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Review

T cell costimulation by CD28, CTLA-4, and ICOS

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= Abstract =

T cells play a central role in the initiation and regulation of the immune response to foreign antigens. Full activation of T cells requires the engagement of T cell receptor complex (TCR) and the binding of a second costimulatory receptor to its ligand expressed on antigen presenting cells (APC). Among the molecules known to provide costimulatory function, CD28 has been the most dominant and potent costimulatory molecule. However, the function of CD28 is becoming more complex due to the recent discovery of its structural homologue, CTLA-4 and ICOS. This review summarizes the biology and physiologic function of each of these receptors, and further focuses on the biochemical mechanism underlying the function of these receptors. Complete understanding of the CD28/CTLA-4/ICOS costimulatory pathway will provide the basis for developing new therapeutic approaches for immunological dieseases.

INTRODUCTION

The immune system is an exceptional defense mechanism found in higher vertebrates. It provides the highly specific and protective responses against the myriad of pathogenic microorganisms that coexist in the world we live. Thus, it has been a long sought after question to understand the mechanism of which the particular immune cells recognize, process, and develops immunity against the foreign antigens. The immune system consists of a wide range of distinct cell types, each with important roles to play. Among these cell types, the lymphocytes play a central role since they determine the specificity of immunity and orchestrates into the development of the effector and memory cells (adaptive immunity). T lymphocytes and B lymphocytes are catagorized into this group as they are able to mount both specificity and memory phenotype against foreign antigens. Cells that interact with these lymphocytes, so called "Antigen Presenting Cells (APC)" are also important since they are able to present antigens to the lymphocytes. These APCs also provide the first and immediate barrier to protect from foreign antigens due to their intrinsic phagocytic ability (non-adaptive immunity). Among the cells in "non-adaptive immunity" are macrophages/monocytes, natural killer (NK) cells, dendritic cells, polymorphonuclear (PMN) cells, neutrophiles, etc.

T cell activation is a critical event in the organization of effective cellular and humoral immune responses. Activated T cells are essential for provision of T cell help, promoting the development of high-affinity antibody production, and the generation of cytotoxic T cell responses. Accordingly, the defects in proteins required for T cell activation give rise to significant infectious pathology and malignancies. However, the decision to allow T cell activation also has potentially dangerous consequences for the host and thus also need to be tightly controlled. Defects in proteins involved in regulating T cell activation therefore often results in autoimmunity. Thus, the major challenge faced in regulating T cell response is how to maintain a

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sufficiently large immune repertoire capable of recognizing all possible foreign antigens, whilst at the same time maintaining T cells in an unresponsive state towards self antigens.

The "two signal hypothesis", composed of specific antigen recognition (signal 1) by the T cell receptor and ligation of additional co-stimulatory signals (signal 2), has been accepted widely now for the requirement of successful T cell activation (1). It is now becoming clear that these co-stimulatory receptors play an important role in controlling the fate of T cell activation, generation and differentiation into effector cells as well as regulating the reactivity against self antigens. Thus, the past decade has been devoted to understand the mechanism underlying the co-stimulatory pathway and apply this concept into the disease situation.

1. CD28

One of the most potent co-stimulators known to date is CD28, a molecule expressed constitutively on the surface of T cells (2). CD28 is composed of two glycosylated 44 kDa chains which are the members of the immunoglobulin superfamily, each containing a single disulfide-linked extracellular Ig variable-like domain. The mature CD28 polypeptide contains 202 amino acids giving a molecular mass of 23 kDa, which is then glycosylated at five sites to give the molecular weight of the mature protein. The extracellular domain is linked via a single transmembrane regions to a 41-amino acid cytoplasmic domain which is presumed to be responsible for initiating costimulatory signals (3). Sequence comparison between human, rat, mouse, and chicken CD28 cytoplasmic domains demonstrates high interspecies conservation, suggesting a crucial role for this domain in signal transduction.

CD28 interacts with two different ligands, B7-1 (CD80) and B7-2 (CD86), both of which are expressed on the surface of APC (2). Ligation of CD28 alone has little effect on resting T cell proliferation, but CD28 ligation in the presence of limited concentration of anti-CD3 or antigen promotes T cell proliferation and IL-2 production by regulating IL-2 mRNA at the level of transcription and translation (4). Co-stimulation via CD28 also mediates strong upregulation of IL-4, IL-5, IL-13, γ-interferon, tumor necrosis factor α, and GM-CSF (5-7). In addition, CD28 upregulates the IL-2 receptor α (8) and β chains (9).

CD28 also has been shown to prevent "anergy", the unresponsive state of T cells *in vitro*. By blocking CD28 interaction using specific antagonists, T cells have been shown to enter into anergic state (10). In contrast, anergic T cells could be rescued from this non-responsive state by the addition of exogenous IL-2 or by CD28 co-ligation, indicating that anergic cells are functionally intact and that unresponsiveness was limited to specific antigen (11). The role of CD28 in controlling T cell responsiveness has also been confirmed by the blocking study which demonstrated the suppression of humoral responses, delaying allograft rejection and blockade of xenograft rejection, and inhibiting the progression of autoimmune diseases (12-14). In addition, a number of *in vivo* experimental systems have suggested the role of CD28 in generating anti-tumor (15). CD28 also has a fundamental role in the early development and differentiaton of both T helper (Th) 1 and T helper (Th) 2 T cell subsets (16-18). In the absence of CD28 signaling, naïve T cells are biased toward Th1 phenotype.

It has been an intensive issue to delineate the signal transduction pathway initiated from CD28 ligation. It is possible that distinct signaling pathways may exist to lead complementation between T cell receptor (TCR) and CD28 to allow full T cell activation. However, it is also feasible that CD28 may enhance the amplitude or duration of a TCR-triggered signal, thereby crossing a threshold to activate downstream signaling cascade. It has been shown that the signaling of CD28 might be regulated by protein tyrosine kinases (19, 20). Using Abs specific to CD28, crosslinking of CD28 resulted in tyrosine phosphorylation of 62kDa, 75kDa, and 100 kDa. However, neither the identity of these proteins nor the role of these tyrosine phosphorylation in the function of CD28 has been shown. Similarly, phospholipase C (PLC)-γ (21, 22) and p21ras pathway were shown to be

activated by anti-CD28 Abs (23) using anti-CD28 Ab crossliking. However, these signaling events could not be reproduced when its natural ligands, B7-1 and B7-2, were used. Thus, the significance for these pathways needs to be validated under the physiological condition. CD28 has also been shown to bind to the p85 regulatory subunit of PI 3-kinase through its phosphotyrosine-SH2 domain interaction of PI 3-kinase. As a result, CD28 engagement resulted in activation of PI 3-kinase and production of D-3 phosphoinositide lipids . However, once again the importance of the PI 3-kinase activation in CD28 function is not clearly understood at present.

2. CTLA-4

CTLA-4 was cloned during a subtractive hybridization screen designed to identify genes involved in cytotoxic T lymphocyte (CTL) function (25). However, despite its origin, CTLA-4 is expressed by both CD^{4+} and CD^{8+} T cells. CTLA-4 is highly homologous to CD28 with overall nucleotide sequence homology of 75% and even shares similar genomic structures with CD28. CTLA-4 possesses the B7 ligand binding motif "MYPPPY" in its extracellular domain, which renders binding to both B7-1 and B7-2. Despite the homology, CTLA-4 has been shown to function as a negative regulator of T cell function. CTLA-4 engagement results in inhibition of T cell proliferation, reduction in IL-2 and IL-2R expression, and blockade in cell cycle progression activation (26, 27). The most compelling evidence for a regulatory function for CTLA-4 has come from CTLA-4 knockout mice that develop fatal lymphoproliferative disease at 3-4 weeks of age (28, 29). This phenotype results from polyconal activation of peripheral T cells that then infiltrate and cause multiorgan destruction. This disease now was shown to be effectively cured by preventing CD28 costimulation using CTLA-4-Ig, B7-1/B7-2 double KO mice, or by crossing onto single TCR transgenic mice (29-31). In addition, the lymphoproliferation has been suggested to be CD^{4+} dependent (32). Very recently, CTLA-4 blockade in normal mice has been shown to give rise to spontaneous autoimmunity (33). Taken together, these studies suggest that one function of CTLA-4 may be to eliminate the weak TCR engaged cells that may cause self-reactivity in the periphery.

One of the fundamental difference between CD28 and CTLA-4 is its expression pattern. While CD28 is expressed constitutively on the surface of resting T cells, CTLA-4 only become expressed after activation. After T cell activation, CTLA-4 message is being upregulated and its protein made within 6 hours. However, the actual detection of CTLA-4 proteins on the cell surface does not begin until 24 hours following activation. It has been shown that in order to mount negative effect, CTLA-4 needs to be engaged prior to 16 hours after TCR stimulation (34). Thus, it seems likely that the low amount of CTLA-4 protein on the cell surface is potent enough to elicit its negative function. One of the reasons for CTLA-4 being highly efficient may be related to its high avidity. It has been shown that CTLA-4 binds to B7 molecules with approximately 20 fold higher avidity (Kd 5-10 nM) than CD28 (35). Thus, upon CTLA-4 expression, B7 binding will be favored toward CTLA-4, not CD28. This may be why the expression of CTLA-4 is more tightly controlled in the cell. Transcription of the CTLA-4 gene, stability of its mRNA, and intracellular trafficking of CTLA-4 proteins synthesized all are under tight activation control. Indeed, CTLA-4 is subject to rapid endocytosis following its surface expression due to its binding to AP-50, a component of AP-2 adaptor complex involved in clathrin-mediated endocytosis (36). Therefore, cell surface CTLA-4 is regulated through multiple pathways.

3. How does the homologous CTLA-4 deliver inhibitory signals?

At least four mechanisms have been proposed to explain the inhibitory activity of CTLA-4: competition for ligand binding to CD28, thus preventing CD28 from binding to B7-1 and B7-2; "stealing" of signaling molecules away from CD28 via the rapid endocytosis of CTLA-4; delivery of a signal that antagonizes CD28 signal; delivery of a signal that antagonizes T cell receptor (TCR)-mediated signal. Using TCR trangenic

mice bred onto recombinase activating gene 2-deficient/ CD28-deficient background, Fallarino et al. have shown that CTLA-4 can potently inhibit T cell activation in the absence of CD28, indicating that antagonism of a TCR-mediated signal is sufficient to explain the inhibitory effect of CTLA-4 (37). Thus, it is likely that CTLA-4 generates active signaling to interfere with TCR-initiated signal transduction cascade. Another piece of evidence for active intracellular signaling comes from the data of Masteller et al (38). In their study, the mutant lacking cytoplasmic domain of CTLA-4 (tailless CTLA-4) was introduced into CTLA-4 deficient mice, which normally dies within 3-4 weeks of age due to severe lymphoproliferative disorder. Interestingly, the CTLA-4 KO mice possessing tailless CTLA-4 were rescued from acute lymphoproliferation and showed no multiorgan destruction, but exhibited lymphoadenopathy and accumulated large numbers of activated T cells similar to those seen in CTLA-4 KO mice. These data suggest that the function of CTLA-4 may be regulated at the two steps; at the level of ligand competition with CD28 and active intracellular signaling by CTLA-4's cytoplasmic domain.

The cytoplasmic tail of CTLA-4 contains 36 amino acids and is 100% conserved among all the species examined. This indicates that the cytoplasmic tail of CTLA-4 is important in initiating its signaling. Indeed, several motifs has been identified within the intracellular domain of CTLA-4 that may contribute to signal transduction. It contains two tyrosine residues both of which are targets for src family tyrosine kinases, including lck and fyn (39). Upon tyrosine phosphorylation, other molecules may bind through their SH2 domains, such as PI 3-kinase (40) and a protein tyrosine phosphatase SHP-2 (41, 42).

We have shown recently that CTLA-4 forms multimeric complexes with T cell receptor (TCR) ζ chain and a tyrosine phosphatase, SHP-2, in activated T cells (39, 42). Using a series of *in vitro* transfection studies, we were able to demonstrate that co-associated SHP-2 can regulate CTLA-4-associated TCR ζ phosphorylation. Indeed, CTLA-4 expressed in activated T cells was shown to be associated with lower phosphorylated species of TCR ζ (mainly p16), but not with the highly phosphorylated p23 TCR ζ. Since TCR ζ phosphorylation serves as a critical proximal signal transducer upon TCR stimulation, we hypothesized that by lowering the extent of TCR ζ phosphorylation CTLA-4 may ultimately antagonize TCR signal transduction, and result in T cell inhibition. This model has been supported by the demonstration that phosphorylation of the two downstream effectors of TCR signaling pathways, LAT and ERK, was significantly reduced in activated T cells upon CTLA-4 crosslinking (42). As complete phosphorylation of TCR ζ into p23 TCR ζ is the critical and very proximal step leading to full T cell activation (43, 44), the ability of CTLA-4 to regulate TCR ζ phosphorylation may be one of the most efficient ways to turn down the T cell activation process.

In an attempt to identify the domains involved in CTLA-4 and TCR ζ binding, we found that the interaction of CTLA-4 and TCR ζ is mediated through their membrane proximal domains, involving lysine-rich positively charged 7 amino acids (KMLKKRS) of CTLA-4 and the proximal tyrosine-containing cytoplasmic tail of TCR ζ. Since the KMLKKRS motif exists in highly charged states at physiologic pH (pI; 11.26), we hypothesized that this motif would interact with the tyrosine-containing motif of TCR ζ that is negatively charged in the cell. The KMLKKRS sequence, which possesses four positively charged amino acids, may form a pocket to favor recruitment of the negatively charged phospho group of ITAM 1 tyrosine motifs or to a lesser extent other ITAM tyrosines in TCR ζ. However, although phosphorylation enhances the binding significantly, it does not seem to be necessary as unphosphorylated TCR ζ still binds to CTLA-4 at the reduced level. Similar electrostatic interaction has been previously reported in Ly-49 binding with DAP-12 in NK cells (45-47) and in binding of CD43, CD44, and ICAM-2 with Ezrin/Radixin/Moesin (ERM) proteins (48).

Interestingly, we found that association of SHP-2 with CTLA-4 is mediated through the distal domain of CTLA-4 involving additional 11 amino acids downstream

of KMLKKRS. Since SHP-2 has been shown to bind to YVKM motif located within this 11 amino acids (41), it is tempting to speculate that SHP-2 binding is mediated through this region. Indeed, Imboden's group have shown that CTLA-4 was not able to function in the T cell line expressing only 7 amino acids (KMLKKR) of CTLA-4 and that the downstream 11 amino acids were critical for optimal CTLA-4 function (49). Thus, our data suggest that TCRζ association alone may not be sufficient for CTLA-4 function, and that the association of SHP-2 or other related tyrosine phosphatase through downstream of KMLKKRS motif is required for optimal function. Therefore, the manipulation of TCRζ and SHP-2 binding sites of CTLA-4 may provide a basis for the novel therapy designed for immunological disorders as well as organ transplantation.

4. ICOS

The role of CD28/CTLA-4/B7 co-stimulatory pathway has been complicated by the recent discovery of the related homologue, ICOS (inducible co-stimulator) (50, 51). Despite its homology with CD28 and CTLA-4, ICOS does not bind to B7-1 or B7-2 but to its own specifc ligand B7-h/B7RP1 (52, 53), GL50 and LICOS (54). B7-h was found to express on APCs such as B cells, macrophages as well as dendritic cells (56). ICOS ligation has been shown to exert co-stimulatory effect on T cells including proliferation, secretion of cytokines (IL-4 and IL-10), upregulation of early T-cell activation markers such as CD69, CD25, and CD71, and effective help for antibody secretion by B cells . However, unlike CD28, ICOS did not induce IL-2 secretion but superinduced IL-10, a B cell differentiation factor. These data suggest that ICOS may play a somewhat overlapping but distinct role as compared to CD28, and may be important in T cell/B cell interaction (53).

Three recent studies specifically addressed the functional role and importance of ICOS by generating ICOS-deficient mice. Both Tafuri et al (57) and McAdam et al (58) have shown that absence of ICOS results in severely deficient T cell-dependent B-cell responses such as lack of germinal center formation and Ig class switching. Similarly, separate studies by Dong et al., (59) have dmonstrated that ICOS has a critical role in T cell activation and proliferation. ICOS-KO T cells are defective in anti-CD3-mediated proliferative responses and failed to produce IL-4 when differentiated in vitro or primed *in vivo*. Consistent with this finding, ICOS was found to be essential in order for mice to mount humoral immune responses after immunization with several antigens (59).

Like CD28, ICOS has also been implicated in Th2 differentiation. McAdam et al. (58) reported that blocking of ICOS/B7-h interaction using ICOS-Ig produced more IFNγ and less IL-4 and IL-10 while stimulation of ICOS using B7h-Ig bound to anti-CD3-coated beads resulted in enhanced proliferation and secretion of IL-4 and IL-10. In addition, ICOS is expressed at higher level on Th2 cells versus Th1 cells, suggesting that ICOS may be important in Th2 cell differentiation. Indeed, recent studies by Coyle et al (60) have demonstrated that ICOS provided co-stimulatory signal to highly polarized Th2 cells, but not to Th1 cells. Thus, a paradigm has developed in which ICOS provides a unique role in Th2 costimulation.

From these data, it is becoming clear that ICOS is essential in mounting successful immune responses. Although ICOS shares many features with CD28, its distinct cytokine profile, effect on T cell proliferation, and its synergistic effect with CD28 costimulation on IL-4 production provide evidence for the existence of potentially distinct signaling pathways of these two related costimulatory molecules (61). In contrast to CD28, ICOS shares a similar pattern of expression with CTLA-4. Both CTLA-4 and ICOS are expressed upon T cell activation and were shown to express on memory cells (53, 62). These data suggest that CTLA-4 and ICOS may play an important role in effector responses whereas CD28 is essential during priming stage.

Despite the accumulating data regarding the role of ICOS in regulating immune responses, the biochemical basis of ICOS function is far from clear. In order to understand the molecular basis underlying ICOS-induced co-stimulation, we have examined the biochemical

characteristics of ICOS in comparison with those known to CD28 and CTLA-4. We showed that ICOS is expressed on both $CD4^+$ and $CD8^+$ T cells upon activation (63). The molecule was localized primarily on the cell surface but significant amounts also exist in intracellular organelles due to its association with AP-50, a component of AP-2 adaptor complexes involved in clathrin-mediated endocyosis. As a result, ICOS is internalized with a temporal kinetics similar to that of CTLA-4. Interstingly, we found that both CTLA-4 and ICOS bind to TCRζ in activated T cells but with distinct specificity, leading to the speculation that ICOS may compete for TCRζ binding with CTLA-4, and either initiate its own signaling or prevent CTLA-4 signaling. Our data showed that unlike CTLA-4 which was associated with lower phosphorylated p18 and p21 TCRζ, TCRζ bound to ICOS contained p23 forms, suggesting that ICOS failed to regulate dephosphorylation of TCRζ. From these data, it is tempting to speculate that ICOS prevents CTLA-4 signaling by limiting the access of TCRζ. In this regard, recent findings by Carl June's group strongly support our hypothesis. They showed that CTLA-4 ligation blocked ICOS costimulation not only by inhibiting cytokine production but also by inhibiting cell proliferation (64). Thus, ICOS represents another class of rapidly internalized regulatory cell surface molecules that may exert its action through competing with CTLA-4.

CONCLUSION

In recent years, our understanding of the T cell-mediated immune response has escalated tremendously. This achievement was possible from the numerous studies in various filed of T cell biology employing the techniques of molecular biology, cellular immunology, cellular biology, biochemistry, etc. It has now become evident that complete T cell activation requires two distinct signals. Signal one requires engagement of the T cell receptor, and signal two requires a second costimulatory signal. For the past decade, many T cell specific molecules exhibiting costimulatory function, such as CD28, LFA-1, SLAM, 4-1BB, OX40, CC27, have been identified (reviewed in (65)). Among these molcules, CD28 has been thought to be a principle mediator of this costimulatory signal. However, the CD28/B7 system is increasingly complex due to the identification of homologous CTLA-4 and ICOS and their ligands with positive and negative signaling activities.

Now it is evident that CD28 plays a critical role in the initial activating phase while CTLA-4 and ICOS are important in regulating later events; effector-memory phase. Exactly how these molecules perform differential functions is still an open question, but one mechanism may be via regulating the threshold for TCR activation. While CD28 lowers the threshold, CTLA-4 increases the threshold for T cell receptor signaling. The function of ICOS as a positive regulator of TCR signaling may become important as the T cell activation and differentiation progresses. These data imply that each costimulator molecule functions at distinct stage of T cell activation and displays the complex interplay during the process, which ultimately leads to the end outcome of T cell responses. Therefore, the manipulation of the CD28/CTLA-4/ICOS pathway has important therapeutic implications and may add to the growing field of selective immunotherapeutics.

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