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Yin-Yang of costimulation: crucial controls of immune tolerance and function

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Summary

In addition to signals from the T cell receptor complex, it has been recognized for many years that a "second" signal, most notably from CD28, is also important in T cell activation. In the recent years, many new members of CD28 family as well as the molecules that are share structural homology to CD28 ligands CD80 and CD86 have been discovered. Interestingly, some of these proteins function to dampen T cell activation and regulate the induction of T cell tolerance. Therefore, positive and negative costimulation are the two sides of the coin to fine tune T cell receptor signaling to determine the outcome of T cell receptor engagement- tolerance vs. function.

Keywords

costimulation; T cells; immunity; tolerance

Introduction

Stimulation of T cells by peptides presented on MHC molecules is accompanied by an array of cell-surface costimulatory molecules that are present on antigen-presenting cells (APC), which engage their corresponding receptors on T cells. For many years, costimulation has underscored the "two-signal" theory. This theory states that: 1) to obtain optimal T-cell activation, costimulation would complement the signal provided by MHC-peptide to ensure productive T-cell activation leading to the effector function; and conversely, 2) the lack of costimulation would result in T-cell tolerance or "anergy" (1). CD28 on naïve T cells is by far the most important costimulatory receptor (2). Naïve T cells costimulated with anti-CD28 have been shown to greatly enhance proliferation and interleukin (IL)-2 production. Consistently, mice deficient in CD28 or both of its ligands (B7.1 and B7.2, hereafter referred to as B7-deficient mice) have been shown to be severely impaired in $CD4^+$ T-cell proliferation (3,4). In addition to CD28, B7.1 and B7.2 also bind to an inhibitory receptor, CTLA4, which crucially controls the immune tolerance (5).

The function and complexity of costimulation has been greatly advanced in the past ten years accompanying genomic and cDNA sequencing projects, which results in a number of novel B7-like molecules. It has become clear that in addition to positive costimulation, numerous inhibitory pathways exist to dampen T-cell function. In this review, we will summarize our work as well as reports in the current literature about the biology of these pathways.

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1. Inducible costimulator (ICOS)

1.1. ICOS and B7h: expression and function

Inducible costimulator (ICOS) is the third member of the CD28 family. It is not expressed by naïve T cells, but induced after T cell activation, which is highly dependent upon CD28 signal (6,7). The ligand for ICOS, B7h (also named B7RP-1, etc) has been described to be constitutively expressed on B cells, macrophages, and can be induced in non-lymphoid tissues and cells by inflammatory stimuli (8,9). Recently, by using mice deficient in B7h and ICOS, we found that, unlike the B7.1/B7.2-CD28/CTLA4 and PDL1/PDL2-PD-1 systems, B7h and ICOS are the only ligand and receptor for the other, respectively (10).

Initially, ICOS costimulation was shown to increase the proliferation of $CD4^+$ T cells, but not IL-2 production (6). In addition, ICOS enhanced the production of various cytokines such as IFN-γ, TNFα (Th1), IL-4, IL-5, IL-10 (Th2) (6,7,11). Lately, analysis of mice deficient in ICOS or B7h has revealed the critical role of ICOS in immune and autoimmune responses (10, 12-15). We and others demonstrated a defect in proliferation and IL-2 production by ICOS-deficient T cells, indicating an important role of ICOS in T cell activation (12,14). Furthermore, ICOS deficient T cells were selectively impaired *in vitro* and in *vivo* in the production of Th2 cytokine IL-4 (12). On the other hand, they were capable of secreting IL-5 (12). Using ICOS- and B7h-deficient mice, we examined the transcriptional mechanisms by which ICOS selectively regulates IL-4 expression by effector Th2 cells (10,16). Our analysis indicates that defective IL-4 production in the absence of ICOS-B7h interaction was functionally associated with a selective deficiency in expression of c-Maf transcription factor that regulate IL-4 gene expression (17,18). c-Maf knockout mice appeared to share a similar phenotype to ICOS-deficient mice (17). Moreover, c-Maf over-expression restored the IL-4 defect in T cells activated in the absence of ICOS-B7h interaction (10,16). Further analysis revealed that ICOS regulates c-Maf expression through activation of IL-4 production. In addition, ICOS costimulation potentates the TcR and CD28 mediated initial IL-4 production, possibly through the enhancement of NFATc1 expression (10,16). These data indicate that ICOS, via enhancing the NFATc expression at an early stage of T cell activation, regulates c-Maf expression and hence IL-4 expression at the effector stage.

Recently, we have examined the signaling mechanisms whereby costimulation regulates NFATc1 expression (19). We found that ICOS synergized with TcR and CD28 signals, resulting in sustained PI3K activity in primary T cells, which is required for NFATc1 up-regulation. Our results indicated that a PI3K-Itk-phospholipase C (PLC)γ1-Ca^{2+} pathway initiated by CD28 and ICOS leads to the induction of *Nfatc1* P1 promoter activity (19).

1.2. ICOS regulation of humoral immunity

ICOS plays an important role in regulation of T-dependant antibody responses and germinal-center reactions. Mice deficient in ICOS or ICOS ligand (B7h) have impared germinal center formation and isotype switching (10,12,14,15,20). Recently, this costimulatory pathway was also found to be important in the generation of $CXCR5^+$ follicular helper T cells (Tfh), which have recently emerged as a unique T cell subset regulating germinal center reactions and humoral immunity (21). ICOS was previously shown to be expressed at high levels on human tonsillular $CXCR5^+$ T cells within the light zone of germinal centers and efficiently supported the immunoglobulin production (22,23). In addition, ICOS deficiency in human and mouse resulted in substantially reduced numbers of Tfh cells, indicating an essential role of ICOS in the differentiation of Tfh cells (24,25). In addition, it has been shown that Tfh cells do not express Th2 cytokines (IL-4, -5, 10) (24), instead they produce IL-21, a cytokine important for B cell maturation (26,27). Consistent with previous reports, we found that Tfh cells have a very distinct gene expression profile (28). Tfh cells did not express the typical markers for Th1

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(IFN-γ and T-bet) or Th2 (IL-4 and GATA3) cells. Although Tfh cells shared IL-21 expression with Th17 cells, they did not express Th17-specific genes such as IL-17, IL-17F, IL-22, or RORγt. Instead, mouse Tfh cells expressed mRNAs for CXCR5 as well as Bcl-6. In addition, Tfh cells preferentially expressed mRNAs for IL-6R and IL-6st (gp130) and also upregulated the expression of IL-21R, suggesting possible regulation of Tfh cells by IL-6 and IL-21 (28). Interestingly, mutation in a RING-type E3 ubiquitin ligase, Roquin, in the mice provoked the development of spontaneous autoantibody production and lupus-like autoimmunity, associated with greatly increased numbers of Tfh cells and enhanced expression of IL-21 and ICOS (29, 30).

The above literature indicates a crucial role of ICOS in regulation of Tfh cell generation and function in promoting humoral immunity. Since B7h is constitutively expressed on B cells, we recently examined whether the generation of Tfh cells required cognate B-T cell interaction (28). Analysis of a B7h conditional knockout mouse deficient in B cell expression of B7h revealed that absence of ICOS ligand in B cells led to a greatly reduced frequency of Tfh cells. In addition to CXCR5 expression, we found that the expression of IL-21 by T cells was also greatly reduced in these mice. ICOS-B7h interaction may thus regulate Tfh cells through production of IL-21. In addition, we found that antigen-specific IgG production and PNA^+ germinal-center B cells were greatly reduced in the absence of B7h expression on B cells. Thus, our data indicate that B7h expression on B cells is necessary for the generation of $CXCR5^+$ Tfh cells, IL-21 production and appropriate antibody responses, suggesting an important function of B cells in vivo as antigen-presenting cells in the generation or maintenance of Tfh cells

1.3. ICOS regulates autoimmunity

Collagen-induced arthritis (CIA) is the most widely utilized mouse model for human rheumatoid arthritis disease, which induced by immunization of genetically susceptible strains of mice such as DBA/1 with chicken type II collagen (CII) proteins. ICOS deficient mice (31) as well as mice receiving anti-B7h blocking antibodies (32) were resistant to collagen-induced arthritis and exhibited absence of joint tissue inflammation. Consistent with the resistance of ICOS-deficient mice to CIA, we found they exhibited greatly reduced levels of IgM, IgG, IgG2a, and IgG2b antibody to CII (31). Interestingly, spleen cells from ICOS-deficient mice showed a remarkably decreased level of IL-17 in response to CII, even though they produced normal amount of TNFα and IFNγ (31).

IL-17 is an inflammatory cytokine secreted by $CD4^+$ T cells and is frequently found in RA synovium (33-35). Base on our data, we hypothesize that IL-17 produced by $CD4^+$ cells in the joint tissue might play a key role in the inflammatory process involved in rheumatoid arthritis. In support of this idea, over-expression of IL-17 in the joints resulted in inflammatory infiltration of the synovium and aggressive cartilage degradation (36). In addition, neutralization of IL-17 with antibodies to IL-17 significantly reduced the joint damage in CIA (37). Moreover, IL-17 knockout mice were also found resistant to CIA (38). Thus, defective IL-17 production in the ICOS-deficient mice may contribute to lack of joint inflammation in these mice. Since IFNγ and IL-17 production is differentially regulated by ICOS, we also reasoned that IL-17-expressing T cells have unique regulatory pathways than those of TH1 cells. Tree years ago, we and Weaver's group demonstrated a new lineage of helper T cells (THi or TH17) that produce IL-17 as an independent lineage from TH1 or TH2 cells (39,40). We indicated that generation of these cells required CD28 and ICOS costimulation but was independent of the cytokine and transcription programs normally associated with TH1 and TH2 differentiation (40). Our further study indicated that in addition to TH17 differentiation, ICOS is also important for IL-17 production in the effector stage after Th differentiation (our unpublished data). Since ICOS ligand, B7h is expressed in inflamed tissues; ICOS-B7h

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interaction may regulate IL-17 local expression in the joint tissue in the CIA model and mediate the inflammatory responses. In addition to CIA, TH17 cells have been shown important in the development of experimental autoimmune encephalomyelitis (EAE), a mouse model for human multiple sclerosis disease (41). In contrast to the pathogenic role in CIA, ICOS-deficient mice exhibited exacerbated EAE (12), and are thus predictable to be normal in TH17 differentiation in this model. The differential roles of ICOS in TH17 differentiation in two different models are likely due to the genetic backgrounds on which these models were applied and/or the type of immunization protocols- in CIA, whole protein was used while EAE only involves MOG peptide.

Recent studies indicate an important role of ICOS in generation of Tfh cells and in production of IL-21 (24,28,29,42). Consistent with this literature, we found that in CIA model, CII-specific IL-21 production was also greatly reduced in ICOS deficient mice (our unpublished data). It has been shown that IL-21 induces T-cell activation and proinflammatory cytokine secretion in rheumatoid arthritis (43). In addition, in CIA model blockade of IL-21 or IL-21R ameliorated disease and also lowered levels of IL-6 and IL-17 (44). Considering that in CIA model, ICOS is important for both IL-17 and IL-21 expression, ICOS may potentially regulates IL-17 production through IL-21 expression. In support of this idea, recently we and other group reported that IL-21 produced by Th17 cells plays a critical role in generation of IL-17-producing cells in vitro and in vivo (45-47). IL-21 in combination with TGF-β promoted the differentiation of TH17 cells (45,47). Loss of IL-21 expression or its receptor resulted in impaired IL-17 production *in vitro* and *in vivo* (45,46). Thus, inducible costimulatory receptor ICOS regulates IL-21 production by $CD4^+$ T cells, which in synergy with TGFβ promotes the IL-17 expression.

1.4. Regulatory T cells and ICOS

The active suppression by regulatory T cells (Treg) has been proposed for maintaining tolerance to both self and foreign antigens. A key phenotype of these suppressors cell is the secretion of IL-10 (48), and ICOS has important role in co-stimulating production of IL-10 (6). Lohning et al. found a correlation between the expression level of ICOS on $CD4^+$ T cells and the type of cytokines produced by these cells (49). Whereas $ICOS^{medium}$ T cells secreted Th2 cytokines such as IL-4, -5 and –13, $CD4^+$ cells expressing high level of ICOS expression predominantly expressed IL-10 (49). The role of ICOS in stimulating IL-10 production may also be important in the generation of Treg cells. Akbari et al demonstrated that ICOS-ICOS-ligand interaction is important in generation of IL-10 secreting Treg cells and in the regulation of allergen-induced airway hyperreactivity (50). Another studies demonstrated the role of IL-10 in the induction of oral and nasal tolerance (51,52). Miyamoto et al. found that ICOS plays an essential role in regulation of mucosal tolerance (53). Thus, ICOS signal plays an important role in controlling IL-10-producing Treg cells and peripheral T cell tolerance. In support of this idea, Herman et al. reported that Treg cells expressing immunoregulatory cytokine IL-10 play a critical role in preventing the rapid onset of diabetes (54). Treg cells coexisted in balance with aggressive effector T cells in pancreatic lesion, and expressed high level of ICOS and IL-10 transcripts in pancreatic tissues compared with draining lymph nodes. Blockade of ICOS resulted in loss of this balance and convert early insulates to aggressive diabetes. These findings suggest that Tr cells regulate development of autoimmunity in ICOS-dependent manner. Recently Gotsman et al. demonstrated the role of ICOS in the regulation of atherosclerosis (55). They found that enhanced atherosclerosis in the ICOS-deficient mice was due to requirement of ICOS for the generation and function of atheroprotective regulatory T cells.

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1.5. Overlapping function of CD28 and ICOS pathways in T cells

CD28 is the most prominent costimulatory receptor for T-cell activation (2). Mice deficient in CD28 or both of its ligands B7.1 and B7.2 were severely impaired in CD4 T cell proliferation (3,4). In these mice, however, reduced levels of effector cytokines were still produced (56-58). Analysis of mice deficient in ICOS or its ligand, B7h, revealed that this pathway, although not globally required for CD4 T-cell activation and effector differentiation, regulates their selective effector function (10,12). To better understand the molecular mechanisms that determine T-cell function or tolerance, we have collectively examined the roles of positive and negative costimulatory molecules (59). We found that $CD4^+$ or $CD8^+$ T cells activated without CD28 and ICOS costimulation were most impaired in proliferation and only underwent approximately 2–3 divisions. However antigen-specific T cells activated in the absence of CD28 and ICOS costimulations could upregulate activation markers and survive over time.

Moreover, T cells activated in the absence of costimulation could had characteristics of anergic cells—hyporesponsiveness to TcR/CD3 restimulation (59). Biochemical characterization revealed that these cells were deficient in TcR signal transduction leading to gene transcription. TcR activation of MAP kinase, NFAT and NF-κB pathways was greatly inhibited in these cells. In addition, the expression of two critical components in TcR signaling such as PKCθ and PLCγ1 was greatly reduced. PKCθ and PLCγ1 were recently identified as targets of ubiquitin-regulated degradation machinery in Ca^{2+}-induced anergic cells (60). On the other hand, expression of two E3 ubiquitin ligases such as cbl-b and Itch was significantly induced in these cells (59). Most strikingly, both $CD4^+$ and $CD8^+$ T cells after activation without CD28 and ICOS signals expressed Grail, an E3 ubiquitin ligase whose expression was previously found to be associated with CD4 T cell anergy *in vitro* and *in vivo* (61,62). Therefore, these $CD4^+$ and $CD8^+$ cells shared some characteristics with anergic Th1 cells previously characterized (1,62-64).

Most interestingly, analysis of effector T cell function revealed that activation of naïve CD4+ and CD8+ T cells T cells in the absence of CD28 and ICOS engagement resulted in complete impairments in their effector function (59). The anergic $CD4^+$T cells did not produce any effector cytokine upon restimulation, whereas $CD4^+$ cells activated in the absence of B7 or ICOS produced low but detectable level of effector cytokines. The anergic $CD8^+$ T cells did not express cytokines and cytolytic enzymes, and could not kill target cells. Moreover, $CD4^+$ and $CD8^+$ T cells activated in the absence of positive costimulatory signals impared in expression of transcriptional factors (T-bet and GATA3 for $CD4^+$ T cells; T-bet and Eomes for $CD8^+$ T cells) that regulate effector differentiation and cytokine expression. PMA/ ionomycin could restore proliferation but not cytokine expression in anergic T cells. These results indicate that the global impairments in effector function in the tolerant cells was not due to simple TcR signaling defects but rather intrinsic absence of an effector gene expression program. Thus, when antigen-specific naïve T cells were activated in the absence of CD28 and ICOS costimulation, instead of differentiating into effector cells, they likely developed into stably tolerized T cells with both TcR signaling and gene transcription defects. Absence of B7 or ICOS costimulation resulted only in defective immune functions, but absolute T-cell tolerance was not observed.

2. PD-L1/PD-L2-PD-1

2.1. Identification of PD-1 and its ligands

PD-1 was first identified by using subtractive hybridization technique to search for genes involved in programmed cell death (65). The extracellular region of PD-1 consists of a single Ig-like variable (IgV) domain, and the cytoplasmic region contains both immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif

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(ITSM) motifs, which is different from other known CD28 family members. PD-1 shares 23% identity with CTLA-4, which rendered the author to hypothesize that the ligand for PD-1 shared high similarity with B7-1 and B7-2 (66). Indeed, PD-L1 was originally identified in a B7 homology-based search of EST databases and named B7-H1 (67). Later, another ligand for PD-1, PD-L2 (also called B7-DC), was also identified by homology-based search (68,69).

2.2. Expression of PD-1 and its ligands

Originally, PD-1 was found to be highly expressed in the thymus, and tissues undergoing activation-induced programmed cell death (65). Later, PD-1 mRNA was found broadly expressed at low levels in T, B, NK-T and myeloid cells, and was further induced upon activation (70,71). The broader expression of PD-1 comparing to other CD28 family members indicates that PD-1 may have functions other than in T cells in the immune system.

The two ligands for PD-1, PD-L1 and PD-L2, have distinct patterns of expression. PD-L1 was expressed at high levels in the heart, thymus, and lung, and low levels in the kidney, spleen, and liver (66); and also reported in pancreatic islets, endothelium, small intestine and placenta (72). At cellular levels, PD-L1 was detected in a variety of lymphohematopoietic cells, including T and B cells, which could be further up-regulated upon activation (71,73). PD-L1 was also expressed on antigen-presenting cells, including activated human PBMC, B cells, macrophages and dendritic cells (DC). In contrast, the expression of PD-L2 was restricted to macrophages and DC, which could be up-regulated upon activation with IFNγ, GM-CSF and IL-4 (71). The distinct expression patterns of PD-1 ligands suggested their differential roles in immune regulations. Indeed, one study showed that PD-L1 up-regulation on activated macrophages depended on TLR4 and STAT1, whereas PD-L2 expression depended on IL-4Rα and STAT6. Thus, PD-L1 and PD-L2 might also have different functions in regulating type 1 and type 2 responses (74).

2.3. Functional Studies on PD-1 and its ligands

PD-1 has been shown to be a negative regulator of T cell activation and is crucial for maintaining immune tolerance. PD-1 deficiency in mouse resulted in spontaneous autoimmune diseases (68,75). Moreover, PD-1 deficiency (76) or blockade (77) accelerated autoimmune diabetes on NOD background. Blocking PD-1 also enhanced experimental autoimmune encephalomyelitis (EAE) disease (78).

It is noteworthy that there were contradictory results regarding the role of PD-1 ligands, PD-L1 and PD-L2, in controlling T cells activation. Both PD-L1-Ig and PD-L2-Ig fusion proteins have been found to either enhanced (67) or inhibited (68) anti-CD3 mediated CD4+ T cell responses. It was also true when blocking antibodies for PD-L1 or PD-L2 were used, both inhibitory (78) and stimulatory (79) effects have been reported.

As noted above, the distinct expression patterns of PD-1 ligands might contribute to their differential roles in immune regulations. In this regards, we found CD11c+CD8α+ DC, which were previously reported to cross-present pancreatic antigens to CD8+ T cells in pancreatic LN, expressed PD-L1 but not PD-L2 (80). It was reported that the PD-L1 protein expressed on islet cells mediated peripheral tolerance and PD-L1 deficiency precipitated the rapid onset of autoimmune diabetes on the NOD background (81). However, an opposite conclusion was drawn on the forced expression of PD-L1 in the islet cells and promoted spontaneous autoimmunity was observed (82). To study the role of PD-1 and its ligands on peripheral tolerance of CD8+ T cells, we used the RIP-mOVA mice, which induce tolerance in transferred OT-I CD8+ T cells. We found an antagonistic Ab against PD-1 provoked destructive autoimmune diabetes in these mice after naïve OT-I cells transferring (80). This effect was mediated by PD-L1 but not PD-L2, and possibly through the CD11c+CD8α+ DC. The blockade

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of PD-1/PD-L1 interaction significantly enhanced OT-I cells cytokine production and CTL activity. Since the blockade did not affect OT-I cell division at the priming phase, we concluded that PD-1-PDL1 interaction crucially controlled the effector differentiation of autoreactive T cell to maintain self-tolerance (80).

To analyze the role of PD-L2 in T cell activation and potential role in tolerance, we generated and analyzed a PD-L2 deficient mouse (83). Antigen-presenting cells from PD-L2 KO mice were more potent in activation of T cells *in vitro*, which depended on interaction with PD-1. PD-L2 KO mice also exhibited increased activation of both CD4+ and CD8+ T cells *in vivo* after immunization. In addition, T cell tolerance to an oral antigen was abrogated in PD-L2 KO mice. Thus, our findings demonstrated that PD-L2 negatively regulated T cells in immune responses and played an essential role in immune tolerance (83). In summary, our findings supported the inhibitory effects of both PD-L1 and PD-L2, which depended on their interactions with PD-1. Furthermore, we reported that both PD-L1 and PD-L2 was important for the maintenance of peripheral tolerance in both CD4+ and CD8+ T cells.

It is not easy to reconcile the contradictory functions of PD-L1 and PD-L2. Kanai et al. suggested that there might be an as yet unidentified receptor for PD-L1 and PD-L2 in inducing T cell activation similar to the B7-1/B7-2:CD28/CTLA (84). Indeed, Wang et al. reported that mutants of PD-L1 and PD-L2 with abolished PD-1 binding capacity could still costimulate proliferation and cytokine production of T cells from normal and PD-1–deficient mice, supporting a putative stimulatory receptor for PD-L1 and PD-L2 (85).

The agonistic or antagonistic effect of the antibodies used in the experiments, or the background of these knockout mice might play a role in the difference. Indeed, PD-1 knockout mice on C57BL/6 background spontaneously developed lupus-like proliferative arthritis and glomerulonephritis, which was worsened by combination with Fas mutation (lpr). PD-1 knockout mice crossed with 2C-TCR on an $H\text{-}2^{b/d}$ background developed chronic and systemic graft-versus-host-like disease (75). PD-1 deficiency mice on a BALB/c background caused dilated cardiomyopathy with severely impaired contraction and sudden death by congestive heart failure (86). Further studies to compare these mice on the same background in the same experiment are necessary.

3. B7-H3

3.1. Identification of B7-H3

Human B7-H3 was first identified by searching the databases with extracellular regions of all published B7 family members. Human B7-H3 locates on chromosome 15, which contains a signal peptide in its N-terminus, single extracellular V- and C-like Ig domain, a transmembrane region, and a cytoplasmic tail (87). We discovered the mouse counterpart on chromosome 9, which shares 88% identity and 93% similarity with the human molecule. Interestingly, we also identified a new isoform of human B7-H3 gene with four Ig-like domains (VCVC), which we named B7-H3b (88). Ling et al. confirmed both VC- and VCVC- forms existed for human B7-H3 (89). Stinberger et al. independently identified the four Ig-like form of B7-H3, and gave a new name to this molecule as 4Ig-B7-H3 (90).

3.2. B7-H3 expression

Human B7-H3 mRNA was broadly expressed in tissues and tumor lines, but not in peripheral blood leukocytes (87). Using antiserum to human B7-H3, it was found that B7-H3 was not detectable on freshly isolated CD3+, CD19+, CD14+ cells and DCs. However, GM-CSF- or IFNγ-activated DC and CD14+ monocytic cells, and PMA plus ionomycin activated CD3+ , CD19+ and NK cells all up-regulated the expression of B7-H3 (87). In most tissues examined, except for brain and placenta, B7-H3b represented the major isoform (88,90).

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Similar to its human homolog, mouse B7-H3 mRNA was widely expressed in all tissues. By using anti-B7-H3 mAbs, we found mouse B7-H3 was expressed on a minor fraction of CD4+ and CD8+ T cells (91). In contrast, nearly all B220+ splenic B cells and splenic CD11c+ DC constitutively expressed B7-H3. Bone-marrow derived DC (BMDC) and peritoneal macrophages all express B7-H3. After LPS stimulation, BMDC exhibited up-regulation of B7-H3 expression. However, B7-H3 expression on purified B cells and macrophages was not altered after the same treatment. Therefore, mouse B7-H3 is mostly expressed by all professional APCs and a subpopulation of T cell population that we have examined.

It has been shown that activated but not resting CD4+ and CD8+ T cells expressed a putative counter-receptor for human and mouse B7-H3 (87,88). Recently, Hashiguchi et al. reported that Triggering receptor expressed on myeloid cells (TREM)-like Transcript 2 (TLT-2) was the receptor for B7-H3. TLT-2 was expressed on freshly isolated CD8+ T cells, and TCR stimulation induced TLT-2 on CD4+T cells. Low levels of TLT-2 were also found expressed on Treg, B, macrophages, NK and DC (92). However, we were not able to reproduce the binding of B7-H3 to TLT-2 (unpublished data).

3.3. B7-H3 function

It was initially reported that human B7-H3 provided a positive co-stimulation to enhance T cell activation (87). However, later studies found that both VC and VCVC forms of human B7-H3 inhibited CD4+ T cell proliferation and downregulated cytokine production upon TCR activation (89). We and others found that mouse B7-H3 protein inhibited T cell activation and effector cytokine production in both CD4+ and CD8+ T cells (91,93). An antagonistic mAb to B7-H3 enhanced T cell proliferation *in vitro* and led to exacerbated EAE *in vivo* (91). B7-H3-deficient antigen-presenting cells showed a capacity to stimulate alloreactive T cells *in vitro*. B7-H3-deficient mice developed EAE earlier and accumulated higher concentrations of autoantibodies to DNA in aged mice (93). Interestingly, B7-H3-deficient mice developed more severe airway inflammation in TH1 polarizing condition, but not in TH2 polarizing or CTL-mediated responses. Cosistent with this, Suh et al. reported that B7-H3 expression was enhanced by IFNγ but suppressed by IL-4 in DC, and thus B7-H3 might provide a negative feedback mechanism for TH1-mediated responses (93). However, there were also evidences that supported the role of B7-H3 in Th2-mediated immune reactions *in vivo*. In a murine experimental allergic conjunctivitis (EC) model, intraperitoneal injection of anti-B7-H3 Ab during the induction, but not effector, phase of EC significantly autmented the severity of EC (94). This notion was further supported by Nagashima et al., who reported that anti-B7-H3 mAb administration during the induction phase significantly reduced airway hyperreactivity in a mouse model of allergic asthma (95).

The inhibitory effects of B7-H3 have been reported in other types of immune responses. Tran et al. showed that reduction of B7-H3 expression on Fibroblast-like synoviocytes (FLS) by RNA interference affected the interactions between FLS and T cells in RA, and influenced the cytokine profiles depended on the activation state of the T cells (96). In prostate cancer patients, strong intensity for B7-H3 was significantly more likely to have disease spread at time of surgery, and at increased risk of recurrence and cancer-specific death (97). B7-H3 was also played a role in non-immune response, such as positive regulatory role in bone formation (98). Given the proposed immune-inhibitory mechanisms of B7-H3 in both CD4+ and CD8+ T cells, this molecule represents an attractive target for therapeutic manipulations.

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4. B7S1 (B7-H4, B7x)

4.1. Identification of B7S1

Our group identified B7S1 by homology search in mouse and human EST databases using amino acid sequences of B7h and B7-H3 (99). Independently, two other groups reported this molecule but named it as B7-H4 (100), or B7x (101). Human B7S1 located on chromosome 1 and mouse counterpart on chromosome 3, which shared 87% amino acid identity. Both human and mouse B7S1 contained an N-terminal leader peptide, two Ig-like domains, and a hydrophobic C terminus, which functioned as a surface GPI-anchor (99,102).

4.2. B7S1 expression

Mouse B7S1 was ubiquitously expressed in both lymphoid and nonlymphoid tissues. By using anti-B7S1 mAb, we found B7S1 protein was expressed by a minor population of cells in the thymus (99). B7S1 was not found on freshly isolated splenic CD4+, CD8+, or CD11C+ cells, but constitutively expressed on B cells. B7S1 expression could be detected on activated macrophages and BMDC. LPS treated peritoneal macrophage or bone marrow-derived DC did not significantly alter the B7S1 expression. On the other hand, LPS, IL-4, anti-IgM and anti-CD40 treatment all resulted in B7S1 downregulation on splenic B cells. This analysis indicated that B7S1 was expressed by a variety of professional APC and might possess regulatory role on T cells.

Human B7S1 mRNA was also ubiquitously expressed at lower levels in tissues examined. However, the human B7S1 proteins expressed on the cell surface was undetectable on the majority of normal human tissues. Similar to mouse B7S1, human B7S1 expression could be induced on T, B, monocytes, and DC after *in vitro* stimulation (100). In addition, a lot of cancer tissues constitutively expressed B7S1, which might used by the tumor to escape the immune surveillance of the host (101,102).

4.3. Functional Studies on B7S1

Recombinant B7S1-Ig protein or forced overexpression of B7S1 on artificial APC inhibited T cell proliferation and cytokine secretion (99), likely by arresting cell cycle (100,101). This inhibitory effect could not be reversed by CD28 costimulation. Administration of B7S1-Ig into mice inhibited CD4+ T cell proliferation, and the maturation and cytotoxicity of CD8+ T cells (100). Blocking mAb to B7S1 enhanced T-dependent immune response and exacerbated EAE *in vivo* (99). B7S1-deficient mice supported the inhibitory function *in vitro*. B7S1-deficient mice displayed increased Th1 responses and lowered parasite burdens upon *Leishmania major* infection (103).

The inhibitory effect from B7S1 has been found to be used by CD4+CD25+ regulatory T cell-mediated suppression, in which Treg cells conveyed suppressive activity of APC through stimulating the expression of B7S1 on APC in an IL-10-dependent manner (104). Aberrant B7S1 expression has been reported in several human cancers, and higher level of B7S1 has been associated with adverse clinical scores, and higher risk of recurrence and cancer-specific death, such as prostate cancer (97) and renal cell carcinoma (105). One of the mechanisms, as reported in the progression of ovarian cancer, was tumor-associated macrophages expressing high levels of B7S1on the surface suppressed tumor antigen-specific T cell immunity (106). In summary, B7S1 represented a critical checkpoint in determining host responses, and joined the negative costimulatory molecules that collaboratively fine-tuned T-cell mediated immunity.

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5. B7S3

5.1. Identification of B7S3

B7S3 was discovered by a homology search of human genome database for novel B7-like genes (107). Human B7S3 located on human chromosome 1, and mouse homologue on chromosome 4. We completely sequenced the mouse B7S3 and found that it contained a leader peptide at its N-terminus, an IgV-like domain, a half IgC-like domain, and a hydrophobic region at the C-terminus, which served as a transmembrane domain. We also cloned a longer form of B7S3, which contained two additional transmembrane domains at its C-terminus. Therefore, we named the short form as B7S3S, and long form as B7S3L.

In addition to the two forms of B7S3, we found 10 close homologues of B7S3 within a 2Mb region on mouse chromosome 4. They all had IgV-IgC-like domains, which shared from 44% to 86% homology with B7S3. Therefore, a B7S3 gene family exists in mice. In human, however, there was only one gene bearing around 60% homology to mouse B7S3 (107).

5.2. B7S3 expression

B7S3S mRNA was widely expressed in tissues we examined (107). Particularly, B7S3S mRNA was found expressed in non-lymphoid tissues, most abundantly in lung and at reduced levels in kidney, heart and stomach; and in lymphoid organs, such as spleen, lymph nodes and thymus. In contrast, the expression of B7S3L mRNA is very restricted, with only low levels detected in the thymus, stomach, intestine and heart. The expression levels of B7S3 in lymphoid cells, including CD4+, CD8+ T cells, B cells, BMDC and peritoneal macrophages, were very low in these cells, and B7S3L almost undetectable. However, the levels of B7S3S in BMDC and peritoneal macrophages were upregulated after different stimuli, including LPS, CpG, TLR2, PolyIC, TNFα and IL-17. These data indicated potential regulation of B7S3 expression by innate and inflammatory stimuli (107).

5.3. Functional studies on B7S3

B7S3-Ig fusion protein bound to its potential receptor(s) on B220+ B cells, CD11c^{hi} DC and Mac-1+ macrophages in spleen as well as peritoneal macrophages (107). The binding of B7S3-Ig to B cells was not affected by LPS stimulation. These results indicate that the B7S3 receptor is constitutively expressed on APCs, distinct from the receptors of other B7 molecules. Freshly isolated CD4 and CD8 T cells, on the other hand, were not bound significantly by B7S3-Ig. After activation by Con A for 48 hours, B7S3-Ig binding was significantly upregulated in both CD4 and CD8 T cells. B7S3 receptor expression on T cells can also be induced by anti-CD3 and anti-CD28 stimulation. Therefore, the B7S3 receptor on T cells shares a similar expression pattern with the receptors for other new B7 family members (107).

B7S3-Ig treatment did not alter the proliferative response of B cells or proinflammatory cytokine production by macrophages in the presence of other stimuli such as LPS and anti-CD40 (107). On the other hand, B7S3-Ig treatment strongly inhibited anti-CD3-activated proliferation and IL-2 production by both CD4 and CD8 cells T cells. Exogenous IL-2 restored the proliferation of B7S3-Ig-treated CD4 and CD8 T cells. These results indicated that B7S3 was a negative regulator of T cell activation, which functions by inhibiting IL-2 expression (107).

Interestingly, Boyden et al. recently reported the genetic mapping of the location for a strain showing loss of $V\gamma5^{+}V\delta1^{+}$ cells (108). Although they named it as skint (for selection and upkeep of intraepithelial T cells 1) gene family, we confirmed that skint 2 was B7S3 by sequence comparison (unpublished data). In this article, Boyden et al. found skint 1 had 2 IgV-IgC-like domains and three putative transmembrane domains, which had a similar structure

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with B7S3L. A single amino acid mutation causing a premature termination of skint 1 before the putative third transmembrane domain was the reason for the deficiency in FVBtac mice (108).

6. BTNL2

6.1. Identification of BTNL2

The major histocompatibility complex (MHC) region, which includes well-defined class I, class II, and class III genes, is composed of clustering of genes involved in the immune response. There are several copies of the B30.2 domain encoded in this region (109), which is also found at the C-terminal of butyrophilin protein. Butyrophilin is a major component of milk fat globule membrane, the expression of which is restricted to mammary gland tissue and regulated lactogenic hormones (110,111). Butyrophilin, together with myelin/oligodendrocyte glycoprotein, and the chicken MHC molecule, B-G, shared sequence similarity to the IgV and IgC domains of B7-1 and B7-2, which have been proposed to be the extended members of the immunoglobulin superfamily (IgSF) (112). By sequence homology search, more than six butyrophilin-like cDNAs have been described in the MHC class II locus (113-116). We confirmed the full-length murine BTNL2 cDNA from a spleen cDNA library. It has four extracellular Ig domains, consisting of two IgV-IgC pairs, unlike most B7s, which only have two. There is a heptad sequence, characteristic for many butyrophilins, which is located between the two pairs of IgV-IgC domains. Unlike many other butyrophilins such as BT3.1, BTNL2 does not have a B30.2 domain in the intracellular region (117). Overall, human and mouse BTNL2 proteins are 63% identical. We confirmed that human BTNL2 mRNA obtained from peripheral blood cells lacked the IgCa domain as reported by Valentonyte et al. (118). As with all B7 members, each domain is encoded by a separate exon. In mice, five other butyrophilin-like proteins are found within this same region; however, these five genes are not conserved in humans (116).

6.2. BTNL2 expression

RT-PCR analysis revealed that BTNL2 is widely expressed in both lymphoid and non-lymphoid tissues. BTNL2 mRNA was expressed in thymus, spleen and lymph nodes. On purified cells, BTNL2 was expressed in T cells, B cells, and macrophages. In non-lymphoid tissues, BTNL2 mRNA was found expressed most abundantly in small intestine and reduced levels in lung and stomach. These findings were confirmed by other group, in which they reported that BTNL2 mRNA is predominantly expressed in digestive tract tissues, in particular small intestine and Peyer's patches (116,119). Analysis by Immunohistochemistry with an anti-BTNL2 polyclonal Ab, Arnett et al further confirms expression of BTNL2 protein in the small intestine. Interestingly, BTNL2 is predominantly expressed in the epithelial cells of the villi but not the crypts, and BTNL2 is localized to cells with the morphological appearance of DC (119).

6.3. BTNL2 function

We found that a BTNL2 receptor was expressed on ConA-activated T cells but not unstimulated T cells (120). The putative receptor was also found constitutively expressed on splenic B220 + B cells, and LPS stimulation further increased a higher level of the receptor. However, BMDC, BM macrophages, periotoneal macrophage, granulocytes, NK cells were not significantly bound by BTNL2-Ig. When CD4+ T cells were activated with anti-CD3 and anti-CD28, a dose-dependent inhibition of proliferation was observed in the presence of plate-bound, but not soluble, BTNL2-Ig. This implies that BTNL2, like PD-L1 (121,122), inhibits proximal TCR signaling events and must be in proximity of the TCR. By using DO11.10 hybridoma, which expressed a receptor for BTNL2 upon activation, we found BTNL2-Ig partially inhibited IL-2 production and activation-induced cell death. We also found BTNL2-

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Ig treatment resulted in at least 50% reduction of anti-CD3-mediated activation of AP-1, NFAT, and NF-κB in this cell line. Inhibition of all three general pathways suggests that BTNL2, like other negative B7-like costimulators, inhibits proximal TCR signaling events.

To compare the inhibitory mechanism of BTNL2 with other B7-like molecules, we prepared BTNL2-Ig, B7S1-Ig, or B7-H3-Ig in the same fashion in our lab. These fusion proteins all effectively inhibited anti-CD3 stimulated T cell proliferation. However, when CD28 costimulation was provided, BTNL2-Ig inhibited T cell proliferation more strongly than B7-H3-Ig but less potently than B7S1-Ig. Similar to B7-H3 and B7S1 (91,99), we found plate-bound BTNL2-Ig moderately reduced IL-2 production when CD4+ T cells were activated with anti-CD3 alone or together with anti-CD28. Notably, the ability of BTNL2-Ig to suppress T cell proliferation was more potent than its effect on IL-2 production. In the absence of CD28 costimulation, proliferation of T cells treated with B7S1-Ig and B7-H3-Ig, but not BTNL2-Ig, was sharply increased by exogenous IL-2. On the other hand, CD28 costimulation greatly increased proliferation in B7-H3-treated T cells, less so in BTNL2-Ig-treated cells, and had no effect on cells stimulated with B7S1-Ig. IL-2 and CD28 costimulation synergistically enhanced T cell proliferation in the presence of B7-H3-Ig or BTNL2-Ig but not with B7S1-Ig. This result suggested differential mechanisms utilized by these three molecules in T cell regulation.

Mutations in human BTNL2 gene have been associated with several autoimmune disease, like sarcoidosis disease (118), myositis (118,123,124) and multiple sclerosis (125). Also, BTNL2 polymorphism exhibited strong linkage disequilibrium with rheumatoid arthritis, type 1 diabetes and systemic lupus erythematosus (126). These findings indicate that BTNL2 is widely used to maintain the peripheral tolerance or to control inflammatory responses in the immune system. More research are needed to understand the function of BTNL2 in guarding tolerance and autoimmunity, and these findings will provide novel therapeutic targets for autoimmune disease such as sarcoidosis and RA.

7. Conclusion and perspectives

Over the past ten years, an extended array of costimulatory pathways have been discovered and characterized. In addition to CD28, ICOS has emerged as an important player in the immune system to fine tune the effector function of T cells. CD28 and ICOS have overlapping function in early T cell activation. On the other hand, many inhibitory costimulatory molecules have also been found. In a recent study we performed, antigen-specific $CD4^+$ or $CD8^+$ T cells activated in the absence of ICOS and CD28 signals were treated with antagonistic antibodies to PD-1, B7-H3 or B7S1 (59). As a result of blocking PD-1, B7-H3 or B7S1, CD4+ and CD8 + T cells exhibited increased proliferative responses to anti-CD3 restimulation, and more significantly, these cells expressed transcriptional factors and produced effector cytokines. Therefore, our *in vitro* data indicate that T cell tolerance resulted from B7 and ICOS blockade required the function of PD-1, B7-H3 and B7S1. Based on these results, we propose that T cell activation is collectively regulated by both positive and negative costimulatory molecules that function to enhance or dampen TcR signaling, respectively.

Although we have achieved significant progress in understanding the complexity of T cell costimulation, there are many questions remained. First, we have still many orphan ligand molecules and conventional approaches have not yet led to the discovery of their receptors. Second, we do not comprehend why there are so many negative costimulatory molecules. Perhaps they have selective function; some regulate immune tolerance in resting states and some are induced on activated antigen-presenting cells to restrict the magnitude of T cell activation. The other possibility is that they each fine tune TcR signaling strength and modulate T cell activation thresholds. Third, we do not understand well the function of costimulatory molecules beyond lymphoid organs. A rich number of these molecules are expressed in

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nonlymphoid tissues, inflammatory sites and in tumor microenvironments. They serve to regulate the function of T cells which dynamically interact with antigen-presenting cells and tissues cells. Lastly, many new costimulatory molecules have receptors on non-T cells. What is their function there? They may modulate the activity of FcR-like or NK receptor-like receptors there.

Modulating T cell costimulation has clinical implications. CTLA4 blockade has been shown to enhance tumor regression in tumor patients (127). However, an autoimmune response was also observed in these patients. While these results provide an exciting application of costimulation blockade, more antigen-specific approaches are desired in conjunction. CTLA4-Ig that inhibits CD28 signaling is also under clinical trials. In the coming years, we anticipate to witness more "human experiments" with costimulation modulation, which may provide a bench to bed switch in the costimulation study.

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