Manipulating Immune Regulatory Pathways to Enhance T Cell Stimulation

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1. Introduction

Cancer immunotherapy aspires to treat malignant disease by activating cancer specific immune responses. It is generally accepted that the latter can only be achieved by an approach in which tumor specific T cells are educated to recognize and kill tumor cells, whilst they are furthermore empowered to overcome immunosuppressive mechanisms present both at peripheral sites and in the tumor environment. Dendritic cells (DCs) have been extensively explored as a cellular vaccine for the stimulation of tumor specific T cells. Several strategies have been devised to manipulate these cells to become strongly activated tumor associated antigen (TAA) presenting cells. Our growing knowledge on the biology of DCs and the co-stimulatory as well as inhibitory molecules expressed by them, provides us with opportunities to generate DCs that are capable of hyper-activating cytotoxic T lymphocytes (CTLs) whilst they impact on regulatory T cells (T_{reg}), which are now well established to be an important contributor to failure of cancer vaccines. In this chapter, we will focus on the cross talk between DCs and T cells mediated by the CD70/CD27 and PD-L1/PD-1 axis as these have been identified as critical pathways in the regulation of immunity versus tolerance.

2. Cancer — A therapeutic challenge

Cancer is a disease in which cells divide abnormally and acquire the ability to invade other tissues. Given the fact that virtually any tissue in the body can become transformed from normal cells towards cancer cells, a large degree of heterogeneity exists. This implies that cancer is an overall name covering a group of over 100 different diseases, which have in

common the occurrence of abnormal cells, which grow out of control and can be present within different parts of the body. Therefore, it is not surprising that cancer is a frequently occurring disease, affecting about one in three people during their lifetime in developed countries [1].

Despite the enormous amount of research and rapid developments in prevention, diagnosis and treatment, cancer remains one of the most difficult diseases to cure. Today, the first line treatment for most solid cancer types is surgical resection or radiotherapy to remove or necrose the primary tumor. Since these strategies do not tackle residual tumor cells or metastases, a commonly encountered problem is disease relapse. Therefore, the aforementioned treatment strategies are often combined with chemotherapy, a systemic but aggressive and non-selective approach to eradicate tumor cells. Despite major improvements in these conventional treatment strategies, the requirement to develop alternative treatment modalities that are tumor cell specific and provide better long-term protection to avoid recurrence of tumor cells remains high [2].

A very promising new approach that fulfills these criteria is immunotherapy. The main premise of cancer immunotherapy is to harness the patients' immune system to specifically recognize and kill tumor cells. This is based on the knowledge that our immune system can discriminate healthy cells from cancer cells as the latter express TAAs [3]. Several strategies have been developed to achieve destruction of TAA expressing cancer cells. These are extensively reviewed in [4]. Of these the exploitation of dendritic cells (DCs), the professional antigen presenting cells of our immune system, to present TAAs to CD4$^+$T helper 1 (T$_H$1) cells and CD8$^+$cytotoxic T lymphocytes (CTLs) is considered to be very promising. Of note, in 2010 the FDA approved a first DC-vaccine, Sipuleucel-T (Provenge®), which was shown to be effective in metastatic, asymptomatic, hormone refractory prostate cancer [5, 6]. Thus, cancer immunotherapy could be a powerful new treatment strategy that oncologists can offer to patients.

It needs to be clarified that cancer development is a complex progressive process that involves a sequence of interactions between cancer cells and immune cells. As mentioned above, the immune system can specifically identify and eliminate cancer cells based on the expression of TAAs. This process was first described by Burnet and Thomas and is generally referred to as tumor immunosurveillance [7]. It occurs physiologically to protect the body from tumor formation. Nevertheless, this process is not perfect and tumors can develop despite tumor immunosurveillance. In fact, the manifestation of malignancy means that the disease has eventually prevailed over immunity. The latter can be explained by the more recently described notion of "tumor immunoediting", a process that comprises three phases [8]. The first phase is the elimination phase in which most immunogenic tumor cells are eliminated by CTLs and natural killer (NK) cells. This phase is followed by an equilibrium phase in which tumor cells that show a reduced immunogenicity are selected for. These tumor cell variants enable the third phase, the escape phase, as they are no longer sensitive to the host immune system. Nonetheless, T cells recognizing these tumor cell variants are often isolated from blood and tumor of cancer patients. Thus the question arises why these cells are unable to eradicate tumor cells?

One of the major hurdles in the fight against cancer is the presence of regulatory T cells (T_{reg}) in the tumor environment as well as in the periphery [9, 10]. Recruitment, expansion and *de novo* generation of T_{reg} is a common theme in cancer (Figure 1). For instance, melanoma cells can secrete transforming growth factor-β (TGF-β) and interleukin-10 (IL-10), factors that are linked to expansion of natural T_{reg} and differentiation of naive CD4$^+$T cells to inducible T_{reg} [11]. Another well-described mechanism is the expression of the enzyme indoleamine 2,3-dioxygenase (IDO) by tumor cells and tumor associated antigen presenting cells, which mediates conversion of CD4$^+$T cells to T_{reg} [12]. The expression of IDO is only one out of many mechanisms that are exploited by DCs to drive CD4$^+$T cell differentiation towards T_{reg} [13]. Nonetheless, it highlights that not only tumor cells drive the stimulation of T_{reg} but that DCs, in particular immature DCs, trapped within the tumor environment, also have an important role in this process. Furthermore, it is well known that T_{reg} prevent full maturation of DCs and as such are at the basis of a vicious circle [14]. The suppressive activity of T_{reg} is mediated by various mechanisms [13]. As mentioned, they hamper full DC maturation [14]. Moreover, T_{reg} can hijack the co-stimulatory molecules CD80 and CD86 from the membrane of DCs through transendocytosis via interactions with cytotoxic T lymphocyte antigen-4 (CTLA-4 or CD152). As such they deprive effector T (T_{eff}) cells from co-stimulatory signals [15]. It has furthermore been shown that T_{reg} influence the character of T cell differentiation by selectively dampening T_H1 responses and CTLs [16, 17]. Importantly, T_{reg} not only hamper DCs in their ability to induce CTL mediated anti-tumor immune responses, they furthermore hamper the functionality of T_{eff} cells or can cause T_{eff} cell death through exocytosis of granzymes and perforin [18]. It has also been described that T_{reg}, which express high levels of the IL-2 receptor CD25, are capable of scavenging IL-2, thus depriving T_{eff} from this cytokine [19]. Finally, T_{reg} can disrupt metabolic pathways through tryptophan conversion [20] or adenosine secretion [21], which leads to the production of suppressive metabolites. In this way, T_{reg} are able to control the induction and/or presence of CTLs as well as their functionality.

Taken together, therapeutic vaccination against tumors takes place in the presence of a milieu that counteracts tumor specific T-cell responses. Therefore, the method of vaccination, *e.g.* DC-based vaccination, should fulfill several requirements. It should result in strong activation of tumor specific CTLs that are able to migrate to the tumor site, recognize and kill tumor cells. Furthermore, the activated CTLs should be refractory to the immunosuppressive mechanisms exerted for instance by T_{reg}. Linked herewith, the vaccination strategy should avoid expansion of T_{reg} or even drive the TGF-β characterized T_{reg} signature into a more favorable IFN-γ signature [22-24].

3. The rationale behind therapeutic anti-cancer vaccination: tumor antigens

Active therapeutic vaccination builds on the power and specificity of the patient's immune system to eradicate cancer cells. The latter is based on the ability of immune cells to discriminate healthy cells from tumor cells, mainly based on the expression of distinct antigens, the so called TAAs. It has to be noted that in contrast to environmentally induced or virus-related tumors (e.g. HPV induced cervix carcinoma, causing genomic mutations
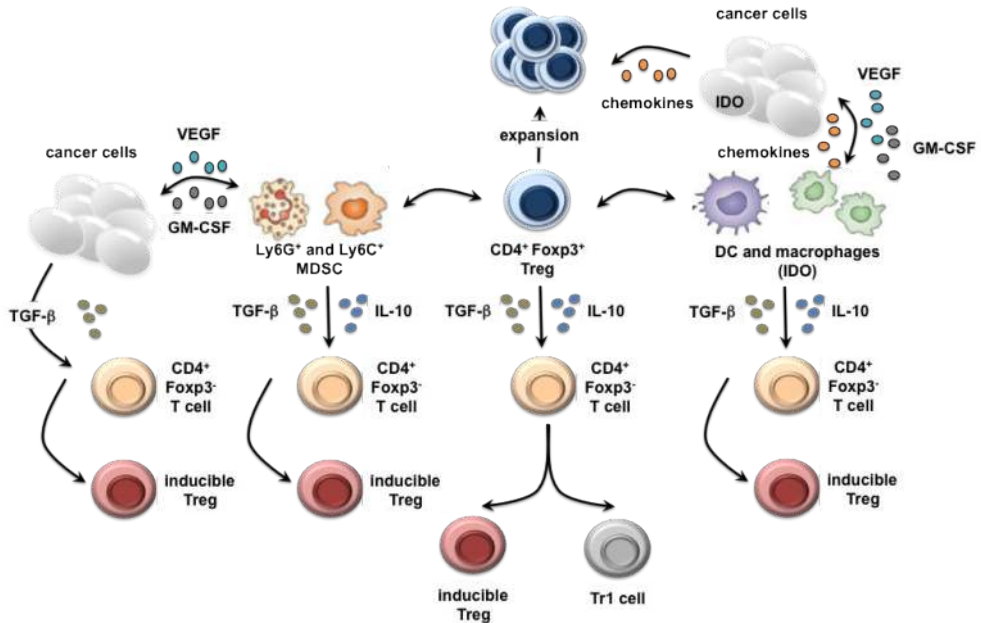
Figure 1. T$_{reg}$ are a major hurdle in cancer. It is well known T$_{reg}$ can be found at elevated numbers in cancers. Three main mechanisms are at the basis of this phenomenon. Firstly, CD4+CD25+Foxp3+T$_{reg}$ or so-called naturally occurring T$_{reg}$ infiltrate the tumor. The latter is driven by the expression of the chemokine receptor CCR4 on the T$_{reg}$ and the expression of its ligand CCL22 in the tumor environment. Secondly, several immune cells found in the tumor environment, including MDSCs, DCs, macrophages and natural T$_{reg}$, as well as tumor cells themselves drive the differentiation of CD4+Foxp3-cells into so-called inducible T$_{reg}$ (Foxp3+) and/or IL-10 producing Tr1 cells (CD4+IL-10+Foxp3-) through the secretion of TGF-β and/or IL-10. Thirdly, it is well known that dysfunctional myeloid cells within the tumor environment further stimulate T$_{reg}$ expansion. Importantly, there is an extensive cross talk between the different immune cells found within the tumor as well as between these immune cells and the tumor cells themselves. This cross talk ensures that the tumor environment remains a sanctuary for immunosuppresive cells, such as the T$_{reg}$.

resulting in strongly immogenic antigens), for most tumors the etiology is unclear and that they are not particularly immunogenic. Nonetheless, several TAAs have been identified as immunological targets. TAAs can be classified in different classes, dependent on their expression pattern. The most important classes are the cancer-testis antigens (e.g. MAGE-family), differentiation antigens (e.g. MART-1/Melan-A, CEA), mutated antigens (e.g. BRAF), antigens that are overexpressed in tumors (e.g. hTERT, WT-1). Examples hereof are carcinoembryonic antigen in colorectal carcinoma [25], α-fetoprotein in hepatocellular carcinoma [26] and members of the melanoma antigen gene (MAGE) family in melanoma [27].

The immune cells that are responsible for the specific recognition of TAAs are T cells. Peptides derived from a TAA can be presented in major histocompatibility class (MHC) molecules to the T-cell receptor (TCR) of T cells offering the opportunity to induce specific and long lasting immune reactivity against TAAs [28, 29]. As mentioned above, CTLs are critical in the

immunological control of cancer cells [30]. Initiation of CTL mediated immune responses requires antigen presentation in MHC I molecules that are present on antigen presenting cells, to the TCR of CD8^{+}T lymphocytes. In particular, DCs are equipped with specialized machinery that promote effective display of antigenic peptide/MHC I complexes. Importantly, DCs also present peptides in the context of MHC II molecules to CD4^{+}T cells, which through delicate interactions with DCs and CD8^{+}T cells help in the induction, expansion and maintenance of CTLs [31]. Although antigen presentation is a first prerequisite for stimulation of CTLs, additional signals, co-stimulation by surface expressed stimulatory molecules and an inflammatory environment provided by the secretion of a plethora of cytokines, are required. Both can be delivered by DCs when these are appropriately activated [32].

Over a hundred TAAs that are recognized by T cells have been identified to date [33]. The question which TAAs has the most potential as a target for therapeutic vaccination was recently addressed in a pilot study in which 75 TAAs were ranked according to predefined and weighted objective criteria, including its therapeutic function, immunogenicity and specificity. This study brought the Wilms' Tumor 1 (WT-1) antigen forward as an excellent, nearly universal TAA [34].

Over the years, several strategies have been developed to introduce TAAs to DCs. Generally these approaches are divided into viral and non-viral strategies. Examples hereof are lentiviral modification and mRNA electroporation of DCs, respectively [35-41]. Both strategies have been shown to be efficient for delivery of TAAs to DCs [42]. Of note, these approaches have later on been fine tuned to furthermore provide immune modulating signals to the DCs [32, 43-46]. A thorough review of the use of lentiviral vectors and mRNA for modification of DCs is provided in references [47, 48] and [49, 50] respectively.

3.1. Dendritic cells guide CD8^{+}T cell activation

Dendritic cells, the most potent antigen presenting cells of our immune system, are at the centre of our immune system. They bridge the innate and adaptive immune system, and orchestrate amongst others T-cell mediated immune responses. Therefore, it is not surprising that DCs are frequently targeted to generate therapeutic immunity against cancer cells [51].

Immature DCs have the intrinsic capacity to scan peripheral tissues for antigens (both self-antigens and foreign antigens) and capture these antigens using several complementary mechanisms [52]. Intrinsically, DCs have a poor T-cell activating capacity as they express low levels of MHC and co-stimulatory molecules. In order to potently stimulate antigen-specific T-cell responses, DCs need to be fully matured, which will be described below. It is however important to note that immature DCs are not immunologically quiescent as they induce T-cell tolerance against self-antigens through several mechanisms such as depletion of T cells, induction of T-cell anergy, T_{reg} amplification or differentiation [53-55]. When immature DCs capture an antigen under pro-inflammatory circumstances, in the presence of pathogen associated molecular patterns or danger associated molecular patterns they undergo a maturation process [56]. This process is characterized by a decreased capacity for phagocytosis and an increased expression of MHC and co-stimulatory molecules, such as CD80 and CD86. In addition, the chemokine receptor CCR7 is up-regulated, allowing DCs to migrate to

lymphoid organs. While DCs migrate to lymph nodes, they process the captured antigens into peptides that primarily bind to MHC II molecules for presentation to CD4+T cells. Importantly, exogenous antigens can enter the MHC I presentation pathway for presentation to CD8+T cells through a process called cross-presentation. Although the mechanisms allowing exogenous antigens to enter the MHC I pathway are not fully understood, two main pathways have been described, leakage of antigens from phagosomes/endosomes to the cytosol and loading of peptides generated by lysosomal degradation onto MHC I molecules that are recycled [57]. The ability to cross-present exogenous antigens, such as TAAs, acquired for for instance dying tumor cells is of utmost importance in cancer immunosurveillance [58].

In the lymph nodes, fully mature, antigen presenting DCs can activate naive CD4+and CD8+T cells. While naive CD8+T cells can differentiate into CTLs, naive CD4+T cells can differentiate into different T_H cell types, of which T_H1 cells are critical in the induction of potent anti-tumor immune responses. In this context, it is important to note that both CD4+and CD8+T cells require a minimum level of MHC/peptide-TCR interactions for proper activation. Too few MHC/peptide-TCR interactions can result in T-cell ignorance or tolerance while persistent interactions can lead to induction of T-cell anergy or deletion [59]. Although recognition of antigen, presented on DCs as peptide/MHC complexes is considered the first signal required for T-cell activation, it is not sufficient. Adequate activation of T cells at least requires co-stimulation, the so-called second signal, delivered by surface expressed molecules. Co-stimulation can promote more efficient engagement of TCR molecules in order to enhance the immunological synapse or can provide additional signals to promote cell proliferation, augment cell survival or induce effector functions such as cytokine secretion or cytotoxicity [60]. Signaling via the TCR and additional co-stimulatory receptors initiates clonal expansion and acquisition of effector functions by primed T cells [61]. The requirement for a third signal, delivered as inflammatory cytokines such as IL-12, to stimulate antigen dependent proliferation is variable. When antigen levels are low, the third signal is critical to induce maximal T-cell proliferation. However, at high antigen levels extensive proliferation can occur in its absence. Importantly, in the absence of a third signal proliferating T cells often fail to develop full effector functions. Thus, proliferation and development of cytolytic function can be fully uncoupled and the absence of the third signal can render T cells functionally tolerant, in the sense that subsequent re-stimulation with a potent stimulus results in limited clonal expansion, impaired IFN-γ production and no cytolytic function. Thus, the presence or absence of the third signal appears to be a critical variable in determining whether antigenic challenge results in tolerance *versus* development of effector function and establishment of a responsive memory population [62]. It is important to note that activated CD8+CTLs disseminate to the periphery (*e.g.* tumor site) and mediate efficient and rapid killing of target cells by various mechanisms, including secretion of cytokines such as tumor necrosis factor-α (TNF-α), exocytosis of lytic granules containing granzymes and perforin, or by ligation of death receptors such as Fas on target cells [63].

3.2. Co-signaling by dendritic cells

Mature DCs express a plethora of diverse co-signaling molecules. These bind to their partnering receptor on T cells and as such deliver various signals that contribute in their own way to the quality of the T cell response. Co-signaling molecules can act on particular aspects of T cell activation, such as survival, cell cycle progression and differentiation to either T_{eff} cell or memory T cells [64]. It is furthermore important to note that intracellular signals may be transduced through both the co-signaling ligand and its receptor, a concept known as bidirectional signaling [65-68]. Consequently, co-signaling can affect both the antigen presenting cell and T cells [69].

The function of a particular co-signaling molecule is strongly related to the timing of its action, as early co-signaling molecules are functionally distinct from those that act late during the T-cell response. It is for this reason that the expression of each co-signaling molecule and/or its receptor is tightly regulated and depends on the activation status of the cell [70].

Another important issue is that the outcome of the signaling is not necessarily stimulatory hence co-signaling molecules can be categorized as being co-stimulatory or co-inhibitory based on the outcome of their signals. Linked herewith, it is worthwhile noting that co-signaling molecules are as essential in determining the expansion and function of T_{eff} cells as they are for the development and function of T_{reg} (defined as $CD4^+CD25^+Foxp3^+$cells) [71].

3.3. Co-stimulation: focus on the CD70/CD27 pathway

There is a growing list of co-stimulatory molecules, of which most belong to either the B7 or the TNF receptor superfamily, and are expressed on DCs and other antigen presenting cells [60, 72, 73]. Some co-stimulatory molecules however, exemplified by CD83 and the CD300 family, which is also expressed on mature DCs, cannot be classified in either of these groups [74, 75].

The co-stimulatory function of B7.1 (CD80) and B7.2 (CD86), which are the founding members of the B7 family and which themselves belong to the immunoglobulin superfamily, have been studied most extensively. These co-stimulatory molecules and their receptor CD28 were extensively discussed in [76] and are therefore not addressed in this chapter. It needs to be highlighted that the B7 family now encompasses several other members, which were more recently described. These include inducible co-stimulator ligand (ICOS-L), programmed death 1 receptor-ligand 1 (PD-L1 or B7-H1), PD-L2 (B7-DC), B7-H3 and B7-H4. The corresponding ligands that are inducibly expressed on T cells are ICOS, programmed death 1 receptor (PD-1), and B and T Lymphocyte Attenuator (BTLA). Although their function in T-cell activation is not yet fully understood, it is clear that these B7 members are not innocent bystanders [77]. In this regard, we will discuss the PD-L1/PD-1 axis in more detail in the section "Co-inhibition: focus on the PD-L1/PD-1 pathway".

The second group of co-stimulatory receptors expressed on T cells consists of the TNF receptor type family and includes CD27, OX40 (CD134), glucocorticoid induced TNF receptor family related protein (GITR, TNFRSF18; CD357) and 4-1BB (CD137). Their corresponding ligands CD70, OX40L (CD252), GITRL (TNFSF18) and 4-1BBL (TNFSF9) are expressed on DCs [73]. A

common theme for the members of this family is their coordinated expression at the interface between T cells and DCs, and their ability to shape the environment that sets the stage for the cross talk between T cells and DCs. In the remainder of this section we will focus on the CD70/ CD27 pathway as this pathway has been identified as a critical regulator of immunity *versus* tolerance.

Similar to the B7 receptor CD28, the receptor CD27 is expressed on naive T cells [78]. Although initially up-regulated upon T cell activation, its expression decreases to undetectable levels after T cells undergo several rounds of division [79]. However, CD27 expression has been reported on central memory T cells [80]. The expression of its ligand CD70 is highly restricted and activation dependent, as it is only transiently expressed on activated T and B cells as well as DCs [81-83]. Studies using CD27 deficient mice have provided insights into the role of CD27 in the generation of T cell immunity. It was indicated that CD27 is of importance in the stimulation of both primary and memory T-cell responses [84]. Although, CD27 seemingly was not required to induce T-cell proliferation, it was shown that CD27 enhances T-cell survival upon successive rounds of cell division [85]. Nonetheless, a role for CD27 in CD8+T cell proliferation was shown in the absence of IL-2, although no functional differentiation was detected [86]. In this regard, CD27 has been linked to the induction of IL-2 and IFN-γ secretion by CD4+T cells, as such instructing the latter to support CD8+T-cell activation [87]. These findings were confirmed in mouse studies using blocking anti-CD70 antibodies [88, 89]. An observation that pinpointed the CD27/CD70 pathway as one of the most important co-stimulatory pathways known today, is the observation that due to their CD70 expression, DEC205+spleen DCs are able to prime CD4+T cells in the absence of a third signal, *e.g.* IL-12 [90]. Moreover, it was demonstrated in mice that stimulation of T cells by DCs that constitutively express CD70 is effective in the absence of signal 3, suggesting that TCR triggering and CD27/ CD70 interaction-at least in mice-is sufficient to promote CTL responses [91]. Many of these studies were performed in the context of infectious diseases. Also in cancer, the CD27/CD70 interaction has been described to help immune cell survival and induction of potent CTL responses [92-95]. Importantly, our group has developed a potent DC-based anti-cancer vaccine based on the use of mRNA to introduce CD70 in addition to two DC activating stimuli CD40 ligand and a constitutive active form of toll-like receptor 4, collectively referred to as TriMix (Figure 2) [43]. It has been demonstrated that these DCs are able to strongly activate allogeneic naive CD4+CD45RA+T cells and when loaded with TAA derived peptides are able to hyper-activate CD8+T cells (>500-fold increase in Melan A-specific CD8+T cells was observed when compared with immature DCs, and a >200-fold increase when compared with DCs activated with a classical cytokine cocktail). Simultaneous introduction of TAA and TriMix mRNA was shown to be feasible [96]. These data formed the basis for clinical evaluation of these so-called TriMix-DCs as a cellular vaccine for immunization of advanced stage melano-ma patients [97]. Extensive immunomonitoring of delayed type hypersensitivity reactions [98] and/or blood samples [99] obtained from these vaccinated patients, demonstrated priming of CD4+and CD8+T cell responses against multiple epitopes of the TAAs present within the DC-vaccine. More importantly, in a small-scale (15 patients) phase IB clinical trial in advanced melanoma, it was shown that combined intradermal/intravenous vaccination with TriMix-DCs induces anti-tumor activity with durable disease control in a number of patients [100].
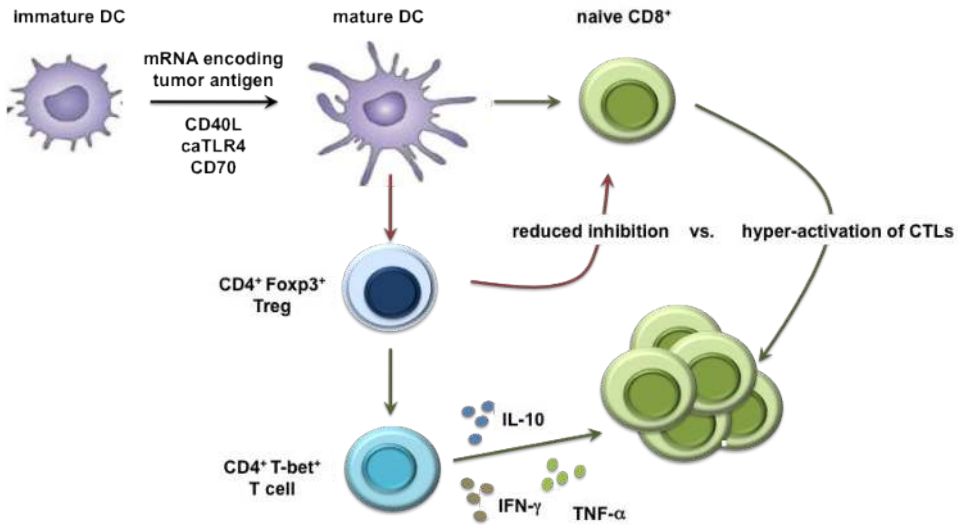
Figure 2. TriMix-DCs hyper-activate CTLs that are partially protected from T_{reg} suppression and drive T_{reg} towards a T_H1 phenotype. Immature DCs can be artificially matured using genetic modification. To obtain strong T-cell responses, DCs can be electroporated with a cocktail of mRNA molecules encoding constitutive active toll like receptor 4, CD40 ligand and CD70, not only maturing the DCs but also providing them with a powerful co-stimulatory molecule. These DCs are able to stimulate both naive CD4+and CD8+T cells. CD8+T cells will become activated to CTLs, while CD4+T_H1 cells will facilitate this process. Moreover, it has recently been shown that these DCs are also able to thwart the function of T_{reg}, whereby their suppressive effect on DCs, CD4+and CD8+T cells is largely incapacitated. As a consequence, the stimulatory potential of these DCs is enhanced because they do not only provide co-stimulation, but also counter immune inhibition.

As mentioned co-stimulatory molecules can also affect T_{reg}. For instance, it was described that triggering OX40 (human and mouse), GITR (mouse) or 4-1BB leads to a reduced Treg suppression [45, 101-106].

Given its important role in T-cell stimulation as described above, it is worthwhile discussing the role of the co-stimulatory CD70/CD27 pathway in the context of T_{reg} suppression. Although it is well described that T_{reg} express high levels of CD27, the effect of CD27/CD70 signaling on the function of T_{reg} has not been studied in detail in the context of cancer [107-109]. Importantly, it is worthwhile to note that different effects of CD70/CD27 signaling on T_{reg} have been described and that these effects seem to be dependent on the context of the CD70 expression. For instance, Claus and colleagues demonstrated that CD70/CD27 interaction in the chronically inflamed tumor environment increased the frequency of T_{reg}, resulting in reduced tumor specific T cell responses and the promotion of tumor growth. The latter was shown to be due to an enhanced survival of T_{reg}, which was linked to the stimulation of CD4+T_{eff} cells to produce IL-2 a well-known survival factor for T_{reg} [107, 110]. It has been suggested by Moser and colleagues that in a pro-inflammatory environment, such as secondary lymphoid organs, priming of T_H1 cells (a necessary component for efficient priming of CTLs) is driven by IL-12 or CD70/CD27 signaling, depending on the function of T_{reg}. They observed that enhanced T_H1

priming in the absence of T_{reg} is CD70 dependent, whereas IL-12 is required for T_H1 development when T_{reg} are at place. The fact that CD70 can exert its co-stimulatory function for T_H1 cells in the absence of T_{reg}, might be explained by their observation that the expression of CD70 on DCs was abrogated by T_{reg} *in vitro* [16]. More recently, we extensively investigated the influence of CD70 expressing TriMix-DCs on T_{reg} (Figure 2). We demonstrated that these DCs do not induce T_{reg} starting from CD4+CD25-T cells. More importantly, the stimulation of CD8+T cells by TriMix-DCs was only marginally influenced by the presence of T_{reg}. In addition, we showed that CD8+T cells that were cultured with TriMix-DCs were partially protected from subsequent T_{reg} suppression. Besides desensitization of CD8+T cells to T_{reg}, we further showed that T_{reg} co-cultured in the presence of TriMix-DCs partially lost their suppressive capacity, a phenomenon that was accompanied by a decrease in CD27 and CD25 expression on these T_{reg}, as well as an increase in the expression of the transcription factor T-bet and secretion of cytokines linked to a T_H1 phenotype. Although the assays were performed *in vitro* and their outcome cannot solely be attributed to CD70, these data further underline the notion that the effect of CD70/CD27 signaling on T_{reg} might depend on its context. Importantly, these data suggest that the potency of CD70 expressing TriMix-DCs is linked to their ability to hyperactivate CTLs that are refractory to T_{reg} as well as on their ability to suppress the function of T_{reg}, even reprogram them to T_H1 cells under certain circumstances [108].

3.4. Co-inhibition: focus on the PD-L1/PD-1 pathway

Over the years, two negative stimulatory (co-inhibitory) receptors on T cells have been extensively studied in the context of cancer. These are CTLA-4 and PD-1, which are both members of the B7 receptor family. Moreover, they are both considered to be critical immune checkpoints that can be exploited to enhance anti-tumor T-cell responses.

The role of CTLA-4 in dampening T cell mediated immune responses has been extensively reviewed in [111, 112]. Briefly, CTLA-4 is up-regulated on activated T cells. At that time CTLA-4 interacts with the same B7 molecules as the CD28 receptor but outcompetes the CD28 receptor due to its higher affinity for these B7 molecules. The net effect of this B7.1 or B7.2/CTLA-4 signaling is a decrease in T-cell activity hence dampening of the immune response. Several mechanisms have been described by which CTLA-4 inhibits effector T-cell functions, including induction of reduced contact period between antigen presenting cells and T cells [113] and by transendocytosis of CD80/CD86, thus compromising the T-cell stimulatory capacity of antigen presenting cells [15]. Furthermore, CTLA-4 is constitutively expressed on T_{reg} and has been shown to be essential for their suppressive function [14]. Today, it is evidenced that unleashing this immunological break through delivery of blocking anti-CTLA-4 antibodies (*e.g.* ipilimumab) is therapeutically beneficial in the fight against cancer [114].

One of the more recently identified B7 receptor family members PD-1 is a distant homologue of CTLA-4, which was originally identified as a gene that was highly expressed by cells undergoing programmed cell death hence its name programmed death 1 receptor [115]. Similar to CTLA-4, PD-1 is up-regulated upon stimulation of T cells, although it has been shown on human T cells that PD-1 can be up-regulated in the absence of TCR triggering

through the addition of cytokines [116]. Importantly, its expression is not limited to activated T cells, as it is also expressed on activated B cells and myeloid cells [117, 118]. Although PD-1 is not present on the surface of freshly isolated T_{reg}, it can be found at high levels intracellularly. Here it is stored for export to the cell surface upon TCR stimulation [119].

The ligands for PD-1 are B7-H1 (PD-L1) and B7-DC (PD-L2). These show a distinct expression pattern. Whereas expression of B7-H1 can be induced on several different cell types, including monocytes, DCs, mast cells, T and B cells, epithelial, endothelial and muscle cells, the expression of B7-DC is more restricted to DCs, macrophages and mast cells [120-122]. Importantly, B7-H1 expression was also found on several human and mouse tumors [123, 124]. Of note, to emphasize that both B7-H1 and B7-DC bind PD-1, they were renamed as PD-L1 and PD-L2, respectively. However, PD-L1 is polyamorous, it can bind to PD-1 as well as B7.1. Therefore, this nomenclature does not reflect the complexity of the signaling pathways that involve PD-L1 [125, 126]. For instance, it was shown that B7.1 (CD80) expressed on T cells transduces an inhibitory signal to T cells following its ligation with B7-H1 *in vitro* [125, 127]. Conversely, B7-H1 expressed on T cells transduced an inhibitory signal to T cells after its interaction with B7.1 (CD80) [125]. So far, it has not been demonstrated that similar T cell-T cell interactions occur *in vivo*. Moreover, it remains to be clarified whether interaction of B7.1 (CD80) expressed on myeloid cells, *e.g.* DCs and myeloid derived suppressor cells (MDSCs), with B7-H1 on T cells would result in a similar inhibitory signal.

The role of the receptor PD-1 is predominantly described as being inhibitory for immune responses (Figure 3), a notion that is supported by the phenotype of PD-1 deficient mice [128]. There is little doubt that PD-L1 is the main partner in this inhibitory function as experiments utilizing strategies to either interfere with PD-1 or PD-L1 (*e.g.* antibodies, genetic down-regulation, *etc.*) often have a similar outcome [70]. Of note, whereas PD-1 deficient mice develop severe autoimmunity, this is not the case in PD-L1 deficient mice [129]. In addition, it needs to be mentioned that contradictory results have been obtained *in vitro* showing both negative and positive signaling of B7-H1 on T cell proliferation and cytokine production. These observations caution us to conclude that PD-L1 is exclusively suppressive.

With regard to suppression of immune responses, it has been described that PD-1 plays a critical role in maintaining peripheral tolerance. In this regard, it has been shown that the co-inhibitory PD-1 receptor plays a role in the conversion of naive CD4$^+$T cells to induced Foxp3$^+$T$_{reg}$ [130]. Moreover, PD-1 is up-regulated on T_{reg} upon TCR stimulation and has been suggested to enhance their proliferation [131]. It is furthermore thought that the interaction of PD-L1 with PD-1 is responsible for the lack of T-cell responsiveness in settings of persistent antigen stimulation such as those encountered in cancer and chronic infectious diseases [132-135]. Several modes of action that result in the corruption of the T_{eff} cell functionality have been described, including inhibition of kinases involved in T cell activation [118], induction of cell death [115] and modulation of the duration of T cell-DC or T cell-target cell contact by down-regulation of the TCR [136-138]. The physiological importance of TCR down-regulation is yet unclear [139], however most of the experimental evidence points out that TCR down-regulation seems to act at different levels to prevent autoimmunity during the onset of immune responses [140].
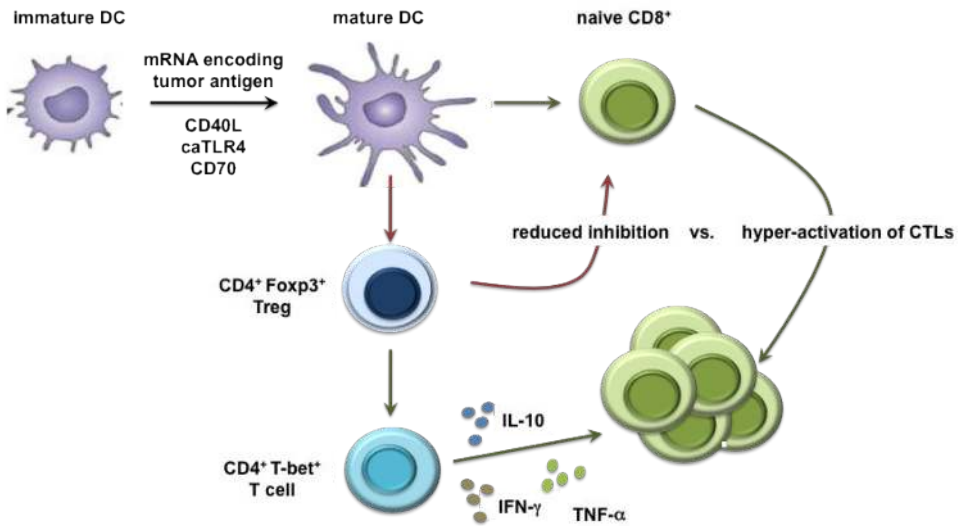
Figure 3. The PD-1/PD-L1 interaction has an inhibitory effect on T cells, while it simultaneously provides survival signals to cancer cells. DCs can be manipulated to stimulate both naive CD4+and CD8+T cells. Immune stimulation of these T cells leads to up-regulation of the receptor PD-1, interacting with PD-L1 on DCs, to terminate the immune response before causing harm to the host. In the tumor environment, however, PD-1 up-regulation will incapacitate T cells before they are able to tackle tumor cells. Moreover, the tumor itself, can up-regulate PD-L1 to avoid T-cell mediated killing. T_{reg} can be induced from naive CD4+T cells. The role of PD-1 in T_{reg} is less documented. Nonetheless it is supposed that T_{reg} are able to express PD-1 and can expand upon PD-1 triggering.

Most studies that have demonstrated negative co-signaling through interaction of PD-1 with PD-L1 during antigen presentation by DCs, have predominantly focused on expansion of T cells and secretion of single cytokines [141]. However, there is a growing appreciation that poly-functional T cells might reflect the effectiveness of the T cells to mediate tumor cell eradication, as was described in the eradication of virally infected cells [142]. In this regard, several studies have found enhanced numbers of tumor-specific poly-functional T cells in patients responding favorably to various forms of immunotherapy, including anti-CTLA-4 antibody therapy [143] and tumor vaccines [144]. In addition, the studies performed by Imai and colleagues [145] suggest that poly-functionality of adoptively transferred CD8+T cells is a sensitive immune correlate for successful immunotherapy against malignancy. Therefore, we recently evaluated whether interference of the PD-1/PD-L1 pathway during antigen presentation by DCs impacted on the ability of CD4+and CD8+T cells to perform multiple functions at the same time [146]. To that end, we genetically engineered monocyte-derived DCs to secrete soluble PD-1 or soluble PD-L1. The latter approach was based on the observation that patients with chronic lymphocytic leukemia that have a splice variants of PD-1, which gives rise to a soluble form of PD-1, have a prolonged survival [147]. Interestingly, we observed that modification of DCs with soluble PD-1 or soluble PD-L1 mRNA resulted in increased levels of the co-stimulatory molecule CD80 and a distinct cytokine profile, characterized by the secretion of IL-10 and TNF-α, respectively. Importantly, these DCs stimulated a significantly

higher number of multifunctional T cells that showed increased cytokine secretion, while it did not induce T_{reg}.

Besides antigen presenting cells, tumor cells use the PD-1/PD-L1 pathway to control anti-tumor immunity. Many human cancers have been shown to aberrantly express B7-H1. Up-regulation of B7-H1 is associated to local inflammation (predominantly the presence of IFN-γ) [148]. Importantly, expression of B7-H1 is a poor prognostic factor in several cancer types, including ovarian cancer [149], renal cancer [150, 151], pancreatic cancer [152] and breast cancer [153]. The latter can be explained by three different mechanisms. First, it has been described that binding of PD-1 (T cells) to B7-H1 on cancer cells provides anti-apoptotic stimuli to these cancer cells [65]. Secondly, it was shown in experiments in which cancer cells were modified to express B7-H1, that these cells become refractory to CTL mediated killing [154, 155]. In another model it was shown that this phenomenon was reversible, when the binding of B7-H1 was prevented by blocking antibodies [156]. Thirdly, tumor associated antigen presenting cells utilize the PD-1/PD-L1 (B7-H1) pathway to control anti-tumor T cell responses using the mechanisms that were described above. This results in the stimulation of T_{eff} cells that show high expression of PD-1, are impaired in their function and resemble the exhausted antigen specific T cells that can be found during chronic viral infection. Interaction between PD-L1 expressed on tumor cells and PD-1 expressed on effector T cells was also shown to directly induce apoptosis of T cells [157].

The knowledge that blockade of PD-1/PD-L1 signaling aids the priming of multifunctional T_{eff} cells, avoids expansion of T_{reg} and facilitates anti-tumor immune responses at the tumor site, has instigated research in which stimulation of T cell responses in tumor bearing mice was combined with the administration of anti-PD-1 or anti-PD-L1 blocking antibodies, demonstrating a synergistic effect of both strategies [152, 154, 156, 158, 159]. Of note, treatment of mice with blocking antibodies without other treatment modalities only had marginal effects. So far, four humanized antibodies have been generated and tested in clinical trials to block the PD-1/PD-L1 pathway. Three of them are blocking anti-PD-1 antibodies, CT-011 [160, 161], MDX-1106 [162] and BMS-936558 [163, 164], whereas the fourth antibody is a blocking anti-PD-L1 antibody called BMS-936559 [165]. The antibody CT-011 was used in a phase I clinical trial in 17 patients with advanced hematological malignancies, demonstrating clinical benefit in about 33% of patients of which one complete response. There was no development of autoimmunity reported [160]. The antibody MDX-1106 was tested in 39 patients with advanced solid cancer, including patients with colorectal cancer, melanoma, prostate cancer, non small cell lung carcinoma and renal cell carcinoma. Manageable toxicity, such as anemia, lympho-penia, colitis and arthritis was reported. Clinical benefit was demonstrate in about 10% of patients of which one complete response, two partial responses and two mixed responses [162]. The same team conducted a large-scale study in a similar patient population (over 260 patients) using the anti-PD-1 antibody, BMS-936558. Objective responses were described in approximately one in four to one in five patients with non-small-cell lung cancer, melanoma or renal cell cancer. Again, the adverse event profile did not appear to preclude the use of the tested antibody. Moreover, a relationship between PD-L1 expression on tumor cells and objective response was suggested in this trial [163, 164]. Also the anti-PD-L1 antibody,

BMS-936559, was evaluated by this group in a phase I trial including over 200 patients suffering from the aforementioned cancers. From this study, it was concluded that antibody mediated blockade of PD-L1 induced durable tumor regression (objective response rate of 6 to 17%) and prolonged stabilization of disease (rates of 12 to 41% at 24 weeks) in patients with advanced cancers, including non–small cell lung cancer, melanoma and renal cell cancer [165].

In conclusion, experimental and early clinical results indicate that manipulation of the B7-H1 (PD-L1)/PD-1 pathway provides novel opportunities in anti-tumor therapy. In particular the combination of PD-L1/PD-1 blockade with vaccination strategies might synergize to initially enhance the stimulation of T_{eff} cells, whilst enabling these T_{eff} cells to exert their function in the tumor environment.

4. Discussion

Progress in our understanding on how immune cells interact with each other and how they are affected by cancer cells has led to the development of immunotherapeutic strategies that aim to harness the potential of CTLs in order to improve the outcome for cancer patients. Although CTLs are critical to fight cancer cells, there is a general consensus that stimulation of CTLs will only be successful when they are able to overcome the immunosuppression exerted by cancer cells and cancer associated immune cells, such as T_{reg}. To achieve the latter, research has focused on two approaches. One approach is designing DCs that are able to stimulate CTLs that are no longer sensitive to immunosuppression and to use these DCs as a cellular vaccine. Another approach is to develop strategies to modulate immunosuppressive mechanisms and use these either alone or in combination with vaccination. Our growing knowledge on the expression pattern and function of individual co-signaling molecules and how these educate T cells to respond or not, has been invaluable for the development of both approaches.

Co-signaling molecules start a signal cascade in T cells, which in turn allows them to sense environmental conditions and consequently respond accordingly. There is a growing list of co-signaling molecules that can either transduce stimulatory or inhibitory signals and therefore are often categorized as such. However, whether co-signaling will be stimulatory or inhibitory is dependent on the level and timing of expression of these molecules hence in reality (when multiple co-signaling molecules are expressed on the antigen presenting cell, be it DCs or cancer cells) the situation is much more complex. Nonetheless, major discoveries on new signaling molecules, their interactions and multilevel functionality have had a tremendous impact on cancer immunotherapy over the years. In this regard, important milestones have been the description of the CD70/CD27 and PD-L1/PD-1 pathways. Although it's early days in their clinical exploitation, the pre-clinical and clinical results described in this chapter demonstrate that manipulation of these pathways is promising. In particular, clinical results such as those obtained with improved DC vaccines, e.g. TriMix-DCs or blocking anti-PD-1 or PD-L1 antibodies, re-fuel the enthusiasm that immunotherapy strategies will find their place as an effective fourth treatment modality for cancer.

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