As part of a fail–safe mechanism to prevent over-active autoimmune responses, T cell anergy may also result if the co-inhibitory receptor cytotoxic T lymphocyte-associate protein-4 (CTLA-4) competes to bind CD28 ligands or if programmed death-1 (PD-1) receptor binds its ligand, PD-L1. A) CD4 antigens on naive CD4+ lymphocytes (activated T cells and T helper cells) bind specifically to type II MHC (MHC-II, which can be expressed on any cell, but interactions with dendritic cells (DCs) are professional antigen presenting cells (APCs) are critical for antitumor immunity; B) CD8 antigens on naive CD8+ cytotoxic lymphocytes (CTLs) bind specifically to type I MHC (MHC-I), which can be expressed on any cell except red blood cells. Tumor cells may present their own antigens on their cell surface via MHC-I, albeit poorly. Other antigen presenting cells, such as M1 macrophages or B cells may also activate CTLs via MHC-I-mediated APC activity.

3. Cancer wars: the tumors strike back

As evident by Coley's observation and subsequent investigations, inflammation and immunosurveillance can cooperate to suppress tumors. However, just as normal cells use cytokines to regulate native immune responses, tumor cells have developed mechanisms to escape them. These effects are not systemic, but localized; tumor cells create immunosuppressive zones within the TME [22,49]. How then is the balance of immunoediting altered in the TME to suppress antitumor immune response and to promote tumor progression? The key to tumors gaining the ability to escape immunosurveillance appears to be four-fold: 1) by altering expression of cell surface markers used by the adaptive immune system to identify and to target them; 2) by acquiring resistance to apoptotic or necrotic mechanisms normally induced by cytotoxic cells as a result of being targeted; 3) by directly suppressing the adaptive and innate immune response of activated T cells and cytotoxic immune cells; and 4) by altering the balance of both adaptive and innate immune cell populations in the TME to maintain tumor-induced immunosuppression. A fifth possible mechanism of tumor "escape" is metastasis. Just as immunoediting can put selective pressure on cancer cells to alter cell surface markers, there is a growing body of evidence that, particularly in advanced cancers, highly effective immunosurveillance in the TME may place sufficient selective pressure on tumor cells populations to result in the emergence of highly mobile tumor cells within the necrotic/apoptotic tumor [50]. The result may be that the remaining cells with metastatic capabilities may attempt to "escape" by evacuating the now hostile TME in search of more tumor-friendly environments.

3.1. Changes in tumor-associated antigens

One possible mechanism is for tumor cells to cease or change expression of tumor-associated antigens (TAAs) that make them easy targets for immunosurveillance. It is even plausible that this mechanism will induce a selective pressure, killing tumor cells that express TAAs and leaving only tumor cells expressing fewer immunogenic surface antigens to proliferate. This was recently confirmed using a genetically engineered mouse model that was used to monitor the onset and growth of immunogenic and non-immunogenic tumors induced in situ [51]. In immune-competent mice, primary tumors in the mice became less immunogenic over time through the selective outgrowth of cells lacking certain TAAs that enabled them to escape immunosurveillance. Moreover, results from mice deficient in antigen presentation of TAAs via MHC-I proved that APC activity was both necessary and sufficient for this immunoediting process to occur. Since tumors themselves can present TAAs via MHC-I expression, loss of molecules important to the innate antigen presentation mechanisms in tumor cells, including mutations or silencing of genes related to IFN-γ signaling (receptors or downstream effectors) can lead to a lack of immune response to IFN-γ [52].

3.2. Changes in susceptibility to cytotoxic response

A related mechanism is for the tumor cell to render themselves less susceptible to the apoptotic and necrotic effects of immune cell targeting. This might include up-regulation of anti-apoptotic proteins

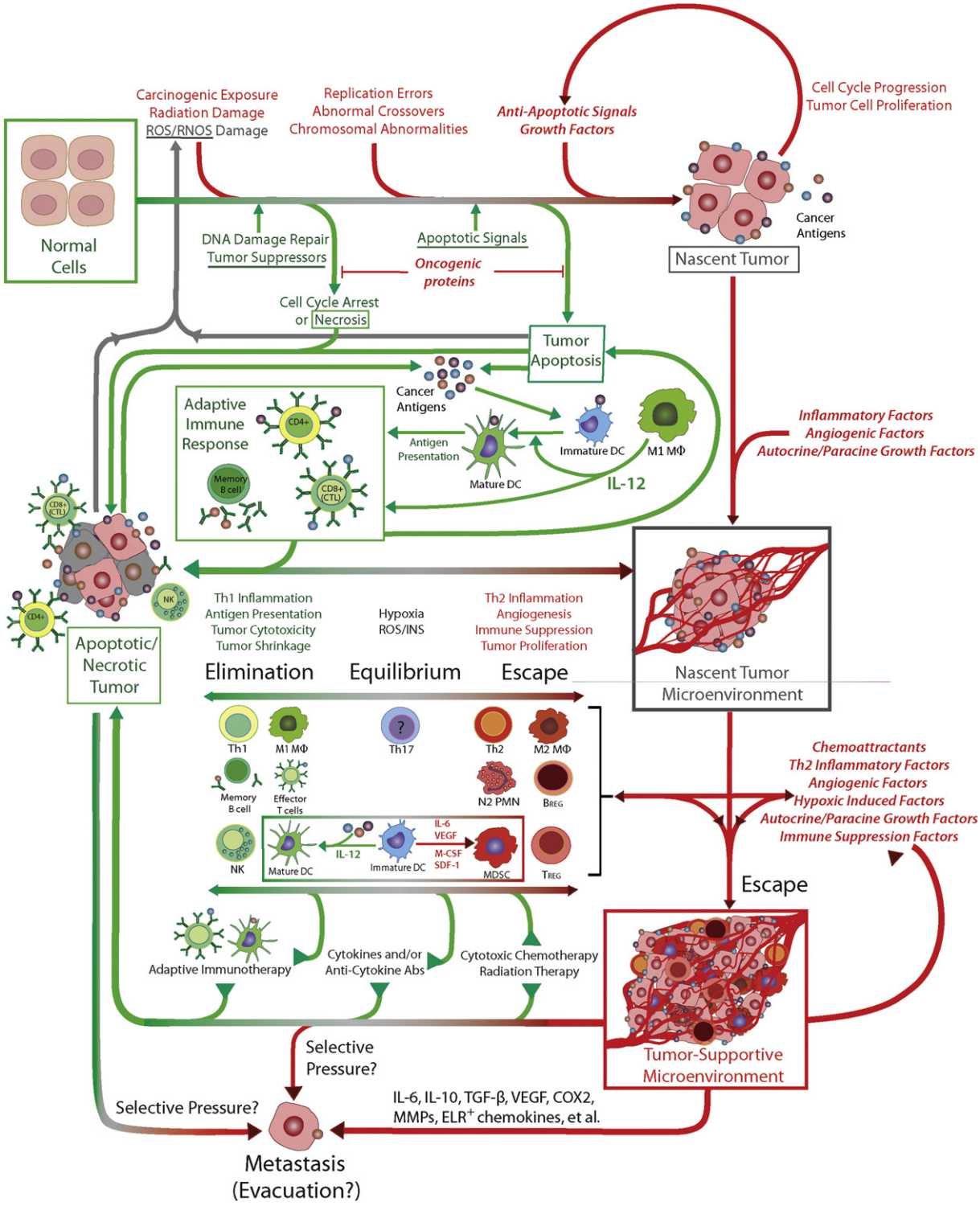

Fig. 3. A model of immunoediting in tumor progression. Normal cells may become nascent tumors by evading tumor suppression after carcinogenic mutation and/or apoptosis that would normally result from gross chromosomal changes. Pro-inflammatory and pro-angiogenic factors can help to establish blood supply for the growing nascent tumor. Activation of the adaptive or native immune response can eliminate the nascent tumor, the tumor may remain in equilibrium as an occult tumor, or the tumor may escape immunosurveillance to create a viable tumor-supportive microenvironment. Innate and adaptive immune responses may still work to eliminate the tumor via immunosurveillance. Tumors may also metastasize to move to another location; this may be an additional mechanism of avoiding immunosurveillance by evacuation of the "hostile" TME. Green color denotes processes potentially leading to tumor eradication, while red color means promoting tumor escape and progression.

or the loss or mutation of death receptors expressed on the tumor's surface, such as TRAIL receptors or Fas. These, of course, are characteristics that are related to avoiding apoptosis, another cancer hallmark, (reviewed in [4,53]). Further details of this process are beyond the scope of this review.

3.3. Direct immunosuppression of activated T cells and cytotoxic cells by tumors

Although B cell-mediated adaptive immunity is an integral part of the antitumor response, mechanisms of T cell-mediated innate and

adaptive immunity appear to be the dominant forces, and, therefore, appear to be the primary targets of tumor-induced immunosuppression. Some of these effects are directly mediated by the tumor cells themselves (reviewed in [45]). For example, many tumor cells have demonstrated increased expression of IDO, which depletes tryptophan (an important amino acid for CLT proliferation and survival of $CD4^+$ T cells) by converting it to kynurenines, which inhibits T cell activation. Secretion of galectins, such as galectin-1 (LGALS1) [54] can impede T cell activity and survival, as well as stimulate angiogenesis.

Shedding of MHC protein homologs A or B (MICA or MICB), which are ligands for NKG2D, a receptor found on NK and other immune cells, can render tumor cells unrecognizable by cells expressing NKG2D and can even decrease NKG2D expression on these cells, as these receptors are subject to endocytosis upon binding their ligand [55]. Tumors can directly inhibit immune cell activities by expression of certain molecules on the cell surface of tumors, such as HLA-G, HLA-E and PD-L1. Another component of the immune system's fail–safe mechanism to prevent over-activation is PD-1, which is expressed on T lymphocytes. If PD-1 on the surface of an activated T cell interacts with its ligand, PD-L1 (also known as B7-H1) concurrently with TCR binding to an antigen presented by MHC, T cell "exhaustion" can result in reduced immune response due to lower cytotoxic activity and/or the induction of T cell apoptosis (Fig. 4). For this reason, PD-1 has also become a target for cancer immunotherapy, which will be further discussed in Section 7.2.

Finally, cytokines that have direct immunosuppressive effects can be secreted by tumor cells themselves, including TGF-β and VEGF; the important roles of these molecules will be discussed in greater depth in Sections 5 and 7. However, it appears that tumor-directed changes in immune cell populations in the TME are the most complex and important mechanisms of tumor-induced immunosuppression.

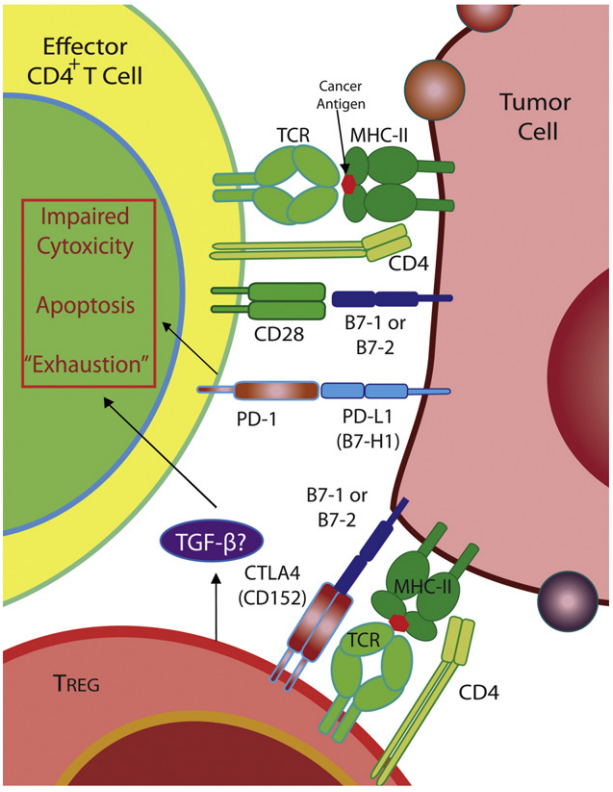

Fig. 4. T cell "exhaustion" via CTLA-4 and/or PD-1. Effector T cells (activated CD4+ T cells or T helper cells) may be "exhausted" (inactivated) by programmed death-1 receptors (PD-1) on their surface binding to PD-L1 ligands on target tumor cells. Also, regulatory T cells (Tregs) can exhaust effector T cells by CTLA-4 on their cell surface binding to B7-1 or B7-2 on tumor cells, which releases a soluble factor that causes effector T cell "exhaustion"; a likely suspect for this effect is TGF-β1.

3.4. Tumors-directed changes in immune cell populations

Tumor cells appear to recruit, "educate" and maintain populations of cancer-associated fibroblasts (CAFs), endothelial cells, and tumor-promoting immune cell types that, collectively, suppress antitumor immune cell types while maintaining sufficient inflammatory and angiogenic potential in the TME to promote tumor growth and progression. Immune cells that tend to promote tumor progression via immunosuppression include type 2 macrophages (M2), Th2 CD4+ cells, regulatory T (Treg) cells, activated B cells, and type 2 neutrophils (N2) [21,22,24,49]. Importantly, there appear to be synergistic and mutually reinforcing cytokine signal networks between tumor-suppressing immune cells, in which Th2 cells, tumor-associated M2 macrophages and N2 neutrophils (TAMs and TANs, respectively), B cells, granulocytic myeloid-derived suppressor cells (MDSCs), and Tregs all appear to play crucial roles, as summarized in Fig. 5 [21,22,25,26,56].

It should be noted that plasticity is an inherent quality of immune cells, as all of them are derived from a relatively small number of progenitor cells that all mature from a single pluripotent hematopoietic stem cell population. Also, as recruited cell populations in tumors can vary between cancer types, the following descriptions of immune cell phenotypes in the proposed model below may not hold true for every type of cancer, and novel, unexpected phenotypes of immune cell populations are likely to be encountered as their interactions in the TME are further elucidated. An example of the plasticity of immune cell phenotypes is the characterization of some unusual peritoneal macrophages (T-PEMs) in tumor-bearing mice by a group at the University of Miami; T-PEMs exhibited increased expression of granulocytic myeloid (Gr-1) markers and reduced CD11b expression, which is more a characteristic of MDSCs than macrophages, and antibody array analysis revealed that their secretion profiles were neither M1 nor M2 and were less differentiated than either phenotype [57]. T-PEMs were deficient in producing M1 inflammatory markers (IL-12 p40 and p70, IL-1β, IL-6, TNF-α, MCP-1/CCL2 and M-CSF/CSF1), but they did not exhibit an increase in expression of M2-related cytokines. The authors noted that TGF-β and prostaglandin E_2 (PGE_2) were likely suspects in the etiology of these unusual monocytes, as treatment of their progenitor cells with TGF-β and PGE_2 synergistically lowered expression of transcription factors NF-κB and CAAT/enhancer binding protein (C/EBP); similarly low levels of NF-κB and C/EBP expression were seen in cultured T-PEMs.

That being said, the immune cell phenotypes described below are employed as a useful oversimplification to illustrate complex interactions among various immune cells with each other and non-immune cell types, resulting in tumor-directed alterations of immune cell populations that suppress the native immune response in the TME.

4. Essential immune cell populations in tumor-induced immunosuppression

4.1. T helper cells: tipping the Th1/Th2 balance

An overview of T helper cell differentiation is presented in Fig. 6. Th1 and Th2 cell populations are both derived from naive, precursor $CD4^+$ T helper (Th0) cells. If Th0 cells are exposed to IL-12, they tend to differentiate into Th1 cells; conversely, Th2 cells arise from the differentiation of Th0 cells exposed to IL-4 and IL-13. Cytokines released by Th1 cells are those typically associated with cytotoxic function: TNF-α, IFN-γ, IL-2 and IL-12. Collectively, these cytokines enhance the cytotoxic capabilities of M1 macrophages, CTLs, NK cells, and NK-T cells. Th1 cytokine responses are typical of those induced by microbial infections. Th2 cells are more commonly associated with asthma, allergic responses, and

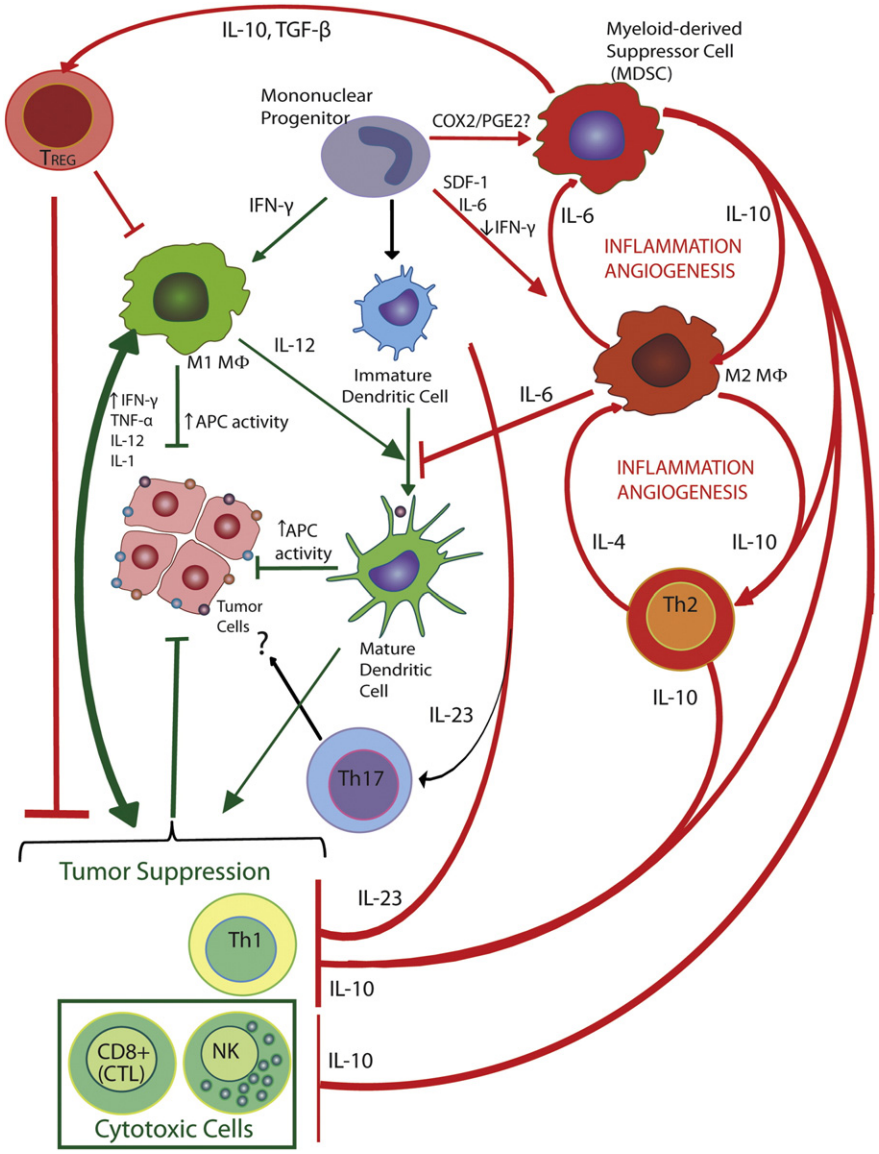

Fig. 5. Tumor-supporting immune cell interactions. Th2 lymphocytes, M2 macrophages and MDSCs mutually reinforce the proliferation and phenotypes of one another, as well as maintaining tumor-promoting inflammation and angiogenesis. These cells, along with T regulatory lymphocytes (Tregs) suppress the activity and proliferation of tumor-suppressing cells, including Th1, M1 and cytotoxic T cells and NK cells. It should be noted that M1 & M2 macrophages can interconvert, but these phenotypes are stable as the M1 and M2 expression profiles reinforce their own macrophage phenotypes, while suppressing the other. Similarly, Th1 & Th2 lymphocytes, as well as Treg & Th17 lymphocytes tend to self-reinforce their own activation profiles and inhibit the other.

immune responses to helminth infections, and Th2-expressed cytokines are characterized by high levels of IL-4, IL-5, IL-6, IL-10, and IL-13.

The ratio of Th1 to Th2 cells is commonly referred to as the Th1/Th2 balance. Importantly, Th1 and Th2 cells produce cytokine profiles that are antagonistic to one another; that is, Th1 or Th2 cells tend to release cytokines that self-reinforce the differentiation and activation of populations of T helper cells of their own phenotype and inhibit the differentiation and activation of the other. As depicted in Figs. 1 and 6, both IL-12 and IFN-γ contribute to a positive feedback loop to reinforce Th1 differentiation. Further, transcriptional factors associated with IL-12 signaling increase transcription of IFN-γ and other Th1 cytokines but inhibit transcription of IL-4, while transcriptional factors activated by IL-4 induce expression of Th2 cytokines and inhibit expression of IL-12 Rβ [58]. Moreover, Th1 and Th2 appear to be terminally differentiated cells with limited plasticity. Thus, once a population of Th1 or Th2 cells is established, they tend to be very stable. In cancer, these signals work in concert not only to suppress Th1 cell maturation and to promote Th2 maturation but also , further, to inhibit T-cell-mediated cytoxicity, to promote the humoral immune responses of B cells, and to educate tumor-associated macrophages (TAMs) [25,59].

4.2. Macrophages: tipping the M1/M2 balance

As we saw in Section 2.2, early in tumorigenesis, macrophages attracted to the TME may exhibit classically activated inflammatory phenotypes (M1) to support immune response to the nascent tumor, resulting in the secretion of large amounts of IL-12, IL-1α, IL-1β, IL-6, TNF-α, and IFN-γ by Th1 lymphocytes (see Fig. 1), as well as the induction of nitric oxide (NO) and ARG1 expression by M1 macrophages, which increases CTL cytotoxicity. However, as the tumor progresses, macrophages in the TME often tend toward an alternatively activated, immunosuppressive (M2) phenotype [60], commonly called TAMs, which have been demonstrated to be induced by exposure to IL-4, IL-13, M-CSF/CSF-1, IL-10 and TGF-β1, among other factors. TAMs support tumor growth, survival, and metastasis and are attracted to the TME by M-CSF/CSF-1, SDF-1/CCL12, and MCP-1/CCL2, which may

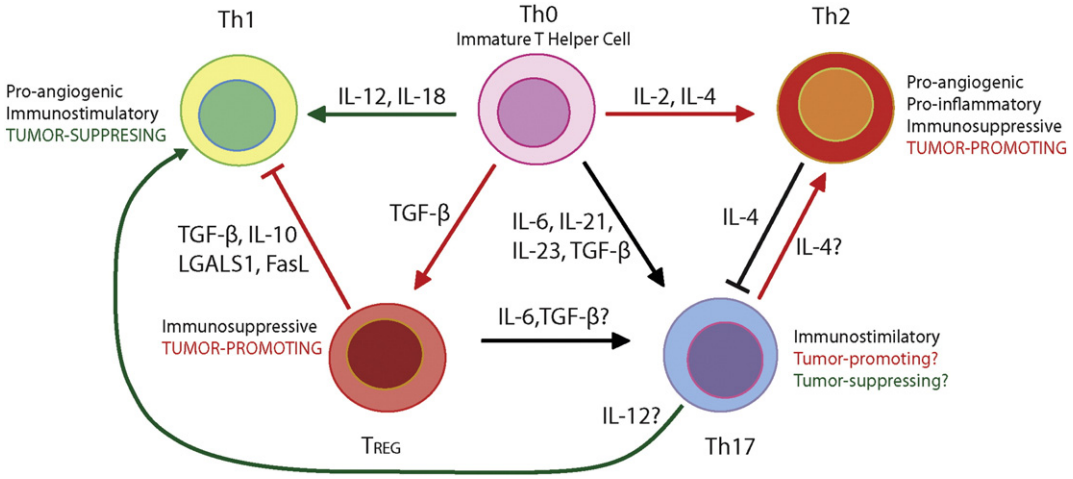

Fig. 6. T-helper cell differentiation. All T helper lymphocytes are differentiated cell types derived from the same common immature T helper progenitor (Th0). Depending upon what cytokines the Th0 cells are exposed to, they may differentiate into one of several different T helper phenotypes: type 1 T helper cells (Th1), type 2 T helper cells (Th2), T regulatory (TREG) or type 17 T helper cells (Th17). Th1 and Th2 cells are generally considered terminal, permanent phenotypes. Some in vitro evidence suggests that TREG and Th17 cells may be somewhat more plastic; the cytokines implicated in these proposed inter-conversions of T helper types are indicated by a question mark (?). However, neither reversion of any of these T helper types back to a Th0 progenitor phenotype nor conversion of Th17 cells to a TREG phenotype have been reported.

be produced by cancer cells, CAFs, or other tumor-associated cells [25,59,60]. Roca et al., examined the regulation of immature monocytes (CD11b + cells) by MCP-1/CCL2 [61]. Using cytokine antibody arrays, they demonstrated that $CD11b^+$ monocytes produce MCP-1/CCL2 and low levels of IL-6. They also discovered that expression of IL-6 and MCP-1/CCL2, two of the most common cytokines in the TME, was mutually reinforcing, wherein stimulation of $CD11b^+$ cells by MCP-1/CCL2 increased IL-6 protein secretion 5-fold and, when induced by IL-6, expression of MCP-1/CCL2 rose 2-fold. This synergistic up-regulation of IL-6 and MCP-1/CCl2 pushed $CD11b^+$ monocyte differentiation toward the M2 macrophage phenotype, and protected both cell types from cell death by inhibiting apoptosis.

M2 macrophages also typically secrete large amounts of growth factors, such as EGF, inflammatory factors (such as COX2) and angiogenic factors, including VEGF and angiogenic chemokines, into the TME. TAMs also play a prominent role in inflammation and angiogenesis, as well as in tumor progression and metastasis (reviewed in [59,62]). Higher densities of TAMs in tumors and overexpression of key instigators of M2 differentiation are considered markers of poor prognosis in a number of cancers [63].

4.3. Dendritic cells: eliminating antigen presentation

Dendritic cells (DCs) play a crucial role in the interface of adaptive immunity by engulfing foreign antigens and by migrating to lymphoid organs to present them to adaptive immune cells. Levels of antigen-presenting DCs in the TME and in peripheral blood are positively associated with good prognosis and inversely correlated with poor prognosis in many cancers [64–67]. An important inhibitor of DC-mediated tumor suppression is PGE_2, the product of COX2. The addition of COX inhibitors or neutralizing antibodies recognizing PGE_2 blocked the immunosuppressive effects of supernates from Lewis lung carcinoma cell cultures on DCs, specifically by increasing DC production of IL-10 and lowering DC expression of IL-12 [68,69]. Previous studies have demonstrated that PGE_2 supports Th2 proliferation and that Th2-associated cytokine expression both limits the production of IFN-γ directly and opposes IL-12 signaling on multiple levels [70]. Several cytokines are also known to impair tumor suppression by DCs as well, including GM-CSF/CSF2, IL-10, and VEGF [71]; all of these appear to work, at least in part, by augmenting COX2- and PGE_2-induced proliferation of MDSCs [72]. Moreover, a positive feedback loop involving COX2 and PGE2 appears to redirect development of immature DCs away from maturation as Th1-inducing DCs (DC1 subtype) and toward their differentiation as tumor-promoting MDSCs [72]. Thus, the inhibition of DC maturation may prove to be a critical aspect of tumor escape, as it blunts antigen presentation and cytotoxic responses by DCs, as well as providing more immature DC progenitors to reinforce M2 macrophage and Th2 T-cell phenotypes in the TME. COX2 and PGE_2 are also major contributors to tumor-promoting inflammation and angiogenesis, two processes in cancer progression that appear to be interdependent and, therefore, inextricably linked (see Sections 5.4 and 7.4) [47,73,62]. Finally, tumor-educated, immature ($CD11c^+/CD11b^+$) DCs are also significant source of TGF-β and appear to be essential to the proliferation of TREGS via the induced secretion of TGF-β [74].

4.4. Immature myeloid cells: MDSCs

MDSCs are elevated in virtually all patients and experimental mice with malignancies and have been linked to both inflammation and tumor-directed suppression of immune response [75]. MDSCs are a heterogeneous group of cells, but in mice, all of them appear to share expression on their surfaces of the granulocytic myeloid (Gr-1) markers Ly6C and/or Ly6G, which are typically characteristic of macrophages and macrophage marker CD11b ($Gr\text{-}1^+CD11b^+$). MDSCs are known to inhibit the proliferation of CD4 + and CD8 + T cells, to block the activation of NK cells, and to polarize T helper maturation to a Th2 (tumor-promoting) phenotype. Recent reports show that COX2, PGE2, and IL-6 may have a role in the differentiation and proliferation of MDSCs, but beyond that, little is known of the cytokine or other secreted factors that may lead to the differentiation of MDSCs from less differentiated monocyte progenitors [72,76]. However, an inverse relationship between MDSCs and DCs is clear. MDSCs and DCs do share a common progenitor: MDSCs can be converted to DCs with all-trans retinoic acid. Proliferation of MDSCs also seem to be inversely correlated with levels of mature DCs in cancer, as was noted by Mattei, et al., in a mouse strain deficient in the transcription factor IRF-8. In IRF-8-deficient ($Irf8^{-/-}$) mice, melanoma cells grew more rapidly, leading to higher number of lung metastases [77]. Also, $Irf8^{-/-}$mice exhibited poor homing of DCs and T cells to tumors, while exhibiting larger numbers of MDSCs in the spleen and tumors. They also confirmed by antibody array and quantitative PCR that IL-3 and IL-6 expressions in splenocytes from $Irf8^{-/-}$ mice were significantly increased, but that expression of IL-10

was decreased. Supplementation of in vitro cultures of melanoma cells with IL-6 and/or IL-27 demonstrated that treatment of with IL-27 could increase IRF-8 production and that co-treatment with IL-6 and IL-27 was synergistic.

MDSCs also have a key role in M1/M2 balance [78]. M1 macrophages are activated by IFN-γ and express low levels of IL-10 and high levels of IL-12, which reinforces Th1 populations and drives maturation of DCs. IL-12 has been shown to reverse M2 phenotypes in TAMs and to induce tumor regression associated with the appearance of activated NK and tumor-specific CTLs [79]. MHC-II also supports a role for macrophages in antigen presentation, which may not be as prominent or important as that for DCs. However, in the presence of IL-10, direct contact between MDSCs and M1 macrophages causes down-regulation of IL-12 transcription and MHC class II (MHC-II) expression in macrophages, which effectively shuts down APC activity in DCs and macrophages, profoundly limiting immunosurveillance by the adaptive immune system [78]. MDSCs also inhibit the cytotoxic functions of NK, B and T cells by the secretion of arginase-1 (ARG1) and NOS2 in the TME [80]. ARG1 depletes L-arginine, a critical factor in T-cell activation and a precursor of nitric oxide (NO) production by NOS2. When NOS2 activity is high and L-arginine levels are low, instead of producing NO, NOS2 starts producing ROS and RNOS, which results in inflammation, T-cell anergy and carcinogenic DNA damage to the surrounding tissue. Some groups have reported that MDSCs promote tumor development by enhancing angiogenesis and by inhibiting T lymphocyte-mediated antitumor immunity [22]. A xenograft model of lung metastases induced by 4T1 breast cancer cells in BALB/c mice confirmed the important role that MDSCs play in tumor immune suppression [81]. ELISPOT analysis demonstrated that MDSCs significantly lowered IFN-γ expression in tumor-bearing tissues. IFN-γ is a potent antitumor cytokine, suggesting that MDSCs play a major role in tumor-induced immunosuppression. MDSCs also help the proliferation of immunosuppressive T cells commonly called TREGS; the clonal expansion of TREGS is dependent on TGF-β and IL-10 secreted by Gr-1$^+$CD11b$^+$ MDSCs [74]. High levels of MDSCs in the TME and peripheral blood have been correlated with poor prognosis and shorter median survival times for several distinct cancer types [76].

4.5. Regulatory T helper cells: TREGS

Regulatory T (TREG) cells play an important role in preventing immune responses to self-antigens, but increased numbers of TREG cells in peripheral blood and TMEs are commonly seen in patients with invasive and metastatic cancers [82]. TREGS are generally identified as T cells that express CD4, CD25 and FOXP3 proteins on their surfaces (CD4$^+$CD25$^+$FoxP3$^+$ T cells) that are normally found in lymphoid tissues; however, they are preferentially recruited to tumors and associated ascites via MDC/CCL22—presumably by binding to CCR4, which is also expressed on TREGS—and are a significant predictor of poor patient prognosis [83]. CD40 expression on MDSCs interacting with CD40L on TREGS in the presence of TGF-β and IL-10 appears to be necessary for the expansion of TREGS, as MDSCs from *Cd40*-deficient mice and mice injected with anti-CD40 were unable to expand TREG populations and exhibited higher levels of tumor-specific T-cell immune response [84]. Further, IFN-γ appears to increase the expression of CD40 [84], IL-10, and TGF-β by MDSCs [85] (see Fig. 7). However, beyond this, little is known of their role in the cytokine network because, the mechanisms by which TREG cells mediate immunosuppression in cancer have been largely ignored [86]. Instead, interest in TREGS has been mainly as an important target for immunotherapy of cancer (see Section 7.1 and 7.2).

5. Key cytokine signaling networks in immunoediting and escape

At the molecular level, TGF-β, Th2 cytokines (including IL-4, IL-5, IL-6, IL-10, IL-12, and IL-13), chemokines (particularly angiogenic chemokines), VEGF, inflammatory factors and GM-CSF, all appear to play major roles in the avoidance of cancer immunosurveillance.

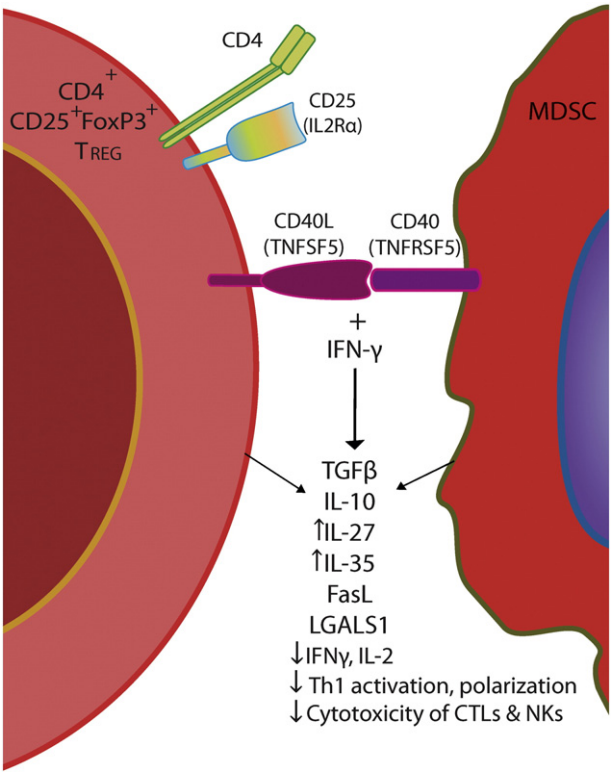

Fig. 7. Thwarting of IFN-γ-mediated immune response by TREGS and MDSCs. Interferon-gamma (IFN-γ) can initiate a powerful immune response as outlined in Fig. 1. However, interactions between CD40 receptors on myeloid-derived suppressor cells (MDSCs) and CD40 ligand (CD40L) on regulatory T cells (TREGS) can short-circuit this response by releasing a number of immunosuppressive cytokines. This results in decreased production of IFN-γ and IL-2 in the TME due to the suppression of Th1 activation and the promotion of Th2 polarization, as well as a concomitant decrease in the activation and cytotoxic activity of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. Additionally, CD40 receptor expression on MDSCs is increased.

Owing to the large number of secreted signals involved, multiplexed immunoassays that screen for changes in protein expression of a wide range of targets have been very helpful in decoding the cytokine crosstalk that promotes tumor-induced immunosuppression. A summary of the key immune cell types and cytokines involved in tumor-induced immunosuppression is presented in Figs. 5 and 8, the latter emphasizing the importance of TGF-β signaling and mediators of inflammation and angiogenesis to maintaining immunosuppressive networks.

5.1. Transforming growth factor beta (TGF-β)

TGF-β signaling has been referred to as a double-edged sword in carcinogenesis [87]. TGF-β signaling is known to induce arrest of the cell cycle in G1 and appears to play a major role in tumor suppression in early tumorigenesis; however, in later stages, TGF-β signaling in the TME is thought to enhance tumor progression. TGF-β directly inhibits the cytolytic activity of NK cells, macrophages, and CTLs and can inhibit the clonal expansion of NK cells and CTLs [88]. Nam et al., utilized cytokine antibody arrays to help reveal the mechanism by which CD8$^+$ T cells, a precursor of cytotoxic T cells, could be subverted in the TME to promote survival of breast and colorectal tumor cells [89]. They also found that CD8$^+$ cells, when exposed to tumor-secreted IL-6 and TGF-β in vitro or in vivo, began to express IL-17, which directly promoted tumor growth and survival. They proposed a model in which TGF-β blocked the differentiation of CD8$^+$ T cells to cytotoxic T cells, which would be likely to attack cancer cells, and IL-6 and TGF-β promoted the expression of IL-17 in CD8$^+$ T cells. TGF-β signaling also appears to play significant roles in tumor immunoediting by affecting the compositions of B cell populations. Although B cells are known to express

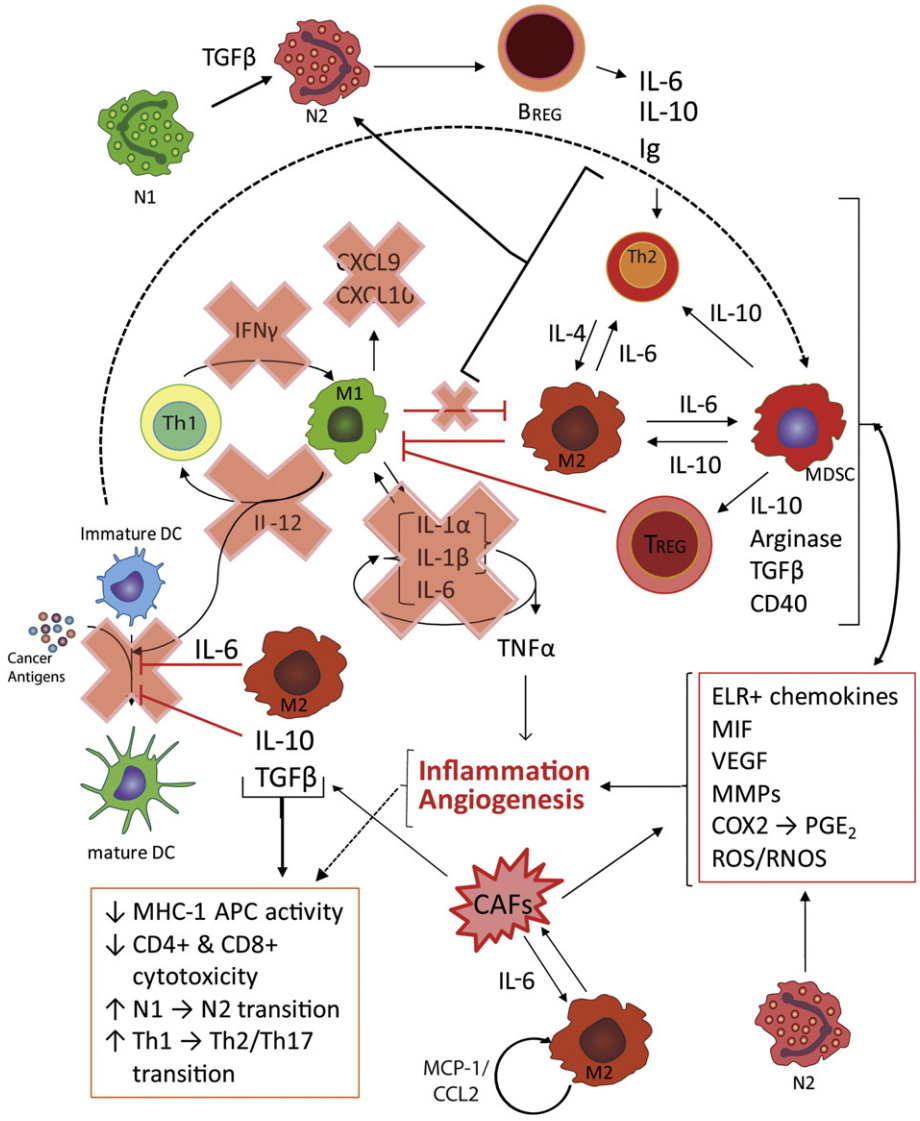

Fig. 8. Cooperativity of cancer-promoting immune cells in the TME. In the TME, a positive feedback loop of cytokine signals that proceeds as follows: First, TGF-β, COX2, PGE2, Th2-associated inflammatory factors and proangiogenic proteins are secreted by cancer cells, CAFs and other cell types in the nascent tumor recruit Th2 lymphocytes, M2 macrophages (TAMs) and N2 neutrophils (TANs). Then, Th2 lymphocytes, TAMs and TANs secrete additional inflammatory and proangiogenic proteins that suppress maturation of DCs and proliferation and activation of cytotoxic cells. As a result, antigen presentation and cytotoxic activities plummet, practically eliminating immunosurveillance in the tumor milieu. Additionally, B cells proliferate, but are not activated, turning them into tumor-promoting BREGS. M2 macrophages recruit MDSCs to the TME, further reinforcing the positive feedback loop of Th2, M2, and N2 proliferation and activation, resulting in substantial increases in tumor-promoting inflammation and concomitant angiogenesis.

TGF-β receptors and to secrete TGF-β, the role of TGF-β in regulating B-cell function, particularly in tumor immunoediting is not well understood [88].

5.2. Th2 inflammatory cytokines

One of the primary effects of TGF-β in cancer immunoediting is in promotion of a Th2 phenotype among T cell populations. TGF-β signaling is mediated by homologs of *Drosophila* SMADs. In an investigation by Kim et al., of the tumor suppressor gene for SMAD4, a central mediator of TGF-β signaling, researchers found that T cells from *Smad4*-deficient mice were skewed toward a Th2 phenotype, with high levels of IL-4, IL-5, IL-6, and IL-13 in supernatants of T cells [90]. Histopathology of the intestines of *Smad4*-deficient mice revealed abnormal accumulation of immunoglobulin A-producing IgA^+ plasma cells infiltrates and massive increases in serum levels of IgA. This correlated with the spontaneous progression of epithelial cancers in the gastrointestinal tract of *Smad4*-deficient mice.

MDSCs also help to maintain Th2 cytokine expression. Returning to effects of $Gr\text{-}1^+CD11b^+$ cells in the xenograft model of lung metastases by Yan et al., a broad-screen cytokine antibody array analysis of lung lysates also demonstrated significant elevation of MMP-9, basic-FGF/FGF2, IGF-1, IL-1β, MCP-1/CCL2, SDF-1/CXCL12, and Th2 cytokines (IL-4, IL-5, IL-9, and IL-10) in the premetastatic lung versus normal lung [81]. They concluded that the shift of the Th1/Th2 balance was due primarily to contributions by MDSCs. Thus, TGF-β has shown to have profound effects on Th1/Th2 balance and myeloid cell lineages, including $Gr\text{-}1^+CD11b^+$ immature myeloid cells, and chemokines, particularly angiogenic cytokines appear to be very important in proliferation of MDSCs.

5.3. Angiogenic chemokines

TGF-β also appears to be a critical driver of chemokine expression in the TME; it has long been known that TGF-β signaling plays an important role in angiogenesis [24,87,88,91]. Thus, it should also be no surprise that many pro-angiogenic chemokines are prominent among the

cytokines expressed by MDSCs in response to TGF-β signaling. Pro-angiogenic chemokines include ELR + CXC chemokines, which contain a signature Glu–Leu–Arg (ELR) sequence upstream of the CXC motif, such as CXCL1-3/GROα-γ, ENA-78/CXCL5, and IL-8/CXCL8 [24]. Other chemokines that are important in promoting angiogenesis include SDF-1/CXCL12 [92] and MCP-1/CCL2 [93,94]. Secretion profiles of MDSCs in a model of mammary carcinoma in mice deficient in the type II TGF-β receptor gene (*Tgfbr2*) provided significant insight in one example of TGF-β signaling supporting tumor-suppression instead of tumor promotion and underscored the importance of immature myeloid Gr-1$^+$CD11b$^+$cells (MDSCs) and chemokines in tumor progression [95,96]. In *Tgfbr2*-deficient mice crossbred with mouse strains prone to spontaneous or inducible tumors, Yang, et al., found that in *Tgfbr2*-deficient mice MDSCs were specifically recruited to the TME, leading to increased tumor cell survival and metastasis; 4 T1 tumor size was proportional to local concentrations of MDSCs, mainly on the invasive front of the tumor [95]. Antibody array analysis revealed increased expression of TGF-β1, ENA-78/CXCL5, myeloperoxidase (MPO), and MMPs in the TME; follow up experiments isolated production of TGF-β to stromal cells, CXCL5 to tumor cells, and MPO and MMPs to Gr-1 + CD11b$^+$ myeloid cells. Flow cytometry demonstrated high levels of CXCR4 expression in MDSCs regardless of *Tgfbr2* status, suggesting a mechanism by which Gr-1$^+$CD11b$^+$ cells can be recruited to growing tumors by SDF-1/CXCL12 in the absence of TGF-β signaling. Thus, MDSCs are such a critical component of tumor progression that cancer cells must recruit them to the TME in the presence or absence of TGF-β signaling via chemokine signaling.

In a companion study from the same lab, RT-PCR analysis demonstrated increases in expression of mRNA for GROα/*Cxcl1*, ENA-78/*Cxcl5*, and COX2/*Ptgs2* in carcinoma cells from *Tgfbr2*-deficient mice, and antibody arrays confirmed increased secretion of a number of chemokines from tumors in *Tgfbr2*-deficient mice, including GROα/CXCL1, ENA-78/CXCL5, CXCL16, "regulated on activation, normal T-cell-expressed and secreted" protein (RANTES/CCL5), MIP-1γ/CCL9, and MIP-3α/CCL20 [96].

5.4. VEGF: a nexus for inflammation, angiogenesis and immunosuppression?

The most prominent angiogenic cytokine, VEGF-A, is also immunosuppressive [96,97]. In fact, VEGF-A was actually the first cytokine ever identified that inhibits DC maturation [96,98], and VEGF signaling is a lynch-pin connecting tumor-promoting inflammation and angiogenesis in cancer [73]. Cytokine array-based detection of secreted proteins from endothelial cells demonstrated that VEGF has an autocrine effect on the release of proinflammatory cytokines via VEGFR2, inducing increased secretion of IL-6, IL-8/CXCL8, and GRO-α/CXCL1 in endothelial cells, but not in leukocytes [99]. Conversely, inflammatory cytokines, primarily TNF-α, IL-1β, IL-6, and IL-8/CXCL8, can induce expression of VEGF [73]. Additionally, hypoxia in the TME not only induces immunosuppression, as we have already discussed, but angiogenesis and inflammation.

The synergistic, mutual reinforcement of expression between inflammatory and angiogenic cytokines is virtually seamless, linking VEGF and COX2 through hypoxia-induced and NF-κB signaling pathways [73,100]. Hypoxic conditions in the TME can induce expression of hypoxia-induced factor 1-α (HIF-1α), which induces COX2; conversely, PGE2, a product of COX2, can induce expression of HIF-1α. Moreover, the expression of IL-8/CXCL8 and other pro-angiogenic and/or pro-inflammatory factors, including TNF, IL-1, IL-6, and IL-8/CXCL8, are upregulated by hypoxic conditions via HIF signaling and hydrogen peroxide production and by NF-κB signaling [73,92]. For example, Sakamoto, et al., used a cytokine antibody array to demonstrate that 67% of colorectal cell lines they tested and 40% of colorectal cancer tissues exhibited constitutive NF-κB activation and noted that prominent effects of NF-κB signaling included increased expression of IL-8/CXCL8, MCP1/CCL2, and GRO-α/CXCL1, which was suppressed by siRNA knockdowns of IκB kinase-γ [101]. VEGF also contributes to inflammation and immunosuppression as a chemoattractant for macrophages, and tumor-educated macrophages are a significant source of VEGF, MMPs, and inflammatory factors, particularly M-CSF/CSF1, which increases expression of VEGF; thus, a positive feedback loop can be established between inflammation/angiogenesis and immunosuppression [59].

Extracellular matrix (ECM) proteases, particularly MMPs can promote both angiogenesis and inflammation by remodeling of ECM proteins in the TME and surrounding tissues as well as releasing VEGF and other angiogenic factors bound within the ECM. CAFs are an abundant source of TGF-β, VEGF, COX2, and MMPs, and, thus are major contributors to angiogenesis and inflammation in the TME [7,102]. Another important contribution of CAFs is in expanding MDSC populations via maintaining predominantly Th2 cytokine secretion patterns in the TME, in which GM-CSF and IL-4 play prominent roles in tumor-directed immunosuppression (see Fig. 8).

6. Unresolved issues in cancer immunoediting

6.1. Mast cells, GM-CSF and IL-4

The relationship of GM-CSF/CSF2 and IL-4 in immunoediting is quite complicated. At low levels, GM-CSF and IL-4 or IL-13 lead to the maturation of DCs, but at higher levels, GM-CSF appears to expand MDSC populations [80]. GM-CSF is abundantly secreted by TAMs (Th2 cells), and mast cells are known to secrete high levels of IL-4, IL-13, and GM-CSF. Although little is known about the role of mast cells in cancer biology, they have been characterized as "Jekyll and Hyde" contributors to cancer growth, and, notably, they have been shown to support polarization of M2 macrophages (TAMs) and to secrete VEGF and IL-8/CXCL8, both of which are potent activators of Th2/M2 inflammation and angiogenesis [103].

However, a study published in Nature Immunology in 2000 suggests that the level of GM-CSF and IL-4 expression may be irrelevant, and the key to subvert these cytokines from the promotion of DC maturation to the expansion of MDSCs may be adding CAFs to the mix. Chomarat et al., demonstrated that, in vitro, monocytes exposed to GM-CSF and IL-4 resulted in their differentiation into antigen-presenting DCs; however when fibroblasts were added to the culture, the fibroblasts began pumping out IL-6 as soon as they contacted the macrophages. This caused the macrophages to up-regulate expression of M-CSF/CSF1 receptors, setting up an autocrine M-CSF loop [104]. In the TME, M2 macrophages are attracted by M-CSF and are a primary source of secreted M-CSF. In a more recent study, targeting CAFs with vaccination for fibroblast activation protein (FAP) in an animal model resulted in the reversal of the predominance of Th2 over Th1 cells and immunosuppressive cells over CTLs and APCs in tumor milieu [102]. This anti-FAP treatment also resulted in lower expression of GM-CSF in the TME, as well as reduced expression of VEGF and another pro-angiogenic factor, placental-derived growth factor C (PDGF-C).

6.2. Neutrophils: N1/N2 balance and TANs

While much research has been done on the roles of TAMs in cancer, until recently, little attention has been given to TANs (tumor-associated neutrophils), which also have tumor-suppressing (N1) and tumor-promoting (N2) phenotypes [105,106]. The roles of these innate immune cells in cancer are not well characterized, possibly due to their being considered "short-lived" compared to other immune cells. However, the prominence of angiogenic chemokines in the TME, which promotes the chemoattraction of N2 neutrophils, argues for their importance [107]. In the innate immune response, neutrophils (also known as polymorphonuclear leukocytes or PMNs) are primarily involved in fighting off microbial infections and wound healing.

Within neutrophils are large cytoplasmic granules containing high concentrations of serine proteinases (including matrix metalloproteinases, or MMPs), antimicrobial peptides (e.g., defensins), and ROS [108]. Because of their cytotoxic contents, release of the contents of neutrophil granules is normally under tight control. N2 neutrophils also secrete high levels of cytokines that induce angiogenesis and inflammation, including VEGF, MMP-9, MCP-1/CCL2, and IL-8/CXCL8. Among human immune cells, CXCR2, a receptor for many angiogenic chemokines, is expressed almost exclusively in neutrophils and mast cells [109]. Additionally, Jamieson et al., demonstrated that blocking CXCR2 activity in a mouse xenograft model, either by *Cxcr2* deficiency or by CXCR1/2 inhibitors, reduced neutrophil infiltration, lowered tumor-associated inflammation and substantially decreased benign and malignant tumor burdens [109].

Thus, TANs are actively recruited to the TME and they likely play an important role in inflammation and angiogenesis, but N2 neutrophils also secrete large amounts of cytokines that suppress immune response, including TGF-β, IL-10, MCP-1/CCL2, MIP-1β/CCL4 and macrophage migration inhibitory factor (MIF) [105,107]. TANs also promote B cell proliferation, maturation, and survival by the expression of "a proliferation-inducing ligand" (APRIL or TNFSF13) and B-cell activating factor/B lymphocyte stimulator (BAFF/BLys or TNFSF13B) [107]. Secretion of these cytokines suggests that TANs may play an important role in cancer immunoediting in general and, specifically, in maintaining and training of B cell populations in the TME. Understanding the role of N2 neutrophils in the "education" of tumor-associated B lymphocytes may provide a large piece of this puzzle, as the role of B cells in tumor progression and escape is not well understood either.

6.3. Regulatory B lymphocytes: enter the Breg

B cells are responsible for the release of antibodies associated with the humoral immune response; activated B cells are crucial in antigen presentation, and B-lymphocyte depletion has been shown to be effective in ameliorating symptoms of auto-immune diseases [110]. Thus, why increased B cell activity would be tumor-promoting rather than tumor-suppressing is not immediately apparent, and although the potential tumor-promoting functions for B lymphocytes was postulated over 50 years ago, their role in cancer progression is still largely unknown [21]. Nonetheless, the inhibition of antigen presentation associated with regulatory B cells (Bregs) may represent a crucial contribution to cancer immunoediting, as underscored by the paradoxical observation that the presence of cancer-related autoantibodies in serum or TMEs is often correlated with poor patient survival [6]. The immunomodulators APRIL and BAFF/BLys, presumably secreted by N2 neutrophils, have been detected in breast carcinomas and other tumors [21,107]. It has been suggested that, in the TME, these cytokines may help to maintain tumor-promoting B cells, which secrete IL-6 and IL-10 [6,21]. Collectively, these cytokines help support M2 polarization in TAMs, inhibit cytotoxic activity and antigen presentation in macrophages, and prevent the maturation of the major contributor to antigen presentation, DCs.

6.4. IL-17, IL-23 and Th17 cells

Th17 cells are a fourth type of T helper cell phenotype that is distinct from Th1, Th2, and Treg cells, but all come from the same immature T helper progenitor, Th0 (see Fig. 6). Th17 cell differentiation is typically induced by TGF-β, IL-6, IL-1α, IL-1β and IL-23, and Th17 cells are characterized by the production of IL-17, IL-2, GM-CSF/CS2, IFN-γ, and TNF-α [111]. Since Th17 cells are best known for their involvement in the pathogenesis of various autoimmune diseases, one might assume that Th17 cells might then play a role in supporting antitumor immunity. However, the role in tumor biology of IL-17 and of the T helper cell type associated with IL-17 and IL-23, Th17 cells, are poorly understood and may be tumor-suppressing [25,112] or tumor-promoting [25], depending upon the context, and the mechanisms of these effects are unclear [25,113].

For example, when Kesselring et al., investigated the role of Th17 cells in patients with head and neck squamous cell carcinoma (HNSCC) [25,114], they found that circulating Th17 cells were elevated in HNSCC patients and represented a large fraction of tumor-infiltrating lymphocytes. A bead-based multiplex ELISA revealed that Th17 cells in the HNSCC tumor milieu secreted IL-1β, IL-6, and IL-23; this was confirmed by ELISA and flow cytometry. Kesselring et al., also noted that proliferation of HNSCC cells in vitro was inhibited and that the apoptosis and necrosis of HNSCC cells increased proportionally with the number of Th17 cells seeded in co-culture. However, antibody array analysis revealed that, compared with monocultures of HNSCC, co-culturing with Th17 cells resulted in a net increase in many pro-angiogenic cytokines and a net decrease in several anti-angiogenic proteins. Most recently, the relationship between Th17 cells and angiogenesis has been strengthened by a report in Nature in which IL-17 and Th17 cells have been implicated in tumor resistance to anti-angiogenic therapy by inducing CAFs to increase expression of granulocyte-specific CSF (G-CSF or CSF3), a known factor in the recruitment and expansion of MDSCs [115].

In unraveling the mystery behind the dual role of Th17 cells in cancer progression and antitumor response, it may be helpful to look at the relationship of IL-12 to IL-23, a cytokine closely associated with the Th17 helper phenotype. With regard to cancer immunoediting, other than TGF-β and IL-6, the only cytokine associated with Th17 cells with a role that is fairly well established is that of endogenous IL-23. A heterodimer of IL-23/p19 and IL-12/p40, IL-23 appears to suppress tumor immune response, while a related cytokine, IL-12, a heterodimer of IL-12/p40 and IL-12/p35, enhances antitumor immunity; these activities are inversely regulated in the TME via activation of STAT3 signaling, resulting in IL-23/p19 production in TAMs being upregulated and in IL-12/p35 gene expression being downregulated in tumor associated-DCs [116].

6.5. Interleukin-2 and interleukin-15

Early in the age of molecular biology, interleukin-2 (IL-2) was considered as a possible cancer therapy to boost immune response to tumor-associated antigens [117]. This made sense, as IL-2 was known as a potent stimulator of the immune response, particularly in the development and expansion of effector T cells. Trials of cancer patients with high doses of IL-2 have been reported in the literature since the mid 1980s [118,119]. Several large clinical studies have examined the effectiveness and adverse events of IL-2 therapy in hundreds patients [120–122]. Considered together, these clinical trials showed a relatively low risk of severe side effects associated with IL-2 therapy in various combinations, but the overall effectiveness was also very low, with only 10–20% of patients showing partial or complete remission of cancers. However, most patients who did respond to IL-2 therapy remained cancer-free for extended periods.

Fever is a common side effect of IL-2 treatment, and this effect has been primarily attributed to induction of TNF-α [123]. This suggests that at least part of the mechanism by which IL-2 exerts clinical antitumor activity is through induction of TNF-α, but, for all practical intents, we know little of the mechanism of IL-2's antitumor activity or of the mechanisms by which normal IL-2 activity is suppressed by tumors. Part of the reason that IL-2 may have poor effectiveness as an immunotherapy of cancer could be that IL-2 appears to increase in vitro expression of FOXP3 (a regulator of immunosuppression) in Tregs and has been shown to induce the expansion of Tregs in peripheral blood in vivo [124]. Additionally, TGF-β and IL-2 are known to induce differentiation of naive Th0 cells to Tregs, as the combined treatment of $CD4^+CD25^-$ cells with TGF-β and IL-2 is necessary and sufficient to induce expression of CD25 and FOXP3 in naive Th0 cells [125]. This suggests that combination therapy of recombinant IL-2 with inhibitors of

TGF-β may be more effective than treatment with IL-2 alone. However, more research needs to be done on the role of IL-2 in cancer immunoediting, and multiplexed immunoassays, such as antibody arrays, could be very helpful in elucidating those mechanisms.

Also, IL-15 is structurally very similar to IL-2, shares some of its receptor components, and can mimic IL-2-dependent T cell proliferation [126,127]. However, its biology is somewhat different and suggests a more pleiotropic role in cancer biology. For example, IL-15 signaling in mast cells and fibroblasts can protect these cells from apoptosis, and in neutrophils, it can also inhibit neutrophil apoptosis and stimulate their expression of IL-8/CXCL8. IL-15 also appears to have potential roles in both innate and adaptive immunity. However, IL-15 therapy has recently emerged as a potentially immunotherapy for cancer; recombinant IL-15 therapy appears to induce differentiation of T, B and NK cells, activate CTL cells, and promote the maturation of DCs [127,128].

7. Implications for cancer immunotherapy

7.1. The importance of antitumor immunity in long-term, progression-free survival

Long-term survival of the patient is considered the "gold standard" of success in cancer treatment, but, from the patients' perspective, disease-free survival is the ultimate goal. In practice, using disease-free survival as an endpoint in a clinical trial is challenging, particularly for advanced and metastatic cancers [73,129,130]. The primary methods of cancer treatment are resection (surgical), small-molecule chemotherapy, radiotherapy and biological therapies (which could include cytokines, antibodies, proteins, genes and vaccines). Many of the approaches to biological therapy employ techniques of immunotherapy. As mentioned in Section 2.1, immunotherapy of cancer is not a new approach. The administration of cytokines, such as TNF-α, IFN-γ, or IL-2, and even the transfer of tumor infiltrating lymphocytes (TLK) or lymphokine-activated killer (LAK) cells to boost antitumor activity has been used since the 1980's [131]. Recently, great strides have been made in immunotherapies, which appear to be finally coming of age and are showing great promise for advancements in cancer survival. However, the relative efficacy of immunotherapy has been greater as an adjuvant in treating patients with operable disease at high risk for postoperative recurrence rather than patients with advanced disease [117].

The question that begs to be answered is whether long-term survival and disease-free survival are largely dependent upon enhancing the patient's own immune system to mount an effective antitumor response and, if so, how to define such a response. As we noted previously, among tumor-bearing mice benefiting from *C. noyvi-NT* infection (Section 2.1) and cancer patients who responded to IL-2 therapy (Section 6.5), antitumor immunity that is sufficient to eradicate tumors endures long after the tumors are gone. Several studies have also noted that the appearance of autoimmune symptoms, prior to diagnosis or as a result of immunotherapy, is a positive indicator of patient survival and prognosis. Among thyroid cancer patients, prior history of thyroid autoimmune disease or the presence of serum thyroid autoantibodies was a statistically significant indicator of positive outcome [132]. One group reported in 2006 that, among melanoma patients treated with IFN-α2b, the appearance of autoimmune symptoms was an independent indicator of both overall survival and relapse-free survival [133], but later admitted that retrospective studies were less conclusive [134]. Most recently, studies with ipilimumab (anti-CTLA-4 treatment) revealed that favorable outcome in patients with stage IV melanoma was associated with "immune-related adverse events" and that these side effects were manageable in most patients [135]. Thus, evidence of a strong immune response to therapy, even to the point of inducing autoimmune symptoms, may be a positive indicator of long-term survival in cancer patients. Another strong indicator that immune response may be essential for disease-free survival is a study of the immune cell infiltrates of tumor biopsies from a large cohort of cancer patients that was published in Oncogene in 2010; in this study, tumor infiltration by larger proportions of memory T cells, particularly Th1 and CTLs, was the strongest prognostic factor for disease-free progression [29].

Another consideration is the extent to which the success of standard (non-immunotherapy) treatments of cancer may be the result of immune response instead of removing or directly killing cancer cells. For example, in one study, radiation or neoadjuvant hormone treatment of prostate cancer resulted in the release of significant levels of autoantibodies detecting TAAs in some patients [136]. An investigation from Nature Medicine in 2007 reported that much of the effectiveness of conventional treatments, such as chemotherapy and radiotherapy, may be due to innate and adaptive antitumor responses in humans and mice via toll-like receptor 4 signaling in dendritic cells, presumably in response to the release of TAAs [137]. These are but two examples in a growing body of evidence that suggest to some cancer researchers and oncologists that, to some extent, the effectiveness of many—if not all—conventional treatments of cancer may be due to their ability to induce an antitumor immune response [138,139].

These same authors argue that, as a logical extension of the supposition that standard cancer treatments may elicit an immune response in some patients, a third consideration is the extent to which immune-boosting therapies can augment standard anti-cancer therapies. Other groups are working on the development of an "immunoscore" to correlate objective measures of antitumor immune response to patient prognosis [29,140,141]. Our model strongly suggests that most, if not all, successful cancer treatments should increase objective measures of antitumor immunity, regardless of the mode of treatment. One study suggests that detection of autoantibodies for TAAs may be an important marker of antitumor immunity [136], which might easily be accomplished using a protein microarray spotted with TAAs to probe patient serum or plasma for autoantibodies recognizing these antigens.

7.2. Targeting Tregs and T cell anergy: anti-CTLA4 and anti-PD-1

The importance of Tregs in tumor-directed immunosuppression also suggests that an increase in cytotoxic cell infiltration of tumors—and therefore cytotoxic antitumor activity—could be accomplished by disruption of the expansion and maintenance of Treg populations and/or the inhibited maturation of DCs that contributes to larger populations of MDSCs. Tregs and MDSCs suppress Th1 polarization without affecting overall T cell proliferation and inhibit cytotoxic effects in CTLs and NK cells, primarily through the expression of IL-10, TGF-β, IL-27, IL-35, soluble Fas ligand (FasL) and galectin-1 (LGALS1) [21,25]. Tregs block Th1 differentiation by inhibiting the production of IFN-γ and IL-2, as well as inhibiting Th1 cell activation by antigen-presenting cells (APCs). The binding affinities of B7-1 and B7-2 with CTLA-4 are somewhat stronger than with CD28, so CTLA-4 expression by Tregs can effectively shut down effector T cell functions if they are in large numbers in the TME (see Fig. 4)—particularly in cooperation with MDSCs (see Fig. 7 and Section 7.3). Depletion or neutralization of Tregs with antibodies to receptors commonly expressed on their surface, such as CD25, glucocorticoid-induced TNFR-related protein (GITR or TNFRSF18) and/or cytotoxic T-lymphocyte antigen 4 (CTLA-4), improves immune-based clearance of tumors and enhances the response to immune-based therapies. Similar results have been seen using cyclophosphoamide therapy, which appears to deplete Tregs, albeit not selectively [80].

CTLA-4 and PD-1 have emerged as prime targets for immunotherapy using antibodies to target immunosuppression by Tregs and PD-L1 expression on tumor cells [47,142]. Ipilimumab (anti-CTLA-4) is the only such antibody that is currently FDA-approved. Clinical trials using a numerous combination therapies with ipilimumab are currently ongoing or planned with a wide range of other therapies, including recombinant cytokine immunomodulators, small molecule immunomodulators, antibodies targeting other proteins, various chemotherapies, and

radiotherapy; most of these studies are evaluating the efficacy and safety of the various ipilimumab combination treatments in melanoma patients. Tremelimumab (also anti-CTLA-4) and nivolumab (anti-PD-1), as well as a number of other antibodies targeting PD-1 and its ligand, PD-L1, are under investigation. As noted in Section 7.1, a number of novel immune related adverse events have been noted with the use of ipilimumab that were not revealed in pre-clinical studies or animal models, and combinations of anti-CTLA-4 and anti-PD-1 antibodies appear to generate overlapping and unique toxicities [142].

7.3. Targeting Treg interactions with MDSCs and Th17 cells

One crucial aspect of targeting Tregs that should not be overlooked is the ability of MDSCs to expand Treg populations in the presence of IFN-γ. CD40 expression on MDSCs interacting with CD40L on Tregs in the presence of TGF-β and IL-10 appears to be necessary for the expansion of Tregs, as MDSCs from *Cd40*-deficient mice and mice treated injected with anti-CD40 were unable to expand Treg populations and exhibited higher levels of tumor-specific T-cell immune response [84]. As noted in Section 7.2, Tregs also block production of IFN-γ and IL-2 and suppress effector Th1 cell activation by antigen-presenting dendritic cells [83]. Further, IFN-γ appears to increase the expression of CD40 [84], IL-10, and TGF-β by MDSCs [85]. Thus, the interactions of MDSCs and Tregs via CD40/CD40L and IFN-γ may help to explain why tumor immunosuppression is resilient—even under treatment with IFN-γ (see Fig. 7). Based upon the model that we have proposed for the relationships between tumor cells and immune cell populations, it is clear that the proliferation of MDSCs and Tregs and the subsequent inhibition of Th1, M1, and APC populations in the TME is a likely explanation of the failure of IFN-γ- and IL-2-based therapies, as the effects of IL-2 are largely based on induction of IFN-γ expression. Thus, targeting MDSCs may be essential to reducing the tumor-supportive immune suppression by Treg cells.

Finally, there may be reason to consider leveraging the apparent inverse relationship between Th17 cells and Treg cells in the TME. Part of the reason that the role of Th17 cells in cancer biology is so fuzzy may be that, in contrast to Th1 and Th2 cells, Th17 and Treg cells appear to be more plastic and potentially inter-convertible [143]. In cancers, Th17 levels in the TME tend to correlate directly with the number of Th1 cells, CTLs, and NK cells and inversely with Tregs [144]. There is also an inverse relationship between the differentiation of Th17 and Treg cells in autoimmune disorders [113]. It has been shown that co-treatment with IL-6 and TGF-β may induce the conversion of mature Treg cells into Th17 cells [145]. Moreover, exposing Th17 cells to IL-12 may re-polarize them to a Th1 phenotype, and treating Th17 cells with IL-4 results in re-polarization to a Th2 phenotype [146]. Crucially, conversion of mature Th17 cells to Tregs has not been reported [143]. Thus, it may be possible that immunotherapy targeting Tregs in an attempt to convert them to Th17 cells or attempting to convert existing or induced pools of Th17 cells in Th1 populations within the TME, directly or indirectly, may be viable strategies, with the net result being increases in both cytotoxic antitumor activity and APC activity.

7.4. Anti-angiogenic/anti-inflammatory co-therapies

In the ideal situation, inflammation should help to enhance antitumor immunity and promote tumor clearance. This much was clear from the results of Agrawal et al., in examining Coley's observation, as noted in Section 2.1 [20]. Based upon the model outlined in this review, it is clear that a primary goal of immunotherapy should be to shift the Th1/Th2 balance toward a Th1 phenotype and macrophage populations toward a predominantly M1 phenotype to enable increased maturation of DCs, which is critical for adaptive antitumor immune responses. Indeed, this has been demonstrated in vivo. However, our model demonstrates that, in the TME, the predominant phenotype of $CD4^+$ cells is Th2, while M2 is the predominant macrophage phenotype in the TME, as N2 is for neutrophils. In addition, TGF-β appears to play a pivotal role in tipping M1/M2 (see Section 4.2) and N1/N2 balances in favor of tumor-promoting inflammation by macrophages and neutrophils, respectively [106].

We have also outlined the close intertwining of VEGF and COX2 in promoting angiogenesis and M2-type inflammation to the maintenance of immune cell populations in the TME that suppress anti-tumor immunity, primarily by inducing the inhibiting the differentiation and activities of APCs and by proliferation of MDSCs (see Figs. 5 and 8). As noted in Section 7.3, MDSCs help to proliferate Tregs in the presence of IFNγ, and Tregs tend to inhibit expression of IFN-γ and IL-2, and Tregs inhibit Th1 and M1 polarization by blunting the expression of IL-12 and increasing expression of IL-10. In addition, TNF-α (a Th1 cytokine) is also a strong inducer of the NF-κB pathway, which is central to the maintenance of tumor-supporting inflammation and angiogenesis, so in the absence of IL-1 and IL-12, TNF-α and IL-6 may be more likely to induce inflammation and angiogenesis than to tip the balances to tumor-suppressive inflammation.

Moreover, considering the crucial role of Th2 and M2 cytokines in inhibiting DC maturation, and the fact that immature DCs are a significant source of TGF-β, as well as the importance of the APC activity of DCs in mounting an effective antitumor immune response, without further investigation it may be presumptuous to assume that the importance of COX2 and PGE_2 in tumor progression or as targets in cancer therapy resides in more their tumor-promoting activities of inflammation and angiogenesis than in immunosuppression. Thus, we propose that the introduction of anti-angiogenic and/or anti-inflammatory therapies should be investigated as co-therapies that may boost the effectiveness of cancer immunotherapies, or, indeed, any mode of cancer therapy. A summary of several therapies suggested by the current model of tumor-induced immune suppression and the predicted results are summarized in Fig. 9. For example, immunotherapies may be enhanced by additionally targeting one or more pro-angiogenic/pro-inflammatory markers, such as COX2, VEGF, or IL-8, or perhaps with inhibitors of IL-6 (a suppressor of DC maturation), or even IL-10.

Based upon the model we have proposed for the nexus of angiogenesis, Th2 inflammation and immunosuppression (Figs. 5 and 9), IL-12 also stands out as a strong potential candidate to break the stranglehold of Th2/M2 and MSDC populations that promote not only immunosuppression to allow escape from immunoediting, but also inflammation and angiogenesis that further support tumor progression. As an example of these types of investigations, a recent publication by Zhang et al., suggests that timing of these combinations of immunotherapy and conventional therapies may be critical [147]. Although IL-12 has been approved for cancer therapy for some time and showed great promise in mouse models, clinical results of IL-12 used as stand-alone therapies resulted in severe toxicities in some cancer patients [148]. However, Zhang et al., may have discovered that timing is a key element to the successful use of IL-12 that may also help to limit its toxicity by narrowing the treatment window. In their in vivo mouse model, IL-12 was administered to each mouse only three times (every other day), as monotherapy or as co-therapy commencing at three, six or nine days after chemotherapy. Treatment with IL-12 alone was ineffective in shrinking either immunogenic or poorly immunogenic tumors, and co-therapy with IL-12 commencing at six or nine days after cyclophosphoamide, gemcitabine or doxorubicin chemotherapy was actually worse than IL-12 alone. However, when IL-12 treatment commenced three days post-chemotherapy, both immunogenic and poorly immunogenic tumors shrank dramatically. According to our model, two possible explanations of this are 1) the ability of IL-12 to enhance antigen presentation by promoting the maturation of immature DCs and 2) binding of IL-12 to IL-12 receptors on activated T cells. Notably, other strategies used to deplete MSDC and Treg populations in this same study were not nearly as effective as IL-12 administered three days after chemotherapy.

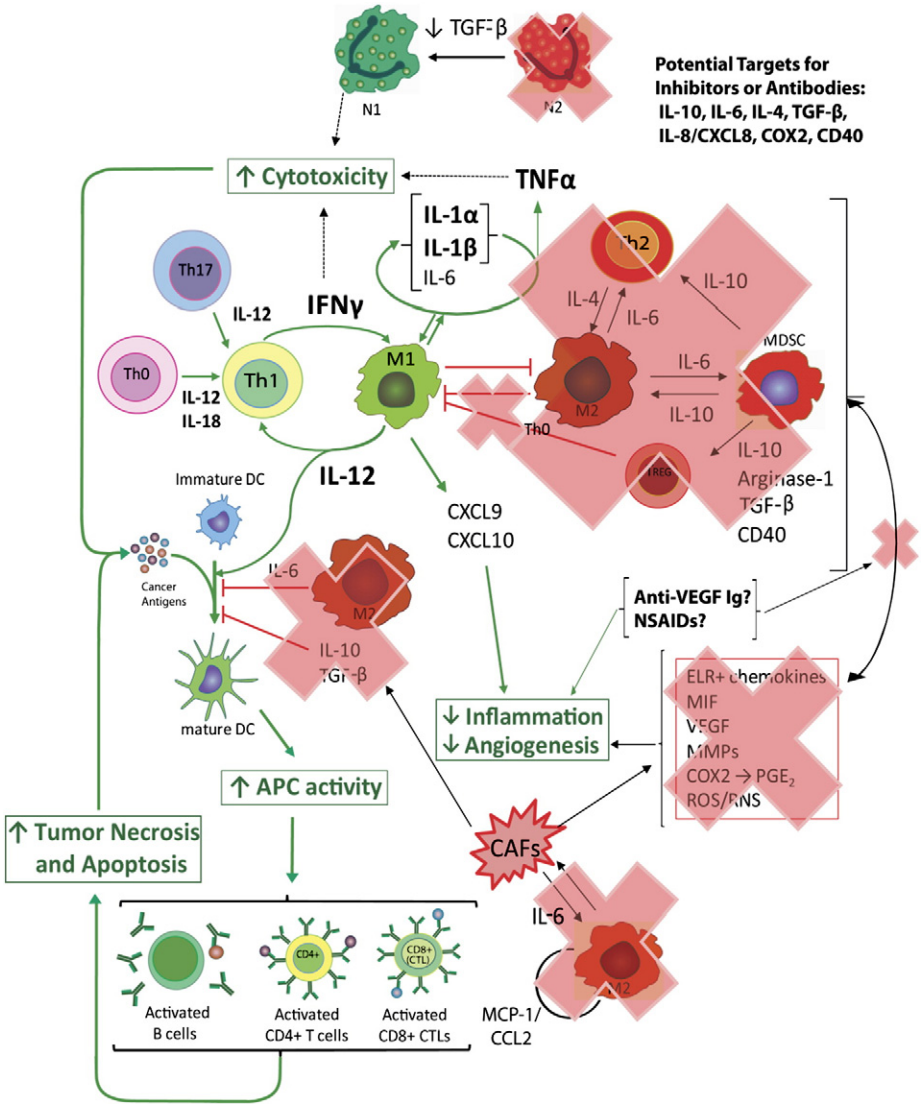

Fig. 9. Implications of tumor-induced immunosuppression for cancer therapy. Since tumor-promoting Th2, M2, N2 and MDSC activities are all mutually self-reinforcing and generally promote inflammation and angiogenesis as well as immunosuppression, therapies that target inflammatory and angiogenic factors (such as anti-VEGF immunoglobulin or NSAIDs) may be synergistic with immunostimulatory treatments, such as recombinant IL-2, IFN-γ, TNF-α,IL-12 or IL-18 (or combinations thereof). Additionally, the central roles of TGF-β, IL-10, IL-6 and IL-4 in the positive feedback loop supporting Th2, M2, N2 and MDSC populations (and inhibiting their tumor-suppressing alternative phenotypes) and inhibition of DC maturation make these potential targets for antitumor therapies. Overall, this model suggests that any successful cancer therapy will likely improve antitumor immune response and that immunotherapies may benefit from complementary strategies for targeting Th2, M2, N2 and MDSC populations and/or their tumor promoting effects on inflammation and angiogenesis.

Regardless of the mode of therapy, it remains clear that altering the dominance of Th2 cells and M2 macrophages in the TME, reinforced and protected by MSDCs, in favor of tumor-suppressing Th1/M1 and mature DC populations represents a necessary prerequisite to any effective antitumor immune response. As Th1 and M1 populations normalize and APC functions are restored, antitumor immune responses should soon follow.

7.5. Interleukin-18 (IL-18)

Since the late 1990s, it has been known that IL-18 can suppress tumor growth in mice by increasing NK and activated CD4 + T cells in a mechanism that was independent of IFN-γ and IL-12 [149]. IL-18, as its alternate name "IFN-γ-inducing factor" suggests, was first identified as an inducer of IFN-γ [150]. Indeed, as depicted in Fig. 6, IL-12 and IL-18 are the two factors that promote the maturation of Th1 cells from immature Th0 progenitor cells, and Th1 cells are significant source of IFN-γ. Also, mature DCs are thought to be a significant source of IL-12 and IL-18, and the current model of tumor-induced immune escape is characterized by a lack of mature DCs and the concomitant expansion of immunosuppressive MDSCs. This suggests (as depicted in Fig. 9) that, in addition to IL-12, treatment of cancer patients with IL-18 may tend to tip the Th1/Th2 balance toward the Th1 (tumor-suppressing) phenotype. However, IL-18 levels tend to be elevated in many cancer patients, and these elevations have been associated with disease progression, poor clinical outcome and survival [151]. In particular, there is evidence that a positive feedback loop between IL-18 and VEGF expression in some cancer cells and that IL-18 can stimulate metastasis and migration [152]. Among other tumor-promoting effects, IL-18 appears to allow certain cancers to escape immune recognition [151,152]. Recently, it was shown that IL-18 can induce expression of programmed cell death 1 (PD-1) in NKs and that the prometastatic effects of IL-18 in nude mice were dependent upon PD-1 receptors [153,154]. Another aspect of the investigation of the role of IL-18 in

cancer immunity is its regulation by a binding protein (IL-18 bp) that appears to be induced by IFN-γ as part of a feedback regulation of IL-18 [155]. In the presence of NK cells, administration of IL-18 bp resulted in metastatic lung tumor regression [155].

Nonetheless, IL-18 may provide an opportunity for cancer immunotherapies. In particular, the safety profile of rhIL-18 is quite attractive [156,160]. Conventional therapies using recombinant IL-18 in combination with other drugs have shown great promise [156,157] and are currently being tested for effectiveness in patients in clinical trials. "Helper" NK cells primed with IL-18 alone or in combination with other immunostimulatory cytokines appear to have great promise, demonstrating significant antitumor activities [156,158,159]. Also, several studies have indicated that co-treatment with IL-18 and IL-2 may enhance cytotoxicity and expansion of NK cells in vivo [156,161].

7.6. TGF-β Inhibitors as part of cancer immunotherapy

TGF-β signaling is arguably the single most important cytokine signal pathway in cancer progression. First, because TGF-β is considered to be tumor-suppressive in primary tumorigenesis but tumor-promoting in later stages [87,162]. Second, TGF-β signaling induces or reinforces the expression of nearly all of the other cytokines and other secreted proteins that help to support not only immunosupression, but also , as we have seen, inflammation and angiogenesis. Moreover, TGF-β and TGF-β-induced factors may also serve as valuable biomarkers for assessing the degree or extent of tissue invasion and metastasis in various cancers. For, example, in a recent investigation by Robert Weinberg's group, (Scheel et al.), screening cultured media with a high-density antibody array detecting 507 proteins revealed several factors related to TGF-β and Wnt signaling that were differentially expressed during epithelial-to-mesenchymal transitions, and follow up experiments confirmed that these were important contributors to both TGF-β and Wnt signaling [87,163], as well as invasion and metastasis [87,163].

The effectiveness of therapies targeting TGF-β is under investigation. Many potential drug candidates targeting TGF-β are currently under development or investigation, including antisense RNAs, sequestering ligands of TGF-β and small molecule inhibitors of TGF-β signaling. For example, tumor vaccine efficiency has been shown to increase in conjunction with inhibition of TGF-β, and no single source of TGF-β has been identified as being critical for this function; therefore global inhibition of TGF-β from many sources may account for the effectiveness of this treatment [87,113]. However, due to the dual nature of TGF-β in cancer tumorigenesis and progression and the pervasive heterogeneity of tumors, much work will be needed to screen for and validate the genetic and proteomic markers that would identify both cancer types and individual patients most likely to benefit from TGF-β inhibitors in combination with other cancer therapies [91].

8. Conclusion

As revealed by high-throughput screening technologies including those based upon antibody arrays, the process of tumor-induced suppression of cancer immunosurveillance is dependent upon recruitment of CAFs, TAMs, TANs, MDSCs, Tregs and other cells that alter the balance of immune cell populations in the TME. The net result is increased inflammation and angiogenesis, as well as increased transition of neutrophil phenotypes from N1 to N2, macrophages from M1 to M2, and T cells from Th1 to Th2, as well as a decrease in the number and activity of CTLs and APCs. Substantially lower populations of mature DCs provide a larger pool of monocyte precursors to support growing M2 and MDSCs populations. Subsequently, the cytokine networks established between and among these immune cell types are mutually reinforcing and serve to collectively maintain immune cell populations in the tumor milieu that are predominantly tumor-promoting instead of tumor-suppressing. Moreover, it appears that TGF-β, VEGF, HIF-1α, COX2 (and its product, PGE_2), angiogenic chemokines, and inflammatory cytokines (particularly Th2-induced cytokines) are all at located the nexus of tumor-induced angiogenesis, inflammation and immunosuppression. This nexus appears to be maintained by the mutual reinforcement of Th2, M2, N2, Treg and MDSC populations via IL-4, IL-6, IL-10, and TGF-β with support by CAFs and Bregs (see Figs. 5, 8, and 9). Absent disruption of mutual reinforcement of the predominant Th2, M2, N2, MDSC and Treg populations in the TME, prospects for mounting an effective antitumor immune response seem dim.

Moreover, our model and a growing body of evidence suggest that the establishment of an effective antitumor immune response in each cancer patient may be essential to the success of any antitumor therapies, regardless of modality. Thus, no matter what modalities or combinations of antitumor therapies are used in the clinical treatment of cancer, it is apparent that monitoring changes in the patient's antitumor immune response is critical feedback to determine the success of those treatments and the ultimate prognosis of the patient. Based upon our model, and the pioneering work of others in tumor immunology, we believe that some kind of "immunoscoring" that evaluates changes in immune response should become part of the routine evaluation of treatment efficacy in every cancer patient.

Much work may still need to be done in vitro, in animal models and in characterizing samples from cancer patients to better fill out the theoretical framework to establish whether this scoring should evaluate the recruitment, differentiation, proliferation, activation/polarization of immune cells in the TME and/or in peripheral blood. As with everything else in cancer biology, strategies may well depend not only on the type of cancer, but also on the actual gene and protein expression profiles as well. Gene expression experiments may be very useful in pinpointing signal transduction pathways that are being activated in patient tumor biopsies or in vitro tumor cultures. Shifts in recruitment, differentiation and proliferation of immune cell populations can easily be determined by cellular markers, using IHC or flow cytometry, but assessing changes in the activation and polarization statuses would be best accomplished by monitoring cytokine expression panels by multiplexed immunoassays. Elucidating the roles of additional cytokines and transcriptional factors that affect cytokine profiling may shed additional light on the complex network of secreted cell–cell signals that shape normal antitumor immunity and pathological, tumor-induced immunosuppression. Cancer antigen arrays should be used to screen for the emergence of higher titers and changes in the diversity of patient autoantibodies to TAAs in response to treatment, regardless of modality. Autoantibodies to specific TAAs themselves may represent biomarkers of prognosis or survival. Finally, all of these investigations should include components to establish correlations between patient prognosis and survival with changes in specific cytokine levels and activation signal transduction pathways, as well as changes in reactivity in patients' autoantibody populations to TAAs and changes in immune cell populations in the both TME and peripheral blood.

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