

B7-1 and B7-2 Selectively Recruit CTLA-4 and CD28 to the Immunological Synapse

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Summary

The reported affinity differences between CD28 and CTLA-4 binding to B7-1 and B7-2 may serve to selectively regulate CD28 and CTLA-4 function by differentially recruiting and/or stabilizing these molecules at the immunological synapse. Here we show that ligand binding is important for the accumulation of both CD28 and CTLA-4 at the synapse. While CD28 is recruited to the synapse in the absence of B7-1 and B7-2 binding, it is not effectively stabilized there, as its localization can be disrupted by CTLA-4. In the case of CTLA-4, ligand binding is critical for its concentration at the synapse. We also demonstrate that the affinity and avidity differences in ligand binding translate into selective recruitment of CD28 or CTLA-4 to the immunological synapse—B7-1 is the major ligand mediating CTLA-4 localization, while B7-2 is the main ligand for CD28 concentration at the synapse.

Introduction

$\alpha\beta$ T cell activation depends on the recognition of specific antigenic peptides bound to major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APC) by the T cell receptor (TCR). TCR engagement alone, however, is insufficient for T cell activation and can instead trigger apoptosis or a state of antigen-specific nonresponsiveness (anergy) (Schwartz, 1990). In addition to TCR ligation, optimal T cell proliferation and acquisition of effector functions require signals by the costimulatory molecule CD28. Upon binding to its ligands, B7-1 (CD80) and B7-2 (CD86), CD28 enhances T cell proliferation by increasing the transcription and the mRNA stability of interleukin-2 (IL-2), as well as by upregulating the antiapoptotic protein Bcl-X_L (reviewed in Acuto and Michel, 2003). A closely related molecule, cytotoxic T lymphocyte antigen 4 (CTLA-4) (Brunet et al., 1987), binds to the same ligands as CD28 with much higher affinity (Collins et al., 2002; Linsley et al., 1994). In contrast to CD28, CTLA-4 restricts T cell activation, as indicated by the fact that CTLA-4 ligation inhibits IL-2 production, IL-2 receptor expression, and cell cycle progression and that CTLA-4-deficient mice die of a lymphoproliferative disorder within 3–4 weeks of age (reviewed in Egen et al., 2002).

Despite the structural similarities between CD28 and CTLA-4, their trafficking patterns and steady-state distributions within the cell are completely different. Recent studies have shown that prior to stimulation, CD28 is uniformly distributed around the plasma membrane. However, when a T cell encounters an APC bearing antigenic peptide-MHC complexes, CD28 concentrates within the immunological synapse in less than a minute (Egen and Allison, 2002). In contrast, CTLA-4 normally resides in intracellular vesicles at the back of the migrating T cell. Upon engagement of the TCR, these intracellular vesicles re-orient toward the APC with a fraction of CTLA-4 eventually localizing to the synapse (Egen and Allison, 2002). Interestingly, the amount of CTLA-4 that translocates to the synapse correlates with the strength of the TCR signal, suggesting that CTLA-4 might preferentially restrict T cell responses to stronger TCR signals (Egen and Allison, 2002; Egen et al., 2002).

The goal of our studies was to determine the requirements for CD28 and CTLA-4 concentration at the synapse. We and others have previously shown that expression of B7-1 and/or B7-2 on the APC is insufficient to induce redistribution of CD28 or CTLA-4 upon contact with a T cell: TCR signaling is required (Bromley et al., 2001; Egen and Allison, 2002). A question that arises is whether ligand binding is then necessary for concentrating CD28 and CTLA-4 at the synapse. This question is especially relevant in light of the recent discovery of CD28 and CTLA-4 isoforms that lack extracellular domains and thus function in a ligand-independent manner (Hanawa et al., 2002; Ueda et al., 2003; Vijayakrishnan et al., 2004). Because the ligand-independent CD28 forms heterodimers with the full-length protein, it was assumed to act exclusively as an amplifier of signals generated by the full-length CD28 (Hanawa et al., 2002). In contrast, the ligand-independent CTLA-4 isoform by itself might be capable of generating a negative signal in T cells (Vijayakrishnan et al., 2004).

The experiments presented here demonstrate that ligand binding is important for the localization of full-length mouse CD28 and CTLA-4 to the immunological synapse. In the case of CD28, a mutant that failed to bind B7-1 and B7-2 concentrated at the synapse of fewer conjugates than the wild-type (wt) molecule. This accumulation defect was alleviated by the presence of endogenous wt CD28 and was exacerbated by high levels of CTLA-4. The analogous CTLA-4 mutant failed to localize to the immunological synapse even in the presence of endogenous wt CTLA-4. Using APC expressing only single ligands, we found that B7-2 is the primary ligand responsible for CD28 concentration at the synapse, while B7-1 is the principal ligand mediating CTLA-4 localization to the synapse. These results are consistent with physicochemical studies that found large avidity differences among the various human receptor/ligand combinations and predicted that the ratio of engaged CD28 to engaged CTLA-4 in the synapse will be higher when B7-2 is expressed on the APC relative to when B7-1 is expressed (Collins et al., 2002). Our experiments verify this hypothesis and show that the

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reported affinity differences in ligand binding translate into selective recruitment of CD28 or CTLA-4 to the immunological synapse.

Results

MYPPPY Mutations Affect CD28 and CTLA-4 Binding to B7-1Ig and B7-2Ig

To evaluate the ligand binding requirements for mouse CD28 and CTLA-4 concentration at the synapse, the second tyrosine in the MYPPPY motif of both molecules was mutated to an alanine (Y123A for CD28 and Y139A for CTLA-4). Previous studies have shown that this mutation abolishes CD28Ig and CTLA-4Ig binding to B7-1-transfected Chinese hamster ovary cells and in surface plasmon resonance (SPR) measurements eliminated CTLA-4Ig binding to both B7-1Ig and B7-2Ig (Morton et al., 1996; Peach et al., 1994). However, it was also reported that the Y123A mutation in CD28 made no difference in the binding of B7-1Ig and only reduced binding of B7-2Ig in experiments using L cell transfectants (Kariv et al., 1996).

To determine whether the Y123A mutation interferes with ligand binding in our system, previously activated 5C.C7 CD28^{-/-} T cells were infected with retroviral vectors encoding either wt CD28-YFP or CD28-Y123A-YFP and stained with anti-CD28 antibodies or B7-1Ig or B7-2Ig. The left panel of Figure 1A shows that while both infected populations expressed comparable levels of CD28 at the plasma membrane, the Y123A mutant failed to bind detectable amounts of soluble B7-1 or B7-2 (Figure 1A, center and right panels). The surface levels of the exogenous molecules were 3–4 times higher than the levels of endogenous CD28 in activated wt 5C.C7 T cells (data not shown).

Similar results were obtained when 5C.C7 CD28^{-/-} T cells were infected with viruses encoding either wt or Y139A CTLA-4-YFP. In these cells, the plasma membrane levels of CTLA-4 were 2–4 times higher than the levels in uninfected 5C.C7 T cells (data not shown). Despite the expression of only three times more CTLA-4 on the surface, as revealed by anti-CTLA-4 antibody staining (Figure 1B, left panel), B7-1Ig staining of wt CTLA-4-YFP-expressing cells was 22-fold higher than that of CTLA-4-Y139A-infected cells (Figure 1B, center panel). In contrast, the difference in B7-2Ig binding to cells expressing wt or Y139A CTLA-4-YFP was comparable to the difference in antibody binding to surface wt or mutant CTLA-4 (Figure 1B, right panel), possibly because of low CTLA-4 levels at the plasma membrane. As CTLA-4 is known to primarily localize to intracellular vesicles rather than the cell surface, these experiments were repeated using wt and Y139A CTLA-4-YFP in which Tyr201, a critical residue in the intracellular localization motif, had been mutated to Phe. This mutation stabilizes CTLA-4 at the plasma membrane by abrogating binding of the clathrin adaptor AP-2 (Baroja et al., 2002). As expected, T cells infected with viruses expressing both wt and Y139A CTLA-4-YFP carrying this mutation had much higher surface levels of CTLA-4 than the original molecules (left panels of Figure 1C versus Figure 1B). Despite this high expression level, CTLA-4-Y139A-YFP failed to bind significant amounts of B7-1Ig or B7-2Ig (Figure 1C, center and right panels). These results dem-

onstrate that in our system, mutation of the second tyrosine in the MYPPPY ligand binding motif of both CD28 and CTLA-4 abolishes binding to B7-1 and B7-2.

Mutation of Y123A Interferes with CD28 Concentration at the Immunological Synapse

To determine whether ligand binding is required for CD28 concentration at the immunological synapse, wt CD28-CFP or CD28-Y123A-CFP were introduced into previously activated primary 5C.C7 CD28^{-/-} T cells via retroviral infection. To determine whether CTLA-4 affects the synaptic concentration of either molecule, the cells were also infected with viruses expressing wt CTLA-4-YFP. The T cells were allowed to form conjugates with peptide-pulsed CH12.Lx cells for 20 min. Conjugates were selected on the basis of protein kinase C- θ (PKC θ) accumulation at the T cell-APC interface because PKC θ localizes specifically to the center of the immunological synapse (Monks et al., 1998). In these experiments, CD28-CFP concentrated at the synapse of 93% (99 of 106) of the cells (Figure 2A). The fact that CD28-CFP did not completely cluster at the synapse (some was present in the rest of the plasma membrane) might be due to its overexpression. However, the fact that this was observed when either CH12.Lx or lipopolysaccharide (LPS)-activated B6 B cells were used as APC (below), despite the fact that the levels of B7-1 and B7-2 on these cells differ by a factor of 5 to 7 (data not shown), suggests that B7-1 and B7-2 are not limiting. The remainder of the conjugates had no detectable accumulation of CD28-CFP—it appeared uniformly distributed around the plasma membrane (Figure 2B). In contrast, CD28-Y123A-CFP concentrated at the synapse of only 25% (25 of 100) of 5C.C7 CD28^{-/-} T cells (Figure 2C). In the majority of the conjugates (56%, 56 of 100), the mutant CD28 was uniformly distributed around the plasma membrane (Figure 2D), while 19% (19 of 100) of the conjugates had excluded CD28-Y123A-CFP from the site of CTLA-4-YFP accumulation (Figure 2E). These results indicate that ligand binding is not absolutely required for CD28 recruitment to the synapse, but it facilitates its concentration there.

The equivalent series of experiments in wt 5C.C7 cells yielded similar results. Fully 97% (97 of 101) of the conjugates had accumulated wt CD28-CFP, while the remainder had uniform CD28-CFP distribution around the plasma membrane. In contrast, 40% (40 of 100) of the T cells had concentrated CD28-Y123A-CFP at the synapse, almost double the number obtained in CD28-deficient T cells. This result suggests that the endogenous wt CD28 facilitates the concentration of CD28-Y123A-CFP at the synapse. Forty-seven percent (47 of 100) of the conjugates had uniformly distributed CD28-Y123A-CFP, while 13% (13 of 100) had excluded the mutant CD28 from the site of CTLA-4 concentration. These results are summarized in the left panels of Figure 2F.

To determine whether CTLA-4 affects the concentration of the wt or the mutant CD28, conjugates were also selected on the basis of CTLA-4 accumulation at the synapse. The data are summarized in the right panels of Figure 2F. In 5C.C7 CD28^{-/-} T cells, 90% (103 of 114) of the conjugates with concentrated CTLA-4-YFP had also accumulated wt CD28-CFP at the synapse. In con-

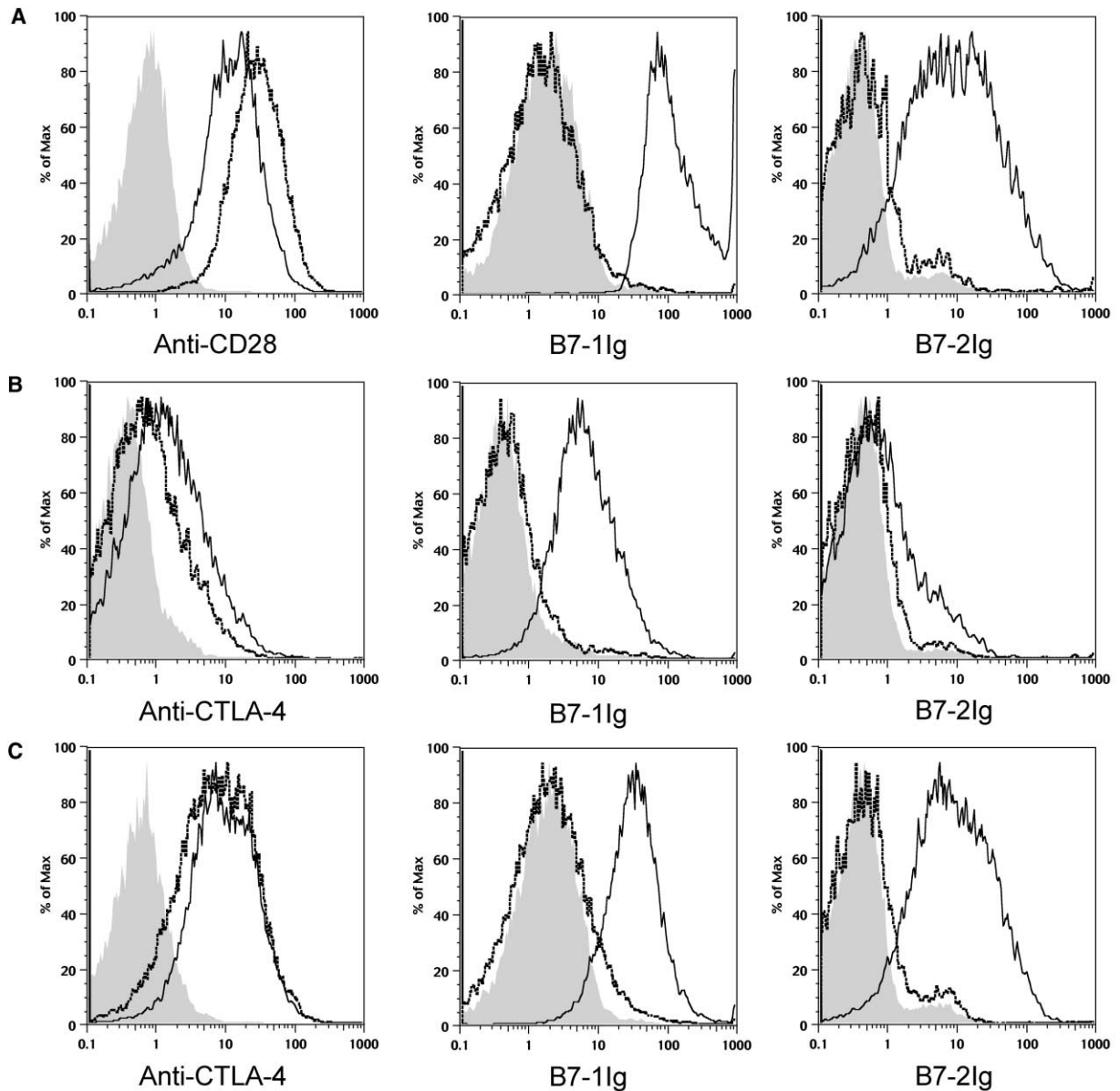


Figure 1. MYPPPY Mutations Affect CD28 and CTLA-4 Binding to B7-1lg and B7-2lg

5C.C7 CD28^{-/-} T cells were infected with either wt (solid line) or mutant (dotted line) CD28 (A) or CTLA-4 (B and C). Infected 5C.C7 CD28^{-/-} cells that did not express YFP were used as a negative control (shaded histogram). Anti-CD28 or anti-CTLA-4 antibodies were used to determine protein surface levels (left panels). Cells were also stained with B7-1lg (middle panels) and B7-2lg (right panels) to evaluate the relative ligand binding capability of the wt and mutant proteins. Both the wt and the mutant CTLA-4 molecules in C contain Y201F mutations, which increase the plasma membrane levels of both proteins.

trast, only 8% (8 of 104) of the conjugates with concentrated CTLA-4-YFP at the synapse concentrated CD28-Y123A-CFP. This is approximately one-third of the number of conjugates that accumulated the mutant CD28 when PKC θ -positive conjugates were counted, suggesting that CTLA-4 can interfere with the concentration of the mutant CD28 at the synapse but not with the accumulation of the wt molecule. It is also possible, however, that these differences represent the heterogeneous nature of T cell activation, so that the cells with concentrated PKC θ are inherently more capable of recruiting the mutant CD28 than the cells with concen-

trated CTLA-4. The majority (63%, 66 of 104) of the remaining conjugates had uniformly distributed CD28-Y123A-CFP, while 29% (30 of 104) had depleted CD28-Y123A from the site of CTLA-4 concentration.

In the studies above, the cells expressed high levels of exogenous CTLA-4-YFP, which could affect CD28-Y123A concentration at the synapse. To determine what happens when CTLA-4 is not overexpressed, experiments were also conducted using wt or CD28^{-/-} 5C.C7 cells that were infected with viruses encoding Ick-YFP (Straus and Weiss, 1992) and either the wt or the mutant CD28 constructs described above. Conjugates were se-

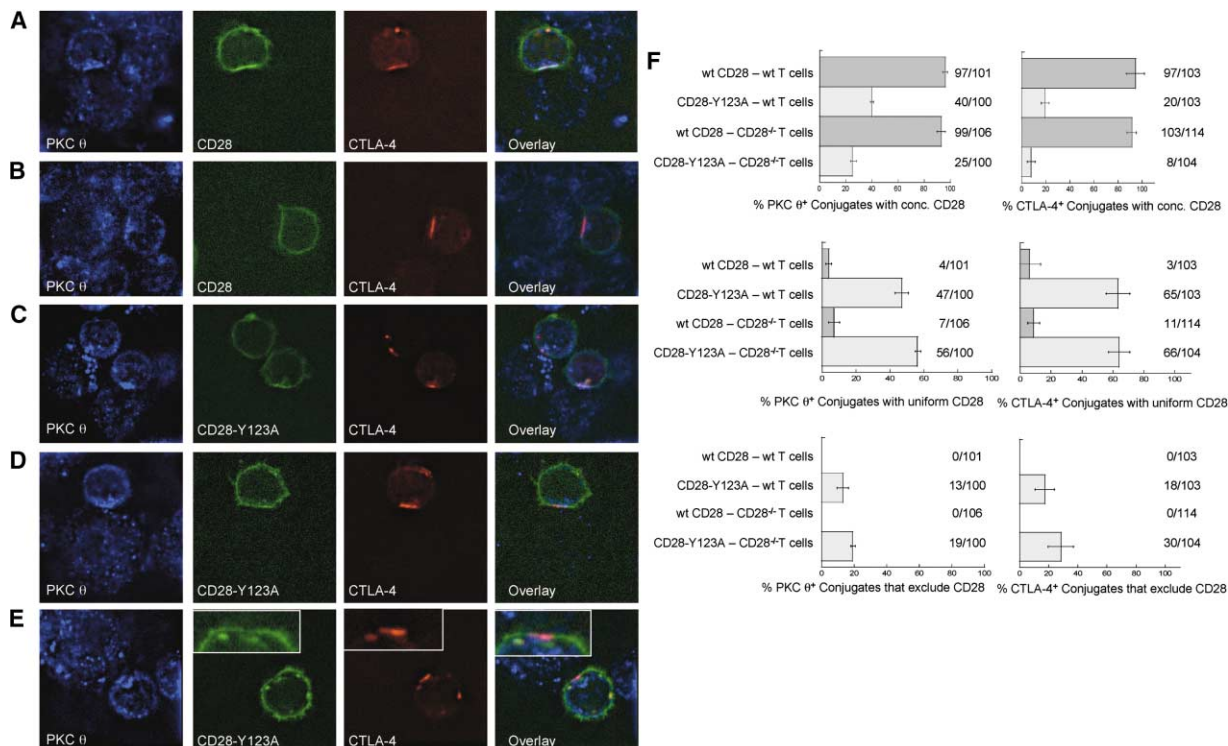


Figure 2. Ligand Binding Is Required for CD28 Concentration at the Immunological Synapse

5C.C7 CD28 $^{-/-}$ T cells were co-infected with wt CTLA-4-YFP and wt CD28-CFP (A and B) or CD28-Y123A-CFP (C, D, and E). Conjugates between MCC-pulsed CH12.Lx cells and infected T cells that had concentrated PKC θ (blue) at the synapse were scored for localization of wt or mutant CD28-CFP (green) to the synapse. CTLA-4-YFP is in red. Representative cells with concentrated wt or mutant CD28 are shown in (A) and (C), while cells with uniform distribution of wt or mutant CD28 are shown in (B) and (D). The cell in (E) is an example where CD28-Y123A is depleted from the site of CTLA-4 concentration. The insets in the top left of the images show the interface between the two cells at a higher magnification. The statistics from three experiments with at least 30 conjugates, both in 5C.C7 wt and CD28 $^{-/-}$ T cells, are shown in (F). Error bars represent standard deviation between experiments.

lected on the basis of the synaptic concentration of Ick-YFP and were subsequently scored for either CD28-CFP or CD28-Y123A-CFP accumulation at the synapse. In both 5C.C7 wt and CD28 $^{-/-}$ cells, 100% of the conjugates with concentrated Ick-YFP (93 for wt and 91 for CD28 $^{-/-}$ cells) also concentrated wt CD28-CFP at the synapse (Figure 3A). In contrast, only 42% (56 of 133) of the conjugates with concentrated Ick-YFP accumulated the mutant CD28-Y123A-CFP in 5C.C7 CD28 $^{-/-}$ cells, while a significantly higher fraction (92%, 93 out of 101) concentrated the mutant CD28-Y123A-CFP in wt 5C.C7 cells (Figure 3B). The remainder of the conjugates had uniform distribution of CD28-Y123A-CFP around the plasma membrane (Figure 3C). The results are summarized in Figure 3D. These data show that while the mutant CD28 can localize to the synapse in a significant number of cells, it does so less readily than wt CD28, indicating an important role for ligand binding in receptor recruitment. Overexpression of CTLA-4 reduces the degree of mutant CD28 localization to the synapse even further, suggesting that CTLA-4 may either physically displace the mutant CD28 or actively reduce its recruitment by generating inhibitory signals.

To examine whether the higher fractions of conjugates with concentrated CD28-Y123A-CFP in wt 5C.C7 versus 5C.C7 CD28 $^{-/-}$ T cells (Figure 2F and 3D) might be due to the formation of heterodimers composed of endogenous CD28 and exogenous mutant CD28-Y123A, 5C.C7

CD28 $^{-/-}$ T cells were co-infected with viruses expressing CD28-YFP and CD28-Y123A-CFP. Fifty-six percent (57 of 101) of the conjugates with concentrated wt CD28 also concentrated the mutant CD28-Y123A (Figure 4A), suggesting that at least some of the effect in 5C.C7 wt T cells might be due to heterodimerization. The rest of the conjugates had uniform CD28-Y123A despite the concentration of wt CD28 (Figure 4B). Furthermore, when the same experiments were performed in wt 5C.C7 cells, a significantly higher fraction (88%, 91 of 103) of the conjugates concentrated both the wt and the mutant CD28, presumably because the additional endogenous wt CD28 heterodimerized with CD28-Y123A-CFP and recruited it to the synapse. It is also possible, however, that the wt CD28 facilitated the generation of a stable synapse either because of its physical properties or because of active signaling. These results are summarized in Figure 4C. Taken together, the data suggest that CD28 can concentrate at the immunological synapse in the absence of ligand; however, B7-1 or B7-2 binding stabilizes CD28 at the synapse, either because the ligands prevent the diffusion of CD28 away from the interface or because they trigger a signaling cascade.

Mutation of Y139A Inhibits CTLA-4 Localization to the Immunological Synapse

To determine whether ligand binding is required for CTLA-4 localization to the immunological synapse,

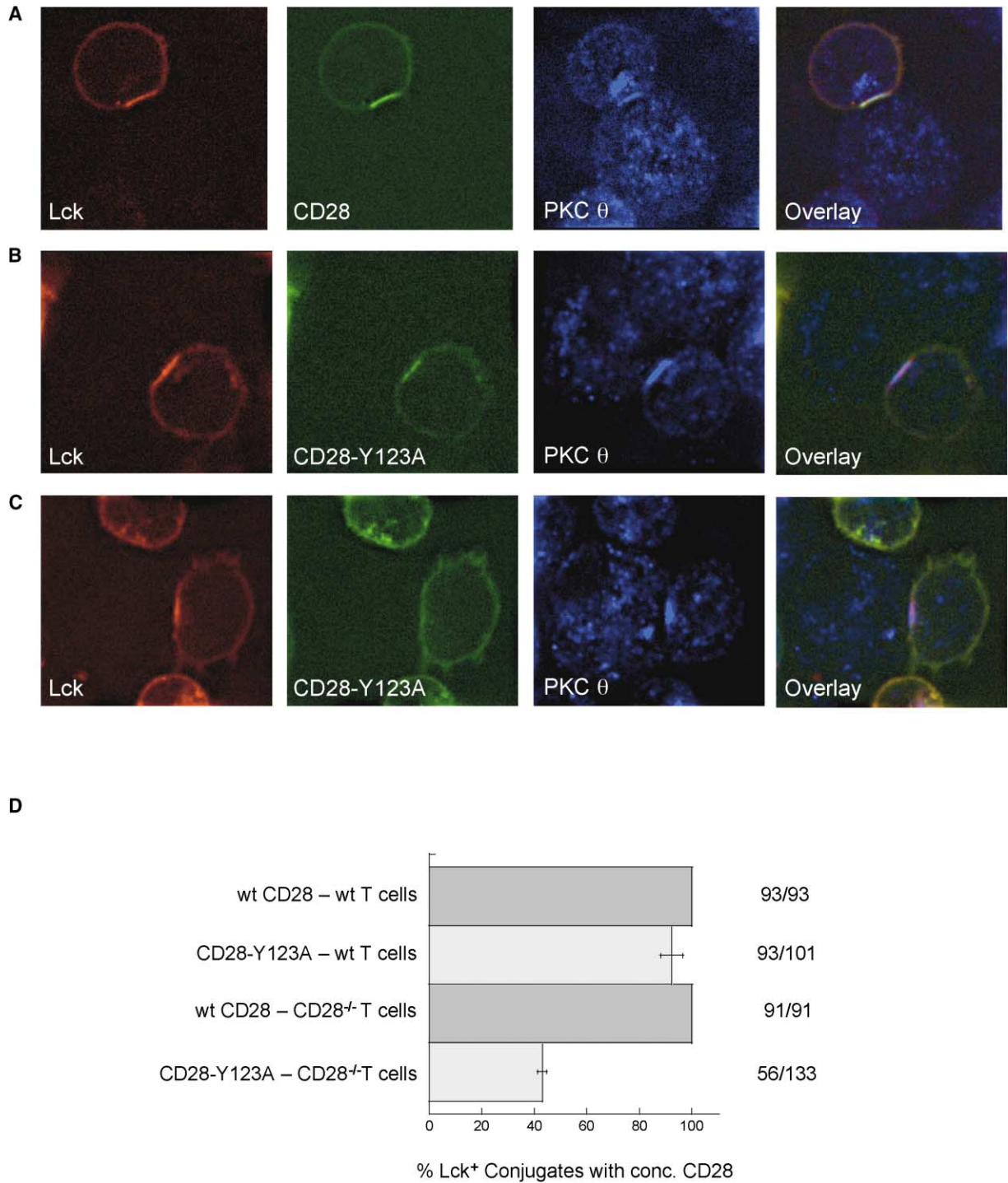


Figure 3. In the Absence of High CTLA-4 Levels, Ligand Binding Is Important for CD28 Accumulation at the Synapse, but It Is Not Absolutely Required

5C.C7 CD28^{-/-} T cells were co-infected with lck-YFP and wt CD28-CFP (A) or CD28-Y123A-CFP (B and C). Conjugates between MCC-pulsed CH12.Lx cells and infected T cells that had concentrated lck-YFP (red) at the synapse were scored for localization of wt or mutant CD28-CFP (green) to the synapse. PKC θ is in blue. Representative cells with concentrated wt or mutant CD28 are shown in (A) and (B), while a cell with uniform distribution of mutant CD28 is shown in (C). The statistics from three experiments with at least 30 conjugates per experiment, both in 5C.C7 wt and CD28^{-/-} T cells, are shown in (D). Error bars represent standard deviation between experiments.

5C.C7 wt T cells expressing either wt CTLA-4-YFP or CTLA-4-Y139A-YFP, in addition to CD28-CFP, were compared. When conjugates were selected on the basis of PKC θ accumulation, 92% (93 of 101) of the cells

localized wt CTLA-4-YFP to the immunological synapse (Figure 5A). In the remainder of the conjugates, CTLA-4 was only present in intracellular vesicles (Figure 5B). In contrast, CTLA-4-Y139A-YFP was concentrated at they

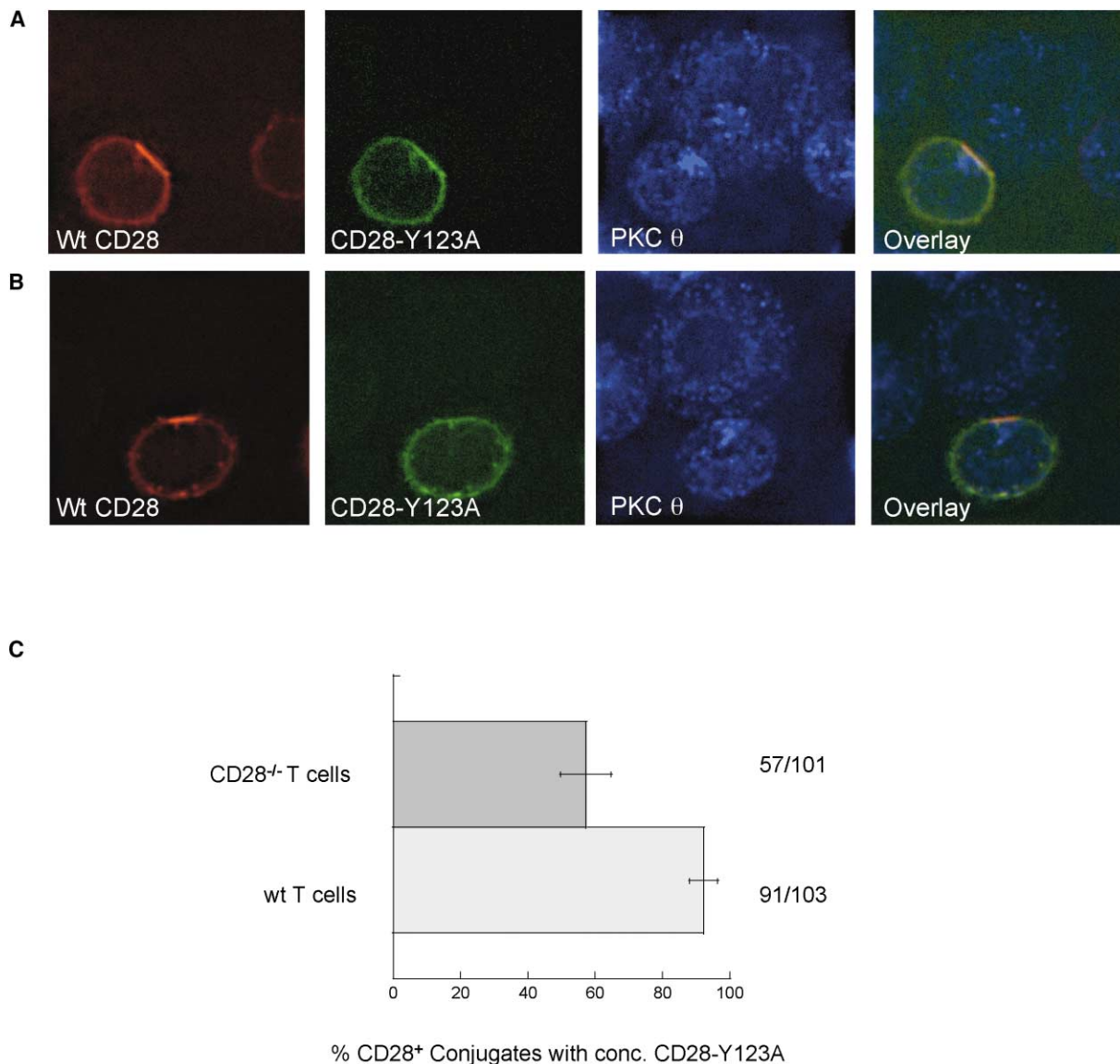


Figure 4. Wt CD28 Facilitates the Concentration of the Mutant CD28 at the Synapse

5C.C7 CD28^{-/-} T cells were co-infected with CD28-YFP and CD28-Y123A-CFP (A and B). Conjugates between MCC-pulsed CH12.Lx cells and infected T cells that had concentrated CD28-YFP (red) at the synapse were scored for localization of CD28-Y123A-CFP (green) to the synapse. PKC θ is in blue. A representative cell with concentrated mutant CD28 is shown in (A), while a cell with uniform distribution of CD28-Y123A is shown in (B). The statistics from three experiments with at least 30 conjugates, both in 5C.C7 wt and CD28^{-/-} T cells, are shown in (C). Error bars represent standard deviation between experiments.

synapse of only 14% (14 of 101) of the conjugates (Figure 5C), while the remaining 86% (87 of 101) had CTLA-4-Y139A-YFP in intracellular vesicles (Figure 5D). The results from the analogous studies in 5C.C7 CD28^{-/-} T cells were very similar (Figure 5E, left panel) and indicate that ligand binding is critical for CTLA-4 localization to the immunological synapse.

When conjugates were selected on the basis of CD28-CFP concentration at the synapse, wt CTLA-4-YFP accumulated in a smaller fraction of cells relative to the numbers for the PKC θ -positive conjugates. This finding could result from differences in the kinetics of CD28 and PKC θ accumulation at the synapse, with the timing of PKC θ recruitment more closely following that of

CTLA-4. The results for CTLA-4-Y139A in CD28-positive conjugates were similar to those in PKC θ -positive ones and are summarized in the right panel of Figure 5E. They demonstrate that ligand binding is critical for the localization of CTLA-4 to the synapse.

B7-2 Is the Major Ligand Mediating CD28 Concentration at the Synapse, while B7-1 Is the Main Ligand for CTLA-4 Localization to the Synapse

The studies described above established that ligand binding is important for CD28 and CTLA-4 concentration at the immunological synapse. However, the use of mutants that completely abolished ligand binding (Figure 1) did not allow us to address the issue of potential

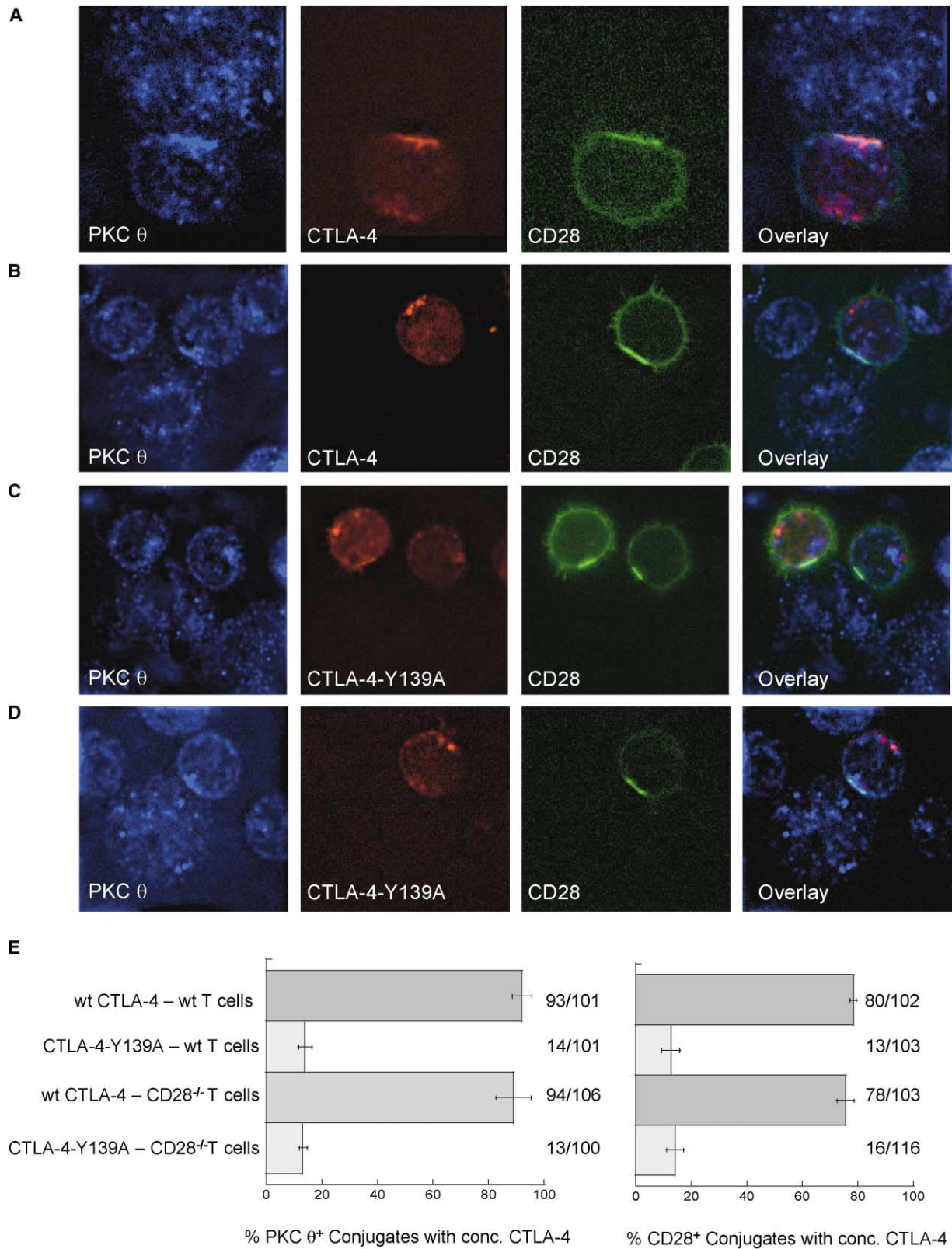


Figure 5. Ligand Binding Is Critical for CTLA-4 Localization to the Immunological Synapse

5C.C7 wt T cells were co-infected with wt CD28-CFP and wt CTLA-4-YFP (A and B) or CTLA-4-Y139A-YFP (C and D). Conjugates between MCC-pulsed CH12.Lx cells and infected T cells that had concentrated PKC θ (blue) at the synapse were scored for localization of wt or mutant CTLA-4-YFP (red) to the synapse. CD28-CFP is in green. Representative cells with concentrated wt or mutant CTLA-4 are shown in (A) and (C), while cells in which wt or mutant CTLA-4 localized only to intracellular vesicles are shown in (B) and (D). The statistics from three experiments with at least 30 conjugates, both in 5C.C7 wt and CD28 $^{-/-}$ T cells, are shown in (E). Error bars represent standard deviation between experiments.

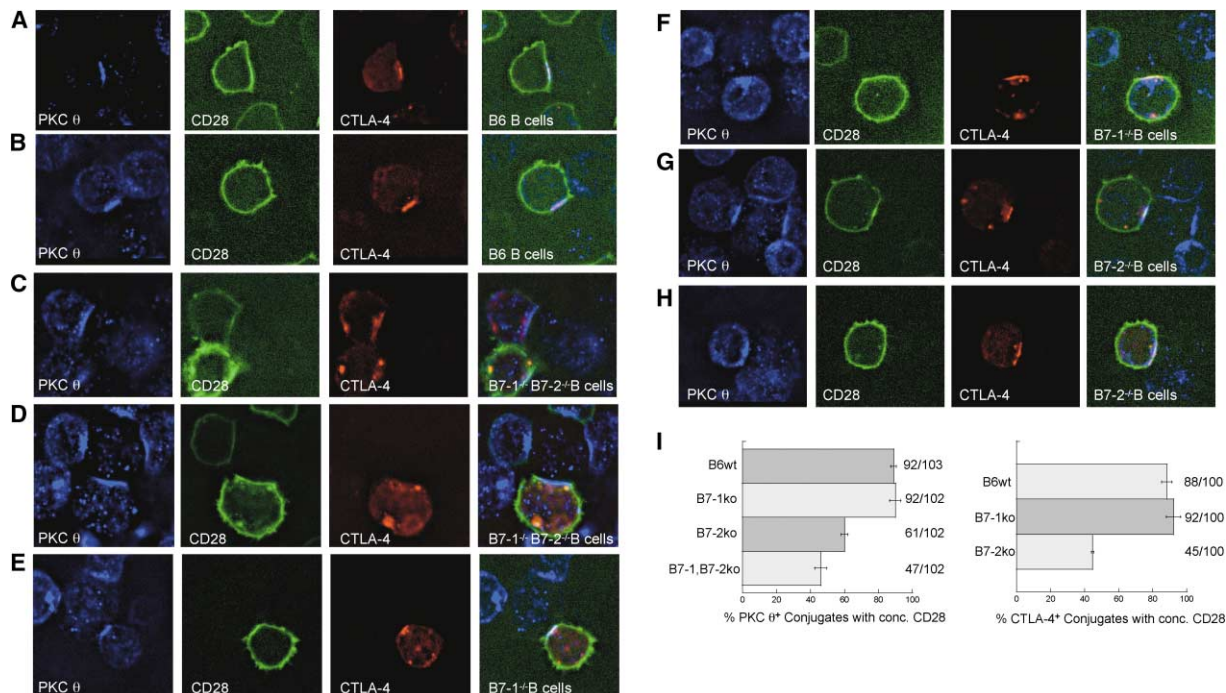


Figure 6. B7-2 Is the Major Ligand for CD28 Concentration at the Synapse

OTII T cells were co-infected with CD28-CFP and CTLA-4-YFP. Conjugates between OVA-pulsed B cells from B6 wt (A and B), B7-1^{-/-} B7-2^{-/-} (C and D), B7-1^{-/-} (E and F) or B7-2^{-/-} (G and H) and infected T cells that had concentrated PKC θ (blue) at the synapse were scored for CD28 (green) localization. CTLA-4 is in red. Representative cells with concentrated CD28 are shown in (A), (C), (E), and (G), while cells with uniform distribution of CD28 are shown in (B), (D), (F), and (H). The statistics from three experiments with at least 30 conjugates are shown in (I). Error bars represent standard deviation between experiments.

differences between B7-1 and B7-2 with respect to recruitment of CD28 and CTLA-4 to the synapse. To this end, we investigated CD28 and CTLA-4 localization at the synapse using LPS-activated B cells from wt, B7-1^{-/-}, B7-2^{-/-}, or B7-1^{-/-}B7-2^{-/-} mice as APC. The B cells had to be LPS activated because they do not express B7-1 until 48 hr post stimulation (Lenschow et al., 1993). The activated B7-1^{-/-} and B7-2^{-/-} B cells had comparable surface levels of B7-2 and B7-1, respectively, to wt B cells. Assuming similar affinities for the anti-B7-1 and anti-B7-2 antibodies used, cells expressed twice as much B7-2 as they did B7-1 (data not shown). Because the B7-deficient animals were on the B6 instead of the B10.A background, we used T cells from OTII TCR transgenic mice rather than 5C.C7.

In order to determine the ligand requirements for CD28 concentration at the synapse, conjugates were selected on the basis of either PKC θ or CTLA-4 accumulation at the T cell-APC interface and the results were compared. When wt B6 B cells were used as APC, CD28 accumulated in 89% (92 of 103) of conjugates with concentrated PKC θ and in 88% (88 of 100) of the cells if they were selected on the basis of CTLA-4 accumulation (Figure 6A). These numbers are comparable to those obtained in the studies of 5C.C7 T cells above. The rest of the conjugates had CD28 uniformly distributed around the plasma membrane, despite the concentration of both PKC θ and CTLA-4 (Figure 6B). Because too few conjugates localized CTLA-4 to the synapse in experiments with B7-1^{-/-}B7-2^{-/-} B cells as APC, the fraction of con-

centrated CD28 was only determined in PKC θ-positive conjugates. In this case, 46% (47 of 102) of the cells concentrated CD28 (Figure 6C), while the rest had uniformly distributed CD28 (Figure 6D). This number is similar to that obtained for CD28-Y123A accumulation in CD28-deficient 5C.C7 T cells in the absence of CTLA-4 overexpression (Figure 3D), again indicating that CD28 can concentrate at the synapse in the absence of ligand, but not as well as when B7-1 and B7-2 are present. When B7-1-deficient B cells were used as APC, the results resembled those for the wt B cells. Eighty-nine percent (92 of 102) of the conjugates with concentrated PKC θ at the synapse and 92% (92 of 100) of the cells with CTLA-4 at the synapse had also concentrated CD28 (Figure 6E), suggesting that B7-1 was not required for CD28 accumulation at the interface. The remaining cells had uniformly distributed CD28 (Figure 6F). In contrast, when B7-2-deficient B cells were used as APC, only 60% (61 of 102) of the conjugates with concentrated PKC θ and 45% (45 of 100) of the cells with concentrated CTLA-4 had also concentrated CD28 (Figure 6G). The data are summarized in Figure 6H. They indicate that B7-2 is the ligand primarily responsible for CD28 concentration at the synapse and suggest that CTLA-4 can outcompete CD28 for binding to B7-1.

When the reciprocal series of experiments were performed, wt B6 B cells were found to induce CTLA-4 accumulation at the synapse of 77% (79 of 103) of the conjugates with concentrated PKC θ and 67% (82 of 123) of those with concentrated CD28 (Figure 7A). These

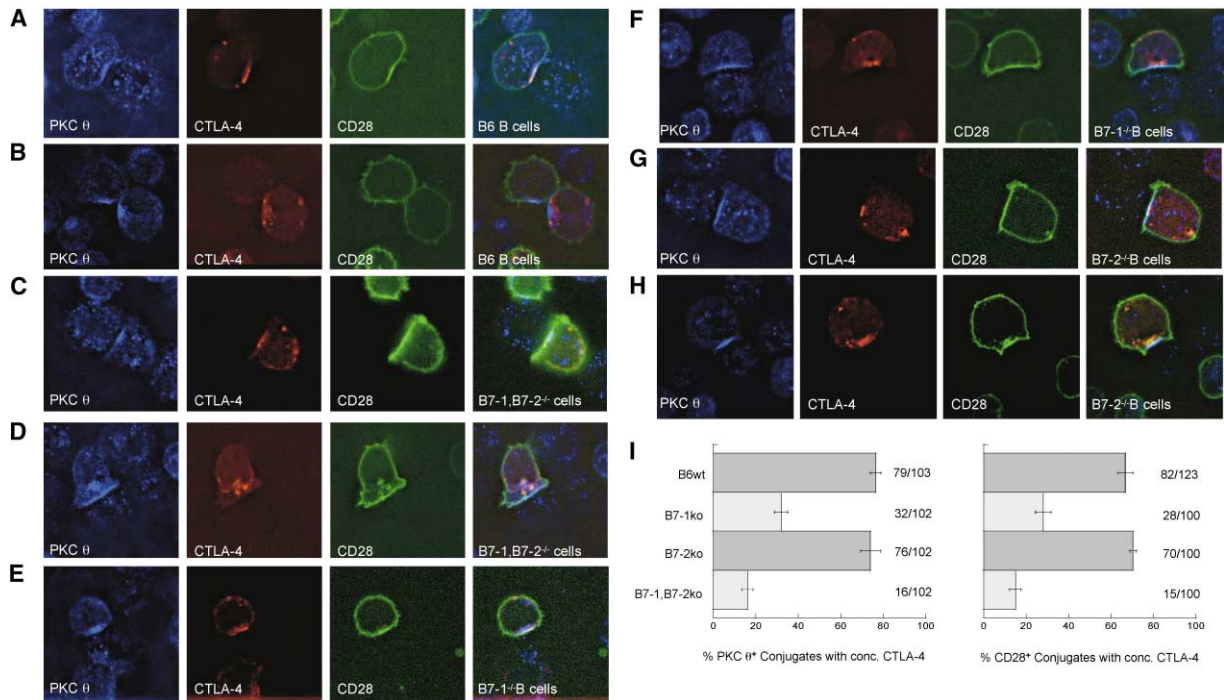


Figure 7. B7-1 Is the Major Ligand for CTLA-4 Concentration at the Synapse

OTII T cells were co-infected with CD28-CFP and CTLA-4-YFP. Conjugates between OVA-pulsed B cells from B6 wt (A and B), B7-1^{-/-}B7-2^{-/-} (C and D), B7-1^{-/-} (E and F), or B7-2^{-/-} (G and H) and infected T cells that had concentrated PKC θ (blue) at the synapse were scored for CTLA-4 (red) localization. CD28 is in green. Representative cells with concentrated CTLA-4 are shown in (A), (C), (E), and (G), while cells in which CTLA-4 localized only to intracellular vesicles are shown in (B), (D), (F), and (H). The statistics from three experiments with at least 30 conjugates are shown in (I). Error bars represent standard deviation between experiments.

numbers are somewhat lower than the ones obtained in the studies of 5C.C7 T cells and CH12.Lx APC, probably because the B6 B cells expressed lower levels of B7-1 and B7-2 than the B cell lymphoma (data not shown). In the conjugates in which CTLA-4 was not concentrated with PKC θ and CD28 at the synapse, it was still evident in intracellular vesicles (Figure 7B). When B cells deficient for both B7-1 and B7-2 were used as APC, CTLA-4 localized to the synapse of only 16% (16 of 102) of the PKC θ-positive and 15% (15 of 100) of the CD28-positive conjugates (Figure 7C). These numbers are similar to those obtained for the mutant CTLA-4-Y139A in 5C.C7 T cells. The rest of the conjugates had CTLA-4 in intracellular vesicles, despite the concentration of both PKC θ and CD28 at the synapse (Figure 7D), indicating that ligand binding is critical for CTLA-4 localization to the synapse. We noted, however, that conjugates with concentrated PKC θ and CD28 seemed less prevalent when the B7-1^{-/-}B7-2^{-/-} cells were used as APC instead of wt B6 B cells, although this effect was difficult to quantify. When B7-1-deficient B cells were used as APC, CTLA-4 localized to the synapse in only 31% (32 of 102) of the PKC θ-positive and in 28% (28 of 100) of the CD28-positive conjugates (Figure 7E), and the majority of the conjugates in both cases had CTLA-4 in intracellular vesicles (Figure 7F). In contrast, when B7-2^{-/-} B cells were used as APC, CTLA-4 localized to the synapse of 75% (76 of 102) of the PKC θ-positive and 70% (70 of 100) of the CD28-positive conjugates (Figure 7G), a frequency close to that obtained with wt cells expressing

both B7-1 and B7-2. These data are summarized in Figure 7I. These results clearly indicate that B7-1 is the preferred ligand for CTLA-4 localization to the synapse.

Discussion

The goal of our study was to determine whether ligand binding is important for CD28 and CTLA-4 localization patterns and whether the differential affinities of CD28 and CTLA-4 for their ligands translate into selective recruitment of the molecules to the immunological synapse. Two independent lines of investigation demonstrated that B7-1 or B7-2 binding is important for CD28 concentration at the synapse, but it is not absolutely required. When the APC lacked both ligands, CD28 accumulated at the synapse of half as many conjugates as when the APC expressed B7-1 and B7-2 (Figure 6). In addition, the ligand binding mutant of CD28 concentrated at the synapse of fewer cells than the wt molecule, especially in the presence of high levels of CTLA-4 (Figures 2 and 3). These results indicate that while CD28 can concentrate at the synapse in the absence of B7-1 and B7-2, ligand binding plays an important role in regulating its localization during T cell-APC interactions.

Data from multiple studies imply that CD28 recruitment to the immunological synapse is initiated by TCR signaling, probably as a result of the reorganization of the actin cytoskeleton. B7-1 and B7-2 binding could stabilize CD28 that has been recruited to the synapse either by preventing its diffusion away from the T cell-

APC contact site or by the generation of a signal that further alters actin dynamics and leads to greater CD28 accumulation at the synapse via a positive-feedback mechanism. The ligand binding mutant of CD28 may either diffuse away from the synapse or fail to generate this potential retention signal. The presence of high levels of CTLA-4 may further enhance this effect by either physically displacing the ligand binding mutant or dampening signals required for its retention.

While ligand binding affects CD28 localization only moderately, the presence of B7-1/B7-2 appears to be a critical factor regulating recruitment of CTLA-4 to the synapse. When it could not bind ligand, either because of the ligand binding domain mutation (Figure 5) or because of absence of B7-1 and B7-2 on the APC (Figure 7), CTLA-4 accumulated at the synapse in a significantly smaller fraction of conjugates than when it could bind ligand. CTLA-4 that localized to the synapse under conditions where ligand binding was not possible could represent a transient population of molecules recently delivered to the synapse from intracellular vesicles as a result of TCR signaling. Alternatively, this CTLA-4 might be derived from a putative small population of molecules present on the cell surface prior to conjugate formation that is recruited to the synapse by the same actin-based mechanism that recruits CD28. Our finding that ligand binding is required for CTLA-4 localization at the synapse brings into question the mechanism by which the ligand-independent CTLA-4 isoform might inhibit T cell function independently of the full-length molecule (Vijayakrishnan et al., 2004). Future studies should address whether the trafficking pattern or the intracellular localization of ligand-independent molecules in primary T cells differ from those of the full-length proteins.

Our experiments with the B7-1- and B7-2-deficient APC clearly demonstrate distinct ligand preferences for CD28 and CTLA-4 localization to the synapse. CD28 concentrated at the synapse equally well when the APC expressed both ligands or had only B7-2, but poorly when the APC expressed only B7-1 (Figure 6). The impaired synaptic localization of CD28 observed with APC expressing only B7-1 was even more pronounced when CTLA-4 was present at the synapse, suggesting that CTLA-4 effectively competes with CD28 for B7-1 binding. Together, the data demonstrate that B7-2 is the preferred ligand for concentrating CD28 at the synapse.

The results for CTLA-4 were exactly the opposite. CTLA-4 localized to the synapse of the same fraction of conjugates when the APC expressed both ligands or had only B7-1, but very poorly when the APC expressed only B7-2 (Figure 7). These results clearly show that B7-1 is the preferred ligand for CTLA-4 accumulation in the synapse.

Our work demonstrates that B7-1 and B7-2 preferentially recruit CTLA-4 and CD28, respectively, to the immunological synapse and as a result may differentially promote inhibition or activation of T cell functions. This was previously suggested based on the delayed surface expression of B7-1 relative to B7-2 (Lenschow et al., 1993), coupled with the delayed expression of CTLA-4 relative to CD28 (Linsley et al., 1992). However, since all four molecules are expressed after activation, it seems unlikely that simple differences in expression kinetics

could by themselves result in differential binding and distinct functional consequences.

Nevertheless, early studies demonstrated that blocking B7-2 on B cells with specific antibodies prevents T cell proliferation, while anti-B7-1 antibodies have no effect (Hathcock et al., 1994). In addition, in several mouse models of autoimmunity, administration of anti-B7-2, but not anti-B7-1, blocking antibodies correlates with amelioration of disease (Lenschow et al., 1995; Nakajima et al., 1995; Peterson et al., 1999; Saegusa et al., 2000). In addition, B7-2-deficient animals are protected from some autoimmune diseases, while B7-1-deficient mice are more susceptible (Girvin et al., 2000; Liang et al., 1999). Furthermore, anti-B7-2 antibodies interfere with the expansion of adoptively transferred T cells (Kearney et al., 1995), prolong allograft survival (Judge et al., 1999), and enhance suppression by CD4⁺CD25⁺ regulatory T cells (Zheng et al., 2004), while anti-B7-1 antibodies have the opposite effect. However, several *in vivo* studies contradict the paradigm that B7-1 is mostly inhibitory while B7-2 is activating (Kuchroo et al., 1995; Li et al., 2000; Miller et al., 1995; Silver et al., 2000). These conflicting sets of data may be the result of changes in the Th1/Th2 balance that affect disease outcome or differential expression of B7-1 and B7-2 on target cells (Salomon and Bluestone, 2001). The majority of the reports, however, as well as our own data, support a model where B7-1 preferentially binds CTLA-4 and as a consequence inhibits T cell function, while by being the favored CD28 ligand, B7-2 can effectively promote T cell responses (Sansom et al., 2003). However, the fact that B7-1 knockout mice do not suffer from the lymphoproliferative syndrome that afflicts CTLA-4-deficient mice underscores the fact that this preference is not absolute, and that B7-2/CTLA-4 binding can be sufficient to prevent lymphoproliferation (Borriello et al., 1997; Freeman et al., 1993).

Recently, several groups have provided a mechanism for differential binding of CD28 and CTLA-4 to B7-1 and B7-2, which derives from structural and physicochemical experiments. Three crystallographic studies have shown that both in the case of human and mouse molecules, the two ligand binding domains of a CTLA-4 dimer lay distal to the dimerization interface, and as a result could promote the formation of a periodic structure where one CTLA-4 dimer binds two dimers of B7-1 or B7-2. These proteins could then extend the hypothetical protein lattice further by binding additional CTLA-4 molecules (Ostrov et al., 2000; Schwartz et al., 2001; Stamper et al., 2001). Subsequent crystallographic data, however, suggested that unlike human B7-1, human B7-2 is monomeric (Zhang et al., 2003). In addition, recent SPR measurements using human proteins demonstrated that while B7-2 exists as monomers, B7-1 tends to form dimers and that with respect to B7 binding, CD28 homodimers are monovalent, while CTLA-4 dimers are divalent (Collins et al., 2002). This study demonstrated that B7-1 markedly favors binding to CTLA-4 over CD28, while B7-2 displays much less bias. Thus, the ratio of engaged CD28 to engaged CTLA-4 at the T cell-APC interface will likely be higher when B7-2 is present on the APC relative to when B7-1 is expressed (Collins et al., 2002). Our own experiments show that B7-2 preferentially concentrates CD28 at the immunological syn-

apse while B7-1 favors the synaptic localization of CTLA-4, indicating that the differences predicted by the binding studies are realized in the assembly of the synapse: while both CD28 and CTLA-4 can bind B7-1 and B7-2, the stronger avidity of CTLA-4 for B7-1 leaves only B7-2 available for binding to CD28.

We have previously shown that the balance of CD28 and CTLA-4 signals can determine not only the level but also the composition and function of the emerging T cell response (Kuhns et al., 2000). Our findings here underscore the complexity of the dynamic mechanisms by which costimulatory signals mediated by CD28 and CTLA-4 can orchestrate T cell responses at the level of individual T cell clones. The relative levels of B7-2 and B7-1 on antigen-presenting cells is determined by the activation status of the APC, which is influenced by components of both the innate (toll-like receptors) and acquired (cytokines and CD40/CD40L) immune systems. The degree to which CTLA-4 modulates the response is in turn affected by the strength of the TCR signal, as well as the relative amounts of B7-1 and B7-2 available for binding. The preferential association of CTLA-4 with B7-1 and CD28 with B7-2, based on differential avidities, provides for a previously unrecognized level for fine tuning of stimulatory and inhibitory signals.

Experimental Procedures

Cells

The mouse B10.A B cell lymphoma CH12.Lx (Arnold et al., 1983), a gift from Dr. William Sha (UC Berkeley), was subcloned, and a line with moderate to high expression levels of H2-I-E^b, B7-1, B7-2, and ICAM-1 was selected. This line and the primary mouse T and B cells were maintained in RPMI 1640 (BioWhittaker), supplemented with 10% fetal calf serum (FCS, Sigma), 2 mM L-glutamine (BioWhittaker), 100 U/ml penicillin and streptomycin (BioWhittaker), and 2 μ M 2-mercaptoethanol (Sigma). The packaging line Phoenix-E (Kinsella and Nolan, 1996) was maintained in DMEM (BioWhittaker), supplemented with 10% FCS, 2 mM L-glutamine, and 100 U/ml penicillin and streptomycin.

Mice, T and B Cell Activation

Recombination activating gene-deficient (RAG^{-/-}) 5C.C7 (V α 11V β 3) TCR transgenic mice on the B10.A background (Taconic), which express a TCR specific for moth cytochrome c peptide MCC (88–103, ANERADLIAYLKQATK) in the context of H2-I-E^b, were bred to homozygosity with B10.A CD28^{-/-} mice, a gift from Dr. Ron Schwartz (NIH), producing 5C.C7 CD28^{-/-} RAG^{-/-} animals. OT-II (V α 2V β 5) TCR transgenic RAG^{-/-} mice (Barnden et al., 1998), which express a TCR specific for hen egg ovalbumin peptide OVA (323–339, ISQAV HAAHAEINEAGR) in the context of H2-I-A^b, were from Dr. William R. Heath (Royal Melbourne Hospital). B10.A, C57/BL6 (termed B6), and B6 mice that were deficient either for CD80 (B7-1^{-/-}), CD86 (B7-2^{-/-}), or both (B7-1^{-/-} B7-2^{-/-}) were purchased from Jackson Laboratories. All mice were bred and maintained in accordance with the animal care and use regulations of UC Berkeley.

5C.C7 or 5C.C7 CD28^{-/-} transgenic lymph node cells were stimulated with 5 μ M MCC peptide in the presence of irradiated (2000 rads) B10.A splenocytes. Similarly, OT-II transgenic lymph node cells were stimulated with 10 μ g/ml OVA peptide in the presence of irradiated B6 splenocytes. T cells were expanded in the presence of 30 U/ml recombinant human IL-2 (Chiron) and infected with retroviruses on days 3 and 4.

B cells were purified from spleens of B6, B7-1^{-/-}, B7-2^{-/-}, or B7-1^{-/-}B7-2^{-/-} animals via negative selection using Mouse pan T (Thy 1.2) Dynabeads (Dyna). They were activated with 30 μ g/ml LPS (Sigma) and used for experiments 60–84 hr later. B7-1 and B7-2 were detected using phycoerythrin (PE)-conjugated hamster anti-B7-1 and rat anti-B7-2 antibodies (BD Biosciences).

CFP/YFP Fusion Constructs and Retroviruses

C-terminal fusions of mouse CD28, CTLA-4, and Ick to CFP and YFP were generated via PCR that deleted their stop codons and introduced a 50-amino-acid flexible linker (10x Ser₃Gly₄) between the proteins and the fluorescent tags. The point mutations in the MYPPPY ligand binding motif of CD28 (Y123A) and CTLA-4 (Y139A), as well as the Y201F mutant of CTLA-4, were generated by PCR using the QuikChange site-directed mutagenesis kit (Stratagene). After sequencing, the protein fusions were cloned into a mouse stem cell virus (MSCV) retroviral vector, a gift from Dr. William Sha (Hawley et al., 1994).

Day 3 activated T cells were infected with mixtures of CFP and YFP retroviruses, produced separately by transient transfections of the Phoenix-E packaging line. To increase virus production, the cells were additionally transfected with plasmids encoding ecotropic envelope and gag-pol proteins, a gift from Dr. Michael Curran (UC Berkeley). Infection was performed by centrifugation at 1200g using filtered retroviral supernatants, supplemented with 4 μ g/ml polybrene (Sigma) and 25 mM HEPES (Irvine Scientific). Infected T cells were used 2–4 days later.

T Cell Staining and Flow Cytometry

A fusion of mouse B7-2 to human IgG1 (B7-2Ig) was produced using an inducible *Drosophila* expression system (Zang et al., 2003). The recombinant mouse B7-1/Fc chimera (B7-1Ig) was purchased from R&D Systems, Inc.

After incubation with the anti-Fc receptor antibody 24G2, 5C.C7 CD28^{-/-} T cells infected with one of the YFP fusions were stained with 50 μ g/ml B7-1Ig or B7-2Ig in PBS-2% FCS for 1 hr at 4°C, followed by a 30 min incubation with PE-conjugated anti-human Ig Fc (Beckman Coulter). To compare protein surface levels, cells were stained with 2 μ g/ml biotin-conjugated hamster anti-mouse CD28 antibody (Caltag) or 2.5 μ g/ml biotin-conjugated hamster anti-mouse CTLA-4 antibody (eBioscience) in PBS-2% FCS for 30 min at 4°C, followed by a 30 min incubation with 1 μ g/ml PE-conjugated streptavidin (eBioscience). The cells were fixed in 4% paraformaldehyde and analyzed on EPICS XL-MCL analytical cytometer (Coulter).

Conjugate Formation and Fluorescence Microscopy

The CH12.Lx B cell lymphoma or activated B cells from wt B6, B7-1^{-/-}, B7-2^{-/-}, or B7-1^{-/-}B7-2^{-/-} mice were incubated for 5 hr either with 5 μ M MCC (CH12.Lx) or 10 μ g/ml OVA peptide (the remainder). Conjugates between the peptide-pulsed APC and the T cells were formed for 20 min at 37°C, subsequent to which the cells were adhered to Superfrost Plus positively charged slides for 5 min (Fisher Scientific), fixed in PBS-4% paraformaldehyde for 30 min, and permeabilized with PBS-0.15% Triton X-100 (Sigma) for 3 min. Unreacted aldehyde groups were quenched with 0.25% NH₄Cl and the cells were blocked overnight at 4°C with PBS-5% normal donkey serum (NDS, Jackson Immunoresearch Laboratories). PKC θ was visualized by 1 hr incubation with rabbit polyclonal anti-PKC θ antibody (C-18, Santa Cruz Biotechnology) in PBS-2% NDS, followed by 1 hr incubation with Cy3- or Cy5-conjugated donkey anti-rabbit serum (Jackson Immunoresearch Laboratories) in PBS-1% NDS. The cells were washed and mounted in Slowfade Light antifade (Molecular Probes).

Cells were imaged on a Leica DMIRB/E inverted microscope using a 1.32 NA 63 \times objective. Fluorescence was excited with a 150W xenon arc lamp (Optiquip). CFP and YFP were detected with XF114 and XF30 filters (Omega Optical), respectively. Image stacks consisting of 10–15 planes spaced 0.5 μ m were collected with a Quantix CCD camera (Roper Scientific), operated by Slidebook software (Intelligent Imaging Innovations). The image stacks were deconvolved using the nearest neighbor algorithm of Slidebook.

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