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CUTTING EDGE

Cutting Edge: CD28-Mediated Transcriptional and Posttranscriptional Regulation of IL-2 Expression Are Controlled through Different Signaling Pathways[1]

Mariano Sanchez-Lockhart, Elides Marin,* Beth Graf,* Ryo Abe,† Yohsuke Harada,† Caitlin E. Sedwick,[2]* and Jim Miller[3]**

***Despite the clear functional importance of CD28 costimulation, the signaling pathways transduced through CD28 have remained controversial. PI3K was identified early as a candidate for CD28 signaling, but conflicting data during the past decade has left the role of PI3K unresolved. In this report, we have resolved this controversy. We show that mutation of the PI3K interaction site in the cytosolic tail of CD28 site disrupts the ability of CD28 to recruit protein kinase C-θ to the central supramolecular activation cluster (c-SMAC) region of the immunological synapse, promote NF-κB nuclear translocation, and enhance IL-2 gene transcription. In contrast, mutation of the PI3K interaction site had no effect on the ability of CD28 to enhance IL-2 mRNA stability. These results suggest that two distinct pathways mediate CD28-induced up-regulation of IL-2 expression, a PI3K-dependent pathway that may function through the immunological synapse to enhance IL-2 transcription and a PI3K-independent pathway that induces IL-2 mRNA stability. The Journal of Immunology,* 2004, 173: 7120–7124.**

CD28 has long been recognized as an important costimulatory molecule that can impact on many events in T cell activation, differentiation, and tolerance. However, the biochemical signaling pathways induced downstream of CD28 are still not completely understood (1, 2). It is thought that the first event in CD28 activation is recruitment of Lck to the proline-rich region at aa 187–192 and subsequent phosphorylation of Y170. Phosphorylation at Y170 creates SH2-binding motifs that can interact with the adapter proteins, Grb-2 and GADS, or with the p85 subunit of PI3K. Activation of PI3K results in the phosphorylation of inositol lipids that serve as plasma membrane docking sites for PH domain-containing proteins, including Akt, Vav, and Itk (3). PI3K is an attractive candidate for the signaling node that CD28 uses to couple with diverse biochemical pathways, because downstream signals generated from these three PH domain-containing proteins have been associated with many of the functional effects of CD28 costimulation. However, the role of PI3K in CD28 signaling has been controversial. In murine tumor cell lines, disruption of the PI3K interaction site in CD28 inhibited IL-2 production, but this was not the case in transfected Jurkat cells. This discrepancy was thought to be resolved when Jurkat cells were found to lack both PTEN and SHIP-1, phosphatases that inactivate the products of PI3K. However, reconstitution of CD28-deficient normal murine T cells with mutated forms of CD28 that fail to activate PI3K demonstrated a significant effect on CD28-mediated up-regulation of the T cell survival factor Bcl-x_L, but only a limited defect in IL-2 expression (4–7). Thus, the role of PI3K in CD28 costimulation has remained unresolved.

One potential site of signal integration between the TCR and CD28 is within the spatial organization of cell surface receptors and associated signaling components within the immunological synapse (IS)[4] (8). The central supramolecular activation cluster (c-SMAC), is enriched for TCR, CD4, CD28, and a minor fraction of CD45 along with associated signaling proteins such as protein kinase C-θ (PKCθ) and Lck, whereas the peripheral region, p-SMAC, contains LFA-1 and associated cytoskeletal components, such as talin. The colocalization of TCR and CD28 within the c-SMAC might reflect the optimal site where both signaling pathways merge. In accordance with this, recruitment of PKCθ to the c-SMAC requires both TCR and CD28 signals (C. E. Sedwick and J. Miller, unpublished data, and Ref. 9). In the absence of CD28 costimulation, PKCθ is recruited to the IS, but it is diffusely distributed across the IS and is not focused into the c-SMAC.

In this report, we demonstrate that CD28 costimulation can induce two separate pathways that can independently impact

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[4] Abbreviations used in this paper: IS, immunological synapse; c-SMAC, central supramolecular activation cluster; p-SMAC, peripheral region SMAC; PKCθ, protein kinase C-θ; WT, wild type; KO, knockout; ARE, AU-rich elements.

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on IL-2 secretion. CD28-mediated activation of PI3K induces recruitment of PKCθ to the c-SMAC region of the IS, nuclear localization of NF-κB, and up-regulation of IL-2 transcription. Disruption of the CD28 PI3K-interaction site, however, has little effect on IL-2 secretion, because CD28 also initiates a PI3K-independent pathway that mediates stabilization of IL-2 mRNA. It is the confluence of these two independent signaling pathways, PI3K-dependent transcriptional regulation and PI3K-independent posttranscriptional regulation, that transduce CD28-mediated costimulation of IL-2 production.

Materials and Methods

Cells

6132 Pro cell transfectants expressing class II (I-A^d) in combination with B7-1 (ProA^d-B7) or ICAM-1 and B7-1 (ProA^d-ICAM-B7) and purification of CD4-positive T cells from DO11.10 TCR-transgenic mice have been described (10). The class II-positive B cell line, A20, that expresses ICAM and B7, as well as other potential costimulatory ligands for T cells, was obtained from American Type Culture Collection (Manassas, VA). All cell lines were maintained in DMEM (Invitrogen Life Technologies, Gaithersburg, MD) supplemented with 10% FCS, 2 mM glutamine, 0.1 mM nonessential amino acids, 40 μg/ml gentamicin, and 50 μM 2-ME. CD28-deficient T cells were stimulated with Ag and 20 U/ml recombinant human IL-2 and 2–3 days later were transduced with retroviruses containing wild-type (WT), TM, N172A, and M173L mutant murine CD28 cDNA clones (11). The viruses contain GFP expressed form an internal ribosome entry site as a marker for transduced cells. Infection efficiency ranged from 10 to 60%, and in experiments where bulk responses were measured (e.g., IL-2 expression), the different populations of T cells were adjusted to the same percentage of CD28$^+$ cells by the addition of CD28 knockout (KO) T cells.

Immunofluorescence microscopy

Peptide-pulsed APC were centrifuged with T cells for 20 s at a relative centrifugal force of 2000 $\times$ *g*. The cell pellet was incubated for 5 min at 37°C, resuspended in DMEM, and plated on poly-L-lysine-coated coverslips for 3 min at 37°C. Cells were fixed in 3% (w/v) paraformaldehyde, permeabilized in 0.3% (v/v) Triton X-100, and stained with primary (Santa Cruz Biotech, Santa Cruz, CA) and secondary Abs (Jackson ImmunoResearch Laboratories, West Grove, PA). For NF-κB localization, the incubation time was increased to 30 min, either in the cell pellet or after plating on coverslips, and nuclei were labeled with Hoechst stain. Samples were analyzed on a Zeiss Axiovert microscope controlled by SlideBook software (Intelligent Imaging Innovations, Denver, CO).

Real time RT-PCR

Activated and retrovirally transduced T cells (1×10^6) were stimulated with Ag presented by ProA^d-ICAM-B7 cells for 5 h, and further IL-2 transcription was inhibited by the addition of 0.5 μg/ml cyclosporine A (Calbiochem, San Diego, CA). Cyclosporine A rapidly inhibits NFAT-dependent IL-2 transcription without affecting IL-2 mRNA stability (12–14). Total RNA was isolated using TRIzol (Invitrogen Life Technologies), and the level of IL-2 mRNA was determined by normalization to a T cell-specific gene, CD3δ, using the $\Delta\Delta C_T$ method for relative quantitation with IL-2 mRNA levels in the absence of Ag as the calibrator. TaqMan probe and primers for IL-2 and CD3δ were obtained from Applied Biosystems (Foster City, CA).

Results

The PI3K-binding site is required for CD28-mediated recruitment of PKCθ to the c-SMAC

To identify the signals that mediate CD28-dependent localization of PKCθ within the IS, we analyzed two point mutations within the YMNM motif of the cytosolic tail of CD28. Mutation of the asparagine at aa 172 to alanine (N172A) disrupts binding of the SH2 domain of Grb-2/GADS but does not affect recruitment of PI3K, whereas mutation of the methionine at aa 173 to leucine (M173L) eliminates the PI3K docking site, without affecting recruitment of Grb-2/GADS (11). Retroviral reconstitution of CD28-deficient T cells with WT CD28, N172A, or M173L resulted in equivalent levels of CD28 surface expression as detected from the endogenous gene in CD28$^{+/+}$ DO11.10 T cells (data not shown). The cytosolic tail truncation of CD28 (TM) was typically expressed at lower levels, even when the GFP expression was equivalent, suggesting that this mutated protein may be somewhat less stable. To avoid any functional redundancy provided by other costimulatory molecule(s), we first analyzed PKCθ recruitment in T cell conjugates with a transfected cell line that expresses I-A^d and B7-1 (ProAd-B7). Stimulation through the TCR alone is not sufficient to recruit PKCθ to the IS (15), as is seen in T cells that cannot signal through CD28 (CD28KO or TM in Fig. 1). Coengagement of the TCR and WT CD28 specifically drives PKCθ to the c-SMAC (DO and WT in Fig. 1, *A* and B). Mutation of the Grb-2/GADS-binding site (N172A) had no apparent effect on PKCθ targeting. In contrast, mutation of the PI3K binding site (M173L) significantly reduced the ability of CD28 to direct PKCθ recruitment to the IS.

To determine the role of CD28 in the context of other costimulatory molecules, experiments were performed using the B cell line, A20, as APC (Fig. 1, *C* and *D*). In this case, other costimulatory molecules, such as LFA-1, can mediate PKCθ recruitment to the IS, but CD28 costimulation is required to direct PKCθ to the c-SMAC (DO and WT in Fig. 1, *C* and *D*). In the absence of CD28 costimulation, PKCθ remains broadly distributed and is not focused into the c-SMAC (KO and TM in Fig. 1*C*). Mutation of the Grb-2/GADS binding site (N172A) did not disrupt CD28-mediated focusing of PKCθ. In contrast, CD28 signaling in the absence of PI3K recruitment (M173L) was unable to direct the localization of PKCθ to the c-SMAC region, even though PKCθ was recruited to the IS. The lack of PKCθ recruitment observed with this mutant cannot be explained by defective distribution of CD28, because M173L was normally localized to the c-SMAC (data not shown). Taken together, these results indicate that the ability of CD28 costimulation to both recruit PKCθ from the cytosol to the IS and to focus the localization of PKCθ to the c-SMAC region is dependent on its ability to activate PI3K.

Nuclear translocation of NF-κB and IL-2 secretion is impaired in M173L-expressing T cells

To determine whether the M173L mutation impacts on PCKθ signaling, we assayed for nuclear localization of NF-κB (Fig. 2). Costimulation mediated through the cytosolic tail of CD28 was required for efficient activation of NF-κB, given that T cells expressing WT CD28, but not CD28KO and TM induced nuclear localization of NF-κB. This function was mediated largely through PI3K, because T cells expressing M173L, but not N172A, had a defect in NF-κB activation. Similar results were obtained when ProAd-B7 (Fig. 2, *A* and *B*) or A20 cells were used as APC (Fig. 2*C*). These results confirm the importance of CD28 costimulation in activation of NF-κB and indicate that this function of CD28 is mediated primarily through activation of PI3K.

To assess whether this defect in NF-κB nuclear translocation resulted in a loss in IL-2 transcription, WT and CD28KO, DO11.10 TCR-transgenic mice were crossed to mice containing a luciferase transgene under the control of the murine IL-2 enhancer (13, 16). The results clearly demonstrated an ability of CD28 to enhance IL-2 transcription and this effect was abrogated by disruption of the PI3K interaction site (M173L in Fig. 3*A*). In contrast, the ability of CD28 to recruit Grb-2/GADS is not required for IL-2 transcription (N172A in Fig. 3*A*). Taken

together, these results suggest that one pathway of CD28 costimulation is mediated through the activation of PI3K and results in the recruitment of PKCθ to the c-SMAC region of the IS, activation of NF-κB, and the up-regulation of IL-2 transcription.

Luciferase transcription promoted by M173L-expressing T cells was higher than that observed with CD28KO or TM T cells, consistent with a low level of PKCθ recruitment (Fig. 1) and NF-κB nuclear localization (Fig. 2) in M173L-expressing T cells. This observation suggests that there is a PI3K-independent pathway that contributes to CD28-mediated up-regulation of IL-2 transcription and that may also be mediated through PKCθ and NF-κB. Nevertheless, the major pathway of CD28-mediated up-regulation of IL-2 transcription appears to be transduced through the recruitment of PI3K.

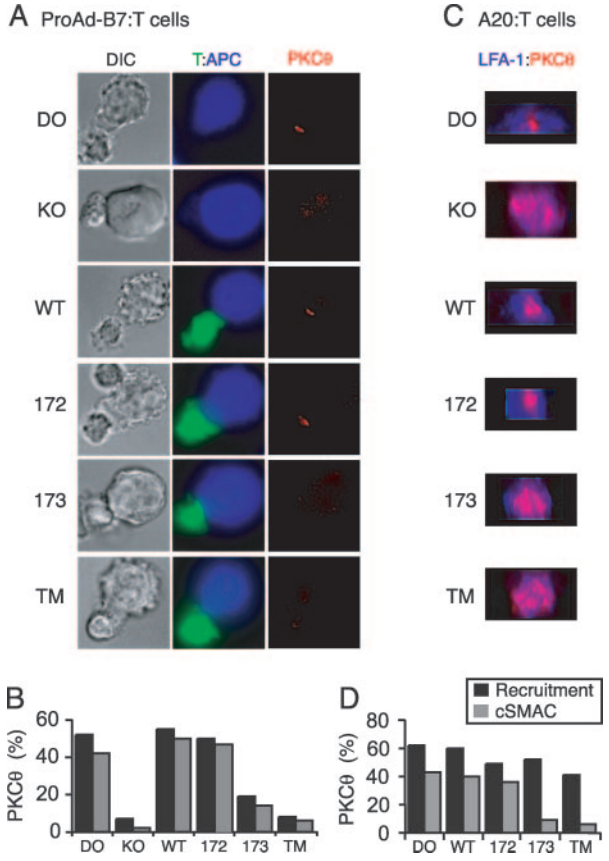

FIGURE 1. Mutation of the PI3K interaction site abrogates the ability of CD28 to recruit PKCθ to the c-SMAC. *A* and *B,* Conjugates between CD28$^{+/+}$ (DO), CD28$^{-/-}$ (KO), or retrovirally transduced CD28KO T cells expressing WT CD28 or the N172A, M173L, or TM mutations and Ag-pulsed ProAd-B7 transfectants were stained for PKCθ (*red*). APC were prelabel with CMAC (*blue*) and retrovirally transduced T cells were GFP positive (*green*). Representative images (*A*) and the frequency of PKCθ recruitment to the IS (*black bars*) or PKCθ focusing to the c-SMAC region (*gray bars*) (*B*; $n = 80$–100 conjugates) are shown. DIC, differential interference contrast. *C* and *D,* T cell conjugates with Ag-pulsed A20 cells were stained for PKCθ (*red*) and LFA-1 (*blue*). Three-dimensional reconstructions and interface projections of the IS for representative images (*A*) and the frequency of PKCθ recruitment to the IS (*black bars*) or PKCθ focusing to the c-SMAC region (*gray bars*) (*B*; $n = 80$–100 conjugates) are shown. PKCθ is recruited to the IS in T cell:A20 conjugates regardless of the presence or absence of CD28 signaling. However, in the absence of CD28 PKCθ is broadly distributed across the IS, and in the presence of CD28 it is focused into the c-SMAC region. This function of CD28 is lost in the M173L mutation.

Mutation of the PI3K interaction site does not diminish the ability of CD28 to promote IL-2 mRNA stabilization

Surprisingly, despite the defect observed in CD28 enhancement of IL-2 transcription, mutation of the PI3K interaction site had little effect on CD28-mediated up-regulation of IL-2 secretion (Fig. 3*B*). This discrepancy between levels of transcription and secretion suggests that M173L is inducing IL-2 mRNA stability. In the absence of CD28 costimulation, IL-2 mRNA rapidly decays with a half-life of ~15 min (CD28KO or TM in Fig. 4). When costimulation is provided by CD28, the half-life increases to ~2 h (DO or WT in Fig. 4). Importantly, the M173L mutation does not impair the posttranscriptional stabilization of IL-2 mRNA that is mediated by CD28. The ability of M173L to induce IL-2 mRNA stability, coupled with TCR-induced IL-2 transcription probably accounts for normal levels of IL-2 secretion in T cells expressing CD28 mutants that cannot activate PI3K (Fig. 3*B*) (4–7). Thus, CD28 can induce a second signaling pathway that is independent of its ability to activate PI3K and this pathway leads to induction of IL-2 mRNA stability.

Discussion

The results reported here support the idea that there are multiple and distinct pathways of CD28 costimulation. Phosphorylation of Y170 leads to the recruitment of PI3K, c-SMAC localization of PKCθ, and activation of NF-κB. This results in the

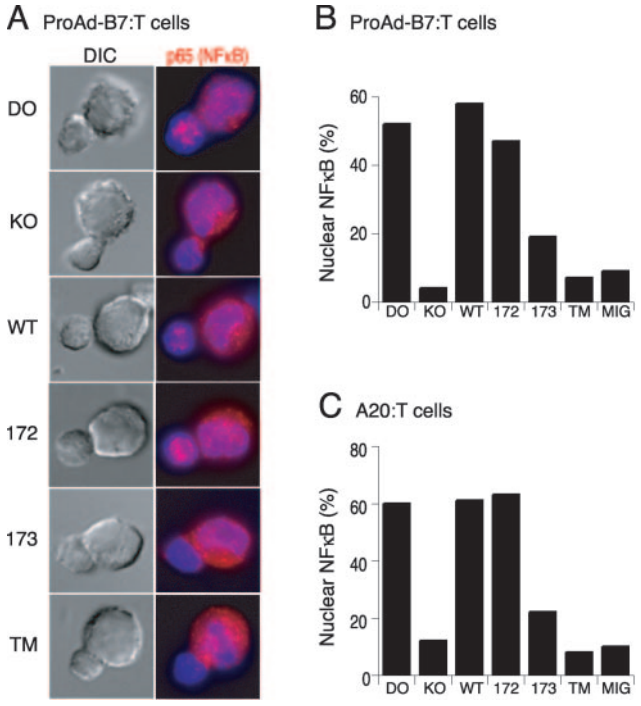

FIGURE 2. Mutation of the PI3K interaction site abrogates the ability of CD28 to drive nuclear localization of NF-κB. *A* and *B,* T cell:ProAd-B7 conjugates were stained for p65 NF-κB (*red*) and nuclei were stained with Hoechst dye (*blue*). T cells are located toward the lower left in each conjugate. Nuclear localization of NF-κB is evident in T cells expressing WT CD28 (DO and WT) and N172A by the appearance of purple staining in the T cell nuclei. The APC constitutively expresses nuclear NF-κB. Representative images (*A*) and the frequency of nuclear localization of NF-κB (*B*; $n = 90$–100) are shown. *C,* T cell:A20 conjugates were scored for the frequency of nuclear localization of NF-κB ($n = 30$–40). MIG, Cells expressing MIGR1 retroviral vector alone.

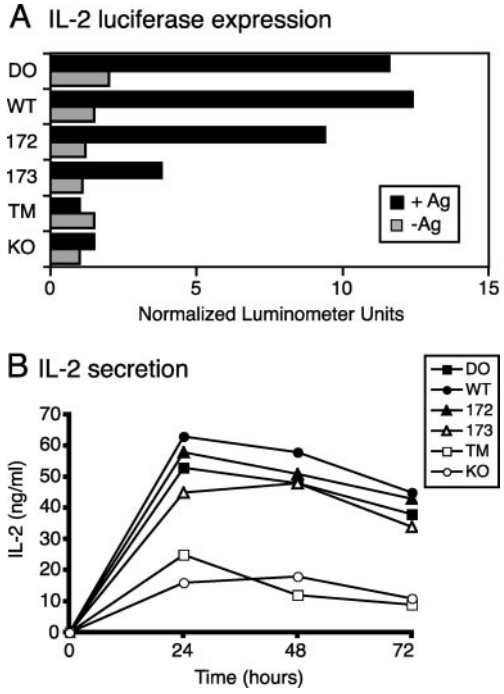

FIGURE 3. The ability of CD28 to promote IL-2 transcription, but not IL-2 secretion, is dependent on PI3K interaction. *A,* IL-2-LUC transgenic T cells were normalized for the percentage of $CD28^+$ T cells and stimulated with ProAd-B7 in the presence or absence of Ag for 24 h. Luciferase activity is shown as fold increase over background for one experiment representative of two. *B,* T cells were normalized for the percentage of $CD28^+$ T cells and stimulated with Ag presented by ProAd-B7 in the presence of excess exogenous human IL-2 to inhibit consumption. Secreted mouse IL-2 was measured by species-specific capture ELISA. This is one representative experiment of three.

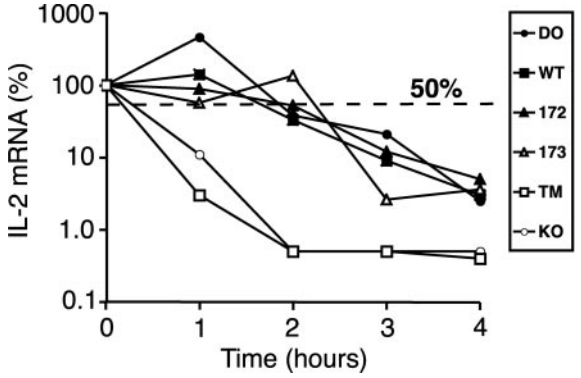

FIGURE 4. CD28-induced mRNA stability is not dependent on PI3K interaction. T cells were normalized for the percentage of $CD28^+$ T cells and stimulated with Ag presented by ProAd-ICAM-B7 cells. After 5 h, cyclosporine was added to block IL-2 transcription, and the levels of IL-2 mRNA were determined at various times by real time RT-PCR. This is one experiment representative of three.

transcriptional up-regulation of a variety of target genes, including IL-2 and Bcl-x_L. Importantly, other signaling pathways emanating from CD28, such as Grb-2/GADS or Lck, cannot compensate for the lack of PI3K activation. For genes such as Bcl-x_L, that depend on CD28-mediated transcriptional regulation, mutation of the PI3K activation site leads to a significant loss in expression (4, 6). In contrast, IL-2 secretion is not affected, because a second, PI3K-independent pathway of CD28 costimulation can lead to IL-2 mRNA stability. This pathway is mediated through the cytosolic tail of CD28 but is not dependent on recruitment of PI3K or Grb-2/GADS. Our preliminary data suggest that the IL-2 mRNA stabilization pathway may be saturated by the very high levels of IL-2 mRNA initially induced after CD28 costimulation. This may account in part for the minimal effect of M173L on IL-2 secretion, despite its large effect on IL-2 transcription. Taken together, our results have resolved a long standing controversy in the literature regarding the role of PI3K in CD28-mediated costimulation, demonstrating that it plays an important role in CD28-mediated up-regulation of IL-2 transcription, but is not required for CD28-mediated induction of IL-2 mRNA stability.

Signal integration between the TCR and CD28-mediated activation of PI3K is likely to occur within the context of the IS. TCR and CD28 are colocalized within the c-SMAC and cooperate to recruit PKCθ to the same region. Activation of PI3K leads to the generation of the D3 phosphoinositides, which are restricted to the inner leaf of the membrane and limited to lateral diffusion from the site of generation. This raises the possibility that these lipid mediators may contribute to the organization of the domains within the IS. Recent studies using pleckstrin homology domain-GFP fusion proteins have localized the product of PI3K, phosphatidylinositol 3,4,5-trisphosphate, to the IS (17–19). In the absence of CD28 costimulation, PKCθ is still recruited to the IS but is not concentrated into the c-SMAC region. Under these conditions, downstream signaling from PKCθ, as detected by NF-κB activation and up-regulation of IL-2 transcription, is attenuated. This correlation between PKCθ localization to the c-SMAC and PKCθ function suggests that the major function of CD28 in this pathway may be to recruit PKCθ to the c-SMAC, allowing for PKCθ interaction with upstream regulators, such as Lck (20), or downstream activators of NF-κB, such as the Bcl10/Malt1/Carma complex (21, 22).

In contrast to the ability of CD28 to induce IL-2 transcription, the ability of CD28 to promote IL-2 mRNA stability is independent of CD28-mediated recruitment of PI3K. The stability of IL-2 mRNA is controlled by the presence of AU-rich elements (ARE) in the 3′-untranslated region (23, 24). The current model is that constitutive ARE-binding proteins, such as tristetraprolin, recruit a multicomponent exosome that degrades the associated mRNA molecule. The signals that interfere with exosome recruitment and induce mRNA stabilization are not well understood. Stabilization of some transcripts has been linked to the p38 MAPK pathway and loss of tristetraprolin binding, through either phosphorylation or competition with non-exosome recruiting ARE-binding proteins, such as HuR. In contrast, IL-2 mRNA stability is not mediated through p38 MAPK and rather appears to be controlled by JNK (14). However, the JNK response element maps to the 5′ end of IL-2 mRNA, so JNK may not have a direct effect on ARE-binding proteins (14, 25). T cell activation leads to an increase in expression of tristetraprolin and HuR (26), but HuR does not recognize the specific AU-rich region in the IL-2 mRNA and another AU-binding protein, NF90, has been implicated in signal-dependent IL-2 mRNA stabilization (27). JNK has been implicated as a potential site for signal integration between TCR and CD28 (28), but JNK1/JNK2 double KOs have little defect in T cell activation (29). If JNK functions primarily in CD28-mediated IL-2 mRNA stabilization and not IL-2 transcription, then, analogous to this report for disruption of the

PI3K/IL-2 transcription pathway, selective abrogation of the JNK/IL-2 mRNA stabilization pathway may still allow for functional levels of IL-2 secretion.

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