



Signaling of the T Cell Costimulatory Receptor CD28: Regulation and Initiation

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**Signaling of the T cell Costimulatory Receptor CD28:
Regulation and Initiation**

A dissertation presented

by

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to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Immunology

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Abstract

T cells provide antigen-specific immunity to pathogens, but are also capable of inducing destructive autoimmunity when responding to self antigens. In order to achieve full activation, T cells must receive two signals: one from the T cell antigen receptor, and a second from a costimulatory receptor, such as CD28. Expression of the ligands for CD28 activation is strongly induced on professional antigen presenting cells under conditions of inflammation or danger-sensing. Hence, CD28 costimulatory receptor acts as a gatekeeper to T cell activation and is essential in maintaining the balance between immunity and self-tolerance.

CD28 function has been implicated in many animal models of T cell-dependent infection control and autoimmune disorders. Intensive study of CD28 signaling biology over more than two decades has elucidated key signaling pathways and transcriptional targets that help to explain the unique CD28-dependent functions in T cells. Some signaling pathways downstream of CD28 activation have been mapped to particular motifs within the CD28 cytoplasmic domain, although no consensus has emerged on the importance of these signaling motifs to CD28 function *in vivo*.

This thesis describes the discovery of a novel motif within CD28 cytoplasmic domain that regulates receptor signal initiation through membrane binding and kinase recruitment, and discusses the new findings in light of existing literature.

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Attributions

The work described in Chapter 2 is reproduced from a submitted manuscript from the following authors:

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Specific contributions were as follows: J.D., E.G. and K.W.W. conceived the study; J.D. and E.G. performed FRET experiments; J.D. and J.P. performed biochemical studies; J.D. and J.G. performed *in vivo* studies with advice from A.H.S., D.A.A.V. and K.W.W.; J.D. and K.W.W. wrote the manuscript.

The work described in Chapter 3 is unpublished, original data, with the exception of Figure 3.1A, which was previously published in Bettini *et al.* 2014 (ref. 131). For this figure panel, M.B. prepared the transduced bone marrow chimeras and isolated T cells, and J.D. performed live cell imaging.

Chapter 1

General Introduction

The T cell costimulatory receptor CD28 is expressed as a disulfide-linked homodimer on the surface of all naïve mouse T cells, ~95% of human CD4⁺ T cells and ~50% human CD8⁺ T cells (1). Early studies conducted over two decades ago established CD28 as a key player in T cell biology, regulating the balance between T cell tolerance and immunity. Many studies since then have elucidated multiple biochemical signaling pathways activated in response to CD28 receptor triggering, some unique to CD28 and some overlapping with T cell receptor (TCR) signaling pathways. Subsequently, studies in a variety of *in vivo* models using both transgenic and knock-in mice expressing various CD28 mutants have yielded a complex picture of the importance of various signaling motifs within the CD28 cytoplasmic domain: the same motifs were found to be either critical to T cell function or dispensable, depending on the experimental model system. However, *src*-family kinase p56^{Lck} (Lck) interaction with CD28 has most consistently been observed to be important for CD28-mediated activity.

This introduction reviews the complex literature surrounding CD28 signaling and *in vivo* biology, with a focus on the role of CD28 in the greater context of T cell activation. Chapters 2 and 3 present new findings that suggest a revised model for CD28 activation and early signaling events. These new findings are discussed in the context of existing literature in Chapter 4.

A Brief History of CD28 Discovery

Early studies of T cell activation revealed the remarkable antigen specificity conferred by the T cell receptor. However, stimulation through TCR or by antibody-mediated activation of the CD3 complex required the presence of accessory cells, which supplied an unknown secondary signal (2). This led to proposal of a two-signal model for T cell activation which endures to the present day: antigen stimulation alone leads to an unresponsive state (anergy) in

order to prevent response to self antigens which might lead to autoimmunity, while antigen stimulation plus costimulation (e.g. through CD28 activation) leads to full T cell activation (3, 4). Early candidates for this secondary receptor included CD2 and CD28 (also known at the time as Tp44) (5). Only stimulation with an antibody against CD28 could substitute for accessory cells in synergizing with the TCR/CD3 signal to promote full T cell activation *in vitro*, which was measured by cell enlargement, robust proliferation, and production of IL-2 (6-8).

The human CD28 cDNA was cloned in 1987 by Aruffo and Seed (9), while the murine variant was isolated in 1990 by J. Allison's lab (10). Early studies into the role of CD28 in T cell activation utilized *in vitro* activation of primary T cells and T cell clones. Broadly, these studies revealed that CD28 costimulation could prevent the anergic phenotype that would be induced by antigen stimulation alone, and that this effect might be due to stabilization of mRNA transcripts for several cytokines, including IL-2, TNF- α and IFN- γ (11, 12). The *Cd28*^{-/-} mouse was developed and characterized by T. Mak's lab in 1993; they observed profound defects in T cell function, including in T helper cell-dependent antibody isotype class-switching *in vivo*, and activation-induced T cell proliferation and IL-2 production *in vitro* (13).

Early Events in the CD28 Signaling Pathway

Once the critical importance of CD28 costimulation to T cell activation was fully realized, a concerted effort involving several research groups began in order to define the distinct biochemical pathways that contributed to CD28 signaling. Several motifs were identified within the CD28 cytoplasmic domain (sequence shown in Figure 1.1) that could recruit early signaling effector molecules, as well as downstream transcriptional targets preferentially activated in response to CD28 signaling.

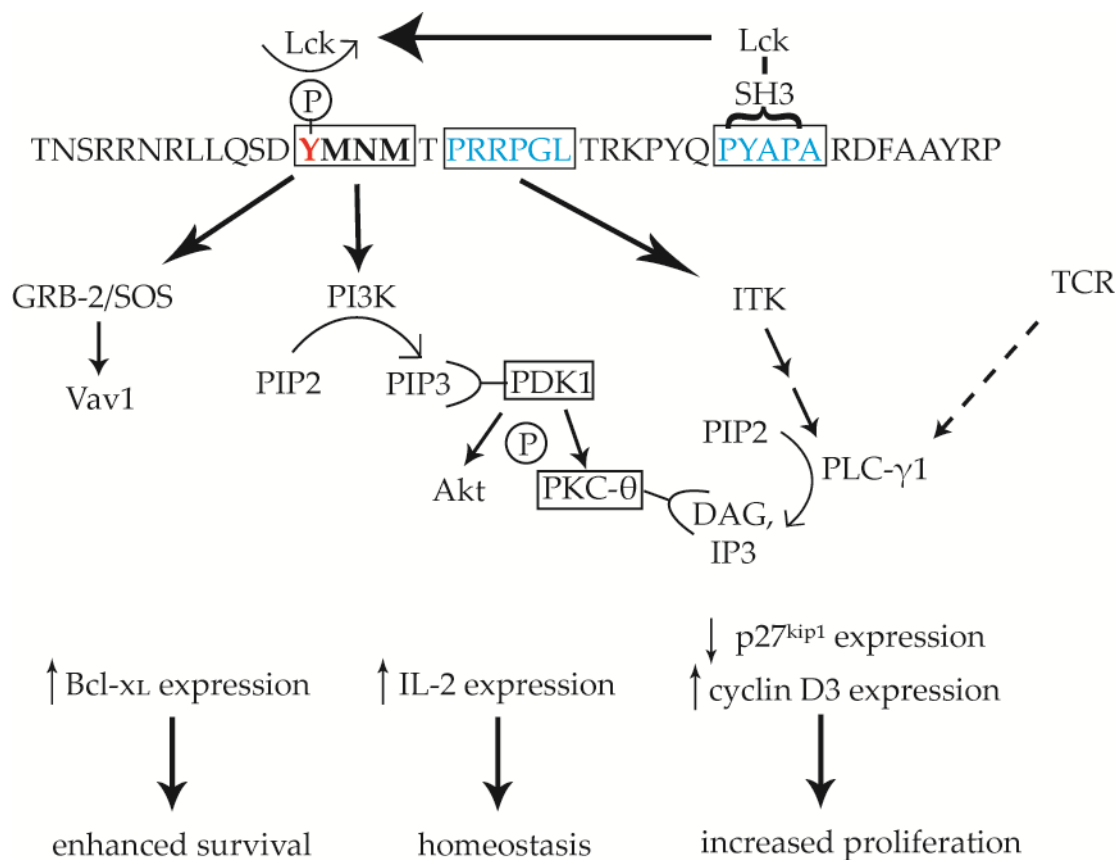


Figure 1.1 CD28 cytoplasmic domain sequence is shown with relevant downstream signaling pathways as understood *circa* 2010. Three sequence motifs of interest within CD28 cytoplasmic domain are highlighted with boxed outlines: the YxxM ITAM-like motif, the PRRPG motif, and the PYAPA motif. Downstream signaling effectors recruited to or activated by these sites are indicated with arrows. Work presented in chapter 2 alters the present understanding of early CD28 signaling events, requiring changes to this signaling diagram in future. Cellular outcomes related to CD28 signaling are also shown.

The Y¹⁸⁹MNM motif is an obvious candidate as a signaling motif, in part because it shares several similarities with the well-characterized immunoreceptor tyrosine-based activation motif (ITAM) found in the cytoplasmic domains of both the CD3 subunits of the TCR and the Ig α and β subunits of the B cell receptor (BCR) (14). The ITAM is characterized by a Tyr residue which is the target of phosphorylation by *src*-family kinases, followed by two irrelevant amino acids, and then a bulky hydrophobic residue such as Leu, Ile, Val, or Met (hereafter referred to as “B”). Two of these YxxB motifs are separated by a 6-8 amino acid spacer to comprise the full ITAM. After Tyr phosphorylation, each [pY]xxB unit can bind to a single SH2 domain to recruit a downstream signaling molecule to the activated receptor. ZAP-70 (in T cells) and Syk (in B cells) kinases contain tandem SH2 domains (one to bind each YxxB unit), which allows recruitment to phosphorylated ITAM-bearing receptors with high affinity and specificity (15, 16).

In the case of the relevant CD28 motif, however, there is only one YxxB unit present, so the YxxM motif is commonly referred to as an ITAM-like motif and is not capable of recruiting ZAP-70 (Figure 1.1). Instead, this motif was shown to bind to the p85 subunit of phosphatidylinositol-3 kinase (PI3K) by co-immunoprecipitation (17, 18). The Y189F mutation severely reduced the binding interaction and the M193C mutation caused a modest reduction while the Y207F mutation (contained within a different motif) had no effect. Together, the results of these studies implicated the YxxM motif in recruitment of PI3K, and the requirement for an intact Tyr residue suggested that one or both of the SH2 domains of the p85 subunit were involved in the interaction. This motif was also shown to directly interact with Grb2 using similar co-immunoprecipitation methods (19). These findings have caused some confusion in the field as the overlap in the interaction sites for both of these signaling mediators would cause

competition for CD28 binding. In general, PI3K has been accepted as the most important interaction partner at this site because this pathway is uniquely activated by CD28 but not TCR signaling. In turn, PI3K activation was shown to activate downstream kinase PDK-1 and eventually PKC θ (20). However, a more recent study reported that PKC θ could be directly recruited to the CD28 cytoplasmic domain at the plasma membrane by physical interaction with Lck as a binding intermediate (21). Hence, there is still considerable confusion regarding the organization of early CD28 signaling events stemming from the YMNM motif, which may need to be resolved by careful comparative analysis within a single study.

Another sequence within the CD28 cytoplasmic domain which has excited interest as a possible binding site for signaling mediators is the P¹⁹⁴RRPG motif (Figure 1.1). Taken at face value, this sequence looks like a potential SH3 domain binding site due to the presence of a PxxP motif. However, analysis of many SH3 domain binding peptides reveals a more complex consensus sequence, which can be divided into two groups: [R/K]xXPxXP or XPxXPx[R/K], where “X” represents a non-glycine hydrophobic residue and “x” represents any amino acid (22). These sequences are nearly mirror images of each other and so they mediate SH3 domain binding in opposite orientations. The expanded CD28 sequence around the motif of interest is MNMTPRRRPGLTRK. This would most likely fall into the second group, in which the charged residue that determines SH3 domain binding orientation is at the C-terminus of the sequence motif. However, this charged residue (R101) is not optimally placed with respect to the 2nd proline of the motif, and the R¹⁹⁶P sequence does not match the expected XP doublet. Hence, this motif within the CD28 cytoplasmic domain represents a very atypical potential SH3 domain binding site. Nonetheless, ITK was shown to co-immunoprecipitate with CD28 from stimulated Jurkat cells (23), and this interaction was shown to be dependent on one of the prolines within

the PRRP motif using *in vitro* binding and activity assays (24).

The final motif of interest to date within the CD28 cytoplasmic domain is another proline-rich motif with sequence P²⁰⁶YAPA (expanded sequence YQP_YAP_{AR}) (Figure 1.1). This sequence more closely matches the consensus SH3 domain binding sequence as described above and falls within the second group, in which the charged residue is C-terminal to the proline motif. The XP doublets of QP and AP also meet the expected criteria. *Src*-family kinase Lck was first shown to associate with CD28 in cells by co-immunoprecipitation studies (25), and a later study focused on the CD28 PYAP motif as a possible interaction partner for the Lck SH3 domain (26). Importantly, the follow-up study did not include any direct binding studies. Instead, the authors relied on kinase activity assays and cellular readouts such as reporter induction and proliferation. They demonstrated defects with both the CD28 P206A/P209A double mutant and the Lck SH3 domain W97A mutant, which supported the straightforward hypothesis that the Lck SH3 domain bound to the CD28 PYAP motif. Another study later suggested that the Lck SH3 domain in fact had very low affinity for CD28 cytoplasmic domain using an *in vitro* binding assay, and that the interaction was instead mediated by the Lck SH2 domain binding to pY207 (also contained within the PYAP motif), but this did not seem to fit with the data from Holdorf et al. (27). A possible explanation arises from our work (presented in Chapter 2) in conjunction with another study demonstrating that the V3 hinge region of PKC θ directly interacts with the Lck SH3 domain (21), which would suggest that the defect previously observed for the Lck W97A mutant (within the SH3 domain) was due to disruption of PKC θ recruitment and activation rather than Lck binding to CD28. In addition, we show that the Lck SH2 domain binds directly to a peptide containing the phosphorylated CD28 P[pY]AP motif and that this interaction is somewhat impaired for the P206A/P209A CD28 mutant peptide. This

might indicate that the defect previously observed for the CD28 mutant in cells was due to disruption of Lck binding through the SH2 domain, not the SH3 domain.

Our studies reveal a fourth, previously unknown motif within the CD28 cytoplasmic domain that is required for its signaling activity: two clusters of basic residues that bind to the Lck kinase domain with high affinity. These studies are presented in detail in Chapter 2. Lck was previously shown to phosphorylate two tyrosine residues within the CD28 cytoplasmic domain (Y189 and Y207) (28), which is in agreement with our results, although another earlier study suggested that Lck could phosphorylate only Y189 (29). Initial binding of Lck to the CD28 basic residue clusters is required for its phosphorylation activity on both Y189 and Y207, creating binding sites for the Lck SH2 domain at pY207 and for the SH2 domains of PI3K and Grb2 at pY189. Thus, we hypothesize that Lck binding to the basic residue clusters within the CD28 cytoplasmic domain is a pre-requisite to all CD28-mediated signaling activity.

CD28 in the T cell Immunological Synapse

T cell activation is a dynamic process that requires a transition from rapid scanning of many antigen presenting cells (APCs) to a complete stop and immunological synapse (IS) formation when an APC bearing cognate peptide:MHC (pMHC) complexes is encountered. Early studies of conjugates between T cells and APCs revealed a repolarization within the T cell that concentrated surface receptors (such as TCR and CD28) and intracellular signaling components (such as Lck and PKC θ) at the IS, which forms at the interface between the T cell and the APC (30-32). This redistribution of signaling molecules to such a small area was thought to support sustained signaling needed to achieve full T cell activation.

The advent of higher-resolution imaging techniques and glass-supported planar lipid

bilayers allowed for a much more detailed examination of the real-time events involved in IS formation (33). The lipid bilayers can be loaded with proteins such as pMHC, ICAM-1 and CD80 at precise molecular densities in order to mimic conditions on the cell surface of an APC. Proteins within the lipid environment retain lateral mobility, which is more physiologically similar to the cellular membrane environment than is a surface bearing immobilized proteins. This minimal system was shown to be sufficient to induce T cell proliferation and cytokine production, supporting its relevance in studying T cell:APC interactions (33, 34). Using this system, microclusters containing TCR and the T cell adhesion molecule LFA-1 were observed as diffuse puncta distributed across a broad contact area between the T cell and the lipid bilayer. These microclusters form within seconds of T cell contact and migrate rapidly towards the center of the contact zone. Over a period of several minutes, a mature IS is formed in which TCR is concentrated in the central supramolecular activation complex (cSMAC) while LFA-1 is segregated into a ring surrounding the TCR, termed the peripheral SMAC (pSMAC). Phosphorylation of TCR-associated CD3 chains and recruitment of downstream kinases and signaling adapters already occurs within early microclusters, whereas signaling molecules become less abundant in the cSMAC (35, 36). Therefore, it has been proposed that TCR signal transduction occurs primarily in the microclusters and may be terminated in the cSMAC in a process that is coupled with TCR internalization.

The importance of CD28 costimulation in full T cell activation is clear, but the role of CD28 in IS formation is more complex. While CD80 is required in addition to pMHC and ICAM-1 to stimulate maximal T cell proliferation using lipid bilayers, CD80 is dispensable in the lipid bilayer for T cell IS formation, which suggests that CD28 activation or recruitment is not required in this process (34). When CD80 is present with pMHC and ICAM-1 in lipid

bilayers, CD28 colocalizes with the TCR in signaling microclusters at early timepoints, but is segregated away from TCR into a ring that lies roughly between the cSMAC and pSMAC in the mature synapse, overlapping somewhat with both (37).

CD28 may not be important for the structural integrity of the IS, but its role in T cell activation may be in synergizing with or potentiating TCR signaling to initiate the full complement of downstream signaling pathways necessary to achieve T cell activation. For example, stable PKC θ recruitment was considered a hallmark of IS formation in early studies of T cell : APC conjugates (32), and this may be a CD28-dependent process as it has since been shown in the lipid bilayer system that PKC θ co-localizes with TCR/CD28 microclusters and then segregates preferentially with CD28 in a distribution pattern that is distinct from that of TCR in the mature synapse (37). CD80-CD28 interaction is required for this process as in the absence of CD80, PKC θ is only transiently recruited and then lost from the mature synapse (37). The CD28 mutant Y123A, which lacks CD80 binding, is similarly defective in stable PKC θ recruitment. An intact CD28 cytoplasmic domain is also required, which implies a physical association between CD28 and PKC θ . This hypothesis was confirmed by co-immunoprecipitation of PKC θ with CD28 from activated T cell hybridomas and primary T cells, the first time that this physical interaction was shown (37). As discussed above, further examination of the CD28-PKC θ interaction revealed that the V3 hinge region of PKC θ binds to the Lck SH3 domain, and that Lck in turn binds to CD28, although the precise motif(s) within the CD28 cytoplasmic domain required for the CD28-Lck interaction were not identified (21).

No signaling molecules other than PKC θ , including PI3K, Grb2, Gads, Vav1 and Itk, were shown to be stably recruited to the IS in a CD28-dependent manner (37). These molecules could still be activated downstream of CD28 signaling, but their activity does not seem to be

spatially coordinated with CD28. Any physical interaction with CD28 may be only transient, or they may be activated secondarily through other CD28-recruited mediators, such as Lck or PKC θ .

In summary, interaction with CD80 allows recruitment of CD28 to early signaling microclusters, where it colocalizes with TCR. CD28 is later partially segregated from TCR in a ring that partially overlaps but is largely distinct from the cSMAC in the mature synapse. The CD28 cytoplasmic domain is required for stable recruitment of PKC θ to the IS.

CD28 Intracellular Functions

The most notable and obvious phenotype of *Cd28*^{-/-} T cells is that they fail to proliferate as strongly as do WT cells in response to stimulation, and this sub-optimal response is accompanied by an increase in cell death in culture. While it remains controversial how CD28 signaling might be distinct from TCR-activated signaling pathways, several biochemical responses in cells have been identified that seem to be dependent on CD28 costimulatory activity.

Perhaps the most well-characterized cellular function attributed to CD28 signaling is enhancement of IL-2 production through stabilization of the *Il2* mRNA transcript (11, 12). Enhancement of secretion of other cytokines, such as TNF- α and IFN- γ , has also been linked to CD28 activity (12). However, it has been difficult to dissect whether this is a direct CD28-mediated effect, or a secondary effect of more robust T cell activation and differentiation in general. This question was addressed by microarray profiling examining changes in mRNA levels in response to either TCR or CD28 stimulation alone (38). This study revealed a consensus motif of AUUUA in the 3' UTR of several mRNA transcripts, including that of IL-2,

that are uniquely induced by CD28 and not TCR.

Bcl-x_L is an anti-apoptotic factor that was also shown to be strongly upregulated in response to synergistic TCR and CD28 signaling when compared to TCR signaling alone (39, 40). Bcl-x_L protects proliferating T cells from activation-induced cell death, which would otherwise result from TCR stimulation in the absence of CD28 costimulation, as well as from Fas receptor-mediated and radiation-induced cell death. CD28 activation was also shown to enhance cell cycle progression (41) and shift cellular metabolism toward enhanced glycolysis, which might support the energetic requirements of the rapidly proliferation T cells (42).

Based on the finding that CD28 is primarily responsible for IL-2 induction following T cell activation and the identification of a CD28 response element consensus sequence motif (43), multiple groups were able to use the *Il2* gene enhancer as a tool to identify CD28-activated signaling pathways that trigger downstream transcription factor activity. In particular, PKC θ is capable of inducing NF- κ B activity on the *Il2* CD28 response element in T cells (44, 45). PKC θ has been shown to interact with CARMA1 at the plasma membrane, which in turn activates IKK β , ultimately leading to nuclear translocation of NF- κ B (46). NF- κ B family members c-Rel and RelA (p65) were both shown to be capable of binding to the CD28 response element in the *Il2* enhancer, and in particular binding of c-Rel was enriched following combined TCR and CD28 stimulation compared to TCR stimulation alone (47, 48). TCR- and CD28-activated signaling pathways overlap at many points biochemically and at the transcriptional level. The *Il2* enhancer shows perhaps the greatest specificity for CD28-induced signals, but still contains an AP-1 binding site, occupancy of which is induced downstream of TCR signaling, that overlaps with the CD28 response element (43, 49). Thus, even with the identification of a CD28 response element that is unique at the molecular level, it has been difficult to tease apart the

distinct contributions of TCR versus CD28 signaling to T cell activation.

In summary, CD28 signaling, whether on its own or by synergizing with TCR signaling, activates a transcriptional program that enhances T cell proliferation, survival and effector differentiation. These biochemical changes help to explain the requirement for CD28 costimulation in bypassing the anergic or apoptotic cell fate that would be the default outcome of TCR stimulation alone. As APCs should only express CD28 ligands CD80 and CD86 (also known as B7-1 and B7-2, respectively) at high levels in the context of inflammation, CD28 maintains the balance between protective immunity and destructive autoimmunity by coupling the T cell response to danger-sensing by innate immune cells.

Role of CD28 *in vivo*: autoimmune and transplantation models

As the net result of CD28 activation is to generally improve T cell viability and function, a role for CD28 can be found in almost any T cell-dependent *in vivo* model system, including development of effective anti-viral T cell responses, priming of T cell-dependent antibody responses, and induction of T cell-dependent autoimmune diseases. The original phenotype identified for the *Cd28*^{-/-} mouse by T. Mak's lab included: 1. defective cellular proliferation and IL-2 production in response to stimulation; 2. skewing of baseline antibody isotype distribution; and 3. reduced induction of neutralizing antibodies in response to viral infection (13). This complemented previous data from *in vitro* stimulation of human T cells, which similarly showed defects in proliferation and cytokine production (particularly IL-2) in response to TCR stimulation without CD28 costimulation (11, 12). However, the knockout mouse provided one of the first opportunities to examine CD28 function on T cells in the context of a complete immune system, in this case in providing help to B cells during the germinal center reaction,

which leads to production of high-affinity neutralizing antibodies. Along those lines, CD28 also proved important in the development of autoimmune diseases in which pathogenesis is mediated by autoantibodies against self antigens, including in collagen-induced arthritis and a mouse model of Lupus erythematosus (50, 51).

In addition to the *Cd28*^{-/-} mouse, the CTLA4-Ig fusion protein is another important tool for studying the role of CD28 *in vivo*. CTLA-4 is an inhibitory receptor whose surface expression is induced on T cells upon antigen stimulation (reviewed in (52-55)). CD28 and CTLA-4 share the same ligands (B7-1 and B7-2), but CTLA-4 has a much higher affinity for both ligands and so is capable of out-competing CD28 for ligand interaction (56, 57). This serves as a negative feedback mechanism to terminate T cell stimulation, both by preventing engagement of CD28 and by inactivating TCR signaling at the plasma membrane through recruitment of phosphatases SHP-1, SHP-2 and PP2A to the cytoplasmic domain of activated CTLA-4 (58-60).

CTLA4-Ig is a soluble fusion protein containing the extracellular domain of CTLA-4 and the constant region of IgG1 that can block CD28-B7 (and CTLA4-B7) interactions, although it lacks the other intracellular inhibitory effects of the full-length CTLA-4 receptor. CTLA4-Ig potently blocks T cell stimulation by APCs *in vitro* (61). The advantage of CTLA4-Ig as a research tool *in vivo* is that CD28 signaling could in a sense be turned on and off during different stages of an experiment (e.g. during T cell priming, expansion and/or contraction) by administering or withdrawing treatment as necessary, although the timing of doses and the half-life of the protein *in vivo* are critical to the success of such an approach. However, the disadvantage compared to working with *Cd28*^{-/-} cells is that a complete interruption of CD28 signaling in all cells cannot be assured *in vivo* and depends on the ability of the fusion protein to

infiltrate all relevant tissues and remain bound at saturating concentrations to all B7 molecules.

CTLA4-Ig was first employed *in vivo* in allogeneic transplantation models, in which recognition of MHC-mismatched donor tissue by recipient T cells normally triggers an inflammatory response and eventual organ rejection. The CTLA4-Ig treatment succeeded in prolonging survival of grafted pancreatic islets and heart (62, 63). Histological analysis of the grafted organs revealed reduced inflammatory cell infiltration and reduced donor tissue destruction. This confirmed the importance of CD28 costimulation in activation of T cells in response to alloantigens *in vivo* and had promising implications for clinical use of CTLA4-Ig as a therapy in transplant patients. A second-generation version of the molecule (trade name belatacept) was approved by the FDA in 2011 to prevent kidney transplant rejection.

Multiple subsequent studies focused on the role of the CD28-B7 pathway in autoimmune disease, in particular in experimental autoimmune encephalitis (EAE), the mouse model of multiple sclerosis. EAE results from immune destruction of the myelin sheath surrounding neuronal axons and can be induced by immunization with spinal cord homogenate or with purified myelin proteins such as myelin basic protein (MBP), myelin proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG) (64). Adoptive transfer of activated T cells specific for myelin-associated proteins into a syngeneic host also confers disease (65). Administration of CTLA4-Ig during T cell priming either *in vivo* (immediately after vaccination) or *in vitro* resulted in less severe disease induction in adoptive transfer recipient mice, confirming the importance of CD28 costimulation during T cell activation (66). However, treatment of recipients after adoptive transfer of previously-activated T cells had no effect on disease timecourse or severity, suggesting that CD28 is not required for maintenance of effector T cells.

Another study utilized an immunization-induced EAE model against MOG₃₅₋₅₅ peptide, which is a known immunodominant T cell epitope on the C57BL/6 background. In this model, both *Cd28*^{-/-} and *B7-1/B7-2*^{-/-} mice were protected from disease, which is consistent with the important role of CD28-B7 interactions in T cell activation (67). *B7-1*^{-/-} and *B7-2*^{-/-} individually provided little to no protection from disease induction, suggesting that the two ligands have overlapping functions in CD28 activation. When T cells were primed in WT hosts and then adoptively transferred, *B7-1/B7-2*^{-/-} recipients exhibited delayed disease induction and reduced peak severity, suggesting that continued costimulation is important for T cell effector function. The discrepancy between results in this model and the CTLA4-Ig treatment adoptive transfer model may be explained by incomplete blockade of B7-CD28 by CTLA4-Ig: if effector T cells are less dependent on costimulation than are naïve T cells, then incomplete blockade may effectively inhibit T cell priming but not dampen an already-primed T cell response. Alternatively, if some costimulation is supplied in the target organ (i.e. the CNS), then CTLA4-Ig may not be able to cross the blood-brain barrier at concentrations sufficient to block effector T cells at that site.

In both EAE models, expansion of self antigen-specific T cells was blunted in CD28-deficient or -blocked conditions, perhaps due to a combination of reduced Bcl-x_L expression, which would lead to an enhanced rate of cell death in TCR-stimulated cells (68), and reduced IL-2 production, which would limit effector cell expansion as T_{eff} cells are highly dependent on IL-2 for their maintained survival and function (69). However, the T_{eff} differentiation program appeared intact as activated cells showed no defect *in vitro* in secretion of IFN-γ and TNF-α under conditions of CD28 deficiency or blockade.

CD28 is also required for the development of lethal lymphoproliferative autoimmune

disorder in *Ctla4*^{-/-} mice (70-72). CTLA-4 deficiency results in massive polyclonal T cell activation and proliferation, lymphadenopathy, splenomegaly, and multi-organ inflammatory cell infiltration accompanied by tissue destruction, with pancreas, liver and heart the most affected target organs (73). Mice typically succumb to the disease within four weeks after birth. Disease progression can be prevented by continuous treatment from an early age with CTLA4-Ig, which prevents the disease-associated aberrant T cell activation, proliferation, and accumulation in tissues. The disease can also be prevented genetically by crossing *Ctla4*^{-/-} onto B7-1 and -2 double knockout, or *Cd28*^{-/-}. This dramatic alteration in phenotype underscores the opposing roles of CTLA-4 and CD28 in maintaining the balance between T cell tolerance and immunity.

CD28 Functions in Regulatory T cells

CD28 has also been shown to be required for development and maintenance of CD4⁺ FoxP3⁺ regulatory T cells (74). Treg development in the thymus may represent an alternative pathway of differentiation for T cells with a moderate affinity toward self antigen, which would otherwise undergo apoptotic cell death during negative selection (75, 76). Costimulation through CD28 or other pathways may have a role in negative selection as *in vitro* stimulation of double positive thymocytes with anti-CD3 and anti-CD28 antibodies triggers apoptotic cell death similar to negative selection, whereas anti-CD3 treatment alone does not (77, 78). In conjunction, these two findings may indicate that combined TCR and CD28 signaling above a certain threshold is required to trigger the Treg cell fate in developing thymocytes. In addition, Treg cells require IL-2 for their survival in both the thymus and the periphery, and enhancement of IL-2 production is a key unique feature of CD28 activation. Adoptive transfer of WT Treg cells into *Cd28*^{-/-} or WT hosts revealed a role for CD28 expression on conventional T cells of the host, which was

required for production of a minimal level of IL-2 in order to support the survival of transferred WT Tregs as they do not make their own IL-2 despite their dependence on CD28 (74). Pre-culture of WT Tregs in IL-2 before adoptive transfer allowed the cells to overcome the IL-2 deficit in *Cd28*^{-/-} hosts. However, Tregs failed to develop from *Cd28*^{-/-} bone marrow even in mixed bone marrow chimeras with WT bone marrow, suggesting that there is also a cell-intrinsic function for CD28 in Treg development (79).

While CD28 deficiency is usually protective in most autoimmune and transplantation models, as described above, it has the opposite effect in the nonobese diabetic (NOD) mouse strain, which develops spontaneous autoimmune insulin-dependent diabetes similar to type 1 diabetes in humans. *Cd28*^{-/-} NOD mice have a higher incidence and greater severity of spontaneous disease compared to WT NOD controls (80, 81). *Cd28*^{-/-} T cells isolated from NOD mice have similar proliferative defects to those observed on C57BL/6 background, but the difference in this model stems from the requirement for CD28 in the development and maintenance of FoxP3⁺ Treg cells (81). The absence of functional Treg cells in *Cd28*^{-/-} NOD mice seems to enhance disease progression, although how *Cd28*^{-/-} T cells circumvent the requirement for CD28-mediated costimulation in this disease setting but not in other autoimmune models remains unclear. Other costimulatory pathways may be able to compensate for lack of CD28 function in this model. Alternatively, this process may be related to the unique peptide binding features of the NOD MHC class II allele (I-A^{g7}), which has a highly unusual peptide binding repertoire and is known to present peptides with low binding affinity at the cell surface (82). In particular, pathogenic CD4⁺ T cells specific for insulin-derived peptides bound to I-A^{g7} have been isolated from NOD mice. Chronic TCR stimulation, which might result from such an unusual pMHC repertoire, has been shown to overcome lack of CD28 costimulation in some

systems (83). However, the exact mechanism of how these self-antigen specific T cells escape negative selection and then become activated in the periphery, and why this process would be independent of CD28-mediated costimulation, remains unclear.

Since CD28 is required for Treg development, studies of CD28 function on mature Tregs have been technically challenging. However, the development of a *FoxP3*^{YFP-cre} *Cd28*^{fl/fl} mouse allowed for conditional deletion of CD28 on Tregs after cell fate commitment (i.e. after expression of the FoxP3 transcription factor, which is the master regulator of the Treg lineage) (84). Thus, Tregs could develop normally in the thymus and then the requirement for CD28 for their continued maintenance and suppressive function could be interrogated independently. There was a slight decrease in CD28-deleted Treg frequency in the thymus, but not the periphery, which could be accounted for by a reduction in both homeostatic and stimulation-induced Treg proliferation. CD28-deleted Tregs also showed a marked disadvantage compared to WT Tregs in a competitive environment (e.g. mixed bone marrow chimeras or adoptive co-transfer). Interestingly, *FoxP3*^{YFP-cre} *Cd28*^{fl/fl} mice developed a spontaneous autoimmune disorder marked by lymphadenopathy, splenomegaly, accumulation of activated (CD44⁺) T cells, and multi-organ inflammatory cell infiltration. The disease presentation is reminiscent of *scurfy* disorder, in which a FoxP3 mutation causes a failure in Treg development. CD28-deleted Tregs showed no defect in suppressive function *in vitro*, but were markedly defective in controlling *in vivo* inflammatory responses in an adoptive transfer-induced colitis model and an immunization-induced EAE model. These results might have been due to reduced expansion of CD28-deleted Tregs *in vivo*, or a failure to home to inflammatory sites in sufficient numbers to restrain inflammation. Thus, in addition to its requirement in T cell development, CD28 is also required to maintain the homeostasis and suppressive capability of the mature peripheral Treg

compartment through support of passive and stimulation-induced Treg cell proliferation.

CD28 Signaling Motifs *in vivo*

The plethora of well-characterized CD28-dependent *in vivo* model systems has afforded ample opportunity to characterize the individual contributions of putative signaling motifs present within the CD28 cytoplasmic domain, which were discussed in detail above. Perhaps due to differential requirements for CD28-mediated costimulation in different model systems, the results of these studies have not yielded a clear consensus on the importance of particular motifs to CD28 function overall.

The Singer laboratory developed a series of transgenic mice bearing several CD28 mutations: Y170F, within the YMNМ motif, reported to recruit PI3K and Grb2; P194A/P197A, within the PRRPG motif, reported to recruit Itk; P206A/P209A, within the PYAPA motif, reported to recruit Lck; and a CD28 truncation (from residue 164) lacking nearly the entire cytoplasmic domain. The transgenic lines were crossed individually onto the *Cd28*^{-/-} background. As expected, T cells from the CD28-tailless mutant behaved similarly to *Cd28*^{-/-} T cells, displaying reduced proliferation and IL-2 production in response to combined TCR and CD28 stimulation *in vitro*, and a reduced frequency of Treg cells *in vivo* (79). The P206A/P209A mutant was phenotypically identical to *Cd28*^{-/-}, while no other mutant exhibited any defect in the assays tested.

These transgenic strains were also crossed onto the *Ctla4*^{-/-} *Cd28*^{-/-} background in order to assess the sufficiency of each CD28 mutant in providing costimulation necessary for activation of autoimmune T cells (85). As expected, the CD28wt transgene restored the lethal lymphoproliferative autoimmune disorder found in *Ctla4*^{-/-} mice, resulting in low body weight,

lymphadenopathy, and a high proportion of T cells displaying an activated phenotype (CD69⁺) and effector differentiation (CD44⁺ CD62L⁻). Mice expressing the CD28-tailless transgene, which should lack signaling capacity similar to *Cd28*^{-/-}, were completely protected, as were those expressing P206A/P209A CD28 mutant transgene. No other CD28 mutants conferred any protection. Thus, in this transgenic system only the CD28 PYAPA motif appears to be required for CD28 costimulatory function, and it behaves like a complete loss-of-function mutant.

In an alternative approach, the Green laboratory developed a targeted knockin mouse strain replacing a portion of the endogenous CD28 locus with the P206A/P209A mutant (86). The knockin approach has the advantage of maintaining transcriptional regulation at the endogenous locus, so mutant CD28 expression perfectly reflects the native WT protein in terms of cell specificity, timing during T cell development, transcription and translation level. Some variation in surface expression could result from the introduced mutation, as it might change protein trafficking to the plasma membrane or endocytosis and recycling rates. However, CD28 surface expression was identical for CD28wt and mutant alleles on T cells from the thymus and spleen, both in the homozygous (mut/mut) and heterozygous (mut/-) genotypes. This was not the case for the transgenic lines discussed above, which all showed slightly elevated CD28 expression compared to the endogenous level in WT C57BL/6 mice (79).

Initial studies with the P206A/P209A knockin mutant revealed a rather complex phenotype that overall seemed to suggest a partial loss of function for CD28 signaling both *in vitro* and *in vivo* (86). T cells from homozygous (mut/mut) mice exhibited normal proliferation in response to anti-CD3/anti-CD28 antibody stimulation, but were markedly defective under the more physiological stimulation condition of co-culture with allogeneic splenocytes, which supply the natural ligands for CD28 activation, CD80 and CD86. Heterozygous (mut/-) T cells

showed defective proliferation at almost the level of $Cd28^{-/-}$ T cells in both stimulation conditions. This might suggest that the P206/P209A mutant retains partial function, which is capable of providing some costimulatory activity, but that defects in the hypomorphic allele become more apparent when the protein is expressed at lower-than-normal levels or when suboptimal stimulation is provided.

In a model of allergic airway inflammation, when sensitized to antigen by immunization and then later challenged intranasally with the same antigen, $Cd28^{+/+}$ mice develop inflammatory lesions in the lungs, which can be identified histologically and by the recovery of eosinophils and neutrophils from bronchoalveolar lavage fluid. $Cd28^{-/-}$ mice are completely protected in this model, showing no evidence of inflammation. No defect was observed for the mutant: heterozygous (mut/-) mice, which exhibited even less costimulatory activity than homozygous (mut/mut) mice, showed normal induction of allergic airway inflammation, similar to $Cd28^{+/+}$ mice (86). However, titers of antigen-specific IgG1 antibodies were dramatically lower in mut/mut and mut/- mice compared to $Cd28^{+/+}$ and $Cd28^{+/-}$ controls. The discrepant results *in vivo* may indicate that different CD28-mediated signaling pathways are responsible for the induction of sensitization and for development of effective humoral immunity through B cell help. The PYAPA motif may be required for signaling through some of these pathways but not others. Alternatively, our results presented in Chapter 2 suggest that CD28-dependent Lck activity is only partially disrupted by mutations in the PYAPA motif, since clusters of basic residues within the CD28 cytoplasmic domain contribute substantially to Lck binding affinity. Hence, the results from this study may be explained as the phenotype of a partial loss-of-function mutant: the defect in this mutant may only become apparent under conditions of sub-optimal stimulation or in processes that have a high threshold requirement for CD28 costimulation.

A subsequent study repeated much of the same analysis but introduced for additional comparison the CD28 Y170F knockin mutant, which is reported to disrupt PI3K binding to CD28 (17, 18). The Y170F mutant behaved like the WT protein in all assays tested, including IL-2 production, proliferation, cell survival in culture, sensitized allergic airway inflammation, and EAE (87). The results for the P206A/P209A were the same as previously described, except that an additional defect for this mutant was shown *in vivo* in an EAE model. A significant reduction in disease severity was observed with homozygous (mut/mut) mice, although not to the same extent as for *Cd28*^{-/-} mice. This provides further evidence that the P206A/P209A double mutant retains some costimulatory function and may represent only a partial loss-of-function due to incomplete disruption of Lck binding.

In order to examine whether residual costimulatory function in the P206A/P209A mutant emanated from the intact YxxM motif, a subsequent study examined knockin mice in which Y170F and P206A/P209A mutations were introduced together (88). This compound mutant displayed a more severe defect than the P206A/P209A mutant alone in terms of IL-2 production, survival and proliferation, but still retained some costimulatory activity compared to *Cd28*^{-/-} cells. Intriguingly, even the compound mutant showed no defect in induction of allergic airway inflammation compared to *Cd28*^{+/+} mice. The authors concluded that one or more other signaling motifs within CD28 cytoplasmic remained to be discovered, which is in agreement with our findings that CD28 basic residue clusters are involved in recruitment of Lck, followed by PKCθ.

CD28 cytoplasmic motif mutants were also rigorously examined for their ability to support antigen-specific CD4⁺ T cell expansion in response to bacterial infection or immunization (89). T cells in *Cd28*^{-/-} mice showed nearly a 10-fold reduction in expansion

compared to that of T cells in wild-type mice in response to bacterial infection. However, in mixed bone marrow chimeras, CD28 Y170F, P206A/P209A or Y170F/P206A/P209A T cells expanded equally well compared to WT T cells in the same bacterially-infected host, indicating intact costimulatory function for all of these mutants.

In summary, these studies reveal a complex phenotype for CD28 cytoplasmic domain mutants. Y170, the recruitment site for PI3K, seems to be largely dispensable for CD28 function *in vivo*. Mutation of the PYAPA motif causes defects in some models but not in others, which might suggest only a partial loss-of-function. Alternatively, different CD28-specific effects might emanate from different motifs within the cytoplasmic domain, and the importance of these biological processes might vary in different models. Clearly, mutants targeting either or both sequences retain some costimulatory function that is attributable to other as-yet-undiscovered motifs.

Current Perspectives

The biochemical processes involved in CD28 signal initiation were heavily investigated more than a decade ago, but this area may be of renewed interest today due to the incorporation of the CD28 cytoplasmic domain into design of some second- and third-generation chimeric antigen receptors (CARs) (reviewed in (90, 91)). These artificial receptors target tumor-associated antigens using a single-chain variable fragment (scFv), which confers specificity, grafted onto a transmembrane domain and one or more cytoplasmic domains, which confer signaling capability. Early designs included for signaling only the CD3 ζ cytoplasmic domain, which contains three ITAMs per chain and as such is the most potent TCR-associated CD3 subunit for recruitment of ZAP-70. However, cells transduced with this first-generation design

did not persist well enough *in vivo* to successfully eradicate tumor (92, 93). As T cells generally require two signals for their full activation, one from the antigen receptor and the other from a costimulatory receptor, a logical follow-up was to incorporate the cytoplasmic domain of a costimulatory receptor in order to support the expansion and survival of CAR T cells *in vivo* (94). The most widely-tested second-generation designs to date utilize either CD28 or 4-1BB with CD3 ζ .

Very little is known about how signal initiation might differ when the cytoplasmic domains of T cell signaling receptors are stitched together in this manner rather than in their native conformations. Intracellular distance from the plasma membrane is likely to be altered in the CAR, and as key effector kinases Lck, PKC θ and PI3K are all capable of membrane association through lipid interactions, accessibility of substrate for kinase activity may be affected. CARs contain two extracellular Ig domains, which matches the size of the TCR. This may be an important design feature as immunological synapse formation involves segregation of positive signaling mediators (TCR and CD28) away from negative regulators (e.g. CD45 and CD148 phosphatases) based on size (95). However, the location of the scFv binding epitope within the target antigen could introduce further variation in the total distance bridged between cells by bound antigen:CAR complex. It is not known whether CAR T cells even form synapses with target cells, and whether such structures would be critical to CAR function. Additionally, CARs may or may not retain the same lateral mobility within the membrane as TCR and CD28, which may be required for efficient signaling in microclusters and for mature synapse formation.

Studies of the membrane binding properties of CD28 and TCR-associated CD3 chains, including the study presented in Chapter 2, may be highly applicable to further investigations of CAR signal initiation. Despite substantial investigation into the biochemical events involved in

CD28 receptor-proximal signaling, studies with *in vivo* model systems have failed to yield a consensus on the importance of various biochemical events to CD28 function at the cellular and organismal levels. It will be important in the future to continue to refine our understanding of the mechanism of CD28 signal initiation, which can in turn inform our understanding of CAR function.

Chapter 2

Membrane Binding by the CD28 Cytoplasmic Domain Inhibits

Lck Recruitment and Signaling

Abstract

The mechanisms controlling initiation of CD28 signaling remain unclear, despite extensive study. Here we show that the CD28 cytoplasmic domain is bound to the plasma membrane in resting cells and that ligand binding favors its release from the membrane. Membrane binding by the cytoplasmic domain requires two clusters of basic residues, which interact with the negatively charged inner leaflet of the plasma membrane. The same clusters of basic residues also serve as interaction sites for Lck, a *src*-family kinase critical for CD28 function. This signaling complex is further stabilized by Lck-mediated phosphorylation of CD28 Y207 and binding of the Lck SH2 domain to this phospho-tyrosine. Mutation of the basic clusters reduces recruitment of PKC θ , which serves as a key effector kinase in the CD28 signaling pathway, to the CD28-Lck complex. Consequently, mutation of either a basic cluster or Y207 impairs CD28 function *in vivo* as shown by reduced thymic differentiation of FoxP3⁺ T_{REG} cells. Based on these results, we propose a new model for the initiation of CD28 signaling.

Introduction

Signaling through the T cell costimulatory receptor CD28 is essential for full activation of naïve T cells and their differentiation into effector cells. T cells from *Cd28*^{-/-} mice exhibit a range of defects, including reduced proliferation and survival in response to stimulation, as well as impaired cytokine production (IL-2 in particular) (13). Consequently, T cells from *Cd28*^{-/-} mice mount poor responses to viral or bacterial challenge, and fail to induce T cell-mediated autoimmunity (67, 85-87). Naïve T cells that are activated through the T cell receptor but do not receive CD28 costimulatory signals either undergo apoptosis or become anergic to further TCR stimulation (96, 97). Intact CD28 signaling is also required for the development and peripheral maintenance of FoxP3⁺ regulatory T cells (T_{REG}) (79, 81), and has a cell-intrinsic role in their ability to exert suppressive functions (84). Germline deletion of CD28 leads to a severe reduction in the frequency of T_{REG} cells in both the thymus and the periphery. Expression of CD28 ligands, CD80 and CD86 (also known as B7-1 and B7-2, respectively), is strongly upregulated on antigen presenting cells (APCs) in response to microbial danger signals (98, 99). Thus, CD28 activation is restricted spatially and temporally by the activation state of APCs, and CD28 is central to the balance of tolerance and T cell-mediated immunity.

Despite intense study, the molecular events downstream of CD28 engagement are not fully understood (1). Lck was shown to phosphorylate Y189 in the YMNM motif, creating a binding site for the SH2 domains of Grb2 and the p85 subunit of PI3K (17-19, 100). It has been proposed that the Lck SH3 domain interacts with the C-terminal PYAP motif (P206, P209) (26), but a recent study showed that the Lck SH3 domain is instead required for recruitment of PKC θ , an essential component of the CD28 signaling pathway that activates NF- κ B signaling through Carma1 (21). The N-terminal poly-proline motif (P194, P197) was proposed to recruit ITK and

TEC, positive regulators of PLC- γ 1 (23, 101). Imaging studies of CD28 microclusters following T cell activation showed no accumulation of Grb2, Gads, Vav1 or Itk and also only transient accumulation of PI3K (37). In contrast, there was robust and sustained recruitment of PKC θ , which required an intact CD28 cytoplasmic domain (31, 37). Thus, no unified model of CD28 signaling has emerged.

Each of these motifs has been characterized in a variety of *in vivo* model systems. Mutation of the PYAP motif results in a significant impairment of CD28 function, as evidenced by reduced development of T_{REG} cells, impaired humoral responses, and protection from both experimental autoimmune encephalomyelitis (EAE) and lethal lymphoproliferative disorder on the *Ctla4*^{-/-} background (79, 85-87). In contrast, mutation of either the YMNМ motif or the N-terminal poly-proline motif had no effect in any of these model systems. More recently, experiments examining *in vivo* proliferation of T cells in response to bacterial challenge revealed a defect with *Cd28*^{-/-} and CD28 tail-less mutants, as expected, but not for mutants targeting either the tyrosine in the YMNМ motif or the two proline residues in the PYAP motif (89). Furthermore, examination of knock-in mice bearing a double mutant targeting both motifs revealed residual CD28 function *in vivo* as assessed by allergic airway inflammatory response (88). Therefore, other signaling motif(s) in the CD28 cytoplasmic domain (CD28_{CD}) may remain to be identified.

Lck activity is critical for the initiation of signaling downstream of both CD28 and the TCR-associated CD3 subunits (102). In resting T cells, Lck is tethered to the plasma membrane through N-terminal myristoylation and palmitoylation sites (103), and a fraction of Lck is associated with CD4 or CD8 co-receptors through a Zn²⁺ coordination site (104). It remains unclear which pool of Lck is involved in CD28 signal initiation in mature T cells. The level of

Lck activity is regulated by phosphorylation of two Tyr residues (105). Lck activity is enhanced following auto-phosphorylation on Y394, located in the kinase domain (106). Phosphorylation of Lck Y505 by Csk inhibits Lck kinase activity through an intramolecular interaction of the Lck SH2 domain with pY505, which induces a closed-clasp conformation (107, 108). CD45 can relieve this inhibition by de-phosphorylation of pY505 (109). Lck still retains some activity when dually phosphorylated, and all four phosphorylation states have been identified in T cells (110).

The cytoplasmic domains of TCR signaling subunits CD3 ϵ and CD3 ζ were both previously shown to interact with the inner leaflet of the plasma membrane in a charge-dependent manner (111-113). CD28 presents an intriguing target for further study because the cytoplasmic domain also has a net positive charge. Here we demonstrate that the CD28 cytoplasmic domain binds to the plasma membrane in live cells at steady state, and that ligand engagement favors dissociation from the membrane. Interestingly, the same CD28_{CD} motifs that mediate membrane binding are also required for initial Lck recruitment and subsequent recruitment of PKC θ , which serves as a key effector kinase in the CD28 signaling pathway. Mutation of these motifs causes a reduction in the frequency of FoxP3⁺ T_{REG} cells similar to the phenotype of *Cd28*^{-/-} mice, emphasizing the *in vivo* importance of these motifs. We also demonstrate that Y207 in the PYAP motif is phosphorylated by Lck and then serves as a binding site for the Lck SH2 domain. Based on these results, we propose a new model for the initiation of CD28 signaling.

Results

Clusters of basic residues in the CD28 cytoplasmic domain mediate interaction with the inner leaflet of the plasma membrane

Based on the high overall positive charge of CD28_{CD} (reflected by an isoelectric point of 10.89), we hypothesized that CD28_{CD} could interact with the negatively charged inner leaflet of the plasma membrane. We utilized a previously reported Förster resonance energy transfer (FRET) approach to examine membrane binding by CD28_{CD} in live cells (*111*). The FRET donor TFP was attached to the C-terminus of CD28_{CD} while the FRET acceptor octadecyl rhodamine B (R18) was incorporated into the plasma membrane (Figure 2.1A, S2.1A). Murine CD28 constructs with WT or mutant cytoplasmic domains were expressed in Jurkat cells, and single cell clones with similar TFP fluorescence intensity were selected for each construct (Figure S2.1B, C). As controls, we utilized a series of fusion proteins with a flexible cytoplasmic linker of variable length (3, 25 or 50 amino acids) and C-terminal TFP (attached to KIR2DL3 extracellular and transmembrane domains). FRET was quantified based on quenching of TFP donor fluorescence upon incorporation of the R18 acceptor into the plasma membrane. 3aa-TFP served as a positive control (FRET efficiency of 55%) because the TFP donor was in close proximity to the plasma membrane (Figure 2.1B). TFP tethered to 25 or 50aa linkers mimicked conditions in which CD28_{CD}, which is 43aa in length, exhibited either partial or no specific membrane interaction, respectively. Due to the flexibility of the linker, a small proportion of TFP molecules were at any time within close proximity to the plasma membrane, resulting in a low level (21%) of TFP quenching with the 50aa linker. We also sequentially imaged TFP-expressing cells without R18 labeling to assess TFP photo-bleaching; the average change in TFP fluorescence was <1% at the end of the time course.

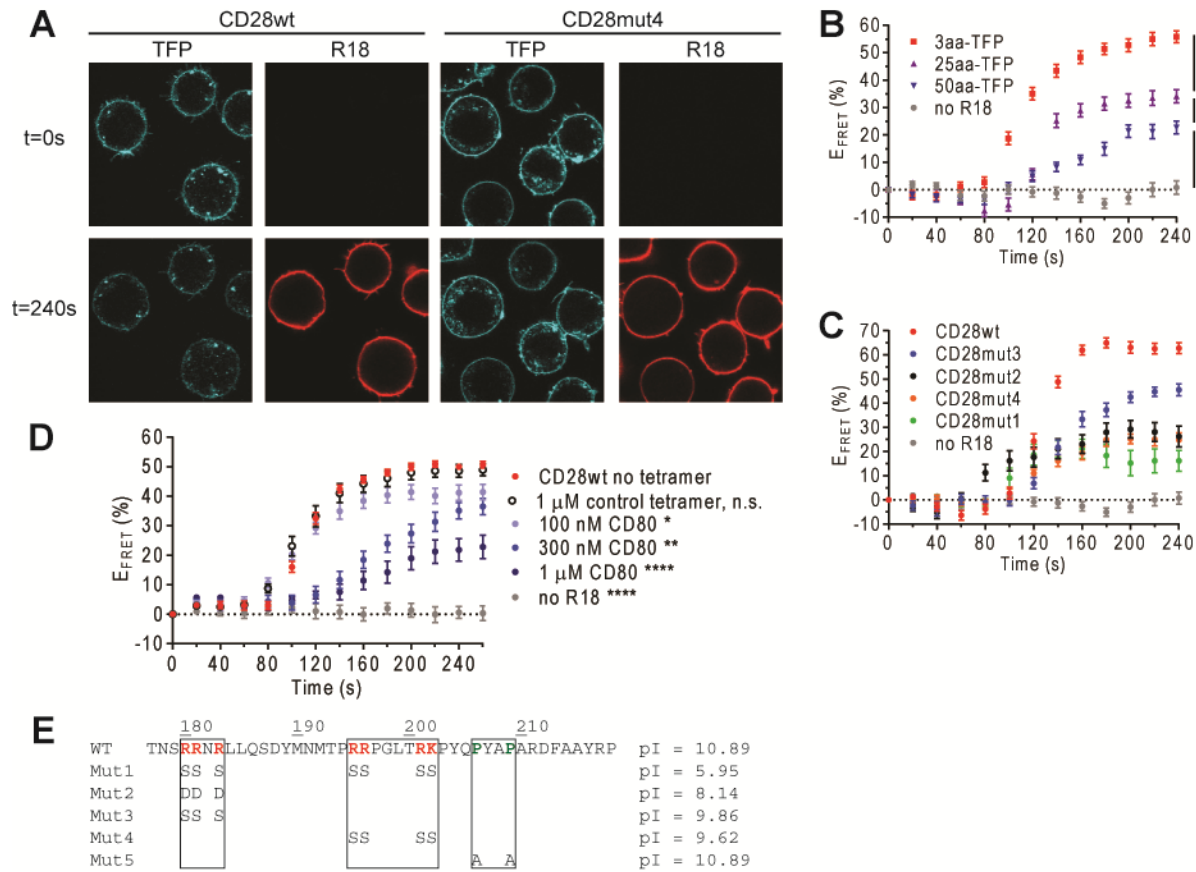


Figure 2.1 The CD28 cytoplasmic domain interacts with the plasma membrane and is released from the membrane upon receptor triggering. (a) Confocal microscopy images are shown of Jurkat cell transfectants expressing CD28-TFP fusion proteins (FRET donor). Images depict cells before (t=0s) and after (t=240s) labeling of the plasma membrane with octadecyl rhodamine B (R18) as a FRET acceptor. (b) TFP fluorescence at the plasma membrane was quantified and used to calculate FRET efficiency (E_{FRET}) for individual cells at each time point. The mean E_{FRET} and SEM for each construct are shown (n=20 cells). Mean E_{FRET} was compared for TFP-expressing cells with no R18 label, a 3aa linker between TFP and the transmembrane domain (3aa-TFP), a 25aa linker (25aa-TFP), and a 50aa linker (50aa-TFP). (*p=0.0187, ****p<0.0001) (c) The mean E_{FRET} was calculated as in (b) for CD28-TFP wild type compared

Figure 2.1 (Continued)

to mutant fusion proteins (* $p=0.0361$, **** $p<0.0001$). (d) CD28WT E_{FRET} was measured after pre-incubation with varying concentrations of CD80 tetramer, a control tetramer that does not bind to CD28 (HLA-DM), or in the absence tetramer. The control without tetramer was compared to each other group (n.s. not significant, * $p=0.0427$, ** $p=0.0012$, **** $p<0.0001$). (e) The sequence and isoelectric point (pI) for WT and mutant CD28 cytoplasmic domain are shown. (Mut5 was studied in other experiments, but not assessed for membrane binding.)

CD28WT exhibited a high quenching FRET efficiency (62%), similar to 3aa-TFP, which indicated that CD28_{CD} was largely bound to the plasma membrane (Figure 2.1C). Membrane binding by CD28WT was confirmed by donor dequenching: photobleaching of R18 restored TFP fluorescence at the plasma membrane to pre-quenching levels (Figure S2.1D, E). CD28 receptor triggering through pre-incubation of CD28WT-expressing cells with CD80 tetramers substantially reduced membrane binding as shown by a concentration-dependent decrease in FRET efficiency, demonstrating that ligand binding induces dissociation of CD28_{CD} from the membrane (Figure 2.1D).

In order to examine the charge dependence of this interaction, we designed several mutants (sequences shown in Figure 2.1E) targeting two clusters of basic residues in CD28_{CD}. Mut1, which targeted both clusters, exhibited the lowest FRET efficiency (16%), indicative of complete loss of membrane interaction. The three arginine residues in the N-terminal cluster were mutated to aspartic acid (introduction of negative charge, Mut2) or serine (charge neutralization, Mut3). Both mutants exhibited a partial loss of membrane binding (FRET efficiency of 27% for Mut2 and 45% for Mut3). Mutation of the central cluster of basic residues to serine (Mut4) also significantly reduced membrane binding (FRET efficiency of 25%) (Figure 2.1C, S2.1A). The degree of membrane binding correlated somewhat with isoelectric point, but there were also positional effects as Mut3 and Mut4 had similar isoelectric points, but Mut4 caused a greater reduction in membrane binding. In contrast, previous studies of the CD3 ϵ cytoplasmic domain had shown that an N-terminal cluster of basic residues made the most significant contribution to membrane binding (*111*). In summary, these results demonstrate that CD28_{CD} is bound to the inner leaflet of the plasma membrane in unstimulated cells in a manner similar to that previously identified for CD3 ϵ and ζ cytoplasmic domains (*111-113*).

Furthermore, CD28 crosslinking by the CD80 ligand induces release of CD28_{CD} from the plasma membrane.

Clusters of basic residues are required for phosphorylation of CD28 dimer by Lck

Initiation of CD28 signaling after ligand binding involves CD28 tyrosine phosphorylation by Lck (101). We investigated Lck phosphorylation of CD28_{CD} in a cell-free environment to assess the impact of mutations of basic clusters without confounding effects of lipid interaction. CD28_{CD} was expressed as a GST-fusion protein and purified following cleavage of the GST fusion partner. Purified recombinant Lck readily phosphorylated CD28WT_{CD} *in vitro* in the presence of ATP and metal co-factors, as shown by anti-phosphotyrosine Western blot (Figure 2.2A, B), while no phosphorylation was detected in control reactions lacking Lck (Figure 2.2B). A cysteine residue was attached to the N-terminus of CD28_{CD} for potential modification, and initial experiments analyzing a mixture of monomers and disulfide-linked dimers formed by brief oxidation with 1mM GSSG revealed that only CD28_{CD} dimer was phosphorylated by Lck, even when the monomer was present in the same sample. This is relevant because CD28 is present in T cells as a homodimer linked by a disulfide bond in the membrane-proximal stalk region (9), and thus the two cytoplasmic domains are likely to be in close proximity.

In order to compare Lck phosphorylation activity on CD28WT and mutant dimers, we prepared mono-biotinylated dimers by conjugating the introduced N-terminal cysteine residue to a short biotinylated bismaleimide linker. Using these dimers as substrates for Lck, we found that mutation of N-terminal (Mut2 and Mut3) and central (Mut4) clusters of basic residues caused a substantial defect in Lck phosphorylation (Figure 2.2B, C). This was a surprising result given that a contribution of basic residues to Lck recruitment or activity was not previously suggested.

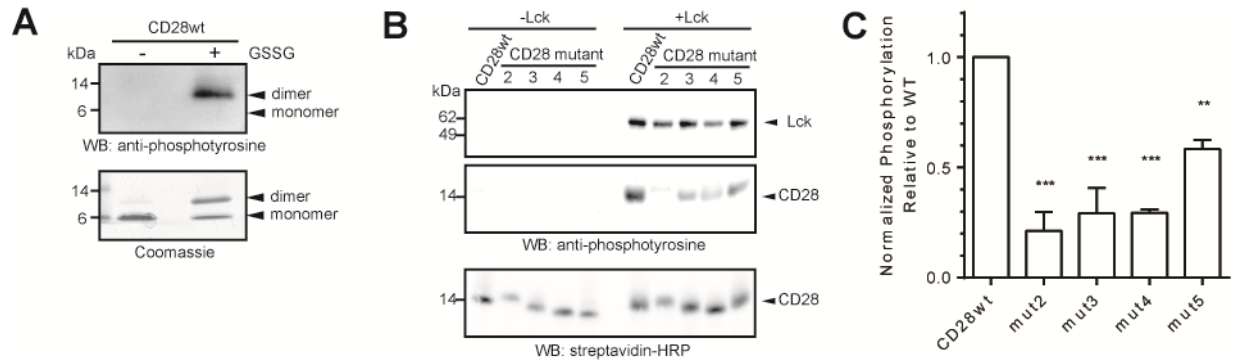


Figure 2.2 Clusters of basic residues in CD28_{CD} are required for phosphorylation by Lck.

(a) Purified CD28WT_{CD} was subjected to an *in vitro* phosphorylation (IVP) assay with recombinant Lck in the presence or absence of oxidizing agent (GSSG). The reactions were visualized by anti-phosphotyrosine WB (top) and Coomassie gel stain (bottom). (b) Mono-biotinylated, N-terminally-linked CD28_{CD} dimers were subjected to IVP assay or a control reaction without Lck. Reactions were visualized by anti-phosphotyrosine WB, which illuminates both Lck and phosphorylated CD28 as indicated by molecular weight standards, and streptavidin WB as a CD28 loading control. (c) CD28 phosphorylation was normalized for loading, and then mutants were compared relative to WT for reactions containing Lck. The average and SD are shown (n=2 experiments). The mean normalized phosphorylation of CD28WT was compared to the mean of each other group for statistically significant differences using one-way ANOVA with Bonferroni correction (***p<0.001, **p=0.0064).

Mutation of the PYAP motif (Mut5) also caused a partial defect in Lck-mediated phosphorylation. Mut1 was not examined in these and further assays because two clusters of basic residues were simultaneously mutated; instead, these clusters were studied individually in Mut3 and Mut4. We further examined CD28WT and Mut5 *in vitro* phosphorylation reactions to determine which tyrosine residues in CD28_{CD} were Lck targets. CD28 dimers were gel-purified after the reaction, subjected to in-gel tryptic digest, and analyzed by LC-MS/MS. Equivalent phosphorylation was detected on Y189 and Y207 for both substrates (Table S2.1). These results corroborate experiments performed by others in cellular systems, which supports the relevance of the *in vitro* system to conditions in cells (28, 29). These findings demonstrate that basic residue clusters within CD28_{CD} contribute to phosphorylation by Lck, and that a dimer of CD28 cytoplasmic domains is the appropriate Lck substrate.

Mutation of CD28 basic residues impairs Lck binding both in vitro and in cells

The reduction in Lck-mediated phosphorylation of CD28 basic residue mutants suggested that these clusters might comprise previously unidentified, direct CD28-Lck interaction sites. We utilized a surface plasmon resonance (SPR) approach to address this question. Lck or an irrelevant control protein (HLA-DM) of approximately the same molecular weight and isoelectric point were captured on a CM4 dextran matrix by amine-coupling. CD28WT or mutant purified disulfide-linked dimers were injected at varying concentrations for 2 min, followed by a 4 min dissociation period (Figure 2.3A, B, S2.2A, B). Signal from the control protein surface was subtracted from the signal of the Lck surface in order to eliminate bulk refractive index changes and background binding. We next immobilized a truncated form of Lck lacking the kinase domain (Lck U-SH3-SH2) and observed minimal binding (Figure 2.3C),

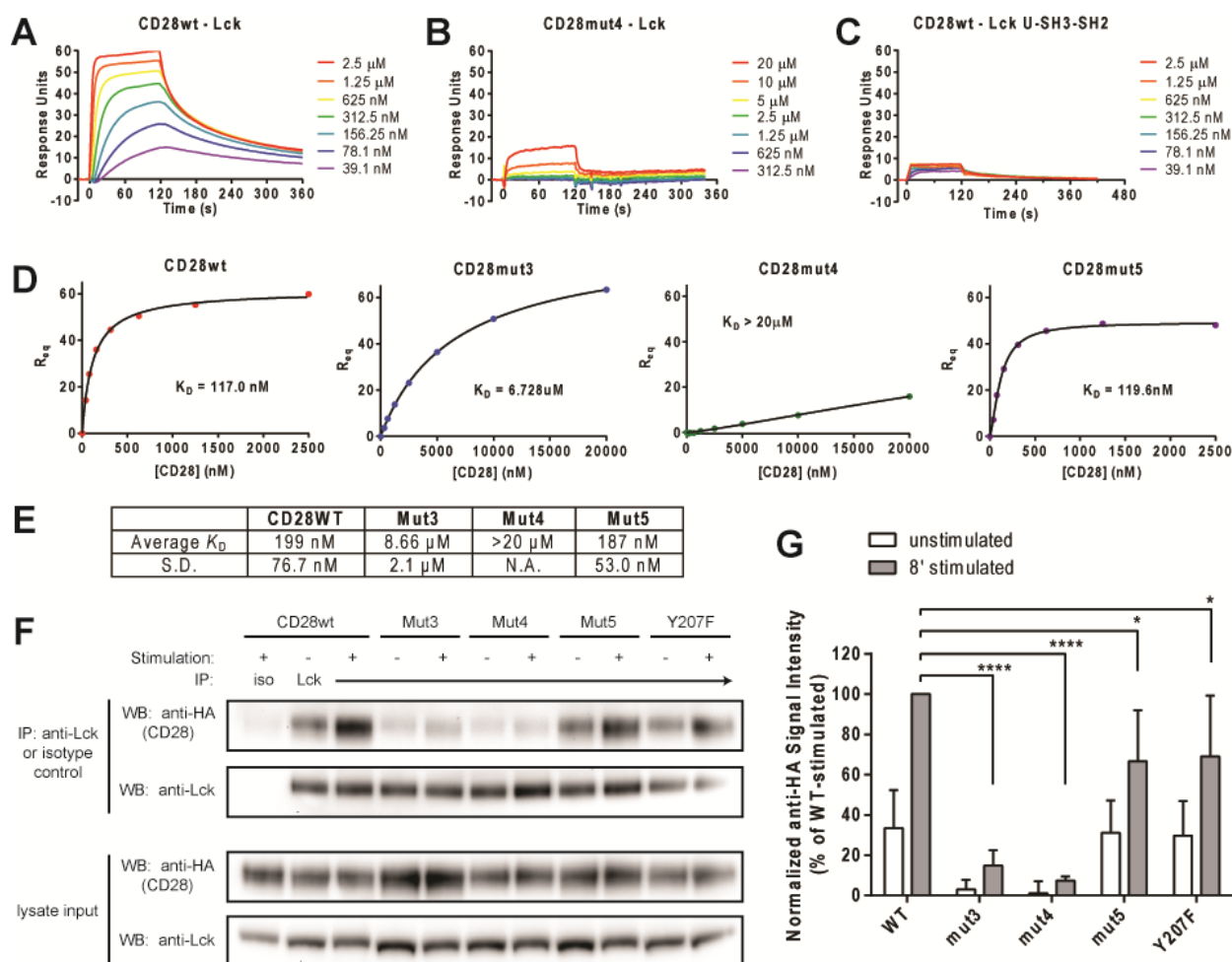


Figure 2.3 CD28-Lck binding interaction requires CD28_{CD} basic residues and the Lck kinase domain. (a,b) The displayed curves depict SPR measurements of varying concentrations of CD28WT (a) or Mut4 (b) disulfide-linked dimers injected over a surface coated with Lck with a 2 min association and a 4 min dissociation period. Bulk refractive index change was subtracted based on the signal from a surface with similar immobilized amounts of an irrelevant protein (HLA-DM). (c) The same concentration series of CD28WT shown in (a) was injected over a surface with immobilized Lck unique-SH3-SH2 domains. (d, e) K_D was determined from a plot of response units bound at equilibrium (R_{eq}) versus CD28 concentration using a saturation binding model with Hill slope. The geometric mean and standard deviation (S.D.) is shown in (e)

Figure 2.3 (Continued)

for n=3 experiments. S.D. was not calculated for Mut4 for because the K_D was too high to estimate accurately (N.A., not applicable). (f) HA-tagged CD28 in complex with Lck was detected by anti-HA WB following IP with anti-Lck antibodies or isotype control from unstimulated or APC-stimulated OT-I⁺ hybridomas. As controls, WB detection of HA-CD28 and Lck was performed on lysate inputs, and Lck detection on IP samples. (g) Densitometry analysis was performed on WBs shown in (f) and HA-CD28 co-immunoprecipitation was normalized within each experiment to the stimulated CD28WT sample (100%). The average and SD are shown (n=5 experiments). Each mutant stimulated sample was compared to CD28WT (*p<0.05, ****p<0.0001).

which indicates that the Lck kinase domain is required for binding to CD28_{CD} dimer.

We estimated the affinity (K_D) of the CD28-Lck interaction from a plot of response units (R.U.) bound at equilibrium versus CD28 concentration by fitting the data to a saturation binding model with Hill slope (114), which is a factor that accounts for cooperativity between multiple binding sites in either the ligand or analyte (Figure 2.3D). Equilibrium affinity measurements were used because the data were not modeled well by kinetic fitting methods, probably due to cooperative effects from the bivalent CD28 analyte. The average affinities were calculated from three experiments for Lck binding to CD28WT dimer (K_D 199 nM), Mut3 (K_D 8.66 μ M) and Mut5 (K_D 187 nM) (Figure 2.3E). Analysis of CD28_{CD} dimers comprising only the C-terminal 25 amino acids (and therefore lacking the N-terminal basic cluster) yielded similar results to those obtained for Mut3, which targets this site by mutagenesis instead of deletion (Figure S2.2C). Accurate affinity estimates require analysis of concentrations above and below the K_D of the interaction, and therefore a higher concentration series was prepared for basic residue mutants (Mut3 and Mut4) and 25mer peptides. The highest concentration measured for Mut4 was 20 μ M, so we can only accurately report that the K_D is above 20 μ M, although the estimated K_D appears to be much higher (>1 mM). In summary, mutation of the N-terminal cluster of basic residues (Mut3) caused a greater than 40-fold reduction in Lck affinity compared to CD28WT, while the central cluster caused an even more substantial reduction despite similar isoelectric points. These experiments were performed under conditions that were non-permissive for phosphorylation (without ATP), indicating that such CD28-Lck binding events could contribute to initial Lck recruitment following ligand binding by CD28.

We further examined CD28-Lck interaction in live cells using OT-I⁺ hybridoma cells transduced with HA-tagged CD28WT or mutant full-length proteins. The OT-I⁺ cells expressed

very little endogenous CD28, which minimized the possibility of pairing of endogenous CD28WT with the introduced mutants (Figure S2.3A). CD28-transduced cells were sorted for matched CD28 surface expression to ensure accurate quantitative comparisons between cell lines (Figure S2.3B). OT-I⁺ cells were either left unstimulated or stimulated with peptide-pulsed DC2.4 cells, which were previously generated from bone marrow-derived dendritic cells (115). Lck was isolated by immunoprecipitation (IP), and co-precipitation of HA-tagged CD28 was analyzed by Western blotting (Figure 2.3F, G). Some CD28-Lck association was observed in unstimulated CD28WT-expressing cells, and stimulation induced an approximately 3-fold increase in Lck recruitment to CD28. Mut3 and Mut4 exhibited a profound reduction in Lck recruitment both with and without stimulation, which is in agreement with the results of the *in vitro* binding assays presented above. Lck association with Mut5 and Y207F in unstimulated cells was very similar to the levels observed in CD28WT-expressing cells, whereas a partial defect in Lck recruitment was detected upon stimulation. Detection of a CD28-Lck interaction in unstimulated cells is consistent with a dynamic equilibrium model in which CD28_{CD} spontaneously associates and dissociates from the membrane; the fraction that is not membrane-bound at a given time could become available for interaction with Lck.

Altogether, these data suggest a model wherein clusters of basic residues are required both for initial binding of Lck to CD28 and sustained interaction, while the PYAP motif influences CD28-Lck interaction only after an initial receptor activation event has occurred.

Lck SH2 domain binds to phosphorylated Y207 in the PYAP motif of CD28_{CD}

Several reports have indicated that the CD28 PYAP motif is critical for signaling function, and it was originally thought to bind the Lck SH3 domain (26). However, this

mutation (Mut5 in our studies) exhibited little to no defect in Lck binding *in vitro*, and a modest defect in CD28-Lck association in cells only post-activation. Other studies have suggested that Lck might associate with this motif using its SH2 domain, not the SH3 domain (21, 27). In order to clarify this controversial point, we examined binding of either Lck U-SH3-SH2 (comprising the unique, SH3 and SH2 domains) or Lck SH2 domain alone to truncated CD28_{CD} peptides representing the C-terminal 25 residues (residues 194-218), which were immobilized on a streptavidin surface. No binding was observed to non-phosphorylated CD28_{CD} peptide (Figure 2.4A, B). However, binding was observed to WT peptide phosphorylated at Y207. The requirement for phosphorylation at Y207 supported the hypothesis that the Lck SH2 domain rather than the SH3 domain was required for this interaction. Equilibrium binding analysis using the isolated Lck SH2 domain confirmed that recognition of phospho-Y207 was indeed mediated by the SH2 domain (Figure 2.4C). However, Y207 phosphorylation did not alter the binding of 25mer CD28_{CD} dimer to full-length Lck (Figure S2.2C), which was largely driven by the central cluster of basic residues. This was likely because recombinant full-length Lck was phosphorylated on Y505 (Figure S2.2D), which would compete with CD28 phospho-Y207 for Lck SH2 domain binding. Covalent coupling of full-length Lck to the dextran matrix in those experiments may also have inhibited conformational mobility, rendering the SH2 domain inaccessible to the competing motif in the CD28 analyte.

We further examined the effect of the two proline residues in the CD28 PYAP motif on Lck interaction using a phosphorylated double mutant (P206A/P209A, analogous to Mut5) 25mer peptide, which showed a 6.37-fold reduction in affinity for the Lck SH2 domain (K_D 63.7 μ M) compared to the corresponding phosphorylated WT peptide (K_D 10.0 μ M) (Figure 2.4D). The individual contribution of each proline residue to Lck SH2 domain binding was assessed

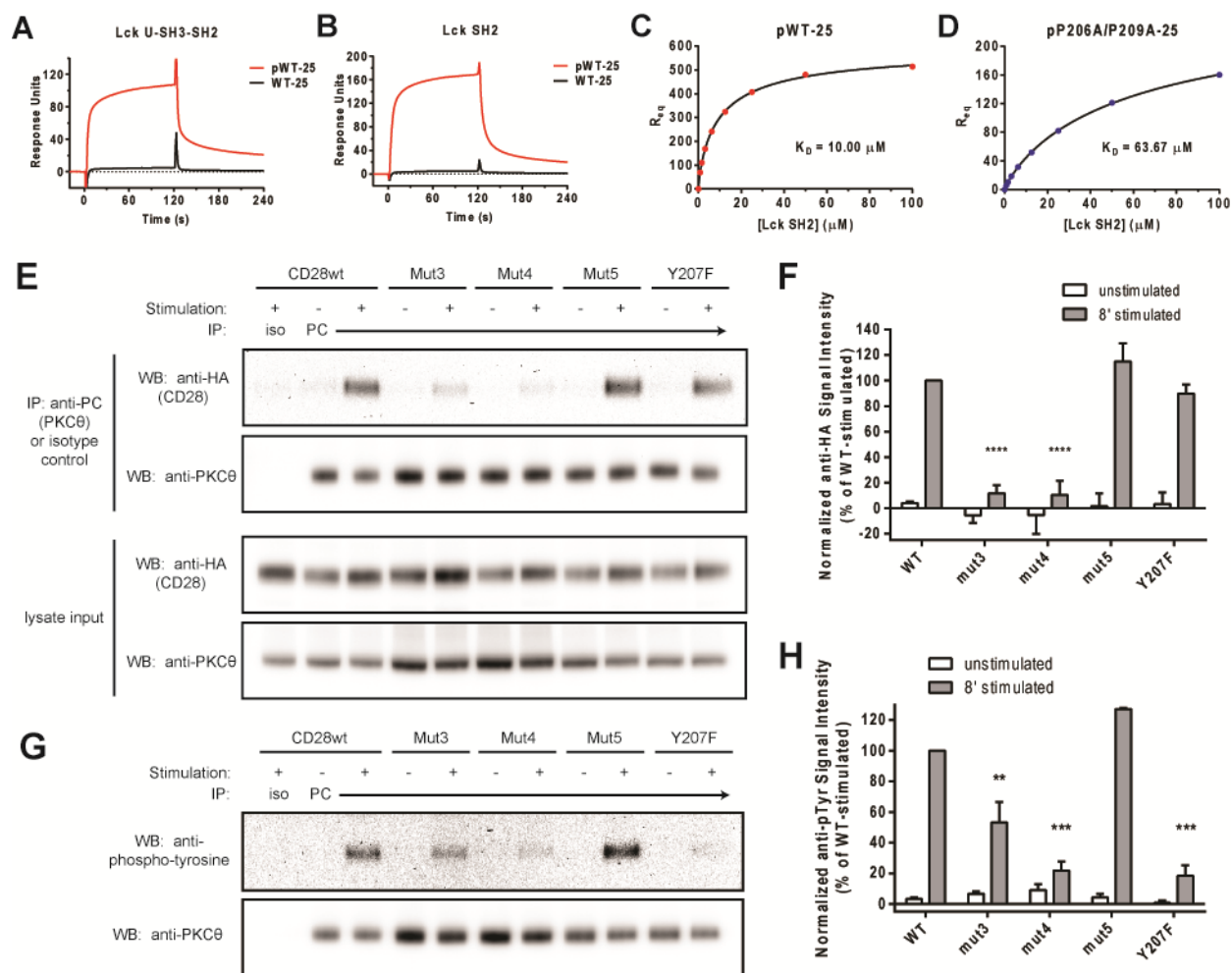


Figure 2.4 Lck SH2 domain binds to the phosphorylated PYAP motif of CD28. Peptides representing the C-terminal 25 residues of CD28_{CD} (with and without phosphorylation at Y207) were coated to a similar density on a streptavidin surface. Lck U-SH3-SH2 (a) or SH2 domain only (b) were injected at a concentration of 3.125 μ M for 2 min association followed by buffer injection. Bulk refractive index change was subtracted based on a control streptavidin surface with no peptide. (c, d) R_{eq} was measured for varying concentrations of Lck SH2 domain. K_D was calculated by fitting to a saturation binding model with Hill slope. (e) HA-tagged CD28 in complex with PC-tagged PKC θ was detected by anti-HA WB following IP with anti-PC

Figure 2.4 (Continued)

antibodies or isotype control from unstimulated or APC-stimulated OT-I⁺ hybridomas. IP reactions were blotted with anti-PKC θ antibodies as a loading control. WB detection of HA-CD28 and PKC θ is also shown for lysate inputs. (f) Densitometry analysis was performed on WBs shown in (e) and normalized within each experiment to the stimulated CD28WT sample (100%). The average and SD are shown (n=3 experiments). Each mutant was compared to CD28WT for stimulated cells (****p<0.0001). (g) Tyrosine-phosphorylated PKC θ was detected by WB following anti-PC (PKC θ) IP from unstimulated or APC-stimulated OTI⁺ hybridomas. (h) Densitometry analysis was performed as in (f) (n=2 experiments, **p=0.0053, ***p<0.001).

using a panel of 15mer peptides (sequences shown in Figure S2.4A). As with the 25mer peptides, binding of Lck U-SH3-SH2 or the isolated SH2 domain to 15mer peptides was only observed upon Y207 phosphorylation (Figure S2.4B, C). Affinity measurements by equilibrium binding analysis revealed that P209 made a greater contribution to Lck SH2 domain binding than did P206 (Figure S2.4D, S2.5).

These results suggest a two-step model for Lck binding to CD28_{CD}: initial binding mediated by clusters of basic residues enables phosphorylation of Y207, which in turn creates a second, lower-affinity binding site for the Lck SH2 domain. Consequently, Mut5 exhibits a defect only under CD28 phosphorylation conditions (i.e. Lck binding to phosphorylated but not non-phosphorylated CD28, and CD28-Lck coIP from stimulated but not unstimulated cells), for which SH2 domain binding is relevant. This interaction may enhance Lck activity by stabilizing the open, active conformation through displacement of the inhibitory Lck phospho-Y505 motif, or by enhancing recruitment of downstream signaling mediators. The impaired *in vivo* function of the double-proline mutant (PYAP to AYAA) may be explained by impaired binding to the Lck SH2 domain as shown here.

Clusters of basic residues are required for recruitment of PKC θ to the CD28 signaling complex

A recent report suggested that PKC θ is directly recruited to the CD28-Lck signaling complex through interaction of the PKC θ hinge region with the Lck SH3 domain (21). Given the importance of CD28 basic residue clusters in binding to Lck, we examined the role of these motifs in recruitment of PKC θ as a critical downstream target of Lck-mediated CD28 signaling. PKC θ was present at very low abundance in OT-I⁺ hybridoma cells used for CD28-Lck studies presented above, and was barely detectable by Western blot in comparison to primary mouse T

cells (Figure S2.4E). Therefore, we transduced the cells to express full-length PKC θ with a PC tag to facilitate immunoprecipitation.

OT-I⁺ cells were either left unstimulated or stimulated with peptide-pulsed DC2.4 cells over-expressing CD80. PC-tagged PKC θ was isolated by IP and co-precipitating HA-CD28 from WT or mutant-expressing cells was detected by Western blotting (Figure 2.4E, F). Although some CD28-Lck complex was detected in unstimulated cells, PKC θ recruitment to CD28 was entirely dependent upon stimulation. Mutation of either the N-terminal (Mut3) or central (Mut4) cluster of basic residues resulted in a significant impairment in CD28-PKC θ complex formation. This phenotype is consistent with the previous report of a direct interaction between CD28, Lck and PKC θ : loss of Lck binding through mutation of basic residue clusters impairs PKC θ recruitment to CD28 because Lck is required as the binding intermediate. However, we cannot rule out the possibility that CD28 basic residues interact directly with PKC θ or through another unknown intermediary.

There was no apparent defect in recruitment of PKC θ to CD28 Mut5 or Y207F. However, since the Lck SH2 domain engages in an intramolecular interaction with phosphorylated Y505 to induce a closed-clasp structure which exhibits reduced kinase activity, we hypothesized that binding to the CD28 phosphorylated PYAP motif might enhance Lck kinase activity by favoring the open, more active conformation. Lck is known to phosphorylate PKC θ on Y90 so we examined the PKC θ phosphorylation status by WB after IP from stimulated or unstimulated OT-I⁺ cells (Figure 2.4G, H) (116). Mut3 and Mut4 exhibited reduced PKC θ phosphorylation, as expected given the defective recruitment of both Lck and PKC θ to these mutants. Although the CD28 Y207F mutant recruited PKC θ to the same level as CD28WT, PKC θ phosphorylation was substantially reduced, suggesting that Lck is indeed less active when

the SH2 domain is not able to bind to the phosphorylated CD28 PYAP motif. Mutation of the two proline residues in the PYAP motif did not reduce PKC θ phosphorylation, even though the affinity of the Lck SH2 domain was reduced ~6-fold by this CD28 mutation. The strong activation signal induced in these studies to facilitate recovery of protein complexes may overcome this modest defect, whereas the level of CD28 signaling elicited *in vivo* may be more sensitive to this mutation.

Mutation of the central cluster of basic residues or Y207 reduces CD28 signaling in vivo

CD28 deficiency causes a severe reduction in the frequency of FoxP3⁺ T_{REG} cells in both the thymus and peripheral lymphoid organs of mice (79, 81). CD28 is known to have a cell-intrinsic function in this process, which made this an ideal model to examine the *in vivo* function of CD28 mutants using transduced bone marrow chimeras without concern over differences in the transduction efficiency (and therefore frequency of CD28-expressing cells) between groups or individual recipients. Bone marrow from *Cd28*^{-/-} mice was transduced with lentiviral vectors driving expression of CD28WT or mutants and used to reconstitute the T cell compartment of lymphopenic recipients after lethal irradiation. Similar CD28 expression levels were observed in peripheral T cells from transduced bone marrow chimeras expressing CD28WT or mutant proteins (Figure S2.6A, B). At 6 weeks post-transplant, spleens and thymi were collected from recipient mice and analyzed for the frequency of CD25⁺ FoxP3⁺ T_{REG} cells among CD3⁺ CD4⁺ GFP⁺ cells or CD4⁺ CD8⁻ GFP⁺ cells, respectively (Figure 2.5A). Fixation and permeabilization caused a decrease in the GFP fluorescence intensity, but transduced cells could nonetheless be detected. We also noted approximately a 10-fold lower frequency of GFP⁺ cells in the thymus compared to the spleen of each recipient. T_{REG} frequency was normal for CD28WT recipients

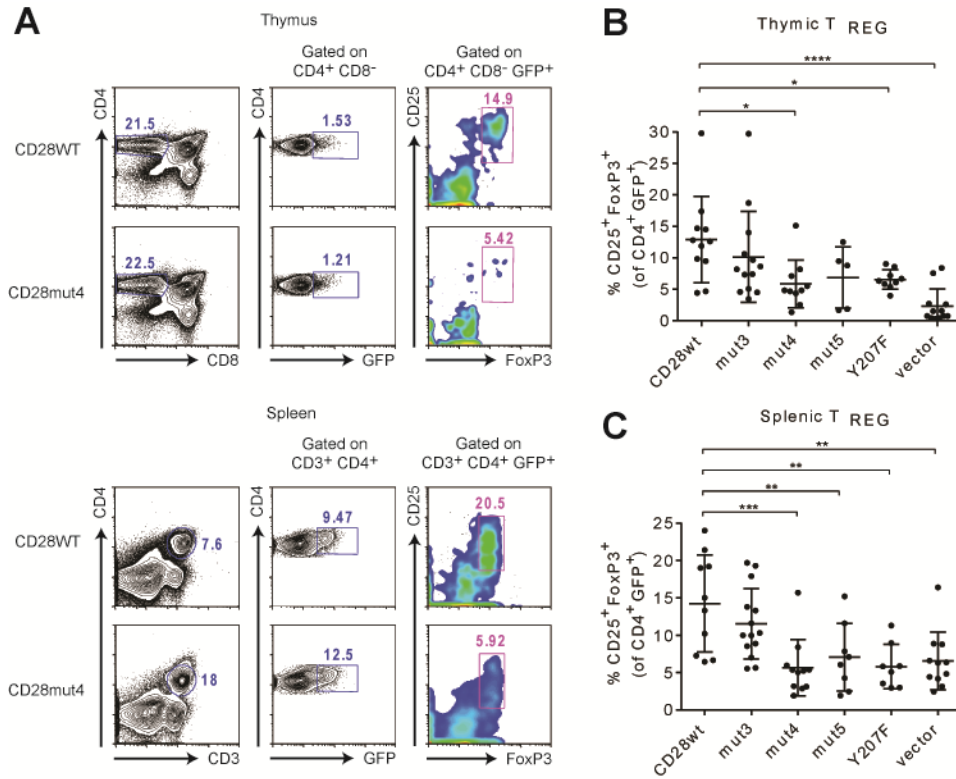


Figure 2.5 Mutation of the central cluster of basic residues impairs the in vivo function of the CD28 cytoplasmic domain. (a) T_{REG} cells from CD28-transduced bone marrow chimeric mice were identified on the basis of CD25 and intracellular FoxP3 expression after gating on CD4⁺ CD8⁻ GFP⁺ thymocytes or CD3⁺ CD4⁺ GFP⁺ splenocytes. Representative plots are shown for one CD28WT- and one CD28mut4-expressing sample. The frequency of T_{REG} cells in the thymus (b) and spleen (c) was analyzed as shown in (a). Mean and SD are shown, and each dot represents an individual mouse with data pooled from four independent experiments. Differences between groups were assessed for statistical significance using one-way ANOVA with Bonferroni correction for multiple comparisons (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

(14% in spleen, 13% in thymus), but the vector-transduced negative control group had a severe defect (7% in spleen, 2% in thymus) as expected for *Cd28*^{-/-} T cells (Figure 2.5B, C). Mut4 and Y207F caused a significant reduction in T_{REG} frequency (6% in spleen and thymus of Mut4 group, 6% in spleen and 7% in thymus of Y207F group), similar to the vector control. We were also able to replicate the defect previously observed for the double proline mutant (Mut5, 6% in spleen and thymus), although only results in the spleen were statistically different from WT. Thus, mutation of either the central cluster of basic residues, the PYAP motif, or Y207 caused a significant defect in CD28 signaling function *in vivo*.

Discussion

Based on these data, we propose a new model for initiation of CD28 signaling (Figure 2.6). We show that CD28_{CD} interacts with the inner leaflet of the plasma membrane in the absence of receptor triggering (step A). CD28_{CD} membrane binding inhibits initiation of signaling because the same clusters of basic residues are required both for membrane binding and for subsequent Lck recruitment. Biological interactions are typically dynamic rather than static, so CD28_{CD} is likely in equilibrium between the membrane-bound and unbound states. We show that ligand engagement shifts this equilibrium toward dissociation from the membrane (step B). This hypothesis is further supported by previous studies of the CD3 ϵ and ζ cytoplasmic domains, which demonstrated membrane binding through similar charge-charge interactions and release from the plasma membrane upon TCR triggering (*117*). Following receptor activation, Lck binding should prevent re-engagement of CD28_{CD} basic residues with the plasma membrane, thus providing a mechanism for signal stabilization. Once bound, Lck phosphorylates Y189 and Y207 within CD28_{CD} (step C). Y207 phosphorylation creates a binding site for the Lck SH2 domain (step D), which may stabilize Lck in an active conformation (by preventing binding of inhibitory pY505 to the Lck SH2 domain), enhancing its activity on other substrates including PKC θ . PKC θ , which is initially targeted to the inner leaflet of the plasma membrane in the immunological synapse through binding to diacylglycerol (DAG) created by the activity of PLC- γ (*118*), can subsequently bind to the SH3 domain of Lck (step F) (*21*), and act a critical effector kinase for CD28 through the NF- κ B pathway.

One report previously suggested that Lck binds first to the PYAP motif of CD28_{CD} using its SH3 domain (*26*), but we did not detect Lck binding to non-phosphorylated 25- or 15-mer peptides containing this motif. Rather, our data show that Lck binds first to clusters of basic

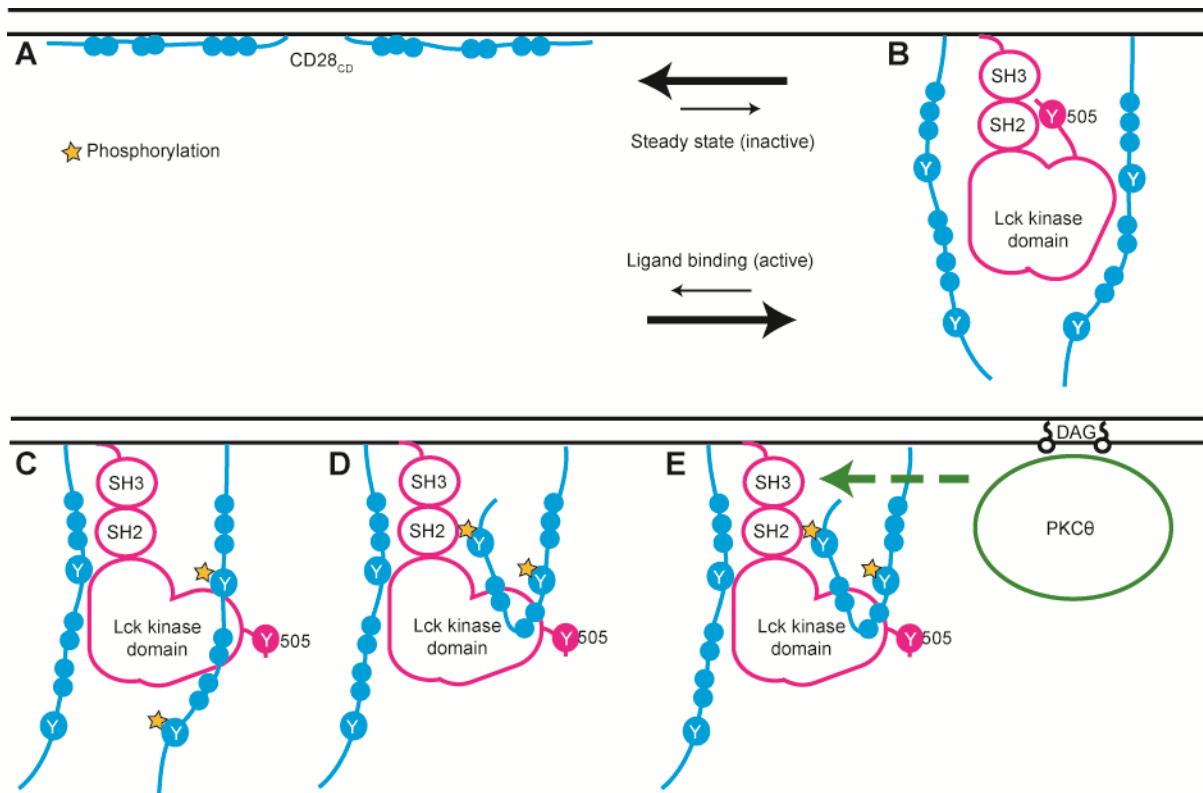


Figure 2.6 Model of early events in the CD28 signaling pathway. (a) Clusters of basic residues in CD28_{CD} mediate interaction with the negatively charged inner leaflet of the plasma membrane in resting T cells. Membrane binding represents a dynamic equilibrium. (b) Ligand binding to CD28 shifts this equilibrium and favors dissociation of CD28_{CD} from the plasma membrane, making clusters of basic residues available for interaction with Lck. A CD28 dimer is required for this interaction. Lck is depicted in a closed conformation with phospho-Y505 bound intra-molecularly to the Lck SH2 domain, although in cells there is likely a mixture of phospho-Lck conformers. (c) Lck phosphorylates two tyrosine residues in CD28_{CD}, Y189 and Y207. Phosphorylation of only one chain of the CD28 dimer is depicted here, although phosphorylation of both chains is likely. (d) CD28 phospho-Y207, located in the PYAP motif, binds to the Lck SH2 domain. (f) The Lck SH3 domain remains free to recruit PKCθ, as

Figure 2.6 (Continued)

reported by others. CD28 basic residue clusters are required for PKC θ recruitment, likely because Lck acts as an intermediary in the interaction. Lck SH2 domain binding to CD28 phospho-Y207 then enhances phosphorylation of PKC θ , possibly through stabilization of the open, more active conformation of Lck.

residues in CD28_{CD}, and that the PYAP motif becomes a binding site for the Lck SH2 domain following phosphorylation of Y207 by Lck. This means that the Lck SH3 domain remains available for interaction with PKC θ , which is then activated through phosphorylation by Lck (116). If the Lck SH3 domain were required for binding to the CD28 PYAP motif, as had been previously thought, then there would be competition for binding to the Lck SH3 domain between CD28 and PKC θ . Rather, the resulting signaling complex composed of CD28_{CD}, Lck and PKC θ is stable, as demonstrated by high-resolution imaging of CD28 microclusters in the immunological synapse (37) and co-immunoprecipitation studies presented here and elsewhere (21). Previous studies did not assess the direct biochemical interaction of the Lck SH3 domain with the PYAP motif, but rather showed that the P206A/P209A mutation impaired CD28 function in cell (26). Our results are in fact consistent with this work, but we show in direct binding studies that Lck only binds to this motif following phosphorylation of Y207 in the PYAP motif. Furthermore, we demonstrate a direct binding interaction between the Lck SH2 domain and peptides phosphorylated at Y207 in the PYAP motif (Figure 2.4A-D, S2.4A-D), which is supported by other published studies (21, 27). The affinity of the Lck SH2 domain for the phosphorylated PYAP motif is quite low (~ 10 μ M), but the local concentration of CD28_{CD}-bound Lck is high, likely resulting in a fast on-rate of binding. Furthermore, recruitment of PKC θ to the Lck SH3 domain is likely to further stabilize the signaling complex. Together, this may explain the strong effect observed *in vivo* for the double proline mutant despite a modest (~ 6 -fold) reduction in affinity for the Lck SH2 domain.

Our studies identify several mechanisms that support specificity of CD28 signaling. Lck binding prior to ligand engagement is inhibited by sequestration of CD28_{CD} basic residues through membrane binding. Furthermore, Lck only phosphorylates a dimer of CD28

cytoplasmic domains. As mentioned above, CD28_{CD} is likely in equilibrium between the bound and unbound states, with the bound form being predominant prior to receptor engagement (as evidenced by high FRET efficiency). Thus, release of only one of the two cytoplasmic domains from the membrane at any given time should not be sufficient to permit receptor phosphorylation, preventing aberrant, spontaneous receptor triggering. Upon stable Lck binding, however, rebinding of the basic residue clusters to the plasma membrane should be prevented due to occupancy by Lck, providing a mechanism for signal stabilization.

Our study also highlights similarities between CD28 and TCR early signaling events. CD28 and TCR co-localize in microclusters during the early stages of T cell signaling, but segregate in the mature synapse (37). Our studies show that the cytoplasmic domains of CD28, like CD3 ϵ / ζ chains, are bound to the inner leaflet of the plasma membrane. Previous studies of CD3 ϵ showed that the interaction occurs primarily between clusters of basic residues and acidic phospholipids, including phosphatidylserine (111). TCR triggering by peptide-MHC complexes results in release of the CD3 ϵ cytoplasmic domain from the plasma membrane, which is associated with a localized reduction in the negative charge and phosphatidylserine density within TCR microclusters (117). Similarly, we observed a charge-dependent interaction of CD28_{CD} with the plasma membrane, and dissociation upon ligand-induced receptor triggering. One of the early events in both TCR and CD28 signaling involves phosphorylation by Lck. Given these similarities, a reduction in phosphatidylserine density in early microclusters may result in coordinated release of both CD3 ϵ / ζ and CD28 cytoplasmic domains. Given the high affinity of CD28 for Lck, CD28 could then facilitate Lck activity on other substrates within the same TCR-CD28 microclusters.

An improved understanding of the role of membrane binding in CD28 signaling could

inform the design of chimeric antigen receptors (CARs) under development for anti-tumor immunotherapy, some of which have incorporated CD28_{CD} as a co-stimulatory signaling module to enhance T cell activation, survival and differentiation (119). Lck recruitment to CD28_{CD} by the two-step mechanism described above may contribute to phosphorylation of linked CD3 ζ chains. Features that could be optimized include the sequence in which ITAM and co-stimulatory signaling domains are linked as well as the distribution of clusters of basic residues, with the goal to minimize ligand-independent receptor activation and enhance ligand-dependent signaling. Finally, an intriguing question is whether the stimulus threshold for inducing receptor triggering (i.e. the required density of CAR antigen on target cells) could be altered depending on the distribution and density of clusters of basic residues that mediate membrane binding.

In summary, we have identified a new motif within CD28_{CD} that mediates membrane interaction in cells, but is also required for Lck recruitment and formation of a signaling complex composed of CD28, Lck and PKC θ . We propose a two-step model for CD28 activation in which initial Lck recruitment is mediated by clusters of basic residues. Subsequent binding of the Lck SH2 domain to the phosphorylated CD28 PYAP motif then stabilizes the open, active Lck conformation and facilitates phosphorylation of recruited PKC θ .

Materials and Methods

Preparation of cell lines

TFP-expressing Jurkat cell lines for FRET experiments were prepared by electroporation of pHAGE vector (Harvard Gene Therapy Initiative) containing murine CD28-TFP or KIR2DL3-linker-TFP cDNA expression constructs into Jurkat cells. For FRET controls, flexible linkers of 3, 25 or 50aa containing repeats of the sequence Gly-Gly-Ser were introduced between KIR2DL3 transmembrane and C-terminal TFP (*III*). Jurkat cells (1×10^7) were incubated on ice for 30min with plasmid DNA (100 μ g), and then electroporated in a Bio-Rad Gene Pulser II at 250V and 975 μ F. Stably-transfected TFP-expressing cells were isolated by multiple rounds of sorting. Clonal populations arising from single cells were analyzed for TFP expression level and cell lines with matched fluorescence were selected for use in FRET experiments.

Full-length CD28WT or mutant cDNA with an N-terminal HA epitope tag was cloned into the pHAGE vector. Lentivirus was produced in HEK293T cells and used to transduce OT-I⁺ hybridoma cells (a gift from E. Palmer, University of Basel). Cells were stained with anti-CD28-PE antibodies (clone 37.51, Biolegend) and sorted for uniform CD28 surface expression. Full-length PKC θ with an N-terminal PC tag was cloned into MSCV vector containing IRES-mCherry reporter, which drives relatively low-level expression in T cells. Retrovirus was produced in HEK293T cells and used to transduce OT-I⁺ hybridoma cells expressing CD28WT or mutant proteins. The same batch of virus was used at an equivalent MOI to ensure equal transduction efficiency across groups. Transduced cells were sorted for similar mCherry reporter expression. Full-length CD80 cDNA was cloned into the pHAGE vector. pHAGE-CD80 lentivirus produced in HEK293T cells was used to transduce DC2.4 cells (a gift from A. Sant, University of Rochester Medical Center). Transduced cells were sorted based on ZsGreen

marker and CD80 surface expression.

Statistical Analysis

Comparisons of the means between multiple groups were made using one-way ANOVA with Bonferroni correction for multiple comparisons in GraphPad Prism software. The multiplicity-adjusted p-value is reported.

FRET measurements

TFP-expressing Jurkat cells were washed with PBS prior to imaging. For experiments involving tetramer labeling, PBS-washed cells were incubated for 5min on ice with the indicated concentration of tetramer before proceeding with the experiment. Cells were allowed to settle on a 15mm glass coverslip for about 5min before assembling the RH-20 flow chamber (Harvard Apparatus). A constant flow of PBS (37°C) was maintained through the chamber. Cells were labeled with 800μL of 1.25μg/mL octadecyl rhodamine B (R18, Life Technologies). For quenching experiments, a time-lapse series was captured on a Leica SP5X confocal microscope with a 60x magnification oil-immersion objective. TFP was excited with a 458nm Argon laser and detected at 466 – 526 nm. R18 was excited with a white light laser tuned to 560nm and detected at 568 – 628 nm. Sequential TFP and R18 images were captured with 16-bit depth at a resolution of 1024 x 1024 pixels and 1x optical zoom.

Microscope settings for dequenching experiments were the same except that a 2x optical zoom was used. Sequential scans of TFP and R18 were performed once before and once after R18 photo-bleaching. Photo-bleaching was achieved by repeated R18 excitation at high laser power with slow scan speed.

Data analysis was performed using ImageJ software (open source, available from the NIH). A region of interest was drawn around the plasma membrane of individual cells and mean TFP fluorescence was measured for each time point of the series. Quenching was calculated using the formula $E_{\text{FRET}} (\%) = (TFP_0 - TFP_x) / TFP_0 * 100$ where TFP_0 is TFP fluorescence at $t=0$, and TFP_x is at $t = x$. $E_{\text{FRET}} (\%)$ was reported as an average of at least 20 individual cells. Dequenching was calculated according to the formula $E_{\text{FRET}} (\%) = (TFP_{\text{DQ}} - TFP_{\text{Q}}) / TFP_{\text{DQ}} * 100$ where TFP_{DQ} is dequenched TFP fluorescence (after R18 photo-bleaching) and TFP_{Q} is quenched TFP fluorescence (before photo-bleaching).

Protein expression and purification

CD28_{CD} was expressed in BL-21 CodonPlus (DE3)-RIPL cells (Stratagene) from the pET41a+ vector (EMD Millipore) as a GST fusion separated by an enterokinase recognition sequence (DDDDK), followed by a cysteine residue that was not part of the native sequence. GST fusion was purified from clarified cellular extracts using GST-Bind™ Resin (EMD Millipore) according to manufacturer recommendations. CD28_{CD} was liberated from GST fusion partner by digestion at a 1:1,000,000 (w/w) ratio of enterokinase to target (New England Biolabs). The peptide was then purified by reversed-phase HPLC using a C18 semi-preparative column (Grace Davison Discovery Sciences). Purified peptides were stored as lyophilized powder at -80°C and reconstituted in appropriate assay buffer immediately before use.

CD28 biotinylated dimer peptides used for *in vitro* phosphorylation assays were prepared from purified lyophilized stocks of unmodified peptide. Lyophilized peptide was reconstituted in water and reduced with TCEP-agarose beads (Thermo Scientific). Beads were removed using a Spin-X filter (Corning) and the peptide stock was adjusted to approximately pH 7.4 with the

addition of 10x PBS. Biotinylated bismaleimide peptide linker (sequence MPA-Gly-Ser-[Lys-biotin]-Gly-Ser-[Lys-MPA]-amide, where MPA indicates 3-maleimidopropionic acid, synthesized by 21st Century Biochemicals) was prepared as a 10mM stock in anhydrous DMSO from single-use aliquots. Linker was added to the reduced peptide in 4 aliquots 5min apart to achieve a 2:1 final molar ratio of CD28_{CD} to linker. The reaction was treated with 0.5mM DTT (to reduce disulfide-linked dimers) and biotinylated dimers were purified by gel filtration under reducing conditions. Unmodified monomers and biotinylated monomers were also separated from the biotinylated dimer in this way.

Murine full-length Lck (residues 6-509), U-SH3-SH2 (residues 6-226), SH2 domain (residues 122-226) were expressed using the Baculovirus pAcDB3 vector (BD Biosciences) with the signal peptide from H-2K^b in order to drive secretion of the mature protein into the cell supernatant, followed by a HA tag (sequence YPYDVPDYA) for purification. Residues 2-5 included modification target sites for membrane trafficking and were removed in order to facilitate protein secretion. Proteins were expressed in SF-9 insect cells (BD Biosciences) and purified from concentrated cell supernatant using anti-HA tag resin (Sigma Aldrich) with elution by competition with excess free HA peptide for 1h at room temperature. The proteins were further purified by gel filtration HPLC. Purified Lck was stored at -80°C in PBS with 25% glycerol, 0.25mM DTT and 0.1mM EDTA for *in vitro* phosphorylation assays or in HBS for SPR experiments.

Murine CD80 extracellular domain (residues 38-246) was prepared similarly with an N-terminal H-2K^b signal peptide and C-terminal BirA recognition sequence followed by an HA epitope tag. After final purification, the protein was buffer-exchanged into 10mM Tris, pH 8 and mono-biotinylated by incubation with BirA enzyme at a 1:20 molar ratio for 2h at 30°C with

0.1mM biotin, 10mM ATP, 10mM magnesium acetate and 50mM bicine pH 8.3. Free biotin was removed by gel filtration purification, and the biotinylated protein was stored in aliquots in PBS at -80°C. Tetramers were prepared by incubation of mono-biotinylated CD80 with ImmunoPure Streptavidin (Thermo Fisher) at a 4:1 molar ratio for 1h at 4°C.

In vitro phosphorylation assays

Purified CD28_{CD} biotinylated dimers (final concentration 1.5μM) were mixed with 100ng of purified Lck in 60mM HEPES pH 7.4, 5mM MgCl₂, 5mM MnCl₂, 3μM Na₃VO₄ and 200μM ATP. The reaction was allowed to proceed for 20min, then separated by SDS-PAGE and transferred to nitrocellulose membrane. Immunoblot was performed with HRP-conjugated anti-phosphotyrosine antibody (Cell Signaling Technology) or streptavidin-HRP (Sigma Aldrich) as a loading control on a separate blot processed in parallel. Chemiluminescent signal was captured using a ChemiDoc XRS (BioRad) with 16-bit depth and densitometry analysis was performed using ImageJ software. The mean phosphorylation intensity normalized to SA-HRP blot for loading was calculated for 2 experiments.

Mass spectrometry analysis of tyrosine phosphorylation

The band corresponding to CD28_{CD} dimer was excised from a Coomassie-stained gel following *in vitro* phosphorylation of WT and Mut5 with Lck. Samples were further processed by the Taplin Biological Mass Spectrometry Facility (Harvard Medical School). Excised gel bands were cut into pieces, treated with 1mM DTT for 30min at 60°C and then alkylated with 5mM iodoacetamide for 15min in the dark at room temperature. Gel pieces were subjected to in-gel trypsin digestion (120), and the extracted peptides were dried and stored at 4°C until analysis.

Samples were reconstituted in 2.5% acetonitrile, 0.1% formic acid and subjected to LC-MS/MS analysis using an LTQ-Orbitrap mass spectrometer (Thermo-Fisher) (121). Peptide sequences were determined by matching protein or translated nucleotide databases with the acquired fragmentation pattern using the Sequest program (ThermoFinnigan) (122). The modification of 79.9663 mass units to serine, threonine, and tyrosine was included in the database searches to identify phosphopeptides. Each phosphopeptide detected by the Sequest program was also manually inspected to ensure confidence in the assignment.

Surface plasmon resonance

SPR experiments were performed on Biacore 3000 and Biacore T200 instruments (GE Healthcare). CM4 chips (GE Healthcare) were used for binding analysis of Lck with full-length and 25mer CD28 dimer. The dextran surface was activated with a 1:1 mixture of 0.4 M EDC and 0.1 M NHS. Full-length Lck, Lck U-SH3-SH2 or HLA-DM were diluted in 50mM citrate pH 4.0 and injected until approximately 400RU of coupling was achieved for full-length Lck and HLA-DM, or 200RU for Lck U-SH3-SH2 (adjusted for lower molecular weight). The remaining active groups were quenched by injection of 1M ethanolamine pH 8.5.

CD28_{CD} dimer stocks were prepared by reconstituting a lyophilized aliquot with HBS containing 1mM GSSG. Serial dilutions were prepared in HBS-EP running buffer (GE Healthcare). CD28 injections were performed at a flow rate of 50 μ L/min with 2min association and 4min dissociation. The surface was regenerated with three 30s injections of 0.5mM DTT. Bulk refractive index changes were removed by subtracting the signal in the HLA-DM flow cell from the Lck (full-length or U-SH3-SH2) flow cell. Binding curves were examined for RU bound at equilibrium (R_{eq}) using BiaEval software (GE Healthcare). Plots of R_{eq} versus

concentration were fitted with a one-site binding model with Hill slope in GraphPad Prism software in order to determine K_D .

Biotinylated 15mer or 25mer peptides containing the PYAP motif (WT sequences YQPYAPARDFAAAYRP and PRRPGLTRKPYQPYAPARDFAAAYRP, respectively, 21st Century Biochemicals) were used for binding studies with Lck U-SH3-SH2 and SH2 domain. CD28 peptides were coated on a SA chip surface (GE Healthcare) to a density of approximately 200RU. Serial dilutions of Lck U-SH3-SH2 or SH2 only were prepared in HBS-EP running buffer and injected at a flow rate of 10 μ L/min with 2min association and 5min dissociation period. No regeneration was needed as the complex dissociated fully. Bulk refractive index change was subtracted based on signal from an empty SA surface. R_{eq} and K_D were determined as described above.

OT-I⁺ stimulation, immunoprecipitation and Western blotting

DC2.4-CD80 cells were treated overnight with 5ng/mL IFN- γ (Pepro-Tech) to induce H-2K^b expression and pulsed for 90min with 1.5 μ M SIINFEKL peptide (GenScript) prior to each experiment. OT-I⁺ cells were stimulated with DC2.4-CD80 cells for 8min at 37°C in complete media. Cells were lysed in 20mM HEPES, 150mM NaCl, 1% Brij-97 (Sigma-Aldrich), 1mM sodium orthovanadate, 1 μ g/mL aprotinin and 1 μ g/mL leupeptin for 45min at 4°C on a rotator. Insoluble material was pelleted and clarified lysates were pre-cleared for 30min with IP beads. Immunoprecipitation was performed with anti-Lck (clone 3A5, Santa Cruz) or anti-PC (clone HPC4, Roche) antibodies and Protein G Dynabeads (Life Technologies) overnight at 4°C. Beads were washed with lysis buffer, transferred to a fresh tube, and then eluted with either high pH buffer (50mM CAPS pH 11.5) for anti-Lck IPs or 5mM EDTA for anti-PC IPs.

Samples were analyzed by Western blotting with either anti-HA-HRP (Roche), anti-phosphotyrosine-HRP (Cell Signaling Technology), anti-Lck (Cell Signaling Technology) or anti-PKCθ (Cell Signaling Technology) antibodies, followed by incubation with mouse anti-rabbit-HRP (Southern Biotech) secondary, if necessary. Chemiluminescent signal was captured using a ChemiDoc XRS (BioRad) with 16-bit depth and densitometry analysis was performed using ImageJ software. Signal for complexed HA-CD28 was normalized for slight differences in CD28 expression, cell counting and IP target recovery according to the formula:

$$\frac{\text{HA coIP} / \text{HA lysate}}{\text{target loading} / \text{target lysate}}$$

where target indicates either the Lck or PKCθ IP target. All samples were compared to the CD28WT stimulated sample within each experiment, and then the mean was calculated across 5 experiments for CD28-Lck or 3 experiments for CD28-PKCθ. PKCθ phosphorylation was normalized to PKCθ loading control and then compared to the CD28WT stimulated sample (n=2 independent experiments).

Transduced bone marrow chimeras

Animal experiments were performed in accordance with a protocol approved by the Dana-Farber Cancer Institute institutional animal care and use committee (IACUC). Bone marrow was harvested from femurs and tibias of *Cd28^{-/-}* donor mice, using 1 donor per 1-2 recipient mice. After red blood cell lysis, bone marrow cells were stained with the Biolegend biotin anti-mouse lineage cocktail. Stained cells were depleted using Dynabeads™ Biotin Binder (Life Technologies). Remaining cells were stained with SA-Pacific Blue (Life Technologies), anti-Sca1-APC antibody (Biolegend), and anti-c-kit-PE antibody (Biolegend).

Lineage⁻ Sca1⁺ c-kit⁺ (LSK) cells were sorted and cultured for 24h with mouse SCF (50ng/mL, Biolegend), mouse IL-3 (20ng/mL, Biolegend) and mouse IL-6 (50ng/mL, Biolegend) in complete DMEM with 20% FBS and 50μM beta-mercaptoethanol. Cells were transduced on retronectin-coated plates with lentivirus derived from the pHAGE vector containing CD28WT or mutant cDNA expression constructs, or empty vector as a negative control. In advance, small-scale transductions were performed for each batch of virus in order to estimate the amount of virus needed to achieve approximately 20% transduction efficiency. At 24h post-transduction, cells were collected, washed and resuspended in PBS with 2% FBS for intravenous injection into lethally-irradiated *Rag1*^{-/-} recipient mice. Recipients were maintained on antibiotic-containing water for 3 weeks after bone marrow transplant to reduce the risk of infection.

Analysis of T_{REG} cell frequency

Spleens and thymi were harvested from transduced bone marrow chimeras at 6 weeks post-bone marrow transplant. 5x10⁶ cells per organ were stained with anti-CD3-PerCP (clone 2C11, Biolegend), anti-CD4-Pacific Blue (clone GK1.5, Biolegend) and anti-CD25-APC (clone PC61, Biolegend) antibodies for splenocytes or anti-CD8-PerCP (clone 53-6.7, Biolegend), anti-CD4-Pacific Blue and anti-CD25-APC antibodies for thymocytes. Cells were fixed and permeabilized using eBioscience FoxP3 staining buffer set according to manufacturer recommendations. Intracellular staining was performed with anti-FoxP3-PE antibody (clone 150D, Biolegend).

CD3⁺ CD4⁺ GFP⁺ gated splenocytes or CD4⁺ CD8⁻ GFP⁺ gated thymocytes were analyzed for frequency of CD25⁺ FoxP3⁺ T_{REG} cells. Results were pooled from 4 independent experiments. Separately, CD28 surface expression was assessed in splenocytes without fixation

and permeabilization by staining with anti-CD3-APC (clone 2C11, Biolegend) and anti-CD28-PE antibodies. CD3⁺ GFP⁺ gated cells were analyzed for CD28 MFI.

Chapter 3

Further Examination of Cytoplasmic Domain Membrane Binding:

Primary Cells and Functional Consequences

Abstract

The inner leaflet of the plasma membrane in mammalian cells is highly enriched in phosphatidyl serine, which bears a negatively charged lipid headgroup. T cell signaling components CD3 ζ , CD3 ϵ , and now CD28 have all been shown to interact with negatively charged lipids, either *in vitro* or in cells. Data presented in this chapter extends analysis of membrane binding to primary T cells, and explores the consequences of disruption of membrane binding by non-mutagenic protein engineering.

Introduction

T cells have extraordinary capabilities in providing antigen-specific protection against foreign invaders of the host, including bacteria, viruses and parasites. However, they also have the potential to cause devastating autoimmune responses when activated inappropriately against self antigens. For this reason, multiple layers of control regulating activation of the T cell receptor (TCR) and costimulatory receptor CD28 are essential in preventing spontaneous T cell activation and destructive autoimmunity.

At steady state *src*-family kinases (principally Lck in T cells) may be prevented from phosphorylating and activating TCR and CD28 through combined activity of the kinase Csk, which phosphorylates the inhibitory motif of Lck at Y505, and various phosphatases that can directly de-phosphorylate activating tyrosine residues within the cytoplasmic domains of TCR-associated CD3 subunits and of CD28 (102, 123). Both CD148 and CD45 have been shown to de-phosphorylate TCR (124, 125), although the activity of CD45 phosphatase is somewhat complicated by the observation that it also de-phosphorylates Lck at both the activating site (Y394) and inhibitory site (Y505) (126). Phosphatases such as SHP-1 and SHP-2 are recruited by inhibitory receptors CTLA-4 and PD-1 to dephosphorylate TCR-CD3 complex and CD28 where they co-localize at the immunological synapse (127-129). CTLA-4 is also capable of interrupting CD28 signaling by competing for binding to shared ligands B7-1 and B7-2, for which CTLA-4 has a higher affinity compared to CD28 (57).

Another proposed mechanism of regulation of TCR signaling stems from the observation that both CD3 ζ and CD3 ϵ cytoplasmic domains interact with negatively charged lipid head groups *in vitro* and in cells. When in complex with lipid vesicles bearing an overall negative surface charge, CD3 ϵ cytoplasmic domain was shown to adopt a partially helical structure in

which the ITAM tyrosine residues were buried within the hydrophobic core of the bilayer-structured vesicles, thus shielding the side chains from Lck activity (*111*). Our results presented in Chapter 2 reinforce this paradigm as we show a dual function for clusters of basic residues in CD28 cytoplasmic domain, mediating both membrane binding and Lck interaction. Therefore, when CD28 is membrane-bound, the Lck binding site is occluded.

The work presented in this chapter extends the analysis of membrane binding by CD28 and CD3 ϵ to primary T cells, an important confirmation of the applicability of previously utilized models to primary T cell biology. We also investigate the function of CD28 membrane binding in cells by attempting to disrupt the membrane interaction through non-mutagenic methods.

Results

Both CD3 ϵ and CD28 are membrane-bound in primary T cells

T cell receptor export from the endoplasmic reticulum requires neutralization of charged residues within the transmembrane domains of the TCR $\alpha\beta$ heterodimer and the CD3 subunits (130). To overcome this problem, the original study of CD3 ϵ membrane binding in cells utilized an engineered protein construct in which the CD3 ϵ cytoplasmic domain was grafted onto the extracellular and transmembrane domains of KIR2DL3 receptor, and then the TFP FRET donor was fused to the C-terminus of the protein construct (111). Membrane binding was later confirmed in the context of a fully-assembled TCR using a human T cell clone, although the ITAM Tyr residues within the CD3 ϵ cytoplasmic domain were mutated to phenylalanine in order to overcome difficulties with expression (117).

We confirmed that CD3 ϵ wt was capable of membrane binding in the context of the fully-assembled T cell receptor complex, and that mutation of two clusters of basic residues within the cytoplasmic domain disrupted membrane interaction (Figure 3.1A, reproduced from (131)). Observed quenching FRET efficiency was markedly lower for CD3 ϵ wt-TFP compared to typical values obtained using the Jurkat cell system (see Chapter 2, (111)), but was nonetheless consistent with values for 3aa-TFP positive control (Figure 3.1B). Based on comparison with 3aa-TFP positive control, ~20% quenching FRET efficiency appears to be the maximum level achievable with primary T cells in this experimental system. This might be due to lower tolerance for protein over-expression in primary T cells compared to Jurkat cells, as we observed rapid cell death within 24 hours after sorting a CD28wt-TFP high-expressing population compared to medium-expressing. We also confirmed membrane binding of CD28wt in transduced primary T cells (Figure 3.1B). These results confirm the relevance of previously-

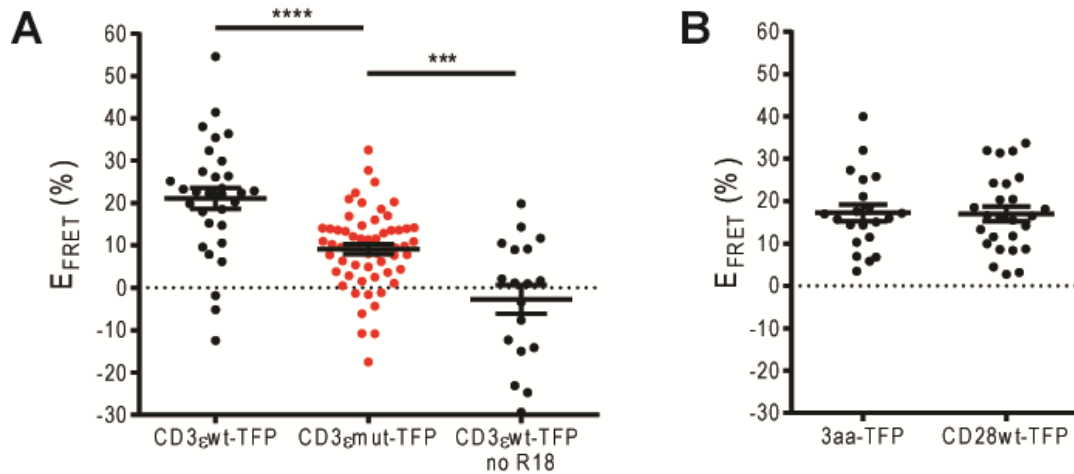


Figure 3.1 CD3 ϵ and CD28 cytoplasmic domains are membrane-bound in primary murine T cells. (a) T cells expressing CD3 ϵ -TFP fusion proteins were isolated from the spleen and lymph nodes of transduced bone marrow chimeric mice. Images were captured for TFP FRET donor and R18 FRET acceptor before and after labeling of the plasma membrane with R18 and used to calculate quenching E_{FRET} (%). Cells were imaged in the same way with no R18 labeling (no R18) as a negative control for photobleaching of the TFP donor. Each data point represents a single cell. (b) T cells were transduced with lentivirus encoding either 3aa-TFP positive control or CD28wt-TFP fusion. Transduced cells were analyzed as in (a) for quenching E_{FRET} (%). **** $p < 0.0001$, *** $p < 0.001$.

utilized *in vitro* and immortalized cellular systems to conditions in primary T cells.

Insertion of a compactly-folded protein domain into CD28 cytoplasmic domain alters its sub-cellular localization

The same clusters of basic residues within CD28 cytoplasmic domain are required for both membrane binding and Lck interaction. These opposing functions present an obstacle to studies of the functional consequences of membrane binding, as mutations that abrogate CD28 membrane binding also reduce or abolish Lck recruitment, and consequently have a negative effect on CD28 signaling activity. Any dysregulation of signal initiation, such as spontaneous receptor triggering, cannot be assessed using membrane binding mutants because they intrinsically lack signaling activity. In order to overcome this problem, we attempted to disrupt CD28 cytoplasmic domain membrane interaction by inserting a compactly-folded protein into the CD28 protein sequence between the transmembrane and cytoplasmic domains (Figure 3.2A). A search was conducted using the RCSB PDB (Research Collaboratory for Structure Bioinformatics Protein Data Bank) for candidate “spacer” proteins according to the following criteria: 1. no internal disulfide bonds or free cysteines; 2. small size (less than 100 amino acids); 3. compact folding in an aqueous environment; 4. N- and C-termini at opposite ends of the structure. Two *E. coli* proteins were selected, phospho-carrier protein HPr (PDB ID 1POH, crystal structure reported in (132)) and cold-shock protein CspA (PDB ID 2L15, crystal structure reported in (133)).

Jurkat single-cell clones expressing CD28wt-HPr-TFP or CD28wt-CspA-TFP constructs were prepared for imaging as previously described (Chapter 2, (111)). The majority of CD28wt-TFP protein typically resides at the plasma membrane, with a small fraction evident in

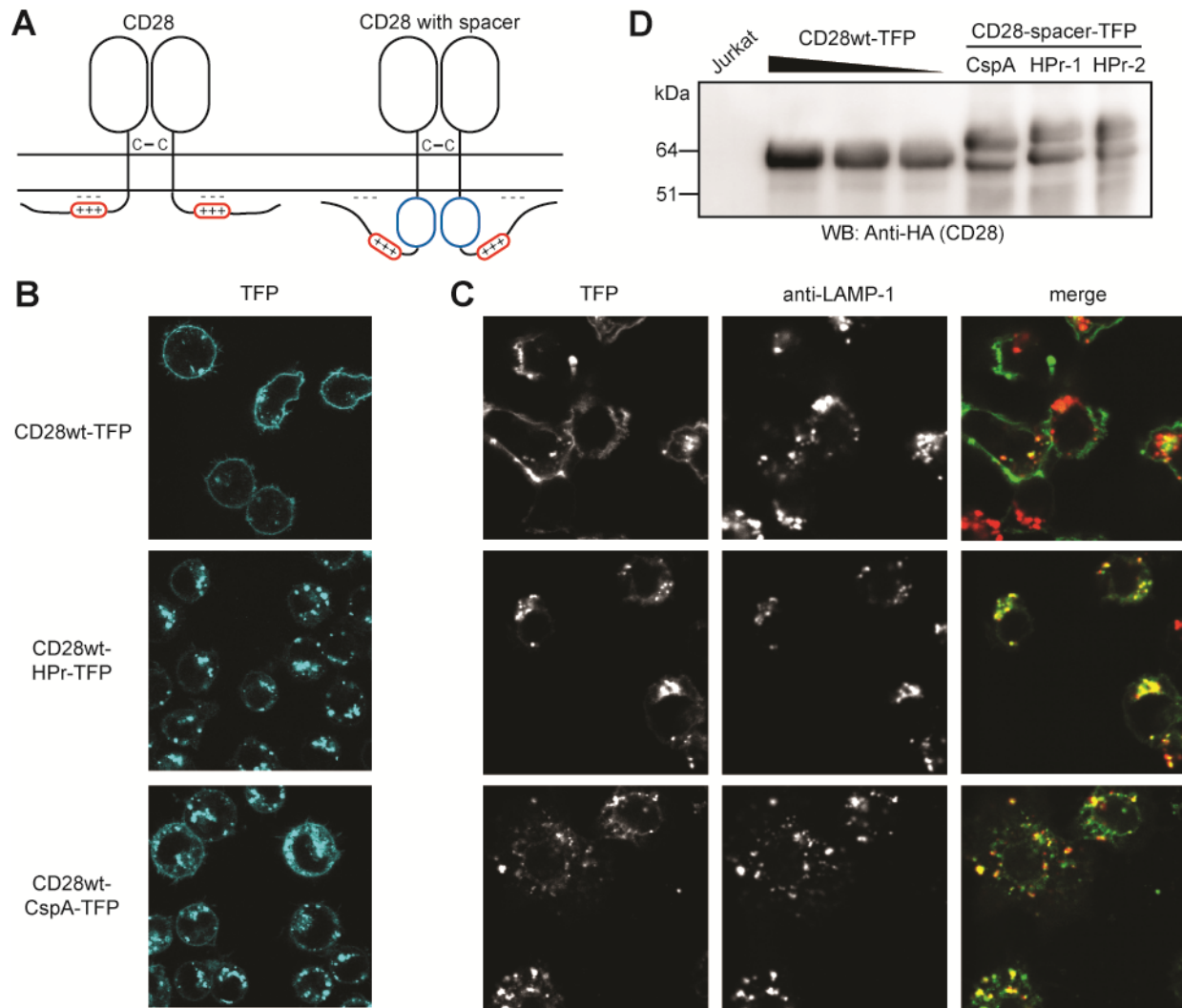


Figure 3.2 Insertion of a compactly folded protein into the CD28 cytoplasmic domain results in altered subcellular localization. (a) Conceptual design is shown for CD28 spacer constructs. A compactly folded *E. coli* protein (either cold shock protein A, CspA, or histidine phospho-carrier protein, HPr) was inserted between the transmembrane and cytoplasmic domains of CD28 in order to alter the binding of basic residues to the inner leaflet of the plasma membrane. (b) TFP distribution pattern is shown in live Jurkat cells expressing either CD28wt-TFP, CD28wt-HPr-TFP, or CD28wt-CspA-TFP. (c) Jurkat cells were fixed, permeabilized, and stained with anti-LAMP-1 antibodies to examine co-localization of TFP with lysosomes.

Figure 3.2 (Continued)

(d) Lysates from Jurkat cells expressing the indicated proteins were analyzed by anti-HA WB.

Two different single cell clones were analyzed for the CD28wt-HPr-TFP construct.

the endoplasmic reticulum (ER), probably newly-synthesized protein in processing before entering the secretory pathway en route to the cell surface, and endo-lysosomal compartments, likely recycled from the cell surface. In contrast, a punctate distribution pattern was observed for both HPr and CspA spacer constructs (Figure 3.2B). As little TFP fluorescence was present at the plasma membrane in the majority of cells, FRET measurements to examine membrane binding were not attempted. However, immunofluorescence staining revealed that the spacer constructs were largely present in lysosomes based on co-localization with LAMP-1, with some co-localization also observed with early endosomal marker EEA-1 (Figure 3.2C, S3.1). A lysosomal distribution pattern could indicate that the spacer domains disrupt protein stability, triggering an unfolded protein response that targets the majority of the protein for immediate degradation (134). Two distinct molecular weight species were observed for the spacer constructs by Western blot (Figure 3.2D). The upper band seems to correspond to the fully-glycosylated, mature polypeptide as the molecular weight matches the single species observed for CD28wt-TFP with a shift of 7.9kDa for the addition of the CspA spacer or 9.6kDa for HPr. This band could also contain a mono-ubiquitinated form as TCR and other surface receptors are known to undergo mono-ubiquitination post-activation in order to direct recycling and degradation through endo-lysosomal compartments (135). The lower band could correspond to either a degradation product, consistent with the observed lysosomal co-localization pattern, or to a pre-glycosylation form, although we do not observe obvious ER retention (Figure 3.2D, S3.1B). The small amount of TFP observed at the cell surface and the co-localization with early endosomes could indicate that some of the protein reaches the cell surface and is then internalized. Receptor triggering is known to cause endocytosis (136). No spontaneous phosphorylation of the CD28 spacer constructs was observed by Western blot, which would

seem to negate the possibility of receptor triggering, but this might have been due to lack of temporal and spatial coordination. The immunological synapse enables robust coordinated phosphorylation by concentrating activated receptors with downstream kinases and other signaling mediators, and simultaneously excluding some negative regulators of signaling activity (such as CD45 and CD148). Low-level basal phosphorylation resulting from loss of membrane binding might not be detectable in the absence of such temporal and spatial coordination. Further investigation using either signaling-deficient CD28 mutants or expression of spacer constructs in Lck-deficient Jurkat cells (JCaM1.6 sub-clone) would be required in order to distinguish between these possibilities.

Discussion

The data presented in this chapter confirm the membrane binding properties of CD3 ϵ and CD28 cytoplasmic domains in primary mouse T cells. Attempts to investigate the consequences of loss of membrane binding were complicated by the observation of an unusual subcellular distribution pattern for CD28 constructs incorporating a compactly-folded spacer domain between the transmembrane and cytoplasmic domains. This result could indicate spontaneous receptor triggering followed by rapid internalization and/or inactivation by phosphatase activity, or that the bacterial spacer proteins are not well-tolerated by mammalian cells.

Some insight into this issue could be gained from a previous study on CAR design, which examined several different combinations of signaling domains linked to CD3 ζ , including some in which full-length Lck was directly linked to its substrates, or could be constitutively bound at steady-state due to inclusion of the CD4 cytoplasmic domain (137). Some constructs were not expressed at the cell surface depending on the ordering of the cytoplasmic domains, but some that included either Lck or CD4 were successfully expressed. Basal phosphorylation of receptors containing Lck could not be directly assessed because phosphorylated tyrosines within Lck itself would confound the interpretation of results. However, basal ZAP-70 association was observed for constructs with linked Lck, suggesting that there was basal phosphorylation of the CD3 ζ ITAMs. Therefore, if spontaneous signaling were to occur in receptors with altered membrane binding, surface expression should still be possible and a phosphorylated species could be captured. However, the regulation of receptor internalization based on phosphorylation state might differ between CD28 and CD3 ζ , as basal CD3 ζ phosphorylation and ZAP-70 occupancy have been observed previously in primary T cells without triggering rapid TCR internalization (138, 139). With these observations in mind, further investigation will be required to determine

whether the phenotype observed for CD28 spacer constructs is due to spontaneous signaling or to flawed design and protein misfolding.

In the latter case, other designs might still be feasible, such as a poly-proline sequence that adopts a rigid helical structure (140). However, a concern with this approach is that flexibility of the protein sequence at the end of the transmembrane domain could simply allow a narrow, helical spacer to fold back and lie flat against the membrane due to the long-range electrostatic force between the positively charged N-terminal basic cluster and the anionic lipid head groups.

The implications of membrane for negative regulation of receptor signal initiation are clear but remain to be proven in intact cells, as occlusion of the Lck binding site and substrate tyrosines as a result of membrane binding have been shown *in vitro* only (see Chapter 2, (111)). However, there is also potential for positive signal regulation. Upon receptor triggering, transient release of the cytoplasmic domain from the membrane would leave the kinase substrate sequence in a membrane-parallel orientation and positioned relatively close to the membrane compared to an endpoint-anchored peptide that is otherwise free in solution. A greater understanding of the functions of membrane binding is essential to our understanding of receptor signal initiation.

Materials and Methods

Preparation of Primary Mouse T cells for FRET Imaging

CD3ε-TFP expressing cells were isolated from spleen and lymph nodes of transduced bone marrow chimeras as described (131). Cells were expanded with three rounds of stimulation with PMA and ionomycin and rested overnight in IL-2 (1 ng/mL) prior to imaging experiments. *Cd28^{-/-}* or WT T cells were cultured in IL-15 (100 ng/mL), IL-7 (5 ng/mL) and IL-2 (2 ng/mL) for 48h and then transduced by spin-infection with CD28wt-TFP or 3aa-TFP lentivirus, respectively. TFP⁺ cells were sorted 96h hours post-transduction and then cultured overnight in IL-2 (1 ng/mL) prior to imaging.

FRET Measurements

Cell preparation and microscope settings were the same as described in Chapter 2, except that a resolution of 512x512 and 3x optical zoom were used in order to achieve adequate membrane definition of primary mouse T cells, which are substantially smaller than Jurkat cells. A higher Ar laser power and line accumulation of 2 were also used in order to enhance TFP fluorescence intensity, which was lower in mouse T cells compared to Jurkat cells. In order to avoid photo-bleaching from the higher laser power, images were captured once before and once after R18 labeling. Quenching FRET efficiency was calculated as described in Chapter 2. Statistically significant differences were assessed using one-way ANOVA with Bonferroni correction for multiple comparisons in GraphPad Prism software.

Design of CD28 Spacer Constructs

The cDNA sequence encoding for HPr (PDB ID 1POH) or CspA (PDB ID 2L15) was

incorporated into the HA-CD28wt-TFP sequence in the pHAGE vector after CD28 residue S179. Based on the Membrane Protein Explorer (MPEx) algorithm, the last residue of the CD28 TM domain is predicted to be I175, with W176 positioned with the lipid headgroups on the inner leaflet of the membrane. Three additional amino acids beyond W176 were included in order to facilitate proper protein folding. Residues W176-S179 were repeated again at the C-terminus of the spacer to act as a flexible linker, followed by the remainder of the CD28_{CD} sequence. Jurkat single cell clones were prepared by electroporation and multiple rounds of sorting as described in Chapter 2.

Immunofluorescence Staining

Jurkat cells at a density of 2×10^6 cells/mL in PBS were allowed to adhere for 20 min to a poly-L-lysine coated coverslip placed in a single well of a 24-well plate. Excess cells were removed by gentle washing twice with PBS. Cells were fixed for 10 min with 2% paraformaldehyde (Thermo Scientific), washed once with PBS, and then permeabilized briefly with 0.05% Triton X-100. Cells were washed three times, blocked for 30 min with 3% BSA in PBS, and then washed again. Primary antibody staining was performed for 1h at room temperature at a concentration of 1 μ g/mL with either anti-EEA-1, anti-LAMP-1 or anti-calnexin antibodies (all from Abcam). Secondary staining was performed with Alexa647-conjugated antibody for 30 min at room temperature at a concentration of 0.33 μ g/mL. Excess liquid was removed from the coverslip, which was then mounted on a slide using ProLong Gold anti-fade mounting medium (Life Technologies). Fluorescence images for TFP and Alexa647 were captured using a Leica SP5X microscope with 60x oil-immersion objective at 3x digital zoom and 512x512 resolution.

Chapter 4

General Discussion

Summary of New Findings and Implications

The starting point of the studies presented in this thesis was the previous observation that the CD3 ϵ cytoplasmic domain interacts with the inner leaflet of the plasma membrane in cells (111). Our initial hypothesis was that this interaction constituted a regulatory mechanism that would function in concert with other known regulatory mechanisms, such as phosphatase activity, to prevent spontaneous phosphorylation and consequent receptor triggering. Alterations in the signaling capability of the TCR-associated CD3 complex would alter T cell thymic selection and lead to a complex mature T cell phenotype that would likely be difficult to interpret (141). Therefore, we chose to focus instead on CD28 costimulatory receptor, which is not required for conventional T cell development, and which we hypothesized would share the membrane binding feature of CD3 ϵ cytoplasmic domain based on its similarly high isoelectric point and clusters of basic residues.

Indeed, we found that CD28 cytoplasmic domain also interacted with the inner leaflet of the plasma membrane in cells, and that ligand-induced triggering caused a reduction in membrane binding, which was also previously observed for CD3 ϵ upon TCR triggering (117). Membrane binding might be a common feature shared by other transmembrane receptors whose cytoplasmic domains are enriched in basic residues. A detailed discussion of candidates for further investigation is presented below.

Surprisingly, we found that mutational disruption of membrane binding had the unintended consequence of also disrupting Lck activity on CD28_{CD} *in vitro*, in the absence of a lipid environment. Further investigation using SPR to examine protein-protein interaction revealed that the same residues responsible for membrane binding also mediated a direct interaction with Lck. The only previously described CD28-Lck interaction was at the CD28

PYAPA motif, reportedly binding to the Lck SH3 domain. However, we could observe no binding by SPR of an Lck U-SH3-SH2 domain construct to a truncated CD28_{CD} peptide lacking the basic residue clusters, until we examined a peptide phosphorylated at Y207 within the PYAPA motif. Although this motif is a good candidate for an SH3 domain binding site, the requirement for phosphotyrosine suggested that the SH2 domain was involved. We confirmed this by examination of the isolated Lck SH2 domain binding to the phosphorylated CD28_{CD} peptide. This finding helped to clarify a recent controversy that had arisen in the field, as the Lck SH3 domain was found to directly recruit PKC θ to form a trimolecular CD28-Lck-PKC θ signaling complex, which would create competition if the Lck SH3 domain were required for binding to both CD28 and PKC θ (21).

Our SPR analysis showed that the affinity of the Lck kinase domain for the CD28 basic residue clusters is considerably higher than that of the Lck SH2 domain for the P[pY]APA motif. Mutation of the PYAPA motif has been the only mutation to consistently yield a loss-of-function phenotype across several *in vivo* models examining CD28 function (see Chapter 1 for a full discussion of these models). In our study, mutation of the basic residue cluster that contributed the most to Lck binding affinity resulted in a complete loss-of-function phenotype, with nearly identical Treg frequencies observed in transduced bone marrow chimeras bearing the basic residue mutant, PYAPA mutants (AYAAA or PFAPA), and empty vector (*Cd28*^{-/-}). The other basic cluster, which did not yield a phenotype when mutated, retains measureable Lck binding affinity. This may be sufficient to meet the threshold for costimulation required in this model. We selected the transduced bone marrow chimera system due to its flexibility in allowing us to test many mutants and construct designs in a relatively short period of time, but use of this system also imposed certain limitations on the phenotypes we could assess. Specifically, the

lymphopenic environment in *Rag1*^{-/-} recipients encourages excessive T cell homeostatic proliferation and alters T cell sensitivity to stimulation, which makes this system ill-suited to studying T cell-mediated inflammatory processes (142-144). Also, transduction rate could not be controlled perfectly between experimental groups, and CD28 expression level could not be perfectly matched to the normal endogenous level. Because of these limitations, it will be essential to examine the basic residue clusters, both individually and with a combined mutation, in a more physiological system such as with knockin mutants or with CRISPR-Cas9-mediated genome editing.

Our results predict a hierarchy of phenotypes for the CD28 mutants we have studied. Combined mutation of both basic residue clusters should completely abolish CD28 signaling. Lck binding is required for phosphorylation of the YMN motif, which is required for recruitment of PI3K, so loss of Lck binding should abrogate both Lck-mediated and PI3K-mediated activity. Itk activity might be able to compensate for this loss, but no phenotype has ever been reported for a mutant targeting the reported Itk binding site. Next, the Y207F CD28 mutant, which abolishes Lck SH2 domain binding, should have a lesser effect because intact Lck binding to the basic residues and phosphorylation of Y189 could still permit PI3K-mediated signaling activity. However, signal amplification through pY189 and possibly other pathways might be impaired in the Y207F mutant, since our results suggest that binding of the Lck SH2 domain enhances kinase activity on downstream targets. Finally, our model predicts that the AYAAA mutant would have an even more mild effect, as we observe only a partial reduction in Lck SH2 domain binding affinity for this mutant. This mutant serves as the current benchmark for evaluating CD28 signaling function *in vivo*, but we observed only slight defects in Lck binding and activity in our cellular and *in vitro* assays. It is possible that the well-documented *in*

vivo defects observed for this mutant are not all due to disruption of Lck activity, but could be attributable to another unknown kinase that we did not investigate in the present study. Again, thorough investigation using appropriate *in vivo* models will be required in order to establish the relative importance of each motif in CD28 signaling function.

Integration of CD28 Signaling Motifs

The various signaling motifs within the CD28 cytoplasmic domain have usually been considered as modules, each independently activating a particular downstream signaling pathway. The YMN_M motif recruits PI3K and Grb2; the PRRPG motif recruits Itk; the PYAPA motif recruits Lck. However, there is greater interdependence than this reductionist description implies. For example, results presented here and elsewhere indicate that Lck is capable of phosphorylating two tyrosines within CD28_{CD} (Y189 and Y207), creating binding sites for the SH2 domains of both Lck itself and the PI3K p85 subunit. Phosphorylation activity by Lck or another kinase is therefore a pre-requisite to any PI3K-mediated signaling activity downstream of CD28 activation.

In addition, we found that Lck binds to a distributed site spanning three regions of CD28_{CD}, which partially overlap with and could occlude binding sites for other signaling effectors. Although the motifs in question are considered distinct from one another and illustrations for simplicity depict simultaneous docking of several signaling mediators, occupancy by one kinase would likely sterically hinder binding of another because these proteins are large compared to CD28_{CD} (e.g. Lck is 56 kDa compared to 5.1 kDa for the CD28_{CD} monomer, 10.2 kDa for the dimer). Even with the cytoplasmic domain fully extended and the kinase compactly folded, it is unlikely that more than two kinases could dock simultaneously on

a single CD28 dimer (one kinase per CD28 cytoplasmic domain). Concentrated CD28 dimers within signaling microclusters likely share several signaling mediators, with the on- and off-rates of binding and the relative local abundance of each kinase dictating its contribution to signal initiation and amplification. A mutation that prevents binding of one kinase would likely enhance recruitment of another by increasing substrate availability, resulting in some compensatory activity.

In summary, mutations targeting one binding site can have both positive and negative effects on other CD28-mediated signaling pathways in addition to the one specifically targeted.

Crosstalk between TCR and CD28 Signaling

The cellular response to combined TCR and CD28 stimulation is much more robust than the response to TCR stimulation alone, with many measurable changes including greater proliferation, cytokine production, survival and metabolic rate. The difference between TCR/CD28 stimulation and TCR stimulation alone is not reflected by the response to CD28 stimulation alone, which results in only a minimal transient response that can mostly be observed at the transcriptional level and typically does not rise to the level of measurable changes in protein expression or cell state. Hence, there seems to be synergy between TCR and CD28 signaling rather than simple additive effects, although it has been difficult to dissect at exactly what stage(s) downstream of receptor activation these pathways might converge or reinforce one another.

As discussed briefly in Chapter 2, TCR and CD28 signaling share several key features. Their signaling seems to be synchronized and co-localized in signaling microclusters that form in the early stages of the immunologic synapse. Although they are segregated at the mature IS,

most of the signaling activity is thought to occur in peripheral microclusters rather than at the cSMAC. Next, both CD3 ϵ and CD3 ζ signaling subunits of the TCR, as well as CD28, have been shown to bind to the anionic lipids present in the inner leaflet of the plasma membrane. Ligand binding has been shown to induce membrane dissociation of both CD3 ϵ and CD28. Finally, the first step in the activation pathway of both TCR and CD28 involves Lck-mediated tyrosine phosphorylation within ITAM or ITAM-like motifs.

These observations suggest that crosstalk between TCR and CD28 signaling could occur at the very first stages of receptor activation. The affinity of Lck for CD28 is relatively high as measured by SPR (100-nM range), and the interaction appears to be stable in cells, as we were able to co-immunoprecipitate the protein-protein complex. Increasing the local concentration of Lck within signaling microclusters could enhance its activity on TCR-associated CD3 subunits. It would be interesting to compare the relative contribution of CD4- or CD8-coreceptor mediated delivery of Lck to TCR versus CD28-mediated delivery. Also, the relative affinity of Lck for the CD3 chains versus CD28 remains to be determined in the same experimental system, which would be an important step in assessing the expected contribution of CD28 to Lck recruitment to microclusters. Our results suggest that binding of the Lck SH2 domain to the phosphorylated CD28 P[pY]APA motif enhances kinase activity on a downstream target, PKC θ . By the same principle, CD28 could deliver the more-active form of Lck to signaling microclusters, enhancing kinase activity on colocalized TCR and other neighboring CD28 molecules.

Multiple studies have previously suggested that a major mechanism of CD28 costimulatory activity is to enhance activation of signaling pathways downstream of TCR (145-147). In one study, blocking CD28-B7 interaction caused a reduction in CD3 ζ phosphorylation, ZAP-70 recruitment, and downstream activation of the ERK, a member of the MAP kinase

family (145). The model of CD28-mediated Lck delivery proposed above would provide a mechanistic explanation for this observed enhancement in early TCR signaling events. PKC θ is known to be activated downstream of TCR indirectly through binding to diacylglycerol (DAG) produced by the activity of PLC- γ 1 (148), but CD28 has also been shown to directly complex with PKC θ through Lck, dramatically enhancing its recruitment to the immunological synapse (37). Thus, CD28-mediated delivery of PKC θ to support TCR downstream signaling is another point of connection which might explain the observed synergy between the two receptors. This would account for the observation that TCR/CD28 stimulation strongly activates CARMA-1 and downstream NF- κ B, even though such activity is not observed following CD28 stimulation alone (46). In summary, TCR and CD28 signaling are inter-connected from their earliest stages, and crosstalk at multiple points in downstream signaling pathways may account for the synergy between them, as evidenced by the dramatic alteration in cellular outcome when both signals are delivered together compared to either signal alone.

Membrane Binding and Receptor Triggering

Discussion of membrane binding by both TCR-associated CD3 subunits and CD28 naturally leads to further questions about how the cytoplasmic domain would dissociate from the membrane in order to become accessible for kinase activity and downstream signaling. The mechanism of receptor triggering has remained an elusive problem despite intensive study over several years, and the discovery of membrane binding has simply added a new wrinkle to this already-complex issue. One model of T cell activation posits that tyrosine phosphatases prevent spontaneous T cell activation by constantly dephosphorylating receptors that become sporadically phosphorylated. Coordinated triggering of many receptors, as upon encounter with

an APC bearing cognate pMHC complexes on its surface, results in enough simultaneous phosphorylation events to overcome the inhibitory activity of phosphatases. In support of this argument, treatment of cells with the pan-phosphatase inhibitor pervanadate results in massive receptor phosphorylation, to a similar or even greater extent than is observed for APC-stimulated cells (149). Building on this principle, the kinetic-segregation model incorporates observations of T cell immunological synapse formation on glass-supported lipid bilayers (95, 150). Multiple transmembrane proteins with intrinsic phosphatase activity in their cytoplasmic domains have extracellular domains that are larger than that of TCR and CD28. When TCR and CD28 congregate in microclusters at high density, the larger phosphatase extracellular domains cannot be accommodated in the juxtamembrane space between the T cell and APC, and the phosphatases are excluded. This physical separation prevents the inhibitory activity of the phosphatases from acting on TCR and CD28, enhancing receptor phosphorylation and signal amplification.

The paradigm of membrane binding as a regulatory mechanism does not conflict with the phosphatase or kinetic-segregation models. Membrane binding could largely shield receptor cytoplasmic domains from inappropriate kinase activity, but transient dissociation might still permit sporadic phosphorylation events. The introduction of negative charge would strongly disfavor rebinding, as the target tyrosine side chains in CD3 ϵ were shown to intercalate with the hydrophobic fatty acid chains of the lipid bilayer. Hence, phosphatase activity would be a necessary control mechanism to reverse aberrant receptor phosphorylation introduced during random transient membrane dissociation.

Such a variation on the phosphatase model would require some mechanism of shifting the equilibrium of the cytoplasmic domain from the bound to the unbound state in order to permit

receptor activation. Previous work has shown that, following T cell stimulation, negatively charged lipids are exposed on the cell surface due to reduced phospholipid flippase activity, which would be expected to reduce the overall negative charge content of the inner leaflet of the plasma membrane (151). In addition, another study showed that the abundance of negatively charged lipids, in particular phosphatidyl serine, is specifically reduced in TCR-CD28 signaling microclusters (117). A reduction in the negative charge content locally within microclusters and generally throughout the inner leaflet of the plasma membrane at the immunological synapse would tend to favor dissociation of positively charged cytoplasmic domains, rendering them accessible to kinase activity. Altered redistribution of lipids between the outer and inner leaflets is an active process, and so this effect would need to be secondary to an initial triggering event of one or a few receptors. This model does not explain the mechanism of triggering of those first few receptors. On the other hand, the model of receptor triggering by negatively charged lipid exclusion from microclusters might or might not require prior activation events, as the mechanism governing lipid dynamics in microclusters is unknown at this time.

A third model evoked to explain the mechanism of receptor triggering involves the transmission of information across the membrane due to mechanical force. Although we often depict TCR:pMHC and CD28:B7 interactions as taking place along static cellular membranes, in reality interacting cells are subject to low-intensity shear forces applied by the flow of lymph through a secondary lymphoid organ or interstitial fluid in a target tissue (152). In addition, scanning T cells are in constant motion as they search from APC to APC for cognate antigen. Even when they undergo arrest and form a stable synapse upon antigen encounter, shear forces continue to exert pressure and could induce microscopic movement. A sudden movement could apply a pulling or tugging force to the protein-protein interaction partners on juxtaposed cells,

which in turn might result in movement of the cytoplasmic domains relative to the membrane, triggering dissociation and exposure to kinase activity. This force would likely be simultaneously applied to many engaged receptors along the interface between the interacting cells, resulting in coordination activation of multiple receptors. As predicted by this model, application of a shear force through manipulation of an antibody-coated bead with optical tweezers has been shown to induce calcium flux in the interacting cell (153). Hence, the mechanical force model provides a possible mechanism for the initial triggering of a few receptors that is compatible with membrane binding as a regulatory mechanism. This model could work in concert with the kinetic-segregation model and charged lipid redistribution, which provide mechanisms for enhanced activation of a secondary wave of receptors, resulting in strong signal amplification.

Historically, most of the studies of receptor triggering in T cells have focused on the T cell receptor. As discussed above, there are notable similarities between TCR and CD28 early signaling events, and their activation may be co-dependent. CD28 is a simpler receptor in that it is expressed as a homodimer rather than a multi-subunit complex. While transduced signals need only pass through the transmembrane domain of CD28 to its cytoplasmic domain, TCR signal transduction involves transmitting information from the TCR $\alpha\beta$ heterodimer to the associated CD3 subunits, likely at the interaction site between their transmembrane regions. There is great opportunity to further our understanding of receptor triggering and signal transduction biology by focusing on CD28 as a reductionist model system.

Future Directions

The most obvious area of future investigation, as discussed above, is to evaluate the *in*

in vivo function of CD28 mutants targeting either or both basic residue clusters using knockin mice or CRISPR-Cas9 edited mutants. Since we predict that basic residue mutants and Y207F would have a phenotype as severe or greater than that of previously investigated mutants, it would be particularly useful to evaluate these in a model in which the AYAAA CD28 mutant retains some costimulatory activity above the null level observed in *Cd28^{-/-}* T cells. These include the experimental allergic airway inflammation model, the EAE model and the bacterial challenge model, which were discussed in detail in Chapter 1. A null phenotype was observed for the AYAAA mutant for Treg development and for the *Ctla4^{-/-}* lethal lymphoproliferative disorder. We confirmed the defect in this mutant for Treg development in transduced bone marrow chimeras, and extended the finding to the more extreme basic residue mutant (Mut4, targeting the central cluster) and to the Y207F mutant. However, as a greater defect than the null phenotype cannot be observed, experiments in these models may not distinguish between the AYAAA, Y207F and basic residue mutants.

A more difficult question remains of how to assess the functional significance of membrane binding. Mutagenic approaches do not seem feasible, as the same sites that mediate membrane binding are also required for Lck kinase activity. We attempted to alter the binding affinity of intact CD28_{CD} for the plasma membrane by inserting a compactly-folded *E. coli* protein to act as a spacer between the CD28 transmembrane and cytoplasmic domains. It is unclear from the altered subcellular localization for this construct whether spontaneous signaling did in fact occur, or whether the design was simply not well-tolerated by mammalian cells. Further investigation, possibly with newly engineered designs for non-mutagenic disruption of membrane binding, will be required in order to understand the functional significance of membrane binding in receptor signaling. CD28 provides a good model system for studying

membrane binding and its functional significance in T cells, as the simple homodimeric structure of CD28 makes experimental manipulation and interpretation of results more feasible than for TCR. Conclusions drawn from experiments with CD28 may have implications not only for TCR, but also for activation of other transmembrane receptors that may be similarly regulated by membrane interaction.

In this vein, another avenue of future investigation would be to assess the potential for membrane binding in other immune receptors. The overall charge content of the cytoplasmic domain, as determined by isoelectric point, represents a good starting point for examining the potential for membrane binding. CD3 ϵ cytoplasmic domain (pI 11.32) has the highest isoelectric point of the CD3 subunits, followed by CD3 ζ (9.56), CD3 δ (9.40), and then CD3 γ (9.23). Membrane binding has been demonstrated for CD3 ζ , even though its isoelectric point is markedly lower than that of either CD3 ϵ or CD28 (11.27). As CD3 ζ_{CD} is substantially longer, clusters of basic residues could interact with the membrane even while other portions of the poly-peptide with lower basic charge content loop out into the cytoplasm. Alternatively, the lower isoelectric point could still be above the threshold required for membrane binding. In our studies, there was a steep drop-off in membrane binding as assessed by quenching E_{FRET} from Mut3 (N-terminal cluster) to Mut4 (central cluster) despite only a slight drop in isoelectric point from 9.86 to 9.62. There could be a hierarchy of affinities for anionic lipids varying as a non-linear function of isoelectric point, with greater change observed in the pI range of 9-10 as opposed to 10-11 and above. Alternatively, there could be positional effects, with membrane-distal motifs playing a greater role. Both scenarios have implications for interpreting membrane binding by CD3 ζ despite its relatively low isoelectric point, and for hypothesizing whether the other CD3 subunits, which have isoelectric points only slightly lower than that of CD3 ζ , might also be candidates for

membrane interaction. It could be highly informative to investigate this point experimentally.

A survey of other costimulatory receptors revealed that CD28_{CD} has the highest isoelectric point. ICOS and OX-40 are somewhat lower, at 9.87 and 9.78, respectively, but would also be good candidates for further investigation. Interestingly, the isoelectric point of 4-1BB is much lower (only 6.40 for the human sequence, and 7.69 for the mouse). As CD28 and 4-1BB are the most commonly-utilized costimulatory domains in second-generation CARs, differences in membrane binding could indicate that such CARs have quite dissimilar signaling biology.

T cell coreceptors CD4 and CD8 α and β have high isoelectric points (10.93, 10.54, 11.57) similar to other membrane-binding cytoplasmic domains. Lck binds constitutively to CD4 and CD8 α through a CxC motif that coordinates a Zn²⁺ ion (104). This interaction might be expected to interfere with membrane association, although the CxC motif is C-terminal to a cluster of basic residues in both coreceptors, which could still mediate some membrane interaction. As Lck contains N-terminal myristoylation and palmitoylation sites that mediate membrane tethering and are very close to the Zn²⁺ coordination site for coreceptor binding, membrane binding by the coreceptor cytoplasmic domains could actually facilitate stable interaction with membrane-tethered Lck by inducing an optimal spatial orientation with respect to the membrane. It would be interesting to examine the potential membrane binding properties of the coreceptors, as the functional significance would be somewhat different than for TCR and CD28 since the coreceptors bear no intrinsic signaling function.

The signaling subunits of the B cell receptor, Ig α and β , also known as CD79, have strikingly low isoelectric points (4.17 and 4.22) with an overall negative charge. These are unlikely to interact with anionic lipids. Hence, membrane binding is not a feature that is likely to

be shared among all tyrosine-based immune receptors. Nonetheless, the receptors mentioned here, as well as others not examined, represent intriguing targets for further study to expand on the paradigm of membrane binding by the cytoplasmic domains of signaling receptors.

The present study also has implications for our understanding of CAR signaling biology. The same principles for TCR-CD28 crosstalk and synergy would apply even more strongly to signaling of CARs that include both CD3 ζ and CD28. Signal activation of the two moieties would be absolutely synchronized due to their physical linkage. In addition, based on the relatively high affinity of CD28 for Lck, CD28 may enhance Lck delivery to and kinase activity on linked CD3 ζ through more rapid and stable recruitment. CD4 and CD8 coreceptors normally facilitate delivery of Lck to activated TCRs due to interaction of the coreceptor extracellular domains with TCR-bound MHC molecules, but as CARs are activated through binding of scFv directly to antigen rather than to MHC, they lack this important mechanism for supporting receptor activation. CD28 may be able to substitute somewhat for coreceptors in providing this function. This could be investigated experimentally by examining alterations in Lck recruitment to CARs or phosphorylation intensity of the linked CD3 ζ cytoplasmic domain with and without CD28 cytoplasmic domain.

As CD28 is N-terminal to CD3 ζ in CAR constructs, it would also be interesting to examine whether CD28 (and CD3 ζ) retains its membrane-binding properties in the context of the longer polypeptide and lower overall isoelectric point, due to the contribution of CD3 ζ with its lower positive charge content. CARs also provide an excellent opportunity to study the effects of altered affinity of the receptor : ligand interaction on membrane dissociation and signal initiation, since part of the pre-clinical development of CARs sometimes involves screening a panel of related scFv's with slight mutations that alter binding affinity for the antigen (154, 155).

This question has been approached for TCR signal initiation using panels of MHC-bound altered peptide ligands, which bear single amino acid changes in the peptide that alter TCR binding affinity (156-158). It would be difficult to examine the effect of membrane dissociation in this system, though, because not all of the CD3 cytoplasmic domains are known to have membrane binding properties. A panel of CARs with variable affinity might provide another opportunity to address this issue, and in turn such a study could begin to address an open question in the field of CAR design: what is the optimal scFv affinity for maximizing on-target CAR function?

Summary

The new research presented in this thesis has demonstrated that the T cell costimulatory receptor CD28, like TCR-associated signaling subunits CD3 ϵ and CD3 ζ , binds to the inner leaflet of the plasma membrane in cells using clusters of basic residues in its cytoplasmic domain. The same motifs responsible for membrane binding also constitute a previously-unknown Lck interaction site. We have developed a new model for the early events in CD28 signal initiation and reinterpreted existing literature in light of these new discoveries. This work also explores questions about the function of membrane binding in regulation of signal initiation not only for TCR and for CD28, but also for CARs under development for immunotherapy.

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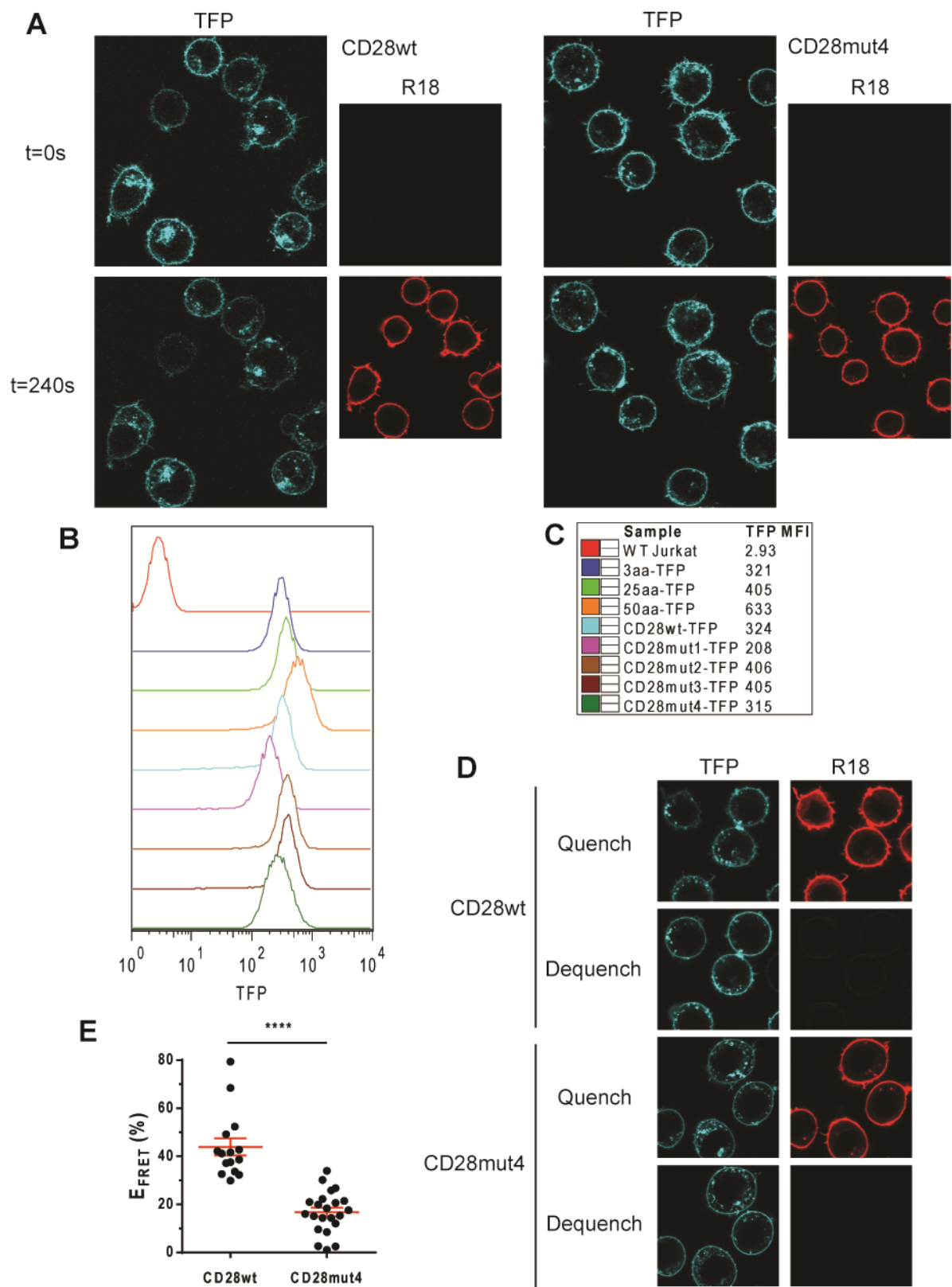
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Appendix

Supplementary Material Related to Chapters 2 and 3

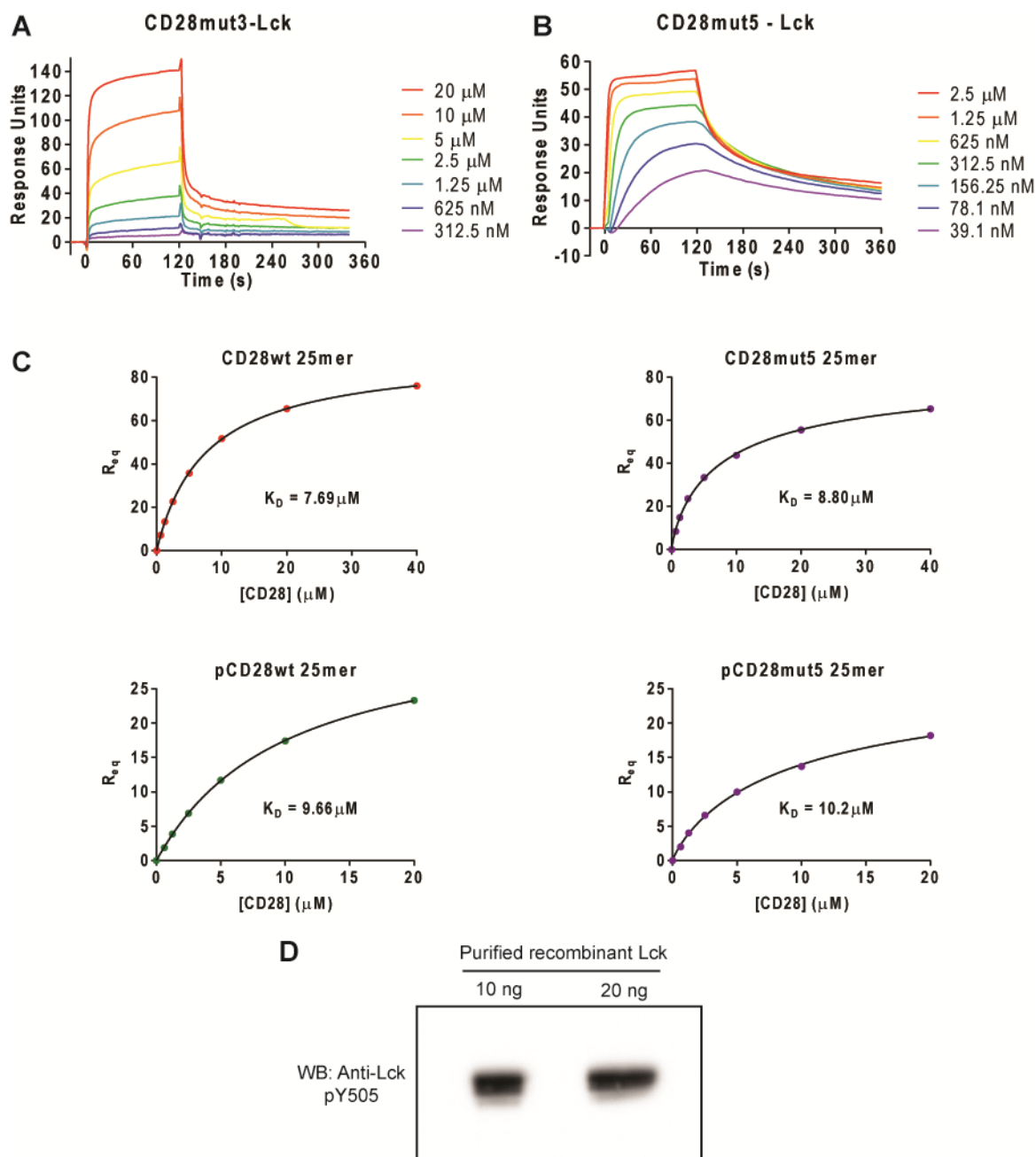
Supplementary Figure 2.1 Characterization of TFP-expressing cell lines and donor dequenching FRET measurements. (a) Raw data for enlarged fields of view are shown for quenching FRET experiments with CD28wt and CD28mut4. (b, c) Overlaid histograms depict flow cytometry analysis of TFP fluorescence for Jurkat single cell clones. Mean fluorescence intensity (MFI) is shown in (c) for each transfectant. (d, e) TFP donor dequenching FRET was analyzed by photo-bleaching of the R18 acceptor. Each point represents an individual cell. Mean E_{FRET} (%) and SEM are also shown. The two groups were compared for statistically significant differences by two-tailed t test. Representative raw data are shown in (d). Images for both TFP and R18 channels were captured before (quench) and after (dequench) selective photo-bleaching of R18. TFP fluorescence measured at the plasma membrane was used to calculate dequenching E_{FRET} .

Supplementary Figure 2.1 (Continued)



Supplementary Table 2.1 Identification of CD28_{CD} tyrosine residues phosphorylated by Lck. CD28WT and Mut5 dimer peptides were normalized for input by OD_{280nm} prior to *in vitro* phosphorylation reaction with recombinant Lck. Reactions were separated by SDS-PAGE and the region corresponding to CD28 dimer was excised. Gel slices were submitted to Taplin Mass Spectrometry Facility (Harvard Medical School) for analysis of phospho-tyrosine modification. Peptides were fragmented by in-gel tryptic digest and the resulting peptide mixtures were analyzed by LC/MS-MS. The table depicts relative phosphorylation of each tyrosine based on the peak area of the phosphorylated fragment compared to the total peak area for that fragment (sum of phosphorylated and unmodified).

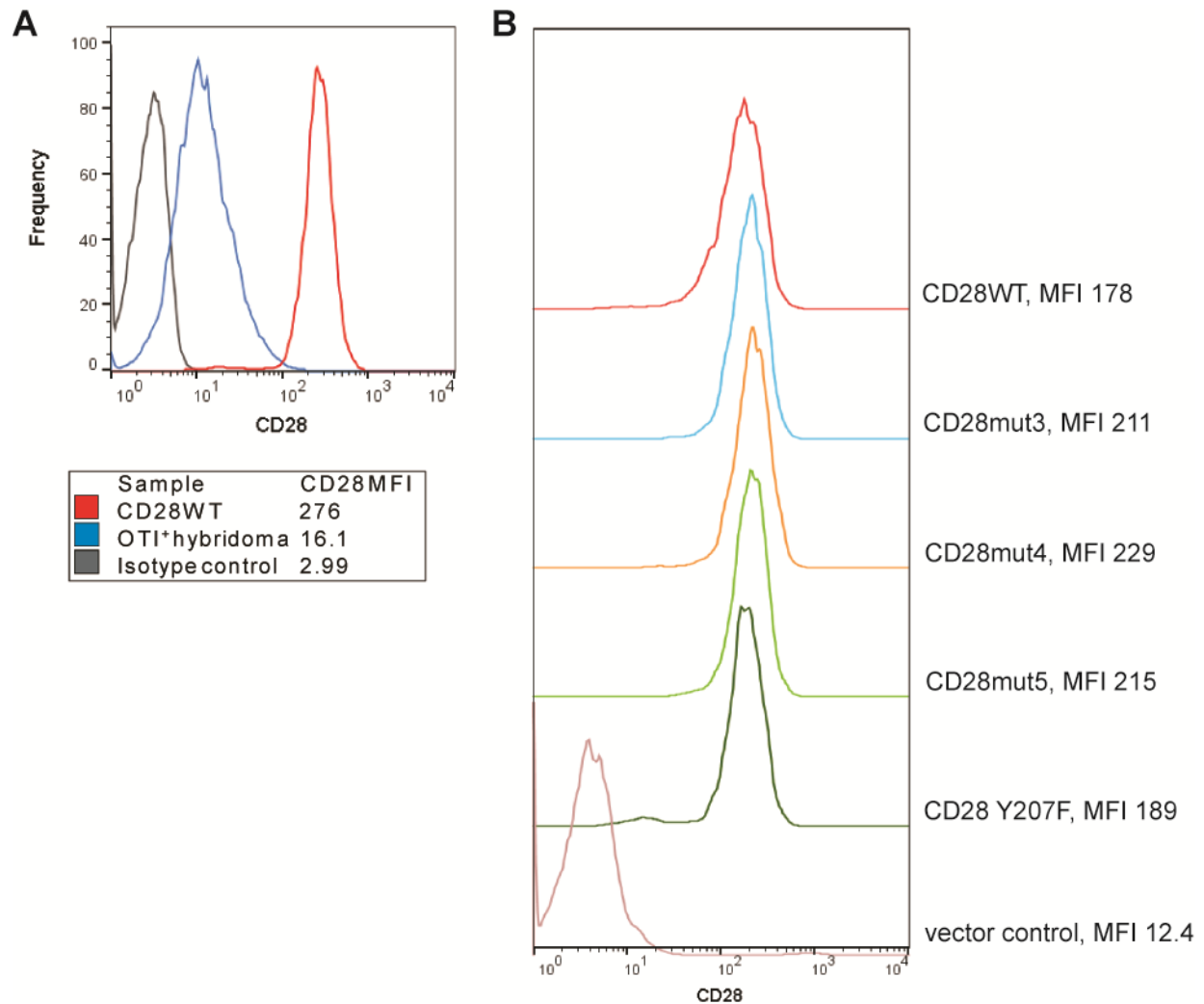
Phosphorylation Site	CD28wt	CD28mut5
Y189 (YMNM)	20.2%	32.8%
Y204	Not detected	2.1%
Y207 (PYAP)	20.9%	35.7%
Y215	2.9%	10.0%



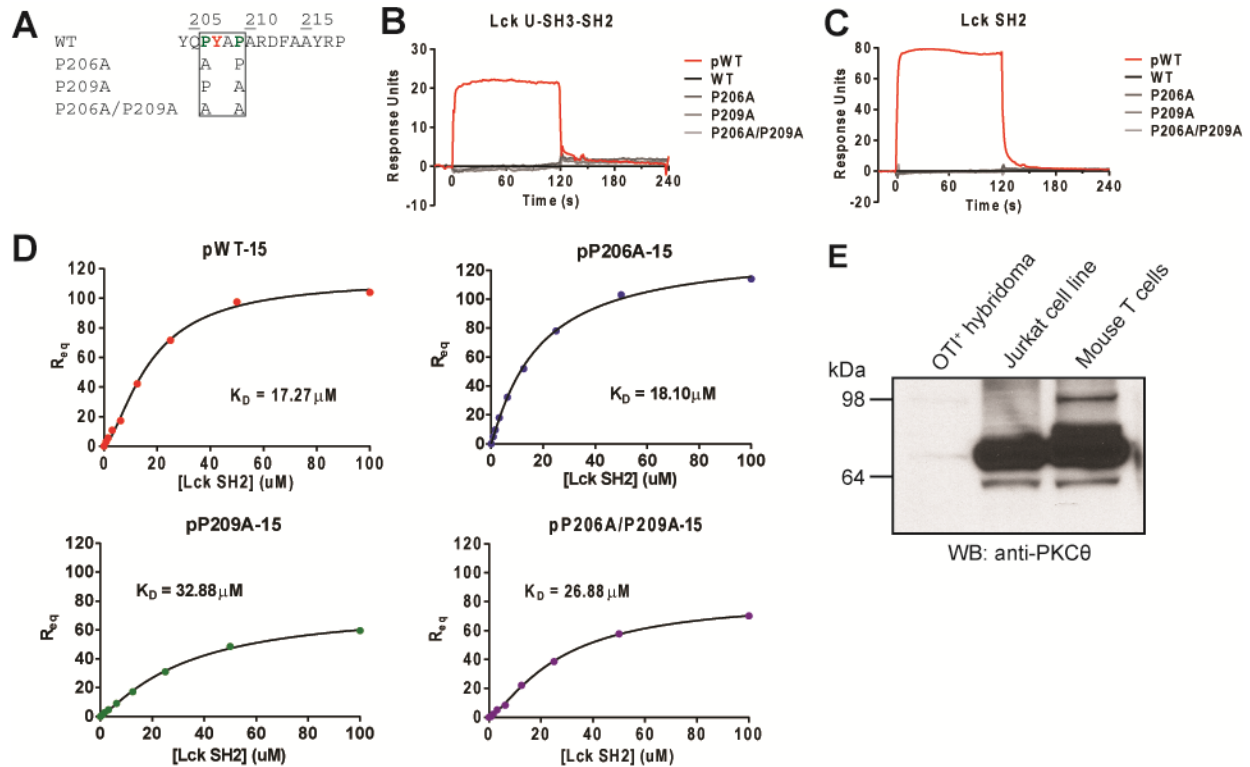
Supplementary Figure 2.2 CD28-Lck binding analysis by surface plasmon resonance. The displayed curves depict SPR measurements of varying concentrations of CD28mut3 (a) or Mut5 (b) disulfide-linked dimers injected over a surface coated with Lck for a 2 min association and 4 min dissociation period. Bulk refractive index change was subtracted based on the signal from a

Supplementary Figure 2.2 (Continued)

surface with similar immobilized amounts of an irrelevant protein (HLA-DM). (c) Equilibrium binding analysis was performed for the indicated CD28 25mer disulfide-linked dimers binding to coated full-length Lck after bulk refractive index subtraction based on a flow cell coated with HLA-DM negative control protein. Plots of R_{eq} at varying concentrations of CD28 are shown. Peptides represented the 25 C-terminal residues of CD28_{CD} (residues 194-218) with or without phosphorylation at Y207. Binding studies were performed in the absence of ATP to prevent phosphorylation of CD28_{CD} by Lck. K_D was calculated by fitting to a saturation binding model with Hill slope. (d) The indicated amount of purified recombinant Lck, which was used for in vitro phosphorylation assays and SPR analysis throughout this study, was analyzed by Western blotting with antibody specific to Lck phosphorylated at Y505.



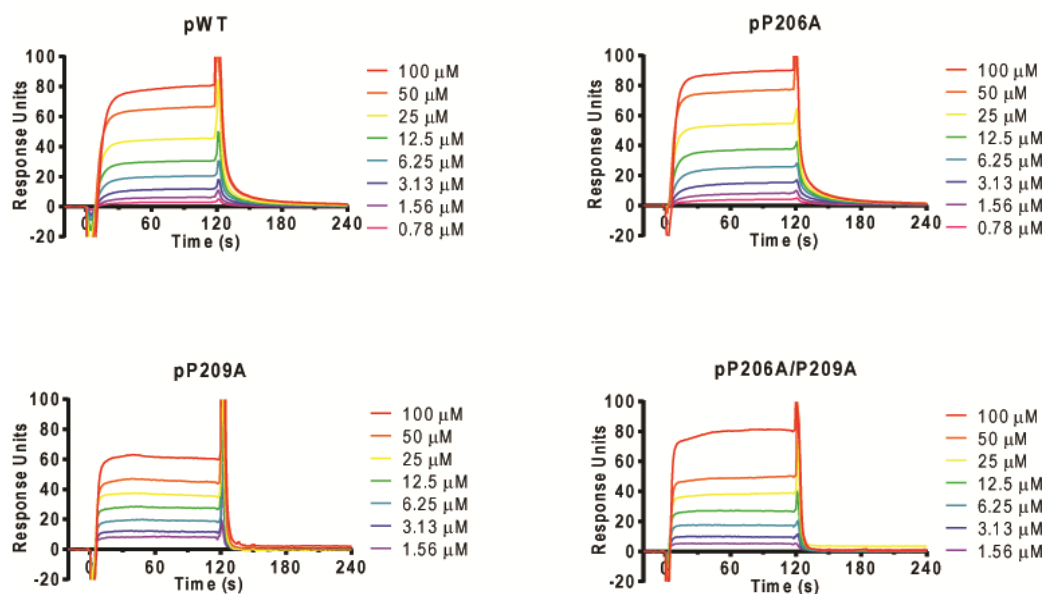
Supplementary Figure 2.3 CD28 surface expression in OT-I⁺ hybridomas. (a) CD28 surface expression is shown for OT-I⁺ hybridoma cells +/- transduction with a CD28WT expression construct. Cells were sorted for uniform CD28 expression. (b) CD28 surface expression is shown for OT-I⁺ hybridoma cells transduced with CD28WT or mutant expression constructs sorted for matched CD28 surface expression, or a vector control sorted based on ZsGreen marker expression.



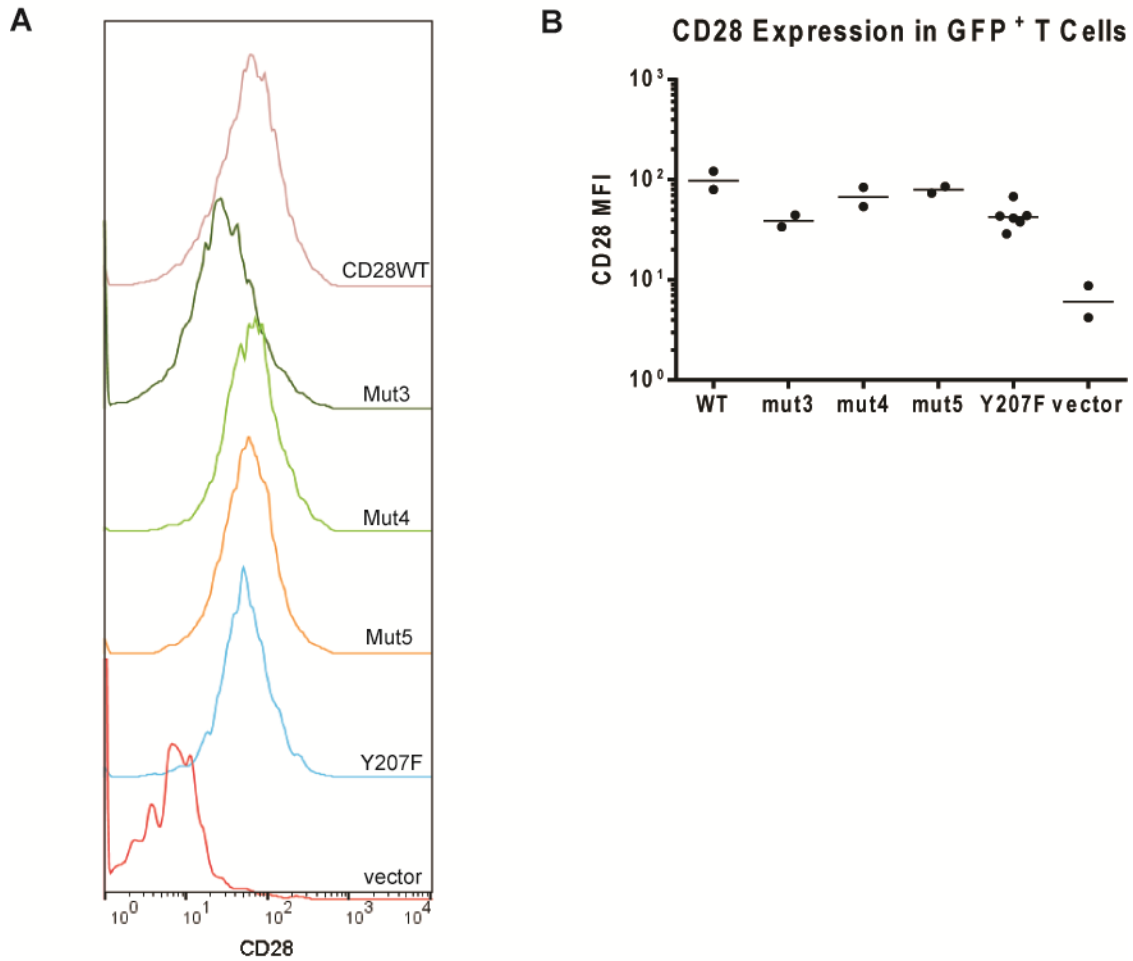
Supplementary Figure 2.4 Analysis of CD28 15mer interaction with Lck SH2 or U-SH3-SH2 using surface plasmon resonance. (a) A panel of peptides was prepared representing the C-terminal 15 residues of CD28_{CD} (residues 204-218). The contribution of P206A and P209A mutations was examined individually and in combination, and each peptide was prepared both phosphorylated (indicated by prefix “p”) and non-phosphorylated on Y207. (b, c) The indicated peptides were coated to approximately the same density on a streptavidin surface and the Lck unique-SH3-SH2 domain construct (b) or Lck SH2 domain (c) was injected at a concentration of 5 μM over each of the surfaces after subtraction of bulk refractive index change based on a streptavidin surface. (d) Purified Lck SH2 domain was injected at varying concentrations over surfaces coated with the indicated peptides. R_{eq} was plotted versus Lck SH2 domain concentration. K_D was calculated by fitting to a saturation binding model with Hill slope.

Supplementary Figure 2.4 (Continued)

(e) PKC θ expression was compared between OT-I⁺ hybridoma (mouse), Jurkat (human) and primary mouse T cells (1×10^5 cells per lane) by Western blot analysis with an anti-PKC θ antibody. Jurkat and mouse T cell lanes were over-exposed in order to reveal the faint signal for OT-I⁺ hybridoma.



Supplementary Figure 2.5 Equilibrium binding measurements of Lck SH2 domain interaction with CD28 PYAP motif. The indicated peptides (sequences shown in figure S2.4A) were coated to approximately the same density on a streptavidin surface. The displayed curves depict SPR measurements resulting from injection of purified Lck SH2 domain at varying concentrations. Bulk refractive index change was subtracted based on a control surface coated with non-phosphorylated WT 15mer peptide, which exhibited no binding to the Lck SH2 domain. Similar results were obtained by subtracting signal from a streptavidin surface.



Supplementary Figure 2.6 Flow cytometric analysis of transduced bone marrow chimeras.

(a) Anti-CD28 surface staining is shown for GFP⁺ T cells from recipients of bone marrow transduced with the indicated CD28 expression constructs or empty vector. (b) CD28 MFI is shown with the geometric mean for multiple samples analyzed as shown in (a).

Supplementary Figure 3.1 CD28 spacer constructs exhibit some co-localization with early endosomes. Jurkat cells expressing CD28-TFP fusion proteins with either no spacer, CspA spacer, or HPr spacer were fixed, permeabilized, and stained with antibodies against either the early endosome marker EEA-1 (a) or the ER marker calnexin (b). Images of the same area of interest are shown for TFP, the indicated marker antibody (labeled with Alexa Fluor 647 conjugated secondary antibody) and the merged image of the two channels.

Supplementary Figure 3.1 (Continued)

