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5.1 Introduction: The Balance of Immune Surveillance in the Tumor

In the beginning of the twentieth century, Paul Erlich was the first to introduce the concept of a vigilant immune system that can be manipulated to counteract tumor development [1]. However, due to lack of experimental evidence, it was not until the 1970s that Frank Macfarlane Burnet postulated the "immune surveillance theory." This theory brings to light a complex immunological mechanism capable of eliminating potentially malignant cells, mainly through recognition of tumor-specific antigens expressed on tumor cells [2]. In later years, several studies describing interactions between the immune system and the developing tumor have further refined this theory [3, 4].

Indeed, strong evidence supporting the key role of immune effector cell populations that are either tumor-specific, including B and T cells able to recognize tumor-associated antigens (TAAs) [5, 6], or non-specific, such as macrophages and natural killer (NK) cells, led to the sophisticated concept of cancer "immune editing," which spans cancer development from tumor immune surveillance to tumor immune escape [7, 8]. According to this concept, cancer development is comprised of three distinct phases [9, 10]: (1) the elimination, (2) the equilibrium, and (3) the escape, which are more extensively reviewed and discussed in separate chapters of this book. Particularly, the phenomenon of tumor immune escape according to which tumors are capable of side-tracking or completely blocking host antitumor immunity through interference with various components of the immune system is of major importance for the development of cancer immunotherapies [11]. Recently, several immune escape mechanisms have been described to hamper antitumor immune responses, either by reducing the homing of immune effector cells to the tumor site or by suppressing antitumor immune functions [12–15]. Therefore, cancer immunotherapies should attempt to stimulate homing and activation of immune effector cells and/or deplete or target pro-tumoral immunosuppressive cell populations and pathways.

Immunotherapy of cancer was selected as the breakthrough of the year 2013, according to Science [16]. Indeed, several groundbreaking clinical trials demonstrated the potency of such therapeutic approaches in patients. Yet, trials have also demonstrated that the responses vary greatly between patients. While in a selected group of patients immunotherapy leads to a full eradication of the tumor, in other patients the same treatment does not evoke a response at all. Currently, tumor immunologists are searching for biomarkers that can be used to describe the "immune signature" of the tumor [17, 18]. Defining the intratumor immunologic profile unique for every tumor type or patient may enable personalized immunotherapeutic strategies for the effective control of tumor progression [19].

This chapter gives an overview of novel strategies for reversing/reducing immunosuppression in the tumor microenvironment, illustrating their targets and the underlying mechanisms responsible for their therapeutic antitumor activity. Prior to this, the immunosuppressive mechanisms most widely encountered in human tumors are briefly addressed.

5.2 The Balance Is Tilted: Mechanisms of Tumor Immune Escape

Tumor immune escape is a consequence of the so-called "immune editing" process driven by the host immune system, through which malignant cells sensitive to immune interventions are eliminated, but in some cases allowing immune-resistant variants to survive and further develop [20, 21]. The mechanisms of tumor immune escape can be functionally divided in two categories: immune tolerance and immunosuppression.

5.2.1 Tolerance Mechanisms

Tumors frequently induce a state of T-cell unresponsiveness toward tumor-associated antigens (TAAs), attributed partly to T-cell ignorance, since tumor cells express mainly self-antigens. Additionally, tumor cells often alter their antigen processing/presentation machinery, mostly toward a defective T-cell priming in the tumor microenvironment [12, 22], but also in adoptive strategies to directly block active immune surveil-

lance, usually with the use of tumor-derived soluble factors [23]. Thus, the main targets of tumor-induced tolerance mechanisms are CD4+ T cells, cytotoxic CD8+ T lymphocytes (CTLs), dendritic cells (DCs), and the antigen presentation machinery. Both the relevance of these immune populations and the tolerance mechanisms they are the targets of are shortly addressed below.

5.2.1.1 CD4+ Helper T Cells and CD8+ Cytotoxic T Lymphocytes: Negative Polarization and Apoptosis

After proper cytokine stimulation, CD4+ mature T helper cells play a crucial role in the initiation and activation of antitumor immune responses. IL-12 polarized, type 1 CD4+ T cells (Th1) provide help to cytotoxic CD8+ T cells by stimulating their proliferation and inducing IFN-γ secretion once antigen-specific immunity has developed [24]. In contrast, IL-4 polarized, type 2 CD4+ T cells (Th2) secrete cytokines which induce neutralizing antibody production by B cells [25], thus directing immunity toward a tumor-promoting Th2 response, prevalent in the context of tumor immunology.

A major mechanism of tumor-induced apoptosis of CTLs is via cross-linking between the overexpressed death receptor FasR (CD95) on the surface of activated effector T cells and its correspondent ligand FasL on the surface of human tumor cells [26, 27]. Direct tolerization of antitumor T cells by tumor cell-induced TGF-β signaling is another highly effective mechanism, leading to a significantly decreased function and frequency of CTLs [23, 28].

5.2.1.2 Defects in the Antigen Presentation Process

The main components of the antigen processing and presentation machinery are the antigen-presenting cells (APCs), TAAs, and major histocompatibility complex (MHC) (or human leukocyte antigen (HLA) in humans) class I antigens. Tumor-induced alterations can affect the functionality of any of these factors via several mechanisms [29].

DCs are the dominant APCs capable in activating T cells but also in tolerizing them, depending on the local microenvironment [30]. Key determinants of DC competence for antigen processing and presentation are their activation and maturation status [31]. In several studies, decreased numbers of mature DCs were detected in the secondary lymphoid organs of tumor-bearing mice [32–34]. This observation is consistent with studies in patients with rapidly growing solid or non-solid tumors which exhibit significantly lower numbers of myeloid mature DCs [35–40]. In addition, isolated DC subsets have phenotypes similar to immature DCs and reduced expression of co-stimulatory molecules [41]. Downregulation of these molecules on the surface of DCs leads to inappropriate provision of co-stimulatory signals required for T-cell activation and interferes with the process of cross-presentation and thus results in death or anergy of antigen-specific CTLs [41, 42]. Moreover, DCs exposed to indoleamine-2,3-dioxygenase (IDO), transforming growth factor-beta (TGF-β) or prostaglandins [29, 43], have been shown to induce tolerance and anergy leading to failure of recognizing tumor cells.

Another means of tumor-mediated immunosuppression, as a result of genetic instability of tumors over time, is the change of their antigenic profile and selective development of "epitope loss" [44–46], by which tumors fail to be recognized and eliminated by the immune system. An additional effect of this genetic instability is a diminished or abolished expression of HLA class I antigens and antigen presentation-associated proteins [25, 47–54], with a frequency of antigenic loss or downregulation ranging from around 15% in melanoma lesions up to more than 50% in primary prostate carcinoma [53, 54].

5.2.2 Immunosuppression Mechanisms

The machinery of tumor-induced immunosuppression is highly versatile, as it has developed to target a large variety of antitumor processes. Within the tumor microenvironment, many cell populations contribute to the generation of an immunosuppressive profile. These include cancer-associated fibroblasts (CAFs), myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), and tumor-associated macrophages (TAMs). Furthermore, various tumor-derived factors with immunosuppressive activities also

contribute to tumor progression. The mechanisms by which these cell populations and factors give rise to tumor-immune escape are addressed below.

5.2.2.1 Cancer-Associated Fibroblasts (CAFs)

CAFs are cells that reside mostly within the tumor mass, or are often found within the tumor stroma. CAFs facilitate the malignant transformation process and promote tumor growth, angiogenesis, inflammation, and metastasis [55]. Similar to normal fibroblasts, CAFs are very heterogeneous [56, 57] and therefore difficult to classify based on expression of specific markers. However, the most widely used markers for CAF classification are α-smooth muscle actin (α-SMA) and fibroblast activation protein (FAP) [58]. Notably, the latter is being studied as a potential biomarker associated with poor prognosis in colorectal cancer [59]. Unlike normal fibroblasts present in healthy tissues, CAFs are more proliferative [60] and secrete various factors that promote tumor growth (such as CXCL12 [61], TGF-β [62]) and modulate the expression of matrix metalloproteinases (MMPs) [63]. Several studies in diverse tumors suggest that CAFs are not only promoting tumor growth and metastasis but can also enhance drug resistance through various mechanisms [64]. In pancreatic cancer, CAFs decrease the sensitivity of cancer cells to chemotherapy and radiotherapy by secretion of soluble factors [65], while in head and neck squamous cell carcinoma, CAFs protect cancer cells through secretion of MMPs [66].

5.2.2.2 Myeloid-Derived Suppressor Cells (MDSCs)

MDSCs ($CD11b^+CD14^-CD33^+$) [67] represent a heterogenic, bone-marrow-derived cell population [68, 69] with an increased frequency in the peripheral circulation and tumors of patients with different malignancies [70–72]. Migration of bone marrow precursors (which are further differentiated to MDSCs) to the tumor zone has been shown to be mainly induced by CCL2 secretd by tumor cells [73]. Once MDSCs arrive, signals derived from the tumor promote their activation [69]. MDSCs are characterized by poor phagocytic activity, continuous production of reactive oxygen species (ROS), nitric oxide (NO), and several anti-inflammatory cytokines [74]. As immune suppressive cells, they have the capacity to inactivate both $CD4^+$ and $CD8^+$ T cells through various mechanisms, including depletion of L-arginine [14], decreased tryptophan levels [75], and production of ROS [76], iNOS [77], and immunosuppressive cytokines, such as IL-10 and TGF-β [78]. Although MDSC-mediated suppression mainly affects T-cell function, it has also been described that MDSCs impair T-cell activation, by inhibiting MHC class II expression [79] and thus leading to decreased antigen presentation.

5.2.2.3 Regulatory T Cells (Tregs)

Similar to MDSCs, Tregs have also been shown to accumulate in tumors of patients with cancer [80]. Intratumoral accumulation of Tregs leads to poor prognosis for patients with gastric [81] and ovarian [80] carcinomas. $CD4^+$ Tregs, characterized by the expression of FoxP3 [82], are a highly immunosuppressive subset of $CD4^+$ T cells. Two major populations of $FoxP3^+$ Tregs have been described to date: one "natural" subset, which differentiates in the thymus, and one "induced," developed in the periphery from conventional $CD4^+$ T cells [83]. Both subsets promote tumor immune escape via the following mechanisms: (1) by secretion of immunosuppressive mediators, including cytokines like IL-10, TGF-β, and IL-35 [84, 85]; (2) by induction of effector T-cell apoptosis [86], as they promote a status of metabolic disruption secondary to IL-2 [87] deprivation; (3) by engagement of contact-dependent mechanisms of immunosuppression (e.g., inhibition of DC maturation, via CTLA-4 interaction with CD80/CD86 on DCs [88]); or by (4) by expression of suppressor molecules, such as LAG-3, CD39, neuropilin 1, or galectin 1 [89].

5.2.2.4 Tumor-Associated Macrophages (TAMs)

TAMs are immune cells that modulate and promote several immunosuppressive factors in the tumor microenvironment [90]. TAMs derive from monocytes that are recruited to the tumor [91] and, in the presence of Th2 cytokines such as IL-4 or IL-13, are polarized toward an M2 ("alternatively activated") non-cytotoxic phenotype [92]. Several studies have underlined their capacity to cause tumor growth both directly, by production

of cytokines that stimulate proliferation of tumor cells [93], and indirectly, by stimulating proliferation of endothelial cells [94]. TAMs are frequently found in solid tumors, where they promote remodeling of the extracellular matrix and secrete growth factors inducing tumor-specific neoangiogenesis [95]. Moreover, TAMs are enriched in hypoxic areas in most of the solid tumors [96], where they support tumor cell proliferation by secreting cytokines and growth factors. Indeed, accumulation of macrophages within the hypoxic tumor areas of patients is correlated with poor prognosis [97]. On the other hand, increasing accumulation of TAMs in the normoxic tumor area supports M1-like macrophages, leading to an antitumor immune response [98], while blocking colony-stimulating factor-1 (CSF-1) signal decreases M2-like polarization and impedes malignant progression resulting in regression of established gliomas [99]. These processes thus underscore the therapeutic relevance of TAM polarization.

Recently, metabolic changes in the tumor microenvironment have gained attention suggesting that, during tumor progression, gradients of extracellular metabolites (like lactate) act as tumor morphogens that promote M2-like polarization [100, 101]. Moreover, it has been suggested that treating TAMs with the glycolysis inhibitor 2-deoxyglucose blocks the development of TAMs with a pro-metastatic phenotype [102]. In the same line, increasing glucose uptake specifically in TAMs outcompetes endothelial cells for glucose usage, thus reducing vascular hyperactivation and decreasing tumor angiogenesis [103], supporting the link between metabolism of TAMs and tumor angiogenesis.

TAM-mediated immunosuppression also affects T-cell function. Under IL-6 and IL-10 stimulation, expression of programmed death-ligand 1 (PD-L1) is induced in TAMs [104], thus impairing T-cell effector activity. Moreover, programmed death 1 (PD-1) expression on the surface of TAMs correlates with decreased phagocytosis [105]. PD-1/PD-L1 blockade increases both effector T-cell activity and PD-1$^+$ TAM phagocytosis, supporting the use of checkpoint inhibitors in cancer treatment. In addition, TAM-derived PGE2, IL-10, and IDO play important roles in the induction of Tregs. Furthermore, TAM-derived CCL17, CCL18, and CCL22 are chemotactic factors for Tregs [87], resulting in the suppression of T cells in the tumor microenvironment. For example, in the HPV16 E6- and E7-expressing TC-1 tumor mouse model, TAMs were shown to cause suppression of the antitumor T-cell response [106], while their secreted IL-10 subsequently induced a Treg phenotype [107].

5.2.2.5 Tumor-Derived Immunosuppressive Factors

Within the tumor microenvironment, signals that stimulate T-cell cytolytic functions can be replaced by inhibitory signals secreted by the tumor itself as a mechanism of immune escape.

Cytokines

The immunosuppressive cytokines TGF-β and IL-10 are produced by Tregs as a means to disbalance T-lymphocyte surveillance of tumor development [108, 109], by inhibiting proliferation of antitumor effector T cells. Granulocyte-monocyte colony-stimulating factor (GM-CSF) is another cytokine with immunosuppressive properties. Due to these properties, GM-CSF facilitates recruitment and expansion of MDSCs in several cancer models [110, 111] and promotes generation and expansion of TAMs [112], despite being described as immunostimulatory in other settings [113]. The GM-CSF receptor (GM-CSF-R) signals through signal transducer and activator of transcription factor 3 (STAT3) [114], which has been linked to elevated PD-L1 expression on myeloid cells [115] and regulation of IDO expression in breast cancer MDSCs [116].

Enzymes

Together with arginase and iNOS, which are central for two of the mechanisms of immunosuppression exerted by MDSCs, IDO and cyclooxygenase 2 (COX2) also present immunosuppressive properties. IDO inhibits T-cell activation by depleting tryptophan [117], one of the essential amino acids necessary for T-cell development, whereas COX2 stimulates PGE2 production, a prostaglandin involved in conversion of human DCs into immunosuppressive MDSCs [118].

Negative Regulatory Factors

Antitumor immune responses are hampered by tumor-induced activation of negative regulatory

pathways (also called checkpoints), either associated with immune homeostasis or actively facilitating tumor immune escape [119–121]. Frequently, antitumor immunity shares characteristics with chronic immune responses, such as T-cell exhaustion [122], mediated by the expression of multiple inhibitory receptors including PD-1 (also known as CD279), cytotoxic T-lymphocyte antigen-4 (CTLA-4, CD152), lymphocyte-activation gene (Lag-3), T-cell immunoglobulin and mucin-domain containing-3 (Tim-3), CD244/2B4, CD160, TIGIT, BTLA, and others [12, 123–128]. Among them, PD-1 and CTLA-4 have been extensively studied and garnered attention due to the clinical success of antibody therapies [129–131]. PD-1 is a member of the CD28 superfamily of T-cell regulators, expressed on activated $CD8^+$ T cells during priming or expansion, and functions mainly in peripheral tissues, where T cells encounter its two corresponding ligands, PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273), members of the B7 family [132]. PD-L1 is expressed in various cell types, including stromal and tumor cells, but also in immune cells after exposure to effector cytokines such as IFN-γ, while PD-L2 is mainly expressed on DCs in normal tissues [133]. In physiological situations, the PD-L1/PD-1 axis is an important negative feedback loop ensuring immune homeostasis through suppression of excessive immune activation [134] and facilitation of immune tolerance to self-antigens [132, 135, 136]. However, in the tumor, the PD-1/PDL-1 axis restricts tumor immunity [129]. Tumor-specific $CD8^+$ T cells that express lower levels of PD-1 showed less exhausted phenotypes [137], as compared with tumor-specific $CD8^+$ T cells with higher PD-1 expression. Similarly high levels of PD-1 have been found on activated $CD8^+$ T cells during chronic infections [138]. Co-inhibitory signaling via PD-L1 (but not PD-L2) is necessary for conversion of naïve $CD4^+$ T cells to adaptive $CD4^+FoxP3^+$ Tregs. In addition, PD-L1 expression in various tumors, including breast, ovarian, colorectal, pancreatic cancer, and hematologic malignancies, has been considered a predictor of poor prognosis [139–143].

Although not as disputed as the PD-1/PD-L1 axis, LAG-3 is also a member of the immunoglobulin superfamily and is expressed on the surface of activated Tregs, $CD8^+$ T cells, B cells, and NKT cells, contributing to tumor immune suppression. Interestingly, Tregs from LAG-$3^{(-/-)}$ mice present reduced regulatory activity [144]. Lastly, CTLA-4 is a receptor expressed on the surface of Tregs and upregulated on activated conventional T cells [145, 146]. CTLA-4 transmits an inhibitory signal for T-cell activation by competing with the co-stimulatory molecule CD28 for binding to their shared ligands CD80 (B7.1) and CD86 (B7.2), with opposing effects [147, 148].

Endothelin Receptors

Aberrant activation of the small bioreactive peptide endothelin 1 (ET1) and its receptors endothelin receptor type A (ETAR) and type B (ETBR), by a large array of stimuli, in a paracrine and autocrine loop [149], has multiple implications in the progression of various solid tumors, including prostate, colon, ovarian, breast, and lung cancer [150–154]. Upon binding of its ligand ET1, ETAR promotes vasoconstriction, tumor cell proliferation, and cell migration [155–158] through phospholipase Cβ and downstream activation of mitogen-activated protein kinase family members, including ERK signaling [150]. ETAR may also play a role in chemoresistance [159]. On the other hand, ETBR was shown to inhibit T-cell homing and adhesion to the tumor by inducing the suppression of intracellular adhesion molecule 1 (ICAM-1) on the endothelial cells [150]. High expression of ETAR has been reported in patients with prostate cancer and bone metastasis [160], HPV-induced neoplasia [156, 161], and renal cell carcinoma [162]. ETBR expression was associated with the absence of tumor-infiltrating lymphocytes and decreased survival of patients with ovarian cancer [163]. Additionally, ETBR overexpression is associated with an aggressive tumor phenotype in melanoma [164, 165] and correlates with tumor progression and metastasis of vulvar squamous cell carcinoma [166].

The above-described spectrum of strategies developed by tumors to evade the cytolytic activity of the immune system illustrates the complexity of the tumor immune escape phenomenon and its capacity to adapt and particularly target distinct mechanisms of the antitumor immune response. Developing tumors are able to use different functions of the immune system to sustain their own growth and to simultaneously build up mechanisms which enable them to hide from an immune-based attack. Different types of tumors develop diverse immune escape mechanisms, translating into various degrees of tumor aggressiveness. Thus, the complexity of the tumor immune escape phenomenon resides in the ability of human tumors to develop unique signatures, which pose a real challenge for development of effective antitumor therapies.

5.3 Shifting the Balance: Strategies to Target Tumor Immunosuppression

Therapeutic approaches against cancer have mainly been oriented on the activation of the immune system to directly eliminate tumor cells, thus decreasing the tumor load. More recently, the importance of cancer-induced immune suppression is being taken into consideration with apparent clinical success of antibodies against immune checkpoints [129]. Despite the therapeutic potency of those immunotherapies, still only a subset of patients exhibit durable responses, suggesting that the main challenge of these strategies is the unique immune signature of tumors, which further translates into a large variability of tumor-induced immunosuppression mechanisms. Hence, the starting point of these strategies consists of mapping this immune signature, followed by a documented selection of uni- or multimodal therapies targeting the predominant immunosuppressive mechanisms developed within each tumor type. Based on their overall target aim, these therapies can be categorized as those which attempt to increase homing of effector T cells to tumors and those that, directly or indirectly, increase antitumor activity of intratumor effector T cells, either by overcoming tumor-induced tolerance or by overriding the immunosuppression mechanisms imposed during tumor development (see Table 5.1).

5.3.1 Strategies Targeting Homing of Effector T Cells

Some of the tumor immune escape mechanisms described above interfere with the proper trafficking of effector T cells from the peripheral circulation or secondary lymphoid organs to the tumor site. A reduced homing of these effector cells to the tumor will give rise to negative regulatory processes leading to tumor progression. Several strategies to block these processes and enhance intratumor homing of effector cells have been proven effective. These include local tumor irradiation, blockade of endothelin receptors, taxane-based chemotherapy, and antibody-mediated targeting of effector CTLs.

5.3.1.1 Local Tumor Irradiation

Local tumor irradiation has long been used as a curative treatment for localized cancer and isolated metastasis, but also as a palliative treatment in patients with widespread disease. Overall, more than 50% of cancer patients receive radiotherapy, often as adjuvant therapy, in association with other therapies such as surgery, hormonal therapy [167], chemotherapy, or bone marrow transplantation. Radiotherapy has been highly effective for certain malignancies, including prostate, endometrial, and cervical cancer. Recently, irradiation has come to the attention of tumor immunologists due to its immunogenic properties and potentially antimetastatic effects [168–174].

A major immunological effect of local tumor irradiation is the induction of cell death [175] that results in release of TAAs and danger signals, which attract immune cells to the tumor site, thus favoring antigen cross-presentation, improved DC function, and therefore enhanced antigen-specific T-cell priming [170, 176, 177]. Furthermore, it has recently been demonstrated that, after irradiation, the remaining cancer cells

Table 5.1 Types of immunotherapy aimed at targeting various mechanisms of tumor-induced immune suppression

Type of therapy	Targeted pathway	Achieved effect
Local tumor irradiation	Antigen presentation and processing Release of tumor-associated antigens Production of proinflammatory cytokines and chemoattractants	Enhanced intratumor homing of effector CTLs[a]
Endothelin receptor blockade	Restoration of ICAM-1[b] expression	
Chemotherapy Taxanes	Inhibition of angiogenesis Induction of programmed cell death Antigen presentation and processing TAMs[c] cytotoxicity	
Ab-mediated targeting of CTLs[a]	Tumor and T-cell concomitant antigen binding	
Depletion/inactivation therapy MDSCs[d] Tregs[e] TAMs[c]	Inhibition of DNA replication Inhibition of tyrosine kinase signaling Enzyme inhibition Inhibition of angiogenesis	Enhanced activity of intratumor effector CTLs[a]
Cytokine therapy IL-15 IL-7 IL-12	T-cell growth factors DCs[f] activation Vaccine adjuvants	
Blockade of negative factors Anti-CTLA-4[g] (Ipilimumab) Anti-PD-1[h]/anti-LAG3[i] Anti-TGFβ[j] Anti CD40/CD40L	Blockade of T-cell checkpoints Inhibition of receptor signaling Induction of T-cell activation Antigen-presenting cell activation	

[a]Cytotoxic T lymphocytes
[b]Intercellular adhesion molecule 1
[c]Tumor-associated macrophages
[d]Myeloid-derived suppressor cells
[e]Regulatory T cells
[f]Dendritic cells
[g]Cytotoxic T lymphocyte-associated protein 4
[h]Programmed cell death protein 1
[i]Lymphocyte-activation gene 3
[j]Transforming growth factor beta

present high levels of co-stimulatory and MHC class I molecules that render them more immunostimulatory and susceptible to T-cell-mediated killing [178]. Other beneficial effects of local tumor irradiation involve the induction of proinflammatory cytokines, such as TNF-α, IL-1β, and TGFβ [168, 179, 180]; expression of chemokines, like CXC-motif chemokines such as CXCL9, CXCL10, CXCL11, and CXCL16 that result in chemotaxis of T cells; and induction of adhesion molecules and death receptors that enhance CTL responses [181, 182]. These changes within the tumor microenvironment facilitate recruitment of effector T cells to tumors via two distinct mechanisms: first, by promoting vasculature normalization [183] and, second, by stimulating overexpression of endothelial adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM-1) [169].

In the last decade, preclinical and human studies brought forward substantial clinical evidence that local tumor irradiation has the capacity to activate the immune system. Notably, combination of immunotherapies and radiation has been shown to enhance antitumor responses. Preclinical studies in tumor-bearing mice displayed that irradiation combined with PD-1 blockade increased overall survival and decreased Treg infiltration [184], when compared with anti-PD-1 treatment alone. Consistent to that combination of anti-PD-L1 antibody and irradiation resulted in substantial tumor regression, together with significant reduction of MDSCs within the tumors and increased $CD8^+$ T-cell infiltration

[185]. Currently, multiple clinical trials are evaluating anti-PD-1 and anti-PD-L1 antibodies in combination with radiation for cancer treatment, but results are not yet published [186]. Additionally, after combination therapy of irradiation and CTLA-4 blockade [187], lung metastasis was inhibited in a mouse 4T1 primary mammary carcinoma. Recently, Vanpouille-Box et al. suggested that, in patients who did not respond to treatment with immune-checkpoint inhibitors, local tumor irradiation may induce tumor-specific CTLs [188]. Clinical studies of combination therapies with anti-CTLA-4 antibodies, such as ipilimumab, demonstrated tumor regression and improved overall survival, primarily in patients with melanoma but also with lymphoma, prostate, or renal cancer [189–194].

Taken together, these preclinical and clinical data illustrate that radiotherapy, alone or in combination with other therapies, effectively stimulates the immune system to fight tumor development. This occurs by facilitating antigen presentation and processing, causing the release of TAAs; increasing production of inflammatory cytokines, chemokines, and receptors involved in recruitment of effector CTLs; and thus enhancing migration of these active effector CTLs to the tumor site.

5.3.1.2 Blockade of Endothelin Receptors

Various studies demonstrated that endothelial cells from a variety of human cancers overexpress the ET1 receptors. Blocking these receptors seems a promising strategy to delay tumor development or stop tumor cell proliferation. In a mouse HPV-induced cervical carcinoma model, blockade of ETAR caused inhibition of tumor growth [165], mediated by an increase in T-cell homing to the tumor site. Moreover, ICAM-1 downregulation, as an effect of ETBR interaction with ET1 [163], is rescued by administration of BQ-788, an ETBR small molecule inhibitor [149]. Neutralization of ETBR by administration of BQ-788, suppressed intercellular communication and growth of melanoma cells in nude mice [165] and significantly increased T cell homing to tumors [149, 163]. In fact, selective ETAR blockade by atrasentan showed delayed progression of hormone-refractory prostate adenocarcinoma [195], enhanced the effect of paclitaxel/docetaxel treatment in prostate cancer [196], and increased the overall survival of patients with chronic lymphocytic leukemia B [197].

5.3.1.3 Taxane-Based Chemotherapy

Conventional chemotherapy is considered to act through direct killing of tumor cells or by irreversible tumor growth arrest. Most chemotherapeutics interfere with cellular processes, such as DNA synthesis and replication, or lead to specific cell cycle arrest through microtubule disruption and apoptosis induction [198]. Originally, taxanes (e.g., paclitaxel, docetaxel) have been categorized as a class of chemotherapeutic drugs which block tumor development upon induction of mitotic inhibition through disruption of microtubule functionality. Other studies suggested additional antitumor mechanisms, such as binding to and blocking the functions of the antiapoptotic molecule Bcl-2 expressed on the surface of tumor cells, thus inducing programmed cell death [199]. More recently, the idea of chemotherapeutic agents, including taxanes, as enhancers of effector CTL homing into the tumor site came into place. The immunomodulatory effects of chemotherapy span both the innate and the adaptive immune systems, highlighting the enhanced potential of chemotherapy in combination with immunotherapy [198]. For example, treatment with the angiogenesis inhibitor paclitaxel resulted in an increased infiltration of circulating effector T cells into the tumor site, in a human xenograft mouse model [200]. Additionally, paclitaxel therapy is associated with tumor regression through direct stimulation of TAM cytotoxicity [201] or indirect activation of DCs, NK, and tumor-specific $CD8^+$ T cells via IL-12, TNF-α, and iNOS secretion by TAMs [202]. Taxanes also promote antigen presentation in murine bone marrow (BM)–DCs and human monocyte-derived DCs (moDCS) in vitro via upregulation of costimulatory molecules and IL-12p70 [203, 204]. Additionally, paclitaxel specifically impairs the viability and the cytokine production of $FOXP3^+$ Tregs [205]. On the other hand,

docetaxel induces maturation of DCs in vitro [206] and selective killing of MDSCs in vitro and in vivo [207, 208].

5.3.1.4 Antibody-Mediated Targeting of Effector CTLs

Monoclonal antibody therapy is a method commonly used to functionally inactivate or deplete suppressive immune populations such as MDSCs or Tregs, as discussed below. However, various studies using bispecific monoclonal antibodies suggest that they can also exhibit antitumor therapeutic potential. These antibodies are artificial proteins composed of fragments of two distinct monoclonal antibodies that can bind to two different types of antigens. In cancer immunotherapies, they are engineered to simultaneously bind to a CTL and a tumor cell. Several examples include engagement of CD3, CD28, or CD137 receptors [209] on the T cells and various tumor cell markers, such as epithelial adhesion molecule, and human epidermal growth factor receptor expressed on the tumor cell [210]. Different studies have shown the therapeutic potency of these strategies in vitro [211] and in vivo [209, 210, 212–214].

5.3.2 Strategies Targeting the Activity of Effector T Cells

Enhancing intratumor homing of immune effector cells will most likely not be sufficient for an effective tumor control, as cells that migrate to the tumor site are often anergic or dysfunctional. As addressed above, multiple mechanisms within the tumor microenvironment, involving a diversity of immunosuppressive cell populations (e.g., MDSCs, TAMs or Tregs), negative regulatory factors (e.g., CTLA-4, PD-1, PDL-1), as well as cytokines and enzymes (e.g., TGF-β and IDO), have been implicated in generating this immune suppressive tumor microenvironment.

To increase the efficacy of immunotherapies and rationally develop novel strategies which enhance the activity of intratumor effector T cells, both inhibition of tolerance mechanisms and restriction of tumor-induced immune suppression should be targeted. To effectively target the above-described negative regulatory mechanisms, several strategies have been studied. An overview of the immunotherapeutic interventions that are most widely studied preclinically as well as in clinical trials will be addressed.

5.3.2.1 Circumventing Activity of Suppressive Immune Populations: Depletion or Inactivation Therapy

One commonly used mechanism to target innate as well as adaptive antitumor immunity is manipulation of the immune suppressive functions of MDSCs, Tregs, or TAMs. A more intrusive alternative, however extremely efficient, is depletion of suppressive immune populations. Different depletion methods, with specificity for the targeted immune population at hand, have been developed.

There are several ways to specifically target and deplete intratumoral MDSCs [215]. Studies using an engineered RNA aptamer that targets IL4 receptor alpha (IL4Rα), upregulated on MDSCs of tumor-bearing mice, showed delayed tumor growth, enhanced T-cell infiltration, and MDSC apoptosis [216, 217]. This strategy may have promising results, since ILRα expression is also elevated in MDSCs in human tumors [218]. Another way to deplete MDSCs is with broad-spectrum tyrosine kinase inhibitors, such as sunitinib [219]. In the TC-1 cervical cancer mouse model, combinations of sunitinib with a cancer vaccine targeting tumor cells expressing the E6,7 oncoproteins of HPV, resulted in MDSC depletion and led to enhanced E7-specific CTL frequencies and subsequent tumor eradication [220]. Consistent to this, sunitinib also induced reversal of Treg elevation, significant reduction of IL4 production, and increased frequencies of IFN-γ-producing T cells [219, 221]. Sunitinib is capable of inducing selective MDSC apoptosis, up to 50%, in patients with metastatic renal cell carcinoma, thus representing one of the most promising drugs for reducing tumor-induced immune suppression [219, 222]. Treatment with chemotherapeutic agents and cytostatic drugs such as 5-fluorouracil [223, 224] or gemcitabine [225,

226], as well as novel strategies, like peptibodies [227], have also been described to deplete MDSCs.

Another immune suppressive population that has been intensively targeted for improving antitumor responses is Tregs. To date, several methods to deplete Tregs have been developed. Depletion of $CD4^+CD25^+$ Tregs by monoclonal antibody therapy has been achieved in both tumor-bearing mice as well as in clinical trials [228, 229]. Selective depletion of $FoxP3^+$ Tregs in transgenic DEREG (depletion of regulatory T cells) mice, in combination with therapeutic immunization against melanoma, greatly enhanced the antitumor effect [230]. However, the potency of a combination of immunization and Treg depletion depends not only on the involvement of Tregs in the tumor model studied but also on the level of Treg induction or activation in the immunization strategy. For example, depletion of Tregs by treatment with an anti-folate receptor 4 antibody did not enhance the immune response induced by immunization with the recombinant viral vector vaccine Semliki Forest virus encoding for the early HPV viral proteins E6 and E7 (SFVeE6,7) in a mouse model of cervical carcinoma [231]. In the clinical setting, a potent method to deplete Tregs by targeting their high CD25 expression is by employing the immunotoxin denileukin diftitox (Ontak™ Ligand Pharmaceuticals), which is approved for clinical use in the treatment of cutaneous T-cell lymphoma [232]. In combination with immunization, it has also been used for treatment of other types of tumors [233]. Daclizumab (Hoffman-La Roche) is another anti-CD25 agent, previously used in patients with T-cell leukemia [234] and, more recently, in combination with a peptide vaccine for treatment of metastatic breast cancer [235] and ovarian cancer [236]. However, anti-CD25 antibodies can also target activated $CD25^+$ effector T cells. Alternatives that circumvent this disadvantage are the use of novel antibodies with human specificity such as anti-glucocorticoid-induced TNF receptor antibodies, or low doses of Treg-depleting cyclophosphamide [237].

Regarding TAMs, selective depletion can be achieved by different approaches, such as blockade of TAM chemoattractant chemokines (e.g., blockade of CCL-2 with the inhibitor molecule bindarit [238] or immunization with a legumain-based minigene DNA vaccine [239]). Notably, the most efficient depletion method in animal models involves the usage of clodronate liposomes. Clodronate liposomes are artificial spheres formed by dispersion of phospholipid molecules into an aqueous solution of clodronate bisphosphonate. Intraperitoneal or subcutaneous administration of clodronate liposomes induced efficient depletion (75–92%) of TAMs in different murine tumor models [240–244]. Furthermore, selective depletion of TAMs is promoted by IL-15 and or TGF-α in human primary colorectal adenocarcinomas [245]. In other studies, IL-15 has been shown to reverse T-cell anergy and to rescue the tolerant phenotype of CD8+ T cells [246]. Several other pharmacological drugs, such as zoledronic acid and sorafenib, may also deplete TAMs and enhance the antitumor responses [247]. Yet it should be noted that nonselective depletion of TAMs also results in the depletion of tumoricidal macrophages, whereby any beneficial effect can be counteracted. Novel strategies that repolarize the protumoral M2-like TAMs to cytotoxic M1-like macrophages should be considered.

5.3.2.2 Immunostimulatory Cytokines: Cytokine Therapy

In addition to the above-discussed IL-15, various other cytokines are viewed as promising immune-restorative drugs. IL-7, a survival cytokine crucial for T-cell development in the thymus and survival of naïve and memory T-cell homeostasis in the peripheral tissues [248], increases the numbers of peripheral $CD4^+$ and $CD8^+$ T cells in patients [249, 250]. IL-12, a cytokine naturally produced by DCs, is a potent immune adjuvant promoting IFN-γ release from immune cells and thus inducing Th1 polarization and proliferation of antitumor effector T cells [251], with encouraging results in preclinical studies on diverse mouse tumor models, including thyroid cancer, bladder cancer, metastatic breast carcinoma, and glioma [252–254].

5.3.2.3 Blockade of Negative Regulatory Factors: Antibody Therapy

Antibody therapy against developing tumors has been employed in the clinics for many years and belongs to the category of "molecular targeted therapy" of cancer. Despite the emergence of a large palette of anticancer monoclonal humanized or chimeric antibodies (MABs), only a small number are approved for patient use by the Food and Drug Administration (FDA). Among them, trastuzumab (Herceptin) is a humanized MAB targeting ERGR activity, specific for HER-2/neu-positive breast cancer and metastatic gastrointestinal cancers [255–257]. Another successful example of MABs is Rituximab (Rituxan), a human/murine MAB targeting CD20 for B-cell lymphoma, lymphocytic leukemia, but also autoimmune diseases [258, 259]. Due to their low toxicity profile and capacity to activate several distinct host effector mechanisms [260], these monoclonal antibodies are seen as very promising anticancer drugs. The mechanisms mainly employed by these antibodies are direct interference with tumor cell progression and cell-mediated cytotoxicity by ligation of Fc receptors expressed on the surface of different immune cells [261].

The blockade of PD-1/PD-L1 interaction by several immune checkpoint inhibitors is currently being used for a wide range of solid and non-solid cancers [262] and has so far exhibited durable responses without serious toxicity in the majority of treated patients. The magnitude of clinical responses achieved with checkpoint inhibitor therapy implies that patients can have preexisting tumor-specific T cells that can be reactivated by blocking the PD-1/PD-L1 interaction. Another antibody that has been approved for treatment of late stage melanoma is ipilimumab (Yervoy), a human monoclonal antibody directed against the CTLA-4 expressed on activated T cells, as discussed above. Due to its capacity to inhibit this negative signaling pathway and contribute to restoration of the antitumor antigen-specific immune response, anti-CTLA4 is nowadays used as a novel therapy for solid tumors [15]. Recently, PD-1 blockade has been shown to increase the induction of effector T cells in the spleen, prolong T-cell proliferation, and enhance recruitment of effector T cells to tumor sites. In multimodality therapy regimens, PD-1 blockade increased therapeutic efficacy of total body irradiation and DC transfer therapy [263]. Also, antibody blockade of LAG-3 in two murine models of self and tumor-tolerance increased the accumulation and effector function of antigen-specific $CD8^+$ T cells [264]. Thus, combination of MAB therapy against PD-1 or LAG-3 with immunization strategies has been recently demonstrated to restore the functions of tolerized antigen-specific $CD8^+$ T cells [265]. Several clinical trials are currently ongoing to evaluate responses in patients with cancer following anti-PD-L1 treatment [266–269]. Several approaches have been employed to induce high avidity effector T cells in an attempt to target the inhibition of tumor-induced tolerance. One such approach involves blockade of TGF-β-induced signaling that has pleiotropic functions in tumor initiation, development, and metastasis. Since cancer cells display dysregulated TGF-β signaling, TGF-β inhibitors act on TGF-β-responsive cells (e.g., fibroblastic, endothelial, and immune cells) in the tumor microenvironment. In a xenograft mouse model of prostate cancer, transfer of tumor-reactive, TGF-β-insensitive $CD8^+$ T cells led to a 50% decrease in average tumor weight, when compared with tumors of mice which underwent transfer of naïve $CD8^+$ T cells [270]. Also, monoclonal antibodies against TGF-β, which are nowadays evaluated in clinical trials, seem to be very promising antitumor candidates as they present little systemic toxicity [271]. Clinical results of TGF-β inhibition in a phase II study performed in hepatocellular carcinoma patients are promising [272]. Additionally, radiotherapy and chemotherapy can induce TGF-β activity, and combined TGF-β inhibition enhances tumor sensitivity to chemotherapy and radiotherapy [273]. Another approach aimed at manipulating TGF-β to improve antitumor immune responses involves generation of TGF-β-insensitive DC vaccines. Transduced DCs, which have been rendered insensitive to TGF-β, maintain their normal phenotype, present

upregulated expression of surface co-stimulatory molecules (CD80/CD86), and induce potent tumor-specific cytotoxic T-lymphocyte responses in vivo [274].

Another target for antibody therapy is the costimulatory molecule CD40 expressed on various APCs and tumor cells. CD40 binds to CD40L expressed on T helper cells, resulting in APC activation as indicated by HLA classs II upregulation and IL-2 production [275, 276]. Agonistic antibodies against CD40 and/or CD40L tested in clinical trials seem to have a promising therapeutic potential [277].

5.4 Concluding Remarks

In the last few decades, major progress has been achieved within the field of cancer immunotherapy, highlighting the underlying therapeutic potential. However, despite the clinical success of antibody therapies against immune checkpoints, especially in the context of CTLA-4 and PD-1/PD-L1 axis blockade, still only a subset of patients shows sustained responses. This illustrates the complexity of tumor immunity and the interplay between antitumor responses, immune tolerance, and immune suppression within the tumor microenvironment. For cancer immunotherapy to be effective, sufficient homing and activation of antigen-specific immune effector cells in the tumor and suppression of immune-suppressive mechanisms is pivotal. This calls for multimodality treatment regimens to achieve long-term tumor regression. A desirable, highly effective immunization strategy should therefore accomplish two purposes. On the one hand, it should aim at increasing both the recruitment of antigen-specific effector T cells to the tumor site and their intratumor arrest for the time necessary to exert their antitumor activity. For this purpose, combinations of immunization regimens with ways to enhance homing of immune effector cells to the tumor site, such as local tumor irradiation, endothelin B receptor blockade, antibody-mediated targeting of effector CTLs, or taxane-based chemotherapy, could be promising strategies. On the other hand, only targeting the homing of vaccine-induced effector T cells to the tumor site might not be enough. We may speculate that once these cells have reached the tumor, they can be anergized or tolerized by diverse immune-suppressive mechanisms developed by the tumor itself or by secondary immune-suppressive populations. To counteract this effect, strategies that aim at maintaining or potentiating the activity of these intratumor antigen-specific effector T cells, such as depletion or functional inhibition of immune-suppressive populations, or blockade of negative regulatory factors are necessary.

Concluding, the development of new multimodality strategies in which immunization therapies are combined with effective antitumor immunological or conventional approaches aimed at increasing homing of immune effector cells to tumors and their intratumor activity is of crucial importance and represents the next step forward in cancer immunotherapy.

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