



# The nature of activatory and tolerogenic dendritic cell-derived signal II

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Dendritic cells (DCs) are central in maintaining the intricate balance between immunity and tolerance by orchestrating adaptive immune responses. Being the most potent antigen presenting cells, DCs are capable of educating naïve T cells into a wide variety of effector cells ranging from immunogenic CD4<sup>+</sup> T helper cells and cytotoxic CD8<sup>+</sup> T cells to tolerogenic regulatory T cells. This education is based on three fundamental signals. Signal I, which is mediated by antigen/major histocompatibility complexes binding to antigen-specific T cell receptors, guarantees antigen specificity. The co-stimulatory signal II, mediated by B7 family molecules, is crucial for the expansion of the antigen-specific T cells. The final step is T cell polarization by signal III, which is conveyed by DC-derived cytokines and determines the effector functions of the emerging T cell. Although co-stimulation is widely recognized to result from the engagement of T cell-derived CD28 with DC-expressed B7 molecules (CD80/CD86), other co-stimulatory pathways have been identified. These pathways can be divided into two groups based on their impact on primed T cells. Whereas pathways delivering activatory signals to T cells are termed co-stimulatory pathways, pathways delivering tolerogenic signals to T cells are termed co-inhibitory pathways. In this review, we discuss how the nature of DC-derived signal II determines the quality of ensuing T cell responses and eventually promoting either immunity or tolerance. A thorough understanding of this process is instrumental in determining the underlying mechanism of disorders demonstrating distorted immunity/tolerance balance, and would help innovating new therapeutic approaches for such disorders.

**Keywords:** activation, tolerance, co-stimulation, co-inhibition, dendritic cells, T cell priming

## INTRODUCTION

The immune system is endowed with the unique capacity to protect against invading pathogens, yet not react to self. Among the different constituents of the immune system, dendritic cells (DCs) play a central role in drawing the thin line between immunity and tolerance. Discovered in 1973 (Steinman and Cohn, 1973), DCs are recognized as the most potent antigen presenting cells (APCs). Their ability to initiate and modulate various forms of T cell responses, earned them the position of being master orchestrators of adaptive immunity (Banchereau and Steinman, 1998). DCs are spread throughout the body, residing in different tissues as sentinels, monitoring their surrounding environment for any signs of danger. Equipped with pathogen recognition receptors (PRRs), DCs are capable of sensing pathogenic invasion (Medzhitov and Janeway Jr., 2002) and self-structures associated with cellular stress (Matzinger, 2002). Upon danger sensing, DCs will undergo functional changes, also known as maturation, crucial for the ensuing induction of T cell responses (Banchereau et al., 2000). A hallmark of DC maturation is the expression of the chemokine receptor CCR7 that allows mature DCs to migrate to draining lymphoid tissues where they activate naïve T cells in a process based on three signals. The first signal results from the ligation of T cell receptors (TCRs) to pathogen-derived peptide antigens that are presented by major histocompatibility complex (MHC) molecules of DCs, which are upregulated upon maturation. This principal

stimulation signal is important to assure antigen specificity of the immune response. Although TCR triggering is crucial for naïve T cell activation, it is not sufficient by itself to initiate an efficacious immune response. The concept of a second co-stimulatory signal was first introduced by Lafferty and Woolnough (1977). They deduced from organ transplantation studies that alloantigens presented by transplanted tissues failed to elicit any immune responses unless accompanied by hematopoietic stimulator cells (Lafferty and Woolnough, 1977). This concept was corroborated by seminal observations by the group of Schwartz, implying that T cells activated solely by TCR engagement were rendered unresponsive and anergic (Jenkins and Schwartz, 1987). This was followed by the discovery of the main elements of co-stimulation: CD28 (Aruffo and Seed, 1987) and CD80 (Freeman et al., 1989), the latter being initially identified as a B cell activation marker and eventually recognized as the ligand of CD28 (Linsley et al., 1990). Subsequently, more pathways contributing to signal II were identified. Based on the nature of their signal, these molecules can be divided into co-stimulatory molecules that promote T cell proliferation, and co-inhibitory molecules that attenuate T cell responses. The nature of signal II is vital in determining the T cell response, which is further defined by a third polarizing signal. This third signal promotes the selective development of naïve T cells into one of the identified types of effector or tolerogenic T cells (De Jong et al., 2005). Although signal III is generally recognized to be mediated

by soluble DC-derived cytokines, there are indications that signal II may also contribute to T cell polarization. A final putative DC-derived signal is suggested to provide polarized T cells with homing directions to the site of infection or injury (Sigmundsdottir and Butcher, 2008). Thus, DCs control the delicate balance between immunity and tolerance through the signals they convey to T cells.

Although the combined effect of all DC-derived signals is important for full blown T cell responses, signal II is key for allowing these responses and licensing them to become either immunogenic or tolerogenic. Here, we shed light on the multifaceted signal II by reviewing current knowledge of to date identified co-stimulatory and co-inhibitory pathways (Figure 1), their mode of action, relation to disease, and any possible clinical applications based on utilizing these pathways.

## CO-STIMULATORY MOLECULES

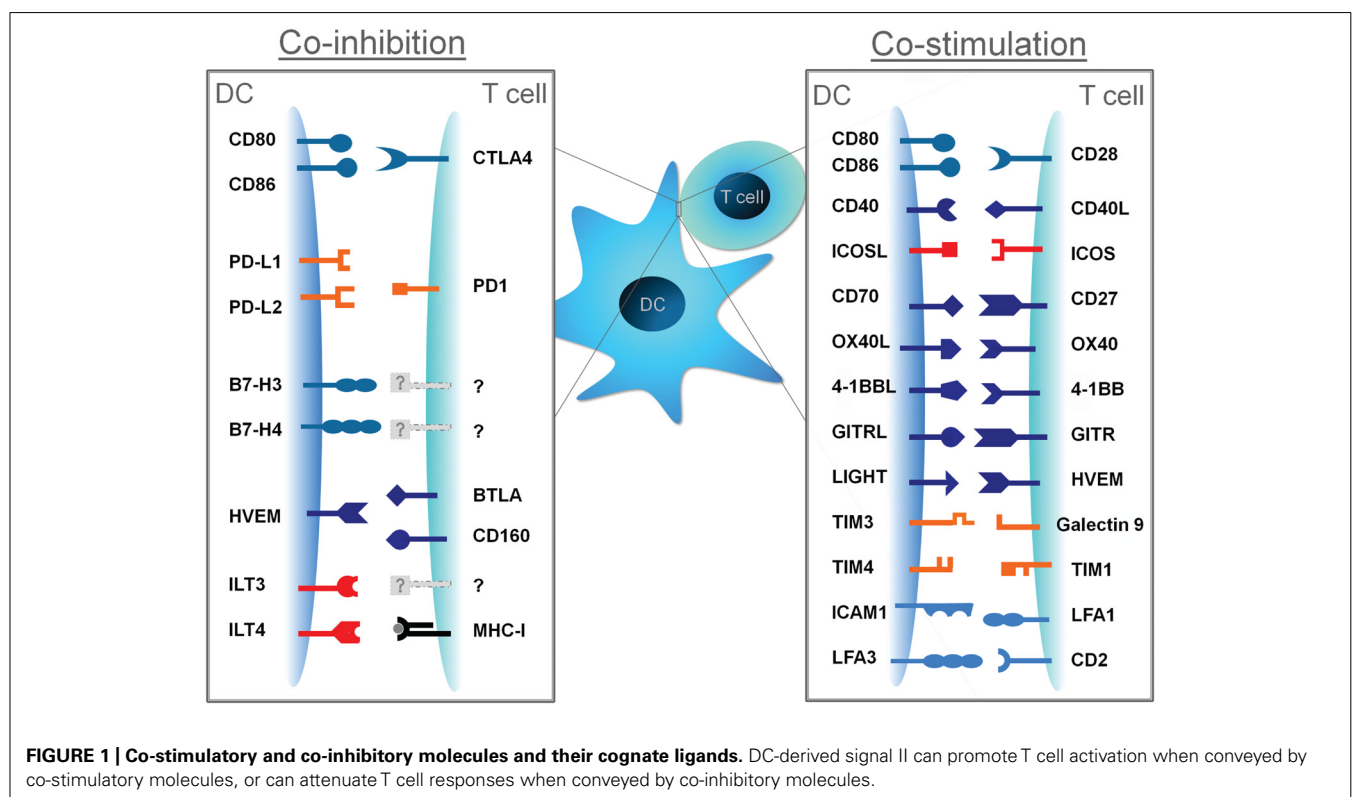
### CD80/CD86/CD28 PATHWAY

Following the discovery of the CD80/CD28 interaction, B7-2 (CD86) was identified as a second ligand for CD28 (Azuma et al., 1993). The CD80/CD86/CD28 pathway was suggested to deliver the strongest co-stimulatory pathway as CD28-deficient cells failed to proliferate in the presence of APCs (Green et al., 1994). The consequences of CD28 engagement by its ligands comprise stimulation of T cell proliferation, dramatic upregulation of IL-2 (Linsley et al., 1991a), promotion of T cell survival by enhancing Bcl-XL expression (Boise et al., 1995), and enhanced glycolytic flux to meet energetic requirements associated with a sustained response (Frauwirth et al., 2002). Those effects were shown to be dependent

on activating the signaling cascades of phosphoinositide-3 kinase (PI3K), protein kinase B (PKB, also known as Akt), and nuclear factor kappaB (NF- $\kappa$ B; Song et al., 2008).

Several reports pointed out a possible role for CD28 signaling in T cell polarization. Murine T cells were shown to produce enhanced levels of IL-4 and IL-5, characteristic for T helper (Th) 2, upon strong CD28 stimulation (Rulifson et al., 1997). Strong CD28 signaling was also demonstrated to inhibit Th17 responses (Purvis et al., 2010). Although it is generally accepted that memory T cells, unlike naives, are less dependent on co-stimulation via CD28, it was shown that this co-stimulatory pathway is important in controlling T cell recall responses (Ndejemi et al., 2006).

In addition to its key role in initiating and sustaining efficient T cell responses, the CD28 pathway is also involved in controlling immune tolerance. Co-stimulation of developing thymocytes by CD28 was shown to induce the expression of Foxp3 and promote the differentiation of regulatory T cells (Tregs; Tai et al., 2005). Furthermore, T cell activation in the absence of CD28 co-stimulation leads to a state of anergy characterized by dramatically reduced production of IL-2 and other effector cytokines upon subsequent TCR triggering (Schwartz, 1997). There is ample evidence that DCs utilize this mechanism to maintain tolerance to self. At steady state conditions, immature DCs present self-derived antigens accompanied by low levels of CD80/CD86 and therefore fail to supply specific T cells with adequate signal II, leading eventually to the deletion, anergy, or regulation of auto-reactive T cells that escaped thymic selection (Steinman and Nussenzweig, 2002). Thus, the CD80/CD86/CD28 pathway is as involved in promoting tolerance as in mediating immunity.



Since many immunogenic tumors lack expression of CD80 and CD86, it was postulated that tumor-infiltrating T cells would receive chronic TCR stimulation without co-stimulation leading to T cell anergy. This hypothesis was tested by inducing the expression of CD80/CD86 molecules on tumor cells prior to injection into mice. Forced expression of CD80/CD86 in tumor cells resulted into CD8<sup>+</sup> T cell-dependent tumor rejection (Townsend and Allison, 1993). However, this method had barely any effect on pre-established tumors (Fallarino et al., 1997), implying that other pathways promoting immune tolerance toward established tumors are involved.

#### CD40/CD40L PATHWAY

CD40 was the first co-stimulatory molecules to be identified from the tumor necrosis factor (TNF) receptor (TNFR) family. First discovered as B cell receptor, CD40 is also expressed by DCs, macrophages, epithelial cells, and even activated T cells. Its ligand (CD40L or CD154), a member of the TNF family, is expressed not only by activated T cells, but also by natural killer (NK) cells and plasmacytoid DCs (pDCs; Quezada et al., 2004). In addition to promoting humoral immunity by activating B cells, the CD40/CD40L pair is pivotal for cellular immunity as it mediates a dialog between T cells and DCs. Indeed, CD40 engagement on DCs was shown to activate NF- $\kappa$ B pathway (Quezada et al., 2004) and consequently inducing DC maturation (Caux et al., 1994) and enhancing DC longevity (Miga et al., 2001). Initially, CD40-induced maturation of DCs was suggested to be sufficient in licensing CD8<sup>+</sup> cytotoxic responses (Schoenberger et al., 1998). However, further investigation in the CD40 pathway revealed that additional signals are necessary for optimal DC activation. CD40 cross-linking alone is not enough to induce IL-12 production, necessary for cytotoxic and Th1 responses, but DC pre-activation by microbial products followed by CD40 ligation dramatically increased IL-12 production (Schulz et al., 2000). This finding indicates that combined triggering of CD40 and PRRs, like Toll-like receptors (TLRs), is critical for DC licensing. The CD40-induced IL-12 also implies a central role for CD40/CD40L pathway in T cell differentiation, by favoring Th1 polarization. Blocking CD40/CD40L interactions lead to abrogated Th1 responses with reciprocal upregulation of Th2 cytokines (Hancock et al., 1998).

The adjuvant effect of CD40 ligation, reflected by DC activation, prompted the application of agonistic anti-CD40 antibodies for cancer therapy. Injecting agonistic anti-CD40 antibodies evoked cytotoxic T cell responses and eradicated the tumor in a mouse model of lymphoma (French et al., 1999). Furthermore, application of fully humanized anti-CD40 agonistic antibody resulted in objective partial responses in 14% of advanced solid tumor patients (Vonderheide et al., 2007). A similar approach was based on the administration of soluble CD40L, which was less efficient as it lead to partial responses in 6% of treated tumor patients (Vonderheide et al., 2001). More clinical trials applying CD40 ligation, singularly or in conjunction with other therapeutic modalities, were carried out and showed promising results (Khong et al., 2012).

Due to its activatory nature, the CD40/CD40L is decisive in regulating tolerance. It was shown that DCs derived from CD40-deficient mice conferred tolerance by priming IL-10 secreting Tregs

(Martin et al., 2003). This effect on tolerance prompted investigating the possibility of exploiting CD40 blocking to enhance allograft survival. Although applying anti-CD40L antibodies as a monotherapy was able to block many effector mechanisms, it failed to induce sufficient allograft tolerance (Jones et al., 2000). However, combinations with other immunosuppressive therapies such as cytotoxic T lymphocyte (CTL)-associated antigen-4-immunoglobulin (CTLA-4-Ig; Larsen et al., 1996) and rapamycin (Li et al., 1998) were shown to result in long-term graft survival. Collectively, CD40/CD40L pathway, in conjunction with other pathways, is vital for initiating active immunity and regulating tolerance.

#### ICOSL/ICOS PATHWAY

The inducible T cell co-stimulator (ICOS) was identified as the third member of the CD28/CTLA-4 family of co-stimulatory molecules (Hutloff et al., 1999). ICOS expression by T cells requires prior TCR activation and CD28 co-stimulation (McAdam et al., 2000). The ligand (ICOSL) is expressed by DCs (Wang et al., 2000), B cells, and a variety of non-hematopoietic tissues (Ling et al., 2000). ICOSL/ICOS pathway exerts its co-stimulatory effects on already activated T cells by supporting proliferation and cytokine production (Hutloff et al., 1999). Additionally, ICOS is proposed to play an important role in T cell polarization. Initially, ICOSL/ICOS was suggested to support Th2 responses. Blocking ICOSL/ICOS interactions was shown to block Th2-lead airway responses without influencing Th1-mediated inflammation (Coyle et al., 2000). Similarly, another study showed that the majority of T cells expressing ICOS *in vivo* co-produced Th2-type cytokines (Lohning et al., 2003). In contrast, disrupting ICOSL/ICOS pathway was found to inhibit Th1-mediated disorders like allograft rejection (Guo et al., 2002) and experimental allergic encephalomyelitis (Rottman et al., 2001). ICOS was shown to be involved driving Th17 responses (Park et al., 2005), further complicating the role of ICOSL/ICOS in T cell polarization. An attempt to resolve this controversy was by showing that engaging ICOS on activated T cells amplified the effector responses of these cells regardless of their polarized state (Wassink et al., 2004).

Benefiting of the activatory effect of ICOSL/ICOS pathway in the context of cancer therapy was evaluated. Induced ICOSL expression on tumor cells was demonstrated to promote tumor regression by inducing CD8 cytotoxicity (Liu et al., 2001). Nevertheless, this strategy was ineffective in case of weakly immunogenic tumors (Ara et al., 2003). Surprisingly, it was recently revealed that tumor cell-expressed ICOSL augments Treg activation and expansion within the tumor local environment (Martin-Orozco et al., 2010). This suggests that triggering ICOSL/ICOS pathway may not be the most optimal option for cancer treatment. On the contrary, blocking its ICOSL/ICOS-mediated suppression may be beneficial in cancer therapy.

The tolerogenic effect of ICOSL/ICOS pathway is not restricted to tumors, as there are indications of its involvement in maintaining immune tolerance. ICOS-deficient mice displayed reduced numbers of natural Tregs (nTregs), which may be owed to a decrease in survival and/or proliferation of these cells (Burmeister et al., 2008). Another indication of ICOS involvement in tolerance

is the finding that ICOS triggering on T cells dramatically increased the production of the anti-inflammatory cytokine IL-10 (Hutloff et al., 1999). Consistently, high ICOS expression by T cells was selectively associated with the anti-inflammatory IL-10 (Lohning et al., 2003). These findings argue for targeting ICOSL/ICOS pathway to induce tolerance for therapeutic purposes. However, it is very important to clearly dissect the conditions under which this pathway induces activation or tolerance.

### CD70/CD27 PATHWAY

CD70 is another member of the TNF family of co-stimulatory molecules. Its ligand CD27 was identified first as a novel T cell differentiation antigen (van Lier et al., 1987). The contribution of CD27 to immunity was later recognized to be dependent on its binding partner CD70, which is expressed under the control of antigen receptors and TLRs in lymphocytes and DCs, respectively (Tesselaar et al., 2003). Similar to CD40, engaging CD27 induced the activation of NF- $\kappa$ B pathway (Akiba et al., 1998). The first indication of the co-stimulatory properties of the CD70/CD27 pathway was provided by triggering CD27, which augmented CD3-induced T cell proliferation (van Lier et al., 1987). This effect was later explained by promoting survival of newly stimulated T cells, in contrast to CD28 that prompts cell cycle entry and induces proliferation (Hendriks et al., 2003). This survival effect relies completely on IL-2 receptor signaling and the autocrine production of IL-2 (Peperzak et al., 2010).

The contribution of CD70/CD27 pathway to T cell polarization is debatable. CD8<sup>+</sup> T cells from CD27 knockout mice maintained the capacity of differentiation into CTLs and interferon-gamma (IFN- $\gamma$ ) production, implying that CD27 is not involved in the development of cytotoxic CD8 responses (Hendriks et al., 2000). On the other hand, transgenic expression of CD70 on steady state immature DCs was found to break CD8<sup>+</sup> tolerance and permit the differentiation of effector CD4<sup>+</sup> and CD8<sup>+</sup> cells from naïve precursors (Keller et al., 2008). Moreover, the murine CD8 $\alpha$ <sup>+</sup> DC subset was revealed to favor the differentiation of Th1 cells in a CD70-dependent and IL-12-independent mechanism (Soares et al., 2007). This is further supported by showing that human Langerhans cells (LCs), an epidermal subset of DCs, are capable of inducing CD8<sup>+</sup> anti-viral responses in a CD70-dependent manner (van der Aar et al., 2011). A recent study also demonstrated that CD70/CD27 pathway impedes the differentiation of Th17 effector cells and attenuates accompanying autoimmunity in a mouse model of multiple sclerosis (Coquet et al., 2012). These findings imply that CD70 involvement in T cell polarization may depend on the type of DCs expressing CD70 and the type of stimuli to which these DCs are exposed.

The activatory effect of CD70/CD27 pathway can be exploited for anti-tumor therapy. Induced expression of both CD70 and CD40L by tumor cells was shown to impede tumor growth and initiate anti-tumor immunity (Couderc et al., 1998). Furthermore, the application of CD70, encoded in a vaccinia virus, was shown to confer protection against introduced tumors (Lorenz et al., 1999). Evidence for possible clinical benefit from mobilizing the CD70/CD27 pathway was provided by a recent clinical trial utilizing DCs expressing CD70, CD40L, and constitutively active TLR4 (TriMix-DC) in the treatment of metastatic melanoma patients.

These TriMix-DCs were able to initiate a broad anti-tumor T cell response, resulting in prolonged progression-free survival (Van Nuffel et al., 2012). This paves the way for a novel strategy in cancer immunotherapy based on mobilizing the CD70/CD27 pathway.

Several reports have implicated CD70/CD27 pathway in autoimmunity. Elevated expression of CD70 by pathogenic T cells was observed in rheumatoid arthritis (Lee et al., 2007) and lupus erythematosus patients (Han et al., 2005). Moreover, blocking CD70/CD27 pathway seems to help ameliorating inflammation in mouse models of arthritis (Oflazoglu et al., 2009) and colitis (Manocha et al., 2009). However, the study reporting Th17 inhibiting effects of CD70 signaling (Coquet et al., 2012) may argue against the blockade of CD70/CD27 pathway, especially since Th17 effector cells are involved in various auto-inflammatory diseases.

### OX-40L/OX-40 PATHWAY

OX-40L and OX-40 belong to the TNF family and TNFR family, respectively. OX-40, also known as CD134, was first described on activated CD4<sup>+</sup> T cells (Paterson et al., 1987). The expression of OX-40 is in fact restricted to recently antigen-activated T cells and not naïve or memory T cells, implying that it is specialized in delivering co-stimulation to activated T cells (Sugamura et al., 2004). The ligand, OX-40L (CD252), is expressed on DCs and macrophages, especially after TLR or CD40 ligation (Ohshima et al., 1997). Additionally, responding T cells express OX-40L themselves (Soroosh et al., 2006). Engagement of OX-40 on T cells promotes long-term survival by inducing the expression of the anti-apoptotic molecules Bcl-2 and Bcl-xL (Rogers et al., 2001). This study suggests that the differential expression kinetics of OX-40 and CD28, the latter being constitutively expressed by T cells, bares functional specialization. Whereas CD28 is essential for the initial priming of naïve T cells into effector T cells, OX-40 is crucial for the expansion (later proliferation) and survival of these effector cells.

Several studies have pointed out a central role for OX-40 in regulating the balance between Th1 and Th2 responses. Co-stimulating T cells through OX-40 was shown to induce IL-4 expression and inhibited IFN- $\gamma$  production (Flynn et al., 1998). Furthermore, DC treatment with thymic stromal lymphopoietin (TSLP), known for its Th2 skewing properties, leads to the expression of OX-40L and the subsequent priming of Th2 cells (Ito et al., 2005). OX-40-favored Th2 response was proposed to be mediated by an initial induction of nuclear factor of activated T cells (NFAT) c1 in an IL-4 receptor-independent manner, followed by an IL-4 receptor-dependent effect on GATA-3 (So et al., 2006). However, it was shown later that DC-derived OX-40L maintained both Th2 and Th1 responses, owed to OX-40-enhanced survival of effector T cells regardless of their polarization (Jenkins et al., 2007). Thus, it seems that the role of OX-40/OX-40L in the differentiation of Th2 cells is restricted to promoting the survival of already established Th2 cells that differentiated under the effect of other DC-derived factors.

OX-40/OX-40L is also involved in controlling immune tolerance. The first evidence of this role is the expression of significant amounts of OX-40 on naturally occurring Foxp3<sup>+</sup> Tregs. OX40 signaling appears to be dispensable for the development of nTregs,



since this population exists in OX-40-deficient mice. However, OX-40 signaling is important for the survival of nTregs as OX-40-deficient mice displayed lower counts of this population of Tregs (Takeda et al., 2004). The effect of OX-40 triggering on the functions of nTregs remains controversial. Whereas one study showed that OX-40 signaling in CD4<sup>+</sup> T cells render them resistant to suppression by nTregs (Takeda et al., 2004), another study reported abrogated suppression following OX-40 triggering on nTregs (Valzasina et al., 2005). Another mechanism by which OX-40L/OX-40 is assumed to contribute to tolerance regulation is by influencing the development of induced Tregs (iTregs). Under conditions promoting iTreg differentiation, OX-40 engagement on T cells was shown to inhibit Foxp3 expression by these T cells (So and Croft, 2007). Nevertheless, the surrounding environment during iTreg differentiation seems to determine the outcome of OX-40 signaling, which was reported to promote the expansion of iTregs if IL-4 and IFN- $\gamma$  were absent from the milieu (Ruby et al., 2009). In conclusion, OX-40L/OX-40 appears to be central in maintaining the survival of T cells in general, but its influence on T cell functions requires further elucidation.

#### 4-1BBL/4-1BB PATHWAY

4-1BB (CD137) is yet another member of the TNFR family. Its expression is induced on T cells following TCR activation (Pollok et al., 1993). The ligand, 4-1BBL of the TNF family, is expressed on activated APCs (Vinay and Kwon, 1998). Engagement of T cell 4-1BB was reported to induce IL-2 production independently of CD28, when accompanied by strong TCR signaling (Saoulli et al., 1998). Furthermore, 4-1BB interaction with its ligand was demonstrated to provide a co-stimulatory signal particularly to CD8<sup>+</sup> T cells, enhancing proliferation, cytotoxicity (Shuford et al., 1997), and survival (Lee et al., 2002). Similar to other TNFR family members, 4-1BB enhanced survival is dependent on NF- $\kappa$ B activation, which in turn induces the two pro-survival molecules: Bcl-xL and Bfl-1 (Lee et al., 2002). When compared to co-stimulation with CD80/CD86, 4-1BBL appears to be more effective in driving CD8<sup>+</sup> memory T cells into a fully differentiated effector state (Bukczynski et al., 2004). Furthermore, 4-1BB ligation was also shown to augment Th1 cytokines and suppress Th2 cytokines, implying a possible role for 4-1BB in T cell polarization (Kim et al., 1998). Collectively, these properties raised the interest in 4-1BBL/4-1BB pathway as potential therapeutic target especially in cancer therapy. Several studies demonstrated a beneficial effect of activating 4-1BB in inducing anti-tumor immunity and tumor regression thereafter (Driessens et al., 2009). Nevertheless, great caution should be taken before transferring these observations into clinical applications especially after reporting possible tolerogenic effects following 4-1BB triggering. Engaging 4-1BB by agonist antibodies was reported to ameliorate the severity of autoimmunity in murine models of experimental autoimmune encephalomyelitis (EAE) (Sun et al., 2002) and systemic lupus erythematosus (Foell et al., 2003), and to inhibit rejections of intestinal allografts in mice (Wang et al., 2003). These findings imply a link between 4-1BBL/4-1BB pathway and tolerance. Indeed, 4-1BB co-stimulation was shown to synergize with IL-2 in promoting nTreg expansion (Elpek et al., 2007). In an experimental model of rheumatoid arthritis, treatment with 4-1BB agonist antibodies

inhibited disease progression, which was attributed to the induction of indoleamine 2,3-dioxygenase (IDO; Seo et al., 2004). Altogether, 4-1BBL/4-1BB pathway contributes to immunity and tolerance, allowing multiple therapeutic applications through this pathway.

#### GITRL/GITR PATHWAY

Glucocorticoid-induced TNFR related gene (GITR) was first discovered as a dexamethasone-induced molecule in murine T cell hybridomas (Nocentini et al., 1997). The expression of the human ortholog was subsequently identified in human lymphocytes and shown to be independent of glucocorticoid treatment. Similar to the TNFR family members OX-40 and 4-1BB, GITR is only expressed on recently activated T cells, implying a role in promoting effector functions rather than involvement in initial priming of naïve T cells (Gurney et al., 1999). The GITR ligand (GITRL) is expressed by APCs and is upregulated upon activation (Tone et al., 2003). GITRL/GITR pathway provides co-stimulation to naïve T lymphocytes demonstrated by enhanced proliferation and effector functions in the setting of suboptimal TCR stimulation (Ronchetti et al., 2004). Additionally, GITR triggering promoted naïve T cell survival through the activation of NF- $\kappa$ B and mitogen-activated protein kinase (MAPK) pathways, though it was not sufficient to inhibit activation-induced cell death initiated by TCR signaling (Esparza and Arch, 2005). GITRL/GITR pathway does not seem to have an impact on T cell polarization. Although applying an agonist antibody against GITR initially enhanced Th2 responses in a mouse model of helminth infection, this effect was short lived and GITR-independent (van der Werf et al., 2011).

A role for GITRL/GITR pathway in immune tolerance was initially demonstrated by the constitutive expression of GITR on Tregs (Shimizu et al., 2002). Factually, Tregs isolated based on the expression of GITR could prevent the development of colitis induced in an adoptive transfer model (Uraushihara et al., 2003). However, engaging Treg-expressed GITR, by agonist antibodies, was shown to abrogate their suppressive capacity (Shimizu et al., 2002). In the beginning, this effect was interpreted by mere activation of Tregs upon GITR stimulation, but this explanation was underscored by the fact that Treg preincubation with anti-GITR did not cause the subsequent loss of suppression (Shimizu et al., 2002). Eventually, it was revealed that triggering GITR on effector T cells rendered them resistant to suppression by Treg (Stephens et al., 2004), providing a plausible explanation for the anti-tolerogenic effects of GITR stimulation. This postulates a model where APC-expressed GITRL would bind GITR on recently stimulated T cells allowing them to resist suppression. Simultaneously, GITR ligation on Tregs would allow their expansion and their subsequent domination at later stages of the immune response (Stephens et al., 2004).

Based on the activatory nature of GITRL/GITR pathway and its characteristic inhibition of tolerance, employing this pathway in cancer therapy was evaluated. The administration of an agonistic antibody against GITR has been shown to augment CD8 anti-tumor immunity (Cohen et al., 2006). In addition to mobilizing anti-tumor responses, triggering GITR was also shown to attenuate Treg-mediated suppression within the tumor (Ko et al., 2005), making GITRL/GITR a promising target for cancer therapy.

## LIGHT/HVEM PATHWAY

The TNFR family member herpes virus entry mediator (HVEM) was initially discovered as a receptor for herpes simplex virus (Montgomery et al., 1996). It is expressed on resting T cells, monocytes, and immature DCs. HVEM has multiple binding partners: LIGHT and lymphotoxin- $\alpha$  (LT- $\alpha$ ) from the TNF superfamily; and CD160 and B and T lymphocyte attenuator (BTLA) from the Ig superfamily. HVEM interaction with these ligands creates a complex network of pathways, which collectively regulates adaptive immune responses (Ware and Sedy, 2011). In this section we will only focus on the co-stimulatory pathway resulting from LIGHT/HVEM interactions. LIGHT is expressed by immature DCs (Tamada et al., 2000a) and is induced upon activation on T cells, in contrast to HVEM (Morel et al., 2000). LIGHT/HVEM interaction was revealed to be required for DC-mediated allogenic T cell responses. Indeed, activating T cell HVEM enhanced T cell proliferation at suboptimal TCR stimulation conditions (Tamada et al., 2000a). Disrupted LIGHT/HVEM interaction was shown to result in inhibited T cell proliferation, further supporting the importance of this pathway in co-stimulation (La et al., 2002). Similar to other TNFR family members, HVEM mediates its effects by activating NF- $\kappa$ B pathway (Harrop et al., 1998). Interestingly, LIGHT/HVEM pathway can also contribute to T cell activation indirectly by inducing DC maturation, reminiscent of the role of CD40 in inducing DC maturation (Morel et al., 2001). LIGHT/HVEM pathway is also suggested to contribute to T cell polarization. T cells co-stimulated through HVEM displayed enhanced production of Th1 cytokines (Tamada et al., 2000b). Accordingly, LIGHT-deficient mice showed reduced IFN- $\gamma$  levels, prolonging allograft survival in these mice (Ye et al., 2002). Due to the complexity of the signaling network of HVEM and LIGHT, reported findings should be interpreted as these observations may involve other pathways.

## TIM FAMILY

In addition to the CD28/B7 and TNFR/TNF co-stimulatory families, the recently identified TIM (T cell Ig domain and mucin domain) family is a new contributor to signal II. This family of genes was initially identified while searching for Th1-specific markers (Monney et al., 2002). In humans, three TIM family members: TIM1, TIM3, and TIM4 have been identified thus far. Mice possess an additional member: TIM2 (Kuchroo et al., 2008). In this section we will only focus on TIM3 and TIM4, which were reported to be expressed by DCs.

TIM3 was first discovered as a specific marker for Th1 cells (Monney et al., 2002), and was shown to induce the death of these cells by binding to its ligand galectin-9 (Zhu et al., 2005). TIM3 expression was also detected on DCs, and its ligation by galectin-9 induced the production of the inflammatory cytokine TNF- $\alpha$ . The absence of TIM3 signaling was shown to result in impaired TLR responsiveness, implying a synergistic relation between TIM3 and TLR signaling pathways (Anderson et al., 2007). Although TIM3 triggering on T cells and DCs leads to ERK (extracellular signal-regulated kinases) phosphorylation and I $\kappa$ B $\alpha$  degradation, different tyrosine phosphorylation patterns in T cells and DCs were detected, providing a plausible explanation for the differential

effects of TIM3 between different cell types (Anderson et al., 2007). Thus far, interactions between DC-expressed TIM3 and T cell-expressed galectin-9 have not been investigated. However, previous findings prompt a model where DC-expressed TIM3 promotes inflammation and the differentiation of TIM3-expressing Th1 cells. IFN- $\gamma$ -induced galectin-9 would interact with TIM3 from other T cells, inducing cell death and thereby self-limiting the immune response. Additionally, TIM3 is suggested to contribute to tolerance. A crucial role for TIM3 in clearing apoptotic cells by phagocytosis was recently revealed. Blocking this function resulted in inhibited cross-presentation of self-antigens and the development of auto-antibodies (Nakayama et al., 2009). In a completely different mechanism, TIM3 expressed by tumor-infiltrating DCs was shown to interact with the alarmin HMGB1, disturbing the recruitment of tumor cell-derived nucleic acids into DC endosomes, attenuating immune responses to these tumors (Chiba et al., 2012).

In contrast to the other members of the TIM family, TIM4 is exclusively expressed by APCs and not by T cells (Meyers et al., 2005). Through binding to TIM1 on T cells, TIM4 was shown to provide T cells with a co-stimulatory signal promoting T cell expansion, cytokine production, and survival. These effects were mediated by induced phosphorylation of the signaling molecules LAT (linker of activated T cells), Akt, and ERK1/2 in stimulated T cells (Rodriguez-Manzanet et al., 2008). Notably, the strength of TIM4 signal is decisive in determining the stimulatory effect, as weak TIM4 signaling inhibits T cell proliferation instead of potentiating it (Meyers et al., 2005). Similarly, TIM4 was shown to inhibit the proliferation of naïve T cells, which lack the expression of TIM1 (Mizui et al., 2008). These data imply that TIM4 has at least two binding partners: an activating ligand (TIM1) and an inhibitory one to be identified. Through these ligands, TIM4 exerts bimodal regulation of immune responses. Analogous to TIM3, the role of TIM4 in regulating immunity is also evident through mediating the engulfment of apoptotic cells. *In vivo* blocking of TIM4 resulted in the development of auto-antibodies (Miyayoshi et al., 2007).

## ADHESION MOLECULES PROVIDING CO-STIMULATORY SIGNALS

Leukocyte adhesion and detachment from other cells is tightly regulated by adhesion molecules. A specific set of these molecules is involved in regulating DC/T cell interactions. This set includes the following molecules: intercellular adhesion molecule 1 (ICAM-1) and lymphocyte function-associated antigen-3 (LFA-3), expressed by DCs, and their respective ligands LFA-1 and CD2, expressed by T cells. The seminal discovery of the involvement of LFA-1 in mediating T cell functions prompted a hypothesis that LFA-1 would act by enhancing adhesion and thereby increasing the range of avidities that can promote antigen recognition (Springer et al., 1982). Subsequently, ICAM-1 was identified as the ligand of LFA-1 (Rothlein et al., 1986). LFA-1 ligation by ICAM-1 was shown to induce proliferation of TCR-stimulated T cells in an IL-2-dependent mechanism, proposing that ICAM-1/LFA-1 interaction as a co-stimulatory pathway (Van Severen et al., 1990). In addition to co-stimulation, ICAM-1/LFA-1 interaction stabilizes the immunological junction (Bleijs et al., 2001) and the ICAM-1/LFA-1 pathway appears to contribute to T

cell differentiation as repeated T cell stimulation with ICAM-1 promoted IFN- $\gamma$  production by these cells (Semnani et al., 1994).

Moreover, blocking ICAM-1/LFA-1 interactions during T cell stimulation drastically increased Th2 cytokines (Salomon and Bluestone, 1998). More recently, ICAM-1/LFA-1 interaction during CD8<sup>+</sup> T cell priming was demonstrated to be essential for the establishment of effective T cell memory (Scholer et al., 2008). The effects of the ICAM-1/LFA-1 pathway are believed to result from influencing multiple cellular signaling cascades. LFA-1 was found to interact with the transcriptional co-activator JAB1, implying an influence on c-Jun-driven transcription (Bianchi et al., 2000).

In parallel, T cell CD2 interaction with its ligand LFA-3 was recognized for contributing to T cell activation by strengthening the adhesion between T cells and APCs and thereby enforcing TCR contact with its ligands (Davis and van der Merwe, 1996). Moreover, CD2 signaling was also shown to restore responsiveness in anergized human T cells (Boussiotis et al., 1994). CD2 blocking *in vivo* was revealed to induce T cell unresponsiveness, further supporting the notion that LFA-3/CD2 pathway contributes to immune activation (Xu et al., 2004). Conversely, specific mobilization of LFA-3/CD2 interactions was demonstrated to induce, single handedly, non-proliferating Tregs secreting high amounts of IL-10 (Wakkach et al., 2001). In light of these contradictions, further characterization of the role of LFA-3/CD2 co-stimulatory pathway is required.

## CO-INHIBITORY MOLECULES

### CD80/CD86/CTLA-4 PATHWAY

Cytotoxic T lymphocyte-associated antigen-4 (CD152) is a CD28 homolog that was discovered in 1987 (Brunet et al., 1987). The closely related structures of these two molecules suggest overlapping functional qualities. Indeed, CTLA-4 binds to CD80 and CD86, though at greater affinities. However, CTLA-4 was the first described co-stimulatory molecule with inhibitory effects in a stark contrast to the activatory properties of CD28 (Linsley et al., 1991b). The effects of CTLA-4 include inhibition of proliferation, cell cycle progression, and IL-2 synthesis (Walunas et al., 1996). Additionally, CTLA-4 seems to have an influence on T cell polarization. T cells lacking CTLA-4 expression were shown to adopt a Th2 phenotype (Bour-Jordan et al., 2003). Furthermore, neutralizing CTLA-4 signaling in T cells was recently shown to enhance IL-17 production and promote the differentiation of Th17 cells (Ying et al., 2010).

The prominent role of CTLA-4 in tolerance is clearly demonstrated by CTLA-4-deficient mice, which succumb at 3–4 weeks of age to massive lymphoproliferative disease (Tivol et al., 1995). Furthermore, the suppressive functions of naturally occurring Tregs, which constitutively express CTLA-4, were dependent on CTLA-4 signaling (Read et al., 2000), corroborating its role in tolerance. CTLA-4 contribution to tolerance is postulated to arise from controlling T cell responses in an intrinsic or extrinsic manner (Rudd et al., 2009). First, CTLA-4 antagonizes the CD28 stimulatory signaling by competing with CD28 on binding to CD80/CD86. Interestingly, CTLA-4 expression on cells is induced in a CD28-dependent mechanism (Alegre et al., 1996), implying that CTLA-4 serves as an internal checkpoint that prohibits excessive stimulation by CD28. Extrinsic inhibitory effects of CTLA-4 are suggested

to be exerted through different mechanisms. CTLA-4 molecules expressed by Tregs were shown to engage CD80/CD86, expressed by DCs, promoting the activity of IDO. The modified catabolic properties of DCs lead to localized deprivation of tryptophan and thereby reduced T stimulatory capacity of these DCs (Fallarino et al., 2003). Another suggested mechanism for the extrinsic effects of CTLA-4 was demonstrated by the capacity of CTLA-4 to capture CD86, expressed by APCs, internalize it for ensuing degradation in a process called *trans*-endocytosis (Qureshi et al., 2011). Tregs were also observed to suppress T cells by establishing a direct interaction through CTLA-4, which binds to CD80 and CD86 expressed by those T cells (Taylor et al., 2004). Finally, unstimulated T cells were revealed to produce a soluble form of CTLA-4, which may possibly convey the inhibitory effects to other cells (Magistrelli et al., 1999). Collectively, CTLA-4 is unequivocally vital for tolerance.

Due to its role in maintaining tolerance, blocking CTLA-4 interaction with CD80 and CD86 was postulated to promote anti-tumor immunity. Indeed, *in vivo* administration of blocking antibodies against CTLA-4 resulted into effective anti-tumor immunity and tumor rejection (Leach et al., 1996). Nevertheless, CTLA-4 blockade efficacy in tumor therapy was correlated with the stage and immunogenicity of the tumor. At early stages small tumors were sensitive to the effects of CTLA-4 blockade (Shrikant et al., 1999), whereas advanced tumors were resistant due to the strongly tumor-induced T cell tolerance (Sotomayor et al., 1999). In an attempt to circumvent this hurdle, anti-CTLA-4 blocking antibodies were tested in combination with other therapeutic modalities. Combined anti-CTLA-4 application and Treg depletion resulted in maximal tumor rejection, which was dependent on the expansion of tumor-specific CD8<sup>+</sup> T cells (Sutmuller et al., 2001). Those promising experimental observations lead to the development of two fully human anti-CTLA-4 antibodies: ipilimumab (Bristol-Myers Squibb, New York, NY, USA) and tremelimumab (Pfizer, New York, NY, USA). Early clinical trials in metastatic melanoma and ovarian carcinoma patients demonstrated that blocking CTLA-4 resulted in extensive tumor necrosis with lymphocyte and granulocyte infiltrates in a large number of patients (Hodi et al., 2003). Further large scale clinical trials have shown irrefutable evidence of the efficacy of anti-CTLA-4 antibodies, leading eventually to FDA approval of these antibodies (Kirkwood et al., 2012). Despite its novelty, this therapeutic strategy is challenged by autoimmune complications resulting from the administration of anti-CTLA-4 antibodies (Sanderson et al., 2005).

The tolerogenic effects arising from CTLA-4 engagement with CD80/CD86 can also be utilized for inducing tolerance toward transplanted tissues. This notion has been supported by observations in animal experimental models. Administration of recombinant CTLA-4-Ig fusion protein after renal or cardiac transplantation enhanced allograft acceptance and reduced inflammatory responses (Azuma et al., 1996). This led to the development of humanized CTLA-4-Ig (Belatacept). Kidney transplantation patients receiving Belatacept showed reduced allograft rejection and maintained better renal functions, compared to patients receiving cyclosporine. These findings resulted in gaining FDA approval for using Belatacept for the prevention of acute rejection post-renal transplant (Vincenti et al., 2011).



### PD-L1/PD-L2/PD-1 PATHWAY

Programed cell death-1 (PD-1) is another member of the CD28 family that is expressed by activated T and B cells (Agata et al., 1996). Two ligands were identified to interact with PD-1: PD-L1 (Dong et al., 1999) and PD-L2 (Latchman et al., 2001). Those ligands are characterized by differential expression patterns. PD-L1 is constitutively expressed and further enhanced on activated lymphocytes, including Tregs and DCs. It is also expressed by a wide variety of non-hematopoietic cell types including the vascular endothelial cells, neurons and pancreatic islet cells. In contrast, PD-L2 expression is restricted to DCs and macrophages under certain conditions (Greenwald et al., 2005). Interestingly, PD-L2 displays three times higher binding affinity to PD-1 in comparison to PD-L1, which on the other hand was also identified to bind to CD80 (Butte et al., 2007). The varying binding and expression properties of PD-L1 and PD-L2 suggest distinct functions in regulating T cell responses. Along with its ligands PD-1, is recognized for its vital role in regulating adaptive immune responses (Sharpe et al., 2007). Indeed, triggering of PD-1 by one of its ligands during TCR signaling can block T cell proliferation, cytokine production and cytolytic activity, and impair T cell survival (Riley, 2009). The intracellular domain of PD-1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) as well as an immunoreceptor tyrosine-based switch motif (ITSM), which are phosphorylated upon ligand engagement. Subsequently protein phosphatases, such as Src homology phosphatase-1 (SHP-1) and SHP-2, are recruited to TSM where they are activated and inhibit proximal TCR signaling events by dephosphorylating key intermediates in the TCR signaling cascade (Chemnitz et al., 2004). Similar to CTLA-4, triggering PD-1 limits glucose metabolism and Akt activation, albeit through different mechanisms (Chemnitz et al., 2004). Consistently, a recent study also demonstrated that PD-1 exerted its inhibitory effects by affecting Akt and Ras pathways and thereby inhibiting cell cycle progression and T cell proliferation (Patsoukis et al., 2012).

The first indication of the importance of PD-1 in immune tolerance came from PD-1-deficient mice, which developed strain-specific autoimmunity. The absence of PD-1 caused the development of cardiomyopathy secondary to the production of auto-antibodies against cardiac troponin in BALB/c mice (Nishimura et al., 1999), while C57BL/6 developed a lupus-like autoimmune disease (Nishimura et al., 2001). In humans, polymorphisms in the PD-1 gene were also associated with susceptibility to several autoimmune diseases including systemic lupus erythematosus (Prokunina et al., 2002), type I diabetes (Nielsen et al., 2003), and multiple sclerosis (Kroner et al., 2005). These observations were supported by functional studies demonstrating the contribution of the PD-L1/PD-L2/PD-1 pathway to central tolerance. In the thymus, interactions between PD-1, expressed by CD4<sup>-</sup>CD8<sup>-</sup> thymocytes, and PD-L1 broadly expressed in the thymic cortex, were deemed crucial in regulating positive selection (Nishimura et al., 2000). PD-1 was also shown to participate in thymic negative selection (Blank et al., 2003). Gene expression profiling studies of central tolerance in non-obese diabetic (NOD) mice also implicated PD-1 and PD-L1 in central tolerance (Zucchelli et al., 2005). PD-L1/PD-L2/PD-1 pathway also contributes to peripheral tolerance through multiple mechanisms.

Self-reactive CD8<sup>+</sup> T cells lacking PD-1 display increased responsiveness to self-antigens presented by resting DCs, suggesting that DC-expressed PD-L1 and PD-L2 may control T cell activation (Probst et al., 2005). PD-L1/PD-L2/PD-1 pathway can also regulate reactivation, expansion, and functions of effector T cells (Keir et al., 2006). Additionally, PD-1 triggering of TCR-stimulated, transforming growth factor-beta (TGF- $\beta$ )-treated T cells profoundly enhanced the *de novo* generation of Foxp3<sup>+</sup> Tregs from CD4<sup>+</sup> naive precursors. Further engagement of PD-L1 on the iTregs sustained Foxp3 expression and enhanced the suppressive capacity of these cells (Francisco et al., 2009). Consistently, PD-L1 was shown to mediate the effects of the immune suppressant vitamin D (VitD). DCs treated with VitD were shown to induce IL-10 producing Tregs in a PD-L1-dependent mechanism (Unger et al., 2009). Interactions between PD-1 and PD-L1 are also proposed to maintain tolerance by modifying DC-T cell contact. PD-1 ligation was shown to inhibit the TCR-induced stop signals, disrupting the stable DC-T cell contact and subsequently allowing tolerized T cells to move freely and prohibiting clustering around antigen-bearing DCs (Fife et al., 2009). Another plausible mechanism for PD-L1/PD-L2/PD-1 pathway-induced tolerance is that PD-L1 expressed by Tregs would engage PD-1 expressed by DCs and modulate DC function and thereby impeding immune responses (Francisco et al., 2010).

The inhibitory effects of PD-L1/PD-L2/PD-1 pathway can be hijacked by tumors to evade anti-tumor immune responses. PD-L1 expression has been confirmed on many tumors including glioblastoma and melanoma as well as cancers of the head and neck, lung, ovary, colon, stomach, kidney, and breast. High expression PD-L1 levels by tumor cells, tumor-infiltrating lymphocytes, or both associated with aggressive tumor behavior, poor prognosis, and elevated risk of mortality (Zang and Allison, 2007). Moreover, DCs generated from peripheral blood of ovarian cancer patients displayed high levels of PD-L1, prompting impaired T cells responses, which were restored by blocking PD-L1/PD-1 interactions (Curiel et al., 2003). *In vivo*, forced PD-L1 expression by squamous cancer cells rendered them resistant to T cell-mediated immunity. This resistance, however, was broken upon treatment with anti-PD-L1 blocking antibodies (Strome et al., 2003). A recent study also revealed that platinum based chemotherapeutics enhanced anti-tumor T cell responses by disrupting PD-L2/PD-1 interactions through reducing PD-L2 levels on both DCs and tumor cells (Lesterhuis et al., 2011). These experimental observations prompted the development of humanized anti-PD-1 and anti-PD-L1 antibodies for clinical application. Early stage clinical trials with these antibodies demonstrated clinical activity, which was characterized by durability accompanied with minimal side effects (Zitvogel and Kroemer, 2012).

There is also evidence that viral infections can make use of PD-L1/PD-L2/PD-1 pathway. Animal models of chronic viral infections had elevated PD-1 expression on exhausted viral antigen-specific T cells. The activity of these T cells was restored following PD-L1 blocking, suggesting a novel strategy for combating chronic viral infections (Barber et al., 2006).

In line with its inhibitory role, PD-L1/PD-L2/PD-1 pathway can be harnessed for the induction of tolerance when needed. Administration of recombinant PD-L1-Ig, with agonistic effect for



PD-1, prolonged the survival of cardiac allografts in mice (Ozkaynak et al., 2002). Furthermore, PD-L1 expression on murine liver allografts is central for spontaneous tolerance (Morita et al., 2010).

### B7-H3 PATHWAY

B7-H3 belongs to the B7 family of co-stimulatory molecules. Similar to other Ig superfamily members, B7-H3 is a transmembrane molecule. It possesses a short cytoplasmic tail with no known signaling domain. B7-H3 is expressed on a wide variety of tissues and tumor cell lines. However, its expression on leukocytes is only detectable following stimulation. B7-H3 expression can be induced on DCs and monocytes by inflammatory cytokines, whereas a combination of phorbol myristate acetate and ionomycin can induce it on T cells. B7-H3 was shown to bind a receptor expressed by activated T cells. This receptor is distinct from CD28, CTLA-4, ICOS, and PD-1 and yet to be identified (Chapoval et al., 2001). Triggering receptor expressed on myeloid cells (TREM)-like transcript 2 (TLT-2), constitutively expressed by CD8<sup>+</sup> T cells and activation-induced on CD4<sup>+</sup> T cells, was proposed to be the binding partner of B7-H3 (Hashiguchi et al., 2008). However, this was strongly refuted by another study providing evidence of non-existing interaction between B7-H3 and TLT-2 (Leitner et al., 2009). Initially, B7-H3 was suggested to be a positive co-stimulatory molecule that induces T cell proliferation, IFN- $\gamma$  production and CTL generation in humans (Chapoval et al., 2001). Nevertheless, this was contradicted by another study demonstrating that B7-H3 is a potentially inhibited T cell stimulation under different conditions and regardless of the stimulation status of the T cells in question (Leitner et al., 2009). This is corroborated by data from murine studies where applying an agonistic fusion protein, B7-H3-Ig, was shown to inhibit proliferation, IL-2 and IFN- $\gamma$  production of TCR-stimulated T cells. This inhibitory effect was demonstrated by exacerbated airway inflammation in B7-H3-deficient mice compared to wild type counterparts (Suh et al., 2003). Moreover, blocking B7-H3 caused enhanced T cell proliferation *in vitro* and worsened EAE *in vivo*. This effect may be explained by the inhibitory influence of B7-H3 signaling over NF- $\kappa$ B, NFAT, and AP-1 that are involved in regulating T cell activation (Prasad et al., 2004). Notably, the effects of B7-H3 were overridden by CD28 co-stimulation, implying that B7-H3 functions optimally in the absence of co-stimulation (Suh et al., 2003). Of interest, tumors are suggested to hijack the B7-H3 to evade anti-tumor immune responses. This is demonstrated by increased disease severity when cancer cells upregulated B7-H3 expression (Hofmeyer et al., 2008). Collectively, further characterization of the B7-H3 pathway is required to resolve functional discrepancies, which may be explained by the existence of two receptors for B7-H3 with opposite functions, yet to be identified.

### B7-H4 PATHWAY

B7-H4 is the last among the B7 family members that was identified. Unlike other B7 family members, which are type I membrane molecules, B7-H4 is characterized by a glycosylphosphatidylinositol (GPI) domain that links to the cell membrane (Prasad et al., 2003). In humans, B7-H4 mRNA was detected in a variety of tissues. However, immunohistochemical analysis did not reveal any

B7-H4 protein expression by these tissues. Likewise, no B7-H4 expression could be detected on freshly isolated T cells, B cells, monocytes, and DCs, but it was induced after activating these cells *in vitro*. The ligand of B7-H4 has not been identified yet, but it is suggested to be expressed by stimulated T cells and to be distinct from other CD28 family members (Sica et al., 2003). B7-H4 is widely regarded as a co-inhibitory molecule. Indeed, treatment of TCR-stimulated T cells by a fusion B7-H4-Ig protein resulted in inhibited T cell proliferation and cytokine production, an effect that required B7-H4 cross-linking (Sica et al., 2003). The inhibitory effects of B7-H4 are proposed to arise from arrested cell cycle progression in T cells (Sica et al., 2003), and impaired induction of JunB, known for its role in inducing IL-2 production in activated T cells (Prasad et al., 2003). A recent study also showed that B7-H4 signaling inhibits phosphorylation of MAP kinases, ERK, p38, Jun N-terminal kinase (JNK), and Akt, usually elicited upon TCR triggering of T cells (Wang et al., 2012a).

In line with *in vitro* findings, mice suffering from graft versus host disease demonstrated prolonged survival upon the *in vivo* application of B7-H4-Ig (Sica et al., 2003). Expectedly, *in vivo* administration of an antagonizing antibody against B7-H4 blocked the inhibitory effect of B7-H4 pathway and led to accelerated disease development in a mouse model of EAE (Prasad et al., 2003). Furthermore, B7-H4-deficient mice showed better control of *Leishmania major* infection as Th1 responses were augmented in these mice (Suh et al., 2006). B7-H4 deficiency also enhanced neutrophils-mediated immunity, implying that B7-H4 may have a role in regulating innate immunity too (Zhu et al., 2009). In addition to its role as a co-inhibitory molecule, B7-H4 seems to mediate the effect of Tregs. It was shown that Tregs, but not conventional T cells, induce high levels of IL-10 production by APCs and consequently trigger B7-H4 expression that renders these APCs immunosuppressive (Kryczek et al., 2006a). The overall tolerogenic effect of B7-H4 can be exploited by tumors to evade immune responses. B7-H4 expression was reported for several tumors including lung cancer, ovarian cancer (Choi et al., 2003), gastric cancer (Jiang et al., 2010), and tumor-associated macrophages (Kryczek et al., 2006b). Blockade of B7-H4 on these macrophages was actually effective in reversing their suppressive effect and restored anti-tumor T cell immunity (Kryczek et al., 2006b). Additionally, manipulating B7-H4 pathway has potential in the field of transplantation. A recent study showed that B7-H4 expression was shown to prolong islet allograft survival in mice (Wang et al., 2012b). Thus, the B7-H4 pathway serves as an interesting therapeutic target in different diseases, though several aspects of this pathway remain elusive.

### HVEM/BTLA/CD160 PATHWAY

As mentioned earlier, the molecules HVEM, BTLA, CD160, and LIGHT interact directly with each other forming a complex pathway network regulating adaptive immune responses. HVEM, expressed by immature DCs, can provide negative co-stimulatory signals through binding to its ligands BTLA and CD160 on T cells (Ware and Sedy, 2011). BTLA belongs to the Ig superfamily and is a structural homolog of CTLA-4 and PD-1. It is also a transmembrane glycoprotein that can be phosphorylated on tyrosines located in conserved cytoplasmic ITIM motif (Watanabe et al.,

2003). T cell expression of BTLA was shown to be very low on naïve cells. However, it is upregulated upon antigen-stimulation peaking at day 2 and declining around day 7 post-stimulation. This expression can be retrieved upon secondary stimulation of activated T cells. Interestingly, anergic T cells and Th1 cells demonstrated high BTLA expression unlike Th2 cells and Tregs that have low BTLA expression (Hurchla et al., 2005). The unique BTLA expression pattern and expression kinetics indicate that BTLA may interfere at certain stages of T cell activation with specificity to certain types of effector T cells.

Herpes virus entry mediator delivers its inhibitory signal to T cells by binding to BTLA, which induces the phosphorylation of its ITIM domain and the recruitment of SHP-2, leading to attenuated antigen-driven T cell activation (Sedy et al., 2005). In addition to inhibiting T cell responses, there is evidence that HVEM/BTLA pathway promotes T cell survival in a mechanism dependent on NF- $\kappa$ B activation (Cheung et al., 2009). Interestingly, BTLA was also shown to mediate Treg suppression by interacting with HVEM expressed by Tregs. This was supported by showing that Tregs from HVEM-deficient mice had lower suppressor activity and that wild type Tregs failed to suppress effector T cells from BTLA-deficient mice (Tao et al., 2008). The inhibitory effects of BTLA are also observed *in vivo*. In an EAE model, BTLA-deficient mice displayed increased severity and persistence of disease when compared with wild type controls (Watanabe et al., 2003). BTLA deficiency was also reported to exacerbate allergic airway inflammation (Dep-pong et al., 2006) and to cause the development of auto-antibodies leading to a hepatitis-like syndrome with advancing age (Oya et al., 2008). Moreover, a single-nucleotide polymorphism (SNP) in the ITIM region of BTLA was reported to associate with increased susceptibility to rheumatoid arthritis (Lin et al., 2006). Another study also revealed an association between another BTLA SNP and rheumatoid arthritis, but not with systemic lupus erythematosus or Sjogren's syndrome (Oki et al., 2011). Similar to B7-H3 and B7-H4, the inhibitory effects of BTLA can be exploited by tumors to evade immunity. Melanoma-specific CD8<sup>+</sup> T cells were shown to persistently express BTLA. Interrupted BTLA signaling, achieved by applying CpG oligonucleotide vaccine formulations, lead to functional recovery of melanoma-specific CD8<sup>+</sup> T cells (Derre et al., 2010).

Herpes virus entry mediator can also interact with CD160, a GPI anchored membrane molecule that is mainly expressed by CD8<sup>+</sup> T cells and activated CD4<sup>+</sup> T cells. Cross-linking CD160 with a specific antibody on stimulated T cells was shown to strongly inhibit T cell proliferation and cytokine production. Similarly, the inhibitory effect of CD160 was also elicited by binding to its ligand HVEM (Cai et al., 2008). Although both BTLA and CD160 bind to the cysteine-rich domain-1 (CRD-1) of HVEM with comparable affinity, CD160 dissociates from HVEM at a slower rate compared to BTLA. Moreover, mutagenesis study of HVEM revealed that CD160 has a distinct binding site on HVEM, albeit overlapping with BTLA (Kojima et al., 2011). Those differences between CD160 and BTLA, though subtle, suggest that these molecules do not have redundant functions. Further delineation of the elusive HVEM/CD160 pathway and its functional implications are required to unravel its specific role in regulating immune responses.

### ILT3 AND ILT4/HLA-G PATHWAYS

The inhibitory receptor Ig-like transcript-3 (ILT3; Cella et al., 1997) and ILT4 (Colonna et al., 1998), both expressed by monocytes, macrophages, and DCs, belong to a family of Ig-like inhibitory receptors that are closely related to the killer cell inhibitory receptors. Both ILT3 and ILT4 were shown to transmit signal through a long cytoplasmic tail containing ITIM motifs, which inhibit cell activation by recruiting the protein phosphatase SHP-1 (Cella et al., 1997; Colonna et al., 1998). In the case of ILT3, the extracellular region consists of two Ig-like domains, which are speculated to contain the putative binding site of the yet to be identified ILT3 ligand (Cella et al., 1997). On the other hand, the binding partner of ILT4 was shown to be the MHC class I molecule human leukocyte antigen G (HLA-G; Colonna et al., 1998). In addition to triggering an inhibitory signal, ILT3 cross-linking was shown to lead to its internalization and delivery into an antigen presenting compartment, suggesting a role in antigen processing (Cella et al., 1997). DC expression of ILT3 and ILT4 was shown to be induced under the effect of CD8<sup>+</sup>CD28<sup>-</sup> alloantigen-specific T suppressor cells (Chang et al., 2002). Immature monocyte-derived DCs (MoDCs) also upregulated ILT3 and ILT4 expression upon treatment with either IL-10 or/and IFN- $\alpha$  (Manavalan et al., 2003). VitD treatment only induced ILT3 expression in MoDCs (Manavalan et al., 2003) and primary human blood BDCA1<sup>+</sup> DCs (Chu et al., 2012). Expectedly, ILT3 expression, by both MoDCs and pDCs, was downregulated following activation (Ju et al., 2004).

Tolerogenic DCs over-expressing ILT3 or ILT4 demonstrated impaired NF- $\kappa$ B activation and consequently reduced transcription capacity of NF- $\kappa$ B-dependent co-stimulatory molecules (Chang et al., 2002). Those DCs were shown to be capable of transforming alloreactive effector T cells into antigen-specific Tregs (Manavalan et al., 2003). Similarly, triggering ILT4 by HLA-G tetramers was shown to impair maturation and T cell stimulatory capacity of human DCs (Liang and Horuzsko, 2003). Interestingly, ILT3 was shown to maintain its T cell inhibitory effect when it was expressed as soluble ILT3-Fc that lacks ILT3's cytoplasmic tail, indicating that ILT3 delivers its inhibitory signal by binding to its partner on activated T cells (Kim-Schulze et al., 2006). Recently it was shown that ILT3 capacity to convert T cells into suppressive cells is dependent on BCL6 signaling in these T cells (Chang et al., 2010). ILT3 is also proposed to be important for controlling inflammation, as silencing ILT3 expression in DCs enhances TLR responsiveness, which is reflected by enhanced secretion of inflammatory cytokines such as IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, and IFN- $\alpha$ . ILT3-silenced DCs could also attract more lymphocytes by secreting high levels of the chemokines CXCL10 and CXCL11 in response to TLR ligation. Eventually, impaired ILT3 expression in DCs rendered them more stimulatory for T cells, which also secreted higher levels of cytokines like IFN- $\gamma$  and IL-17 (Chang et al., 2009). Another suggested mechanism by which both ILT3 and ILT4 contribute to tolerance is by possibly mediating the effects of IDO. DCs cultured in tryptophan-deprived local environment upregulated the expression of ILT3 and ILT4, favoring the development of Foxp3<sup>+</sup> Tregs (Brenk et al., 2009). Finally, ILT4 was shown to be central for the development of type I Tregs, induced by IL-10-treated DCs (Gregori et al., 2010).

The effects of ILT3 and ILT4/HLA-G pathways are also evidenced *in vivo*. Immune modulation exerted by ILT4/HLA-G interactions is believed to mediate maternal tolerance toward the semi allogenic fetus (Hunt et al., 2005). Moreover, *in vivo* treatment with VitD was shown to upregulate the expression of ILT3 on DCs in healing psoriatic lesions. Nevertheless, ILT3 was revealed to be dispensable for the induction of Tregs and completely overridden by the inhibitory effects of VitD (Penna et al., 2005). Consistently, maternal VitD intake during pregnancy was found to enhance ILT3 and ILT4 gene expression levels in cord blood, pointing out a plausible mechanism for early induction of immune tolerance (Rochat et al., 2010). Enhanced ILT3 and ILT4 levels were also observed at an early stage of venom-specific immunotherapy, implying a possible role in inducing tolerance toward allergic reactions (Bussmann et al., 2010). Owing to its inhibitory effects, ILT3 is suggested to be employed by tumors as a mean of evading anti-tumor immunity. Indeed, soluble ILT3 protein was found at high levels in the serum of patients with melanoma, and carcinomas of the colon, rectum, and pancreas produce. This soluble ILT3 was active in inducing suppressor CD8<sup>+</sup> T cells that block anti-tumor immunity, which was restored upon blocking or depleting ILT3 (Suciu-Foca et al., 2007). A similar mechanism is also utilized by viruses, as demonstrated by a point mutation in one of HIV Gag epitopes that increased binding to ILT4 and consequently programmed myelomonocytic cells to become tolerogenic (Lichterfeld et al., 2007). The inhibitory effects of ILT3 can also be harnessed for allograft acceptance. Indeed, soluble recombinant ILT3-Fc was shown to suppress T cell-mediated rejection of allogenic islet transplants in mice (Vlad et al., 2008). In correlation to its inhibitory effect, blood monocytes during multiple sclerosis relapses demonstrated lower ILT3 expression, which was restored upon treatment with IFN- $\beta$ , unraveling a plausible therapeutic target in the treatment of multiple sclerosis (Jensen et al., 2010). Similarly, a SNP in the ILT3 extracellular region was correlated with low surface expression and increased serum cytokine levels in lupus patients (Jensen et al., 2012).

### CONCLUDING REMARKS AND FUTURE PROSPECTS

Since the identification of the CD80/CD86/CD28 classical co-stimulatory pathway, the concept of DC-derived signal II was dramatically expanded to accommodate the ever increasing number of newly discovered co-stimulatory and co-inhibitory pathways. An increasing body of reports reflects the complexity of these pathways and implies possible interactions to form a sophisticated network controlling adaptive immune responses. The existence of multiple co-stimulatory and co-inhibitory pathways postulates for overlapping functions. Nevertheless, this notion of redundancy should be considered carefully. The components of these pathways have distinct expression patterns and kinetics, which means that these pathways are not simultaneously operative. In addition, mobilizing these pathways can trigger distinct signaling cascades and thereby leading to variable outcomes.

Dendritic cell expression of co-stimulatory and co-inhibitory molecules is dictated by several factors. The specific type of DC is a major determinant of this expression. In humans, DCs are classified into groups based on origin, specific expression of certain surface markers, and functional properties. For example, human

blood DCs are divided into two major subsets: pDCs and myeloid DCs (myDCs). The latter can be further divided into three subsets: BDCA1<sup>+</sup> DCs, BDCA3<sup>+</sup> DCs, and CD16<sup>+</sup> DCs. In parallel, skin DCs are also classified into epidermal LCs, dermal CD1a<sup>+</sup> DCs, and dermal CD14<sup>+</sup> DCs. Similar classification can be expected in other tissue-resident DCs. Most of the findings concerning co-stimulatory and co-inhibitory molecules in humans were based on experiments performed on the *in vitro* generated MoDCs, which serve as a great tool for delineating immunological functions and mechanisms. However, there are strong indications of differential expression of co-stimulatory and co-inhibitory molecules among different DC subsets. These variations can be partially related to the intrinsic qualities of every DC subset. For instance, pDCs and LCs lack the expression of TLR4, and consequently they are not able to upregulate CD80 and CD86, observed in other subsets in response to lipopolysaccharide (LPS).

Another central determinant of co-stimulatory and co-inhibitory molecules expression by DCs is the type of stimulus, to which DCs are exposed. As mentioned earlier, DCs respond to pathogen stimulation by upregulating CD80 and CD86. However, there are indications that certain co-stimulatory molecules are strictly expressed upon activation with a specific class of pathogens. A clear example is CD70 expression by LCs upon TLR3 triggering by double-stranded RNA derived from viruses, granting LCs advantage in eliciting strong anti-viral CD8<sup>+</sup> T cell responses. Although dermal DCs and MoDCs express TLR3, they do not upregulate CD70 in response to double-stranded RNA, implying a combined effect of the type of stimulus and the type of DC in inducing CD70 expression. Similarly, pDC stimulation with CpG B, a TLR9 ligand, induced the expression of CD70, which was not observed using another type of stimulation or in other DC subsets (Shaw et al., 2010). Another example demonstrating the effect of pathogenic stimulation is the upregulation of OX40L only upon exposure to the soluble egg antigen from the parasite *Schistosoma mansoni*. Furthermore, DC treatment with certain immune modulating agents can influence the expression of co-stimulatory and co-inhibitory molecules. VitD-treated DCs displayed induced expression of PD-L1 and ILT3, concurrently with inhibited expression of CD80 and CD86. On the other hand, DCs under the influence of IL-10 had normal expression levels of CD80 and CD86 but over-expressed ILT3 and ILT4. It is also evident that DCs are strongly influenced by cues derived from the local environment. The well-documented effect of VitD, the major component of local skin milieu, is a clear example. The influence of other known tissue-related environmental factors on co-stimulation requires further elucidation. Thus, optimal understanding of the role of DC-derived signal II requires determining the total repertoire of co-stimulatory and co-inhibitory molecules expressed by different DC subsets and under different conditions.

In addition to the differential DC expression of co-stimulatory and co-inhibitory molecules, the respective ligands of these molecules are also described to be expressed by T cells following different kinetics. Some of these ligands are constitutively expressed, like CD28, whereas others are restricted to recently TCR-activated T cells such as 4-1BB and GITR. Furthermore, some of these ligands were shown to be exclusively expressed by



certain types of effector T cells, like the Th1-specific expression of TIM3. Taken together, the different expression modalities of the co-stimulatory and co-inhibitory pathway constituents imply that these pathways are mobilized at certain stages of T cell priming and under specific conditions.

Despite the stimulatory or inhibitory nature of signal II, there are some indications pointing out a role in T cell polarization, typically undertaken by cytokine-based signal III. For instance, OX-40/OX-40 and 4-1BBL/4-1BB pathways are proposed to promote the differentiation of Th2 and Th1 effector cells, respectively. Nevertheless, the observed polarizing effect was in many occasions revealed to be the mere outcome of promoted T cell survival rather than active polarization signaling mediated by these co-stimulatory or co-inhibitory molecules. Therefore, reported contributions of signal II to T cell differentiation should be interpreted carefully and further investigated.

The vast immunological consequences of signal II have transformed its pathways, both stimulatory and inhibitory, into therapeutic targets for the treatment of a wide variety of diseases. Mobilizing co-stimulatory pathways and blocking co-inhibitory interactions showed promising results in promoting anti-tumor immunity and it is proposed to be beneficial for the treatment of chronic viral responses. Assuming that mature DCs provide optimal positive co-stimulatory signals while priming anti-tumor T cells, blocking co-inhibitory pathways may augment the efficacy of these T cells. In that respect, concurrent targeting of multiple co-inhibitory pathways might be necessary. Neutralizing the

key inhibitory check point CTLA-4 permits extensive primary T cell activation, but by itself is not sufficient for driving an anti-tumor immune response, especially in the case of advanced tumors. However, the additional circumvention of yet another co-inhibitory check point, which is dictated by the tumor itself, may solve this problem. Selecting the second inhibitory target would highly depend on the type of the treated tumor, as different types of tumors were revealed to preferentially express certain co-inhibitory receptors (PD-L1, PD-L2, B7-H3, etc.). The synergistic effects of such a combinatorial blocking strategy may not only mount efficient anti-tumor T cell responses, but also allow the persistence of such responses within the local tumor environment.

On the other hand, promoting tolerance by blocking activation and mobilizing co-inhibitory pathways is a promising strategy for raising allograft tolerance. Similarly, immune suppressant agents were also revealed to manipulate these pathways in a comparable manner to induce tolerance. Nevertheless, these therapeutic modalities should be applied with great care to avoid any possible adverse effects like inducing susceptibility to infection or autoimmune reactions. Targeting these therapies to a specific pathway or a specific cellular compartment, like a certain DC subset, may be an option to bypass any possible complications.

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