Published in final edited form as:
Immunol Endocr Metab Agents Med Chem. 2012 September ; 12(3): 224–235.

MODULATING CO-STIMULATION DURING ANTIGEN PRESENTATION TO ENHANCE CANCER IMMUNOTHERAPY

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Abstract

One of the key roles of the immune system is the identification of potentially dangerous pathogens or tumour cells, and raising a wide range of mechanisms to eliminate them from the organism. One of these mechanisms is activation and expansion of antigen-specific cytotoxic T cells, after recognition of antigenic peptides on the surface of antigen presenting cells such as dendritic cells (DCs). However, DCs also process and present autoantigens. Therefore, antigen presentation has to occur in the appropriate context to either trigger immune responses or establishing immunological tolerance. This is achieved by co-stimulation of T cells during antigen presentation. Co-stimulation consists on the simultaneous binding of ligand-receptor molecules at the immunological synapse which will determine the type and extent of T cell responses. In addition, the type of cytokines/chemokines present during antigen presentation will influence the polarisation of T cell responses, whether they lead to tolerance, antibody responses or cytotoxicity. In this review, we will focus on approaches manipulating co-stimulation during antigen presentation, and the role of cytokine stimulation on effective T cell responses. More specifically, we will address the experimental strategies to interfere with negative co-stimulation such as that mediated by PD-L1 (Programmed cell death 1 ligand 1)/PD-1 (Programmed death 1) to enhance anti-tumour immunity.

Keywords

Cancer; Co-stimulation; Dendritic cell; Immunotherapy; Signalling; T cell; B7; PD-L1; PD-L2; CTLA4; PD-1; CD80; MAPK; NF-κB

INTRODUCTION

A key drawback for effective immunotherapy of cancer is to stimulate effective T cell responses against tumour antigens. This is mainly caused by the natural tolerance towards tumour-associated antigens (TAAs), which are in most cases mutated forms of self-proteins. Therefore, a plausible strategy leading to effective cancer immunotherapy would be to present TAAs to antigen-specific T cells in a context that can overcome the endogenous

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tolerogenic mechanisms. TAA-specific T cells are present in physiological conditions, but their effector activities are strongly suppressed. However, these T cells can be activated and effectively exert therapeutic anti-tumour immune responses [1]. Therefore, cancer immunotherapy is currently being developed in two fronts. The first one, induction of T cells with high affinity TAA-specific T cell receptors (TCRs), and the second, the enhancement of antigen presentation by manipulating the immunological synapse between antigen presenting cells and T cells.

Dendritic cells and antigen presentation

DCs comprise a heterogeneous population of antigen-presenting cells (APCs), which regulate the priming of innate and adaptive immune responses [2]. DCs derive from cluster of differentiation (CD)34 hemapoietic progenitors, and so far, 3 main sub-populations have been described according to the expression of characteristic surface markers [3]. Generally, DCs are classified as myeloid (or conventional) DCs which express CD11c, or CD11c negative plasmacytoid DCs. There is evidence that these two linages derive from myeloid or lymphoid progenitor cells, respectively [4]. In addition, a third DC sub-population originates from differentiated monocytes, which have lost the expression of the monocyte marker CD14 [5].

DCs are generated in the bone marrow and remain in peripheral tissues in an immature stage, where they phagocytize and process a wide range of antigens by proteosomal or endosomal pathways, depending on the nature of the antigen. These antigens also include self-proteins or innocuous substances, but several endogenous tolerogenic mechanisms are in place to prevent autoimmune responses. However, when these DCs encounter pathogens, “danger molecules” such as cellular heat shock proteins or inflammatory mediators, they migrate to secondary lymphoid organs and acquire strong antigen presenting capacities (DC maturation) [6-12]. There, DCs present antigenic peptides to either CD4 or CD8 T cells, depending on the major histocompatibility complex (MHC) context in which these peptides are presented. For example, intracellular antigens (such as viral and tumor antigens) are degraded in the proteasome, and the resulting peptides are preferentially loaded into MHC class I molecules (p-MCH) at the endoplasmic reticulum. These p-MHC I complexes are transported and exposed on the cell membrane [9]. Antigen-specific CD8 T cells recognise these p-MHC I molecules, followed by activation and differentation to cytotoxic T cells. On the other hand, to activate CD4 T cells, antigenic peptides must be presented in the context of MHC class II molecules. This is usually (but not exclusively) accomplished after antigen internalisation by phagocytosis and degradation in endosomes. These CD4 T cells will then differentiate into specific subsets which regulate immune responses in different ways. Thus, these T cells are usually called “helper” (Th cells) and can either stimulate cytotoxic responses (Th1, Th17), antibody responses (Th2), or inhibit immune responses (regulatory T cells, or Tregs). The fate of differentiation will strongly depend on the type of cytokines and chemokines present during antigen presentation.

The “three-signal” hypothesis of T cell activation

Possibly, one of the key roles of DCs is to control and direct T cell activation at the level of antigen presentation, as they are capable of priming naïve T cells [10]. As explained above, antigens have to be processed into peptides and presented to specific T cells in the immunological synapse [13-16]. It is within the immunological synapse where certain signals are delivered to the T cells resulting in their activation, expansion and acquisition of effector activities. The first signal, or signal 1, refers to the engagement between the TCR and p-MHC complexes on the surface of the APC (Figure 1). This engagement is a necessary, but not sufficient, requirement for T cell activation. In fact, triggering of the TCR alone usually leads to T cell anergy, that is, limited expansion followed by unresponsiveness

after reencounter with antigen [17, 18]. To effectively activate T cells, further signals have to be provided to T cells in the immunological synapse. These signals are generally called “co-stimulation” and they are delivered by the binding of certain receptors on the T cell surface with their ligands on the DC surface. Overall, co-stimulation is considered as a second signal or “signal 2” (Figure 1).

Co-stimulation plays a fundamental role in regulating T cell functions at multiple levels, particularly during antigen presentation. Antigen-presenting DCs can engage naïve T cells and provide “positive” (activatory) or “negative” (inhibitory) co-stimulation. Ultimately, these signals are provided by a wide panel of co-stimulatory receptors expressed by DCs. For example, binding between CD80/CD86 in DCs and CD28/CD27 in T cells causes T cell proliferation and acquisition of cytotoxic activities [19]. On the other hand, CD80/86 can also engage inhibitory receptors on T cells such as the cytotoxic T-lymphocyte antigen 4 (CTLA4), and deliver inhibitory signals leading to anergic T cells or Tregs (Figure 1) [15]. As another example, programmed death-ligand 1 (PD-L1) in DCs binds to its receptor PD-1 on T cells. This binding inhibits T cell activities at multiple levels [20-22]. Thus, it is not surprising that tolerogenic immature DCs exhibit low levels of co-stimulatory molecules and up-regulate inhibitory molecules [23, 24]. After encounter with pathogen-derived molecules which trigger pathogen pattern recognition receptors such as toll-like receptors (TLRs), DCs strongly up-regulate co-stimulatory molecules and p-MHC complexes. As both “positive” and “negative” co-stimulation takes place during antigen presentation, there is a downstream integration of these signals, which ultimately determines the “strength” of the second signal and controls T cell activation [19].

As discussed above, two distinct signals need to be delivered for T cell activation; signal 1 (antigen recognition) and signal 2 (co-stimulation). However, although these two signals may activate T cells, an additional signal has to be provided which will polarise T cell differentiation. This is particularly important for CD4 T cell activation, since they can regulate CD8 T and B cell activities.

Signal 3 or cytokine priming

DCs amongst other cell types secrete different cytokines while undergoing antigen presentation to T cells, depending on the particular stimuli that they encountered at the site of infection or inflammation. These cytokines will influence the differentiation profile of T cells activated by signals 1 and 2. This is generally referred to as the “third signal”, “signal 3”, or “cytokine priming” (Figure 1) [25-27]. When this occurs, T cells differentiate into different effector T cells such as CD4 Th1, Th2, Th17, Treg cells or enhance CD8 T cell responses to weakly immunogenic antigens (Figure 1) [28]. As a matter of fact, extensive T cell proliferation can only occur if Ag levels are high in the absence of signal 3. The third signal contributes most to T cell proliferation when Ag levels are low [28]. Moreover, these naïve T cells that proliferate in the absence of a third signal fail to develop cytolytic effector functions. Thus, the presence or absence of a third signal is essential in determining whether stimulation of DCs by Ag results into tolerance or into development of effector function [29].

The detailed characterization of the polarization potential of cytokines allows the rational design of new vaccination protocols for cancer immunotherapy (Figure 1). For example, cytokines interleukin (IL)1 and IL-12 stimulate differentiation towards Th1 and enhancement of CD8 cytotoxic activities [29-35]. Accordingly, CD8α DCs produce high levels of IL12 and they are particularly effective for CD8 T cell priming [36], and stimulation of clonal expansion via expression of Bcl-3 in proliferating T cells, an anti-apoptotic molecule [37, 38]. In certain situations, IL1-β, IL17, IL6, IL23 and transforming growth factor (TGF)-β can polarize T cell differentiation in a pro-inflammatory T helper

subset named Th17 [39, 40]. These T cells express IL17, which triggers an accelerated and strong inflammatory reaction. This subset of T cells has been implicated in the development of autoimmune diseases since it seems to promote the loss of tolerance towards allergens and auto-antigens [41-44]. On the other hand, IL10 and TGF-β will drive T cell differentiation towards Th2 or immunosuppressive Tr1 or Foxp3+ Tregs [23, 24, 45-48].

Requirement of the third signal *in cis* during antigen presentation

The third signal can be provided by DCs to antigen presenting cells in two distinct situations. The first one, by direct activation (activation *in cis*) by triggering pattern recognition receptors such as TLRs (Figure 2). This recognition strongly activates DCs leading to their phenotypic maturation and secretion of different cytokine patterns. The type of expressed cytokines will depend on the particular TLR that is triggered, which will differentially activate distinct intracellular signaling pathways. For example, TLR2 activation by yeast zymosan activates extracellularly regulated protein kinase (ERK) and stimulates secretion of IL10 that will result in immune suppression [49, 50]. On the other hand, TLR4 stimulation by a wide variety of ligands results in DC maturation, IL12 production and immune stimulation with anti-tumour properties [3, 51-53].

The second mechanism for providing signal 3 is indirectly *in trans* through the exposure to inflammatory mediators by neighboring cells during an immune response (Figure 2). This suggests that inflammation itself could substitute pathogen recognition for the induction immune responses [6, 54, 55]. Although from a theoretical point of view this concept could be effectively applied to immunotherapy, there is increasing evidence that indirectly activated APCs after cytokine exposure behave very differently compared to cytokine-secreting, directly activated APCs [56-58]. Indirectly activated DCs up-regulate MHC molecules and are capable of providing co-stimulatory signals, leading to T cell clonal expansion. However, as indirectly activated DCs do not provide the third signal in the immunological synapse, the engaged T cells do not differentiate to particular subsets (Figure 2) [56, 59]. Therefore, inflammation can amplify immune responses, DCs have to provide inflammatory mediators themselves to initiate effective immune responses [56, 60, 61].

These observations demonstrate the importance of developing the right adjuvants to optimize the efficacy of vaccines for immunotherapy [59]. In fact, this could explain the disappointing outcomes of certain cancer immunotherapy clinical trials using CpG as an adjuvant. CpG is recognized by TLR9 and it is a potent inflammatory mediator, although it is absent in conventional human DCs [62, 63]. In addition, CD8α DCs provide strong third signals during antigen presentation, they express TLR3 but not TLR7 [59, 64-66]. Consequently, the right choice of adjuvants could potentiate the current formulations of vaccines for immunotherapy by specifically targeting particular DC subsets.

Modulation of co-stimulation to enhance immunotherapy

The manipulation of the immunological synapsis opens attractive possibilities to control T cell activation and differentiation for the treatment of cancer and autoimmune disorders. To manipulate co-stimulation, the expression levels of co-stimulatory molecules in DCs can be modified. An effective way to achieve this is to specifically activate intracellular signalling pathways in DCs belonging to the TLR signal transduction pathways. The main pathways involved in DC maturation are the nuclear factor (NF)-κB and mitogen activated protein kinases (MAPKs) ERK, p38 and JNK1 [67-73]. This strategy ensures the up-regulation of co-stimulatory, adhesion and major histocompatibility molecules together with cytokine expression, which will provide strong signals 1, 2 and 3.

Most pro-inflammatory genes are controlled by promoters responding to NF-κB dimmers, and thus, this pathway is one of the main controllers of pro-inflammatory responses [8, 74-76]. Its activation is required for up-regulation of co-stimulatory molecules, MHC and pro-inflammatory cytokines, particularly IL6, IL12, tumour necrosis factor (TNF)-α [74, 77-81]. There is also a considerable body of evidence linking MAPKs to enhancement of DC function by up-regulation of co-stimulatory molecules and secretion of pro-inflammatory mediators, although most of these studies use chemical inhibitors rather than specific activators. The p38 MAPK is activated in virtually all cell types by cellular stress and TLR signalling [82-84]. The inactivation of MKK3 in mice, one of the upstream kinases of p38, resulted in a complete lack of IL12 production by macrophages and DCs, and in general a decrease in pro-inflammatory responses [85]. Both p38 and NF-κB contribute of up-regulation of co-stimulatory and MHC molecules in monocyte-derived DCs as shown after LPS treatment in the presence of phosphatidyl inositol 3 kinase (PI3K), p38 and NF-κB inhibitors [8]. In fact, p38 activation induces histone phosphorylation, leading to opening of the chromatin structure allowing NF-κB dimmers to bind to promoters controlling transcriptional up-regulation of pro-inflammatory genes [75]. Actually, p38 activation rather than the JNK pathway seems to be critical for human DC maturation after a wide variety of pro-inflammatory stimuli including cytokines, TLRs and other immunostimulatory agents [69]. However, JNK may act in cooperation with the other MAPKs to induce pro-inflammatory responses and immune activation [86-88]. MAPK p38 has been clearly shown to be critical for anti-viral CD8 T cell responses after CD83 up-regulation in human DCs stimulated *via* CD40-CD40L. CD40 ligation results is strong secretion of pro-inflammatory cytokines such as IL6 and IL12 [80], which could lead to Th1 and Th17 responses. However, there are still some studies using chemical inhibitors in human DCs that may challenge the absolute requirement of p38 over JNK regarding DC maturation [71].

In the case of the MAPK ERK, most of the evidence points out to a regulatory rather than a stimulatory role, which includes inhibition of DC maturation in mouse and human cells [70]. Chemical inhibition of ERK results in up-regulation of co-stimulatory makers and MHC molecules, accompanied by increased expression of pro-inflammatory cytokines such as TNF-α and IL-12 [70]. ERK inhibition also results in p38 activation, which overcomes ERK-mediated immunosuppressive activities, at least in macrophages [89]. However, some evidence suggests that ERK activation is also required for production of some pro-inflammatory cytokines such as TNF-α [90], IL23 and augmentation of natural killer (NK) responses [86, 91]. This could explain why ERK is in most cases phosphorylated after TLR activation. The timing/kinetics of ERK activation in combination with other signal transduction pathways may influence its role either in immune suppression of immune activation [92-95].

Thus, as TLR activation leads to activation of NF-κB, MAPKs and several other signalling pathways, TLR agonists are currently being used in clinical trials for the treatment of infectious diseases and cancer [96-99]. For example, TLR agonists can be combined with inhibitors of immunosuppressive signalling pathways such as PI3K to enhance differentiation of polyfunctional T cells [100]. The advantage of using synthetic TLR agonists for immunotherapy is the control of the exact composition of the adjuvant in the vaccine [101, 102]. This simplifies vaccine design and prediction of therapeutic outcomes. Concluding, all this body of evidence indicates that activation of NF-κB, p38 and possibly JNK, combined with inhibition of suppressive pathways such as PI3K and ERK, would increase anti-tumour capacities possibly by enhancing signals 1, 2 and 3.

Instead of using TLR agonists as vaccine adjuvants, selective activation/inhibition of signalling pathways can also be applied using gene therapy approaches. Thus, DC activation has been achieved by expression of constitutive activators of signalling pathways in human

and mouse DCs [103]. Constitutive activation of NF-κB by expression of Kaposi's sarcoma-associated human herpesvirus (KSHV) vFLIP protein using lentivectors, or by over-expression of the NF-κB inducing kinase (NIK) using adenoviral vectors effectively up-regulates CD80, CD86, CD83, CD40, ICAM I, MHC class I and II [77, 79]. In addition, this is accompanied by strong secretion of the Th1 polarising cytokines IL12, TNFα and other chemokines, ensuring the delivery of a strong signal 3. In fact, constitutive NF-κB activation in mouse DCs was particularly efficient to stimulate Th1 immune responses [79]. As expected, vFLIP expression clearly enhanced anti-tumour immune responses in a mouse lymphoma model and anti-parasitic immunity in a leishmaniasis model [77, 78]. Similarly, activation of p38 and JNK1 pathways in mouse and human DCs by expression of constitutively active mutants of MAPK kinase kinase (MKK)6 (the upstream p38 kinase) or a fusion protein between MKK7-JNK1 produced similar results [24]. These included up-regulation of CD80, CD40 and ICAM-I, although without significant expression of Th1 cytokines IL12 or TNFα. Nevertheless, these DC activators proved to be effective in immunisation, and exhibit anti-tumour activities at least for the p38 activator [22, 24]. In this case, the lentivector preparation could have directly provided adjuvant activities *in vivo* through TLR3 and TLR7 engagement, at least in mouse models of vaccination [12]. On the other hand, activation of the ERK pathway in mouse and human DCs by expression of a constitutively active MEK1 mutant effectively down-modulated co-stimulatory molecules, particularly CD40 and MHC molecules [23, 24]. This ensures that "positive" co-stimulation does not take place. Moreover, ERK activation in mouse DCs stimulated the production of bioactive TGF-β. This effect was particularly effective in human DCs, for which this third signal drove T cell differentiation towards immunosuppressive Foxp3 Treg cells [23]. Therefore, signals 1, 2 and 3 can also be modulated to achieve immune suppression and tolerance with therapeutic applications towards autoimmune diseases such as inflammatory arthritis [23].

B7 co-stimulatory molecules as targets for immunotherapy

Recently, much attention has been paid to blocking negative co-stimulation during antigen presentation to enhance immunotherapy for cancer and chronic infectious diseases. More specifically, inhibition of the B7 co-stimulatory family with T cell inhibitory properties [104-112]. One of such inhibitory interactions takes place between either PD-L1 or PD-L2 on the surface of DCs with PD-1 on the T cell surface. PD-L1 is a member of the B7 family of co-stimulatory/co-inhibitory molecules widely expressed by many cell types including T cells and DCs [20, 21, 113]. PD-1 is up regulated in activated T cells during antigen presentation and its binding to PD-L1 recruits SHP1 and SHP2 phosphatases to its cytoplasmic domain, which terminate TCR signalling [114-116]. As a matter of fact, PD-L1 expression rather than PD-L2 in peripheral tissues is critical to keep immune tolerance [20, 21].

Therefore, interference of PD-L1/PD-1 using either systemic administration of blocking antibodies or silencing PD-L1 has been applied to enhance positive co-stimulation and improve immunotherapy, both in mouse models or in human DCs [105, 106, 111, 117, 118]. Interestingly, blockade or silencing of PD-L1 significantly enhances recruitment and association between CD8 T cells to DCs *in vivo* and *in vitro* in mouse models [22, 119, 120]. This stronger and prolonged association suggests that signal 1 and signal 2 provided to T cells in the immunological synapse are significantly reinforced. Interestingly, PD-L1 silencing in antigen presenting myeloid mouse DCs with a PD-L1-specific short hairpin (sh)RNA decreased TCR down-modulation in activated T cells after antigen presentation [22, 121, 122]. This lack of TCR down-modulation most likely ensured TCR signal transduction to T cells with a concomitant stronger signal 1. In addition, the elimination of negative co-stimulation would in principle increase positive co-stimulation provided by

CD80/CD86 and CD40. Therefore, a stronger activatory signal 2. Accordingly, PD-L1 silencing in antigen presenting DCs induced the expansion of a population of hyper activated pro-inflammatory TCR^{high} CD8 T cells, with increased levels of interferon (IFN)γ and IL17 [22]. This is particularly interesting for antitumor immunotherapy, considering the key role of cytotoxic CD8 T cells against cancer cells. Cellular vaccination with these PD-L1-silenced DCs inhibited tumour growth in a mouse lymphoma model and prolonged survival. Surprisingly, overall long-term cure rates did not improve in comparison with vaccination using unmodified DCs [22, 121]. This suggested that PD-L1 inhibition accelerated tumour-specific T cell responses, although additional co-stimulatory signals might be needed to enhance T cell cytotoxic activities. We propose that additional manipulation of signal 3 during antigen presentation could improve the results obtained by interference with PD-L1/PD-1 blocking. This strategy could be particularly important to overcome immunosuppressive tumour microenvironments. Removal of PD-L1-based regulation combined with a potent signal 3 provided by cytokine secretion could generate enhanced T cell immunity in a therapeutic setting. While elimination of the PD-L1/PD-1 inhibitory interaction hyperactivates T cells and accelerates their expansion, specific cytokine combinations could potentially modify the type of immune response to be achieved as long as there are provided *in cis* by antigen presenting DCs.

Expression of other B7 family members by APCs or cancer cells has also been associated to inhibition of T cell responses and poor prognostic in cancer patients. Therefore, their blockade in many instances may be also used to enhance co-stimulation and manipulate T cell differentiation. The B7-H2 co-stimulatory molecule, or inducible T cell co-stimulator (ICOS) ligand (ICOS-L), is expressed in physiological conditions amongst others by immature human DCs [123], human airway epithelial cells [124]. B7-H2/ICOS co-stimulation is required for T cell activation and recall of T and B cell responses [125-127]. However, it has been recently demonstrated that B7-H2 is also a ligand for both CD28 and CTLA-4, involved in activatory and inhibitory signalling in T cells, respectively [128]. Therefore, it could be possible that depending on the context of co-stimulation, B7-H2 may enhance or inhibit immune responses. Thus, B7-H2 expression is up-regulated in a number of cancer cells such as in leukaemia [129] and myeloma cells [130], although possibly linked to increased tumour cell proliferation and survival rather than immune evasion. Finally, the involvement of ICOS-L/ICOS co-stimulation on T cell differentiation is unclear. While some reports suggest that it enhances human Th1/Th17 responses with anti-tumour activities [131], other reports suggest that it is required for Th2 but not Th1 or Th17 differentiation [132, 133].

B7-H3 is preferentially a negative regulator of T cell responses that is expressed by a wide range of cells including DC, monocytes [134-137], T cells [138] and tumour cells [139, 140]. Its up-regulation by cancerous cells confers a mechanism to escape immune responses [139, 141-144]. Therefore, B7-H3 blockade may also enhance tumour immunotherapy by preventing cancer cell escape. However, the role of B7-H3 as an inhibitor of immune responses is still unclear, as many studies also link B7-H3 co-stimulation with increased T cell responses, Th2 differentiation and anti-tumour immunity [145-149]. However, many of these discrepancies might be due to the different experimental systems (most of them murine), as B7-H3 co-stimulation of human T cells seems to be inhibitory, and its expression by tumour cells shows a poor prognosis [150]. Nevertheless, the precise role of B7-H3 in immune activation or inhibition remains highly controversial, and its therapeutic exploitation unclear [147, 151-154].

B7-H4 is another member of the B7 family of co-stimulatory molecules [155] with immunosuppressive activities, similarly to PD-L1. B7-H4 is also expressed by a wide range of cell types including DCs, macrophages, endothelial and mesenchimal cells, and it plays a

key homeostatic role by inhibiting T cell functions to maintain immunological tolerance [156-158]. Its expression by tumour cells inhibits T cell cytotoxic activities and provides a means for immune escape [159-163]. Similar strategies as those used for inhibiting PD-L1 co-stimulation could also be applied for B7-H4 to enhance anti-tumour activities, and blocking antibodies are being developed for this purpose [164].

CTLA-4 blockade for tumour immunotherapy

CTLA-4 is a well-known T cell inhibitory B7-receptor that is expressed by activated T cells and particularly by Tregs [165-168]. This receptor constantly traffics between endosomes and the T cell surface [169], and it has a higher affinity than CD28 for their common ligand, CD80 [170]. Several mechanisms of action have been proposed to explain its potent immunosuppressive activities, ranging from competition with CD28 for CD80, delivery of an inhibitory intracellular signal or by CD80/CD86 transendocytosis from the membrane of antigen-presenting cells [171-174]. Nevertheless, independently of its mechanism of action, antibody-mediated CTLA-4 blockade has been largely successful in animal models of cancer. Its blockade clearly enhances T cell cytotoxic responses, inhibits Treg activities and induces differentiation of cytotoxic CD4 T cells [175-177].

Interestingly, CTLA-4 blockade can also be combined with other immunotherapy approaches such as inhibition of inhibitory co-stimulation [178], enhancement of activatory co-stimulation [179, 180], cytokine treatments [181] or superantigens [182]. These approaches turn the balance towards effector T cell activities instead of Tregs [183]. More important, clinical activity is evident and CTLA4 blocking shifts the T cell differentiation balance towards Th1/Th17 in human cancer patients [184]. Clinical efficacy with an approved blocking antibody (ipilimumab) has been reported in a wide range of cancers such as melanoma [185, 186], prostate cancer [187], non-small cell lung cancer [188] amongst others [189]. However, although clinical efficacy might not be apparent after single use, such as in pancreatic adenocarcinoma, significant clinical responses are obtained in combination with other immunotherapeituc strategies, both in mouse models and in humans [190, 191].

This last case on blocking negative co-stimulation, or inhibitory interactions with blocking antibodies is the demonstration and immunotherapy can actually work, with significant clinical efficacy for a wide range of cancers [192]. Immunotherapy will surely be fundamental for the treatment of advanced cancers, due to the capacity of the immune system to detect, reach and eliminate microscopic metastasis.

CONCLUSIONS

The three-signal hypothesis of T cell activation is a convenient mechanistical model that explains the requirements needed for T cell activation and the initiation of immune responses. Thus, T cells require at least three “types” of signals to expand and exert their effector activities. The first one is direct antigen recognition. The second one, co-stimulation, is provided after integration of activatory and inhibitory interactions between receptors and ligands within the immunological synapse. The outcome of these interactions will determine the “activation” status and effector capacities of T cells. However, a third signal is also required to modulate T cell differentiation into different subsets that will control the type of immune responses. This ensures that immune responses are adequate to fight the particular type of pathogen, including cancer. This third signal is provided by a combination of cytokines, chemokines and other inflammatory mediators. Interestingly, there is evidence suggesting that for efficient immune responses, the three signals have to be given to T cells *in cis*, that is, by the APC directly associated to the T cell. This is a critical

observation for designing adequate vaccination regimes, as many adjuvants used for vaccination protocols provide the “third signal” mainly *in trans*.

Nowadays, it is relatively straightforward to modify DCs to efficiently provide the three signals to T cells, and even modulate co-stimulation to control the strength and type of immune response [3, 103]. It is also possible to induce immune suppression and tolerance [23]. Manipulation of signals 1 and 2 is relatively simple by the co-expression in DCs of antigens, activators/inhibitors of maturation pathways or blocking antibodies that interfere with negative co-stimulation. It is rather more complicated to manipulate signal 3 so that DCs giving the first two signals to T cells can also produce the desired cytokine/chemokine profiles in the immunological synapse. The modulation of the three signals in a directed, targeted way would allow a fine-tuned control of immune responses leading to better immunotherapy treatments.

Blocking antibodies targeted to inhibit negative co-stimulation to T cells are promising for the future of cancer immunotherapy. There are several of these antibodies already approved for human therapy, which are showing clinical efficacy. However, these will probably have to be combined with other immunotherapeutic approaches, most likely because of the necessity of strengthening co-stimulatory signalling and cytokine priming.

Acknowledgments

ID is funded by an ERASMUS (EU) scholarship. KB is funded by the Fund for Scientific Research-Flandes. DE is funded by an arthritis research UK career development fellowship (18433).

LIST OF ABBREVIATIONS

APC	Antigen presenting cell
CD	Cluster of differentiation
CTLA4	cytotoxic T-lymphocyte antigen 4
DC	Dendritic cell
ERK	extracellularly regulated protein kinase
ICOS	Inducible T cell co-stimulator
IFN	Interferon
IL	Interleukin
KSHV	Kaposi’s sarcoma-associated human herpesvirus
MAPK	mitogen activated protein kinases
MKK	MAPK kinase kinase
MHC	major histocompatibility complex
NF	Nuclear factor
NK	Natural killer
PD-L1	Programmed cell death 1 ligand 1
PD-1	Programmed death 1
PI3K	phosphatidyl inositol 3 kinase
p-MHC	peptide-MHC complex

TAA	tumour-associated antigen
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor

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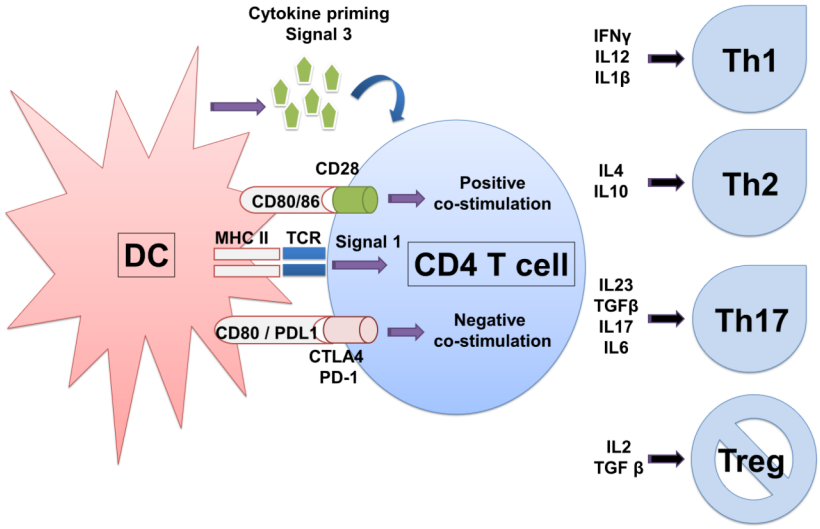

Figure 1. Antigen presentation in the immunological sypnase

The scheme depicts a DC (left) presenting antigen in the context of MHC molecules to a CD4 T cells. The peptide-MHC engages to the T cell TCR delivering signal 1 as shown within the T cells. Simultaneously, co-stimulatory or co-inhibitory ligands on the DC surface (CD80/86, or CD80/PDL1) engage with their receptors on the T cell, leading to either positive stimulation or negative stimulation. The resulting signal from the integration of these co-stimulatory interactions will provide a second signal to T cells. In this scheme, the DC produces cytokines within the immunological synapse, giving a third T cell polarising signal. On the right, the most representative T helper subtypes are shown, and the cytokines promoting these T cell subtypes are also indicated.

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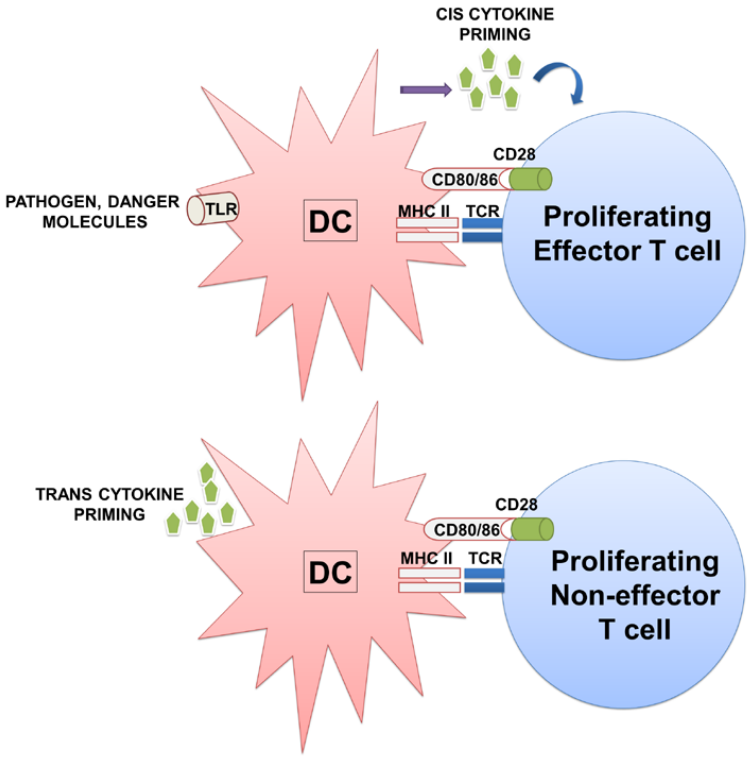

Figure 2. Effects of directly or indirectly activated DCs on antigen presentation and T cell polarisation

On top, a directly activated DC through TLR engagement (left) presents antigen to T cells and provide the three activatory signals to the T cells (right). In this case, cytokine priming occurs *in cis*, leading to T cell proliferation and acquisition of effector activities. On the bottom, an indirectly activated DC through cytokine priming *in trans* (i.e. cytokines produced by neighbouring cells within an inflammatory environment, left of the scheme). In this situation, cytokines are not produced within the immunological synapse and the resulting T cell proliferates, but does not acquire significant effector activities or a distinct differentiation profile.