Seminars in Immunology 28 (2016) 54–63

Contents lists available at ScienceDirect

Seminars in Immunology

journal homepage: www.elsevier.com/locate/ysmim

Review

Enhancing T cell therapy by overcoming the immunosuppressive tumor microenvironment

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ARTICLE INFO

Article history:
Received 30 November 2015
Received in revised form 25 January 2016
Accepted 26 January 2016
Available online 10 February 2016

Keywords:
Immunosuppression
Cancer
Microenvironment
TIL
Infiltration
MDSC

ABSTRACT

Immune response to tumors can be successfully oriented for therapeutic purposes, as shown by the clinical efficacy of checkpoint blockade in extending the survival of patients with certain solid and hematologic neoplasms. Nonetheless, numerous patients do not benefit from these new treatments. Tumor-specific $CD8^+$ T lymphocytes, either endogenously revived by checkpoint interference or adoptively transferred after in vitro expansion and retargeting, can be extremely efficient in controlling metastatic disease but have to overcome a number of restraints imposed by growing tumors. This immune escape relies on a profound modification of the tumor environment, which is rendered less permissive to lymphocyte arrival, persistence, and functional activity. We review here emerging findings on the main negative circuits limiting the efficacy of cancer immunotherapy, as well as novel and conventional approaches that can translate into rational combination therapies.

1. Introduction

Immunotherapy of cancer is rapidly emerging as a frontline approach to cancer therapy due to the recent success of checkpoint inhibitors and adoptive T cell therapy (ACT) of cancer in the clinic. Checkpoint inhibitors such as antibodies blocking PD1/PDL1, achieved long-term survival and durable remissions in subsets of cancer patients [1–4]. However, still many patients show a short-term benefit or no benefit at all. On the other hand, ACT with in vitro-expanded, tumor infiltrating lymphocytes (TIL) or T cells from peripheral blood genetically modified to target the tumor [5–7], has shown best results so far in metastatic melanoma treated with TILs [7], and in acute lymphoblastic leukemia for genetically-modified T cells [8]. There is still ample room for improvement of efficacy, in particular in solid malignancies other than melanoma. Two elements can be modified to achieve better therapeutic effects from adoptively transferred T cells: the potency of the T cells themselves, and the often T cell-refractory tumor microenvironment in which T cells must exert their anti-tumoral function. It is likely that therapeutic intervention directed at the tumor microenvironment would be particularly important for such cases when the T cell quality is suboptimal [9]. The tumor microenvironment can be a formidable hurdle for therapeutic effect of endogenous or adoptively transferred T cells by limiting T cell infiltration [10], and by counteracting T cell activity with a myriad of immunosuppressive mechanisms [11].

Evidence that improved survival [12–14] and clinical responses to immunotherapy e.g. with checkpoint inhibitors correlate with the presence of T cells in the tumor microenvironment [15], has been the driving force to look for the genetic mechanisms dictating T cell infiltration or exclusion from tumors [16,17]. One such tumor cell intrinsic mechanisms involve the activation of the WNT/β-catenin oncogenic pathway, which resulted in T cell exclusion by repressing the expression of CCL4 chemokine and subsequent infiltration of $CD103^+/CD8\alpha^+$ dendritic cells (DC) [17]. Other genetic mechanisms explaining differences in tumor infiltration by T cells concern non-malignant cells in the tumor, such as endothelial cells (see below). Strategies to increase tumor infiltration have traditionally targeted tumor vasculature and/or manipulated the chemokines present in the tumor

Abbreviations: ACT, adoptive T cell therapy; TLS, tertiary lymphoid structures; MDSC, myeloid derived-supressor cells; Tfh, T follicular helper; NSCLC, non-small cell lung cancer; MSR, mesoporous silica rods; PSC, pancreatic stellate cells; TIL, tumor-infiltrating lymphocytes; TAM, tumor-associated macrophages; RNS, reactive nitrogen species; IDO, indoleamine 2,3 deoxygenase; CAF, cancer associate fibroblast; IR, ionizing radiation; COX-1, Cyclooxigenase-1; DPP4, dipeptidyl peptidase-4; ATRA, all-trans retinoic acid; TBI, total body irradiation; 5-FU, 5-fluouracil; CTX, cyclophosphamide.

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http://dx.doi.org/10.1016/j.smim.2016.01.002
1044-5323/

microenvironment. New approaches take advantage from the advancements in biomaterials using porous scaffolds, which can deliver or attract immune cells upon implantation in vivo. Current active research on the formation of ectopic tertiary lymphoid structures (TLS) might provide translational clues to generate de novo TLS in tumors, potentially making use of such biomaterials.

Immunosuppressive mechanisms in tumors include T cell intrinsic control mechanisms such as the up-regulation of molecules that decrease T cell function (e.g. PD1), and T cell extrinsic mechanisms including cellular elements such as myeloid-derived suppressor cells (MDSC) and fibroblasts, and molecules such as indoleamine 2,3 deoxygenase (IDO), among many others. Either blocking such inhibitory mechanisms, or promoting immunostimulatory mechanisms that are downregulated or absent in tumors, could revive a more favorable environment for T cells. The discovery of checkpoint blockade and the encouraging results achieved in the clinic with mAbs interfering with rheostats of T cell activation represent the first tangible demonstration that it is possible to reverse cancer-induced T cell dysfunctions and this is sufficient to cause tumor eradication, at least in some patients affected by specific malignancies. On a general note, it appears that currently explored checkpoint inhibitors can intercept at least two different routes: one affecting systemic tolerance (CTLA4) and another acting on local, intra-tumoral restraints (PD-1/PD-L1). While CTLA4 blockade can enhance $CD8^+$ T cell infiltration in treated patients [18] and hence predispose the tumor environment to the activity of PD-1/PD-L1 blockade, the response to the last checkpoint inhibitors is dependent on pre-existing, tumor-infiltrating $CD8^+$ T cells [19]. In experimental melanoma models, the combination of mAbs anti-CTLA4 and anti-PD-L1 increased the intratumoral ratio of effector T cell to both Treg and MDSC [20], findings that might explain the recent confirmation of the additive activity of combination therapy in a randomized, phase III study in metastatic melanoma patients [21].

Preclinical data support the notion that checkpoint inhibitors could be synergistic with ACT [22–27]. This is reasonable as ex vivo "revived" or genetically manipulated T cells will likely decrease their function upon prolonged exposure to the tumor microenvironment. Since different reviews have recently addressed the biology and clinical significance of checkpoint blockade [28,29], we will focus our attention on emerging strategies to overcome suppressive mechanisms in cancer that can boost the efficacy of tumor-specific $CD8^+$ T lymphocytes. Specifically, we will discuss the regulation of myelomonocytic cell recruitment, reversion of the enzymatic inhibition of T cell activation and targeting of the fibroblastic tumor stroma. We will also discuss novel ways to overcome immune suppression by promoting immune stimulation in the tumor via STING/type I IFN, or systemically, by manipulating gut microbiota.

In summary, here we will review therapeutic approaches, including conventional cancer therapies, i.e. radiotherapy and chemotherapy, that result in increased T cell infiltration or reversed immunosuppression within the tumor microenvironment and could therefore be combined with T cell therapy for enhancing its impact on cancer patients.

2. Strategies to increase tumor infiltration by T cells

2.1. Generation of lymphoid structures inside tumors

Tertiary lymphoid structures (TLS) are ectopic lymphoid formations that display an organization very similar to that of lymph nodes, including a T cell zone with mature DC, a germinal center with proliferating B cells, follicular DC and T follicular helper (Tfh) cells (reviewed in [30]). TLS develop under conditions of chronic inflammation and cancer, and their presence has been associated with improved prognosis in lung and colorectal cancers [31–33]. Clinical responses to vaccinations against HPV in patients with advanced cervical intraepithelial neoplasia were associated with generation of TLS [34]. However, not all lymphoid aggregates in tumors reminiscent of TLS are necessarily indicative of a strong adaptive immune response against the tumor, but certain qualitative requirements seem to exist. For example, in non-small cell lung cancer (NSCLC) patients, high density of CD8 T cells per se is not a prognostic value unless combined with a high density of mature DC [35]. Similarly, the generation of TLS after vaccination with a GMCSF-secreting vaccine in pancreatic cancer patients, did not correlate with improved survival unless it showed a suppressed T regulatory (Treg) and enhanced Th17 gene signature [36]. Expression of CCL21 by cancer cells in a mouse tumor model resulted in the generation of lymphoid-like structures that contained mostly Treg cells but no B cell germinal centers, and fueled immune tolerance and tumor progression [37]. In breast cancer, the presence of Tfh in germinal centers predicted high lymphocytic infiltration, survival and response to chemotherapy [38]. Therefore, therapeutic strategies should be directed at the generation of TLS with all the elements of germinal centers. Although generation of TLS for immunotherapy of cancer has not been experimentally tested yet, a conceptually close approach pursuing the generation of ectopic lymphoid tissue to promote antitumor T cell responses is based on the use of LIGHT. LIGHT (TNFSF14) is a member of the TNF family that can act as a costimulatory molecule through binding to HVEM on T cells [39]. LIGHT can also be secreted as a cytokine and bind lymphotoxin-β receptor (LTβR) expressed on non-lymphoid cells. LTβR signaling promotes the organization and maintenance of lymphoid structures including lymph nodes, through its ability to induce the expression of various chemokines such as CCL21, as well as adhesion molecules [40]. Overexpression of LIGHT by cancer cells in preclinical models dramatically increased T cell infiltration of T cell-void tumors [41]. This correlated with an increase in expression of CXCL10, CXCL9 and CCL21, as well as MAdCAM-1 in the tumor. Exogenous administration of an adenovirus encoding LIGHT before surgical excision of the tumor generated CTL that eliminated distant metastases [42].

2.2. Use of porous scaffolds

Novel strategies linking material engineering and immunology exploit organic or inorganic scaffolds implanted in mice to manipulate the trafficking of immune cells [43,44]. Macroporous scaffolds have been generated from polymerized alginate that integrate a collagen-mimetic peptide to facilitate T cell migration, plus macroparticles with soluble IL15/IL15Rα fusion protein, and lipid bilayer-bound anti-CD3, -CD28 and -CD137 antibodies to promote T cell activation and survival [44]. Surgical implantation of such organic scaffolds preloaded with anti-tumor T cells resulted in dramatically improved anti-tumoral effects when compared to systemic administration of the T cells, in a model of residual disease following surgical resection of breast cancer, and in an aggressive ovarian cancer model [44]. The antitumor effects were accompanied by strong proliferation and heavy infiltration of tumors by T cells delivered by the scaffold. In a similar approach, inorganic mesoporous silica rods (MSRs) have been used to create scaffolds that achieved sustained release of bioactive molecules and recruited high numbers of DC [43]. A unique feature of these MSRs is that they are delivered by needle injection and spontaneously assemble to form 3D structures (the "scaffolds"), therefore bypassing the need for surgical implantation. MSRs technology was proven to generate strong antibody and T cell responses when used for vaccination. Although T cell infiltration of tumors in a therapeutic setting was not assessed, it is conceivable that the recruitment

of DC to peritumoral/intratumoral sites, combined to proper activation signals that could be delivered in the MSRs scaffolds might promote higher recruitment of T cells into the tumor.

2.3. Manipulation of chemokines in the tumor microenvironment

Chemokine gradients govern T cells migration into healthy tissues and tumors and therefore represent an obvious target when seeking to increase tumor infiltration by T cells. Recent studies in mice make use of pharmacological agents already available in the clinics for that purpose. In an example of drug repurposing, Barreira da Silva et al. [45] found that treatment of tumor-bearing mice with Sitagliptin, currently used in type II-diabetes, increased tumor infiltration by T cells. Sitagliptin inhibits the enzyme dipeptidyl peptidase-4 (DPP4). Membrane-bound or soluble DPP4 mediate in vivo truncation of the CXCL10 chemokine among other proteins. In this study, the effect of Sitagliptin in augmenting T cell infiltration seemed specific of the CXCR3/CXCL10 axis. Treatment with Sitaglaptin was synergistic with checkpoint inhibitors anti-CTLA4 and anti-PD1 administered together. In another study, Ene-Obong et al. observed that in patients with pancreatic ductal adenocarcinoma, poor survival correlated with lower densities of CD8 T cells in the stroma adjacent to tumor cells [46]. Activated pancreatic stellate cells (PSC) present in non-adjacent stroma, secreted CXCL12 that "sequestered" CD8 T cells away from the tumor, preventing their access to the tumor regions. Consistently, treatment of mice bearing fully developed pancreatic tumors with ATRA (all-trans retinoic acid), which renders PSC quiescent, increased CD8 T cell infiltration in juxtatumoral stroma. However, the effect of ATRA might be not mediated only by a decreased production of CXCL12, since quiescent PSC produce lower levels of other proteins that can prevent T cell infiltration such as fibronectin [46]. Notably, increasing levels of CXCL12 in the tumor itself would not necessarily increase T cell infiltration, since too high levels of this chemokine can cause repulsion of T cells [47], as further discussed below.

2.4. Targeting tumor vasculature

A number of studies focused on the peculiarities of tumor vasculature to reverse poor T cell infiltration of tumors. Specific expression of certain molecules by tumor endothelial cells (FasL, Endothelin B receptor) [16,48] or functional abnormalities causing poor perfusion [49], resulted into paucity of CD8 T cells infiltrating tumor tissue. Administration of low-dose TNF directed to tumor vessels caused vascular "normalization" and increased tumor T cell infiltration in a murine model of pancreatic cancer. Reprogrammed macrophages in treated tumors produced angiopoietin 2 that sensitized endothelial cells to VCAM induction by low-dose TNF, which promoted leukocyte adhesion [50]. Interestingly, vessel normalization by low-dose TNF, but not vascular destruction by IFNγ improved survival. Low-dose TNF also enhanced the efficacy of therapeutic vaccination and adoptive T cell transfer.

Targeting of VEGF/VEGFR pathway in preclinical studies resulted in higher CD8 T cell infiltration that was associated with enhanced effects of cancer cell vaccines [51,52] and adoptive T cell therapy [53]. In particular, low doses of anti-VEGF receptor 2-antibody [52] showed very similar effects to low-dose TNF. The immune-promoting effects of VEGF blockade were therefore initially attributed to vessel normalization [52,53]. However, a recent study suggests an alternative mechanism involving FasL [48]. VEGF and Cyclooxigenase-1 (COX-1) co-expressed in human ovarian cancer, cooperate with IL10 to induce FasL expression specifically in tumor endothelium. Endothelial FasL killed CD8 T cells preferentially compared to Tregs, due to higher expression of c-FLIP in Tregs. Pharmacological blockade of VEGF and COX1 with anti-VEGF antibodies and acetylsalicylic acid respectively, limited FasL expression on endothelium, increasing CD8 T cell infiltration and enhancing the anti-tumoral effects of adoptive T cell transfer. Therefore, the increased T cell infiltration found after anti-angiogenesis therapy could be due to a reduction of FasL-mediated killing of CD8 T cells.

A search for molecules differentially expressed by endothelial cells from ovarian tumors with or without tumor-infiltrating lymphocytes (TILs), revealed endothelial B receptor (ET_BR) as another key molecule that can be pharmacologically targeted to increase T cell influx into tumors [16]. High expression of ET_BR associated with absence of TILs in ovarian cancer patients. The mechanism suggested involved increased NO production resulting in decreased expression of ICAM1 by endothelium. In vivo treatment of mice with a specific ET_BR-inhibitor peptide, BQ-788, increased T cell homing, and synergized with prophylactic and therapeutic vaccination protocols in preclinical models in which the vaccine showed no therapeutic benefit by itself.

3. Strategies to overcome suppressive mechanisms in the tumor microenvironment

3.1. Modulation of cytokine and chemokine microenvironment to regulate myelomonocytic cell recruitment

MDSC and tumor-associated macrophages (TAM) recruited or differentiated within the tumor by tumor-derived factors inhibit the innate and adaptive antitumor immune response and aid tumorigenesis by invasion of nearby tissues, stroma remodeling, and promotion of cell proliferation, among others. Thus, targeting their recruitment or functionality has potential for cancer treatment [54].

The simplest way to inhibit immune regulatory myelomonocytic cells in cancer is to cut their recruitment to the tumor stroma since many of the stromal cells are constantly replenished by blood circulating precursors. For example, the vast majority of TAM originates from the monocyte pool, and their recruitment is primarily determined by the CCL2-CCR2 axis. Among chemokines, CCL2 represents the natural candidate, and preclinical studies have shown that CCL2 blockade can be used also to boost anti-tumor immunity, in addition to exert an anti-metastatic activity. In fact, administration of anti-CCL2/CCL12 mAbs combined with a recombinant vaccines for different NSCLC mouse models augmented efficacy and enhanced and resulted in cure of about half of the mice [55]. Moreover, the combined treatment increased the number of activated intratumoral $CD8^+$ T cells. Even when used as single agent in models of NSCLC, antitumor and anti-metastatic activity of CCL2 blockade was lost in immunodeficient mice [56]. Interestingly, the treatment did not alter the number of TAM but their function, which lead to the activation of $CD8^+$ T lymphocytes within the tumor. In agreement, with these results, we observed that tumors expressing a strong antigen can be spontaneously rejected in *ccl2* gene ablated mice [57]. The effect depends, in part, on the creation of a tolerogenic environment in the spleen of tumor-bearing hosts. In fact, through the CCR2/CCL2 axis the spleen of tumor-bearing mice accumulate $Ly6C^{hi}$ monocytic cells in the marginal zone: these cells share markers with granulocyte–macrophage progenitors and show properties in common with hemopoietic precursors, since they can give rise to both mononuclear phagocytes and granulocytes [57]. Interestingly, CCL2 serum levels in cancer patients correlate with accumulation of myeloid progenitors and predict overall survival of patients who respond to cancer vaccines [57]. Targeting CCL2/CCR2 axis with a monoclonal antibody (carlumab, CNTO 888) showed initial but modest effects as single therapeutic agent in patients with metastatic castration-resistant prostate cancer [58,59], paving the way to possible combination strategies with immune modulators. However, the sudden discontinuation

of the therapy in mice resulted in unwanted and severe rebound effects, with an increased mobilization of monocytes from bone marrow, overproduction of IL-6 and VEGF-A that caused increased metastatic disease and death [60]. An interesting evolution of targeting CCL2/CCR2 axis is based on the delivery of small interference RNA targeting CCR2 mRNA to monocytes, by means of optimized lipid nanoparticles: repeated treatment with these nanocarriers resulted in a reduced accumulation of TAM in two different mouse tumor models [61]. In addition to the adverse action on metastases, targeting CCL2 for long time might have secondary effects, since the CCL2-CCR2 axis is required for monocyte recruitment in infection and normal homeostatic replenishment of the intestinal macrophage pool.

The intra-tumoral production of reactive nitrogen species (RNS), such as peroxynitrite, can induce post-translational modifications of chemokines and cytokines by nitration/nitrosylation of specific amino acids, as shown for human and mouse CCL2. As a result of these changes, the modified CCL2 could no longer attract tumor-specific $CD8^+$ T lymphocytes, but could still recruit myeloid cells to the tumor site [62]. This is an additional mechanism contributing to create a cold tumor environment, which can be reversed by pharmacological intervention. In fact, in vivo administration of a novel drug, the [3-((aminocarbonyl)furoxan-4-yl]methyl salicylate (AT38) that blocks intratumoral RNS production, ablated post-translational, RNS-induced modification of CCL2, promoted a robust T cell infiltration within the tumor, and allowed the adoptively transferred, tumor-specific $CD8^+$ T lymphocytes to reject solid tumors [62].

Another cytokine controlling monocyte recruitment and conversion to macrophages in the inflamed tumor environment is CSF-1. The pharmacologic inhibitor of CSF-1R signaling, GW2580, abrogated tumor recruitment of $CD11b^+Gr\text{-}1^{lo}Ly6C^{hi}$ monocytic (MO)-MDSC in mice bearing a lung carcinoma model and decreased the expression of both proangiogenic and immunosuppressive genes [63]. A brain-penetrant tyrosine kinase inhibitor of CSF-1R tyrosine kinase activity (BLZ945) blocked tumor progression and increased mouse survival in a model of autochthonous, PDGF-B-driven glioma [64]. Even though circulating myeloid cells and microglia were reduced in mice exposed to BLZ945, tumors had similar numbers of TAM, another example suggesting that redundant chemoattractants might be responsible for TAM recruitment. In glioma-bearing mice, BLZ945 modified the M2 polarization of TAM, increasing their killing and phagocytic function and blocking glioma cell-macrophage heterotypic signaling. Of translational value, gene signatures of mouse TAM reconditioned by BLZ945 therapy associated with enhanced survival in patients affected by proneural glioblastoma multiforme [64]. The clinical relevance of blocking CSF-1/CSF-1R signaling was also confirmed in an experimental setting in which radiotherapy was followed by an increase in CSF-1 expression in immunocompetent mice bearing prostate cancer; in this setting, CSF-1R inhibition with GW2580 and PLX3397 greatly affected myeloid infiltration and significantly delayed tumor regrowth after irradiation [65].

The role of CSF-1R in promoting the immunosuppressive tumor microenvironment was confirmed by recent clinical studies. Patients with locally advanced tenosynovial giant cell tumor (PVNS), not amenable to surgical treatment were treated with RG71555, a human monoclonal antibody that inhibits the dimerization of CSF-1R. Patients showed clinical responses associated with profound reduction of $CSF\text{-}1R^+$ and $CD163^+$ macrophages in tumor tissues accompanied by an increase in $CD8^+$ T cells, which reproduced results in transplantable tumor models characterized by robust TAM infiltration [66]. Adverse effects were modest (mostly periorbital edema) but a fast and persistent depletion of human $CD14^+CD16^+$ but not $CD14^+CD16^-$ monocytes was observed [66]. To confirm the systemic effects on normal macrophages, the RG7155 mAb reduced $CSF\text{-}1R^+$ and $CD68^+CD163^+$ macrophages in the liver and colon of cynomologus monkeys, whereas alveolar macrophages in the lung were marginally affected [66]. Another monoclonal antibody against CSF-1R (IMC-CS4) is undergoing a phase I clinical trial (NCT01346358) to assess safety and pharmacokinetic profile in patients with advanced solid tumors, either refractory to standard therapy or for whom no standard therapy is available. Moreover, PLX-3397, a small inhibitor of CSF-1R is tested in ongoing phase I/II clinical trials both in solid and hematological tumors.

3.2. Reversing the enzymatic inhibition of T cell activation.

The myelo-monocyte compartment conditioned by developing cancers can alter the functionality of tumor-specific $CD8^+$ T lymphocytes also by imposing metabolic restraints. Many enzymes with immune regulatory activity are up-regulated, either in tumor environment or draining lymph nodes, such as arginase 1, inducible nitric oxide synthase and IDO, along with an increased production of nitric oxide and reactive oxygen and nitrogen species [54]. Among these enzymes, targeting IDO for therapeutic interventions is advancing more rapidly to the clinic. This enzyme catabolizes L-tryptophan and can be constitutively expressed in the tumor cells or upregulated in myeloid cells in response to local inflammatory signals, including the IFN-γ produced by tumor-infliltrating T lymphocytes [67]. This counter-regulatory response, which normally intervenes in the host to control tissue homeostasis during acute T lymphocyte activation, can become detrimental for the anti-tumor activity of effector T lymphocytes. Thus, combination of IDO-inhibitor drugs with emerging immunotherapeutic approaches may be synergistic, as indicated by preclinical studies of combination therapies with anti-CTLA-4, anti-PD-1 or adoptively transferred T cells [68–71].

3.3. Targeting cancer associated fibroblasts

Cancer associate fibroblast (CAF) can contribute to impede diffusion of soluble molecules and trafficking of immune cells to the tumor in virtue of their ability to produce components of the extracellular matrix. However, CAF expressing the fibroblast activation protein-a (FAP) on their membrane can release the chemochine CXCL12, which can contribute in T cell exclusion from tumor environment (see Section 2.3). It has been advanced that, once released in the environment, CXCL12 can coat cancer cells, masking them from the immune attack through a not yet identified mechanism [72]. CAF can be deleted by conditional knockout in mice bearing an autochthonous pancreatic cancer and this synergizes with anti-PD-L1 treatment in releasing the antitumor activity of pre-existing T cells [72]. Since widespread depletion of FAP^+ stromal cells might have catastrophic consequences due to their function in regulating tissue architecture homeostasis [73], CAF must be targeted by alternative approaches, such as their reprogramming by administration of a vitamin D analog [74] or interfering with CXCL12 chemokine. The latter treatment opened the environment of pancreatic cancer, allowing the accumulation of T cells and tumor growth control in association with anti-PD-L1 [72].

3.4. Enhancing innate immune recognition of tumors by using STING agonists

Engagement of type I IFN receptor 1 (IFNAR1), recognizing both IFN-α and -β, in tumor-infiltrating $CD8\alpha^+/CD103^+$ DC is required for a full cross-presentation of tumor antigens and activation of tumor-specific $CD8^+$ T cells, both in primary tumors and after radiation therapy [75–78]. It is likely that the tumor antigens involved in a successful cross-presentation and activation of therapeutic $CD8^+$

T cell response are generated by passenger non-synonymous mutations in cancer genes [79]. Type I IFN production by DC requires the sensing of tumor-cell derived DNA in the cytosolic DC compartment through the STING-mediated pathway. In brief, cytoplasm detection of DNA by cGAMP synthase generates canonical 2′ to 5′ cyclic GMP-AMP, which binds to STING and promotes the recruitment and phosphorylation of TANK-binding kinase 1 and interferon regulatory factor 3 (IRF3). IRF3 translocate to the nucleus where it controls transcriptional programs leading to type I IFN production, this cytokine acts in autocrine and paracrine fashion by binding to receptors on tumor-associated DC [75–78].

Recent evidence indicates that STING pathway can be exploited for enhancing intra-tumoral immunity. First, interference with CTLA-4 and PD-1 rheostats is therapeutically ineffective in mice lacking STING, suggesting that STING might be an essential downstream hub for checkpoint blockade therapies [76]. Moreover, the STING activator cGAMP was unsuccessful alone but synergized with radiation to decrease tumor burden and increase survival in wild type but not STING-deficient mice, which suggest that cGAMP might be used as novel drug to decrease tumor resistance to radiation [78]. Last, intra-tumoral injection of non-canonical cyclic dinucleotides, appropriately designed to activate human in addition to mouse STING, demonstrated therapeutic effects in multiple mouse tumor models, including melanomas, breast, and colon cancers; mice that regressed tumors developed a long lasting memory $CD8^+$ T cell response and rejected a second tumor challenge [80].

3.5. Promotion of anti-tumor immunity by manipulation of gut microbiota

Myeloid cell distribution and function within the tumor microenvironment is partly influenced by the gut microbiota, to the extent that chemotherapy and active immunotherapy can be severely impaired in microbiota-depleted mice [81]. In particular, gut microbiota seems to release unidentified signals that maintain a functional tone in myelomonocytic cells and allow them to respond with diversified cancer killing programs to either platinum therapy or the immune stimulatory combination of intra-tumoral injection of mAbs blocking IL-10R and the TLR9 agonist CpG-oligonucleotides [81]. A multifaceted relationship also connects microbiota and checkpoint blockade therapy. Either antibiotic-treated or germ-free mice bearing transplantable melanomas did not respond to anti-CTLA4 therapy unless distinct *Bacteroides* species were introduced back in the gut. T cell responses specific for *B. thetaiotaomicron* or *B. fragilis* associated with the efficacy of CTLA-4 blockade in both mice and patients. The therapeutic response to checkpoint therapy was related to anti-CTLA4-induced subclinical colitis, which caused bacterial colonization of the mucosal layer, induction of Th1 immune responses in the tumor draining lymph nodes and maturation of intratumoral DC [82]. Another commensal, *Bifidobacterium* was responsible for the resistance of mouse strains housed in different mouse facilities, and oral administration of this bacterial species was sufficient to improve melanoma tumor control and synergize with anti-PD-L1 therapy. Analogously to the previous case, the increased DC functionality at the tumor site and the consequent enhancement of $CD8^+$ T cell priming and accumulation in the tumor microenvironment were found to be responsible for the microbiota-driven help [83].

4. Conventional therapies as immunostimulants

The rising of immunotherapy has also directed investigators to study the interactions of conventional therapies, i.e. radio and chemotherapy, with the immune system. In the case of radiation, it is increasingly clear that the immune function of the host will determine the extent of therapeutic response [84–86]. Recent preclinical work has focused on high-dose “ablative” radiation, uncovering a role for STING-mediated [78] activation of class I-interferon production by the immune system [87] in the immune-stimulating effects of radiation. These findings lay out the rationale for combination therapies, such as checkpoint inhibition or ACT plus radiotherapy. Similarly, cumulative evidence indicates that the efficacy of chemotherapy relies at least partially in the immune system, either by promoting the recognition of cancer cells, or by eliminating suppressive mechanisms [88].

4.1. Radiotherapy

Local treatment of tumors with ionizing radiation (IR) acts at several levels to promote both T cell infiltration and function. Single doses of radiation increased in several animal models the expression of adhesion molecules in endothelium, in particular VCAM-1 [89–91], whereas ICAM-1 and E-selectin were upregulated in patients receiving conventional fractionated radiotherapy [92]. Low doses (2 Gy) of local IR were sufficient to induce this response [91]. IR can also induce vascular normalization [93], in particular when used in combination with adoptive T cell therapy [91,93]. Furthermore, IR can induce the expression of T cell attractive chemokines such as CXCL9, CXCL10, CXCL16 and CCL5 in the tumor microenvironment [90,91,93,94]. All these mechanisms result in an increased influx of endogenous T cells at day 5–14 post IR [91,94–97], whereas the recruitment of adoptively transferred T cells can occur even at day 1–3 post-IR [89,94]. A possible explanation for this delay in the increase of endogenous when compared to adoptively transferred T cells, might be a higher dependence on the activation/reprogramming of innate immune cells, such as DC [87,95] and TAM [91], which takes place quite rapidly after IR (day 1–3) [87,91,95].

Besides increasing tumor infiltration by T cells, radiation can also activate the immune function. The mechanisms described by which radiation can favor immune recognition of cancer cells include: increased MHC expression [89,98,99], increased antigen expression [100,101], up-regulation of Fas [102] and calreticulin [103] on cancer cells. Furthermore, radiation can induce stromal “loading” and subsequent recognition by T cells in tumors expressing strong antigens [87,104]. Since most of the studies have used high-dose hypofractionated radiation, it is not clear at this point whether conventional fractionated radiation is equally immune-stimulatory. One of the few studies analyzing this question showed that local low-dose gamma irradiation can alter myelomonocytic cells in the tumor environment. In a mouse model of pancreatic cancer, low dose irradiation “reprogrammed” TAM toward an M1-like phenotype characterized by enhanced secretion of pro-inflammatory cytokines like IL-12 and nitric oxide output; reconditioned macrophages supported anti-tumor Th1 responses, endothelial activation and CTL recruitment to tumors [91].

With this background, a synergistic effect could be expected from the combination of radiation with checkpoint inhibitors. For the anti-CTLA4/radiation combination, a prominent effect in systemic disease was observed in preclinical models of metastatic breast cancer [105]. In the first thorough analysis of the combination between radiotherapy and checkpoint blockade in patients with metastatic melanoma [106], regression of lesions occurred in a number of patients receiving anti-CTLA4 and hypofractionated stereotactic radiation but the majority of them did not benefit from the therapy. Analyzing mice bearing tumors, the adjuvant activity of local radiation on overall antitumor response related to the broadened diversity of the TCR repertoire of intra-tumoral as well as splenic and circulating T cells. Resistance was attributed to upregulation of PD-L1 on melanoma cells and consequent T cell exhaustion [106]. Radiation can increase the expression of

PDL-1 in the tumor microenvironment [107], therefore the blockade of the PD-L1/PD-1 pathway might be necessary for optimal local and systemic effects of checkpoint inhibitor/radiation combinatorial therapies, as suggested by preclinical studies [106–108].

IR is also used systemically (total body irradiation, TBI) as preconditioning regime for adoptive T cell transfer, sometimes in conjunction with chemotherapy [109]. TBI works through mechanisms still incompletely understood, although increased availability of T cell homeostatic cytokines resulting in homeostatic expansion [110,111], decreased numbers of regulatory T cells [111], and systemic innate immunity stimulation through TLR activation [112], have been suggested. In mice, higher doses of preconditioning TBI before adoptive transfer correlated with better anti-tumoral effects [113]. In patients, adding 12 Gy TBI to chemotherapy as preconditioning regime increased the levels of pro-T cell survival cytokines IL-7 and IL-15, and resulted in high response rates [114]. The mechanisms discussed above regarding local radiation could also contribute to a higher infiltration of tumors by adoptively transferred T cells after TBI, because the tumor is also irradiated. The effects of TBI are long lasting, since they can still result in higher infiltration when T cell transfer is delayed 3 weeks since TBI [93].

4.2. Chemotherapy

The antitumor activity of chemotherapy can rely on several off-target, beneficial effects directed toward the host innate and adaptive immune system, which cooperate for successful tumor eradication [115] by influencing both tumor immunogenicity and tumor-induced suppression. Cancer cells dying after being exposed to particular antineoplastic agents, such as anthracyclines and oxaliplatin, either present on their surface or release extracellularly damage-associated molecular pattern molecules, including calreticulin, ATP, and high mobility group box 1 protein, which in turn can heighten the functions of DC [116]. The molecular mechanisms underlying this "immunogenic cell death" are different but translate into enhanced phagocytosis and antigen presentation by DC, DC maturation, activation of NLRP3-inflammasome-dependent release of IL-1β and TLR4- and Myd88-dependent inflammatory response, all contributing to various extent to enhance the priming of anti-tumor T lymphocytes [116]. The activity of anthracyclines on intratumoral DC can further extend to the point of guiding them toward dying cancer cells, allowing the expression of formyl peptide receptor 1 (FPR1) and its tight association with the ligand annexin 1. The genetic inactivation of *Fpr1* impaired DC spatial distribution in cancer, as well the ability to present antigens from dying cells and prime T cell responses toward tumor antigen [117]. In clinical support of these findings, a loss-of-function allele of the gene coding for formyl peptide receptor 1 (FPR1) associated with poor metastasis-free and overall survival in breast and colorectal cancer patients undergoing adjuvant chemotherapy [117].

Anthracyclines can also induce in cancer cells a TLR3-dependent recognition of endogenous RNA leading to the autocrine release of type I IFN and downstream production of the chemokine CxCL10 [118]. Thus, both autocrine and paracrine activity of IFN underlie the in vivo efficacy of chemotherapy on the adaptive immunity.

Chemotherapy can exert negative effect on the tumor environment by increasing the macrophage-assisted chemoresistance of cancer cells (reviewed in [119]). However, depending on the tumor and likely the stage of the disease, chemo and radiotherapy can reshape the stromal components by acting locally but also in tumor distant sites. Conventional chemotherapeutic agents, such as gemcitabine [120–122] and 5-fluouracil (5-FU) [123], can be extremely cytotoxic for MDSC; in particular, M-MDSC are particularly susceptible to low doses of these therapeutic agents, doses with limited or no impact on cancer cells, but able to cause a long-lasting depletion of the splenic M-MDSC pool [57]. The elimination of M-MDSC relieved tumor-dependent immunosuppression and unmasked the therapeutic activity of otherwise ineffective, adoptively transferred, tumor-specific T cells [57]. This selectivity can be further explored to improve ACT. We recently found that lipid nanocapsules (LNCs) loaded with a modified liposoluble form of gemcitabine targeted the immunosuppressive M-MDSC subset. Subcutaneous administration of gemcitabine-loaded LNCs reduced the percentage of spleen and tumor-infiltrating M-MDSC in lymphoma and melanoma-bearing mice, with enhanced efficacy when compared to free gemcitabine; thus relieving tumor-associated immunosuppression. Very low dose administration of gemcitabine-loaded LNCs increased the efficacy of adoptive T cell therapy in tumor-bearing mice (Sasso et al., unpublished observations).

The effect of chemotherapy on myelomonocytic cells can also heighten negative regulation of anti-tumor response, in part by hyper-activation of the innate immunity pathways. Gemcitabine and 5-FU, in fact, can trigger the inflammasome complex in MDSC through the intracellular lysosomal permeabilization and the release of cathepsin B. The resulting IL-1β production promoted the secretion of IL-17 by $CD4^+$ T cells, which had a negative impact on the overall anticancer efficacy of the combined chemotherapy regimen. Interestingly, anakinra, an IL-1 receptor antagonist approved for the treatment of rheumatoid arthritis, could restore the therapeutic window by interfering with the effect of IL-1β released from MDSC [124].

The selective depletion of monocytes in blood and lymphoid organs, including splenic immunosuppressive M-MDSC, was recently shown for trabectedin, a drug approved in Europe (by the EMEA) for the treatment of sarcomas and ovarian carcinomas, which also reduced TAM infiltration in multiple mouse cancer models [125]. Interestingly, trabectedin selectively triggered cell apoptosis in monocytes, but not in granulocytes and lymphocytes [125]. The increased susceptibility to trabectedin of both human and mouse monocytes was found to depend on the higher expression of TRAIL death receptors in these cells, which mediated the cleavage of caspase-8 and the activation of the extrinsic apoptotic pathway [125]. The importance of apoptosis regulation in MDSC biology is highlighted by recent findings. The development of M-MDSC and polymorphonuclear (PMN)-MDSC subsets, in fact, requires the continuous inhibition of different apoptotic pathways, notably the caspase-8 dependent extrinsic pathway in M-MDSC, and the mitochondrial intrinsic pathway in PMN-MDSC [126].

Despite the promising results of checkpoint blockade combination therapy, many patients with melanoma are refractory and this percentage increases when other neoplasms are considered. Interestingly, preclinical data support a rationale for combining anti-PD-1 and anti-CTLA4 treatment with epigenetic-modulating drugs, such as inhibitors of either DNA methyltransferase or histone deacetylase. This protocol was able to cure the majority of mice bearing large transplantable tumors of poorly immunogenic and metastatic colon and mammary carcinomas, by mainly affecting the number of tumor-infiltrating and splenic PMN-MDSC. Further investigation about the molecular target of the epigenetic regulation pointed to phosphatidylinositide 3-kinases: highly active inhibitors mimicked the effect on PMN-MDSC without affecting $CD8^+$ T cell activity [127].

These data also reinforce the concept that cancers resistant to immune checkpoint blockade can benefit from MDSC elimination. Cisplatin preconditioning changed the percentages of myeloid cells by increasing DC and eliminating MDSC and enhanced cytokine-induced killer cell responses in melanoma-bearing mice [128]. Paclitaxel, an agent inhibiting microtubules disassembly, enforces MDSC differentiation to DC [129], whereas docetaxel, a drug with similar action, selectively kills M2 macrophages and polarizes MDSC toward an M1-like phenotype possibly through

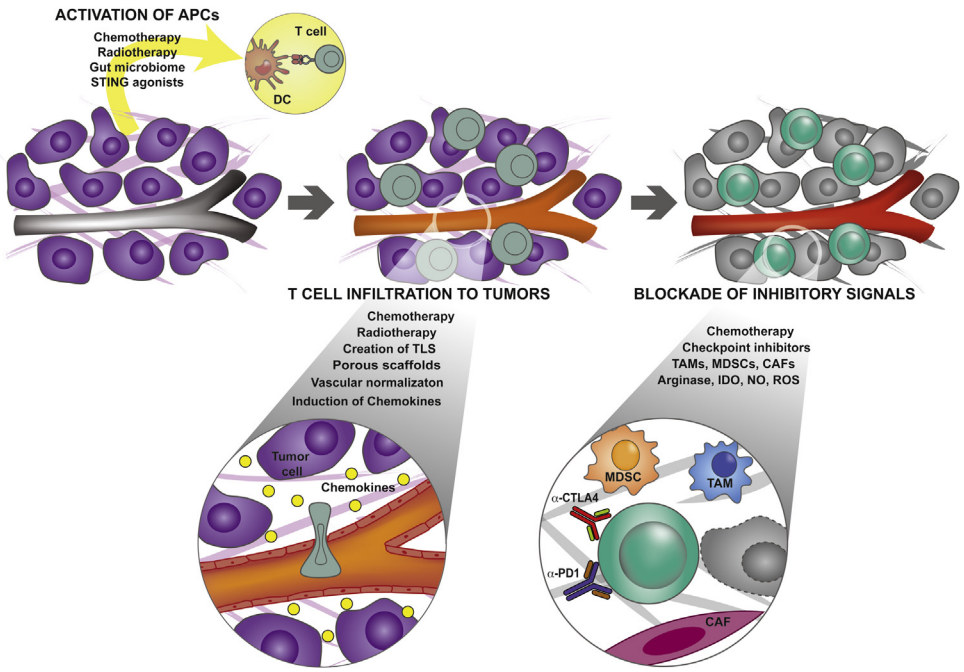

Fig. 1. Different tumor environments require distinct approaches to improve cancer immunotherapy by T lymphocytes. From left to right, three main tumor environments can be envisioned based on current evidence. Left panel: in some tumors, T cells are absent and vascularization is compromised, restricting the arrival and survival of incoming T lymphocytes; approaches triggering a local activation and recruitment of APCs might be necessary to overcome these limitations to T cell therapy. Middle panel: deranged vascularization might allow a limited infiltration of tumor-specific T cells, but treatments are necessary to promote additional and protracted influx of effectors. Right panel: in other tumors, vascularization is not the limiting factor, T cells can reach the environment but they are subject to a negative influence of tumor-induced inhibitory pathways. These represent diagrammatic and summarizing scenarios, which might coexists under some circumstances. In the future, a full characterization of the immune profile of the patient's tumor might help identifying the prevalent landscape(s). Abbreviations are indicated in the main text.

interference with STAT3 signaling [130]. Cyclophosphamide (CTX) seems to induce complex and sometimes conflicting effects on MDSC. In preclinical model of colon cancer the metronomic CTX administration in combination with gemcitabine mitigated Treg- and PMN-MDSC-mediated immunosuppression, increased intratumoral IFN-γ production and triggered anti-tumor immunity in vivo [131]. In clinical studies, however, CTX in combination with doxorubicin increased the number of circulating, immature MDSC ($Lin^{-/Lo}$, HLA DR^-, $CD33^+CD11b^+$) in newly diagnosed solid tumor patients [132]. However, it should be considered that in this study the patients were also receiving G-CSF, which might affect the systemic accumulation of myeloid cells independently from tumor [132].

Clinical data suggest that the activity of novel targeted therapies might also affect myelomonocytic cells in cancer patients. For example sunitinib, a multi-kinase inhibitor acting on multiple pathways, including VEGFR and c-kit signaling, induced a strong reduction in $CD33^+$/HLA-DR^- M-MDSC in renal cell cancer (RCC) patients, even if no correlation between MDSC levels and tumor burden was observed [133]. One cycle of bevacizumab, a monoclonal antibody directed against VEGF-A, was sufficient to decrease immature myeloid cells ($CD45^+lin^-$HLA-DR^-) and increase DC frequency in the blood of lung, breast and colorectal cancer patients [134]. However, in RCC patients, bevacizumab alone had no effect on MDSC levels, whereas an increase in circulating MDSC was observed after addition of IL-2 to bevacizumab regimen; this discrepancy could be attributed to tumor burden, an important variable, since RCC patients usually receive anti-VEGF therapy for advanced tumors, normally associated with higher MDSC levels [135]. The treatment of melanoma patients with vemurafenib, a highly specific inhibitor of mutant B-RAF V600E, which constitutively activate the MAP kinase pathway and it is detected in about 60% of cutaneous melanomas, reduced the frequency of both $CD14^+$ HLA-DR^- MDSC and $CD14^-CD66b^+$ARG-1^+ MDSC [136].

In considering the interplay between chemotherapy and $CD8^+$ T cells, however, it must be pointed out that the combination of either immune stimulators (vaccines, checkpoint inhibitors) or adoptive cell transfer of effector T cells with chemotherapeutic agent has the potential to affect negatively T lymphocyte expansion. A personalized peptide vaccine boosted immunological cellular and humoral responses in unresectable pancreatic cancer patients and gemcitabine did not affect the immune reactivity [137]. However, while resting T cells are spared by administration of a low dose gemcitabine regimen sufficient to alter systemically the MDSC numbers and functions, antigen-activated T cells can be killed in vivo by this treatment following their adoptive transfer (Sasso et al., unpublished observations), notably narrowing the therapeutic window. Therefore, the type of drug, dose and schedule must be carefully tested in clinical trials exploring the adjuvant activity of chemotherapy on ACT.

5. Concluding remarks

The outcome of T cell therapy is determined by the quality of the T cells and the immunosuppressive characteristics of the particular tumor microenvironment. Here we reviewed emerging and conventional therapeutic strategies aimed at changing the tumor microenvironment into a more favorable environment for T cell infiltration and function (Fig. 1), to be used in conjunction with ACT or checkpoint inhibitors. One obvious issue is the hierarchy and the rationale for combining different treatments and moving them forward to clinical translation. Biomarkers are urgently needed to guide this process. As we move deeper toward personalized medicine and better molecular profiling and classification of cancers, we should also make efforts to characterize the immune context of tumor microenvironment. We need to define which are the prevailing mechanisms restraining T cell effectiveness in different subgroups of patients. Tumors with different histology might

share immune regulatory pathways, i.e. different mutations and epigenetic modifications might shape the surrounding stroma in converging ways. If this were the case, cancers from patients could be classified according to their immune escape profile and thus be treated with the most appropriate therapeutic approaches.

Acknowledgments

This work was supported by grants from the Italian Ministry of Health; Italian Ministry of Education (FIRB cup: B31J11000420001), Universities, and Research; Italian Association for Cancer Research (AIRC, grants 6599, 12182 and 14103) to V.B, as well as the Ludwig Foundation (A.A.). L.C. is supported by a Cancer Research Institute Irvingtonpostdoctoral fellowship.

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