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Two pathways of costimulation through CD28

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Abstract CD28 is recognized as the primary costimulatory molecule involved in the activation of naïve T cells. However, the biochemical signaling pathways that are activated by CD28 and how these pathways are integrated with TCR signaling are still not understood. We have recently shown that there are at least two independent activation pathways induced by CD28 costimulation. One is integrated with TCR signaling in the context of the immunological synapse and is mediated through transcriptional enhancement and the second is mediated through the induction of mRNA stability. Here, we review the immunological consequences and biochemical mechanisms associated with CD28 costimulation and discuss the major questions that need to be resolved to understand the molecular mechanisms that transduce CD28 costimulation.

Keywords Antigen presentation · CD28 · Costimulation · Immunological synapse · mRNA stability · Signal transduction · T cells · Transcriptional regulation

T cell activation requires the recognition of specific peptide-major histocompatibility complexes (MHC) displayed on the surface of antigen presenting cells. Because foreign protein antigens must compete with self-proteins for binding to MHC, T cells have evolved to recognize very low numbers of specific peptide-MHC complexes. This low number of receptor/ligand interactions and the relatively low affinity of T cell antigen receptor (TCR) for peptide-MHC complexes are not sufficient to allow for intercellular interactions. Effective T cell activation requires the participation of a variety of cell surface accessory molecules that form receptor/ligand pairs between T cells and antigen presenting cells (APC). These accessory molecules mediate two important functions. First, they provide

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adhesion to allow for the formation of stable T cell:APC conjugates. Second, they provide costimulatory signals that work in concert with TCR signaling to promote T cell activation and differentiation. Clearly all proteins that interact between the two cells will provide some contribution to intercellular adhesion. In contrast, only a subset of the accessory molecules have been documented to provide effective costimulatory functions. Although the basic concept of costimulation is well established, the specific molecular mechanisms whereby costimulatory molecules influence T cell activation and differentiation events are not fully understood.

CD28 costimulation

The two signal model and the concept of costimulation are well engrained in our understanding of the regulation of T cell activation and tolerance. T cell encounter with peptide-MHC ligands in the absence of an ongoing innate immune response generally does not lead to effective T cell activation and rather favors the induction of tolerance. One of the key consequences of the innate immune response is the upregulation on dendritic cells of CD80 and CD86, the ligands for CD28. Because CD28 is the major costimulatory molecule expressed on naïve T cells, CD28 can be viewed as the T cell-associated receptor for detection of the presence of a pathogen. At the same time, dendritic cells in the T cell zones of the lymph nodes can express high levels of CD80/CD86. However, as we and others have recently shown, engagement of CD28 requires continued stimulation through the TCR [1, 2]. Thus, this synergistic cross talk between TCR and CD28 provides a mechanism for coincidence detection to regulate T cell activation and control the initiation of T cell immune responses.

CD28 has been shown to have important functional consequences on T cell activation [3–6]. CD28 costimulation leads to a dramatic upregulation in IL-2 expression mediated by enhanced transcription and mRNA stabilization [7, 8]. CD28 costimulation also plays an important role in T cell survival, inducing expression of the anti-apoptotic protein Bcl-XL [9], and can regulate the metabolic activity of T cells [10]. T cell activation in the absence of CD28 leads to T cell anergy rather than activation, and costimulation through CD28 can protect against anergy induction [11, 12]. CD28 plays a key role in the generation of Th2 responses [13]. Finally, CD28 is required for the thymic maturation of NKT cells [14] and T regulatory cells [15]. Because CD28 is expressed on naïve T cells, it plays a critical role in initial T cell priming. Once T cells are activated, additional costimulatory molecules are upregulated, including ICOS, OX40, and 41BB that can enhance T cell survival, expansion and/or effector function [16, 17].

The impact of CD28 costimulation on T cell function can sometimes appear paradoxical. For example, CD28 costimulation is required to protect T cells from the induction of anergy, but is also required for tolerance induction [18]. CD28 is not required for thymic maturation of conventional CD4 and CD8 T cells, but is essential for the development of NKT and regulatory T cells [14, 15]. CD28 costimulation plays an important role in promoting Th2 differentiation, but is not required for Th2 effector cytokine secretion. The opposite is true for Th1 cells, which can differentiate in the absence of CD28, but require CD28 for subsequent IL-2 secretion. Thus, although the importance of CD28 costimulation is well recognized, the molecular mechanisms whereby CD28 can regulate T cell development, activation, expansion, and differentiation and how these molecular mechanisms are integrated with signals from the TCR are not understood.

Attenuation of the immune response during aging

The most striking changes in the immune response in the elderly are in the reduction of immune response to newly encountered antigens. This creates a major health problem in this population because of the frequency of exposure to new variants of highly mutable viruses, such as influenza and RSV, emerging pathogens, such as SARS, opportunistic bacterial infections, and possibly cancer. The reduced immune response during aging is a multifactorial process, including reduced hematopoietic stem cells and lymphoid precursors, thymic involution, reduced T and B cell proliferation, germinal center formation, and effector cell maturation, decreased cytokine signaling, increased ratio of memory to naïve T cells, and overproduction of regulatory T cells [19–27]. In spite of the potential complexity of understanding the relative importance and potential interplay among all of the factors associated with immune senescence, a major component of the reduced immune response in the elderly may be associated with a failure to provide CD28 costimulation. In mice, adoptive transfer experiments have identified an intrinsic defect in activation of naïve CD4 T cells [28–32]. Interestingly, activation of the innate immune response and, presumably, associated CD28 costimulation can restore antigen responses in T cell from old mice that have been adoptively transferred into young animal [31]. In addition, the defect in T cell proliferation and effector T cell generation in vitro can be rescued by the addition of exogenous IL-2 [33], an analogous phenotype to T cell activation in the absence of CD28 costimulation [34]. In humans, aging is associated with a downmodulation of CD28 expression [35]. CD28-null T cells accumulate with age and up to 70% of CD4 and 95% of CD8 T cells do not express CD28 [35]. Even in young adults 20–30% of CD8 T cells can be CD28-null. CD28-null cells are thought to be generated by repeated antigen stimulation and so represent a form of memory T cell. These T cells are replicatively senescent with highly eroded telomeres, but are also resistant to apoptosis, which allows them to accumulate to high numbers. Functionally, CD28-null T cells are hyporesponsive, have defects in effector function, and may be immunosuppressive [35, 36]. Taken together these results from both animal models and human samples indicate that diminished CD28 costimulation may contribute to attenuation of immune responses in the elderly.

Biochemical events associated with CD28 signaling

In spite of the current understanding of T cell signaling events and long-term interest in CD28 costimulation, the biochemical events associated with CD28 costimulation are still not well understood. CD28 signaling is associated with several different protein interaction motifs in the cytosolic tail that can mediate recruitment and activation of downstream signaling proteins (Fig. 1). The most studied signaling pathway downstream of CD28 is activation of PI3K through SH2 domain interactions of p85 with phospho-Y170 within the YxxM motif [37, 38]. PI3K activation is predicted to impact on T cell activation through the enhanced recruitment of PH domain proteins to the membrane, including Itk and Akt. Itk phosphorylates and activates PLCγ, increasing the calcium and PKC responses [39, 40]. Sustained calcium signaling leads to calcineurin-dependent nuclear localization of NFAT. PKCθ plays an important role in the upregulation of NF-κB and AP1 [41]. Akt is a central regulator of cell activation, proliferation, and survival [42]. In T cells, Akt is thought to mediate the CD28-mediated, PI3K-dependent inhibition of the cell cycle regulator, $p27^{KIP}$, that promotes cell cycle entry and progression [43], as well as the pro-survival factor,

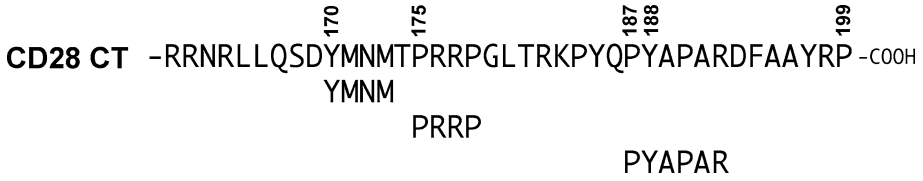

Fig. 1 Signaling motifs in the cytosolic tail of CD28. The sequence of the cytosolic tail of CD28 and three potential docking sites for cell signaling molecules are shown below. The amino acid number of the start of the motif relative to the mature CD28 protein is shown above. Note that in some publications the amino acid positions in CD28 have been numbered to include the 19 amino acid leader sequence. Phosphorylation of Y170 creates a docking site for SH2 domains which has been shown to bind to PI3K (YxxM) and Grb2 (YxN). The YxxM motif mediates recruitment of PKCθ to the cSMAC, upregulation of IL-2 transcription, and induction of Bcl-XL expression. The polyproline motif starting at 175 has been shown to interact with the SH3 domain of Itk and GADS. The polyproline motif starting at 187 can bind the SH3 domains of Lck and Grb2. The Lck SH2 domain can also bind to phosphorylated Y188. CD28-filamin interaction is also dependent on this polyproline motif. Y188 is important for CD28 localization to the cSMAC. The polyproline motif starting at P187 mediates IL-2 mRNA stability and contributes to cytokine expression, humoral responses, and the reorganization of lipid rafts. See text for references and further discussion

Bcl-XL [44]. Akt also plays a role in CD28-mediated glucose uptake, which may play a key role in CD28 regulation of T cell metabolism [45, 46]. Although the precise linkage is not yet established, Akt can cooperate with PKCθ in CD28-mediated regulation of a NF-κB reporter construct in Jurkat T cells [47]. Akt can also inhibit GSK-3, the kinase that opposes calcineurin and drives NFAT back to the cytosol. Thus, Akt leads to sustained NFAT activation. All of these transcription factors, NF-κB, AP1, and NFAT, play an important role in the upregulation of IL-2 transcription. Although it is clear that CD28 can promote activation of PI3K, the functional role of PI3K activation in CD28 costimulation remains controversial. In murine tumor cell lines, disruption of the PI3K interaction site in CD28 inhibited IL-2 production [37, 38], but this was not the case in transfected Jurkat cells [48, 49]. This discrepancy was thought to be resolved when Jurkat cells were found to lack both PTEN and SHIP-1, phosphatases that inactivate the products of PI3K [50, 51]. However, transgenic or retroviral reconstitution of normal murine T cells from CD28-deficient mice with CD28 mutations in the YxxM motif had a limited phenotype [52–56]. A defect in Bcl-XL induction and T cell homing was noted [52, 54, 57], but there was little overall impact on the immune response and T cell activation resulted in normal levels of IL-2 secretion. This discrepancy was resolved when we showed that the YxxM motif was required for CD28-mediated recruitment of PKCθ, activation of NF-κB and upregulation of IL-2 transcription, but that this did not impact on IL-2 secretion because the primary pathway for CD28 induction of IL-2 secretion was mediated through mRNA stability [56, 58] (Wang X and Miller J, unpublished data). Although the YxxM motif appeared to be functionally relevant for CD28 costimulation, recent data indicate that this may not be mediated through activation of PI3K as originally proposed [59].

CD28 can also recruit the adaptor proteins Grb2 through SH2 and SH3 domains and GADS through SH3 domains [60–63]. Both adaptor proteins can recruit SOS and so activate Ras. However, CD28-mediated activation of Ras appears to be limited to antibody cross-linking and is not induced by B7 [64]. Interestingly, GADS also interacts with SLP76 and LAT, which can bind the guanine nucleotide exchange factor Vav, and the Grb2 SH3 domain can interact with Vav directly [65]. Vav has been implicated downstream of CD28 costimulation, and PKCθ has been shown to interact with Vav in Jurkat T cells [66–69]. Mutation of the Grb2/GADS binding site on CD28 leads to a decrease in Vav

phosphorylation and can inhibit IL-2 production [60]. CD28 costimulation can also enhance TCR signaling downstream, most notably through activation of JNK [70] and ERK, possibly through inhibition of the Ras antagonist, Rap1 [71, 72]. CD28 has been shown to bind to the SH3 domain of Itk [40, 73]; however, the functional significance of this interaction and of the polyproline motif starting at 175 is not clear. Recently, filamin A has been shown to be recruited to the immunological synapse and may play a role in lipid raft organization or PKCθ recruitment [74, 75]. Interestingly, filamin A recruitment to the immunological synapse is dependent on the polyproline motif starting at 187 [74]. Filamins are large, actin binding scaffolds that have been shown to interact with over 20 different proteins associated with cell migration and signaling [76, 77]. Finally, CD28 can interact with both the SH2 and SH3 domains of Lck [78, 79]. The original model was that recruitment of Lck to CD28 resulted in CD28 activation, because Lck was shown to mediate phosphorylation of Y170, creating the binding site for the SH2 domains of PI3K and Grb2 [79, 80]. However, more recently, Lck has been considered as an effector of CD28 costimulation. Double mutation of the prolines at 187/190 results in a defect in the thymic maturation and peripheral differentiation of T regulatory T cells, whereas mutation of Y170 has no effect [15, 81, 82]. Likewise, we have shown that the ability of CD28 to enhance IL-2 mRNA stability requires the polyproline motif, but not the YMNM motif [56, 58] (Wang X and Miller J, unpublished data). In vivo this translates to a more dramatic effect of the P187/P190 mutation compared to the Y170 mutation [52, 83]. Although the functional relevance of this polyproline motif is clear, whether any or all of these effects are mediated though Lck recruitment is not known.

CD28 costimulation within the immunological synapse

One of the major complications in dissecting CD28 costimulation is that most of the specific pathways that are activated by CD28 costimulation are shared with TCR signaling. Both protein profiling of signaling intermediates [84] and genetic profiling of changes in gene expression [85, 86] have suggested that CD28 costimulation functions primarily to modify those signaling pathways that can be regulated by the TCR itself and it has been difficult to identify a unique contribution of CD28. One potential site where CD28 could impact on TCR signaling is within the cSMAC of the immunological synapse [87–90]. The proteins that are recruited to the immunological synapse between a T cell and APC are not randomly distributed. They segregate into at least three subdomains, called supramolecular activation clusters [91]. The central region, cSMAC, is enriched for cell surface proteins such as the TCR, CD4, CD28, and a minor fraction of CD45, along with associated signaling proteins such as PKCθ and Lck; the peripheral region, pSMAC, contains the integrin, LFA-1, and associated cytoskeletal components, such as talin; and an outer region that contains the majority of CD45 [92–100]. Additionally, some proteins such as CD43 are excluded from the interface altogether [101]. In addition to the spatial arrangement of proteins within the immunological synapse, there is a dramatic temporal organization as well. Initially, TCR and CD4 form small clusters that coalesce into the cSMAC, while LFA-1 moves out into the pSMAC [94, 95]. CD28 is also recruited to these TCR clusters in the immature immunological synapse [102]. This remodeling provides a brief colocalization of CD4 and the phosphatase, CD45, possibly accounting for the initial activation of the CD4-associated Src family kinase, Lck [96, 99]. Sustained TCR signaling is thought to take place within microclusters that form at the periphery of the synapse and transduce signals while being transported through the pSMAC enroute to the cSMAC [103–105].

It was recently shown that CD28 can also be recruited to these microclusters and these may provide an additional site for TCR/CD28 signal integration [106].

The functional relevance of the localization of proteins to specific domains within the immunological synapse is not clear. The best example is for CD45, the cell surface phosphatase that plays an important role in Lck activation (by removal of the inhibitory phosphate at Y505), but can also inhibit T cell activation by removal of the activating phosphate at Y354 of Lck and possibly other signaling molecules. CD45 is polarized toward the APC, but largely excluded from the immunological synapse. Thus, CD45 is at high concentration at the site of TCR microcluster formation, possibly enhancing association of TCR/CD4 with activated Lck. Microclusters then move through the pSMAC, which is a region of very low CD45 [96, 99, 105]. We have shown that the exclusion of CD45 from the immunological synapse is dependent on LFA-1 expression and the failure to exclude CD45 correlates with reduced calcium signaling [107]. Thus, the pSMAC may regulate the magnitude of sustained TCR signaling by segregating CD45 from activated TCR complexes. Whether TCR signaling is restricted to microclusters or persists after localization in the cSMAC is still controversial. Initially it was proposed that TCR signaling occurred within the cSMAC region; however, recent data indicate that the cSMAC is a site for TCR downregulation [108]. More recently it was shown that under conditions of suboptimal TCR engagement, signaling may persist in the cSMAC and so the cSMAC may be a site for regulating the threshold of TCR signaling for optimal T cell activation [109, 110]. Finally, CD28 signaling has been associated with localization of CD28 and PKCθ to the cSMAC. Although T cells express a number of PKC isoforms, PKCθ is selectively activated and recruited to the immunological synapse, where it is colocalized with TCR and CD28 in the cSMAC [93, 97]. PKCθ plays an essential role in transducing TCR-mediated activation of NF-κB [41, 111, 112]. Expression of CD28 is required for the targeting of PKCθ to the cSMAC and in the absence of CD28 PKCθ is recruited to the immunological synapse, but it is diffusely distributed across the synapse and is not focused into the cSMAC [56, 113]. This disruption in PKCθ localization in the absence of CD28 correlates with a loss in PKCθ-dependent induction of NF-κB and IL-2 transcription. Interestingly, all of these functions of CD28 (recruitment of PKCθ to the cSMAC, activation of NF-κB, and upregulation of IL-2 transcription) are lost by a single amino acid mutation of the PI3K interaction site in the cytosolic tail of CD28 [56]. In addition, we have mapped the cSMAC localization signal in the cytosolic tail of CD28 [1]. Mutation of a single amino acid (Y188F) reduces the efficiency of CD28 recruitment to the immunological synapse and disrupts localization of CD28 to the cSMAC. Interestingly, localization of PKCθ mirrors CD28 localization, indicating that CD28, and not other signals within the cSMAC, is the primary signal for PKCθ localization within the synapse. Finally, the Y188F mutation also results in reduced activation of NF-κB, suggesting that mislocalization of CD28 and correspondingly PKCθ may reduce the magnitude of CD28 costimulation. Taken together these findings suggest that the magnitude of TCR signaling and the integration between TCR and CD28 signaling may occur within and through the spatial organization of proteins in the immunological synapse.

CD28 costimulation through the upregulation of mRNA stability

Although TCR and CD28 signaling normally occur within the context of the immunological synapse, the ability of CD28 to upregulate IL-2 mRNA stability can be transduced in *trans*, i.e. from a separate site on the cell surface from TCR engagement [114]. This argues that TCR and CD28 signaling integration can take place downstream from plasma

membrane-associated events. Furthermore, we have shown that the CD28 induced IL-2 transcription and mRNA stability are mediated through independent motifs within the CD28 cytosolic tail (Wang X and Miller J, unpublished data). The induction of mRNA stability is transduced through a polyproline-like motif; however, the specific signals that are activated by this motif are not clearly identified. The regulation of mRNA stability is largely controlled by AU-rich elements (ARE) within the 3′ UTR of the mRNA [115–117]. ARE-mediated mRNA degradation plays an important role in the regulation of many genes [118, 119], including cytokines [7]. RNA stability can also be regulated by microRNA, although the relationship between microRNA and ARE-mediated mRNA decay are not well established [120]. The current model for regulated mRNA stability is that AU-binding proteins that induce mRNA instability, such as TTP, bind to the 3′ UTR in unstimulated cells [121]. T cell activation leads to an increase in expression of TTP, and TTP can bind to the AU-rich region in the IL-2 3′ UTR and drive IL-2 mRNA degradation [122, 123]. TTP recruits the multi-component exosome, allowing for deadenylation and 3′ exonuclease digestion of the mRNA. In some cases, mRNA molecules can also be targeted by a 5′ de-capping enzyme allowing for 5′ exonuclease digestion as well [117, 124]. In the absence of ARE-mediated mRNA degradation, either by genetic disruption of TTP expression [125] or the deletion of the ARE from TNF [126], overexpression of TNF results in the induction of autoimmune inflammatory diseases. The stability of ARE-containing mRNAs can be enhanced during cell activation events, although the mechanisms that mediate this stabilization are not well understood [115–117]. One model that has been proposed is that cell signaling induces the recruitment of different AU-binding proteins, such as HuR, that may compete with TTP for binding to the 3′ UTR and, thus, interfere with TTP-dependent recruitment of the exosome. Although HuR can bind to the ARE in IL-2 mRNA, HuR does not appear to be involved in CD28-mediated mRNA stabilization [127]. Alternatively, MAP kinase activation has been implicated in the induction of mRNA stability. JNK can induce IL-2 and IL-3 mRNA stability [128–130]; p38 has been implicated in the stabilization of IL-2, IL-6, IL-8, and TNFα mRNA [131–133]; and ERK can stabilize COX-2 mRNA in smooth muscle cells. One potential target of MAP kinase activation is TTP itself. Phosphorylation of TTP appears to increase the stability of TTP, probably through the association with 14-3-3. However, whether phosphorylation itself directly impacts on TTP binding affinity for ARE or whether 14-3-3 association regulates the ability of TTP to recruit the exosome degradation machinery remain controversial [134–136].

The initial focus on the regulation of mRNA stability in T cells was the JNK pathway. A JNK-response element was identified by mutagenesis in the 5′ end of IL-2 mRNA and two proteins, YB-1 and nucleolin, were found to bind to this element [129]. YB-1 and nucleolin are ubiquitously expressed multifunctional nucleic acid binding proteins [137, 138]. However, these proteins alone are not sufficient to induce mRNA stability and require at least one protein that might bind to the 3′ UTR. In addition, the ability of CD28 to enhance JNK activity [70, 139] and the importance of JNK in IL-2 expression [140] remain controversial. More recently the focus has been on NF90, which was originally identified as a transcription factor associated with NFAT [141], and is now recognized as an RNA-binding protein. NF90 can compete with TTP for binding to the AU-rich region of the IL-2 mRNA [142]. NF90 is localized in the nucleus in resting cells, but T cell activation results in movement of the majority of NF90 to the cytosol and this nuclear-cytosolic shuttle event is linked to IL-2 mRNA stabilization. Recent analysis of NF90 knock out T cells has confirmed an important role of NF90 in the regulation of IL-2 secretion, but it is not clear how much of this effect is mediated through NF90's effect on

IL-2 transcription or mRNA stability [143]. The signals that regulate NF90 localization/function, how CD28 might impact on regulation of NF90, and how NF90 might interact with other factors that impact on mRNA stability are not understood.

Two pathways of CD28 costimulation

Recent attempts to dissect the different roles of CD28 in normal T cells have suggested that distinct functions might be mediated by different signaling pathways [55, 56]. We have defined two independent pathways that impact on the ability of CD28 to upregulate IL-2 expression [1, 56, 114] (Wang X and Miller J, unpublished data). It is well established that CD28 costimulation can enhance cytokine secretion through an increase in transcription and through the induction of mRNA stability. But the relationship between these molecular events and their relative impact on the levels of cytokine secretion were not understood. We have recently shown that these two mechanisms involved in the upregulation of IL-2 secretion are mediated through independent signaling pathways. First, mutation of M173 in the cytosolic tail of CD28, a mutation that disrupts the ability of CD28 to recruit and activate PI3K, results in a failure to recruit PKCθ to the cSMAC, drive nuclear localization of NF-κB, and enhance IL-2 transcription [56]. Disruption of this site also results in a loss in Bcl-XL upregulation, a defect in the generation of graft-versus-host responses and an alteration in T cell trafficking, but has little effect on overall cytokine expression [52–54, 57]. However, this mutation did not affect the ability of CD28 to promote IL-2 mRNA stability and had little effect on the level of IL-2 protein secretion. Second, mutation of a polyproline motif (PYAPARDF) disrupts the ability of CD28 to induce IL-2 mRNA stability (Wang X and Miller J, unpublished data). This element is associated with Lck and Grb2 recruitment and mutation of the proline residues interferes with T regulatory cell development and results in a general reduction in cytokine expression and humoral responses [15, 60, 61, 79, 81–83, 144]. Importantly, the two pathways are independent. Mutation of the YMNM motif blocked upregulation of IL-2 transcription without affecting mRNA stability, whereas mutation of the PYAPARDF motif blocked the induction of mRNA stability without affecting transcription. In addition, disruption of mRNA stability had a greater effect on the levels of IL-2 secretion than did disruption of CD28-enhanced IL-2 transcription. Understanding these pathways and the biochemical events associated with signal transduction will provide important insight into how CD28 costimulation can impact on so many aspects of T cell activation, differentiation, and tolerance.

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