An Enigmatic Tail of CD28 Signaling

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CD28 costimulation regulates a wide range of cellular processes, from proliferation and survival to promoting the differentiation of specialized T-cell subsets. Since first being identified over 20 years ago, CD28 has remained a subject of intense study because of its profound consequences on T cell function and its potential for therapeutic manipulation. In this review we highlight the signaling cascades initiated by the major signaling motifs in CD28, focusing on PI-3 kinase-dependent and -independent pathways and how these are linked to specific cellular outcomes. Recent studies using gene targeted knockin mice have clarified the relative importance of these motifs on in vivo immune responses; however, much remains to be elucidated. Understanding the mechanism behind costimulation holds great potential for development of new clinically relevant reagents, a fact beginning to be realized with the advent of drugs that prevent CD28 ligation and signaling.

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OVERVIEW OF CD28-MEDIATED COSTIMULATION

A critical component of the immunological response to foreign protein is the activation of T lymphocytes. T-cell receptor (TCR) recognition of peptide loaded MHC molecules provides antigen specificity and initiates the required steps for T-cell activation, although additional signals are needed for complete T-cell activation. A two signal model for lymphocyte activation was first proposed by Bretscher and Cohn in 1970 as a mechanism for self:nonself discrimination in B cells (Bretscher and Cohn 1970) and was subsequently expanded to incorporate CD8 T-cell activation by Lafferty et al (Lafferty and Cunningham 1975). Using T-cell clones, Schwartz and Jenkins showed that engagement of the TCR by peptide loaded MHC in the absence of other signals is insufficient to activate the T cell, and in fact may render it unresponsive to further antigenic stimulation, a condition termed anergy (Quill and Schwartz 1987; Mueller et al. 1989; Schwartz et al. 1989; Jenkins et al. 1990; Linsley and Ledbetter 1993). They further showed that provision of additional, non-MHC restricted signals provided by professional APC, resulted in the T cell becoming fully activated leading to clonal expansion and development of effector function. Thus, the two-signal model, where signal one is defined as the binding of peptide/MHC complexes and signal two refers to the additional, costimulatory signal(s), provided a potential mechanism for peripheral tolerance (June et al. 1990; June et al. 1994; Bluestone 1995). The most studied and well characterized costimulatory receptor/ligand

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complex is the CD28/B7 interaction (as reviewed in Rudd and Schneider 2003; Miller et al. 2009; Paterson et al. 2009; Rudd et al. 2009; Sharpe 2009).

CD28 was first identified by antibodies recognizing a 44 kDa protein on the surface of human T cells that synergized with PHA in the induction of a proliferative response (Hansen et al. 1980; Gmünder and Lesslauer 1984). The cDNA was expression cloned in 1987 by Aruffo and Seed and shown to encode a 23 kD type 1 transmembrane protein (Aruffo and Seed 1987). CD28 is expressed on the cell surface as a glycosylated, disulfide-linked homodimer (Aruffo and Seed 1987). In humans, CD28 is expressed on approximately 80% of CD4+ T cells and 50% of CD8+ T cells and has been detected on plasma cells, neutrophils, and eosinophils, although the function in these latter cell types is unclear (Lee et al. 1990; Venuprasad et al. 2001; Woerly et al. 2002). In contrast to humans, in mouse 100% of both CD4 and CD8 T cells express CD28 (Gross et al. 1990).

Most important in the initial activation of naïve T cells, ligation of CD28 on CD4+ T cells has diverse and profound consequences. CD28 signaling increases the sensitivity of the T cell to antigen receptor engagement, and as a result proliferation is induced at otherwise submitogenic concentrations of antigen (Gmünder and Lesslauer 1984; Damle et al. 1988; Damle and Doyle 1989; Shahinian et al. 1993b; Bachmann et al. 1996; Bachmann et al. 1997). Cytokine production is greatly increased, most significantly IL-2. CD28 costimulation results in a 50-fold increase in IL-2 secretion by both transcriptional and posttranscriptional regulation of expression (Lindsten et al. 1989; Fraser et al. 1991). Interestingly, under some conditions, this has been found to be resistant to suppression by cyclosporine, suggesting engagement of a novel signal transduction cascade (Thompson et al. 1989; Ledbetter et al. 1990). In addition, cell survival is enhanced by CD28 costimulation, in part by inducing expression of anti-apoptotic proteins including Bcl-X_L (Boise et al. 1995; Radvanyi et al. 1996; Sperling et al. 1996). Thus, by a variety of mechanisms, CD28 mediated costimulation greatly enhances the T cells effector response to antigen

Whether CD28 can activate signaling independent of TCR engagement or function only in a TCR dependent manner has been controversial. The ability of certain "superagonistic" anti-CD28 antibodies to independently activate T cells suggests that in some circumstances CD28 can function in the absence of a TCR derived signal (Tacke et al. 1997; Luhder et al. 2003). Furthermore, microarray analysis of T cells stimulated only through CD28 supported TCR independent signaling; however, this effect appeared to be relatively short lived (Riley et al. 2002; Marinari et al. 2004). Importantly, the existence of both TCR dependent and independent pathways is not mutually exclusive, and it is likely that both are operative and important.

CD28 is the founding member of the immunoglobulin (Ig) family of costimulatory receptors (Hansen et al. 1980; Gmünder and Lesslauer 1984), that now includes the receptors: cytotoxic T lymphocyte-associated antigen 4 (CTLA-4, CD152) (Dariavach et al. 1988), inducible costimulator (ICOS) (Hutloff et al. 1999; Tezuka et al. 2000), programmed death receptor 1 (PD-1) (Ishida et al. 1992), and B- and T-lymphocyte attenuator (BTLA) (Watanabe et al. 2003). Although CD28 and CTLA-4 both bind the ligands B7-1 (CD80) and B7-2 (CD86) (Freeman et al. 1989; Balzano et al. 1992; Azuma et al. 1993; Freeman et al. 1993) and are located on human chromosome 2q33 (Naluai et al. 2000) (mouse chromosome 1 (Gross et al. 1990; Howard et al. 1991)), CTLA-4 forms bivalent homodimers between CD80 molecules which contributes to its higher affinity for CD80 (12 compared to a 200 nM Kda value for CD28) (van der Merwe et al. 1997; Evans et al. 2005). CD28 and ICOS predominately enhance T-cell activation unlike CTLA-4, PD-1 and BTLA which are inhibitory (Rudd and Schneider 2003; Miller et al. 2009; Paterson et al. 2009; Rudd et al. 2009; Sharpe 2009). This review will cover the diverse functions of the CD28-B7 interaction highlighting the complex signaling events that define CD28 function.

THE MOTIFS BEHIND CD28 SIGNALING

The extracellular domain of CD28 binds to B7 proteins using a MYPPPY motif and this interaction initiates the costimulatory signal transduction cascade (Freeman et al. 1989; Balzano et al. 1992; Azuma et al. 1993; Freeman et al. 1993; Rudd and Schneider 2003). CD28 has a highly conserved, relatively short cytoplasmic tail (41 aa in human, 38 aa in mouse) that has no intrinsic enzymatic activity. However, several motifs have been identified including: four tyrosine residues, four serine and two threonine residues, two PxxP motifs, and two lysine residues each of which may be important in function (Rudd and Schneider 2003; Rudd et al. 2009). Phosphorylation of the tyrosine residues provides docking sites for src homology-2 (SH2) domain containing proteins whereas the proline-rich motifs can bind src homology-3 (SH3) domain containing proteins. The serine and threonine residues can also be phosphorylated and finally the lysine residues provide potential sites for ubiquitination. Interestingly CD28, CTLA-4, and ICOS share an YxxM motif (Rudd and Schneider 2003) a consensus binding site for the p85 subunit of the lipid kinase phosphatidyl-inositol 3-kinase (PI3K) (August and Dupont 1994; Pagès et al. 1994; Prasad et al. 1994). CD28 contains the sequence YMNM, where the Asn residue in the +2 position confers additional specificity for Grb2/GADS binding (Songyang et al. 1993; Schneider et al. 1995b), whereas the methionine at the +3 position confers p85 specificity (Takeda et al. 2008). The ability to bind Grb2 is absent from both ICOS and CTLA-4, which may in part explain the signaling and functional differences between these coreceptors (Rudd and Schneider 2003; Rudd et al. 2009). Early work has shown that the cytoplasmic tail of CD28 is tyrosine phosphorylated presumably by the Src family kinases Fyn and Lck (Raab et al. 1995; King et al. 1997), as well as phosphorylated on serine/threonine residues (Hutchcroft and Bierer 1994; Hutchcroft et al. 1996; Parry et al. 1997a). These events initiate specific protein–protein interactions and subsequent activation of downstream signaling cascades that define the costimulatory functions of CD28.

Motif Specific Protein–Protein Interactions

In the simplest format, CD28 signaling is initiated by activation of two dominant signaling cascades, one that requires phosphorylation of a tyrosine residue within the membrane proximal YMNM motif and subsequent binding of the p85 subunit of PI3K whereas another pathway is initiated by the more distal proline-rich regions (Rudd and Schneider 2003; Miller et al. 2009; Paterson et al. 2009; Rudd et al. 2009; Sharpe 2009) (Fig. 1). Each of these bind distinct sets of proteins, although there is

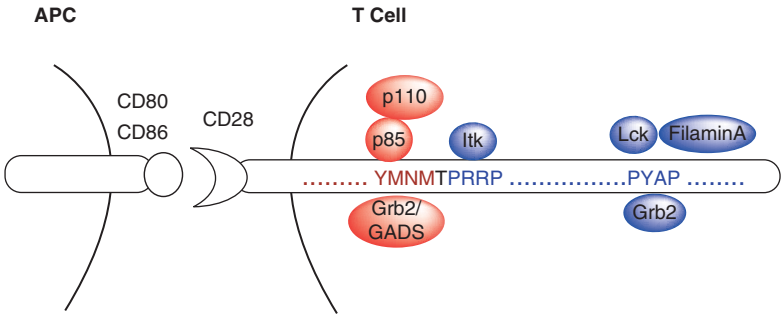

Figure 1. *Motif specific protein:protein interactions with the cytoplasmic tail of CD28.* Engagement of CD28 by CD80 or CD86 expressed on the antigen presenting cell (APC), initiates signal transduction cascades dependent on specific association of proteins with the cytoplasmic tail of CD28. The proximal YMNM motif when phosphorylated via Src family kinases binds the SH2 containing (indicated in red) proteins p85, a subunit of PI3K, and Grb2 or GADS via their SH2 domain. The distal proline-rich motifs bind the SH3 containing (indicated in blue) proteins Itk, at the sequence PRRP, and Grb2 (via its SH3 domain), filamin-A and Lck at the sequence PYAP.

significant overlap and potential for interaction. The observation that PI3K activation was enhanced via CD28 signaling led to the discovery that the p85 subunit of PI3K directly binds the YMNM motif via an SH2 interaction following phosphorylation of the tyrosine residue (Pagès et al. 1994; Truitt et al. 1994; Cai et al. 1995; Raab et al. 1995). Although PI3K-dependent signaling is likely the major pathway initiated by the proximal motif, its relative importance in CD28 function has been highly controversial. Initial in vitro CD28 signaling results should be interpreted with caution as the Jurkat T-cell line lacks both the PTEN and SHIP-1 phosphatases that function to inactivate the products of PI3K; therefore, the threshold to fully activate PI3K is reduced (Shan et al. 2000; Freeburn et al. 2002). Other proteins that can bind this region include Grb2 and GADS via their SH2 domain (Raab et al. 1995; Schneider et al. 1995a; Kim et al. 1998; Ellis et al. 2000). Competition between Grb2, GADS and PI3K for binding to the YMNM motif may be important in determining downstream CD28-dependent signaling events

Signaling by the distal PYAP motif is likely initiated by binding and activation of the Src family kinase Lck (King et al. 1997; Holdorf et al. 1999) although Fyn, Grb2, and GADS (via their SH3 domains) can also bind this region (Okkenhaug and Rottapel 1998; Ellis et al. 2000). The association of IL-2-inducible T-cell kinase (Itk) directly with the proximal proline-rich region (PRRP) of CD28 via its SH3 domain (Marengere et al. 1997) has been shown; however, if this event requires PI3K-dependent production of D3-lipids remains poorly understood. The PYAP motif also associates with filamin-A, an actin binding protein that functions as a scaffold for lipid raft formation (Tavano et al. 2006).

The generation of two specific knockin mice by our laboratory, the CD28 Y170F and CD28 AYAA mice, has clarified the role(s) of these two motifs in CD28 function (Friend et al. 2006; Dodson et al. 2009). Mutation of the proximal YMNM motif, although abrogating PI3K binding and subsequent protein kinase B (PKB)/Akt activation has no discernible in vivo effect (Dodson et al. 2009). On the other hand, mutation of the distal proline motif (PYAP) resulted in impaired CD28-dependent functions that included proliferation, IL-2 and other cytokine secretion, and adaptive immune system defects (Friend et al. 2006; Dodson et al. 2009). These data provide evidence that the distal proline motif initiates a critical, nonredundant signaling pathway required for CD28 function, whereas the proximal tyrosine based motif may be dispensable.

PI3K-DEPENDENT SIGNALING PATHWAYS

Activation of PI3K induces the production of the D3-lipids, phosphatidylinositol (3,4)-bisphosphate (PIP2) and phosphatidylinositol (3,4,5)-triphosphate (PIP3) where PIP3 is the main TCR/CD28 induced D3-lipid (Sasaki et al. 2000; Wang and Rudd 2008) (Fig. 2). These D3-lipids recruit pleckstrin homology (PH) domain containing proteins including phosphoinositide-dependent kinase 1 (PDK1), PKB/Akt, and possibly Wiskott Aldrich Syndrome Protein (WASP), a sorting nexin (SNX9), Itk and Vav (Parry et al. 1997b; Costello et al. 2002; Harriague and Bismuth 2002; Badour et al. 2007). PKB can be phosphorylated and activated by PDK1 and is a particularly important PI3K-dependent effector protein in CD28 signaling (Vanhaesebroeck and Alessi 2000). Downstream targets of PKB are diverse and include glycogen synthase kinase 3 (GSK3), mTOR, cAMP responsive element binding protein-1 (CREB), Bcl-2 antagonist of cell death (BAD), Bcl-X_L, FOXO family of transcription factors, and inhibitor of nuclear factor-κB (IκB) (Vanhaesebroeck and Alessi 2000). Thus, the PI3K-dependent signaling pathway has the ability to regulate a diverse array of CD28 functions that include cell cycle progression, apoptosis, cellular metabolism, and IL-2 transcription.

SIGNALING PATHWAYS INITIATED BY BINDING OF ADAPTOR PROTEINS

In addition to the PI3K-dependent pathway, binding of adaptor proteins (i.e., Grb2 or GADS) to the proximal motif can initiate

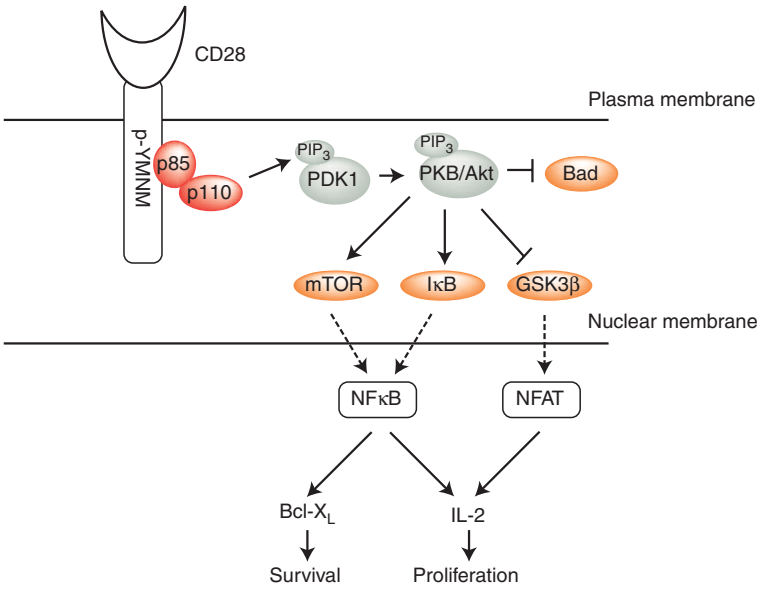

Figure 2. PI3K dependent signal pathway. Tyrosine phosphorylation of the YMNM motif via Src family kinases initiates the binding of the p85 subunit of PI3K. PI3K activity leads to the production of D-3 lipids, which recruit proteins via their pleckstrin homology domain (PH), including PDK1 and PKB/Akt. Once PKB is phosphorylated by PDK1, PKB phosphorylates its downstream targets including mTOR, IκB, GSK3β and Bad. Active mTOR and IκB result in increased NF-κB transcriptional activity whereas the phosphorylation of Bad and GSK3β results in increased survival and NFAT transcriptional regulation, respectively. The activation of NF-κB and NFAT (indicated by the dotted lines) induces the transcription of both Bcl-X_L, a prosurvival factor, and IL-2, an important T-cell cytokine required for proliferation as well as other genes.

signaling (Fig. 3). Grb2 recruits Son of Sevenless (Sos), a guanine nucleotide exchange factor and activator of GTPase $p21^{ras}$, and Vav, also an exchange factor (Schneider et al. 1995a; Collins et al. 1997; Bustelo 2000). A mechanism by which CD28 via Grb2 binding to the PxxP motif mediates JNK activation is plausible where Grb2 recruits Sos and Vav which in turn phosphorylate and activate Ras-related C3 botulinum toxin substrate (Rac1), PKCθ and cell division cycle 42 (CDC42) (Collins et al. 1997; Bustelo 2000). Rac1 and CDC42 activate the MEK4/MEK7 to MEKK1 signaling cascade culminating in JNK activation (Su et al. 1994). In support of this model, CD28 induces a GTP-RAC complex, possibly through the phosphorylation of Vav by ZAP-70 (Salojin et al. 1999), and activates MEKK1 in a Vav-dependent manner (Marinari et al. 2002). CD28-dependent activation of JNK (Su et al. 1994) is inhibited by mutant forms of Vav that lack functional GTP-GDP exchange activity and a dominant negative mutant of MEKK1 (Marinari et al. 2002; Wood et al. 2006). GADS potentially interacts with SLP76 and LAT in a signaling complex thereby providing CD28 a means to modulate TCR mediated signaling (Rudd and Schneider 2003; Rudd et al. 2009). Although these studies imply Grb2/GADS, Vav, PKCθ, and downstream MAPK signaling are linked to the PxxP motifs in CD28, this cascade has yet to be fully defined.

REGULATION OF IL-2 PRODUCTION BY CD28

Transcriptional Regulation of the IL-2 Gene by CD28

The regulation of IL-2 by CD28 is complex involving both increased gene transcription and post-transcriptional stabilization of mRNA

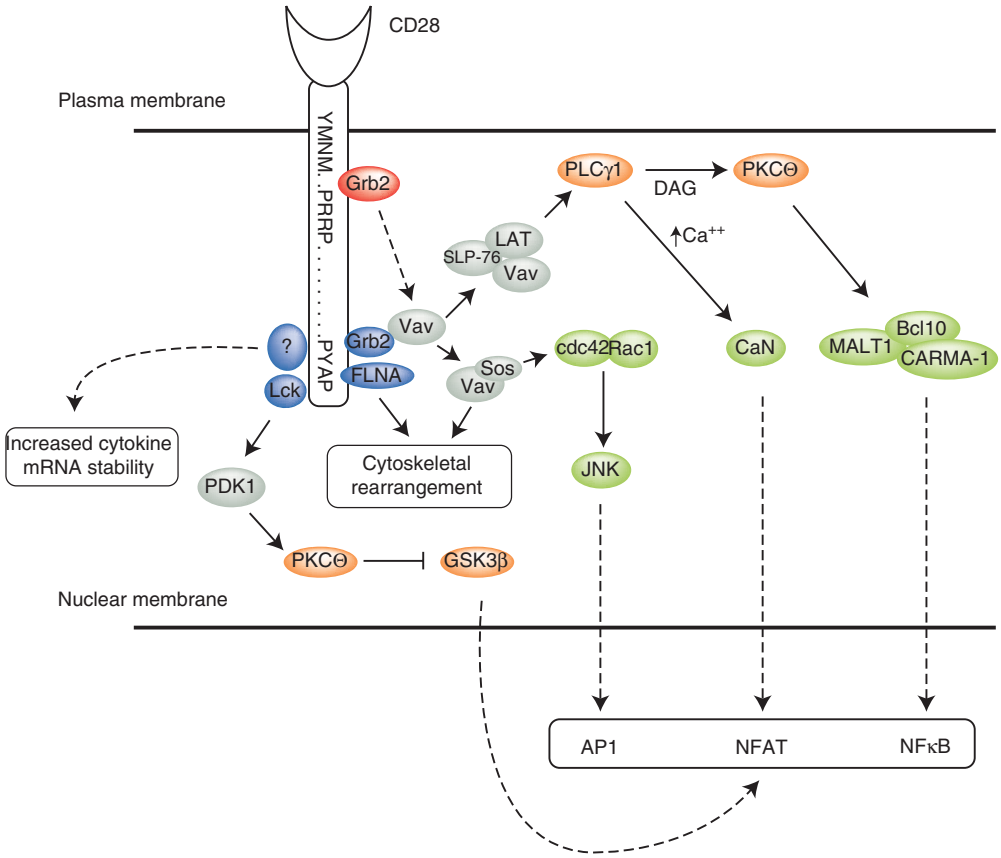

Figure 3. *Adaptor* protein intiated signaling events. The binding of the adaptor protein Grb2 to CD28 occurs via its SH2 domain at the proximal YMNM motif or its SH3 domain at the distal PYAP motif. Grb2 through an SH3 interaction may subsequently bind Vav which has the potential to initiate two signaling complexes. The Vav-Sos complex results in cdc42/Rac1 activation an initiator of cytoskeletal rearrangement and downstream MAPK activation (JNK) that induces the formation of the AP1 transcriptional complex. Vav also binds the SLP-76-LAT complex, which activates PLCγ1 a kinase that increases intracellular Ca^{2+}, through IP3 production, and activates PKCθ via DAG. The increase in intracellular Ca^{2+} results in calcineurin activation, a phosphatase that acts on NFAT allowing for its nuclear translocation. PKCθ activation leads to the formation of a multi-protein complex with Bcl10, MALT1 and CARMA-1 that induces NF-κB transcriptional activation. The distal PYAP motif also binds Lck a Src kinase that phosphorylates PDK1 which phosphorylates and activates PKCθ and subsequently inactivates GSK3β via phosphorylation resulting in enhanced transcription of NFAT–dependent genes. Stabilization of cytokine mRNA is also dependent on signals originating at the PYAP through a mechanism that may involve Lck binding (indicated via the dotted arrow) or an unknown protein whereas cytoskeletal rearrangement at the PYAP motif is filamin-A (FLNA) dependent.

(Lindsten et al. 1989; Bohjanen et al. 1992). The IL-2 promoter has binding elements for members of the NFAT, NF-κB, Oct-1, and AP-1 (c-fos/Jun) families of transcription factors and CD28 regulates the expression and activity of NFAT, NF-κB, and AP-1 (Fraser et al. 1991; Granelli-Piperno and Nolan 1991; Shapiro et al. 1997; Miller et al. 2009). Research pioneered by the Weiss laboratory identified a specific region in the IL-2 promoter, termed the CD28-response element (CD28RE) that binds transcription factors in a CD28-dependent manner

thus providing a site for signal integration (Fraser et al. 1991; Shapiro et al. 1997). Analyses of the CD28RE/AP-1 complex determined that CD28 signaling activated both IκBα and IκBβ resulting in increased transcription of IL-2, which was inhibited by dominant negative mutants of these IκB proteins (Lai and Tan 1994; Harhaj et al. 1996; Harhaj and Sun 1998; Zhou et al. 2002). The phosphorylation of the IκB complex, which targets IκB for degradation through ubiquitination, via upstream serine/threonine kinases called the IκB kinases (IKKs), results in NF-κB translocation to the nucleus (Lai and Tan 1994; Harhaj et al. 1996; Harhaj and Sun 1998). CD28-dependent IKK activation was inhibited by a dominant negative MEKK1 and IKK deficient cells have defective IL-2 production implicating CD28 as an upstream mediator of the IKK complex and NF-κB activation (Harhaj et al. 2000; Tuosto et al. 2000). Through a mutational screen, mice identified as defective in antigen receptor activation of JNK and NF-κB were subsequently determined to also be defective in caspase recruitment domain-containing membrane associated guanylate kinase protein-1 (CARMA-1) activation (Jun et al. 2003). CARMA-1 binds Bcl10 via CARD-CARD binding domains and activation of CARMA-1 is enhanced by PKCθ phosphorylation (Gaide et al. 2002; Takeda et al. 2008). Bcl10 deficient T cells also show impaired NF-κB activation; therefore, a CD28-dependent signaling cascade can be predicted for NF-κB that includes PKCθ, Bcl10 and CARMA-1 proteins (Gaide et al. 2002; Takeda et al. 2008). The series of events that initiate this complex is not well understood, but the oligimerization of the CARMA-1 protein through its coiled-coil domain (CC2) and its localization via its CC1 domain are involved (Tanner et al. 2007) along with a lysine residue at 808 of CARMA-1 (Wang et al. 2004).

Analyses using CD28 deficient T cells determined that CD28 is required for proper segregation of PKCθ into the immunological synapse and the failure to localize PKCθ correctly results in poor activation of NF-κB and IL-2 gene transcription (Huang et al. 2002; Sanchez-Lockhart et al. 2004; Sanchez-Lockhart and Miller 2006). Mice or T cell lines deficient in PKCθ have a pronounced defect in NF-κB activation through TCR-CD28 signals (Sun et al. 2000; Matsumoto et al. 2005). In Jurkat T cells Vav has been shown to bind PKCθ and acts synergistically to enhance CD28-dependent activation of NF-κB (Coudronniere et al. 2000; Dienz et al. 2000; Hehner et al. 2000a; Hehner et al. 2000b; Villalba et al. 2000a). Results indicate the CARMA-1, Bcl10, and mucosa-associated lymphoid tissue (MALT) lymphoma translocation gene 1 (MALT1) complex is dependent on CD28-dependent PKCθ translocation and activation, possibly through Vav and Grb2, to regulate NF-κB activation (Wang et al. 2002; Hara et al. 2003; Jun et al. 2003; Wang et al. 2004; Tanner et al. 2007). CD28 activation of NF-κB may involve RIP-2, a CARD containing adaptor also called CARDIAK or RICK, as RIP-2 deficient T cells have impaired IL-2 production, proliferation, and NF-κB transcription (Kobayashi et al. 2002). CD28 mediated activation of NF-κB may include the role of PKB, NIK, and Cot; however, this remains unclear as these experiments used overexpression of mutant proteins or resulted in partial inhibition of CD28 functions (Kane et al. 1999; Lin et al. 1999; Hehner et al. 2000b; Yamada et al. 2000; Bauer et al. 2001).

Regulation of NFAT by CD28 is another costimulatory pathway that augments IL-2 expression (Fraser et al. 1991). TCR and CD28-dependent signaling pathways cooperate in the activation of PLCγ-1 leading to increased intracellular calcium levels, PKC phosphorylation and activation of calcineurin (Ca^{2+}-dependent Ser/Thr phosphatase, CaN), which then dephosphorylates NFAT enhancing its nuclear translocation (Fraser et al. 1991). The immunosuppressant drugs cyclosporine and tacrolimus block CaN activation and thereby prevent NFAT translocation (Fraser et al. 1991). In contrast, CD28 derived signals induce the phosphorylation and inactivation of GSK3β, a kinase that promotes the nuclear export of NFAT (Diehn et al. 2002; Pan et al. 2007). By this mechanism, CD28 leads to NFAT nuclear trapping and increased transcription of NFAT-dependent genes,

including IL-2 (Diehn et al. 2002; Pan et al. 2007).

Postranscriptional Regulation of IL-2 mRNA Expression

In addition to transcriptional control of the IL-2 gene, CD28 regulates the stability of IL-2 and other cytokine mRNA (Lindsten et al. 1989; Miller et al. 2009). Specific AUUA sequences in the 3′ untranslated region destabilize the message (Bohjanen et al. 1992). Briefly, there are two general models of mRNA stability 1) microRNA and 2) AU-rich elements (ARE) within the 3′ UTR of the mRNA that affect degradation of the RNA especially that of cytokine genes including IL-2 (Lindsten et al. 1989). In unstimulated cells AU-binding proteins bind the 3′ UTR and recruit a multicomponent exosome that deadenylates and digests the 3′ UTR of the mRNA via exonucleases (Ogilvie et al. 2005). T-cell stimulation induces TTP activation, an AU-binding protein, and subsequent binding to the ARE within the 3′ UTR of IL-2 mRNA that induces degradation (Ogilvie et al. 2005). The absence of TTP results in increased IL-2 expression after T-cell activation (Ogilvie et al. 2005). To counter TTP induced degradation, a CD28-dependent mechanism may require HuR translocation to the cytoplasm (Seko et al. 2004) and/or elements of MAPK activation (i.e., JNK and p38) (Chen et al. 1998; Winzen et al. 1999; Chen et al. 2000). As CD28 regulates JNK activation (Su et al. 1994; Marinari et al. 2002; Wood et al. 2006), YB-1 and nucleolin, JNK induced proteins, stabilize the IL-2 transcript at the 5′ end; however, these proteins are not sufficient and may require another protein that binds to the 3′ UTR (Chen et al. 2000). One possible candidate, NF90, a transcription factor associated with NFAT and IL-2 activation (Corthesy and Kao 1994), has been shown to compete with TTP for binding at the ARE of IL-2 mRNA (Shim et al. 2002). NF90 knockout T cells have decreased IL-2 secretion but whether this effect is on the regulation of IL-2 transcription or mRNA stability, as well as, how CD28 signaling affects NF90 function is unclear (Shi et al. 2007). T-cell activation shuttles NF90 from the nucleus to the cytoplasm that dissociates TTP from the ARE and results in IL-2 mRNA stabilization (Shim et al. 2002; Shi et al. 2007).

Attempts to dissect the signaling pathway from CD28 to IL-2 using a mutational approach have yielded inconsistent results. Early experiments in Jurkat cells showed an association of PI3K with CD28 but the dependency of IL-2 secretion on this association were conflicting (Stein et al. 1994; Truitt et al. 1994; Crooks et al. 1995; Lu et al. 1995; Truitt et al. 1996; Sadra et al. 1999). A role for the YMNM motif in IL-2 secretion was suggested using murine cell lines but whether this was mediated through Grb2 or PI3K was not clear (Pagès et al. 1994; Cai et al. 1995; Kim et al. 1998; Watanabe et al. 2006). The role of the YMNM motif in IL-2 secretion remained unclear from studies in transgenic mice or retroviral reconstitution on the CD28-deficient background (Burr et al. 2001b; Harada et al. 2001; Okkenhaug et al. 2001; Andres et al. 2004b). These data showed either no effect on proliferation and IL-2 secretion (Okkenhaug et al. 2001; Andres et al. 2004b), a partial inhibition (Burr et al. 2001b), or inhibition of proliferation and IL-2 secretion at early time points (Harada et al. 2001). A caveat of these experiments is that CD28 expression is not under endogenous control but that of a heterologous promoter.

Recent data has supported the PYAP motif as the dominant signaling motif determining the net output of IL-2 following CD28 costimulation. In a retroviral reconstitution system, the proline to alanine mutation within the PYAP motif resulted in either decreased CD28-dependent proliferation and IL-2 production (Burr et al. 2001b) or no effect unless this mutation is combined with the tyrosine mutation in the YMNM motif (Andres et al. 2004b). In transgenic mice, the PYAP motif had impaired CD28-dependent proliferation whereas the Itk binding proline motif (PRRP) did not (Gogishvili et al. 2008). Interestingly, in a PI3K mutant mouse ($p110\delta^{D910A/D910A}$) CD28-dependent proliferation was maintained whereas Vav deficient primary T cells had defective proliferation (Gogishvili et al. 2008). These results predict

that the PYAP motif mediates CD28-dependent proliferation through Vav and Lck independently of PI3K, although the role of Grb2 or GADS (Watanabe et al. 2006) was not directly addressed.

We directly tested the relative importance of the YMNM and the PYAP motifs in regulating CD28-dependent IL-2 production using gene targeted knockin mice. This approach benefits from the study of truly naïve T cells in which CD28 expression is controlled by endogenous elements. Under these conditions the PYAP motif has a critical role in CD28 mediated proliferation and IL-2 secretion (Friend et al. 2006; Dodson et al. 2009). Importantly, these studies also showed that CD28 expression level is an important determinant of the outcome of engagement as mice with a single AYAA allele had greater impairment in both proliferation and IL-2 secretion than mice with two AYAA alleles. In contrast, the Y170F mutation had little appreciable effect independent of gene dosage (Dodson et al. 2009). There is segregation of transcriptional control of IL-2 expression and regulation of mRNA stability by the YMNM and PYAP motifs. The Y170F mutation impairs IL-2 transcription but has little effect on net IL-2 secretion and no effect on mRNA stability whereas mutation of the PYAP motif has the reciprocal effect (Sanchez-Lockhart et al. 2004; Miller et al. 2009) (X. Wang, J. Green and J. Miller, unpublished data). The PYAP motif can mediate Grb2 binding and downstream JNK activation; therefore, these data give credence to the theory that CD28-dependent JNK activation is the dominant pathway resulting in IL-2 mRNA stability.

The CD28 knockin mice also provided an opportunity for us to directly examine components of the signaling cascade leading to IL-2 production in naïve, primary T cells. Previous studies showed that the YMNM motif was required for CD28-dependent activation of PKB, which in turn led to phosphorylation and inactivation of GSK3β resulting in enhanced IL-2 gene transcription (Ohteki et al. 2000; Appleman et al. 2002; Diehn et al. 2002; Wood et al. 2006). However, data from the knockin mice showed the phosphorylation of both PKCθ and GSK3β were dependent on the PYAP and not the YMNM motif (Dodson et al. 2009). An alternative mechanism of the phosphorylation of GSK3β has been proposed where PKC isoforms directly phosphorylate GSK3β possibly through Vav or PDK1 (Lee et al. 2005) independent of PKB (Goode et al. 1992; Fang et al. 2002; Vilimek and Duronio 2006), a model supported by the knockin data.

CD28 SIGNALING AND THE IMMUNOLOGICAL SYNAPSE

The organization of specific proteins within the contact site of the APC and T cell during T-cell activation is referred to as the immunological synapse (Grakoui et al. 1999; Dustin and Chan 2000; van der Merwe 2002). Proteins segregate into supramolecular activation clusters (SMAC), which contain three distinct domains: the cSMAC ("c" indicates central), enriched in TCR, CD4, CD28, and signaling components such as PKCθ and Lck, the pSMAC ("p" indicates peripheral), enriched in LFA-1 and other cytoskeletal components and an outer region that contains mostly CD45 (Grakoui et al. 1999; Bromley et al. 2001; Bunnell et al. 2002; Freiberg et al. 2002). Initially, TCR and CD4 form small microclusters that also contain CD28 within the cSMAC whereas LFA-1 moves into the pSMAC (Grakoui et al. 1999; Krummel et al. 2000; Andres et al. 2004a). Although how the activation of Lck and its role in CD28 signaling is poorly understood, the colocalization of CD45, a phosphatase that activates Lck by removing an inhibitory phosphate (Y505), with CD4 associated Lck and CD28 in these microclusters provides a possible model of Lck activation and association with CD28 (Johnson et al. 2000; Freiberg et al. 2002).

CD28 is required for the localization of PKCθ to the immunologic synapse (Monks et al. 1998; Bromley et al. 2001; Andres et al. 2004a). Mutational analysis mapped this requirement to the YMNM motif, suggesting a PI3K-dependent mechanism (Sanchez-Lockhart et al. 2004; Sanchez-Lockhart and Miller 2006; Yokosuka et al. 2008). In recent

experiments, the ability of CD28 to recruit PKCθ to the immunological synapse was subsequently determined to be independent of PIP3 generation (Garcon et al. 2008). Interestingly PKCθ colocalizes with filamin-A, and when filamin-A is knocked down via siRNA, PKCθ activity, as well as, NF-κB, NFAT/AP-1, and IL-2 transcription are reduced (Hayashi and Altman 2006). As CD28 has the ability to modulate the cytoskeletal components, Rho (Kaga et al. 1998) SLP76, Rac1 (Michel et al. 2000; Raab et al. 2001), and PKCθ (Villalba et al. 2000a) in a Vav-dependent mechanism (Villalba et al. 2000b) and Vav has been shown to enable the TCR/CD3 complex to cluster (Fischer et al. 1998; Holsinger et al. 1998; Krawczyk et al. 2002), a CD28-dependent molecular signaling scaffold may be postulated at the immunological synapse. CD28 is tethered to the lipid raft via binding of filamin-A (Tavano et al. 2006) where CD28, through the YMNM motif, recruits PKCθ via PI3K activation (Sanchez-Lockhart et al. 2008). Thus, CD28 actin rearrangement functions through the binding of filamin-A, the downstream activation of Vav and PKCθ and the Vav effectors CDC42 and Rho-GTPases (Tavano et al. 2006). These data predict the movement of CD28 to the immunological synapse occurs before the majority of signaling events, through its PYAP motif, and is critical for CD28's ability to function.

After the PYAP motif has recruited CD28 to the immunological synapse, the binding of PI3K to the YMNM motif also modulates CD28 receptor mediated endocytosis. CD28 undergoes internalization, an event that requires the YMNM motif and binding of PI3K, which results in degradation via the lysosome or a recycling back to the cell surface (Cefai et al. 1998). CD28 internalization also requires the recruitment of WASP, via its SH3 domain, and a sorting nexin (SNX9) (Snapper and Rosen 1999; Badour et al. 2007). An SNX9 mutant that lacks the phagocyte oxidase homology (PX) domain that interacts with the p85 subunit of PI3K and PIP3 failed to induce CD28 internalization and NFAT transcription, whereas the overexpression of SNX9 increased these processes (Badour et al. 2007). These results indicate that CD28 recruitment and signaling is functionally linked to the active signaling environment of the immunological synapse.

CD28 ENHANCES T-CELL SURVIVAL

A major consequence of CD28 signaling is protection from cell death (Boise et al. 1995; Noel et al. 1996; Radvanyi et al. 1996; Sperling et al. 1996). The two dominant pathways of apoptosis in T cells are (1) receptor mediated (i.e., TNF family) or (2) mitochondrial-associated proteins (i.e., the Bcl family) both of which are modulated via CD28 signaling (Rudd et al. 2009). CD28 costimulation prevents activation induced cell death (AICD) by inhibiting the expression of CD95L expression (*cis* mediated death) on the T cell and by decreasing the formation of the death inducing signaling complex (DISC) (Kirchhoff et al. 2000). CD28 stimulation increases the protein expression of c-FLIPs which compete with procaspase 8 for binding to the death domains of CD95 and FADD thereby preventing the conversion of procaspase 8 to active caspase 8 and the induction of cell death (Kirchhoff et al. 2000). CD28-deficient cells are more sensitive to Fas-mediated cell death, which could be reversed by the overexpression of PKB (Jones et al. 2002). The overexpression of PKB prevented the binding and downstream conversion of procaspase 8 to caspase 8 through inhibition of the DISC complex (Jones et al. 2002). CD28 also enhances targets of glycolysis, including Glut1 expression and glucose uptake, all required for increased metabolism during cellular proliferation through the activation of PKB (Frauwirth et al. 2002).

CD28 stimulation also induces the up-regulation of antiapoptotic factors including Bcl-X_L (Boise et al. 1995). The overexpression of Bcl-X_L in CD28-deficient mice restores cellular survival but not proliferation (Dahl et al. 2000); thereby, separating these two CD28-dependent functions. Bcl-X_L, an NF-κB inducible gene, requires CD28-dependent IKK activation through the activation of PKCθ and formation of the CARMA-1, Bcl10, and MALT1 complex (Wang et al. 2002; Hara et al. 2003; Jun et al. 2003;

Wang et al. 2004; Tanner et al. 2007; Takeda et al. 2008). CD28-deficient mice reconstituted with a disrupted YMNM motif failed to induce Bcl-X_L whereas CD28-deficient mice reconstituted with disrupted proline rich motifs of CD28 retained the capacity to induce Bcl-X_L (Burr et al. 2001b; Harada et al. 2001; Okkenhaug et al. 2001). The overexpression of PKB led to constitutively elevated amounts of Bcl-X_L protein and inhibited FAS mediated apoptosis (Jones et al. 2000; Jones et al. 2002). These data suggested a central role for the YMNM motif, via PKB activation, in the regulation of cell survival by CD28. These results held true until the generation and analysis of CD28 knockin mice, which surprisingly indicated that neither mutation in the YMNM or PYAP motifs alone prevented CD28-dependent up-regulation of Bcl-X_L (Dodson et al. 2009). However, the expression of Bcl-X_L is dependent on both PKCθ and NF-κB activation, events that are regulated by CD28 and may not be exclusively downstream of the YMNM motif (Khoshnan et al. 2000; Marinari et al. 2004; Li et al. 2005; Manicassamy et al. 2006).

CD28 REGULATES T-CELL SUBTYPE DEVELOPMENT AND DIFFERENTIATION

Although the peripheral T-cell repertoire is not markedly altered in the absence of CD28, a role in negative selection has been suggested (Shahinian et al. 1993b; Lucas et al. 1995; Sperling et al. 1996; Noel et al. 1998). However a more dramatic phenotype has been uncovered with the identification of regulatory T cells (Tregs) (Boden et al. 2003; Tang et al. 2003; Tai et al. 2005; Guo et al. 2008). CD28-deficient mice have drastically reduced numbers of thymically derived Tregs (Salomon et al. 2000; Tang et al. 2003; Lohr et al. 2004; Tai et al. 2005). CD28 could contribute to Treg development enhancing IL-2 production (Guo et al. 2008) or by a cell intrinsic mechanism (Tai et al. 2005; Tao et al. 2005). However, the failure of wild-type cells to support the generation of CD28-deficient Tregs suggests that exogenous IL-2 is not sufficient in the absence of other CD28-dependent signals (Tai et al. 2005). Additional data recently obtained using the knockin mice supports that CD28, partially via the distal carboxy-terminal proline motif, provides an intrinsic signal necessary for the generation of cytokine responsive Treg precursors (C Lio, C Hsieh, and J Green, unpublished data).

As the differentiation of T cells into distinctive populations (TH0, TFh, TH1, TH2, and TH17) are defined in part by their cytokine secretion profiles, a role for CD28 in the generation and maintenance of these populations has been implicated. Although CD28 regulates multiple cytokines, it is neither absolutely required nor sufficient for the generation of any distinct T-cell subset. CD28 is important for TH2 generation but not the secretion of TH2 cytokines (King et al. 1995; Rulifson et al. 1997); however, TH1 generation does not require CD28 but the secretion of IL-2 by these cells requires CD28 (Shahinian et al. 1993a; Lucas et al. 1995; Villegas et al. 1999). Transgenic overexpression of PKB in CD28-deficient mice restored the production of IL-2 and IFN-γ but not IL-4 and IL-5 the cytokines required for the generation of TH2 cells (Kane et al. 2001). CD28-deficient mice show a defect in IL-4 production on receptor stimulation, which is partially restored by the overexpression of PDK1 (Andres et al. 2004b; Nirula et al. 2006). Although PDK1 is an upstream activator of PKB, in this case PDK1 was determined to be dependent on PKA and NFAT (Nirula et al. 2006). CD28-deficient and IL-4 deficient mice fail to induce germinal center formation and upregulation of CD40L in an Ova model of stimulation (Reiter and Pfeffer 2002). Interestingly, AYAA knockin mice also have defects in IL-4 production, germinal center formation, and decreased activation of PDK1 (Friend et al. 2006; Dodson et al. 2009). These data illustrate the complex role of the YMNM and PYAP motifs in the activation of PKB and PKA in TH2 type T cells and in the secondary immune response.

The development of a specialized cell type called NKT cells, as characterized by the expression of both a T-cell marker, invariant TCR (Vα14-Jβ18), and an NK marker (NK1.1) that respond to glycolipid α-galactosylceramide

when presented by CD1d, is also dependent on CD28 signals (Williams et al. 2008). Through the use of CD28-deficient and B7-deficient mice, a distinctive role of CD28 in the thymic generation of these NKT cells was identified, as the absence of either CD28 or its ligand B7 resulted in severely decreased numbers of NKT cells (Williams et al. 2008). Paradoxically, the overexpression of B71 in the thymus of transgenic mice also resulted in decreased NKT cells (Williams et al. 2008). These results suggest that not only the presence of CD28 but the strength of signaling through CD28 coordinate in the development and differentiation of cell types.

THE ROLE OF CD28 COSTIMULATION IN DISEASE

Perhaps surprisingly, mutations in CD28 have not been identified as the genetic basis for any human disease. However, mouse models of disease have been shown to depend on CD28 function. The use of CD28- and B7-deficient cells has implicated this pathway in the generation of a TH2 response required for the destruction of islet cells (autoimmune diabetes in the NOD mouse model) (Lenschow et al. 1996) and in the activation of newly recruited T cells responsible for epitope spreading (relapsing EAE in the EAE mouse model) (Miller et al. 1995). CD28 has been shown to be required in a model of allergic airway inflammation (Harris et al. 1997a; Harris et al. 1997b; Keane-Myers et al. 1997; Mathur et al. 1999; Burr et al. 2001a). A variety of transplantation studies showed that a lack of CD28 could prolong graft acceptance, but in most cases the organs were eventually rejected (Turka et al. 1992; Sayegh and Turka 1998; Harada et al. 2001). However, when combined with blockade of CD40:CD40L interactions, indefinite graft acceptance was achieved (Larsen et al. 1996).

Examination of the role for the specific motifs of CD28 in disease models are limited; however, the PYAP motif was important in a model of autoimmune disease using CTLA-4-deficient mice (Tai et al. 2007), a functional YMNM motif is needed to generate a graft versus host response (Harada et al. 2001) whereas disease severity in experimental allergic encephalomyelitis is reduced in CD28-deficient mice and the CD28-AYAA knockin mice, but not the CD28-Y170F knockin mice (Friend et al. 2006; Dodson et al. 2009). In the absence of CD28 or in the CD28-AYAA knockin mice, antibody production and germinal center formation is markedly reduced (Friend et al. 2006; Dodson et al. 2009). Thus, in a number of model systems, CD28 plays a critical role.

An understanding of costimulation, and the mechanism by which it regulates immune responses provides new avenues for the development of therapeutics. As CD28 decreases the threshold required to activate naïve T cells, as well as increasing the survival of activated T cells, one can envision development of drugs that either potentiate or down-regulate an immune response. Although global inhibition of CD28 function would be anticipated to profoundly inhibit T-cell function, more specific targeting of specific regions of the extracellular or intracellular regions of CD28 (i.e., the B7 binding domain or the signaling motifs YMNM or PYAP) via antibodies, small molecule mimetics or siRNAs could potentially alter some aspects of CD28 effects whereas leaving others intact. This is beginning to be realized with the approval and marketing of a humanized version of CTLA4Ig (abatacept, Orencia™) for the treatment of rheumatoid arthritis in adults and Stills disease in children (Genovese et al. 2005) and is being explored for other conditions (Linsley and Nadler 2009; Podojil and Miller 2009). Second generation agents have shown promise in the prevention of transplant rejection (Vincenti et al. 2005). The potential for the manipulation of costimulatory signals as an approach for cancer immunotherapy is currently an area of active study (Waldmann and Morris 2006). Although the exact mechanism of action of CTLA4Ig and related compounds is not fully understood these agents have promise for providing greater specificity in the manipulation of the immune response with less toxicity than many currently used drugs.

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