Tumor Immune Escape Mechanisms that Operate During Metastasis

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Abstract: Immune cells actively influence, among other factors, each step of tumor development determining the chance of a cancer cell to survive in a threaten microenvironment. Antitumor immune-mediated mechanisms are activated as soon as the first cancer cell is detected and operate both during primary tumor formation and during metastasis. However, when both innate and adaptive immunity becomes impaired, tumor development occurs. In this sense, compelling evidences indicate that tumor cells employ mechanisms that circumvent or thwart the immune response to enhance their own growth. These mechanisms include the secretion of immunosuppressive factors and the induction of distinct regulatory lymphoid or myeloid cells and, as occur with the immune response, they operate both during primary tumor formation and metastasis. Interestingly, cellular and molecular mechanisms of the immune response are important components of the tumor microenvironment and have the ability to promote or suppress tumor progression depending of the context of each cell interaction. In that sense, researchers are focusing their attention in the study of the influence of the tumor microenvironment in tumor growth and metastasis to better understand cancer biology and to formulate novel therapeutic approach. This review will focus on the present knowledge about interaction between immune cells and tumors in the context of metastasis, discussing the participation of different components of innate and adaptive immune response in the process of metastasis formation and dissemination.

Keywords: Antitumor immunity, cancer, immune escape, tumor microenvironment.

ROLE OF THE IMMUNE RESPONSE AGAINST TRANSFORMED CELLS WITHIN THE TUMOR MICROENVIRONMENT

Antitumor immune-mediated mechanisms operate by identifying in the host new cancerous or precancerous cells, even when those transformed cells are still invisible to the current detection systems in the clinic [1]. Moreover, the activation of immune cells significantly influence each step of tumor development determining the chance of a cancer cell to survive in a threaten microenvironment. In this sense, tumor immunosurveillance mechanisms function during primary tumor formation where they attempt to maintain cellular homeostasis and tissue architecture by destroying neoplasic cells and forcing the phenotype of the resulting tumor through a less immunogenic form. As expected, this immune based prevention machinery subsequently also alters the metastatic process where it influences, not only the detachment of the malignant cell, but also the seeding at a distant organ to consolidate a metastasis [2]. Thus, it is believed that immune response not only keeps under control newly transformed cells, but also it can promote tumor growth and metastasis depending of the immune cell population that is activated once the tumor is consolidated.

The thought about the existence of an immune-mediated mechanism that controls the development of cancer disease shaped, a long time ago, the *immunosurveillance theory*. For decades however, this attractive idea was challenged countless times, until experimental and clinical evidences indicated undoubtedly that both innate and adaptive immune response were key players in the control of cancer; being immune components as significant contributors to tumor progression as tumor suppressor genes or DNA repair mechanisms. However, the *immunesurveillance theory* faces the fact that tumors do arise in a healthy and immunocompetent host, in spite of the implicit capacity of the immune system to prevent it. Therefore, in a more complete explanation, the *cancer immunoediting hypothesis* [3], developed later, full filled the existing gaps and redefined the immunosurveillance theory, thus integrating the different mechanisms of tumor-immune escape with the premises originally conceived. Accordingly, the immunoediting hypothesis supports the idea that immune system not only protects the host against tumor development but also can promote tumor growth, by shaping and selecting tumor escape variants with reduced immunogenicity [3]. Immunoediting process was envisaged to be composed of three phases, termed "The three Es of cancer immunoediting": elimination, equilibrium and escape. The first phase, elimination, correspond to the original concept of cancer immunosurveillance, in which cells of the innate and adaptive immune response operate to recognize and destroy developing tumors, protecting the host against cancer. Transformed cells that are not destroyed during the elimination course proceed to the second phase named equilibrium. Equilibrium is an extended period in which the tumor and the components of the immune system enter into a dynamic balance. During this phase, the immune system was predicted to control tumor cell growth but with-

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out completely eliminating the transformed cells. As a consequence of the constant interaction of the immune system with tumors over a long period of time, the phenotype of developing tumors is edited or sculpted, resulting in the selection of tumors variants that have been shaped into a less-immunogenic status. Up to now, equilibrium was a poor understood phase, principally because researchers had to deal with the difficulty to identify early neoplastic lesions that are subjected to immunological control. Very recently, the existence of the equilibrium phase was proved by direct evidences [4]. Intriguingly, by using an attenuated dose of the carcinogen methycholantrene (MCA), the authors succeed at recognized small lesions containing tumor cells in an equilibrium state. This suboptimal dose caused an initial wave of tumors affecting a small proportion of mice, which were then separated of the experiment. The apparently healthy, surviving mice showed no evidence of growing tumors during an extensive time; however, when the state of equilibrium was disrupted by specific immunosuppression the dormant tumors escaped from immune control, rapidly grew out and killed their hosts. These results finally demonstrated that dormant tumors exist and they are kept in check by the immune system during the equilibrium phase. After this immune selection process, the escape phase takes place, in which tumor variants now develop into clinically apparent tumors that grow progressively and proceed to a metastatic phenotype [5].

Cancer cells in primary tumors are surrounded by a complex multitude of stromal cells, such as endothelial cells, lymphatic cells, fibroblast and a variety of bone marrow-derived cells (BMDCs) including macrophages, dendritic cells (DCs), myeloid derived suppressor cells (MDSCs), neutrophils, mast cells and mesenchymal stem cells (MSCs) [6]. All together, these stromal cells and the extracellular matrix proteins comprise the tumor microenvironment that has become the focus of renewed effort to explore the heterotypic interactions regulating tumor initiation, progression and metastasis. Immune cells take active participation within the tumor microenvironment influencing each step of tumor development and determining, among other factors, the possibility of a cancer cell to survive in adverse conditions [6]. The dynamic and reciprocal interactions between tumor cells and immune cells within the tumor microenvironment orchestrate events critical to tumor evolution toward metastasis. Nowadays the most accepted concept indicates that in general, cellular components of the immune system are capable of rejecting tumors; however once the tumor is formed these components are instructed by cancer cells to promote their growth and invasion. In line with this, a number of studies have shown that stromal cells populations have the ability to promote [7] or suppress tumor progression depending of the context of each cell interaction [8]. Among them, one of the most attractive and dynamic interactions associated with cancer progression involves the tumor with the immune system cells. The alliance between these two populations is reciprocal; each immune component influences the cancer cell, whiles the tumor mass affects and dictates the behavior and the composition of the stroma. This review will focus on the present knowledge about interaction between immune cells and tumors, particularly in the context of metastasis formation and dissemination. We attempt to integrate the understanding of immune system-metastasis interaction with ongoing work in immune mediated anticancer therapies. The effects of the diverse components of the immune response in others steps of cancer disease have been extensively reviewed previously and will not be discussed in this opportunity.

INNATE AND ADAPTIVE COMPONENTS OF THE ANTITUMOR IMMUNE RESPONSE

Tumor formation is certainly under the control of the immune machinery, either by cellular or soluble components. Several studies have shown that components of both innate and adaptive immune system participate in the process of immunoedition and basically during the elimination phase [1, 3]. A schematic explanation of the elimination process is illustrated in Fig**. (1**).

In sharp contrast to what was previously believed, the innate compartment is highly compromised in the supervision against tumors [9, 10]. Initiation of the antitumor response occurs when components of the innate immunity are alerted with danger signals or alarmins released in the tumor microenvironment by the growing tumor, either as a consequence of stroma remodeling or by the use of chemotherapeutics drugs [11]. The term "alarmin" is meant to identify the endogenous molecules that signal tissue and cell damage and are rapidly released following nonprogrammed cell death [12]. Interestingly, alarmins are believed to recruit and activate receptor-expressing cells of the innate immune system, including DCs, and thus directly or indirectly to promote adaptive immunity responses. Additionally, early inflammation events or cellular transformation signals induce the activation of Natural Killer (NK) cells, NK T Cell (NKT), γδT cells, and macrophages [13] that, in turn, activate the production of IFN-γ, a common outcome that is critical for a successful antitumor response. NK cells recognize tumor cells but not normal cells mainly by employing two strategies. As a general rule tumor cells down-regulate class I major histocompatibility complex (MHC) molecules, thus releasing the NK cell from the inhibition provided by class I MHC-specific inhibitory receptors ('missing self recognition'). Additionally, a stimulatory receptor NKG2D expressed by NK cells, T cells and macrophages recognizes ligands (MHC class I chain related [MIC], H6O, retinoic acid early inducible [Rae1] and UL16 binding proteins [ULBP]) that are up-regulated on tumor cells and virally infected cells but are not expressed well by normal cells. Ectopic expression of these ligands on tumor cells leads to the potent rejection of the tumors *in vivo*. NKT cells are a relatively newly recognized member of the immune community. They are true T cells with a T cell receptor (TCR) but recognize lipid antigens presented by CD1d, a nonclassical MHC molecule [14]. In cancer, type I NKT cells, are mostly protective, by producing IFN-γ to activate NK and CD8+ T cells and by activating DC to make IL-12 [14]. In contrast, type II NKT cells primarily inhibit tumor immunity. γδT cells are T cells with a less frequent TCR (γδ chains instead of αβ chains), they are particularly enriched at epithelial surfaces where they respond to self-molecules that signal potential danger or cellular stress and shown antitumor and immunoregulatory activities [15]. As mention before, the common outcome of the activation of these cellular compo-

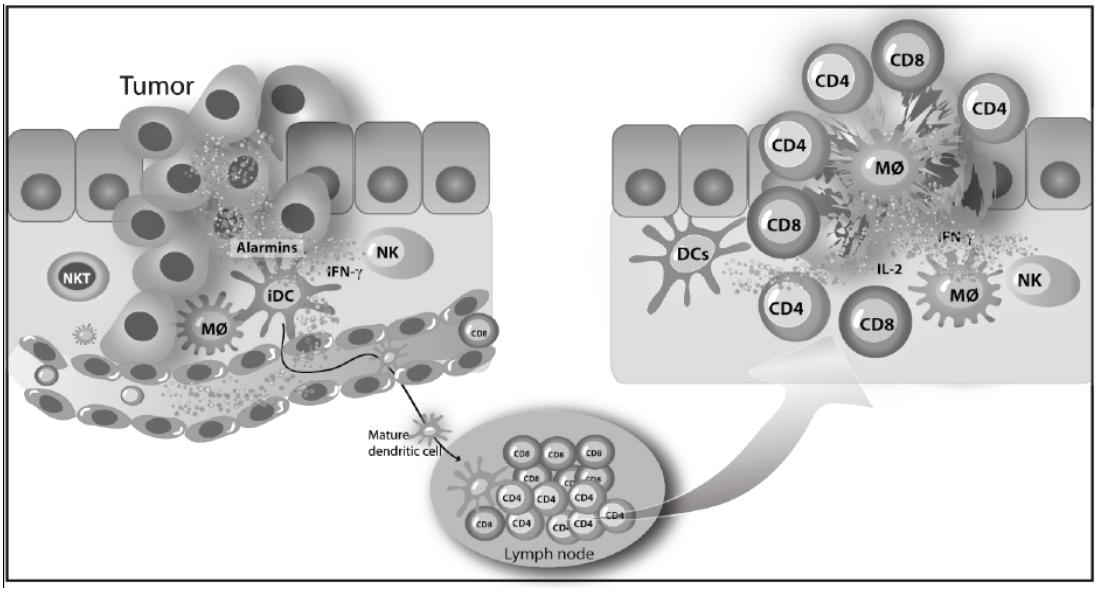

Fig. (1). The components of both innate and adaptive immune response participate in the process that eliminates a cancer cell. Initiation of the antitumor response occurs when components of the innate immunity detect danger signals released in the tumor microenvironment by the growing tumor. DC, NKT, NK cells, γδT cells, and macrophages recognize early cellular transformation signals and activate the production of IFN-γ. The destruction of the tumor cell promotes the release of tumor antigens that are engulfed by immature DCs, recently recruited to the tumor. DCs migrate to the lymph node and induce the proliferation of Th1 $CD4^+$ and $CD8^+$ tumor-specific T cells. Afterwards, tumor-specific $CD4^+$ and $CD8^+$ T cells travel from the lymph node to the tumor site where they recognize, through their specific T cell receptor (TCR), antigen-positive tumor cells and mount an antitumor immune response. Th (T helper), DC (dendritic cell), NKT (natural killer T cell), NK (Natural killer).

nents of the innate immune response is the release of IFN-γ, which as a positive loop, also activates macrophages and more NK cells. Concomitantly, tumor elimination by macrophage and NK cells occurs by the action of tumoricidal products as oxygen reactive and nitrogen reactive intermediates [16], or by TRAIL [17, 18] or by perforin [19] dependent mechanisms, respectively. As a consequence of the destruction of the tumor cell in hands of the innate immune components, tumor antigens are now exposed, and the adaptive immune system is recruited and activated [20]. Immature DCs that are enlisted to the tumor site become activated and gain the ability to engulf tumor antigens and migrate to the draining lymph nodes where they mature [21, 22]. Once in the lymph node, antigen-loaded DCs contact and induce the activation of naïve tumor-specific T helper 1 (Th1) $CD4^+$ T cells. Th1 cells facilitate the development of tumor-specific $CD8^+$ citotoxic T lymphocytes (CTL) via cross presentation of antigenic tumor peptides on DCs MHC class I molecules [23]. Subsequently, these tumor-specific $CD4^+$ and $CD8^+$ T cells travel from the lymph node to the tumor site where they recognize, through their specific T cell receptor (TCR), antigen-positive tumor cells and mount the antitumor immune response. In addition, polarized Th1 $CD4^+$ T cells produce large amounts of IL-2 and IFN-γ thus enhancing the activity of tumor-specific $CD8^+$ T cells, macrophages and NK cells that, in concert, will destroy tumor cells by direct or indirect mechanisms [24]. For instance, CD8+ CTLs kill tumor cells by direct release of perforins and granzymes or by activating the death receptor- death ligand pathways. Regarding IFN-γ– mediated tumor elimination several mechanisms have been proposed. On cancerous cell IFN-γ enhances immunogenicity, hence promoting tumor recognition and elimination by up-regulating components of the MHC class I antigen processing and presentation machinery [25]. On the other hand IFN-γ has a profound effect on the host immune system where it promotes $CD4^+$ T cell polarization towards Th1 functional phenotype assisting in this manner, the appropriate type of cellular immune response required for tumor rejection [26].

An extensive amount of experimental data derived from various mouse models of cancer, together with convincing clinical data from human patients has provided unequivocal evidence that cells of the immune system, both from the innate and adaptive compartments are required for the prevention of cancer [1, 27]. For instance, depletion of NK and NKT cells resulted in an increase rate of tumor formation after a carcinogen insult [28]. In addition, pioneering experiments from Schreiber's group showed that the disruption of IFN-γ function, either by blocking its receptor IFNGR1 or the cytokine itself, abrogated tumor immune rejection [29]. Interestingly, mice lacking either the ligand binding subunit of the IFNGR1 or the IFN-γ develop tumors with a higher frequency than their wild types counterpart after MCA injection [26, 30]. Furthermore, mice deficient in STAT1, the transcription factor activated in response to IFN-γ, also showed an increase occurrence of tumor formation after the carcinogen treatment mentioned [26]. Hence, IFN-γ is a key cytokine defending the host against tumor formation. The existence of a lymphocyte dependent cancer immunesurveillance process was demonstrated using *RAG2* $^{-/-}$ mice [24]. These severe immunecompromised mice are deficient in the recombinase activating gene (RAG2), and thus consequently, they lack B, and T lymphocytes and NKT cells, serving as an exceptional model to evaluate the importance of an intact

immune system in the control of tumor development [31]. Following challenge with the carcinogen MCA, *RAG2*$^{-/-}$ mice developed tumors with a higher incidence than wild type immunocompetents mice [24]. Remarkably, the authors also showed that after a long time all immunodeficient not treated mice developed spontaneous tumors, while only a 25% of the immunocompetent aged mice presented any class of neoplasic growth; thus underscoring the critical role that the cellular immune response plays controlling cancer. Finally, perforin deficient mice *(pfp* $^{-/-}$*)* were found to be more susceptible to MCA-induced sarcomas [30] and spontaneous B lymphomas [32] as compared with their wild type counterparts. Perforins are contained in the cytolitic granules of cytotoxic lymphocytes, CTL cells and NK cells, and it has been fully demonstrated that are secreted to kill target cells [32].

Even when experimental evidences emphasize the importance of the immune response against the transformed cells, the study of the antitumor immune response should be performed taking into account the tumor microenvironment since tumor mass progresses as a whole and not as an isolated identity.

THE POWER OF THE TUMOR MICROENVIRONMENT TO CONFER IMMUNE PRIVILEGE

Tumor microenvironment clearly affects the behavior of the transformed cell [6]. In this context, it is conceivable to believe that it may additionally influence the malignancy of metastatic cells. A variety of stromal cells in the surrounding environment are recruited to tumors, and these not only promote growth of the primary tumor but also facilitate its metastatic dissemination [6]. Thus, it plausible to hypothesize that both, the detachment of an active tumor cell from the primary tumor and/or the reactivation of an isolated dormant cell to form a clinically detectable metastasis may occur, in part, through perturbations in the tumor microenvironment. For example genotoxic agents as free radicals or inflammatory cytokines can alter the tumor microenvironment to promote tumor initiation and progression [33]. Metastatic consolidation depends on the capacity of the cancer cell to colonize distinctive microenvironments with a different composition of stromal cells, cytokines and growth factors. In particular, a wide variety of immune cells is recruited to the tumor site and participates actively to determine the metastatic success at each step in the metastatic cascade: the primary tumor, systemic circulation and the final metastatic destination. In the next paragraphs we will summarize the present knowledge about heterotypic interactions between the cancer cells and the immune cells, in the context of tumor immune privilege Fig. (**2**), including the secretion of immunosuppressive factors and the induction of distinct regulatory lymphoid or myeloid cells such as regulatory T cells, DCs, myeloid derived suppressor cells (MDSCs) and macrophages and how these microenvironmental events influence tumor invasion and metastasis.

Immunosuppressive Strategies that are Employing by Tumor Cells

Tumors employ different strategies to thwart the immune attack and create an immune privileged microenvironment through the promotion of active immunosuppression. These include the production of immunosuppressive cytokines such as transforming growth factor-β (TGF-β) and interleukin-10 (IL-10), or the expression of pro-apoptotic and inhibitory signaling molecules including Fas Ligand (Fas-L) and programmed death ligand-1 (PD-L1), which may act in concert to dampen the activity of effector T cells and antigen-presenting cells [5, 34]. IL-10 is an anti-inflammatory cytokine that participates in the regulation of the immune response at several levels [35]. IL-10 inhibits cytokine production of activated T and NK cells and of macrophages and it blocks the antigen presenting activity of DCs [35]. High IL-10 production was found to be associated with the immunosuppressive activity frequently observed in tumor bearing hosts, and it was speculated that overproduction of IL-10 could be responsible of tumor cell evasion of immune responses [36]. TGF-β is another cytokine that inhibits activation, proliferation, and activity of lymphocytes [37]. High levels of this immunosuppressive cytokine are often found in several malignancies and have associated with poor prognosis and lack of response to immunotherapy [38]. Recent evidence indicates that TGF-β acts on cytotoxic T lymphocytes (CTL) to specifically inhibit the expression of different cytolytic gene products; namely perforin, granzyme A, granzyme B, Fas ligand, and IFN-γ, which are collectively responsible for CTL-mediated tumor cytotoxicity [39]. Consistently, blockade of TGF-β signaling allows the generation of a potent antitumor immune response [40]. PD-1 is an inducible receptor expressed on $CD4^+$ and $CD8^+$ T cells following activation. The expression of one ligand for PD-1, designated PD-L1 or B7-H1, on tumor cells of a variety of histological origins has suggested a potential mechanism for tumor-immune escape [41]. It has been demonstrated that cancer cell-associated PD-L1 increases apoptosis of antigen-specific human T cell clones *in vitro* and *in vivo* [42]. Thus, blockade of PD-L1 (B7-H1) might also be a complementary therapy to augment tumor-specific T cell responses. In addition, accumulating evidence underlines the importance of negative regulatory pathways in tumor cells [43]. Signal transducer and activator of transcription (STAT) - family proteins are latent cytoplasmic transcription factors that convey signals from cytokine and growth factor receptors to the nucleus. It has been demonstrated that STAT3 signaling in tumor cells suppresses both innate and adaptive antitumor immune responses, further enhancing tumor progression [43]. Moreover, proof-of-concept studies in cell culture and animal models have validated STAT3 protein as a promising molecular target for novel cancer therapies, including small molecule inhibitors of STAT3 signaling [44].

Evidences indicate that tumor-mediated immunosuppressive strategies can either be preexisting, arise through outgrowth of escape mutants, or take place during tumor-sculpting actions by the immune system [3]. It is generally assumed that the synchronous blockade of these inhibitory signals might be effective in combination with other immunotherapy strategies to overcome immunological tolerance, promote tumor regression or avoid metastatic disease [45]. Thus, in depth understanding of the mechanisms devised by tumors to counteract specific T cell responses may contribute to the rational design of effective immunotherapeutic strategies.

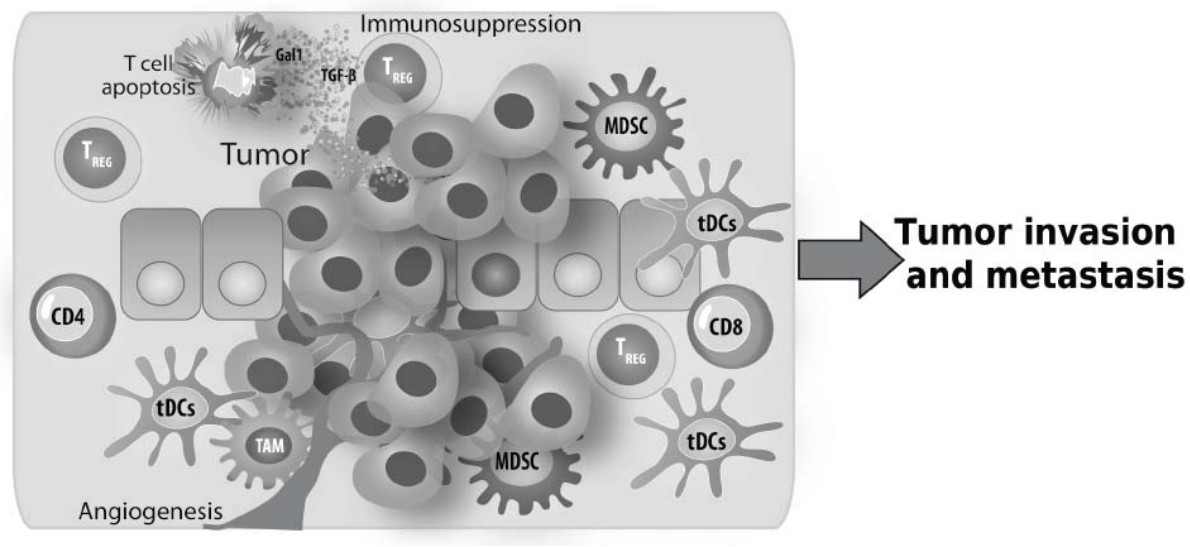

Fig. (2). Heterotypic interactions between the cancer cells and the immune cells in the tumor microenvironment that contribute to invasion and metastasis. Cancer cells in primary tumors induce the modification of tumor microenvironment either by secreting immunosuppressive factor as Gal-1 and TGF-β that, in turn, promote T cell apoptosis or the differentiation of regulatory T cells (Treg); or by attracting or activating many stromal cells including endothelial cells, myeloid-derived suppressor cells (MDSCs) tumor associated macrophages (TAMs), and tolerogenic dendritic cells (tDC) and more regulatory T cells. Consecutively, tumor microenvironmental events will support tumor progression by inducing angiogenesis, invasion and metastasis. T: T lymphocyte.

Protein-Glycan Interactions within the Tumor Microenvironment

In recent years the importance of protein-glycans interactions in the progression of cancer has gained renewed attention. Glycans decorate the surfaces of all mammalian cells, and the extracellular matrix with which they interact [46]. Research over the past decade has demonstrated that the differential glycosylation of cell surface glycoproteins or glycolipids can control critical immunological processes, including T-cell activation [47], migration [48], apoptosis [49, 50] and cytokine synthesis [51]. Furthermore, malignant transformation is largely associated with abnormal glycosylation resulting in the synthesis of altered carbohydrate determinants in the tumor microenvironment [52]. Therefore, the glycosylation pattern of a cell is a code for cellular physiology and pathology, a phenomenon that has already inspired novel methods to distinguish neoplastic from healthy tissues and anticipates novel therapeutic opportunities. The combinatorial possibilities of glycan structures of each cell provide enormous potential for information display, which together integrates the so-called 'glycome'. The responsibility of deciphering the biological information encoded by the glycome is assigned, at least in part, to a large number of endogenous glycan-binding proteins or lectins whose expression and function are regulated during tumor progression [53, 54].

Galectins are endogenous lectins characterized by their ability to recognize N-acetyllactosamine sequences, which may be displayed on both N- and O-glycans on cell surface glycoconjugates [55, 56]. All galectins contain at least one conserved carbohydrate-recognition domain (CRD) that is responsible for carbohydrate binding. So far, fifteen mammalian galectins have been identified in a wide variety of tissues and species. Galectin-1, a proto-type member of this growing family, has recently emerged as a key regulator of immune cell homeostasis, particularly during cancer [56]. Through cross-linking cell surface glycoproteins and forming protein-saccharide lattices, galectin-1 promotes T-cell apoptosis [57-59], modulates cytokine secretion [60] and controls the physiology of antigen-presenting cells [61, 62]. *In vivo*, therapeutic administration of galectin-1 suppresses Th1-dependent chronic inflammation in experimental models of autoimmunity by increasing the susceptibility of this subpopulation of CD4+T cells to activation-induced cell death and skewing the balance of the immune response toward a Th2 cytokine profile [60, 63]. Recent work from our laboratory provided a rational explanation for this effect, demonstrating that Th1 and Th17 effector cells express the repertoire of cell surface glycans that are essential for galectin-1 binding and subsequent cell death. In contrast, Th2 cells are resistant to galectin-1 binding through differential α2,6-sialylation of cell surface glycoproteins [50]. In addition, gene microarray analysis recently showed that galectin-1 is selectively overexpressed in $CD4^+CD25^+$ $FoxP3^+$ regulatory T cells and substantially contributes to the immunosuppressive activity of these cells [64]. More recently, we have provided evidence that galectin-1-glycan interactions favor the differentiation of IL-27-producing tolerogenic DCs which in turn promote the expansion of a population of IL-10-producing $FoxP3^-$ regulatory T cells [61].

Galectin-1 is overexpressed in a wide range of tumors, including astrocytoma, glioblastoma, melanoma, prostate, breast and ovary carcinomas and in their tumor-associated stroma [65]. Commonly, galectin-1 expression is associated with poor prognosis and the acquisition of metastatic phenotype [66-68]. During the last years researchers have made considerable effort to provide the 'poor prognosis signature' to identify tumor- and metastasis-related genes and to predict more precisely the clinical outcome of the disease. Interest-

ingly, gene and protein expression profiles using microarrays or proteomic analysis had recurrently led to the identification of galectin-1 as a typical protein, whose expression is upregulated in a plethora of tumors and metastatic lesions, as compared with their non-transformed counterparts [67, 69, 70]. Using proteomic analysis of human breast carcinoma it was found that galectin-1 expression is upregulated in cancerous versus non-neoplastic tissues [67]. Immunohistochemical staining for galectin-1 in more than one hundred breast cancer specimens showed significant correlation between galectin-1 expression in cancer-associated stromal cells and axillary lymph node metastasis. These observations are in agreement with a recent proteomic study that categorized galectin-1 as a metastasis-associated protein in the human breast carcinoma cell line MDA-MB-435 [69] and in gastric carcinoma cells [70]. In this regard, another study revealed a statistically significant relationship between high expression of galectin-1 and poor prognosis in patients with high-grade glioblastoma compared with patients who survived longer and had lower expression levels of galectin-1 mRNA [71]. In the same study the authors also demonstrated in a xenograph model that during *in vivo* growth, tumors express galectin-1 preferentially at the leading edge or periphery of expanding tumors. Furthermore, silencing galectin-1 resulted in greater sensitivity to cytotoxic chemotherapy, an effect which could have direct implications in clinical outcomes. As a whole these experimental evidences indicate that galectin-1 might be an appropriate candidate to selectively target, in the treatment of metastatic cancer disease.

As stated above, galectin-1 inhibits T-cell effector functions by several mechanisms [58, 72-75]. Thus, it has been hypothesized that expression of this endogenous lectin may contribute to create a tolerogenic and immunosuppressive microenvironment at sites of tumor growth. This intriguing hypothesis has been confirmed in human and mouse melanoma [76]. Targeted inhibition of galectin-1 gene expression within the tumor microenvironment rendered mice resistant to tumor challenge and stimulated the generation of a tumor-specific Th-1 response in tumor-draining lymph nodes [76]. Subsequently, these findings were confirmed in human malignant samples of head and neck squamous cell carcinoma (HNSCC) patients [77], supporting a role for galectin-1 in negative regulation of antitumor responses. Furthermore, galectin-1 expression was found to be upregulated in metastatic mammary adenocarcinoma cells by the action of the immunosuppressive cytokines TGF-β1 [78].

Very interestingly, Juszczynski *et al* [79] recently found that Reed Sternberg cells in classical Hodgkin lymphoma selectively overexpress galectin-1 to damp antitumor immune response. Moreover, galectin-1 treatment of activated T cells favored the secretion of Th2 cytokines and the expansion of CD4 +CD25+FOXP3 + regulatory T cells [79]. Altogether, these results suggest that galectin-1 may contribute to confer a status of immune privilege and metastatic advantage to tumors and allow envisaging that targeting galectin-1-glycan interactions may help to potentate cancer immunotherapeutic strategies.

Regulatory t Cells and the Development of Metastasis

In the past years, a subset of regulatory T cells expressing CD4 and CD25 and the transcription factor FoxP3 has gained considerable attention as key regulators of T cell tolerance and homeostasis [80]. This population of T cells is specifically engaged in the maintenance of immune self-tolerance and the control of aberrant immune responses to foreign antigens. Foxp3 is the master transcription factor that induces regulatory T cell differentiation, although regulatory T cells are additionally characterized by the expression of CTLA-4, GITR and the chemokine receptor CCR4 [80]. Overexpression of Foxp3 in conventional CD4+ T cells converts them to a regulatory T cell phenotype and endows them with suppressive activity [81]. Remarkably, regulatory T cells have been implicated in tumor cell evasion of immune responses [82, 83] by suppressing T cell mediated antitumor immunity through the release of inhibitory cytokines as TGF-β and IL-10 or by directly blocking T cell proliferation. TGF-β is an immunoregulatory cytokine with mayor relevance in tumor progression and metastasis. Interestingly, TGF-β it was shown to be a key inductor of Foxp3 expression and hence a master regulator of regulatory T cell differentiation [84].

Consistent with these findings a growing body of evidence indicates that, in later stages of cancer development, TGF-β is actively secreted by tumor cells, contributing not only to cell growth, invasion, and metastasis but also decreasing host-immune response, in part, by converting $CD4^{+}CD25^{-}$ T cells into regulatory T cells [85, 86]. Additionally, it was demonstrated that TGF-β suppresses CTL function by inhibiting the expression of perforin, granzymes, FAS ligand and IFN-γ [39]. Hence, the existence of a tight relationship among the production of TGF-β, the differentiation of regulatory T cells, and the progression to a metastatic phenotype deserves a particular interest. TGF-β is also one of the most recognized epithelia mesenchymal transition (EMT) inducers, being Snail the most relevant transcription factor involved in this process [87]. EMT represents a key step that tumor cells must undergo to acquire cell motility and ability to metastasize. Indeed, it is considered a transdifferentiation process characterized by a decrease in the expression of epithelial markers and an increase in mesenchymal markers. Very recently, it was shown that murine and human melanoma cells undergoing EMT induced considerable increase in regulatory T cells number and impaired DCs function *in vivo* and *in vitro* [88]. Notably, studies performed in mice implanted with $Snail^{+}$ tumors showed almost no infiltration of antitumor effector cells in the local tumor site of and no $CD8^{+}$ responses specific for tumor antigens were induced. This, in turn, resulted in enhanced tumor metastasis in various organs. Intriguingly, the authors explained that these events could be mediated by induction of regulatory T cells with high Foxp3 expression and DCs with low costimulatory molecule expression and high IDO (indoleamine 2, 3-dioxygenase) production [88]. IDO is a catalyzing enzyme of tryptophan in the kynurenine pathway and blocks the proliferation of T lymphocytes by the local depletion of tryptophan [89]. In line with these findings, it was reported that IDO^{+} cells may promote regulatory T cells development in the tumor draining lymph nodes [90, 91]. Hence, this novel result indicates that Snail-induce EMT accelerates cancer

metastasis utilizing not only the enhanced invasive ability, but also induction of multiple immunesuppression and immunoresistance mechanisms including immunosuppressive cytokines, regulatory T cells, impaired DCs and cytotoxic lymphocytes resistance.

In keeping with the idea that the metastatic tumor cell may influence the antitumor immune response it was demonstrated that metastatic tumor draining lymph nodes (mTDLN) extirpated from patients with cervical cancer in early stages were significantly enriched in $CD4^+Foxp3^+$ [91, 92] and tended to contain lower number of effector memory $CD8^+$ T cells. Hence, the increased recruitment of suppressor type cells concomitant with the scarcity of cytotoxic type cells suggest that in mTDLN the presence of tumor cells could shift the balance against anti-tumor immune response facilitating the survival of metastatic tumor cells and possibly contributing to systemic tolerance. This phenomenon was proved to be true also in other type of cancers. In breast cancer patients draining lymph nodes positive for micrometastasis- have an induced regulatory T cells abundance as compared to nodes negative for metastasis [93]. In patients with gastric cancer the population of $CD4^+CD25^+$ regulatory T cells in regional lymph nodes was significantly higher in comparison to those in control lymph nodes [94]. In addition, a similar suppressive population of regulatory T cells was found to be overrepresented in metastatic lymph nodes in patients with melanoma. In accordance with their suppressive phenotype these $CD4^+CD25^+Foxp3^+$ cells inhibit *in vitro* proliferation and cytokine production (IL-2 and IFN-γ) of infiltrating $CD4^+CD25^-$ and $CD8^+$ T cells through a cell contact dependent mechanism, and secret IL-10 and TGF-β [95].

Collectively these findings, lead to suspect that the immune suppressive regulatory T cells population, present in tumor-bearing hosts, might contribute to the failure of some immune based therapies. It is therefore attractive to consider strategies that would selectively counteract the suppressive activities of $CD4^+CD25^+$ regulatory T cells without inhibiting other immune cell functions.

Tolerogenic Dendritic Cells and the Development of Metastasis

DCs are vital for the generation and maintenance of antitumor immune responses [96]. DCs can engulf apoptotic or necrotic tumor cells, process and present tumor-associated antigens on their surface to activate a tumor-specific T cell response. Tumor cells, either in the primary tumor or in the metastasis, contain a large number of antigens that can be recognized by the host immune system, and hence are able to elicit T cell responses. Some of them are identified [97] but many others remain to be characterized. It has been largely known that DCs are derived from hematopoietic progenitor cells and most of them differentiate along the myeloid lineage however, they can also differentiate along the lymphoid lineage [96]. This alternative lineage composes a rare subset of DCs known as plasmacytoid DCs (pDCs) [98]. DCs that leave the bone marrow are defined as immature DCs (iDCs). Upon antigens capture in resident tissue, DCs migrate to draining lymph nodes and differentiate from immature cells to mature cells (mDC). Mature DCs express major histocompatability complex (MHC) Class II, CD86, CD54, and CD83 molecules [99, 100]. Mature human DCs express Human leukemic D-related antigen (HLA-DR), which is the MHC Class II for human, they additionally express CD80, CD86, and CD40 that are critical to induce T cell activation [99, 100]. Antigen-presentation in general results in the manifestation of antigen-specific immune responses, including Th1 responses, known to be beneficial for antimetastatic effect [101]. Very recently, it was demonstrated that DC can also induce peripheral tolerance by inhibiting the development of an effective T cell response and promoting the differentiation of regulatory T cells, including $CD4^+CD25^+$ $Foxp3^+$ regulatory T cells and IL-10 secreting Tr1 cells [102]. These tolerogenic/regulatory DCs are instructed by an immunosuppressive noninflamatory microenvironment that includes recognition of apoptotic cells[103], interaction with stromal cells [104] and with $CD4^+CD25^+Foxp3^+$ Treg cells [92] and exposure to soluble factors such as IL-10 [105], Vasoactive Intestinal Peptide [106] and 1,25-dihydroxyvitamin D3 [107]. In this sense, we have recently demonstrated that galectin-1, either exogenously supplied or endogenously regulated, drove the differentiation of DCs with a regulatory function; these DCs promoted T cell tolerance, blunted Th17 and Th1 responses through mechanisms involving IL-27 and IL-10 [61]. Remarkably, this tolerogenic context is commonly found within the tumor milieu what determines the existence of a defective DC system and the induction of a state of T-cell unresponsiveness during tumor progression [108]. As a consequence, impairment of DC function is considered one of the main factors responsible for tumor immune escape [109].

In patients with different types of cancers it was documented that tumors contain a higher proportion of iDCs [110-113]. For example, DCs isolated from melanoma metastasis and basal cell carcinomas are minimally activated, have reduced allostimulatory activity, and express low levels of the costimulatory molecules such as CD80 and CD86 [110-112]. Consistent with these observations, iDC were found to be present in the primary tumor region of HNSCC, whereas mDCs rarely infiltrate the tumor [113]. Very interestingly, other study has intended to clarify the DCs activation status in the draining lymph nodes before metastasis developed, in presence of a primary tumor [114]. They showed in patients with breast carcinoma, that DC maturation and Th1 response appeared to be less active in SNs (sentinel lymph nodes) compared with non-SNs (defining SNs as DLN that are targeted to be reached first by tumor cell). Not surprisingly, once metastasis was established in SNs, DC maturation was triggered and was followed by the upregulation of Th1 responses, which may reflect antigen-specific immune responses in SNs. However, induction of Th2 cytokines and regulatory T cells response developed in parallel, thus counteracting both DC maturation and Th1 responses in SNs and contributing to a dominant tolerogenic surrounding [114]. Also in breast cancer patients it was shown that SNs were mostly occupied by mDC (stained as $DC\text{-}Lamp^+$) as compared to the non-SN suggesting that in this study DC maturation appeared to be more active in SNs [115]. Thus, whereas an increased proportion of mDC in the SN may be indicative of a possible enhanced immunoreactivity with a potential benefit, it is not clear yet, whether it may represent or not a good prognosis factor for breast can-

cer patients. In this regard, two independent groups indicated that a higher number of mDC-Lamp$^+$ cells in lymph nodes was correlated with a lower rate of macrometastasis [116, 117], while a third group found that the presence of mDC-Lamp$^+$ cells in primary tumors was associated with an increased frequency of metastasis and a poor outcome [118]. Despite some discrepancies, in general evidences indicate that the immune surveillance activities of the SLN in melanoma and breast cancer are thought to be suppressed, whereas in cancers of gastrointestinal-tract, the presence of T cells in the SLN has not been shown to suppress the host's immune function [119]. Thus a more in depth analysis of the immunological state of the SN is required to clearly determine whether maturation status of DC is an indicative factor of an increase risk of metastasis in breast cancer patients.

In addition to iDC accumulation, tumor tissues can induce the recruitment pDCs with the potential to suppress T cell response [120-122]. Particularly in HSCC the presence of CD123$^+$ pDCs was associated with poorer outcome [113]. Despite being identified in primary tumors, pDCs are also found in mouse TDLN. Interestingly, this subset of pDC express the immunoregulatory enzyme IDO and are able to activate resting CD4$^+$CD25$^+$Foxp3$^+$ Tregs promoting a potent suppressor activity [90].

Overall, through the secretion of multitude inhibitory factors, tumor cells not only influence the normal processes of myeloid cell differentiation, affecting the capacity of a DC to mature and fully activate T cell response, but also determine the recruitment to the tumor microenvironment of subpopulations of DCs, iDC and pDC, with regulatory/tolerogenic phenotype.

Myeloid Derived Suppressor Cells and Tumor Associated Macrophages in the Development of Metastasis

As mention above MDSC belong to the group BMDCs that together with other stromal cells compose the primary tumor microenvironment. In mouse, MDSCs represent a heterogeneous population of immature myeloid cells comprising immature macrophages, granulocytes, DCs and other myeloid cell poorly differentiated, that can be identified as positive for Gr1$^+$CD11b$^+$ markers. In contrast to bone marrow, where they are found in high proportion representing a reservoir of precursors for myeloid cells lineage, in peripheral organs MDSC´s function is mainly suppressive. It has been demonstrated that MDSC impair tumor immunity and thereby facilitate carcinogenesis and tumor progression by inhibiting T and NK cell activation, and by polarizing immunity toward a tumor promoting Th2 phenotype. When there is a tumor, MDSCs tend to accumulate in the secondary lymphoid organs influencing their maturation to a suppressive phenotype. Moreover, it is widely documented that in tumor bearing mice MDSCs accumulate in spleen, lymph nodes and peritumoral areas [123, 124]. Tumor derived factors along with other factors released by host cells, can stimulate the generation and recruitment of MDSCs. To date a number of cytokines, chemokines or soluble factors have been identified including colony stimulating factor (CSF-1), IL-6 [125], VEGF[126], granulocytes- macrophages colony-stimulating factor (GM-CFS) [127] and IL-13[128], among others. In keeping with this, the metastatic mouse 4T1 mammary carcinoma cell line, transfected with the proinflammatory cytokine IL-1β in order to produce a chronic inflammatory microenvironment at the tumor site, showed increased numbers of immature splenic Gr1$^+$CD11b$^+$ MDSCs [129]. Moreover, dampening the inflammation in the tumor microenvironment resulted in a decrease accumulation of MDSCs cells, a reduction in tumor progression and a extend survival of tumor bearing mice [130].

Once fully activated MDSCs can suppress antitumor immune response directly via TGF-β production [131] [132], L-ARG (Arginina) metabolism [133], hyperproduction of reactive oxygen species [134], or indirectly by expanding CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells [135]. Very recently it was demonstrated that MDSCs inhibit T cell activation also by consuming cystine and sequestering cysteine, thereby depriving T cells of this essential amino acid, required for activation and function [136]. Another mechanism by which MDSCs inhibit antitumor immunity is the down regulation of L-selectin levels on naive T cells, hence decreasing their ability to home to sites where they would be activated [137].

Numerous evidences indicate that MDSCs can promote tumor growth not only by suppressing the antitumor immune response but also by promoting tumor angiogenesis through the secretion of VEGF [138]. When Gr1$^+$CD11b$^+$ cells were co-injected with tumor cells into mice, an increment in tumor growth and angiogenesis was observed, hence suggesting that Gr1$^+$CD11b$^+$ cells have a pro-angiogenic role in tumors [139]. Furthermore, it has been hypothesized that the intrinsic resistance of tumors to antiangiogenic therapy may be mediated by an increased number of Gr1+CD11b+ cells, which are a source of VEGF and supplement the lack of VEGF following anti-VEGF treatment. These results indicate that immune cellular components of the tumor microenvironment, as MDSCs, may promote tumor growth via cooperative immune escape and proangiogenic mechanisms.

The role of BMDCs and hence of MDSCs in tumor angiogenesis and growth is not only limited to the growth of primary tumors but also metastasis. A large number of macrophages and hematopoietic progenitor cells accumulate in pre-metastatic niche in which chemoattractants are produced by distant primary tumors serving as metastatic soil [140]. In direct relation with this, it was shown that inflammatory chemoattractants S100A8 and S100A9, whose expression is induced by distant primary tumors, can attract myeloid cells in the premetastatic lung by inducing serum amyloid A (SAA) [141, 142]. Intriguingly, Sihna and col [143] demonstrated that Gr1$^+$CD11b$^+$ MDSCs are, in fact, induced and attracted by these proinflammatory proteins S100A8/A9 and that MDSCs also synthesize and secrete S100A8/A9 thus creating a positive loop that potentate accumulation of MDSC. These interesting groups of results suggest that tumor-mediated upregulation of chemoattractants like S100A8/A9 and SAA promote the recruitment of MDSCs to the premetastatic niche and predetermines lung metastasis.

Tumor associated macrophages (TAM) are one of the most important inflammatory cells that surround and infiltrate tumors [144]. They mainly promote tumor progression and metastases [145] and show potent immunosuppressive features [146, 147]. Interestingly, Gr1$^+$CD11b$^+$ immature

myeloid cells may represent a source of tumor-associated macrophages (TAM) since they can easily maturate in F4/80+ macrophages-like cells with enhance immunosuppressive activity [148]. Macrophages can be classified in classical activated macrophages (M1) or in alternatively activated macrophages (M2) depending of the phenotype and effector function. M1 macrophages mainly release proinflammatory cytokines and are central elements in cellular immunity against infections and tumors. On the contrary, M2 macrophages are essential for humoral immunity and correspond to a predominant Th2-type cytokine profile. In most cases functions associated with TAMs are consistent with M2 macrophages [149]. TAMs facilitate tumor growth by diverse mechanisms that include the induction of tumor immune suppression [150], the release of angiogenic factors [145]and the promotion of metastasis [144, 145]. Regarding the later, Pollard and col. have hypothesized that TAMs contribute to tumor invasion by breaking the basement membrane surrounding the primary tumor, allowing tumor cells to escape and invade normal tissues [144]. This idea is consistent with the fact that TAMs produce proteases that destroy the basement membrane [151]. Very interestingly, experimental studies have demonstrated that TAMs in primary mammary adenocarcinomas can regulate late-stage carcinogenesis by virtue of their proangiogenic properties [152] as well as foster pulmonary metastasis by providing epidermal growth factor (EGF) to neoplasic mammary epithelial cells and thus enhancing their invasive behavior [144]. Additionally, TAMs can be instructed by others components of the acquired and cellular immune response to promote invasion and subsequent metastasis [153]. IL-4-expressing C4D4+ T lymphocytes directly regulate the phenotype and effector function of $Gr1^{-}$ $CD11b^{+}$ $F4/80^{+}$ TAMs that, in turn, enhance lung metastasis through activation of EGFR signaling in malignant mammary epithelial cells. After this last section we can conclude that MDSCs and TAMs share the capacity to facilitate tumor progression by halting antitumor immunity or by promoting invasion and metastasis and both can be considered cellular-mediated tumor immune escape mechanisms. Thus, it is possible to consider that depleting or altering MDSCs and TAMs phenotype could shift the immune balance to a tumor rejecting microenvironment that fosters antitumor activity.

CONCLUSION AND FUTURE DIRECTION TO DRUG DISCOVERY

Convincing data presented in this review support the concept that the immune system is important in the control of tumor development and progression. Based on the recent progress achieved in human tumor immunology, various active immunization trials have been attempted in cancer patients. However the efficacy of these trials has been limited to date. One of the major problems is the local and systemic immunosuppression in cancer patients. As stated above, malignant tumor cells employ a variety of mechanisms to circumvent tumor-specific immunity and thwart immunotherapy strategies, mainly through the production of diverse array of tumor-derived factors which directly blunt effector T cell responses or influence the recruitment of tolerogenic immune cell populations. Understanding the paradigms by which tumors escape immune attack, might provide new targets and tactics for improving the response to active immunotherapy.

Given the major role that tumor microenvironment has in every step in metastasis and the broad spectrum of immunoregulatory molecules involved, numerous candidates have been postulated as new targets for anticancer therapies. Several molecules, pathways and immunosuppressive cells populations have been shown to be relevant. For instance, blockade of different inhibitory signals (PD-1/PD-L1, IDO, TGF-β and IL-10) together with conventional chemotherapy, vaccination, or adoptive transfer of effector CTLs [45]. In line with this, the identification of galectin-1 as a fine-tuner of immune escape mechanisms in cancer capable of tilting the balance toward an immunosuppressive microenvironment at sites of tumor growth, disabling tumor-specific immunity and thwarting potential immunotherapy strategies, led to a new avenue of therapies targeting protein glycans interaction. Thus, treatment of patient with the neutralizing galectin-1 antibody as an adjuvant therapy after surgery might contribute to halt cancer progression by reinforcing immunosurveillance mechanisms that protect distant organs from the development of metastasis, as soon as the primary tumor has been removed. Additionally, the depletion or inactivation of regulatory T cells using anti-CD25 antibodies to evoke effective antitumor immunity in mice or to enhance efficacy of cancer vaccination has shown to be successful in several tumor models. Furthermore, several clinical trials have indicated the potential role for CTLA-4 blockade in cancer immunotherapy [154]. In addition, *in vivo* depletion of TAMs using a DNA-vaccination against legumain, an endopeptidase overexpressed in TAMs but not in M1 macrophages, impaired tumor growth and metastasis by reducing both angiogenesis and the suppressive properties of tumor microenvironment [155]. Importantly, the success of this strategy was demonstrated in murine models of metastatic breast, colon, and non-small cell lung cancers. Another therapeutic strategies to deplete MDSCs included the use of Gemcitabine that reduced substantially the number of splenic MDSCs in tumor bearing mice without affecting the number of $CD4^{+}$, $CD8^{+}$, NK cells, macrophages or B cells [156].

Thus targeting components of the tumor immune escape machinery, either selecting to intrude the tumor cell itself or depleting tumor associated cells of the immune system, may also lead to a new area in tumor immunology research to finally increase treatment efficacy and revert resistance to immunotherapy. Elucidation of the cellular and molecular mechanisms involved in tumor-immune system interactions will open new opportunities in biomedical research, attempting to delineate novel therapeutic strategies in cancer diseases and particularly in metastatic cancers, which nowadays are rarely cured.

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