CTLA-4 Differentially Regulates the Immunological Synapse in CD4 T Cell Subsets

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CTLA-4 Differentially Regulates the Immunological Synapse in CD4 T Cell Subsets

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Primary murine Th1 and Th2 cells differ in the organization of the immunological synapse, with Th1 cells, but not Th2 cells, clustering signaling molecules at the T cell/APC synapse site. We sought to determine whether differential costimulatory signals could account for the differences observed. We found that Th2 cells express higher levels of CTLA-4 than Th1 cells, and demonstrated that Th2 cells lacking CTLA-4 are now able to cluster the TCR with the same frequency as Th1 cells. Furthermore, reconstitution of CTLA-4 into CTLA-4-deficient Th2 cells, or into Th1 cells, inhibits the clustering of the TCR. We have also shown that Th2 cells, but not Th1 cells, show variations in the organization of the immunological synapse depending on levels of expression of CD80/CD86 on the APC. These studies demonstrate a unique role for CTLA-4 as a critical regulator of Th2 cells and the immunological synapse.

In these studies, we sought to understand the mechanism controlling the differential organization of the immunological synapse in Th1 and Th2 cells. Specifically, we asked whether differential costimulatory signals, particularly CD28 and CTLA-4, could be responsible for the observed differences in synapse organization in these two effector subsets. We have shown in this study that Th2 cells express higher levels of CTLA-4 than Th1 cells and that this increased CTLA-4 expression is responsible for the observed differences in synapse organization. In the absence of CTLA-4, Th2 cells that fail to cluster the TCR upon activation in the presence of CTLA-4 now cluster the TCR with the same frequency as Th1 cells. Furthermore, reconstitution of CTLA-4 into CTLA-4-deficient Th2 cells, or into Th1 cells, inhibited the clustering of the TCR. In addition, our data indicate that Th2 cells, unlike Th1 cells, vary the organization of the immunological synapse depending on the levels of CD80/CD86 expression on the APC, suggesting that the type of APC, or the degree to which that APC is activated, might determine the type of synapse that is formed.

**Materials and Methods**

**Antibodies**
Anti-CD4 (GK1.5), anti-CD8 (TIB 210), anti-Thy-1 (Y19), anti-class II MHC (212A1, TIB 92, or 14.4.4), anti-CD3/216 (24G2), anti-IFN-γ (XMG1.2), and anti-B220 (TIB 164) were purified from culture supernatants on protein G columns and dialyzed against PBS before use. Biotin and FITC anti-β2-microglobulin (K252), purified anti-CTLA-4 (UC10-4F10-11), PE anti-IFN-γ (XM1G2), allophycocyanin anti-IL-4 (11B11), FITC anti-Ve11 (RR8-1), biotin and FITC anti-CD8 (RA3-6B2), allophycocyanin anti-CD11c (HL3), PE anti-I-Eα (14-4-4S), PE anti-ICAM-1 (32E), FITC anti-CD80 (16-10A1), purified anti-CD80 (1G10/B7), purified and FITC anti-CD86 (GL1), PE anti-CD86/2 (53-5.8), PerCP anti-CD4 (RM4-5), and biotin anti-hamster IgG (G70-204 and G94-56) were purchased from BD Pharmingen; purified anti-talin (C20) was purchased from Santa Cruz Biotechnology. Alexa-488 anti-FITC, streptavidin Alexa-488, Alexa-594 anti-goat, Alexa-647 anti-mouse, and streptavidin Alexa-647 were purchased from Molecular Probes.

**Mice**

B10.BR mice were obtained from The Jackson Laboratory. The AND TCR transgenic mice have been previously described (24). C57BL/6 CTLA-4−/−, CD80−/−, and CD86−/− mice were received from A. Sharpe (Harvard Medical School, Boston, MA). These mice were crossed onto B6 AND TCR transgenic and B6 Rag2−/− mice to generate B6 Rag−/− AND TCR Tg CTLA-4−/− and B6 Rag−/−AND TCR Tg CTLA-4−/− mice. All mice used to generate CD4 T cells in these studies were 6–10 wk old. All animal protocols were approved by Yale’s Institutional Animal Care and Use Committee 2004-10393.

**Peptides**

The peptide used in these studies is derived from moth cytochrome c (MCC; peptide 81–103). MC3Q = VFAGLKKTRANERALAYLQKATK. Peptide was synthesized and purified by HPLC by the W. M. Keck Foundation Biotechnology Resource Laboratory.

**Preparation of APCs, CD4+ T cells, and bone marrow-derived dendritic cells (BMDCs)**

T cell-depleted APC were prepared by Ab-mediated complement lysis of B10.BR splenocytes, as previously described (25). CD4+CD8− T cells from lymph nodes and spleens of transgenic mice were isolated using immunomagnetic negative selection, as previously described (25). BMDCs were generated from B10.BR bone marrow cultured with GM-CSF, as previously described (26). These cells were cultured without further stimuli and used on day 7, by which time the cells have begun to mature.

**Generation and activation of effector T cells**

Th1 and Th2 cells were generated by in vitro cytokine skewing, as previously described (27). Effector T cells were restimulated by coculture with T-depleted splenocytes from B10.BR mice pulsed with 50 μM indicated peptide, as previously described (13).

**B cell activation**

T cell-depleted splenecells were treated with 30 μg/ml LPS for 72 h and used to restimulate effector T cells, as described above.

**CD80/CD86 blocking**

T cell-depleted APC were incubated with a mixture of anti-CD80 and anti-CD86 Abs at a concentration of 5 μg/ml (unless specified) for 15 min at room temperature, following peptide pulse and before use in stimulation of T cells.

**Immunocytochemistry and microscopy**

T cells were mixed with peptide-pulsed APC, as described above, for 10 min at 37°C. T cell/APC conjugates were then placed on Alcian blue-coated coverslips in serum-free medium and incubated for 30 min at 37°C to permit adherence. Cells were fixed in ethanol, permeabilized with saponin, and stained, as previously described (28). Analysis was done using a conventional Zeiss microscope and the Openlab software (Improvision).

**Flow cytometry analysis**

For intracellular cytokine staining, cells were restimulated for 4 h with 0.1 μg/ml PMA and 0.75 μg/ml ionomycin in the presence of GolgiStop and permeabilized using the BD Cytofix/Cytoperm kit following their standard protocol (BD Biosciences). Ethidium monoxide was used to distinguish and exclude dead cells (Invitrogen Life Technologies).

**Retroviral transduction**

CTLA-4 cDNA was purchased from American Type Culture Collection (accession BC042741), and modified by PCR to introduce BglII (5′) and 24 bp (3′) restriction sites to subclone into pMSCV retroviral expression vector (provided by K. Murphy, Washington University, St. Louis, MO), which contains a cassette with GFP and an internal ribosome entry site permitting the translation of two open reading frames from one mRNA. The plasmids containing CTLA-4 internal ribosome entry site-GFP or GFP alone were transfected into Phoenix Ecotropic packaging cells (provided by G. Nolan, Stanford University, Stanford, CA), and 24 h later, were transferred to 30°C for 48 h and low viral production. The retroviral supernatants were used to spin infect Th1 or Th2 effector cells (2000 rpm for 90 min) 48 h following restimulation with peptide and APCs. Cells were then incubated at 37°C for 96 h, rested for 48 h, and sorted for GFP-positive cells using the FACSVantage SE cell sorter (BD Biosciences). Approximately 10% of cells were GFP+. Cells were analyzed for CTLA-4 expression by flow cytometry. Sorted GFP+ cells were then restimulated with MCC-pulsed syngeneic APC and analyzed by fluorescence microscopy, as described above.

**Generation of Th1 and Th2 cells in vivo**

Sensitization and challenge were performed, as previously described (29–31). For intranasal sensitization, mice were lightly anesthetized with isoflurane and challenged intranasally with 100 μg of pOVA protein (grade V; Sigma-Aldrich) in 50 μl of PBS on days 0, 1, and 2, as previously described. For low-dose LPS treatment, 0.05 μg of *Escherichia coli* LPS O55:B5 (Sigma-Aldrich) was used on each day of sensitization. For high-dose LPS treatment, 20 μg of LPS was used. All mice were lightly anesthetized with isoflurane and challenged intranasally with 25 μg of pOVA in 50 μl of PBS on days 14, 15, 18, and 19, and sacrificed on day 21. Mediastinal lymph node cells were isolated and allowed to rest in culture for 5 days. These cells were then restimulated with PMA and ionomycin in the presence of GolgiStop for 4 h, then fixed, permeabilized, and stained for intracellular IL-4, IFN-γ, and CTLA-4, as described above.

**Statistical analysis**

To demonstrate significance of TCR clustering quantitation, 95% confidence intervals were calculated and are shown as error bars. Nonoverlapping error bars indicate significance at α = 0.05 level. Values of p were calculated using binomial distribution analysis. For conjugate quantitation, a minimum of 10 fields and 50 conjugates was counted for each group.

**Results**

Th1 and Th2 cells differ in immunological synapse organization

As shown in Fig. 1, and as we have previously published, unlike Th1 cells, Th2 cells do not cluster the TCR, CD4, and PKCθ at the site of T cell/APC contact when stimulated with primary B cells and peptide (13). In these studies, Th1 and Th2 cells were prepared from AND TCR transgenic mice and tested for their ability to
Therefore, whereas both Th1 and Th2 cells are able to form active APC conjugates, not Th2/APC conjugates (data not shown).

**FIGURE 1.** Th1 and Th2 cells differ in their organization of the immunological synapse. Th1 and Th2 cells were generated by cytokine skewing of CD4 T cells from AND TCR transgenic mice, as previously described (27), and were stimulated with peptide-pulsed APCs. T cell/APC conjugates were stained with anti-Vβ3, anti-talin, and anti-B220. TCR and talin localization in T cell (Vβ3)+/B cell (B220+) conjugates (A) and T cell/B220+ cell conjugates (B). Conjugates that clustered talin at the T cell/B cell or T cell/B220+ cell synapse site were examined for TCR clustering. Shown here is the percentage of these conjugates in which the TCR clusters at the T cell/APC synapse site. Difference in TCR-clustering frequency between Th1 and Th2 cells is significant with p < 0.0001. Error bars mark 95% confidence intervals. For all experiments, a minimum of 10 fields and 50 active T cell/APC conjugates was counted. This experiment was repeated more than five times.

Cluster the TCR, PKCθ, and talin at the T cell/APC interface. Talin is a molecule that activates LFA and links it to the cytoskeleton, and is used in this study as a functional indicator of active conjugation between the T cell and its APC (32–35). Of the conjugates between Vβ3+ and B220+ cells defined by clustered talin, only 17% of Th2 cells had clustered TCR as compared with 55% of Th1 cells (Fig. 1, A and C). There was no difference in the number of conjugates observed between Th1 and Th2 cells, with both subsets clustering talin at the T cell/B cell synapse site with equal frequency (data not shown). Similarly, PKCθ only clustered in Th1/APC conjugates, not Th2/APC conjugates (data not shown). Therefore, whereas both Th1 and Th2 cells are able to form active conjugates with resting B cells, only Th1 cells are able to cluster the TCR and PKCθ under these conditions.

**Th2 cell synapse organization varies with APC type**

We have observed that B220+ APCs from the spleen appear to be capable of signaling both Th1 and Th2 cells to cluster the TCR at the synapse site (Fig. 1, B and C). In our splenic APC preparations, these events are rare (only ~2% of the cells are B220+ and class II MHC+), making it difficult to gather quantitative information on synapses with these cells. Therefore, we asked whether Th2 cells could cluster the TCR at the T cell/APC interface upon activation with other APCs, such as dendritic cells (DCs). To address the ability of Th2/DC conjugates to aggregate the TCR at the conjugation site, we stimulated Th1 and Th2 cells with peptide-pulsed BMDCs and examined conjugates between CD4 T cells and class II MHC-positive BMDCs. Similar to what we observed with T cell/B cell conjugates, both Th1 and Th2 cells clustered talin at the T/DC contact site (Fig. 2A). In contrast to the results observed with resting B cells, the TCR (Vβ3) localized to the T/DC contact site with both Th1 and Th2 cells (Fig. 2, A and B). As has been seen previously, it was common to see multiple T cells actively conjugated with a single DC in which all or many of the T cells had clustered the TCR at the site of contact (36).

Because other studies have shown clustering of signaling molecules at the CD4 T cell/APC site between Th2 clones and B cell lymphomas (1), we asked whether our primary Th2 cells would cluster the TCR when stimulated with activated B cells. B cells were activated with LPS for 72 h, pulsed with peptide, and then used to stimulate Th1 and Th2 cells. Under these conditions, unlike what was seen with resting B cells, both Th1 and Th2 cells clustered the TCR at the CD4 T cell/APC contact site with the same frequency (Fig. 2, C and D). Therefore, although Th2 cells do not cluster the TCR at the conjugation site with resting B cells, they are able to do so when stimulated with either mature BMDCs or activated B cells.

**CD80/CD86 regulate synapse organization**

We observed aggregation of the TCR at the synapse site in Th2 cells only when stimulated with peptide presented by activated B cells and BMDCs, but not resting B cells. Because CD80/CD86 interactions with CD28 and CTLA-4 have been shown to be important in T cell activation and cell membrane organization, we asked whether differential CD80/CD86 expression might be responsible for the observed difference in TCR clustering in Th1 and Th2 cells. In keeping with previous accounts, we observed that both resting and activated B cells, as well as activated BMDCs, express high levels of class II MHC and ICAM-1, but differ in CD80/CD86 expression, with activated B cells, B220+ splenocytes, and class II MHC+ BMDCs expressing higher levels of both CD80 and CD86 than resting B cells (data not shown). Because costimulatory molecules have been shown previously to be required for TCR clustering at the site of conjugation in naive T cells (17, 18, 37), we looked to see whether CD80/CD86 was required for TCR clustering in Th1 or Th2 cells. T cell-depleted splenocytes were cultured with LPS for 72 h, pulsed with peptide, then preincubated with either a mixture of anti-CD80 and anti-CD86 Abs or isotype control Abs before being used to stimulate T cells. Active T cell/B cell conjugates were examined by microscopy for clustering of the TCR. As seen in Fig. 3A, both Th1 and Th2 cells clustered the TCR at the site of contact, as expected, when the activated B cells were preincubated with an irrelevant isotype control Ab (54% of Th1 cells and 53% of Th2 cells). When anti-CD80 and anti-CD86 Abs were used to block the interaction of CD80 and CD86 with their ligands, the frequency of cells in which clustering of the TCR at the site of conjugation was observed was significantly reduced for both Th1 and Th2 cells (Fig. 3B). This shows that costimulatory molecules (CD80/CD86) are required for efficient aggregation of the TCR at the conjugation site in both Th1 and Th2 effector cells. Furthermore, these data suggest that whereas Th1 cells are able to cluster the TCR at the site of
conjugation when stimulated with APCs that express low levels of CD80/CD86, Th2 cells require higher levels of costimulation from their cognate APCs.

To confirm that Th2 cells require higher expression of CD80/CD86 on their cognate APC than Th1 cells to cluster the TCR at the T cell/B cell synapse site, CD80/CD86 availability was varied by titrating the blocking CD80/CD86 Abs in vitro. LPS-stimulated B cells were preincubated with a mixture of anti-CD80 and anti-CD86 Abs at either 5, 0.01, or 0 μg/ml. Active T cell/B cell conjugates were examined by microscopy for clustering of the TCR, as described above. As was observed in Fig. 3B, in the absence of blocking Abs, both Th1 and Th2 cells clustered the TCR at the T cell/B cell synapse site, and in the presence of the highest concentration of blocking Abs, neither subset clustered the TCR efficiently (Fig. 3C). At the intermediate concentration of blocking Ab, Th1 cells maintained clustering at the synapse site, but Th2 cells did not (Fig. 3C). This confirms that whereas both Th1 and Th2 cells require CD80/CD86 on their cognate APC for optimal TCR clustering, Th2 cells require a higher expression level of these costimulatory molecules than Th1 cells.

**Th2 cells express higher levels of CTLA-4**

Because Th1 cells have a lower threshold than Th2 cells for CD80/CD86 signals required for TCR clustering, we investigated how CD80/CD86 might be playing a differential role in immunological synapse organization. Because CD80 and CD86 interact with both CD28 and CTLA-4, we examined expression of CD28 and CTLA-4 in Th1 and Th2 cells. CD28 levels were comparable in Th1 and Th2 cells (data not shown). CTLA-4 has been shown to rapidly relocalize to the cell surface (20), so we examined CTLA-4 levels by intracellular staining as surface levels alone might under-represent available CTLA-4 protein. As seen in Fig. 4A, CTLA-4 expression was significantly higher in resting Th2 cells (right panel) compared with resting Th1 cells (left panel). The same differences were observed following multiple rounds of stimulation (data not shown).
The expression of CTLA-4 on the cell surface has been shown to be tightly regulated, with CTLA-4 localized primarily in intracellular compartments in resting T cells (38–42). To determine whether any of the CTLA-4 present in Th1 or Th2 cells was on the cell surface, nonpermeabilized Th1 or Th2 cells were also stained for CTLA-4. As seen in Fig. 4B, resting Th2 cells constitutively expressed low levels of CTLA-4 on the cell surface (right panel), whereas no CTLA-4 was observed on the cell surface in Th1 cells (left panel). Relative mean fluorescent intensities of both surface and total CTLA-4 were calculated, expressed as a fold increase over the values for the matched isotype control (Fig. 4C). These values suggest that ~25% of the total CTLA-4 present in Th2 cells is expressed on the cell surface. Furthermore, the small amount of CTLA-4 expressed in Th1 cells appears to be exclusively intracellular.

FIGURE 3. CD80/CD86 molecules are critical in regulating the type of synapse formed. A, CD80/CD86 interactions were blocked on LPS-activated B cells using anti-CD80 and anti-CD86 Abs or isotype control Abs. These cells were used to activate Th1 and Th2 cells. B, Quantitation of TCR clustering at the T cell/B cell site with disruption of CD80/CD86 binding (as described in Fig. 1C). Difference in TCR-clustering frequency with isotype control vs blocking Abs is significant for both Th1 and Th2 cells with p < 0.0001. This experiment was repeated more than five times. C, A titration of CD80/CD86-blocking Abs was used to examine partial block of CD80/CD86. B cells were incubated with anti-CD80 and anti-CD86 Abs at 0, 0.01, or 5 μg/ml concentrations, and then used to stimulate Th1 and Th2 cells. Quantitation of TCR clustering at the T cell/B cell site with partial block of CD80/CD86 (as described in Fig. 1C). Difference in TCR-clustering frequency between Th1 and Th2 cells with 0.01 μg/ml Ab block is significant with p < 0.0001.

FIGURE 4. Th2 cells express higher levels of CTLA-4. A, Intracellular stain of resting Th1 and Th2 cells for CTLA-4 (gating on live T cells); isotype control in gray. B, Surface stain of resting Th1 and Th2 cells for CTLA-4; isotype control in gray. C, Relative mean fluorescent intensity of surface (black) and total (gray) CTLA-4 normalized to matched isotype control. D, Intracellular stain for IL-4” and IL-4” T cells. D, Intracellular stain for IL-4” and IL-4” T cells were compared (0.4% of Th1 cells and 27.3% of Th2 cells).
IFN-\(\gamma\) is in comparison with gating on the non-IL-4-producing cells (Fig. 4E). These cells were pooled, restimulated with PMA and ionomycin, and stained intracellularly for IL-4, IFN-\(\gamma\), and CTLA-4. A. Expression of IFN-\(\gamma\) and IL-4 in CD4\(^+\) cells. Isotype controls for these stains are shown on the left. Gates show cells used to compare CTLA-4 expression in B. B, CTLA-4 (right panel) and isotype control (left panel) stains of the IFN-\(\gamma^+\) and IL-4\(^+\) cells.

To confirm that we were looking at Th2 cells, and not contaminating Th1 or undifferentiated CD4 T cells, we examined levels of CTLA-4 expression in Th2 cells as defined by IL-4 expression. To accomplish this, we restimulated the Th1 cells (Fig. 4D, left panel) and Th2 cells (Fig. 4D, right panel) for 4 h and examined levels of CTLA-4 and IL-4 intracellularly. Gating on IL-4-producing Th2 cells (Fig. 4D, right panel), we analyzed expression of CTLA-4 on those cells (Fig. 4E, right panel). We observed enrichment for CTLA-4 expression on the IL-4\(^+\) cells (Fig. 4E, right panel). This is in comparison with gating on the non-IL-4-producing cells (Fig. 4E, right panel, IL-4\(^-\)), which expressed little CTLA-4. Therefore, in considering Th2 cells defined by IL-4 production, Th2 cells express higher levels of CTLA-4 than non-IL-4-producing cells. Th1 cells express very little IL-4 or CTLA-4. However, gating on the contaminating IL-4-producing cells from the Th1-skewed cells (only 0.4\% of the cells (Fig. 4D, left panel)) demonstrated that these cells also express high levels of CTLA-4 (Fig. 4E, left panel). Taken together, these data clearly demonstrate higher expression of CTLA-4 in Th2 cells than in Th1 cells.

To confirm our finding of higher expression of CTLA-4 in Th2 cells in vivo, we used a murine model of asthma using high- and low-dose LPS during sensitization to skew the immune response toward either a Th1 or a Th2 response, as previously described (29–31). Briefly, BALB/c mice were intranasally sensitized with OVA and either a high or low dose of LPS, and then challenged 2 wk later with intranasal OVA. Cells were harvested from the mediastinal lymph nodes and allowed to rest in vitro for 5 days; then the high- and low-dose groups were pooled and restimulated with PMA/ionomycin before examination for IFN-\(\gamma\), IL-4, and CTLA-4 expression by flow cytometry. IL-4-producing cells were compared with IFN-\(\gamma\)-producing cells for CTLA-4 expression. As can be seen in Fig. 5, the IL-4-producing cells expressed higher levels of CTLA-4 than the IFN-\(\gamma\)-producing cells. These results clearly demonstrate that Th2 effector cells generated in vivo express higher levels of CTLA-4 than Th1 effector cells.

**CTLA-4 regulates Th2 cell synapse**

To investigate whether the increased expression of CTLA-4 in Th2 cells interfered with the clustering of the TCR when stimulated with resting B cells, we generated Th1 and Th2 cells from CTLA-4-deficient TCR transgenic mice. CTLA-4-deficient mice develop lymphoproliferative disease if left untreated (43, 44), but CTLA-4,
CD80, and CD86 triple-knockout mice are healthy (45). CTLA-4, CD80, and CD86 triple-knockout mice were bred onto AND transgenic Rag2 knockout mice. Effective Th1/Th2 generation was confirmed using intracellular cytokine staining for IL-4 and IFN-γ (data not shown). Th1 and Th2 cells from CTLA-4-deficient and CTLA-4 heterozygous littermates were restimulated with resting B cells and then examined for TCR clustering. In the cells generated from theCTL A-4 heterozygous littermates, Th1 cells clustered the TCR at the site of T cell/B cell conjugation, but Th2 cells did not (Fig. 6, upper two rows, and B). In contrast, both Th1 and Th2 cells generated from the CTLA-4-deficient mice clustered the TCR with equal frequency (Fig. 6, A, lower two rows, and B).

To confirm the role of CTLA-4 in Th2 synapse organization, CTLA-4 was reintroduced into Th2 cells generated from CTLA-4-deficient mice. A retroviral construct was prepared containing wild-type murine CTLA-4 coexpressed with GFP. Th2 cells were generated from CTLA-4-deficient TCR transgenic mice, transduced with the viral construct during restimulation, and allowed to rest. GFP⁺ cells were sorted, stimulated with peptide-pulsed resting B cells, and examined for TCR clustering. Successful reintroduction of CTLA-4 was confirmed by flow cytometry (Fig. 7A). Th2 cells given the control virus (GFP alone) still clustered the TCR at a frequency consistent with previous experiments (Fig. 7, B and C). In contrast, Th2 cells in which CTLA-4 was restored lost the ability to cluster the TCR (Fig. 7, B and C). Therefore, CTLA-4 expression is both necessary and sufficient for the observed block in Th2 TCR clustering.

To investigate whether CTLA-4 could block the clustering of the TCR in Th1 cells, the same CTLA-4 construct was introduced into wild-type Th1 cells. GFP⁺ wild-type Th1 cells were sorted and stimulated with peptide-pulsed B cells. Interestingly, GFP⁺ cells had only a very small increase in CTLA-4 expression (Fig. 7D), but even with this small increase, there was a corresponding reduction in the frequency of TCR clustering when stimulated with resting B cells (Fig. 7, E and F).

To better understand the quantity of CTLA-4 that is required to block TCR clustering at the synapse site, we compared levels of expression of CTLA-4 under conditions in which the TCR did, or did not cluster. We used the viral transduction experiments
because they provide matched synapse and CTLA-4 expression data, and included both the Th2 and Th1 experiments. To allow for comparison between experiments, we normalized the mean fluorescent intensity of CTLA-4 against the matched isotype control stain in each experiment, providing a value representing relative expression of CTLA-4. We then averaged the values for conditions in which the TCR did, or did not cluster at the synapse site, which were 1.5 and 4.5, respectively. This represents a 3-fold increase, which is consistent with our observed differences in CTLA-4 expression between Th1 and Th2 cells at both the protein and RNA levels (data not shown).

**Discussion**

In this study, we have demonstrated that whereas both Th1 and Th2 cells cluster the TCR at the site of contact with activated APCs, only Th1 cells are able to do so with resting B cells. Although both Th1 and Th2 cells require CD80/CD86 for optimal TCR clustering, the difference in synapse organization can be explained by the higher expression level of CTLA-4 in Th2 cells, including expression at the cell surface. This increased expression of CTLA-4 inhibits TCR clustering. This suggests a model in which low-level expression of CD80/CD86 on resting B cells is sufficient to provide the activating signals via CD28 on Th1 cells needed for aggregation of the TCR. Higher levels of CTLA-4 expression on Th2 cells, however, lead to inhibitory signals when CD80/CD86 expression levels on the APC are low, resulting in a loss of TCR clustering. When the expression of CD80/CD86 is increased, as is observed on activated B cells or DCs, dominant CD28 signaling is restored, leading to clustering of TCR molecules.

We have demonstrated in this study a new role for CTLA-4 as the critical regulator of Th2 immunological synapse organization and are the first to demonstrate a differential role for CTLA-4 in different subsets of primary T cells. In contrast to wild-type Th2 cells, which fail to cluster the TCR when stimulated with resting B cells, TCR clustering occurs in Th2 cells generated from CTLA-4-deficient mice. The subsequent loss of TCR clustering when CTLA-4 is reintroduced into these cells demonstrates that this is a function of CTLA-4. We have also shown that APC types vary in their ability to cluster the TCR at the site of conjugation with Th2 cells, which may have important implications in vivo. This is critical, because many studies on immunological synapse formation have used cell lines as APCs, or other artificial stimuli, which may fail to detect these differences. For example, in some of the early synapse studies, B cell lymphomas were used to stimulate D10 cells (a Th2 clone) (1). Given our data with activated B cells, it is not surprising that a B cell lymphoma would yield similar results, masking the differences we have observed between Th1 and Th2 cells.

This model fits with what we know about the differing abilities of CTLA-4 and CD28 to bind to their shared ligands, CD80 and CD86. CTLA-4 is able to bind to both CD80 and CD86 with higher affinities and avidities than CD28. Binding studies have found that CTLA-4 has ~20-fold higher affinity for CD80 and ~8-fold higher affinity for CD86 when compared with CD28 (23). In addition, both structural and binding studies have shown that whereas CD28 forms monovalent dimers, CTLA-4 forms bivalent dimers that enable it to form highly stable lattice structures with CD80 and CD86, resulting in an increase in its avidity for these molecules by ~100-fold (21–23). The combination of this increased avidity and affinity gives CTLA-4 a binding advantage of ~3 orders of magnitude over CD28. This accounts for our observation at limiting CD80/CD86 molecule expression where CTLA-4 inhibition of clustering occurs.

The difference we observed in CTLA-4 levels between Th1 and Th2 cells may play additional roles in the differential regulation of signaling and activation between these two effector subsets. We have looked at cytokine production by wild-type Th2 cells stimulated with resting vs activated B cells, and also compared cytokine production between CTLA-4-deficient or -intact Th2 cells, to see whether there is a correlation between synapse organization and cytokine production. As expected, we found that increased IL-2 production correlated with increased CD28 signals, as has been previously published (46), but saw no additional differences (data not shown). In addition to differences in cytokine production, Th2 cells have been shown to have defects in tyrosine phosphorylation of Fyn and ZAP70, in sustained calcium mobilization, and in ability to respond to low-affinity peptide/MHC (13, 47–50). Some of these differences could be the result of differences in relative levels of CTLA-4 in effector Th2 vs effector Th1 or naive CD4 T cells. We have shown that the higher levels of CTLA-4 in resting Th2 cells block the clustering of the TCR at the B cell conjugation site. This lack of clustering could lead to a reduction in avidity that would block full activation of the Th2 cell when the TCR affinity is low. Th2 differentiation has been associated with lower affinity TCR interactions (13, 51, 52). If the normal Th2 TCR repertoire is of lower specificity, then Th2 cells might only be able to be fully activated by APCs that have received innate activation signals leading to increased expression of CD80/CD86 that enable them to trigger clustering of the TCR. In this way, CTLA-4 may act as an additional level of control over Th2 responses. Our work suggests that the APC may influence other Th2 signaling events, and that the differences in signaling observed between Th1 and Th2 might be overcome when different APCs are used to stimulate.

Our findings provide an interesting mechanism of control unique to Th2 cell/B cell interactions, in which a B cell must first receive an activation signal itself before it is able to signal the clustering of the TCR at the site of contact with its cognate Th2 cell. This extra activation step is not required in Th1 cells, suggesting a unique function for immunological synapse organization in Th2/B cell interactions, and possibly a need for tighter control via innate immune signals.

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**Disclosures**

The authors have no financial conflict of interest.

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