Spatiotemporal Regulation of T Cell Costimulation by TCR-CD28 Microclusters and Protein Kinase C θ Translocation

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SUMMARY

T cell activation is mediated by microclusters (MCs) containing T cell receptors (TCRs), kinases, and adaptors. Although TCR MCs translocate to form a central supramolecular activation cluster (cSMAC) of the immunological synapse at the interface of a T cell and an antigen-presenting cell, the role of MC translocation in T cell signaling remains unclear. Here, we found that the accumulation of MCs at cSMAC was important for T cell costimulation. Costimulatory receptor CD28 was initially recruited coordinately with TCR to MCs, and its signals were mediated through the assembly with the kinase PKCθ. The accumulation of MCs at the cSMAC was accompanied by the segregation of CD28 from the TCR, which resulted in the translocation of both CD28 and PKCθ to a spatially unique subregion of cSMAC. Thus, costimulation is mediated by the generation of a unique costimulatory compartment in the cSMAC via the dynamic regulation of MC translocation.

INTRODUCTION

T cells require two distinct signals for activation and differentiation: an antigen (Ag)-recognition signal transmitted through the T cell receptor (TCR) upon recognition of the Ag peptide-major histocompatibility complex (MHCp) on an antigen-presenting cell (APC), and a costimulatory signal transmitted through costimulatory receptors upon binding ligands expressed on APCs. Costimulatory signals are essential for the full activation of T cells for cytokine production, proliferation, survival, and functional differentiation. In order to induce proper activation of T cells through costimulation, expression of costimulatory receptors and their signals should be regulated in appropriate strength and timing in a dynamic and quantitative fashion. Modulation of costimulatory signals for T cell activation has been attempted in the clinic setting, including the modulation of autoimmune diseases, such as rheumatoid arthritis and psoriasis, prevention of graft versus host disease in transplantation, effective vaccinations, and augmented antitumor immunity (Riley and June, 2005; Salomon and Bluestone, 2001).

CD28, which is constitutively expressed on both naive and effector T cells, plays a predominant role in costimulation by binding its ligands, CD80 (B7-1) and CD86 (B7-2) (Acuto and Michel, 2003; Chambers et al., 2001; Rudd and Schneider, 2003; Sharpe and Freeman, 2002). CD86 is constitutively expressed at low amounts on most professional APCs and is rapidly upregulated after activation, whereas CD80 is inducibly expressed at a later time. Studies of genetically modified mice lacking CD28 or CD80/CD86 have confirmed that CD28 signals augment a variety of T cell functions, including cell-cycle progression, antiapoptotic function, cytokine production, T helper polarization, cytokotoxic T cell differentiation, and humoral immunity maturation. In addition to these direct functions, CD28-mediated signals upregulate other costimulatory, cytokine, and chemokine receptors, consequently inducing secondary costimulatory responses. Although CD28 downstream signals have been extensively analyzed for more than a decade, the precise mechanisms are not clearly understood. Many molecules, such as phosphoinositide 3-kinase (PI3K) (Harada et al., 2003; Pages et al., 1994), lymphocyte-specific protein tyrosine kinase (Lck) (Holdorf et al., 1999; Liu et al., 2000), growth factor receptor-bound protein 2 (Grb2) (Raab et al., 1995), Grb2-related adaptor protein (Gads) (Watanabe et al., 2006), IL2-inducible T cell kinase (Itk), the guanine-nucleotide exchange factor Vav (Villalba et al., 2000), protein kinase B (PKB) (also known as Akt) (Kane et al., 2001), protein phosphatase 2A (PP2A) (Alegre et al., 2001; Chuang et al., 2000), and protein kinase C θ (PKCθ) (Villalba et al., 2000), have been implicated in CD28-mediated costimulatory signals. However, because these molecules also function in TCR downstream signals, it is difficult to parse CD28-specific cascades qualitatively and quantitatively.
Immune responses are initiated by the communication between Ag-specific T cells and APCs. An immunological synapse (IS) is formed at the T cell-APC interface and is made up of a central supramolecular activation cluster (cSMAC) containing the TCR-CD3 complex and a peripheral SMAC (pSMAC) containing the integrin LFA-1 (Garakou et al., 1999; Monks et al., 1998). An IS is dynamically generated at the interface between a T cell and a B cell or a glass-supported planar bilayer, whereas there was a report describing a multifocal pattern of TCR, not cSMAC, between a T cell and a dendritic cell (DC) (Brossard et al., 2005). We have recently reported that T cell activation is spatiotemporally regulated by TCR microclusters (MCs) containing receptors, kinases, and adaptors (Bunnell et al., 2002; Campi et al., 2005; Saito and Yokosuka, 2006; Yokosuka et al., 2005). As the initial step of T cell activation, TCRs form small clusters at the interface between a T cell and an APC or a planar bilayer. Because these clusters contain both TCRs and phosphorylated proteins, and intracellular calcium concentrations are increased when a few MCs are generated, TCR MCs appear to function as signalosomes for T cell activation. As a subsequent step, only TCRs translocate to the center of the interface to result in cSMAC formation. TCR MCs containing kinases and adaptors are observed only at the peripheral edge of a T cell and not in cSMAC. These findings mainly from the planar bilayer system imply that TCR MCs are responsible for Ag recognition and the induction of activation signals both at initial contact and at a later stage for maintenance of activation, and that cSMACs are unable to sustain signaling (Varma et al., 2006). This concept of MCs serving as a key signalosome in T cell activation has raised important questions on how CD28-mediated costimulation is spatially and temporally regulated in the relationship with TCR MCs for the full activation of T cells.

CD28 was previously reported to traffic into the T cell-APC interface and to colocalize with PKCζ upon Ag recognition (Egen and Allison, 2002; Pentcheva-Hoang et al., 2004). Regarding the localization of CD28 in the IS, CD28 was reported to be localized at cSMAC (Bromley et al., 2001) or to be segregated from TCR in IS (Andres et al., 2004a; Huang et al., 2002; Tseng et al., 2005). Similar to TCR signals, CD28 signals were suggested to precede cSMAC formation because CD28 upregulated TCR-induced intracellular calcium within seconds after T cell-APC conjugation. It is also inferred that CD28 localized in lipid rafts and augments the recruitment of TCR downstream molecules to lipid rafts. However, the dynamic regulation of CD28 and its related signaling molecules is poorly understood.

We report here the results of molecular-imaging analysis of CD28-mediated costimulation. CD28 was recruited coordinately with TCR to MCs, and then PKCζ was recruited to TCR-CD28 MCs by association with CD28, thereby resulting in the initial activation of T cells. Furthermore, CD28 played a role in retaining PKCζ at a spatially unique and dynamic subregion of cSMAC, leading to sustained signals for T cell activation.

RESULTS

Cluster Formation of CD28 at TCR MCs and cSMACs
To examine the dynamic movement of CD28 on a live T cell, we visualized CD28 during T cell activation on a glass-supported planar bilayer by objective total internal reflection fluorescence microscopy (TIRFM) (Tokunaga et al., 1997). We performed retroviral infection to introduce EGFP-tagged CD28 (EGFP-CD28) into CD4+ T cells from AND TCR-transgenic (AND-Tg) (specific for moth cytochrome c 88–103 [MCC88-103] on I-E^d) CD28-deficient (CD28−/−) mice. The T cells were allowed to settle onto a planar bilayer containing glycosphatidylinositol (GPI)-anchored I-E^d loaded with MCC88-103, ICAM-1-, and CD80-GPI (I-E^d-ICAM-1-CD80) (Bromley et al., 2001; Grakoui et al., 1999), and EGFP-CD28 movement in the entire process of T cell-bilayer contact was imaged. The densities of I-E^d, ICAM-1, and CD80 were adjusted to equal those of lipopolysaccharide (LPS)-stimulated B cells, i.e., approximately 250, 100, and 60–120 molecules/μm², respectively (Figure 1A).

CD80/CD86 binding has been shown to enhance both T cell proliferation and IL-2 production as costimulatory functions. Because we previously demonstrated that CD80-GPI enhances IL-2 production by naive T cells on a planar bilayer (Bromley et al., 2001), we applied silica beads coated with the same I-E^d-ICAM-1-CD80-containing liposomes to stimulate T cells. AND-Tg naive T cells showed marked increases in IL-2 production, proliferation, and CD69 expression upon bead stimulation (Figures 1B–1D). Similar results were obtained with effector T cells (Figure S1, available online).

Similar to the clustering of TCR-CD3 (Campi et al., 2005; Yokosuka et al., 2005), CD28 was found to generate clusters at the T cell-bilayer interface as soon as a T cell attached, and the number of clusters increased during cell spreading. CD28 MC formation was completely dependent on the presence of CD80 (Figure 2A and Movies S1 and S2). After cSMAC formation, CD28 MCs continued to be generated at the periphery of the cell-bilayer interface and accumulated in the central region (Figure 2B and Movies S3 and S4). Then, we examined whether CD28 MCs move in a manner similar to TCR MCs, namely, colocalization initially with TCR MCs and later with cSMACs. Surprisingly, CD28 MCs colocalized with TCR MCs within the first few minutes of contact (Figure 2C). The average number of CD28 MCs is 136 ± 39.4 in ten representative cells at 2 min. Almost all of the CD28 MCs (93.9% ± 7.4%) initially overlapped with CD3. After cSMAC formation, a majority of CD28 translocated to the center but, unexpectedly, segregated from TCR; high-density regions of CD28 (CD28hi) and CD3 (CD3dim) segregated from each other, although the CD28hi region exhibited dim CD3 staining (CD3dim) and the CD3hi region exhibited dim CD3 staining (CD3dim) (Figures 2C and 2D). Compared to the cSMAC observed in the absence of CD28–CD80 interaction, CD3hi regions were less massive because CD28hi regions interfered with the formation of massive CD3hi regions. Collectively, CD28hi regions appeared to be localized at relatively outer regions of cSMACs. We also utilized a planar bilayer containing Cy5-labeled ICAM-1-GPI as a marker for pSMAC to investigate whether CD28hi regions localized in cSMAC or pSMAC. The ICAM-1 ring merged with LFA-1 and surrounded both CD28hi and CD28dim regions, indicating that these CD28 clusters are localized in a previously unexplored outer region of cSMAC (Figure 2E). Collectively, these data show that CD28 initially accumulates and localizes at TCR MCs upon Ag stimulation, and is later sorted to the outer region of cSMAC as a unique compartment in IS.
Ligand-Dependent but Signal-Independent Formation of CD28 Clusters

To examine whether particular downstream signals of CD28 are required for MC formation, we reconstituted various EGFP-tagged wild-type (WT) or mutant CD28 into AND-Tg Cd28^−/− /C0^−/− /C0^−/− T cells. Each EGFP-CD28 mutant contained a mutation in CD80/CD86 binding site (Y123A), dimerization (C142S), or PI3K, Grb2, and Gads binding site (Y189F), or a deletion of cytoplasmic 16 amino acids critical for lipid-raft association (D16) or the entire cytoplasmic tail (DCP) (Andres et al., 2004b; Holdorf et al., 1999; Pages et al., 1994; Schneider et al., 1995). The surface expression of all CD28 mutants was nearly equal on activated T cells (data not shown). We found no substantial differences in CD28 MC formation and distribution both at initial contact and after cSMAC formation among all CD28 mutants except Y123A. Y123A failed to form CD28 MCs, whereas even the CD28^DCP mutant formed MCs (Figure 3), indicating that CD28 MC formation depends on CD28-CD80 binding but not on downstream signals of CD28. Nonetheless, the CD28 cytoplasmic domain may still recruit or associate with specific signaling molecules for costimulation.

CD28 Cluster and Its Downstream Signals

We analyzed a set of candidate molecules that move and colocalize with CD28 on a planar bilayer. TCR-proximal signals were first analyzed by staining for phosphorylated CD3ζ (Figure S2) or the kinase Zap70 (data not shown), but their phosphorylation status was not altered by the presence of CD80-GPI (Herndon et al., 2001). Then, we analyzed PI3K as a likely candidate for mediating costimulation because PI3K is clearly demonstrated to bind to the “YMNM” motif of CD28 cytoplastic tail and to augment IL-2 production (Harada et al., 2003; Truitt et al., 1994). When AND-Tg T cells expressing EGFP-p55ζ as an indicator of type I PI3K were plated on an I-E^K-ICAM-1-iCD80 planar bilayer, p55ζ clusters were transiently observed at the entire cell-bilayer interface. However, they disappeared soon after moving toward the center and did not accumulate at cSMAC, similar to Zap70 and the adaptor SLP-76 (Yokosuka et al., 2005) (Figure S3A and data not shown). The presence of CD80-GPI on the planar bilayer resulted in a slight but significant enhancement of area (0.050 ± 0.026 μm^2 with CD80-GPI; 0.037 ± 0.024 μm^2 without CD80-GPI, p value < 0.001 with Student’s t test) and fluorescence intensity of EGFP-p55ζ clusters (0.282% ± 0.160% with CD80-GPI; 0.182% ± 0.109% without CD80-GPI, p < 0.001 with Student’s t test) (Figure S4), confirming that PI3K is recruited to and enriched at TCR-CD28 MCs through CD28-CD80 binding during initial activation. However, these movements of PI3K clusters did not simply correlate with the accumulation of CD28 at cSMAC. Two-color analysis of AND-TCR T cell hybridomas expressing both ECFP-CD28 and EYFP-p85ζ confirmed this result—the initial colocalization of PI3K with TCR-CD28 MCs and the dissociation from CD28 in cSMAC later (Figure S3B). Catalytic subunit p110ζ showed similar movement to p55ζ and p85ζ (data not shown). Furthermore, we analyzed cluster formation of Grb2, Gads, Vav1, and Itk in AND-Tg T cells expressing each EGFP fusion protein because these molecules have been suggested to functionally correlate with CD28 costimulation. However, we observed no accumulation at cSMACs regardless of the presence of CD80-GPI (data not shown).

CD28-Mediated Translocation of PKCθ to T Cell-APC Interface

We then analyzed PKCθ movement by using the assay described above, because PKCθ is activated after stimulation
through both TCR and CD28 (Berg-Brown et al., 2004; Pfeifhofer et al., 2003; Sun et al., 2000), is segregated in IS with CD28 costimulation, is recruited to lipid raft, and plays a critical role in NF-%kappa%B activation. AND-Tg T cells expressing ECFP-CD28 and EYFP-PKC\(\text{q}\) were conjugated with a dendritic cell line, DC-1, expressing I-E\(\kappa\), ICAM-1, and transfected CD80, and imaged by confocal microscopy. Histograms on right panels show fold fluorescence intensities of EGFPCD28 (green) and CD3 (red) on the diagonal yellow lines in DIC images. (D) Fluorescence intensities of CD3 and EGFPCD28 in CD28 (-) or CD3 (-) clusters in cSMAC or pSMAC (p) were compared to the intensities of the entire interface in the cell in (C) bottom two rows (n = 10). *, p value < 0.001 with Student’s t test. Scale bars represent 5 \(\mu\)m. A representative of three independent experiments is shown. (E) AND-Tg T cells expressing EGFP-CD11a (top) or EGFP-CD28 (bottom) were stained with Alexa Fluor 546-labeled anti-TCR\(\beta\) Fab and were plated on a planar bilayer containing I-E\(\kappa\), CD80, and Cy5-labeled ICAM-1 (prepulsed with MCC88-103). Cells were imaged real time by confocal microscopy 20 min after contact. Histograms on the right panels are described as in (C). Scale bars represent 5 \(\mu\)m. A representative of two independent experiments is shown.

We further analyzed the kinetics and distribution of PKC\(\text{q}\) by using a planar bilayer and AND-Tg T cells expressing EGFPCD28. PKC\(\text{q}\) clusters were transiently observed even in the absence of CD80-GPI (Figure 4D, top and Movie S5). In contrast, PKC\(\text{q}\) accumulated to form clear clusters and persisted on the bilayer with CD80-GPI at initial activation (Figure 4D, bottom and Movie S6). Furthermore, after cSMAC formation, PKC\(\text{q}\) clusters accumulated at the central region of the cell-bilayer interface in an annular form, which lasted for at least 60 min (Figure 4E and Movies S7 and S8). These data demonstrated that CD28-CD80 interaction enhanced the accumulation of PKC\(\text{q}\) both at MCs initially and at the central region of cell-bilayer interface after cSMAC formation.
Next, we examined whether PKC\(\theta\) clusters colocalized with TCR or CD28 during initial and sustained activation. Costaining of PKC\(\theta\) clusters with CD3 revealed that almost all PKC\(\theta\) clusters (95.2%, \(n = 1302\) in ten cells) merged with TCR MCs at initial contact (Figure 5A, top). After cSMAC formation, a majority of T cells (76.5%, \(n = 132\)) showed PKC\(\theta\) accumulation in the CD3\(^{\text{dim}}\) region in an annular form and exclusion from the CD3\(^{\text{hi}}\) region in cSMACs (Figure 5A, bottom and Figure 5B). Because this PKC\(\theta\) accumulation resembles that of CD28 clusters, we examined the colocalization of CD28 with PKC\(\theta\) by using AND-Tg Cd28\(^{-/-}\) T cells expressing both EYFP-PKC\(\theta\) and ECFP-CD28 on a planar bilayer. Depending on CD28-PKC\(\theta\) interaction, almost all cells (93.4%, \(n = 61\)) initially exhibited colocalization of PKC\(\theta\) with TCR-CDC28 MCs (Figures 5C and 5D, and Figure S5A). Thereafter, PKC\(\theta\) translocated to and accumulated at the center of the interface. After cSMAC formation, PKC\(\theta\) persistently localized with CD28 at the outer region of cSMAC by segregating from core CD3 (Figures 5E and 5F, and Figure S5B). A majority of the cells with the annular form of CD28 clusters (81.8%, \(n = 33\)) colocalized with both PKC\(\theta\) clusters and the CD3\(^{\text{dim}}\) region. As shown in Figure 2E, the annular form of PKC\(\theta\)-CD28 clusters settled in cSMACs (Figure 5G). PKC\(\theta\) and CD28 clusters in the annular form were also imaged at the interface between EGFP-PKC\(\theta\)-expressing AND-Tg T cells and DC-1, although the frequency was lower and the images were less clear compared to those in the planar bilayer, probably as a result of membrane ruffling and variety expression of costimulatory receptor ligands on APCs (Figure S6 and Movie S9).

To understand the mechanism of CD28-mediated PKC\(\theta\) recruitment, we examined the physical association between CD28 and PKC\(\theta\). Indeed, we found that PKC\(\theta\) was coprecipitated with CD28 in lysates from T cell hybridomas expressing both ECFP-CD28 and EYFP-PKC\(\theta\) (the same cells as those for imaging in Figures 4A and 4B) upon PMA stimulation (Figure 5H, Figure 3).
more importantly, this physical association was identified in both effector and naive T cells from mice upon PMA stimulation (Figure 5I and data not shown). The CD28-PKC\(_\text{q}\) assembly appears to be weak because the association was detectable only with mild detergent, such as digitonin (Figure S7). These results suggest that CD28 recruits PKC\(_\text{q}\) by forming the assembly, although there is no evidence of the direct interaction of CD28 with PKC\(_\text{q}\).

5C.C7-Tg T cells, whose Ag specificity is the same as that of AND-Tg T cells, generate smaller cSMACs because they have lower TCR affinity than AND-Tg T cells upon stimulation with the same concentrations of Ag on a planar bilayer. Under the condition in which cSMACs were not well formed by 5C.C7-Tg T cells with lower concentrations of MCC88-103 (1 and 0.1 \(\mu\)M), PKC\(_\text{q}\) still accumulated in an annular form (Figure S8). This result suggests that the annular accumulation of PKC\(_\text{q}\) clusters takes place without strong TCR-MHCp binding, which leads to clear cSMAC formation, but is dependent on CD28-CD80 binding.

Dynamic Maintenance of CD28-PKC\(_\text{q}\) Clusters at cSMAC

The critical role of CD28 in retaining PKC\(_\text{q}\) at cSMAC was confirmed by interrupting CD28-CD80 binding, via Ig fusion proteins of WT cytotoxic T lymphocyte antigen-4 (WT CTLA-4 Ig). The formation of both CD28 and PKC\(_\text{q}\) clusters in an annular form was diminished by the addition of WT CTLA-4 Ig but not Y139A (YA) CTLA-4, a binding mutant for CD80/CD86. The percentage of cells forming annular CD28 and PKC\(_\text{q}\) clusters was reduced from 87.2% to 32.4% and from 42.3% to zero, respectively (Figures 6A and 6B). Severe reduction of IL-2 production was observed when CD28-CD80 binding was blocked by WT CTLA-4 Ig at 2 min after stimulation, when only TCR-CD28-PKC\(_\text{q}\)-containing MCs were generated, as well as at 10 min after initial contact, in (E), and the interval between images is 10 s. Scale bars represent 5 \(\mu\)m. A representative of five independent experiments is shown. Real-time images in (D) and (E) are available in Movies S5–S8.
between T cells and I-Ek-ICAM-1-CD80 planar bilayers without MCC88-103. PKC(1) was mainly localized in cytosol without Ag stimulation; however, it efficiently translocated to CD28 clusters upon PMA stimulation (Figures 6D and 6E). These results suggest that CD28 clusters recruit and retain PKC(1) and induce the formation of the annular form of PKC(1) clusters.

TCR clusters in cSMAC were found to be relatively stable on the basis of a fluorescence recovery after photobleaching (FRAP) study (Grakoui et al., 1999). We investigated the dynamics of annular forms of CD28 and PKC(1) clusters in cSMAC by FRAP (Figures 6F and 6G and Movies S10 and S11). Indeed, both CD28 and PKC(1) showed rapid recovery of their clusters, indicating active maintenance of the structure and dynamic exchange within cSMAC. PKC(1) exhibited more rapid and complete recovery than CD28, probably as a result of its higher mobility as a cytoplasmic protein. These data suggest that the cytoplasmic tail of CD28 functions to dynamically recruit and concentrate PKC(1) by specific association, and they indicate that the annular form of PKC(1) clusters is dynamically generated and maintained by recruiting CD28-PKC(1) from peripheral TCR-CD28 MCs.

Furthermore, PKC(1) clusters in the annular form were stained with anti-phospho-PKC(1) at the outer region of cSMAC in a CD28-CD80-dependent manner in normal T cells, although a question on the antibody specificity has been raised (Lee et al., 2005) (Figure S9). The result suggests that PKC(1) that accumulated in the annular form was active in the induction of costimulatory signals.

Regulation of PKC(1) Translocation to TCR-CD28 MCs by CD28 Signals

To examine the requirement of the cytoplasmic tail of CD28 for PKC(1) translocation to TCR-CD28 MCs, we established a series of AND-TCR T cell hybridomas expressing EYFP-PKC(1) and various ECFP-CD28 mutants. First, we analyzed the translocation of PKC(1) to the T cell-APC interface by using DC-1 with or without CD80 expression (Figures 7A and 7B). WT CD28 exhibited colocalization with PKC(1) at the T cell-APC interface upon Ag stimulation 2 min and 30 min after conjugation. As in Figure 4A, 2 min after T cell-APC conjugation, the translocation of PKC(1) to the interface in T cells expressing CD28 with the mutated or deleted cytoplasmic tail (Y189F, Δ16, and ΔCP) was similar to that in T cells expressing WT CD28. In contrast, PKC(1) patches that colocalized with CD28 showed a decrease in number in T cells expressing CD28 with the mutated or deleted cytoplasmic tail (Y189F, Δ16, and ΔCP) 30 min after conjugation (Figure 7B).

The colocalization of CD28 with PKC(1) was further analyzed on the planar bilayer with AND-Tg Cd28<sup>−/−</sup> T cells (Figure 7C) and AND-TCR T cell hybridomas (Figure 7D and Figure S10). Whereas CD28 MCs were generated independently of the cytoplasmic tail of CD28, PKC(1) was not clearly recruited and did not form MCs if the cytoplasmic tail was mutated or deleted (Figure 7C and Figure S10). These data demonstrate that CD28 MC formation is independent of, but lasting recruitment of PKC(1) to CD28 MCs is completely dependent on, the cytoplasmic tail of CD28. Quantification of the number of AND-TCR T cell hybridomas exhibiting colocalization of PKC(1) clusters with WT or mutant CD28 clusters is shown in Figure 7D. Consistent with these imaging data, biochemical analysis revealed that the physical association between PKC(1)- and CD28-bearing cytoplasmic tail mutations was much weaker than that between PKC(1) and WT CD28 (Figure 5H). To clarify the function of the colocalization of CD28 with PKC(1) within the same TCR MCs, we reconstituted AND-Tg T cells from Cd28<sup>−/−</sup> mice with these CD28 mutants and stimulated with anti-CD28 plus PMA to analyze costimulatory function (Figure 7E). We noted a strong correlation among CD28-PKC(1) colocalization, physical association, and CD28-mediated costimulation, suggesting that CD28-mediated costimulation for the full activation of T cells is mediated by the recruitment of PKC(1) to TCR-CD28 MCs.

DISCUSSION

In this study, we illustrated the dynamic aspects of CD28-mediated costimulation in relation to TCR MCs. CD28 colocalizes with TCR to form TCR-CD28 MCs together with signaling molecules as signalosomes, where CD28 plays a critical role in recruiting PKC(1) to MCs at initial activation, and then to the outer region of cSMAC, possibly for sustained activation to exhibit effector functions. Previous studies have indicated that TCR MCs generated at the periphery of IS induce activation signals, whereas cSMAC may not be responsible for transducing activation signals, because phosphorylated proteins are detected almost in peripheral MCs and not in cSMAC (Yokosuka et al., 2005), and that Ca<sup>2+</sup> signaling is not sustained in the absence of peripheral MCs (Varma et al., 2006). However, the present result of CD28-PKC(1) signaling at the outer region of cSMAC leads to the idea that cSMAC also provides signal competency for costimulation and that different signal clusters are responsible for TCR-mediated and costimulatory signals for the maintenance of T cell activation.

The translocation of CD28 to the T cell-APC interface before cSMAC formation was previously reported (Andres et al., 2004), but we demonstrated that CD28 colocalizes with TCR and its downstream molecules as a TCR-CD28 MC. This colocalization of TCR with CD28 in the same MC coincides with the findings that CD28 shares most of the downstream molecules with TCR. We searched for a molecule that contributes specifically to CD28 signals by colocalizing initially with TCR MCs and then translocating to CD28 MCs at the outer region of cSMAC. Although CD28 signal enhanced the colocalization of PI3K with CD28, PI3K clusters differ from that of CD28. Finally, we found that PKC(1) met the criteria for the specific mediator of CD28 signals: accumulation at TCR-CD28 MCs initially and at cSMAC later.

PKC(1) is known as a downstream molecule of CD28. PKC(1)- and CD28-deficient animals revealed similar functional defects (Pleilhofer et al., 2003; Sun et al., 2000), although the physical interaction has not been shown. We provided evidence of the functional assembly between CD28 and PKC(1) in three aspects: (1) PKC(1) is contained within early activation signalosomes as TCR-CD28 MCs and translocates to cSMAC with CD28; (2) the physical association of CD28 with activated PKC(1) is demonstrated by coimmunoprecipitation; and (3) the early localization of PKC(1) at MCs and the late at cSMAC are functionally associated with IL-2 production. The similar regulation was observed in B cells; CD19 enhanced B cell functions through its colocalization with B cell receptors (BCRs) and Syk as BCR MCs (Depoil et al., 2008).
PKC\(\alpha\) has been considered as a marker for cSMAC during T cell activation (Monks et al., 1998), whereas CD28 was suggested to play a role in segregation of PKC\(\alpha\) in IS (Huang et al., 2002; Tseng et al., 2003). In addition to the finding of colocalization of CD28-PKC\(\alpha\) in TCR MCs, we identified a unique functional subregion of cSMAC where CD28 and PKC\(\alpha\) accumulated. The generation of this subregion appears to depend on both localization and density of CD28 because PKC\(\alpha\) was localized in CD28\(^q\) but not TCR-CD3\(^q\) region. Furthermore, CD28 cluster formation depends on the density of CD28 and CD80/CD86 on T cells and APCs, and on the avidity of TCR, through the competition between CD28-CD80/CD86 and TCR-MHCp (unpublished data). We propose a functional region of cSMAC, CD3\(^{dim}\)CD28\(^q\), as “costimulatory signalosome” for T cell activation. We demonstrated by FRAP analysis that no signal molecule translocated to the CD3\(^q\) region, and the CD3\(^{dim}\) region was more flexible and dynamically regulated than the CD3\(^q\) region (unpublished data). The CD3\(^{dim}\) region within cSMAC may be a negative regulatory compartment for T cell activation as suggested (Varma et al., 2006). The CD3\(^{dim}/CD3^q\) ratio appears to vary depending on the strength of TCR stimulation. A strong stimulus may induce more CD3\(^{dim}\) region for endocytosis or degradation of TCR complexes, whereas a weak stimulus may result in more CD3\(^{dim}\) regions that are susceptible to CD28-PKC\(\alpha\)-mediated costimulation. This is consistent with previous data suggesting negative regulatory functions of cSMAC by signal-strength-dependent TCR degradation (Cemerski et al., 2007). Our results suggest that cSMAC may inherit yin and yang functions for the regulation of T cell activation: endocytosis or degradation of the TCR complex for negative regulation through the CD3\(^q\) region, but CD28-PKC\(\alpha\) accumulation for sustained T cell signaling through the spatially distinct region.

Our data depicted both spatial and temporal differences in the dynamics of PKC\(\alpha\) and Zap70-SLP-76 for MC formation. PKC\(\alpha\) may be recruited to the plasma membrane via diacylglycerol (DAG) or in a phospholipase C-\(\gamma\)1 (PLC\(\gamma\)1)-DAG-independent manner (Villalba et al., 2002) and may be functionally activated by membrane translocation (Bi et al., 2001), whereas Zap70 and SLP-76 require phosphorylation for retention on the cell surface by associating with CD3 and LAT, respectively. Thus, PKC\(\alpha\) may associate with TCR-CD28 MCs via two steps—initial recruitment to the cell surface by DAG binding, followed by lateral movement to TCR-CD28 MCs through the association with CD28. The frequency of cells with PKC\(\alpha\) translocation in CD28 Y189F, PI3K-binding mutant was lower than that in \(\Delta 16\) mutant, in both T cell-APC and T cell-bilayer experiments, whereas stimulation of Y189F CD28-expressing cells with PMA plus anti-CD28 augmented the proliferation, suggesting the involvement of PI3K in PKC\(\alpha\) membrane translocation and the partial rescue of T cell response by PMA-mediated PKC\(\alpha\) relocation.

CD28-mediated membrane translocation and the colocalization of CD28 with PKC\(\alpha\) could be explained by several mechanisms including recruitment into lipid raft and DAG-gradient. However, we failed to obtain the evidence to support these ideas (see Supplemental Data). Moreover, cell adhesion by CD28 may contribute to the functional recruitment of PKC\(\alpha\) to CD28 clusters through cytoskeletal rearrangement (Kaga et al., 1998). In this context, because filamin-A was reported to translocate into IS, associate with actin and PKC\(\alpha\), and enhance IL-2 production in a CD28-CD80-dependent manner (Hayashi and Altman, 2006; Tavano et al., 2006), this mechanism might be involved in the colocalization of CD28 with PKC\(\alpha\).

During T cell activation, the functional enhancement of T cell responses by CD28-mediated costimulation is prominent for T cell activation with weak TCR stimulus. Imaging studies have suggested that CD28 supports IL-2 production if no cSMAC is generated (Purtic et al., 2005) or if APC has a low concentration of Ag or self-Ag (Wulfing et al., 2002). In relation to these studies, we observed that CD28-PKC\(\alpha\) clusters could be generated even if cSMAC were not clearly generated with lower-affinity TCR, suggesting further differential regulation of two spatially distinct signaling clusters: Peripheral MCs and annular clusters in cSMAC may lead to different signaling pathways, such as Ca\(^{2+}\)-NFAT and NF-\(\kappa\)B activation. The spatiotemporal regulation of TCR-CD28 MC formation in cSMAC may explain the complexity of intercommunicating molecules downstream of TCR and CD28.

Whereas CD28 localization in IS had been extensively investigated, nano-scale imaging of costimulatory receptor CD28 and its signal-transduction partners PI3K and PKC\(\alpha\) by TIRFM has...
provided a new vision of the two costimulatory mechanisms that are functionally critical: the early recruitment of PKCζ and PI3K to TCR-CD28 MCs and the late formation of a dynamic subregion in cSMAC enriched with PKCζ but depleted of TCR and PI3K.

**EXPERIMENTAL PROCEDURES**

**Reagents**

Antibodies and reagents were purchased from the following suppliers: anti-mCD28, biotinylated anti-mCD3ε, FITC-anti-mCD11c, phycoerythrin (PE)-anti-mMHC class II, PE-anti-mCD69, PE-anti-mCD80, PE-anti-mCD86, and PE-isotype matched control Ig were from eBioscience; anti-PKCζ and anti-phospho PKCζ, from Santa Cruz; Alexa Fluor 647-anti-CD3 and Alexa Fluor 647-anti-phospho CD3ζ, from BD Pharmingen; Alexa Fluor 488-streptavidin, Alexa Fluor 647-anti-rabbit IgG, and Alexa Fluor 548-anti-hamster IgG, from Molecular Probes; and goat polyclonal anti-CD28 and rabbit polyclonal anti-PKCζ, from Santa Cruz. HRP-anti-GFP was from Miltenyi Biotec and phorbol 12-myristate 13-acetate (PMA) was from Sigma. Anti-mouse CD28 was provided by R. Abe (Science University of Tokyo, Japan).

**Mice and Cells**

AND-Tg and 5C.C7-Tg mice on Rag2−/− background were provided by R.N. Germain (National Institutes of Health, NIH) and K. Yasutomo (Tokushima University, Japan), respectively, and Cd28−/− mice were purchased from Jackson Laboratories. The DC line, DC-1, was provided by J. Kaye. T cell hybridomas expressing both ECFP-CD28 and EYFP-PKCζ were plated on a planar bilayer containing I-Ek, ICAM-1, and CD80 without MCC88-103 and were allowed to develop CD28 clusters for 15 min. Translocation of EYFP-PKCζ in the same cells was imaged before and after 5 μM PMA stimulation (D). The average intensities of ECFP-CD28 (left) and EYFP-PKCζ (right) in cSMAC in the cells in (D) were compared to those of the entire images (E), n = 11. Scale bars represent 5 μm. A representative of two independent experiments is shown.

(F and G) Dynamism of CD28 and PKCζ clusters in an annular form. AND-Tg T cells expressing EGFP-CD28 (F) or EGFP-PKCζ (G) were plated on a planar bilayer as in (A) for 20 min. Recoveries of CD28 and PKCζ were analyzed by FRAP at specific regions (white square) of the annular clusters. Images were obtained every 2.5 s by confocal microscopy, and fluorescence recovery ratios were calculated (F), n = 28; (G), n = 30. Green lines in graphs depict the recovery ratios of cells in top panels. Mathematical formulas are approximated curves involving 28 or 30 cells. Scale bars represent 5 μm.

A representative of two independent experiments is shown. Real-time images in (F) (G) are available in Movies S9 and S10.

**Primary Cell Culture and Transduction**

Expression constructs were transiently transduced into Phoenix packaging cells (provided by G. Noran, Stanford University), via LipofectAmine Plus (Invitrogen). Retroviral supernatants were concentrated tenfold by centrifugation at 8000 x g for 12 hr. CD4+ T cells were purified from AND-Tg Rag2−/− or AND-Tg Cd28−/− mice and stimulated with 5 μM MCC88-103 (ANERA-DLIAYLQATK) and irradiated spleen cells from B10.BR mice. One day after stimulation, the cells were suspended in the retroviral supernatant and
Figure 7. Function of CD28 Cytoplasmic Tail in the Translocation of PKC\(\theta\) to T Cell-APC Interface and TCR-CD28 MCs

(A and B) AND-TCR T cell hybridomas expressing EYFP-PKC\(\theta\) (yellow) and ECFP-WT or mutant CD28 (cyan) were conjugated with MCC88-103-pulsed DC-1 without [(A), top row] or with CD80 [(A), bottom seven rows]. Cells were imaged real time at the indicated times by confocal microscopy. *, DC-1. Scale bars represent 5 \(\mu\)m. In fixed samples, conjugating pairs with PKC\(\theta\) translocated to cell-cell interface were counted [(B), n = 60, 68, 60, 115, 68, 65, 73, and 122 from left to right]. A representative of two independent experiments is shown.

(C) AND-Tg T cells from \(Cd28^{-/-}\) mice were reconstituted with ECFP-WT or mutant CD28 (cyan) and EYFP-PKC\(\theta\) (yellow), plated on a planar bilayer containing Cy5-I-E\(k\) (red) and ICAM-1 plus CD80 (prepulsed with MCC88-103), and imaged by confocal microscopy in real time 2 min or 30 min after cell-bilayer contact. Scale bars represent 5 \(\mu\)m. A representative of four independent experiments is shown.

(D) AND-TCR T cell hybridomas in (A) were plated on a planar bilayer as in (C). Images were obtained in real time by confocal microscopy immediately after contact (Figure S10), and the percentages of cells containing PKC\(\theta\) clusters are counted (n = 38, 26, 70, 42, 70, 83, 59, and 44 from left to right). A representative of two independent experiments is shown.

(E) AND-Tg T cells from \(Cd28^{-/-}\) mice were reconstituted with WT or mutant CD28 and stimulated with PMA in combination with anti-CD28. T cell proliferation was measured 48 hr after stimulation. The graph shows mean ± SD (n = 3) obtained by three independent experiments.
centrifuged at 1000 x g for 90 min in the presence of 8 μg/ml polybrene (Sigma) and 200 U/ml human recombinant interleukin 2 (Ajinomoto). At day 3 or later, 20%-80% of T cells were transduced on the basis of EGFP, ECFP, or EYFP expression and sorted with FACSAria (BD) to obtain populations with homogeneous fluorescence intensity. Cells were maintained for 5–14 days in RPMI1640 medium containing 10% FCS supplemented and recombiant IL-2.

Planar and Spherical Bilayers

GPI-anchored proteins of mouse I-Ek, ICAM-1, and CD80 were transfected into and purified from Chinese hamster ovary (CHO) or baby hamster kidney cells and were incorporated into dioleoyl phosphatidylcholine liposomes (DOPC, Avanti Polar Lipids). I-Ek- and ICAM-1-GPI were labeled with Cy5 mono NHS ester (Amersham Biosciences). Planar bilayers containing I-Ek-, ICAM-1-, and CD80-GPI were generated in a flow cell chamber system (Biotech). The expression levels of I-Ek, ICMA-1, and CD80 on the planar bilayer were quantified with 5-μm-diameter silica beads (Bangs Laboratories). Silica beads were loaded with DOPC containing I-Ek-, ICMA-1-, and CD80-GPI at the same concentrations as those of the planar bilayer, stained with FITC-labeled anti-I-Ek (14-4-4), ICMA-1 (YN1/1), or CD80 (16.10A1), and analyzed by FACSCalibur (BD). The densities of I-Ek, ICMA-1, and CD80 on the bilayer were calculated on the basis of standard FITC beads (Bangs Laboratories) and adjusted to approximately 250, 100, and 60–120 molecules/μm², respectively. Planar bilayers were loaded with 10 μM MCC88-103 in citrate buffer, pH 4.5, for 24 hr at 37°C, blocked with 5% nonfat dried milk in PBS for 1 hr at 37°C, and left to stand in the assay medium. All planar bilayer experiments were performed in HEPES-buffered saline containing 1% FCS, 2 mM MgCl₂, and 1 mM CaCl₂.

TIRF Imaging

Cells were imaged with TIRFM as previously described (Tokunaga et al., 1997). A solid-state laser (488 nm, 20 mW; Sapphire 488-20-OPS, Coherent) and a solid-state laser (488 nm, 20 mW; Sapphire 488-20-OPS, Coherent) and an inverted microscope (IX-81, Olympus, Japan) were used. Images were captured with an electron-bombarded charge-coupled device camera (C-7190-23; Hamamatsu Photonics).

Fluorescence Imaging with Planar Bilayer

Cells transduced with various fluorescent-tagged proteins or stained by Fab antibodies were real-time imaged by TIRFM or confocal microscopy. For immunofluorescence staining, cells on a planar bilayer were fixed with 4% paraformaldehyde and stained with indicated Abs in 1% BSA PBS 0.3% saponin for 30 min at 25°C after blocking with 1% BSA PBS 0.3% saponin. All images were collected on a Leica DMIRES2 system, and data were analyzed with Leica confocal software.

T Cell-APC Conjugation Assay

DC-1 cells were prepsulted overnight at 37°C with 5 μM MCC peptide and washed before assay. AND-TCR T cell hybridomas (2.5 x 10⁵) expressing EGFP-CD28 and/or EYFP-PKCI were cultured with DC-1 on a glass-bottomed dish for real-time imaging, or on 12 mm coverslips for fixing and staining.

T Cell Stimulation with Silica Beads

CD4⁺ T cells were prepared from AND-Tg mice on Rag2⁻/⁻ background by magnetic cell sorting (Miltenyi Biotec). Naïve or effector CD4⁺ T cells (5-10 x 10⁵) were stimulated with silica beads coated with lipid bilayer containing I-Ek-, ICAM-1-, and CD80-GPI prepulsed with MCC88-103 in a 96-well round-bottomed dish. At 6 hr after stimulation, cells were stained with anti-CD69 and analyzed by FACSCalibur. At 48 hr, IL-2 in supernatants was measured by ELISA. Also at 48 hr, the cells were pulsed with 2 Ci/well of ³H-thymidine for 12 hr, and the incorporated radioactivity was measured with a Microbeta scintillation counter (Amersham Pharmacia Biotech).

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and ten figures and can be found with this article online at http://www.immunity.com/cgi/content/full/29/4/589/DC1.

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REFERENCES

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