Cytotoxic CD8+ T cells in cancer: to be or not to be

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When developing cancer, the hosts immune response against the tumor can be attenuated through various mechanisms, such as immune cell based suppressive mechanisms, factors deriving from the interaction of tumor cells with immune cells, and tumor cell based suppressive factors. To create an effective immune response against the tumor, these barriers need to be overcome. Recent studies within the field aim, which at elucidating the molecular mechanisms employed by these factors, create opportunities for targeting them and thus enhancing the antitumor immune response. This review aims to give an overview of the most important immune suppressive factors within the tumor microenvironment and their underlying molecular mechanisms. More importantly, it aims to describe the most efficient and widely used therapeutic strategies for blocking these factors.

1. General introduction

Since the 1960's, more and more studies pointed towards the capacity of the immune system to recognize and eradicate arising tumors. Burnet termed this process the cancer immunosurveillance theory [1]. Although various studies from the field challenged this concept [2], within the last decennium the discussion has revived. Preclinical experiments with mouse models lacking components of the IFN-γ pathway or perforin showed that these mice were more prone to tumor development than their wild-type counterparts [3]. In time, the concept of immunosurveillance has been extended, thus this term was recently found to be no longer sufficient. Therefore, the broader concept of immunoediting was proposed. This concept takes into account the protection of the host against the tumor, as well as shaping of the tumor by the immune system of the host [3]. Because the immune system cannot always induce a sufficient response against the tumor, immunotherapy is a promising way to give this natural response a hand. It functions by strengthening as well as enhancing the natural immune response against the developing tumor. This response is based on antigen-specific immune effector cells, especially CD8+ T cells. Although substantial progress has been made, the process of T cell activation through immunotherapy regimens is far from perfect. The aim of this review is to discuss some of the most widely encountered mechanisms by which the functions of CD8+ effector T cells are diminished due to immunosuppressive factors or the tumor microenvironment itself. An emphasis is set on describing the most effective and novel strategies used nowadays to target immunosuppression in the tumor microenvironment, illustrating their targets and the underlying mechanisms responsible for their therapeutic anti-tumoral activity.

2. Immune effector T cells – The final frontier

Cytotoxic CD8+ T lymphocytes

Cytotoxic CD8+ T lymphocytes (CTLs) are one of the major immune cell subsets of the adaptive immune system. As a subgroup of T lymphocytes, their function is to recognize antigens of intracellular microbes and either help phagocytes kill these cells or kill them themselves. Furthermore, they are able to kill tumor cells or cells that are damaged in some other way. Originally, CD8+ T cells are formed in the thymus, along with all other T lymphocytes.

These naïve CD8+ T cells, that have not yet encountered antigens, are then activated in peripheral tissues, through interaction with antigen presenting cells (APCs).
Subsequently, they are able to differentiate into central memory CD8+ T cells (Tcm), effector memory CD8+ T cells (Tem) or effector CD8+ T cells, which are also termed cytotoxic T lymphocytes (CTLs). Tcm have limited effector function, but upon secondary stimulation can proliferate into CTLs. Tem on the other hand, are able to mediate inflammatory functions by producing effector cytokines [4]. Cytotoxic lymphocytes exert their function by releasing cytokines such as perforin and granzyme B, thus inducing apoptosis in tumor cells, allogeneic targets and virus- infected cells [80]. Upon repeated antigen stimulation or after recognition of self- antigens, T lymphocytes eventually enter apoptosis themselves. This process occurs either through the mitochondrial (intrinsic) or death receptor (extrinsic) apoptotic pathway.

Contributive factors to CD8+ T lymphocyte activation

As mentioned earlier, naïve CD8+ T lymphocytes can be activated in peripheral tissues. The T cell receptor (TCR) on the surface of the CD8+ T cell interacts with the peptide-bound MHC I molecules on the surface of the antigen presenting cells (APCs). In addition, a co- stimulatory signal is required. The T cell surface receptor CD 28 binds to the co- stimulatory molecule CD80 and CD86, expressed on APCs. Another co- stimulatory signal can be provided by activated helper T cells (CD4+ T cells) expressing CD40 ligand, a member of the TNF superfamily of molecules. Binding between this ligand and its correspondent receptor CD40 on the surface of APCs activates these cells and causes cytokines such as IL-12, a promoter of T cell differentiation, to be released. IL-2, another factor for T lymphocyte differentiation, is produced mainly by CD4+ T cells upon antigen stimulation. In response to activation of the TCR or in response to IL-12 or IL-18 [5], IFN-γ increases expression of MHC-I, thereby enhancing recognition of infected cells by CTLs.

3. Immune suppressive factors

3.1 Immune cell based suppressive factors

3.1.1 *Myeloid derived suppressive cells (MDSCs)* represent one of the main immune cell populations of myeloid origin and consist of a diversity of phenotypes, ranging from granulocytic and monocytic origin to DCs and macrophages [6]. Under non –pathological conditions, MDSCs are present in the bone marrow and are involved in myelopoiesis. In reaction to various pathological conditions such as cancer, sepsis and infectious diseases, MDSCs are expanded rapidly and accumulate in lymphoid tissues [7]. In mice, MDSCs are generally characterized as CD11b+Gr1+ [8]. Two main populations of MDSCs have been repeatedly reported so far: monocytic MDSCs (M-MDSCs) and granulocytic MDSCs (G-MDSCs). This granulocytic MDSCs population can be found both in mice and in humans as the major subset of circulating MDSCs [8]. Over time, MDSCs have developed various mechanisms to suppress activity of various T cell populations. First of all, generation of reactive oxygen species (ROS) by the NADPH oxidase complex suppresses T cell response by affecting protein functions in target cells [9]. While this mechanism is mainly induced by G-MDSCs, M-MDSCs produce mainly NO [10]. Secondly, by depletion of nutrients that are required for lymphocyte growth and development, such as L-arginine [11] and L- cysteine [12], T cell activation is inhibited.

Thirdly, studies have shown that MDSCs can promote the activation and expansion of other immune suppressive populations such as regulatory T cells, through secretion of immunosuppressive factors like IL-10, TGF- β or IFN-γ [13] or immune activating factors like CD40 [14].

3.1.2 *A regulatory T cell (Treg)* can be defined as a T cell that inhibits the normal immune response by influencing the activity of another cell type [15]. The classic Tregs derive from the thymus and can be identified as CD4+CD25+FOXP3+ T cells [16]. They include naturally occurring Tregs (nTregs) and inducible Tregs (iTregs). [56]. In various cancers, such as ovarian, breast, lung and pancreatic cancer, an increased number of Tregs was found [reviewed in 16]. Tregs can suppress the antitumor immune response in various ways. A number of cytokines secreted by Tregs, such as IL-10, IL-35 and TGF-β, inhibit the activity of effector T cells [17]. These secreted cytokines inhibit cytokine production, expansion and effector functions (cytolysis) of effector cells [18]. An additional mechanism by which Tregs inhibit the function of effector T cells is by disrupting the effector cells metabolically. By depleting IL-2 in effector T cells, these cells go into apoptosis [19]. Moreover, the catalysis of ATP, normally involved in energy transfer in cells, to adenosine has been shown to suppress effector T cell function as well [20]. Additional mediators of the suppressive effect of Tregs on the antitumor immune response are among others granzyme B [21] and the TRAIL [22] pathway. The activation of these pathways causes the effector T cells to go into apoptosis. Finally, indoleamine 2,3-dioxygenase (IDO) is upregulated in DCs, through cell-cell mediated signaling of CTLA-4 on the Tregs and CD80 on DCs. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) is the main inhibitory signal of T cells [16]. IDO blocks antigen-specific T cell proliferation, by depleting one of the essential aminoacids involved in the metabolic process of effector T cells, tryptophan [23].

3.2 Interaction of tumor cells with immune cells

Programmed Death-1 (PD-1) is a cell surface glycoprotein that belongs to the B7 family of co-stimulatory molecules [24]. PD-1 has two ligands on which it can bind: PD-L1 and PD-L2. PD-L1 is upregulated on many cell types, such as hematopoietic, endothelial and epithelial cells. PD-L2 is upregulated on macrophages and dendritic cells [25]. Normally, PD-1 plays a major role in limiting the activity of T cells during inflammation and also in limiting autoimmunity, by maintenance of tolerance [25]. Upon binding with PD1 on T or B cells, PD-L1 on tumor cells causes inhibition of T cell receptor- mediated lymphocyte proliferation and cytokine secretion [26].

One other pathway that induces negative regulatory activity is by using the LAG-3 glycoprotein, expressed on activated natural killer cells (NK cells), T cells and B cells [31,32]. Using LAG-3 (-/-) mice, it was shown that LAG3 negatively regulates active T cell expansion [33, 34], thereby playing a role in tumor immunosuppression.

Finally, recent studies brought up two other ligands, B7-H3 and B7-H4 (B7x). They have been found to be related to B7-H1 and B7-H2 and bind on so far unknown receptors on activated T cells [27,28]. It has been shown that B7-H3 inhibits T cell proliferation and cytokine production, although the receptors are not yet known [29], while B7-x negatively regulates T-cell activation [30].

3.3 Tumor cell based suppressive factors

3.3.1 *Fas ligand (FasL),* a transmembrane protein belonging to the TNF family and normally involved in apoptosis, is produced by NK cells and activated T cells. Upon binding to its correspondent receptor FasR, expressed on the surface of the target cell, the target cell undergoes apoptosis [35]. Pitti et al. reported the existence of soluble decoy receptors, such as DcR3 , that bind to FasL and inhibit FasL-induced apoptosis [36]. It has been proposed that decoy receptors play a role in immune evasion by cancer cells. Indeed, malignant gliomas was one of the tumor types that has been shown to express DcR3 [37]. Injection with recombinant DcR3 blocks FasL- mediated apoptosis *in vitro* and *in vivo* [38].

3.3.2 Immune suppressive cytokines

Transforming growth factor-β (TGF-β) is an important and well-studied member of regulatory cytokines and almost all cells produce TGF-β and have a receptor for it. Normally, it regulates proliferation and differentiation of cells, embryonic development, wound healing and angiogenesis [39]. Various studies have proposed three types of TGF-β that have an important role in regulation of immune functions: TGF-β1, TGF-β2 and TGF-β3 [40]. Furthermore, three TGF-β receptor proteins are identified as well: TβRI – III [reviewed in 41]. In the early stages of tumors, TGF-β acts as a tumor suppressor by inhibiting cell growth, promoting cellular differentiation or promoting apoptosis. Cells that are becoming malignant, become (partially) resistant towards this growth inhibition. This could be due to mutations or loss of genes involved in the TGF-β signaling pathway [41]. Through production or activation of TGF-β, cancer cells use this pathway in their advantage; to promote immune tolerance. TGF-β targets CD4+ effector T cells (Th1 and Th2), CD8+ cytotoxic T cells, DCs, NKs cells and macrophages. Furthermore, it stimulates the generation of Tregs [42]. Thomas and Massagué showed that TGF-β inhibits five cytolytic gene products of CTLs: perforin, granzyme A, granzyme B, Fas ligand and IFN- γ, thereby promoting tumor progression [43].

4. Targeting CD8+ T cell suppressive factors

4.1 Targeting immune cell based suppressive factors

4.1.1 MDSCs

In general, three main ways of targeting the suppressive functions of MDSCs are currently studied. First of all, various chemical treatments are being used to deplete MDSCs. Sunitinib inhibits signaling through receptor tyrosine kinases, such as vascular endothelial growth factor receptors (VEGFR), both at the level of the bone marrow, thus decreasing production of hematopoietic stem cells, which are MDSCs precursors, as well as the level of tumor microenvironment, limiting local recruitment of already circulating MDSCs. Patients with metastatic renal cell carcinoma who received sunitinib treatment showed a reduction in MDSCs, as well as a reversal in Treg cell elevation [44]. Two other cytotoxic agents that were able to induce a major decrease in the number of MDSCs in tumor mouse models were gemcitabine [45] and 5- fluorouracil [46].

A second strategy of targeting MDSCs is by promoting differentiation of these immune suppressive cells into mature non-suppressive cells that can also mediate an anti-tumor response. All trans retinoic acid (ACTA) is a derivative of vitamin A and has been shown to induce MDSC differentiation *in vivo* and *in vitro* [47,48]. In a clinical study, 18 patients with metastatic renal cell carcinoma, which presented elevated levels of MDSCs, received a high (>150 ng/mL) or a low plasma concentration (<135 ng/mL) of ATRA. Patients receiving a high concentration were found to have a lower number of MDSCs [49]. Finally, the immunosuppressive functions of MDSCs can be inhibited. As mentioned before, MDSCs can inhibit T-cell activation by depleting the cell of L-arginine. Arginase-1 (ARG-1) and nitric oxide synthase-2 (NOS2) are the key enzymes in this process [50]. In mice, phosphodiesterase-5 (PDE-5) inhibitor, an agent currently being used for non-malignant conditions such as erectile dysfunction, suppressed this pathway in MDSCs: ARG1 and NOS2 were downregulated [51]. Furthermore, PDE-5 inhibitor sildenafil decreased MDSC amounts and immunosuppressive functions [52]. Sinha et al. showed that prostaglandin E2 (PGE2) induces MDSCs and thus promotes tumor progression, by use of the enzyme cyclooxygenase2 (COX-2) [53]. In a mouse model of glioma, COX-2 inhibitors blocked PGE2 production, reduced MDSCs and delayed glioma development [54]. One other mechanism of MDSCs that can be targeted is the generation of ROS. In tumor bearing mice, CDDO-Me, a synthetic triterpenoid, was shown to completely abrogate the immune suppressive effect of MDSCs by reducing ROS [55].
When combining therapies targeting MDSCs with radiotherapy, only moderate results have been shown. In a mouse glioma model, combination of sunitinib and low-dose radiotherapy only modestly improved survival. Combining sunitinib with high-dose radiation therapy resulted in fatal toxicities [104]. Clinically, success has only been shown on a case by case basis [105,106].

4.1.2 Tregs

Various ways to block the inhibition of the anti-tumoral immune response of Tregs have been studied. Targeting CD25 expression on Tregs with anti- CD25 monoclonal antibodies has been found to reduce the amount of Tregs in an *in vivo* murine model [57]. Daclizumab (Zenapex ®) is an anti-human CD25 mAb already approved for use in autoimmune diseases, transplantation and cancer [58]. In metastatic breast carcinomas, patients treated with daclizumab showed a reduced amount of Tregs. After vaccination with cancer antigen peptides, more cancer-specific CTLs were observed as well [59]. One problem arises however with the use of anti- CD25 because of its observed non-specificity. Anti-CD25 treatment can eradicate activated T cells in the tumor as well, thereby decreasing anti-tumoral activity of these cells [58]. Furthermore, certain chemotherapy regimens have been found to affect the amount of Tregs. Paclitaxel-based chemotherapy has shown to decrease the size of the Treg population, rather than other subsets, including effector T cells [60].
Blockade of the suppressive function of Tregs can occur in many ways. One way that has been studied, is the use of a monoclonal antibody against CTLA-4. As mentioned before, CTLA-4 is the main T-cell inhibitory signal used by Tregs [16]. In several mouse models, tumor immunity and tumor regression were improved using CTLA-4 mAb [61,62]. Although it induces cancer regression in patients with advanced cancer as well [63,64], the mechanism between CTLA-4 mAb and Tregs is not yet fully elucidated. One study showed that CTLA-4 mAb did not induce an intra-tumoral depletionof Tregs in patients, suggesting an increased T cell activation rather than an inhibition of Tregs [65].
Human Tregs also express receptors such as vascular endothelial growth factor receptor-3 (VEGFR-3) that could be blocked by certain molecules, such as sunitinib [66].

Patients with renal cell carcinoma (RCC) treated with sunitinib showed a decline in peripheral blood Tregs [67]. This effect may be indirect however, due to negative effects on MDSCs [44].
Glucocorticoid-induced tumor necrosis factor receptor (GITR) stands at the basis of another mechanism by which Tregs exert their suppressive function. Indeed, anti-GITR antibody has been shown to counter Treg function *in vivo* and *in vitro* [68]. Moreover, treatment of advanced tumors with a combination of anti-GITR mAb and anti-CTLA-4 mAb has shown to have a synergistic effect, leading to eradication of more advanced tumors. In contrast, treatment with a combination of anti-CD25 mAb and anti-GITR mAb was less effective than anti-GITR treatment alone. This was due to the fact that anti-GITR depleted CD25+ activated effector cells as well [69].
Demaria et al. showed that by using anti- CTLA-4 antibody alone, no effect on primary tumor growth or survival was seen in a mouse model of breast cancer. Combining the antibody with irradiation, mice showed a statistical significant survival advantage [107]. Furthermore, in a recent phase III trial, patients with metastatic melanoma received a monoclonal antibody against CTLA-4 in combination with chemotherapy, or chemotherapy alone. Patients receiving the combination therapy had a significantly improved overall survival compared with patients receiving chemotherapy alone [108].

4.2 Targeting the interaction of tumor cells with immune cells

The fact that studies have shown that PD-L1 is upregulated on tumor cells, thereby inhibiting anti-tumoral T cell responses, provides a good rationale for treatment of PD-L1 with monoclonal antibodies [24, 70]. Indeed, after blocking PD-1 signaling alone, tumor progression or prolonged host survival was seen in several *in vivo* mouse models [71,72]. In human *ex-vivo* studies, treatment with anti-PD-L1 or anti-PD-1 mAb caused an increase in T-cell expansion and proliferation, as well as an increased cytokine production and enhanced cytolytic activity [73,74]. Several clinical studies investigating this effect are ongoing [reviewed in 75].
Using two murine models of self- and tumor tolerance, Grosso et al. showed that antibody blockade of LAG-3 resulted in increased accumulation and effector function of antigen-specific CD8+ T cells [76]. Combining LAG-3 mAb and PD-1 mAb treatment in a mouse model, it has been found that it reduces tumor growth , due to enhanced anti-tumor immunity. More importantly, dual-antibody-treated mice demonstrate more robust immune responses than either single- treated group [77].
Even though receptors for B7-H3 are not known, several studies encourage investigating this mechanism. *In vitro* analysis in mouse models showed that by using an anti- B7-H3 monoclonal antibody, T cell proliferation was enhanced [78]. Furthermore, Xu et al. have established a monoclonal antibody that suppresses the effect of B7-H3 in solid tumors in the central nervous system [79]. Because tumor- expressed B7-H4 negatively regulates T cell activation as stated before, the blockade of B7-H4 represents a novel target for tumor immunotherapy. Although efficient neutralizing antibodies specific for human B7-H4 are not yet available [81], small interfering RNA (siRNA) [82] and antisense oligonucleotides [83, 84] specific for B7-H4 have been used to block expression of B7-H1. Blocking B7-H1 in tumor associated macrophages inhibited their suppressive function, it enabled tumor specific effector T cell function and it suppressed tumor growth in human ovarian cancer xenografts [83,84].

4.3 Targeting tumor cell based suppressive factors

4.3.1 Decoy receptors

By inhibiting FasL-induced apoptosis by activated T cells or NK cells, decoy receptors provide a target for therapeutic intervention. Even though this method of inhibiting immunosuppression is not studied intensively, several studies have been done. The resistance of human pancreatic cancer cells to FasL has been shown to be overcome by DcR3-specific small interfering RNA [99,100]. Furthermore, triptolide (TPL), used as a natural medicine in China for years, has been shown to effectively reduce DcR3 expression and increase FasL expression in pancreatic cancer cells [101]. Denbinobin, another biologically active, natural product isolated from *Ephemerantha lonchophylla* decreases DcR3 expression as well, synergistically enhancing FasL-induced apoptosis in human pancreatic cells [102]. Xu et al. showed that knockdown of DcR3 by RNA interference enhances apoptosis and inhibits growth of gastric cancer cells. This down-regulation of DcR3 enhanced sensitivity of gastric cancer cells to 5-FU, a cytotoxic agent decreasing the number of MDSCs, and increased expression of Fas and FasL [103].

4.3.2. Immune suppressive cytokines

Even though targeting TGF-β or its signaling components creates a challenge because of the dual nature of this pathway, numeral (pre-)clinical studies have been done. Three major approaches have been established to inhibit TGF-β or its signaling components: anti-TGF-β antibodies, antisense TGF-β oligonucleotides and TGF-β receptor kinase inhibitors.

Because monoclonal antibodies targeting the TGF-β pathway directly inhibit access of the ligand to its receptor, they are extensively being used in many studies targeting cancer [85]. In preclinical models, monoclonal antibody 2G7 and 1D11 have been thoroughly tested. Both have been found to bind with high affinity to the TGF-β isoforms I, II and III and inhibit tumor growth [86,87]. On the basis of strong preclinical data, three human monoclonal antibodies have been developed, lerdelimumab (CAT-152)[88], metelimumab (CAT-192)[89], fresolimumab (GC-1008)[90]. These three antibodies have been used in various preclinical and clinical studies. Despite passing safety tests, lerdelimumab [88] and metelimumab [89] failed to show efficacy and were discontinued. Fresolimumab showed safety and efficacy in phase I trials [reviewed in 90]. However, clinical development of fresolimumab for oncology trials has been suspended. After a takeover of Genzyme by Sanofi, the company decided to focus on the fibrotic applications of the drug.

Antisense oligonucleotides (ASOs) are single-stranded polynucleotide molecules that are designed to hybridize to complementary RNA sequences and inhibit translation of these molecules [91]. Antisense Pharma developed one such nucleotide termed AP12009, which has been shown to inhibit the human TGF-β2 pathway. Phase I and II clinical trials showed that the drug was well tolerated with no severe side effects [92]. Furthermore, phase I/II trials in patients with advanced pancreatic cancer, malignant melanoma and colorectal carcinoma showed encouraging survival results [93]. ASOs are also being used in combination with tumor vaccines. A dose-related survival advantage in patients which received the combined vaccine was seen in a phase II clinical trial, therefore allowing the start of a phase III clinical trial [94].

TGF-β receptor kinase inhibitors provide another way to target the immunosuppressive function of TGF-β. Preclinical models using kinase inhibitors targeting the type I receptor have proven effective, leading to decreased tumor growth and increased immunogenicity in a murine glioma model [95]. Treatment of human glioma cell lines led to a reduction of cell proliferation and motility and a reduction in expression of angiogenic factors [96]. Clinical trials are currently on their way. Furthermore, combined therapy provides a promise here as well. Using a chemotherapeutic agent and a TGF-β kinase inhibitor, metastasis was significantly blocked in a breast cancer mouse model [97]. Treatment of gemcitabine-resistant pancreatic cancer cells with a TGF-β1 inhibitor sensitizes these cells to drug treatment [98].

5. Concluding Remarks

Since the rising of the concept of immunosurveillance, extensive research has been done in order to achieve a final goal: the use of cancer immunotherapy in patients, selectively targeting and eradicating the tumor. It has become clear during preclinical and clinical trials that different obstacles have to be overcome in order to be able to successfully use cancer immunotherapy. Therefore, countering different pro-tumoral pathways and immunosuppressive factors is necessary for achieving this goal. Anti-tumoral treatment may be aimed at three different subsets of immunosuppressive factors. First, immune cell based suppressive factors, such as MDSCs or Tregs, have been shown to create many opportunities to counter the effect of tumor cells on the immune system. Thus, several clinical trials are currently being performed. Secondly, the interaction of tumor cells with immune cells provides another targeting point. Treatment with anti-PD-1 and LAG-3 antibodies have shown to be successful in mouse models, and clinical trials are on their way as well. Recently, research has been done to highlight two new family members of the B7 family: B7-H3 and B7-H4. Thirdly, tumor cell based suppressive factors present another obstacle. Amongst them, some highly versatile ones are the well known TGF-β, as well as the relatively new decoy receptors.

A challenge provided by the use of cancer immunotherapy is that the immunosuppressive mechanisms of the tumor may be different within tumor types and may also depend on the tumor progression and tumor microenvironment. Therefore, typing of the specific tumor and its immunosuppressive and pro-tumoral pathways would be necessary in order to determine the types of treatment that would be efficient to use.

Concluding, combination of current treatments such as chemotherapy or radiotherapy with targeting different immunosuppressive factors and pro-tumoral pathways, taking into account the unique immune signature of the specific tumor, could create individualized treatments against cancer.

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