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Mitogenic CD28 Signals Require the Exchange Factor Vav1 to Enhance TCR Signaling at the SLP-76-Vav-Itk Signalosome[1]

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Almost all physiological T cell responses require costimulation—engagement of the clonotypic TCR with MHC/Ag and CD28 by its ligands CD80/86. Whether CD28 provides signals that are qualitatively unique or quantitatively amplify TCR signaling is poorly understood. In this study, we use superagonistic CD28 Abs, which induce T cell proliferation without TCR coligation, to determine how CD28 contributes to mitogenic responses. We show that mitogenic CD28 signals require but do not activate the proximal TCR components TCRζ and Zap-70 kinase. In cell lines lacking proximal TCR signaling, an early defect in the CD28 pathway is in phosphorylation of the adaptor molecule SLP-76, which we show is essential for recruitment of the exchange factor Vav leading to Ca^{2+} flux and IL-2 production. Point mutations in CD28 that result in diminished Vav phosphorylation also result in defective Ca^{2+} flux, IL-2 production, and Tec-kinase phosphorylation. Using Vav1-deficient mice, we further demonstrate the importance of Vav1 for efficient proliferation, IL-2 production, and Ca^{2+} flux. Our results indicate that CD28 signals feed into the TCR signaling pathway at the level of the SLP-76 signalosome. *The Journal of Immunology,* **2007, 178: 1363–1371.**

Almost all physiological T cell responses require costimulation (1). Ag specificity is provided by recognition of cognate peptide in the context of appropriate MHC by the clonotypic TCR, whereas the interaction of CD28 with its natural ligands CD80/86 provides the most important costimulatory stimulus for activation of naive T cells (2–4). Although the signals transduced by CD28 have been extensively studied, the mechanisms of TCR and CD28 signal integration are poorly understood. In particular, it is not clear whether CD28 contributes qualitatively unique signals, separate and independent from TCR signaling (5–8), or signals that quantitatively amplify TCR signaling (3, 9–11), or both (12).

We have previously generated two functionally distinct classes of CD28 Abs (13, 14). Conventional CD28 Abs recognize an epitope adjacent to the CD80/86 binding site, and like natural ligands induce proliferative responses only when combined with TCR stimulation. By contrast, superagonistic CD28 Abs recognize a membrane proximal epitope and fully activate primary peripheral T cells in the absence of TCR ligation (13, 14). Stimulation with

such Abs thus provides an exception to the costimulatory requirement of T cell responses, and a unique tool to define CD28-specific events leading to mitogenic responses.

Mitogenic CD28 signaling can also be induced after stimulation with the recombinant natural ligand CD80 (15). However, such activation only occurs if the physiologically monovalent CD28 extracellular region is replaced with a bivalent receptor such as the extracellular domains of CTLA-4. Ligation of such bivalent CD28 chimeras leads to an artificially stable complex in a receptor lattice structure resulting in mitogenic responses. Studies of binding stoichiometry, as well as crystallographic evidence suggests that superagonistic CD28 Abs similarly induce the formation of a receptor lattice structure (15, 16). Taken together, these results suggest that CD28 superagonists induce a physiologically relevant, although dysregulated signaling pathway at the extremity of CD28 costimulatory signaling, in the same way that ligation with CD3 Abs or high-avidity lymphocytic choriomeningitis virus Ags induce particularly strong, and therefore costimulatory independent signaling at the extremity of the TCR pathway (1, 17). In this study, we have used superagonistic Abs to dissect CD28 signals at the extremity of costimulatory signaling, and thus in the absence of signaling events induced by TCR ligation.

We have previously shown that stimulation of primary T cells with CD28 superagonists induces phosphorylation of the exchange factor Vav and adaptor SLP-76, activation of phospholipase C (PLCγ)[6] leading to delayed but sustained Ca^{2+} flux, and activation of the PKCθ-NF-κB pathway (18). Surprisingly, these events occur in the absence of inducible phosphorylation of the proximal TCR signaling components TCRζ, Zap-70 kinase, and linker for activation of T cells (LAT), suggesting that CD28 can contribute autonomous and qualitatively unique mitogenic signals (13, 18, 19). Using a genetic approach, we show in this study that this is not the case. Mitogenic CD28 signaling is not autonomous but dependent on constitutive proximal TCR signaling. We demonstrate that

[6] Abbreviations used in this paper: PLC, phospholipase C; LAT, linker for activation of T cell; WT, wild type; PKB, protein kinase B.

constitutive TCR signals are required for CD28-mediated phosphorylation of the adaptor SLP-76, and that CD28 signals feed into the TCR signaling pathway at the level of the SLP-76 signalosome, with Vav1 as an essential component of this complex.

Materials and Methods

Abs, cell lines, and constructs

Generation of Abs to rat $\alpha\beta$TCR (R73), conventional (JJ319), and superagonistic (JJ316) Abs to rat CD28 have been described previously (14, 20). Superagonistic (5.11A1) and conventional (7.3B6) Abs to human CD28 (13) was provided by TeGenero or purchased (CD28.2) (BD Biosciences). Mouse anti-human TCR Vβ8 (16G8), anti-human CD3 (UCHT1), anti-human Itk (2F12), and anti-phosphotyrosine (4G10) were obtained from BD Biosciences. Mouse anti-human Zap-70 (3.3.1) (18) was provided by Dr. A. D. Beyers (University of Stellenbosch, Matieland, South Africa), and polyclonal anti-CD3ε Ab was obtained from DakoCytomation. Rabbit anti-Vav (C-14), mouse anti-TCRζ (6B10.2), and anti-SLP-76 (F-7) were obtained from Santa Cruz Biotechnology, and rabbit anti-phospho-Akt(S473), rabbit anti-phospho-PLCγ1(Y783), and rabbit anti-PLCγ1 were from Cell Signaling Technology. Sheep anti-mouse IgG was from Roche, and rat anti-mouse IgG, goat anti-mouse IgG-peroxidase, and goat anti-rabbit IgG-peroxidase were from Dianova.

Jurkat P116 and J14 cell lines (21) were provided by Drs. R. T. Abraham (The Burnham Institute, La Jolla, CA) and B. Schraven (Institute of Immunology, Magdeburg, Germany), Jurkat E6.1 and TCR-deficient JRT3-T3.5 were provided by Dr. W. Kolanus (University of Munich, Munich, Germany), and TCRζ-deficient 3A9 cells were obtained from Dr. D. Vignali (St. Jude Children's Research Hospital, Memphis, TN). The TCR$^-$ T cell hybridoma cell line 58, which expresses low levels of endogenous CD28, was described previously (13, 22). TCR-negative 58 cells expressing chimeric CD25-TCRζ (23) were obtained from Dr. E. Vivier (Centre d'Immunologie, Marseille-Luminy, France). Kinase-defective human Zap-70 (Zap-70 D461N KD in pSRa) from Dr. F. Michel (Institut Pasteur, Paris, France) and SLP-76 wild-type (WT) and Y3F constructs (24) provided by Dr. G. Koretzky (University of Pennsylvania, Philadelphia, PA) and Dr. B. Schraven were cloned into the retroviral vector pczCFG5IEGN. Chimeric CD8-CD3ε (25) from Dr. J. Sancho (Instituto de Parasitologia y Biomedicina, Granada, Spain) was cloned into the EcoRI/BamHI site of pczCFGIZ. NKT Vα14 TCR invariant and $\gamma\delta$TCR in pczCFG5IEGN/IZ/IEGZ were provided by Dr. T. Herrmann (Institute for Virology and Immunobiology, Würzburg, Germany).

The generation of chimeric CD28 comprising the extracellular region of rat CD28 and the transmembrane and intracellular regions of mouse CD28 in pczCFG5IZ, and full-length human CD28 in pczCFG5IZ have been described previously, as has transduction of TCR-negative 58 cells with chimeric rat/mouse CD28 and with myelin basic protein-specific $\alpha\beta$TCR (13, 26). The CD28 tailless mutant was generated by PCR adding a stop codon at amino acid position 165. The Δ25 mutant was generated by PCR with the chimeric rat/mouse CD28 construct as template by adding a stop codon at position 176. All point mutants were generated using the QuickChange kit (Stratagene) according to the manufacturer's instructions, with the chimeric construct serving as template (used primers on request). All constructs were verified by sequencing and cloned into the EcoRI/BamHI site of pczCFG5IZ. Constructs in retroviral vectors were transduced into 58 or Jurkat cells as described previously (13).

Stimulation, proliferation assays, FACS analysis, immunoprecipitation, and Western blotting

Spleen and lymph nodes were homogenized through nylon cell strainers to obtain single-cell suspensions. CD4$^+$ T cells were isolated using a mixture of hybridoma supernatants against CD8 (53-6.73), CD19 (1D3), MHC class II (2G9), and NK1.1 (4D11) for 25 min on ice, and cells were then washed twice in cold PBS. BioMag anti-rat IgG coupled to magnetic beads (Qiagen) was added for 25 min at 4°C, and CD4$^+$ T cells were collected by negative selection using magnetic cell separation. Primary cells were stimulated as described previously (18). Flow cytometric data were collected on light scatter-gated live cells using CellQuest software. Cytoplasmic Ca^{2+} levels were measured as described previously (18) using a FACSVantage flow cytometer and CellQuest and FloJo software (BD Biosciences). Stimulation of cells for biochemical analysis, immunoprecipitation, and Western blotting were as described previously (18).

Generation of CD28 transgenic mice

The chimeric rat/mouse CD28 construct was cloned into the SalI/NotI site of the SVA$^+$ Vector, which harbors the human CD2 minigene including a
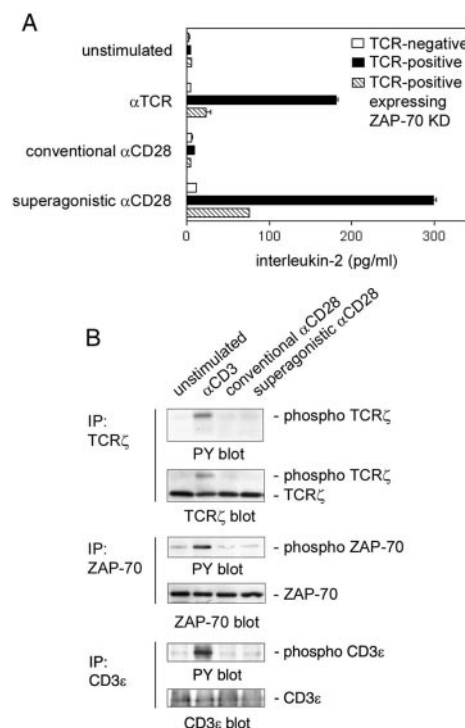

FIGURE 1. Mitogenic signals through CD28 require but do not activate proximal TCR signaling components. *A*, Fifty-eight cells expressing CD28 were reconstituted or not with $\alpha\beta$TCR of defined specificity, and TCR$^+$ cells were additionally transduced with dominant-negative, kinase-defective Zap-70. Cells were stimulated for 2 days with the indicated rat specific Abs, and IL-2 production was measured by ELISA. After stimulation with PMA/ionomycin, the three cell lines produced 511 ± 4, 538 ± 15, and 449 ± 3 pg/ml IL-2, respectively. *B*, Jurkat E6.1 cells were stimulated with the indicated human-specific Abs before secondary cross-linking, lysis, and precipitation of TCRζ, Zap-70, or CD3ε. Samples were resolved by SDS-PAGE, Western blotted, and membranes were probed with phosphotyrosine, TCRζ, Zap-70, or CD3ε-specific Abs as indicated.

locus control region (27). After linearization and elimination of the prokaryotic part of the vector, transgenic mice were generated using standard techniques by oocyte injection into C57BL/6 × DBA2 F2 oocytes. Five founder mice were identified that expressed comparably high levels of the transgene— ~10-fold higher compared with endogeneous CD28 as determined by FACS analysis (data not shown). Chimeric CD28 was expressed on virtually all T cells, with comparable expression on CD4$^+$ and CD8$^+$ T cells, and with lower expression levels on B cells, NK cells, and NKT cells (data not shown). A founder line was chosen, backcrossed four times to C57BL/6 mice, and subsequently crossed to Vav1$^{-/-}$ mice, which also were on the C57BL/6 background (28). All animal studies have been reviewed and approved by the local institutional review committee (Bezirksregierung Unterfranken).

Results

Mitogenic CD28 signals do not activate but require proximal TCR signaling components

We have previously demonstrated that stimulation of primary peripheral T cells with superagonistic CD28 Ab induces proliferation without phosphorylation of the proximal TCR signaling components TCRζ and Zap-70. Based on this result, we addressed whether proximal TCR signaling components are dispensable for T cell activation by CD28 superagonists (Fig. 1). TCR-deficient 58 cells, which express only low levels of endogenous CD28, were transduced with a chimeric CD28 construct comprising the rat extracellular domain and mouse transmembrane and intracellular domains (referred to hereafter as CD28 WT), and reconstituted or not with TCR$\alpha\beta$ of defined specificity (13, 26). TCR-positive and

-negative cells produced comparable IL-2 after stimulation with PMA/ionomycin, but only TCR-positive cells responded to CD28 superagonist stimulation (Fig. 1A). Importantly, none of the cell lines described in this study responded after stimulation with conventional CD28 Ab, which is analogous to ligation with the natural ligands CD80/CD86, indicating that these cell lines are not dysregulated in their costimulatory responses as has been described for other cell lines (29).

To further dissect this surprising requirement of proximal TCR signals for mitogenic CD28 signaling, we used kinase defective Zap-70 as a dominant-negative construct to block early TCR signaling. IL-2 production was again severely diminished in TCR-positive cells expressing kinase defective Zap-70 after CD28 superagonist stimulation (Fig. 1A). Given the specific role of Zap-70 in TCR signaling, and taking into account that even in unstimulated T cells there is some degree of TCRζ and Zap-70 phosphorylation as part of tonic signaling (30, 31), constitutive proximal TCR signals are essential for mitogenic CD28 signaling.

To extend these results beyond 58 cells and rat CD28, we made use of other cell lines stimulated with the recently generated anti-human CD28 superagonistic Ab (13). The same results were obtained using TCR-deficient lines JRT3-T3.5 and 3A9, as well as Jurkat cell lines either deficient in Zap-70 (P116) or expressing kinase defective Zap-70 (data not shown). Reconstitution of TCR-negative cells with γδTCR or NKT Vα14 TCR restored responses, indicating that TCR specificity plays no role in activation by CD28 superagonists (data not shown). However, reconstitution of TCR-negative cells with surrogate TCR receptors such as CD25-TCRζ (23) or CD8-CD3ε (25) restored responses upon TCR/CD3 chimera ligation but not after stimulation with CD28 superagonists, suggesting that integrity of the TCR/CD3 complex is required for mitogenic CD28 signaling (data not shown). More importantly, in WT JE6.1 Jurkat cells, stimulation with CD28 superagonist did not induce TCRζ, Zap-70, or CD3ε phosphorylation (Fig. 1B), confirming and extending our findings in primary cells. These results indicate that mitogenic CD28 signals require, but do not activate proximal TCR signaling.

Phosphorylation of the adaptor SLP-76 is required for IL-2 production and Ca^{2+} flux during mitogenic CD28 signaling

To define the defect that prevents mitogenic CD28 signaling in cells lacking proximal TCR signaling, we further analyzed membrane proximal signaling events. In primary T cells, superagonistic CD28 stimulation induces prominent phosphorylation of the adaptor molecule SLP-76 (18). This occurs in the absence of increased Zap-70 phosphorylation, suggesting that a kinase distinct from Zap-70 can phosphorylate SLP-76. To determine whether SLP-76 phosphorylation is defective in the CD28 pathway in cells lacking proximal TCR signaling, we compared responses in JRT3-T3.5 cells reconstituted or not with TCR (Fig. 2A). Because of the low levels of endogenous CD28 expression relative to that on Jurkat (JE6.1, P116, or J14) cells, these cells were transduced with human CD28 before TCR reconstitution. Stimulation with superagonistic CD28 Abs induced prominent SLP-76 phosphorylation in TCR-positive cells, which was severely diminished in TCR-negative cells. We also compared JE6.1 WT cells with cells expressing kinase defective Zap-70, or with P116 Jurkat cells that express neither Zap-70 nor Syk (21) (Fig. 2A). Stimulation with CD28 superagonist induced prominent SLP-76 phosphorylation in JE6.1 WT cells, but diminished phosphorylation in cells expressing kinase defective Zap-70. SLP-76 phosphorylation in response to CD28 superagonist was also severely diminished but still detectable in P116 cells, suggesting that a kinase apart from, and likely downstream of Zap-70 or Syk can phosphorylate SLP-76 (Fig.
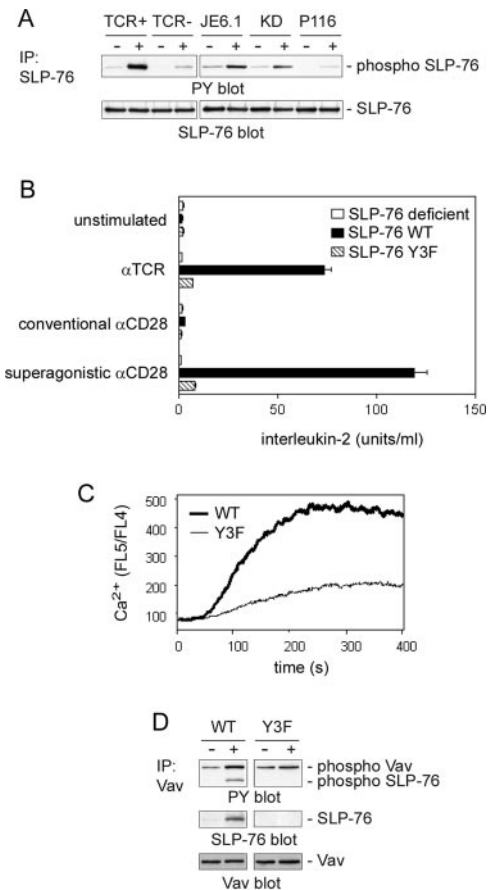

FIGURE 2. SLP-76 phosphorylation is required for Ca^{2+} flux and IL-2 production during mitogenic CD28 signaling. *A*, SLP-76 phosphorylation after superagonistic stimulation is defective in cells lacking proximal TCR signaling. JRT3-T3.5 cells transduced with human CD28 and reconstituted (+) or not (−) with TCR, Jurkat E6.1 WT, Zap-70 kinase-defective transduced (KD), and P116 Zap-70-deficient cells were stimulated with superagonistic anti-human CD28 Ab before secondary cross-linking, lysis, and precipitation of SLP-76. Western blotted membranes were probed with phosphotyrosine and SLP-76-specific Abs as indicated. *B*, SLP-76 phosphorylation is required for IL-2 production during mitogenic CD28 signaling. J14 SLP-76-deficient cells reconstituted with WT or mutated Y3F SLP-76 were stimulated with the indicated human-specific Abs, and IL-2 production was measured. After stimulation with PMA/ionomycin, SLP-76 WT and Y3F cells produced 82 ± 11 and 80 ± 3 U of IL-2, respectively. *C*, SLP-76 phosphorylation is required for Ca^{2+} flux. J14 cells reconstituted with SLP-76 WT or Y3F were loaded with Indo-1, stimulated with superagonistic anti-human CD28 Ab, and intracellular Ca^{2+} levels were measured over time. *D*, Assembly of the SLP-76-Vav signalosome is defective in SLP-76 Y3F cells. J14 SLP-76 WT and Y3F cells were stimulated with CD28 superagonist and Vav was precipitated. Membranes were probed with phosphotyrosine SLP-76 and Vav Abs as indicated.

2A). Regardless of the kinase responsible, SLP-76 phosphorylation after CD28 stimulation is defective in cells lacking proximal TCR signaling.

Next, we tested whether SLP-76 is indeed in the CD28 pathway by determining if defective phosphorylation of SLP-76 prevents mitogenic CD28 signaling. J14 SLP-76-deficient cells were reconstituted with either SLP-76 WT or a nonphosphorylatable SLP-76 Y3F mutant (24) at comparable levels. After CD28 superagonist stimulation, neither SLP-76-deficient nor SLP-76 Y3F cells produced IL-2, in contrast to SLP-76 WT cells which produced high levels of IL-2 (Fig. 2B). All three cell lines produced comparable

IL-2 after stimulation with PMA/ionomycin, and no IL-2 was produced after stimulation with conventional CD28 Ab. SLP-76 expression and phosphorylation are thus required for IL-2 production after CD28 superagonist stimulation.

A function of the three tyrosines mutated in the SLP-76 Y3F construct is to recruit the exchange factor Vav and Tec-family kinases, forming a multimolecular complex with PLCγ leading to its enzymatic activation (32). Generation of inositol triphosphate by PLCγ in turn results in Ca^{2+} flux, which we measure in this study as a sensitive assay of PLCγ activation. In primary cells superagonistic, but not conventional CD28 Abs, induce PLCγ phosphorylation and delayed but sustained Ca^{2+} flux (18). Similarly, CD28 superagonist stimulation induced delayed and sustained Ca^{2+} flux in SLP-76 WT cells, but only low levels of Ca^{2+} could be induced in SLP-76-deficient and SLP-76 Y3F cells (Fig. 2C and data not shown). To directly show that assembly of the SLP-76 signalosome is required for Ca^{2+} flux, the interaction of SLP-76 with Vav was examined. We precipitated Vav from SLP-76 WT and Y3F cells, and looked for coprecipitation of SLP-76 (Fig. 2D). Although Vav phosphorylation was induced in both cell lines after CD28 superagonist stimulation, SLP-76 coprecipitated with Vav in stimulated SLP-76 WT but not Y3F cells. Ca^{2+} flux thus requires SLP-76 phosphorylation and assembly of the SLP-76 signalosome, pointing to the crucial role of the SLP-76 signalosome for downstream events during mitogenic CD28 signaling.

Integrity of the Grb-2 binding site in CD28 is required for IL-2 production and Vav phosphorylation during mitogenic CD28 signaling in 58 TCR$^+$ and Jurkat cells

Next, we asked what proximal events occur at the level of the CD28 molecule itself. Because different regions of the cytoplasmic tail of CD28 have been implicated in CD28 signaling, we generated constructs in which either the whole cytoplasmic tail of CD28 (CD28 tailless) or the C-terminal region including the two proline-rich regions and three tyrosine residues were deleted leaving only the YMNM motif intact (CD28 Δ25) (Fig. 3A). Mutated constructs were expressed in 58 TCR$^+$ cells, which were stimulated either with anti-TCR, superagonistic, or conventional anti-CD28 Abs. As expected, 58 TCR$^+$ cells expressing the CD28 tailless mutant produced only low levels of IL-2 in response to CD28 superagonists, underscoring that IL-2 production in this system indeed relied on signaling events and not on adhesion as described in other systems (9, 33) (Fig. 3B). IL-2 production by CD28 Δ25 mutant cells was comparable with that from CD28 WT cells, demonstrating that in this system the YMNM motif is sufficient for mitogenic CD28 signaling (Fig. 3B), which we went on to dissect in the next step (Fig. 3, A and B). The Y at position 170 is important for the binding of different effector molecules. Mutation of this amino acid to F resulted in a considerable reduction in IL-2 production, further underscoring the importance of this motif for signaling in this system (Fig. 3B). Mutation of flanking amino acids leads to differential abolishment of binding to downstream effector molecules. Thus, the N residue at the +2 position is crucial for the binding of the adaptor molecules Grb-2 and GRID/Gads (34, 35), whereas the M residue at the +3 position is important for the binding of the p85 subunit of PI3K (36). Mutation at the +3 position resulted either in unchanged (CD28 M173A) or enhanced (M173L) IL-2 production compared with the CD28 WT cells, whereas the IL-2 production of CD28 N172A cells was found to be significantly reduced and comparable to the CD28 Y170F mutant (Fig. 3B and data not shown). Similar results were obtained with Jurkat cells expressing CD28 WT, N172A, M173A, and M173L (data not shown). Abolishment of binding of the p85 subunit of PI3K to CD28 173 mutants was tested by immunoprecipitation and Western blotting.
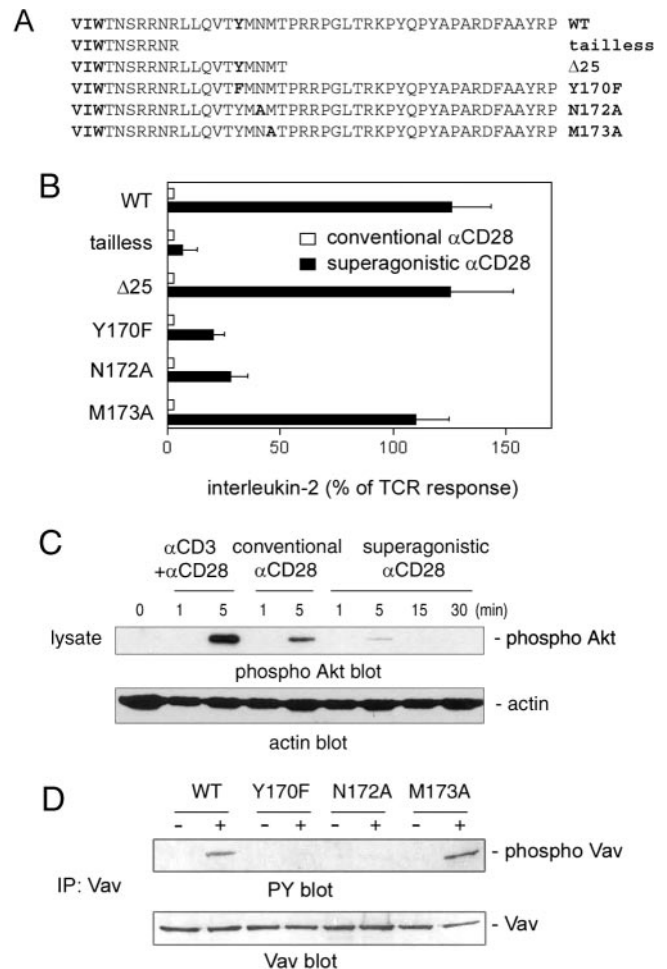

FIGURE 3. Vav phosphorylation requires integrity of the Grb-2 binding site and correlates with IL-2 production during mitogenic CD28 signaling. *A*, CD28 mutants used in this study. The C-termini of the transmembrane regions are in bold as is Y170 and point mutants in the YMNM motif. *B*, Integrity of the Grb-2 binding site is required for IL-2 production. TCR plus 58 cells expressing comparable levels of the WT, Y170F, N172A, or M173A CD28 were stimulated for 2 days with the indicated rat-specific Abs. IL-2 production measured by ELISA was normalized to that after TCR stimulation in each cell line. *C*, PKB phosphorylation is weaker after superagonistic CD28 stimulation compared with costimulation. Primary rat T cells were stimulated with anti-CD3 in combination with anti-CD28, a conventional anti-CD28 Ab (JJ319), or the CD28 superagonist (JJ316) for the indicated times followed by lysis and Western blot. Membranes were probed with a phosphotyrosine PKB or β-actin-specific Ab. *D*, Integrity of the Grb-2 binding site is required for CD28-specific Vav phosphorylation. TCR plus 58 cells expressing WT, Y170F, N172A, or M173A CD28 were stimulated with superagonistic rat CD28-specific Ab before secondary cross-linking, lysis, and precipitation of Vav. Western blotted membranes were probed with phosphotyrosine or Vav-specific Abs as indicated.

Confirming previous results (36), p85 could be immunoprecipitated together with CD28 from stimulated 58 TCR$^+$ cells either expressing CD28 WT or N172A, but only baseline levels could be detected in cells expressing CD28 M173L and M173A (data not shown). Therefore, the binding of PI3K to CD28 is not of major importance for IL-2 production in this cell line system, whereas the binding of adaptor molecule(s) at the +2 position is essential.

Protein kinase B (PKB) (Akt) is a major target of PI3K and its activation is one of the signaling pathways of CD28 costimulation (37). To investigate the importance of PI3K for superagonistic

CD28 signaling in more detail and in a physiologically more relevant system, we assessed PKB activation in primary rat T cells after superagonistic stimulation. PKB was phosphorylated within 5 min after costimulation and also after ligation with a conventional anti-CD28 Ab alone in line with previously reported results (38) (Fig. 3*C*). In contrast, PKB was only weakly phosphorylated 5 min after superagonistic stimulation, and phosphorylated PKB could not be detected at later time points ruling out delayed activation kinetics (Fig. 3*C*). Therefore, it seems that the PI3K/PKB pathway does not play a major role for CD28 superagonist signaling in bulk T cell preparations.

Vav is a major substrate of kinases activated during CD28 ligation (39), and such phosphorylation is dependent on the integrity of the Grb-2 binding site in CD28 (34). Because mutation of this site resulted in defective IL-2 production after superagonistic CD28 stimulation, we tested whether Vav phosphorylation would also be affected by this mutation. We found, indeed, that after stimulation Vav was prominently phosphorylated in CD28 WT, M173L, and M173A cells, but only low levels of phosphorylation could be detected in CD28 Y170F and N172A cells (Fig. 3*D* and data not shown). Importantly, phosphorylation of Vav after TCR ligation was comparable in CD28 WT and N172A cells (data not shown). Therefore, Vav phosphorylation in our system is a CD28-mediated event, which mirrors the IL-2 production in cell lines expressing mutated CD28 constructs.

Vav phosphorylation correlates with Ca²⁺ flux and Itk phosphorylation during mitogenic CD28 signaling

Although these data do not provide any new mechanism of how Vav is recruited to CD28, we used the CD28-mediated Vav phosphorylation to determine correlations with other signaling events. One of the functions of Vav is formation of a multimolecular complex with SLP-76 and the Tec-family kinase Itk leading to phosphorylation and activation of PLCγ (32). PLCγ phosphorylation was indeed severely reduced in CD28 N172A Jurkat cells in comparison to CD28 WT cells (Fig. 4*A*). Furthermore, we measured Ca^{2+} flux as a sensitive assay for PLCγ activation. Substantial Ca^{2+} flux was induced in CD28 WT, M173L and M173A Jurkat cells after superagonistic CD28 stimulation and was comparable in these cell lines, but it was severely defective in CD28 N172A cells (Fig. 4*B* and data not shown). Therefore, the CD28-mediated phosphorylation of Vav correlates with Ca^{2+} flux, an event indicative of PLCγ activation.

Given the fact that assembly of the SLP-76 signalosome is essential to induce Ca^{2+} flux and IL-2 production (Fig. 2), we defined which defective or missing component of the SLP-76 signalosome could cause subsequent defective responses observed in CD28 N172A cells. First, we determined whether assembly of the Vav-SLP-76 complex was defective in cells expressing CD28 N172A. Consistent with data using 58 TCR⁺ cells, Vav was not inducibly phosphorylated in Jurkat cells expressing CD28 N172A (Fig. 4*D*). However, SLP-76 was comparably phosphorylated in CD28 WT and N172A cells, and could be coprecipitated equally well with Vav from stimulated Jurkat cells expressing CD28 WT or N172A (Fig. 4, *C* and *D*). The recruitment of Vav to SLP-76 in CD28 N172A cells is therefore not the defective event upstream of PLCγ activation and Ca^{2+} flux.

Key to the activation of PLCγ is its phosphorylation by Zap-70/Syk- and Tec-family kinases (32, 40). Given that Zap-70 is not inducibly activated by superagonist CD28 stimulation, we determined whether Tec-family kinases could be activated and lead to PLCγ activation. Phosphorylation of Itk in stimulated Jurkat cells expressing CD28 WT or M173A was detectable (Fig. 4*E* and data not shown). However, no such phosphorylation could be detected in cells expressing CD28 N172A. The defective Vav phosphory-
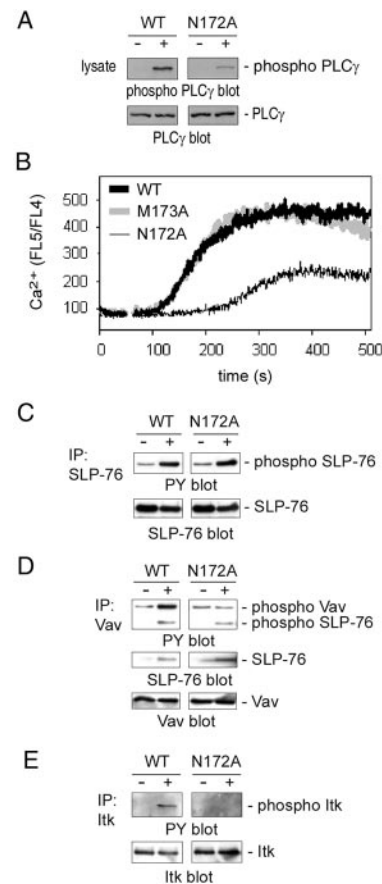

FIGURE 4. Vav phosphorylation correlates with PLCγ activation, Ca^{2+} flux, and Itk phosphorylation, but not SLP-76 phosphorylation during mitogenic CD28 signaling. *A*, The Grb-2 binding site is required for effective PLCγ activation. Jurkat E6.1 cells expressing WT or N172A CD28 were stimulated for 1 min with the superagonist, and PLCγ phosphorylation was investigated after lysis, blotting of whole protein lysates, and probing with a phospho-PLCγ-specific Ab. Defective PLCγ phosphorylation in CD28 N172A cells could also be observed at later time points (data not shown). *B*, The Grb-2 binding site is required for efficient Ca^{2+} flux. Ca^{2+} levels of Jurkat E6.1 cells expressing WT, N172A, or M173A CD28 were followed over time. *C*, SLP-76 phosphorylation does not require integrity of the Grb-2 binding site. Jurkat E6.1 cells expressing WT or N172A CD28 were stimulated with CD28 superagonist followed by secondary cross-linking, lysis, and precipitation of SLP-76. Western blotted membranes were probed with phosphotyrosine Ab or SLP-76-specific Ab as a loading control. *D*, Vav recruitment to SLP-76 does not require the integrity of the Grb-2 binding site. After stimulation of Jurkat E6.1 WT or N172A cells with superagonistic CD28 Ab Vav was precipitated. Membranes were probed with phosphotyrosine and SLP-76-specific Abs and a duplicate membrane with Vav-specific Ab to ensure comparable loading. *E*, Itk phosphorylation requires integrity of the Grb-2 binding site. After stimulation of Jurkat E6.1 WT or N172A cells with CD28 superagonist Itk was precipitated. Membranes were probed with phosphotyrosine Ab followed by Itk Ab to ensure comparable loading.

lation observed in N172A cells therefore correlates with defective Itk phosphorylation, PLCγ activation, and Ca^{2+} flux, and subsequently defective IL-2 production. These results are consistent with defective Itk phosphorylation and Ca^{2+} flux observed in Vav1⁻/⁻ thymocytes (41).

Vav1 expression is required for mitogenic CD28 signaling in primary peripheral T cells

To investigate whether the data presented above using cell lines are also relevant to activation of primary peripheral T cells, we
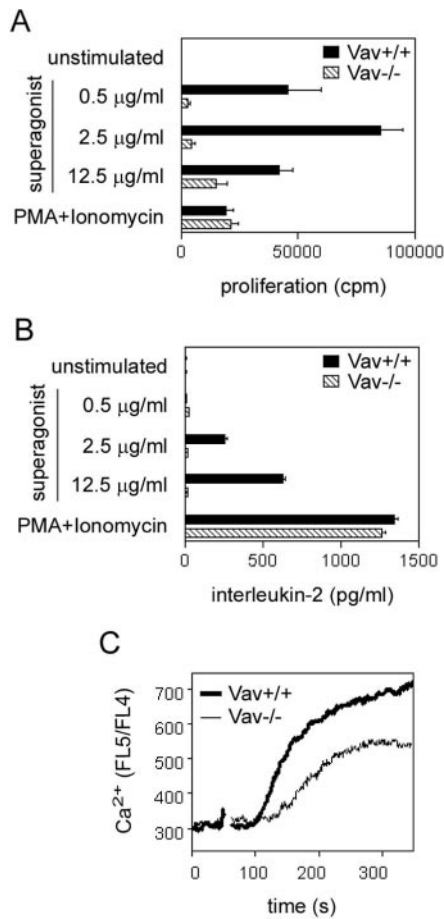
FIGURE 5. Vav1 is required for efficient proliferation, IL-2 production, and Ca^{2+} flux during mitogenic CD28 stimulation. *A* and *B*, Purified T cells from rat CD28 transgenic $Vav1^{-/-}$ or $Vav1^{+/+}$ cells were stimulated as indicated for 3 days. Proliferation as measured by [^{3}H]thymidine incorporation, and IL-2 production were determined. Stimulation with conventional CD28 Ab JJ319 did not induce proliferation or IL-2 production (data not shown). *C*, Ca^{2+} levels of purified T cells from rat CD28 transgenic $Vav1^{-/-}$ or $Vav1^{+/+}$ cells were followed over time. Stimulation with conventional CD28 Ab JJ319 did not induce Ca^{2+} flux in primary cells (data not shown).

exploited a transgenic mouse model. In these mice, the chimeric rat/mouse CD28 molecule is expressed at high levels in all T cells and at a slightly lower level in B cells and other lymphocyte populations (data not shown).

These mice were crossed with $Vav1^{-/-}$ mice to generate CD28tg $Vav1^{-/-}$ mice. After superagonistic CD28 stimulation, proliferation of peripheral T cells purified from these mice was severely reduced in contrast to cells from $Vav1^{+/-}$ and $Vav1^{+/+}$ CD28tg mice (Fig. 5*A* and data not shown). However, at higher concentrations of stimulating Abs significant proliferation of $Vav1^{-/-}$ cells was observed, suggesting either that there is a pathway independent of Vav, or functional compensation by Vav2/3. IL-2 production was also severely reduced, and Ca^{2+} flux was defective in CD28tg $Vav1^{-/-}$ cells (Fig. 5, *B* and *C*). Therefore, we were able to recapitulate the important features of experiments with cell lines expressing the CD28 N172A mutant, in which Vav phosphorylation was defective, using primary peripheral T cells from CD28tg $Vav^{-/-}$ mice. These results point to a crucial role of Vav in the pathways of mitogenic CD28 signaling leading to PLCγ activation and subsequently Ca^{2+} flux as well as proliferation and cytokine production.

Discussion

Despite extensive studies on signals provided by CD28, the mechanisms of TCR and CD28 signal integration during costimulation are poorly understood (9). In this study, we make use of a novel approach to define CD28-specific signals contributing to T cell activation by using superagonistic CD28 Abs. The advantage of this approach is that only one receptor is ligated resulting in a mitogenic signal, and that signals proceed without the interference of inducible TCR-specific signaling events (13, 18, 19). We show two previously unexpected results: 1) mitogenic CD28 signaling is not autonomous, but requires constitutive proximal TCR signaling; and 2) signal integration is a proximal event occurring at the SLP-76 signalosome, requiring expression of Vav1 and involving functional cooperation of Vav with the Tec-family kinase Itk.

A major question regarding the contribution of CD28 to costimulation is whether CD28 delivers qualitatively unique signals, autonomous and separate from TCR signaling (5–7), or signals that quantitatively amplify proximal TCR signaling (3, 9). Given that we trigger only CD28 with superagonists, we expected that CD28 would deliver qualitatively unique, autonomous signals in our system. Instead, we find that mitogenic CD28 signals are not autonomous but dependent on constitutive proximal TCR signaling. Both the expression of a functional TCR and the intact kinase activity of Zap-70 (but not the inducible activation thereof) are absolutely required and a prerequisite of superagonistic stimulation. We uncover an event that technically could not be shown by analysis of TCR and CD28 costimulatory signaling: the CD28-mediated phosphorylation of SLP-76, which is essential for mitogenic CD28 signaling. Our results indicate that mitogenic CD28 signals feed into the TCR signaling pathway at the level of the SLP-76 signalosome, delivering quantitative signals to amplify TCR signaling.

Given that Zap-70 and LAT are not inducibly phosphorylated during mitogenic CD28 signaling, it is unlikely that Zap-70 itself phosphorylates SLP-76 (18). Moreover, SLP-76 phosphorylation after CD28 ligation is detectable, although severely diminished, in P116 cells that express neither Zap-70 nor Syk. These results suggest that neither of these kinases is directly responsible, but that activation of the SLP-76 kinase is downstream of Zap-70/Syk activity, and hence dependent on proximal TCR signaling. The most likely candidates fall into the Tec-kinase family, whose activation is not only dependent on Syk-family kinases, but which typically have substrate specificity overlapping with that of Syk-family kinases (42). For example, Itk has recently been shown to phosphorylate LAT, and another Tec-family kinase Rlk/Txk phosphorylates SLP-76 in overexpression systems (43, 44). Both of these substrates were until recently thought to be specific Zap-70 substrates. Tec-family kinases have therefore been proposed to amplify Ag receptor signals by maintaining phosphorylation of substrates initially phosphorylated by Syk-family kinases (42). In thymocytes and Jurkat cells, constitutive or tonic TCR signaling occurs without the need for TCR engagement but requires expression of the adaptors LAT and SLP-76 (31). In this respect, the requirement of constitutive Zap-70 activity for superagonistic CD28 stimulation may be to create a nucleation site required for subsequent amplification. Low levels of SLP-76 phosphorylation by Zap-70 during constitutive signaling may be required for the recruitment of a kinase activated by superagonistic CD28 stimulation. Once recruited, this kinase could phosphorylate SLP-76 leading to a signal amplification loop. Although this remains speculative, it should be noted that a similar mechanism may be used during signaling through NKG2D-DAP10, which also induces SLP-76 phosphorylation in the absence of increased Syk-family kinase activation and

requires Vav1 expression (45, 46). Furthermore, increases in SLP-76 phosphorylation, which depend on CD28-signaling and not adhesion-dependent enhancement of Zap-70 activation, have been previously observed after costimulation (9). These data suggest that a CD28-dependent kinase phosphorylates SLP-76 during costimulation.

Using site-directed mutagenesis, we show that the proximal CD28 signals required for IL-2 production are mainly generated by the YMNM motif in the CD28 cytoplasmic tail. Interestingly, recent results have shown that transplanting the CD28-derived YMNM motif to either ICOS or CTLA-4, which both have a YxxM motif in their cytoplasmic tails and are therefore able to bind and activate PI3K, could restore CD28 function, especially IL-2 production (47–49). Mutation of the +3 residue, which is critically involved in the binding of the p85 subunit of PI3K, had no influence on IL-2 production in our cell line systems. The dysregulated turnover of D3 phosphoinositides in Jurkat and other cell lines (50) makes it impossible to stipulate whether PI3K is required for the generation of mitogenic CD28 signals in this system. In primary rat T cells the phosphorylation of PKB, a downstream target molecule of PI3K, was much weaker after superagonistic CD28 stimulation compared with costimulation and even ligation with a conventional anti-CD28 Ab, suggesting that the PI3K/PKB pathway is not of major importance for mitogenic CD28 signaling. What we can conclude is that integrity of the +2 residue of the YMNM motif is essential for mitogenic CD28 signaling, and correlates with phosphorylation of the exchange factor Vav.

PI3K was even implicated as negative regulatory factor in one study (36), based on results with the M173L mutation. We could reproduce the enhanced CD28 signaling and IL-2 production using cells harboring this mutation. One has to consider, however, that in this case a new motif, YxxL, has been generated that is known to bind preferentially Src family kinases. This could result in enhanced signaling effects without PI3K implication, and, in fact, in the M173A mutant these positive effects could not be reproduced.

Although Vav has long been appreciated as the major tyrosine kinase substrate induced by CD28 ligation, its role in CD28 signaling has not been clearly demonstrated (5, 7, 8, 51, 52). We show in this study using transgenic mice that Vav1 is essential for efficient proliferative responses, IL-2 production, and Ca^{2+} flux during mitogenic CD28 signaling. Our results contradict a study showing that Vav1 expression is not required for signaling through CD28 (53). This study, however, equated responses after stimulation with PMA and a conventional CD28 Ab with CD28-specific costimulatory responses, a questionable assumption given that Vav1 is upstream of PLCγ activation and generation of 1,2-diacylglycerol, the physiological equivalent of PMA.

The nature of the adaptor molecule(s) requiring asparagine at the +2 position of the YMNM motif remains unclear. Both Grb-2 (34, 36) and GRID/Gads (35) have been proposed, potentially bridging the YMNM motif to Vav phosphorylation. We could, however, not detect a direct association between CD28 and Grb-2 in immunoprecipitation experiments (data not shown). Very recently, however, it was shown by others that in the case of NKG2D-DAP10, which also contains a YxNM motif, both PI3K and a Grb-2/Vav1 intermediate are necessary and sufficient for phosphorylation of SLP-76, PLC-γ2, and cytotoxic function of NK cells (54). Therefore, it is tempting to speculate that the link between CD28 and Vav1 consists of a similar pathway. PP2A has been shown to bind to both CD28 and CTLA-4 also at the YXXM motif (55) and additionally was shown to be a key regulator of the stimulatory response using a bispecific tandem single-chain Fv ligand for CTLA-4 (56). Therefore, PP2A would also be a potential candidate for a link between CD28 and Vav. However, tyrosine phosphorylation of CD28 negatively regulates association with PP2A (55), and Vav was not phosphorylated in a situation where CD28 cannot be phosphorylated at Y170. For these reasons, it seems unlikely that PP2A is the linker between CD28 and Vav. Apart from the YMNM motif, other motifs in the CD28 cytoplasmic tail have been implicated in signaling (57–60). We could not provide evidence for the involvement of regions other than the YMNM motif, which may well be because we used cell lines and not primary cells, and that the primary read-out was restricted to IL-2 production.

The importance of Tec-family kinases for phosphorylation and activation of PLCγ leading to sustained Ca^{2+} flux has been demonstrated (40, 61, 62), and Tec-kinase family members have previously been implicated in CD28 signaling (9, 29, 63). In thymocytes, Itk and Tec kinase phosphorylation and activation is dependent on expression of the exchange factor Vav1 (41). Given that Ca^{2+} flux correlates with Vav phosphorylation during mitogenic CD28 signaling, we asked whether Itk could also be involved in PLCγ activation. We show that Vav and Itk phosphorylation are indeed correlated, both being dependent on the integrity of the +2 residue in the YMNM motif. These results, together with the data from others (41), strongly suggest functional cooperation of Vav and Itk during mitogenic CD28 signaling.

In summary, from our results emerge the following picture. During mitogenic CD28 signaling, Vav is phosphorylated in a pathway that involves a Grb-2-like adaptor binding to the asparagine residue in the CD28 YMNM motif. In parallel, SLP-76 is phosphorylated by an unidentified kinase, which is likely to be downstream of Zap-70. Phosphorylation and activation of Itk correlates with Vav phosphorylation in that it is dependent on integrity of the Grb-2 binding site in CD28, and may depend on Vav expression and activation as demonstrated in double-positive thymocytes (41). These three components (phosphorylated SLP-76, Vav, and Itk) act in concert to transmit costimulatory signals leading to PLCγ activation, Ca^{2+} flux, and IL-2 production.

Lastly, induction of NF-κB and Bcl-X_L by superagonistic but not conventional CD28 Abs (13, 18, 19, 64) suggests that these events also result from CD28 and TCR signal integration, in direct contrast to recent reports (5, 7). Perhaps the simplest way to resolve these differences will be to analyze CD28-dependent effects in mice with defective proximal TCR signaling, such as SLP-76-deficient mice transgenically expressing SLP-76 Y3F (24). Finally, whether these pathways of mitogenic CD28 signaling are also relevant for apparently qualitatively unique effects such as IL-4 induction in memory T cells (65), IL-8 induction (7) or L-plastin phosphorylation (66) remains to be investigated.

Acknowledgments

Disclosures

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