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Structural immunology of costimualtory and coinhibitory molecules

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The T cell costimulatory pathways are central to regulating immune responses, and targeting these pathways represents one of the most promising approaches for achieving immunotherapy. The molecular structures of costimulation revealed invaluable mechanistic insights underlying costimulatory receptor/ligand specificity, affinity, oligomeric state, and valency, which provided the bases for better manipulation of these signaling pathways. The incredible growth of this field led to identification of new members and unexpected interactions, revealing a complicated regulatory network of immune responses. The advances in structural biology of costimulation will promise unprecedented opportunities for furthering our understanding and therapeutic application of T cell costimulatory pathways.

costimulatory molecules; T cells; molecular structure; immunotherapy

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The immune system has the remarkable ability to defend against the array of "non-self" dangers in the environment including microbial pathogens and transformed cells, yet not respond to self. The high specificity of the immune responses is determined by recognition of specific antigens by antigen receptors on lymphocytes. Accumulating evidence indicated that the interaction of T-cell receptor (TCR) with antigenic peptide/major histocompatibility complex (MHC) alone is not sufficient to drive the activation of naive T cells leading to optimal immune responses [1,2]. Productive T-cell activation requires a second antigen-independent co-signal, the "costimulatory signal", provided by cell surface molecules on T cells. In the absence of costimulation, TCR-mediated activation of T cells results in antigen-specific unresponsiveness (termed as T-cell suppression, tolerance, anergy or exhaustion), rendering the T cells unable to respond to subsequent exposure to antigen [3,4]. The critical roles of costimulatory molecules in regulating the immune response have given impetus to the study of costimulation and resulted in incredible growth of this field. A group of such "costimulatory molecules" have been identified to have the opposite function, i.e. they are actively suppressing T cell responses in the presence of TCR signal. These molecules are termed "coinhibitory molecules" [5].

The identified receptors and ligands involved in T-cell costimulation belong to a broad array of cell surface or soluble proteins. The most extensively studied costimulatory molecules include the members of the B7/CD28 (Figure 1) and tumor-necrosis factor (TNF)/TNFR families (Figure 2). Typically, the ligands are expressed on antigen-presenting cells (APCs) or peripheral tissues, and the receptors are expressed on T cells. However, the ligands could often serve as receptors to deliver biologically significant signals. The specific binding between the paired ligands and receptors forms a delicate network to regulate and control initiation, expansion, and differentiation of ef-

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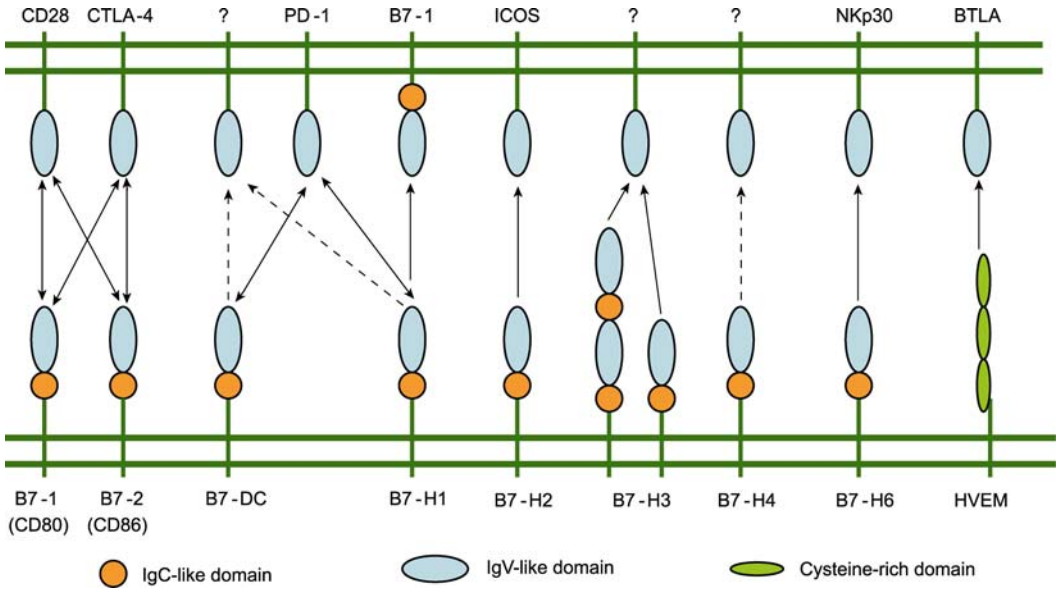

Figure 1 Structural organization of co-stimulatory B7/CD28 superfamily members. The names of the receptors (up) and ligands (bottom) are indicated. BTLA binds to the TNFR family member HVEM. Ligand B7-H1 binds to another ligand B7-1. B7-H1 and B7-DC are thought to have another unknown receptor. B7-H3 and B7-H4 are still two orphan ligands. The ligands of B7/CD28 family are Ig superfamily members with an IgV-like and an IgC-like domains in the extracellular portion, whereas the receptors are Ig superfamily members with a single IgV-like domain in the extracellular portion. B7-H3 exists in the 2Ig form in mice and in the 4Ig form in humans.

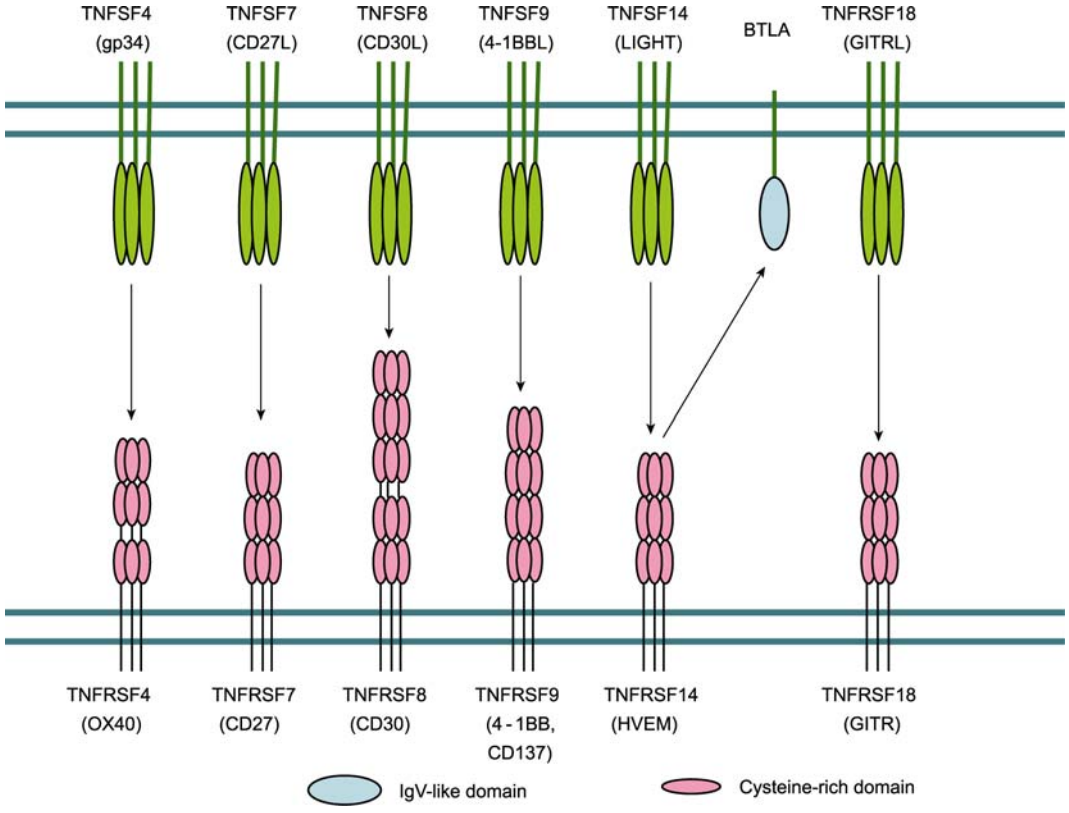

Figure 2 Strutural organization of costimualtory TNF/TNFR family members. Common (in parentheses) and standardized TNF/TNFR nomenclature are provided. LIGHT also interacts with LTβR and the decoy receptor DcR3/TR6; HVEM also interacts with LTα3 and co-stimulatory CD28 family member BTLA. The TNF ligands (top) are shown as homotrimeric type II transmembrane proteins. The TNFRs (bottom) are depicted as type I transmembrane proteins, characterized by cysteine-rich motifs in their extracellular domain. They are expressed as monomers that are thought to associate in trimers when interacting with their ligands.

fector and memory T cell responses as well as T cell homeostasis at multiple stages of the immune responses. It has become a critical immunotherapy approach to manipulate these co-signaling pathways to promote immune responses against viral infection and cancer or to reduce graft rejection and autoimmune diseases. Here, we will describe structures of co-signaling molecules in the context of their functions and therapeutic applications.

2 Molecular structure of costimulatory molecules

2.1 The B7/CD28 family members

All of the B7-like molecules and their receptors are type I transmembrane glycoproteins and are members of the immunoglobulin (Ig) superfamily. Members of the B7 family share 20%–35% identity in their amino acid sequences. Despite such a low homology in primary amino acid composition, these molecules share a similar secondary structure: single or multiple Ig V- and Ig C-like extracellular domains. Cysteine residues, which are involved in the formation of the disulfide bonds of the IgV and IgC domains, are well-conserved. A hallmark of the B7 family molecules is their uncharacteristic intracellular domain which does not share homology with highly diverse amino acid composition. The receptors for the B7 family are members of the CD28 family, and possess a single Ig V-like extracellular domain. Their cytoplasmic tails contain putative SH2- and SH3-motifs thought to be involved in signal transduction [6]. By crystallography and molecular modeling, tertiary structures for ligands and receptors of the B7-CD28 family have been determined.

The interactions of receptor--ligand pairs are mediated predominantly through residues in their IgV domains. In general, IgV domains are described as two-layered-strands with “front” and “back” sheets. The front and back sheets of CTLA-4 IgV domain consist of strands A′GFCC′ and ABEDC″, respectively, whereas the front and back sheets of the B7-1/B7-2 IgV domains are composed of strands AGFCC′C″ and BED, respectively. The binding faces between CTLA-4/CD28 and B7-1/B7-2 are dominated by the interaction of the CDR3 analogous loop from CTLA-4/CD28, centered on the MYPPPY motif, with the surface formed predominantly by the conserved residues between B7-1 and B7-2 on the G, F, C, C′ and C″ strands [7,8]. The strictly conserved MYPPPY sequence motif within the CDR3 analogous loops of CTLA-4 and CD28 is critical for recognition of theirs ligands B7-1 and B7-2. However, this hexapeptide motif is not conserved in other known B7/CD28 family receptors. The ligand binding site in ICOS was mapped to a region overlapping yet distinct from the ligand binding sites in CD28 and CTLA-4. A related FDPPPF sequence motif in the analogous position is identified as a major determinant for the binding of ICOS to B7-H2 [9]. The X-ray crystal structure of PD-1 shows that the β-strands of the Ig superfamily fold are well conserved between CTLA-4 and PD-1. However, the CDR3 loop in PD-1 is loosely ordered and does not have conserved amino acids. None of the PD-1 CDR3 amino acids was found to be important for binding PD-1 ligands by scanning mutagenesis [10].

Like B7-1 and B7-2, both B7-H1 and B7-DC bind to receptor PD-1. We [11] generated molecular models of the IgV-type domains of B7-H1 and B7-DC by homology (or comparative) modeling based on X-ray coordinates of human B7-1 and B7-2. Significant conservation of surface residues is observable on the IgV BED faces of B7-H1 and B7-DC, while the opposite A'GFCC'C" faces do not display significant residue conservation. By the site-directed mutagenesis, the PD-1 binding sites were mapped to the A'GFCC'C" faces closely corresponding to the CD28/CTLA-4 binding sites in B7-1 and B7-2. The residues forming the receptor binding sites are not conserved in B7-H1 and B7-DC, indicating significant structural heterogeneity of the interactions of PD-1 with B7-H1 and B7-DC.

CD28, CTLA-4, and ICOS are covalently linked homodimers due to the interchain disulfide mediated by the quivalent of Cys122 in CTLA-4. Crystal structures of CTLA-4/B7complexes contain bivalent homodimers of CTLA-4 with B7-binding sites located distally to the CTLA-4 dimeric interface, which suggests that the CTLA-4 homodimer binds to non-covalent homodimers of B7-1 or B7-2 to form a lattice of CTLA-4/B7 interactions [7,8]. Formation of such a lattice is thought to trigger the formation of stable signaling complexes as part of the immunological synapse. The structure [12,13] of the B7-H1/PD-1 and B7-DC/PD-1 complex highlights the overall similarity of binding of B7-H1 and B7-DC to PD-1. Despite the low sequence identity between CTLA-4 and PD-1, the PD-1/PD-1L and CTLA-4/B7 complexes exhibit gross similarities in overall organization, albeit with significant detailed differences. As observed in the CTLA-4/B7 complexes, the PD-1/PD-1L interfaces are formed by the front β-sheets of both the PD-1 and PD-1L IgV domains. The PD-1/PD-1L interfaces are formed by residues distributed over the front sheets of both molecules, in contrast to the proline-rich CDR3 loops in CD28, CTLA-4, and ICOS that make the majority of the contacts with their B7 ligands. Furthermore, consistent with the primary sequence differences (e.g. the lack of the proline-rich motif), the CDR3 loop in PD-1 makes few and no contacts with B7-H1 and B7-DC, respectively. Of particular note, the more acute angle between IgV domains in the PD-1 receptor-ligand complexes results in more compact assemblies with end-to-end distances spanning approximately 76 Å, as compared to the CTLA-4/B7 complexes that span approximately 100 Å. These differences in dimension must be accommodated in order to support the localization of both types of inhibitory complexes to the central zone of the immunological synapse. Notably,

the linker regions connecting the ectodomains and transmembrane segments are longer for PD-1 (20 residues) and B7-H1 (10 residues) and B7-DC(11 residues) than those present in CTLA-4 (6 residues) and B7-1 (9 residues), and could easily allow the PD-1/PD-1L complexes to span an end-to-end distance comparable to the linear dimensions of the TCR/MHC complex and other pairs of signaling molecules (i.e. approximately 100–140 Å) within the immunological synapse. Recruitment of CD28 to the immunological synapse is independent of B7-1 and B7-2 binding, although ligand binding is important for the accumulation of both CD28 and CTLA-4 at the synapse [14]. However, PD-1 extensively accumulates at the synapse only when T cells interact with dendritic cells (DCs) expressing high B7-DC levels, while B7-H1 is critically important when the DCs have little B7-DC [15]. Moreover, PD-1 can not form a covalent dimer as it lacks the analogous Cys residue. Whether PD-1 requires dimerization to transduce signals is unclear.

2.2 The tumor necrosis factor receptor/ligand superfamily members

There are six interactions in the TNF/TNFR superfamily that have gained prominence as positive regulators of T cells—that is,OX40 ligand (OX40L)/OX40, 4-1BBL/4-1BB, CD70/CD27, CD30L/CD30 and LIGHT/HVEM (herpes-virus entry mediator), GITRL/GITR. The TNF family ligands are type II (i.e. intracellular N terminus and extracellular C terminus) transmembrane proteins, characterized by a conserved C-terminal domain coined the 'TNF homology domain' (THD). The THD is a 150 amino acid long sequence containing a conserved framework of aromatic and hydrophobic residues. Among the co-stimulatory TNF family ligands, only the atomic-level THD structure of TNF is available [16]. The known THDs share a virtually identical tertiary fold and associate to form trimeric proteins. The THDs are β-sandwich structures containing two stacked β-pleated sheets each formed by five anti-parallel β strands that adopt a classical 'jelly-roll' topology. The inner sheet (strands A, A′, H, C and F) is involved in trimer contacts, and the outer sheet (strands B, B′, D, E and G) is exposed at the surface. Trimeric THDs are ~60 Å in height and resemble bell-shaped, truncated pyramids with variable loops protruding out of a compact core of conserved anti-parallel β strands. The trimer is assembled such that one edge of each subunit (strands E and F) is packed against the inner sheet of its neighbour to form large and mostly hydrophobic interfaces, resulting in a very stable interaction.

The TNF family receptors are primarily type I (extracellular N terminus, intracellular C terminus) transmembrane proteins. The extracellular domains are characterized by the presence of cysteine-rich domains (CRDs), which are pseudo-repeats typically containing six cysteine residues engaged in the formation of three disulfide bonds [17]. The number of CRDs in a given receptor varies from one to four, except in the case of CD30 where the three CRDs have been partially duplicated in the human but not in the mouse sequence. The repeated and regular arrangement of CRDs confers an elongated shape upon the receptors, which is stabilized by a slightly twisted ladder of disulfide bridges. The sequence alignment of TNF receptor family members in the absence of structural information is difficult because the spacing of cysteine residues is not always conserved between receptors. Each CRD contains two basic structural modules [18].

The first complex crystal structure of TNF/TNFR family that ligand (LTα) bound to its cognate receptor (TNFR1) reveals no contact between individual receptor chains [19]. Based on this, it was inferred that the ligands recruited or "cross-linked" three receptor monomers into the final 3: 3 complex. The functions of TNF/TNFR superfamily members depend on obligatory 3-fold symmetry that defines the essential signaling stoichiometry and structure. The regions of contact between ligands and receptors are very diverse among family members and contribute to the specific interactions of ligand–receptor pairs. However, prediction of receptor–ligand interactions is not straightforward as different ligands bind the same receptor (e.g. both TNF and LTα bind TNF-R1) and almost identical ligands bind different receptors.

3 Structure, functions and therapeutic application of T cell co-stimulatory molecules

3.1 The B7--CD28/CTLA-4 pathway

The most extensively characterized co-signaling pathway is B7--CD28/CTLA-4 pathway, in which both B7-1 and B7-2 ligands bind to receptors CD28 and CTLA-4 [6, 20]. Engagement of CD28 on naive T cells by either B7-1 or B7-2 ligands on antigen-presenting cells (APCs) provides a potent costimulatory signal to T cells activated in the presence of T cell receptor (TCR) engagement, which results in productive T cell responses. CTLA-4 is not expressed on naïve T cells, but rapidly up-regulated following T cell activation. Meanwhile, CTLA-4 exhibits an affinity for B7-1/B7-2 that is 10 times that for CD28. Once CTLA-4 is expressed on activated T cells, CTLA-4 competes with CD28 for binding to B7s to block stimulatory signal of CD28, or directly delivers negative signals to activated T cells to limit or terminate the immune responses and prevent autoimmune diseases. The function of CTLA-4 as a negative regulator of T cell activation has been demonstrated in CTLA-4-deficient mice, which die within 3–4 weeks of birth from massive lymphocytic infiltration and tissue destruction in critical organs [21,22]. A study showed that infusion of human CTLA-4- neutralizing mAb into patients with melanoma induced a broad spectrum of autoimmune-like responses [23]. Abatacept, which is the CTLA-4-immunoglobulin (Ig)

fusion protein (CTLA-4Ig), is an approved drug for the treatment of rheumatoid arthritis by the Food and Drug in 2006 and by the European Medicines Agency in 2007 [24].

Although the early studies demonstrated that CTLA-4-Ig was efficacious at preventing transplant rejection in various murine models [25], the subsequent studies in primates did not yield the same robust results [26]. *In vivo* studies suggested that CTLA-4Ig did not completely block T-cell activation and proliferation, and large doses of CTLA-4Ig were needed to produce an effect [27]. While CTLA-4 expressed on the cell surface binds with much higher avidity to both B7-1 and B7-2 than dose CD28, CTLA-4Ig is ~100 fold less potent in inhibiting B7-2-mediated costimulation than B7-1 [28]. Because of the identification of the contact residues between CTLA-4 and B7-2 [29], modification of these residues with the intent of increasing the affinity of CTLA-4Ig for B7-2 became theoretically possible. For these reasons, mutagenesis and screening were performed on individual amino acids within the CDR1- and CDR3- analogous loops and the region C-terminal to the CDR3- loke loop, as these regions compose the B7-2 binding domain. A CTLA-4Ig variant, LEA29Y (belatacept), was developed with two amino acid substitutions at L29Y and L104E. LEA29Y binds approximately two fold more avidly to B7-1 and four fold more avidly to B7-2, resulting in 10 fold more increase in its ability to inhibit T-cell activation in vitro when compared with CTLA-4Ig [30]. LEA29Y significantly prolongs the renal allograft survival in a preclinical primate model as compared to CTLA-4Ig. LEA29Y also showed efficacy as the mainstay maintenance immunosuppressive agent in a non-human primate model of neonatal porcine islet xenotransplantation [31]. Based on these encouraging results in non-human primate models, LEA29Y (belatacept) moved into phase II clinical trials to evaluate the efficacy of this drug in kidney transplant recipients.

3.2 The B7-H1/B7-DC—PD-1 pathway

B7-H1 was first identified as a T cell costimulatory molecule that augments human T cell proliferation in the presence of either anti-CD3 or alloantigens *in vitro* [32]. Later, a closely homolog, B7-DC was also found to have similar costimulatory effects, and both bind to a receptor, programmed death-1 (PD-1) expressed on activated T cells [33]. However, their receptor PD-1 is believed to be an inhibitory receptor because of the phenotypes of lymphoproliferative/autoimmune diseases in PD-1-deficient mice [34,35]. Several studies indicated a negative regulatory role for B7-H1 and B7-DC in T cell responses *in vitro*. Costimulation of T cells with B7-H1 or B7-DC inhibits TCR-mediated proliferation and cytokine (IL-2, IL-4, IL-10, and IFN-γ) production and results in cell cycle arrest [36,37]. These seemly contradictory data, however, could be best interpreted by expression of the additional costimulatory receptor on T cells other than PD-1. This hypothesis is supported by the study that costimulation of B7-DC in conjunction with B7-1 for cytokine production is a PD-1 independent event [38]. Using computer modeling and a site-directed mutagenesis approach, we identified mutants of B7-H1 and B7-DC with abolished PD-1 binding capacity. Interestingly, these non-PD-1 binding mutants are still able to costimulate proliferation and cytokine production of T cells from normal or PD-1–/– mice at a comparable level to the wild type of B7-H1 and B7-DC [11]. These studies suggest that B7-H1 and B7-DC costimulate T cell growth through a receptor other than PD-1. Unexpectedly, B7-H1 was found to bind to B7-1 in both mouse and human cells, but B7-DC did not. Even the affinity of the association of B7-1 and B7-H1 is higher than that between B7-1and CD28, but lower than that between B7-1 and CTLA-4 and between B7-H1 and PD-1. However, upon engagement by the other molecule, both B7-H1 and B7-1 appear to be able to deliver inhibitory signals in the T cells that express them [39]. These results blur the current definitions of receptors and ligands and introduce an additional level of complexity in the interaction between the costimulatory molecules and the inhibitory pathways that they control.

B7-H1 and B7-DC have distinct expression profiles and biological roles *in vivo*. B7-H1 is broadly expressed in the lymphoid and non-lymphoid tissue while B7-DC is restrictedly expressed in DCs and monocytes. B7-DC has been suggested to promote tumor immunity via a PD-1-independent mechanism in an animal tumor model, while B7-H1 is highly expressed by human tumor tissues [40]and correlates with poor prognosis in ovarian cancer, renal cancer, urothelial carcinoma, pancreatic cancer, non-small cell lung cancer, and gastric carcinoma. Blocking B7-H1 by monoclonal antibodies enhances the anti-tumor immune response against B7-H1 expression tumor in mice [41]. PD-1 is highly expressed on exhausted T cells in chronic viral infection, which contributes to dysfunction (exhaustion) of viral specific T cells and lack viral control. Blocking B7-H1/PD-1 interactions *in vitro* reverses the exhaustion of HIV, HBV, HCV, and SIV-specific T cells and restores their functions, and *in vivo* substantially reduces the viral burden in chronic LCMC infection [42,43]. These results suggest that the B7-H1/PD-1 pathway is a potentially attractive therapeutic target for treatment of tumor and chronic viral infections.

B7-H1 and B7-DC may positively or negatively regulate T cell responses by binding to the same and different inhibitory or stimulatory receptors. The more selective enhancing or preventing binding of B7-H1/B7-DC to different receptors will be more crucial for manipulation of this costimulatory pathway for therapeutic application. Although the crystal structure highlights the overall similarity between the B7-H1/PD-1 and B7-DC/PD-1 complexes, receptor PD-1 binding residues are considerably more variable in the B7-H1 and B7-DC. Thus, PD-1 interactions with B7-H1 or B7-DC must vary significantly at the molecular

level of detail. Considering the unusual degree of variability among residues crucial for binding to the same receptor, interactions between receptors and ligands should be more permissive to modulation. In this case, two single-point mutants of the PD-1 receptor were generated to exhibit novel biochemical properties. The mutant A99L exhibited two- and three fold higher affinity for B7-H1 and B7-DC, respectively. Another mutant L95R exhibited wild type affinity for B7-DC, but essentially no binding to B7-H1 [10]. We have also generated B7-H1 mutant proteins with three- to four-fold higher affinity for PD-1 than wild-type [11]. The remarkable uncoupling of ligand recognition provides a novel opportunity to dissect the distinct roles of the two PD-1 ligands. Soluble forms of these mutant proteins (e.g. Ig fusions) are supporting mechanistic studies by allowing for the manipulation of the PD-1/PD-1L pathways in cell-based and animal model systems, and may afford new targeted therapeutic approaches.

4 Perspectives

We have realized that costimulatory pathways not only regulate the initial activation of naive T cells but also control effector, memory, and regulatory T cells. Cancer cells or pathogens utilize the inhibitory signaling pathways to impair the host's defensive immune responses. The critical roles of costimulation in regulating immune responses resulted in exponential growth of the studies in this field. More and more co-signaling pathways have been identified. A costimulatory molecule could promote or inhibit T cell responses by binding to specific stimulatory or inhibitory receptors. A receptor can be engaged with different ligands. The interactions between two ligand molecules or between the members of B7—CD28/CTLA-4 and TNF—TNFR family have also been identified. With so many overlapping positive and negative signaling pathways, a hierarchy may exist in the orchestration of these signals by different interaction avidity of ligand and receptor binding except different dynamic expression of the costimulatory molecules. These provide more opportunities for specific and selective manipulation of costimulation. The more structural understanding of these events will provide a basis for studying the biological functions of the costimulatory pathways and encourage their application in therapy.

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