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Mariano Sanchez-Lockhart, Minsoo Kim and Jim Miller

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Cutting Edge: A Role for Inside-Out Signaling in TCR Regulation of CD28 Ligand Binding

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Efficient T cell activation depends on the engagement of both TCR and CD28, although the molecular mechanisms that control this signal integration are not fully understood. Using fluorescence resonance energy transfer, we show that T cell activation can drive a reorientation of the cytosolic tails of the CD28 dimer. However, this is not mediated through CD28 ligand binding. Rather, TCR signaling itself mediates this conformation change in CD28. We also show that TCR signaling can induce CD28–ligand interactions. Although the CD28 dimer appears to bind ligand monovalently in solution, we show that both ligand binding sites are required to efficiently recruit CD28 to the immunological synapse. These results suggest, that analogous to the cross-talk from TCR that regulates integrin activation, TCR-initiated inside-out signaling may induce a conformational change to the extracellular domains of CD28, enabling ligand binding and initiating CD28 signaling. The Journal of Immunology, 2011, 187: 000–000.

Materials and Methods

Cells

CD4-positive lymph node T cells were purified by negative selection from wild-type (WT) or CD28-deficient (CD28KO) DO11.10 TCR transgenic mice, as previously described (16), and activated in vitro with OVA peptide 323–339 presented by irradiated BALB/c splenocytes and 10 U/ml recombinant human IL-2. The 6132 Pro cell transfectants expressing I-Ad, ICAM-1, and CD80 (6) were used as APCs in all experiments, except where transfectants expressing I-Ad and ICAM-1 were used as B7-negative APCs. Cells were maintained in DMEM (Invitrogen Life Tech) supplemented with 10% FCS, 2 mM glutamine, 0.1 mM nonessential amino acids, and 50 U/ml penicillin–streptomycin.

T cell transfection

Monomeric cyan fluorescent protein (CFP) and monomeric yellow fluorescent protein (YFP) N1 plasmids were described previously (17). Murine CD28 was mutated at Y104A, within the ligand binding site, by overlapping PCR, and the mutation was confirmed by DNA sequencing. Mouse and human WT CD28 and mouse Y104A CD28 were cloned upstream of the fluorescent proteins with a 4 aa linker (RSTG), to produce the CD28–monomeric CFP or CD28–monomeric YFP fusion proteins. For bimolecular fluorescence complementation, the monomeric variant of Venus or N-terminal (1–154) or C-terminal (155–238) fragments of Venus was cloned downstream of mouse WT or Y104A CD28. Previously activated T cells were rested for 7–14 d and electroporated using Amaxa Nucleofection (Lonza Cologne). Briefly, 4 μg plasmid DNA was transfected into 5 × 10^6 cells; the cells were rested for 5 h before analysis.

The online version of this article contains supplemental material.

Abbreviations used in this article: CD28KO, CD28-deficient; CFP, cyan fluorescent protein; FRET, fluorescence resonance energy transfer; WT, wild-type; YFP, yellow fluorescent protein.

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Reprint requests should be addressed to Dr. Jim Miller, Center for Vaccine Biology and Immunology, University of Rochester, 601 Elmwood Avenue, Box 609, Rochester, NY 14642. E-mail address: jim_miller@urmc.rochester.edu

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Fluorescence resonance energy transfer

For T cell:APC conjugates, APCs were preloaded with or without 2 μg/ml OVA peptide, centrifuged (20 s at 2000 × g) with T cells, incubated for 5 min at 37˚C, resuspended in complete media, and plated in α T dishes. For anti-CD3 cross-linking, T cells were incubated with 1 μg/ml biotinylated anti-CD3 (2C11; BD Biosciences) for 5 min at 37˚C, plated in α T dishes, and incubated for 10 min in 1 μg/ml streptavidin-Cy5 (Jackson Immunoresearch Laboratories). TCR signaling was inhibited by the addition of 5 μM PP2 (Axon, San Diego, CA). Live cell imaging was done at 37˚C on a Nikon E2000-E microscope with a 60× oil immersion objective, as described (17). CFP and YFP images were captured before and after YFP photobleaching. Image analysis was done within an optical segment of the plasma membrane. After subtraction of background, fluorescence resonance energy transfer (FRET) efficiency was calculated as: FRET Efficiency = [(CFPpost − CFPpre)/(CFPpre)*100.

CD28 localization

T cell:APC conjugates were formed as described above or in combination with 4.5-μ latex beads coated with 1 μg/ml anti-CD3 (2C11) or anti-MHC class I (34-2-12; BD Biosciences) and plated on poly-l-lysine–coated coverslips. Samples were fixed in 3% paraformaldehyde and were imaged at room temperature on a Zeiss Axiovert microscope with a 63× oil immersion objective. Conjugates were scored for recruitment of CD28 to the B7-positive cell and shown as percent of conjugates. For bimolecular fluorescence complementation, the efficiency of CD28 localization to the immunological synapse was determined on no-neighbor deconvolved images and represented as a ratio of Venus fluorescence within the immunological synapse over Venus fluorescence outside the immunological synapse.

Results and Discussion

To address the molecular events associated with CD28 activation, we have established a FRET-based assay to detect changes in the orientation of the cytosolic tail domains of CD28. Both CD28-CFP and CD28-YFP fusion proteins were expressed at the cell surface (Fig. 1A) and restored CD28 function in CD28KO T cells (Supplemental Fig. 1). Resting T cells coexpressing both CD28-CFP and CD28-YFP showed a high FRET efficiency (Fig. 1B). The level of energy transfer was independent of the overall levels and ratio of CFP and YFP fluorescence, indicating that FRET was likely mediated by intramolecular interactions within the CD28 homodimer, rather than by CD28 clustering (Supplemental Fig. 2A–D). Mouse and human CD28 do not efficiently form interspecies heterodimers (18). High FRET efficiency was detected in both mouse/mouse and human/human homodimers, but significantly reduced when mouse CD28-CFP and human CD28-YFP were coexpressed (Supplemental Fig. 2E). These results and the relatively high levels of FRET detected indicated that the C termini of the CD28 cytosolic tails are in proximity within the CD28 homodimer (Supplemental Fig. 2F).

Both CD28-CFP and CD28-YFP were efficiently recruited to the immunological synapse in T cell:APC conjugates (Fig. 1A). Strikingly, CD28 FRET efficiency was significantly reduced within the immunological synapse (Fig. 1B). These results indicate that some reorientation of the cytosolic tail domains within the CD28 homodimer takes place during T cell activation.

Surprisingly, the reduction of CD28 FRET was not dependent on CD28 ligand binding (Fig. 2). CD28-CFP and CD28-YFP were mutated within the CD28 ligand binding site (Y104A) (19, 20). When CD28 cannot bind ligand, it is neither recruited to nor excluded from the immunological synapse, so CD28 remains evenly distributed across the plasma membrane (Fig. 2A). Therefore, we measured CD28 FRET efficiency of CD28 that was present at the interface with the APC and within the pool of CD28 that was localized outside the contact region. FRET efficiency was significantly reduced even in the absence of ligand binding, but only within the pool of CD28 that was localized within the immunological synapse (Fig. 2B). These results were confirmed with WT T cells and B7-deficient APCs, where the change in CD28 FRET efficiency was dependent on Ag stimulation, but not CD28–B7 interactions (Fig. 2C). Taken together, these results suggest that the reduced FRET between the cytosolic tail domains in CD28 reflects a conformational change within the CD28 homodimer that is induced prior to CD28 ligand binding and signaling.

These data indicate that the reorientation of cytosolic tail domains within the CD28 homodimer is not mediated by CD28 ligand binding, but rather by some proximal signaling event associated with T cell activation. To address the role of TCR signaling, we activated T cells expressing CD28-CFP and CD28-YFP by anti-CD3 cross-linking. T cells stimulated with anti-CD3 showed a substantial decrease in FRET around the entire T cell plasma membrane (Fig. 2D). The difference in the distribution of FRET change within the plasma membrane of T cells stimulated by Ag presentation (Fig. 2B, 2C) or anti-CD3 cross-linking (Fig. 2D) could reflect differences in the magnitude or kinetics of TCR signaling or simply the delivery of polarized (APC) or nonpolarized TCR signals (anti-CD3 cross-linking). Inhibition of TCR signaling by
addition of the Src-family kinase inhibitor PP2 blocked the anti-CD3–induced reduction in CD28 FRET (Fig. 2D). These results indicate that the reorientation of the CD28 cytosolic tail domains detected during T cell activation can be mediated by TCR signaling.

The ability of TCR signaling to drive a change in the orientation of the cytosolic tail domains of CD28 is reminiscent of inside-out signaling that regulates integrin activation and ligand binding (1, 2). We and others have previously shown that inhibition of TCR signaling after a functional immunological synapse is formed results in a rapid loss of CD28 polarization from the synapse, even though the T cell:APC conjugate remains intact and CD28 ligands on the APC are still available for CD28 binding (14, 15). To determine whether TCR signaling could induce the initiation of CD28–ligand binding, we formed conjugates with B7-positive cells in the absence of Ag (Fig. 3). Under these conditions in the absence of any TCR signaling, CD28 is not recruited to the B7-positive cell, despite the availability of CD28 ligand (Fig. 3A). Strikingly, T cell activation with anti-CD3 coated beads induced the rapid redistribution of CD28 toward the B7-positive cell (Fig. 3A, 3B). Control anti-MHC class I–coated beads (Fig. 3B) and addition of anti-CD3–coated beads in the absence of an interacting cell had no effect on CD28 localization (not shown). Thus, TCR signaling at a localized site (anti-CD3–coated beads) can induce CD28–ligand interactions at a distal site (B7-positive cell), suggesting that TCR signaling can enhance CD28 ligand binding.

One potential mechanism whereby TCR signaling could impact CD28–ligand interaction is to alter the valency of CD28 ligand binding. CD28 is expressed as a disulfide-linked homodimer; however, biochemical and structural analysis of recombinant, soluble CD28 indicates that it can only interact with ligand monovalently (21, 22). Although both binding sites in the CD28 dimer are accessible to ligand, their orientation is thought to preclude simultaneous ligand binding, because of steric interference at the distal end of the ligand. However, when CD28 is expressed as a transmembrane protein, TCR inside-out signaling may transduce a conformational change to the ligand binding domains, enabling bivalent binding. To address the role of bivalent CD28 binding during recruitment of CD28 to the immunological synapse, we used bimolecular fluorescence complementation (23) to selectively label CD28 heterodimers that express only...
a single, functional ligand binding site. WT CD28 was fused to intact Venus, the N-terminal fragment of Venus (CD28-VN), or to the C-terminal fragment of Venus (CD28-VC). Coexpression of WT CD28-VN and WT CD28-VC results in acquisition of Venus fluorescence, and CD28-VN/CD28-VC dimers were efficiently recruited to the immunological synapse (Supplemental Fig. 3). These results indicate that reassembly of the fragments of Venus on the C terminus of CD28 does not interfere with CD28 expression, ligand binding, or synapse localization.

When CD28 Y104A-VN and Y104A-VC were coexpressed, the heterodimer reconstituted Venus fluorescence and was expressed at the plasma membrane, but this dimer does not have a ligand binding site and was not recruited to the immunological synapse (Fig. 4). To determine whether a single ligand binding site was sufficient to drive CD28 localization to the immunological synapse, T cells were cotransfected with WT CD28-VN and Y104A-VC. In this case, Venus fluorescence was reconstituted only in CD28 dimers that contained a single functional ligand binding site. Consistent with previous results (20), coexpression of WT CD28 did facilitate recruitment of Y104A CD28 to the immunological synapse. The selective labeling of CD28 dimers containing two, one, or no ligand binding sites, using bimolecular fluorescence complementation, allowed us to quantify the relative recruitment efficiency of these dimers. This analysis revealed a clear defect in the recruitment of dimers with only a single ligand binding site (Fig. 4). Thus, the presence of two functional ligand binding sites is critical for efficient recruitment of CD28 dimers to the immunological synapse.

In this report we show that TCR signaling can drive reorientation of the cytosolic tail domains of CD28 and induce CD28 ligand binding, suggesting that TCR may regulate bidirectional CD28 signaling. The ability of cell surface receptor signaling to dynamically regulate ligand binding of a different receptor is well established in the regulation of integrin activation (1, 2). For integrins, TCR-dependent activation of RAP-1 leads to recruitment of cytosolic proteins to the integrin cytosolic tails. This drives separation of the integrin cytosolic tails, which can be detected as a loss in FRET efficiency (24), and a corresponding change in the conformation of the extracellular domains that increases the affinity for ligand and allows for the induction of integrin signaling. Whether TCR-induced changes in the orientation of the CD28 cytosolic tail domains is also mediated through downstream signaling or some localized change within the plasma membrane is not known. Our results suggest that inside-out signaling from TCR may regulate both the primary adhesion molecule, LFA-1, and the primary costimulatory molecule, CD28, involved in T cell activation and provides a molecular paradigm to coordinate the sequential receptor activation events that control complex cellular processes.

We also show that CD28 depends on two functional ligand binding sites for efficient recruitment to the immunological synapse. Our results favor a model whereby TCR-induced inside-out signal through the CD28 cytosolic tail domains drives a corresponding conformational change in the extracellular domain of CD28 that facilitates bivalent ligand binding. Of interest, bivalent ligand binding to CD28 through superagonist mAb or CTLA-4/CD28 fusion proteins results in T cell activation in the absence of TCR engagement (25), raising the possibility that TCR can activate CD28 both to enhance ligand binding and to potentiate CD28 signaling. Although historically CD28 has been thought to modulate TCR signaling, our results support the idea that TCR signaling can also strongly regulate CD28 function. Under superoptimal situations, both TCR and CD28 alone can activate T cells, so both receptors can signal independently. Under normal situations, however, synergistic cross-talk between TCR and CD28 provides a mechanism for coincidence detection to regulate T cell activation and control the initiation of T cell immune responses.

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**Disclosures**

The authors have no financial conflicts of interest.

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