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A Role for CD28 in Lymphopenia-Induced Proliferation of CD4 T Cells

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The peripheral mechanisms that regulate the size and the repertoire of the T cell compartment during recovery from a lymphopenic state are incompletely understood. In particular, the role of costimulatory signals, such as those provided by CD28, which have a critical importance for the immune response toward foreign Ags in nonlymphopenic animals, has been unclear in lymphopenia-induced proliferation (LIP). In this study, we show that accumulation of highly divided CD4 T cells characterized by great potential to make IFN-γ is significantly delayed in the absence of B7-CD28 costimulation during LIP. Furthermore, CD28-sufficient CD4 T cells show great competitive advantage over CD28-deficient CD4 T cells when transferred together into the same lymphopenic hosts. Administration of CTLA-4-Ig removed this competitive advantage. Interestingly, CTLA-4-Ig treatment resulted in modest inhibition of LIP by CD28-deficient responders, suggesting that some of its effects may be independent of mere B7 blockade. The Journal of Immunology, 2004, 173: 3909–3915.
Adoptive transfer and cell preparations

Unless otherwise specified, donor T cells were collected from secondary lymphoid tissues (axillary, brachial, cervical, mesenteric, and inguinal lymph nodes, and spleen) and labeled with CFSE (Molecular Probes, Eugene, OR) by using a technique previously described (12). CD44low CD4 T cells were purified in two stages—first, CD4 T cells were prepared by negative selection against CD8, MHC class II, and CD11b (all Abs labeled with FITC) using anti-FITC BioParticles (Polysciences, Warrington, PA), followed by depletion of CD44hi cells using magnetic microbeads (Miltenyi Biotec, Auburn, CA), as previously described (5). Briefly, the purified CD4 T cells were labeled with anti-CD44-FITC (eBioscience, San Diego, CA), 0.004 µg per 10^7 cells suspended in 100 µl of labeling buffer (2% FCS in PBS), washed, and labeled with anti-FITC magnetic microbeads. The negative fraction was collected following Miltenyi Biotec magnetic column separation. The same protocol was used to prepare CD44low CD8 T cells. CD25+ CD4 T cells (>95% purity) were prepared using positive selection with anti-CD25 biotinylated mAb, PC61, and streptavidin-labeled magnetic microbeads (Miltenyi Biotec). The control CD25− CD4 T cells were prepared by further depletion of the CD25− fraction for CD8, MHC class II, CD11b, DX5, and glucocorticoid-induced TNF-related gene using biotinylated Abs and streptavidin-labeled magnetic microbeads. The DTA-1 hybridoma, specific for glucocorticoid-induced TNF-related gene, was a generous gift from Dr. S. Sakaguchi (Kyoto University, Kyoto, Japan).

In some of the experiments, mice were treated with CTLA-4-Ig or anti-CTLA-4 mAb (4F10), both reagents administered i.v.; 250 µg per mouse, every 5 days. Some mice were depleted of the last 2 h of stimulation. The cells were then washed and fixed in 2% formaldehyde, and intracellular staining was done in a permeabilization buffer containing 0.3% saponin and 25% FCS. Ag-induced cytokine production by DO11.10 T cells was measured directly ex vivo. Splenic and lymph nodes were taken from euthanized animals 1 h after they received an i.v. injection of the OVA323−339 peptide, and immediately fixed in 2% formaldehyde. The cells were stained for flow cytometry in the permeabilization buffer with PE-labeled anti-IL-2, IFN-γ, IL-4, or isotype control Abs (eBioscience; BD Biosciences, Mountain View, CA); PerCP-cyanin 5.5-labeled anti-CD4 (BD Biosciences), and allophycocyanin-labeled KJ1-26 (Caltag, Burlingame, CA).

Results

CTLA-4-Ig treatment inhibits LIP by CD4, but not CD8 T cells

The role of CD28 costimulation in LIP was previously tested using CD28-deficient responder T cells (5) or B7 blockade with CTLA-4-Ig (7). Although both experimental approaches showed that CD28 signals are not essential for LIP, CTLA-4-Ig treatment inhibited the high degree of proliferation (greater than six to eight cell divisions) exhibited by a subset of naive DO11.10 T cells. One formal possibility that could explain this result is that DO11.10 TCR had some cross-reactivity to a true Ag. In that case, B7 blockade may have had no effect on pure LIP, but merely blocked Ag-driven proliferation. This possibility is unlikely for several reasons—the investigators used DO11.10 RAG−/− responder T cells, the fraction of highly divided cells was inversely proportional to the starting population (7), and the proliferative burst of DO11.10 responders occurs following a prolonged (up to 2 wk) period of relative inactivity (11). To test whether this pattern is generalizable, we tested susceptibility to CTLA-4-Ig treatment of CFSE-labeled responder T cells from another TCR Tg, HA RAG−/−. Although certain features distinguish the pattern of proliferation for HA T cells when compared with DO11.10 T cells, e.g., shorter latency period before the first cell division and prominent presence of intermediate peaks for CFSE content (11), the size of the highly divided fraction was again noted to be inversely proportional to the number of transferred cells (Fig. 1A). Furthermore, administration of CTLA-4-Ig markedly inhibited the rate of cell division, and especially reduced the size of the highly divided fraction (Fig. 1B).

To test whether effects of CTLA-4-Ig can be attributed solely to blockade of CD28 signals, we measured proliferation of CD28-deficient and CD28-sufficient DO11.10 T cells transferred into RAG−/− recipients. As reported previously (5), proliferation of CD28−/− DO11.10 appeared generally similar to CD28+ DO11.10 T cells (Fig. 2). However, as reported by Gudmundsdottir and Turka (7), CTLA-4-Ig treatment inhibited LIP of wild-type DO11.10 RAG−/− cells, and virtually eliminated the high-divider fraction (Fig. 2). Interestingly, treatment with CTLA-4-Ig also resulted in relatively limited, but very consistent inhibition of LIP by CD28−/− responder CD4 T cells, again affecting most prominently the fraction of most highly divided cells (Fig. 2). A similar experiment was done using polyclonal CD44low CD4 T cell responder T cells, where wild-type (Thy1.1) and CD28−/− (Thy1.2) CD4 T cells were cocentransferred into RAG−/− recipients. Although the two
were left untreated, and some of which were treated with CTLA-4-Ig. Histograms show CFSE content of indicated responder cells on day 8 after adoptive transfer. The numbers above the histograms show the geometric mean fluorescence intensity in FL-1. The figure also shows the relative ratios of transferred wild-type and CD28−/− CD4 T cells on the indicated days in the spleens of CTLA-4-Ig-treated and untreated animals. Similar data were seen in the lymph nodes. This experiment is representative of three independent experiments done using C57BL/6 and BALB/c strains.

FIGURE 3. CTLA-4-Ig treatment inhibits LIP of polyclonal naive CD4 T cells. Purified CD44low CD28-sufficient and CD28-deficient CD4 T cells were mixed together, labeled with CFSE, and transferred into CD45.1 RAG−/− mice (4 × 10^6 total CD4 T cells per recipient), some of which were left untreated, and some of which were treated with CTLA-4-Ig. Histograms show CFSE content of indicated responder cells on day 8 after adoptive transfer. The numbers above the histograms show the geometric mean fluorescence intensity in FL-1. The figure also shows the relative ratios of transferred wild-type and CD28−/− CD4 T cells on the indicated days in the spleens of CTLA-4-Ig-treated and untreated animals. Similar data were seen in the lymph nodes. This experiment is representative of three independent experiments done using C57BL/6 and BALB/c strains.

Presence of B7 promotes LIP of CD28-sufficient, but not CD28-deficient CD4 T cells

To focus our attention solely on the role of B7 costimulatory signals and considering that CTLA-4-Ig treatment may work via additional inhibitory mechanisms, we compared the ability of polyclonal CD44<sup>low</sup> CD28-sufficient and CD28-deficient CD4 T cells to proliferate in irradiated wild-type, B7-deficient, and CD28-deficient hosts following a cotransfer. Both responder populations were distinguished from host cells and each other by congenic markers, CD45.1 and Thy1.1, respectively. Once again, the CFSE fluorescence intensity in FL-1. The figure also shows the relative ratios of transferred wild-type and CD28−/− CD4 T cells on the indicated days in the spleens of CTLA-4-Ig-treated and untreated animals. Similar data were seen in the lymph nodes. This experiment is representative of three independent experiments done using C57BL/6 and BALB/c strains.

Potential role for regulatory CD25<sup>+</sup> CD4 T cells in controlling LIP

In the experiments just described, we consistently found a greater degree of LIP in irradiated CD28-deficient hosts compared with wild-type hosts. We considered that at least one reason for the difference may be inhibitory effects of the regulatory CD25<sup>+</sup> CD4 T cells, which are present in greatly reduced numbers in CD28-deficient animals (8, 13). However, depletion of CD25<sup>+</sup> CD4 T cells with the anti-CD25 mAb PC61 before irradiation did not appear to enhance the ability of responder CD4 T cells to proliferate. Unfortunately, interpretation of the anti-CD25 depletion experiments is difficult, because CD25 is up-regulated on responders, especially within the high-divider fraction, and residual PC61 depletes those cells (Fig. 7).

Therefore, we tested whether CD25<sup>+</sup> CD4 T cells can inhibit LIP of polyclonal wild-type naive CD4 T cells. Irradiated CD28-deficient mice were injected with 5 × 10^6 wild-type CD45.1 CD25<sup>+</sup> CD4 T cells, equal numbers of CD45.1 CD25<sup>−</sup> CD4 T cells, or left untreated, before transfer of 2 × 10^6 responder naive polyclonal Thy1.1<sup>+</sup> CD4 T cells. Indeed, CD25<sup>+</sup> CD4 T cells were more potent inhibitors of LIP than CD25<sup>−</sup> CD4 T cells (Fig. 8, left panel), despite their own relatively limited ability to undergo LIP when compared with CD25<sup>−</sup> CD4 T cells (data not shown). In addition, we found that CD25<sup>+</sup> CD4 T cells also dramatically enhance the ability of responder CD4 T cells to proliferate (Fig. 7).

CD28<sup>−/−</sup> CD4 T cells do not sense B7 deficiency

Because the use of B7-deficient recipients and treatment with CTLA-4-Ig both had an equalizing effect on wild-type and CD28<sup>−/−</sup> responder CD4 T cell populations undergoing LIP, it is likely that B7 blockade is at least one mechanism of inhibition mediated by CTLA-4-Ig. However, the cotransfer experiments did not exclude the formal possibility that CTLA-Ig treatment may have additional effects, including blockade of another B7 ligand. Thus, we tested the ability of CD28-deficient CD4 T cells without cotransferred wild-type cells to undergo LIP in irradiated B7-deficient and CD28-deficient hosts. We felt that the CD28-deficient mice represented the closest B7-sufficient controls compared with B7-deficient mice, both strains having a similar degree of immune deficiency and paucity in the numbers of regulatory CD25<sup>+</sup> CD4 T cells (8, 13). We expected to see greater proliferation in irradiated B7-sufficient hosts, if CD28-deficient T cells express another B7 ligand that plays a positive role in LIP. However, this was not found to be the case (Fig. 6).
inhibited LIP of HA (Fig. 8, middle panel) and DO11.10 (Fig. 8, right panel) Tg CD4 T cells in RAG-deficient hosts, whereas CD25+CD4 T cells had absolutely no effect. Finally, CD25+CD4 T cells modestly inhibited LIP of CD8 T cells, whereas CD25−CD4 T cells actually enhanced LIP of CD8 T cells (data not shown). Taken together, these results suggest that regulatory CD25+CD4 T cells can inhibit LIP, although do not necessarily explain the greater degree of LIP observed in CD28-deficient hosts. The latter may also be simply attributed to relatively inefficient endogenous competitors. Nevertheless, it is possible that comparison of LIP in wild-type and B7-deficient hosts may underestimate the importance of B7 signals because B7-deficient hosts have a marked deficiency in regulatory CD25+CD4 T cells.

Responder CD4 T cells in the highly divided fraction acquire the ability to produce IFN-γ.

It has been noted in multiple studies that, during LIP, highly divided T cells acquire memory-like phenotypic characteristics (4, 6, 14, 15). This includes the ability to make IFN-γ following recall stimulation in the case of CD4 T cells (6, 7). This property may be useful not only for the neonate (6) but also following recovery from a lymphopenic state by the adult, because it could ensure existence of a relatively diverse repertoire of memory-like T cells capable of rapid response against potential pathogens. However, this property may also pose a danger of autoimmunity if the most highly divided and differentiated T cells will be those with greatest affinity for self-Ags. It has been long recognized that absence of

FIGURE 4. Minimal effects of CTLA-4-Ig treatment on LIP of naive CD8 T cells. Purified mixed CD44low CD8 T cells from wild-type (Thy1.1) and CD28−/− (Thy1.2) mice were labeled with CFSE and transferred into CD45.1 RAG−/− mice (3 × 10^6 total CD8 T cells per recipient), some of which were left untreated, and some of which were treated with CTLA-4-Ig. Histograms show CFSE content of indicated responder cells in spleens and lymph nodes of recipient animals at the indicated times after adoptive transfer. The numbers above the histograms show the geometric mean fluorescence intensity in FL-1. The figure also shows the relative ratios of transferred wild-type and CD28−/− CD8 T cells. This experiment is representative of three independent experiments done using C57BL/6 and BALB/c strains.

FIGURE 5. Wild-type CD4 T cells have a competitive advantage over CD28−/− CD4 T cells in B7-sufficient hosts. Purified mixed CD44low CD4 T cells from wild-type (CD45.1) and CD28−/− (Thy1.2) mice were labeled with CFSE and transferred into sublethally irradiated (700 cGy) wild-type, B7−/−, and CD28−/− recipients (all CD45.2, Thy1.2). Solid-line histograms show CFSE content of wild-type responder CD4 T cells and hatched histograms show CFSE content of CD28−/− responder CD4 T cells in spleens of indicated mice 1 and 2 wk after adoptive transfer. The bar graph shows the relative ratios of transferred wild-type and CD28−/− CD4 T cells in the spleens of recipients at the indicated times. Similar data were seen in the lymph nodes. This experiment is representative of three independent experiments.
costimulation during responses to conventional responses can lead to induction of tolerance. Therefore, although our experiments clearly show that LIP is not entirely independent of B7:CD28 costimulation in terms of the ability of responders to proliferate, we also wished to test whether costimulation has additional effects on functional differentiation of CD4 T cells during LIP. We found that the ability of responder CD4 T cells to become potential producers of IFN-\(\gamma\) correlated highly with their ability to undergo a high degree of proliferation. Although the rate of accumulating cells in the most highly divided fraction was considerably slower in the absence of B7:CD28 signals (Fig. 9), the fraction was not eliminated. Polyclonal cells in this fraction did show the potential to produce IFN-\(\gamma\) when restimulated in vitro with PMA/ionomycin. Furthermore, CD28-deficient DO11.10 T cells in the fraction of most highly divided cells also produced IL-2 and IFN-\(\gamma\) when restimulated by the OVA peptide directly in vivo (Fig. 10). The cells did not acquire the ability to produce IL-4 (data not shown).

**Discussion**

Although there is a clear long-standing consensus on the importance of B7:CD28 costimulation in primary responses of T cells to foreign Ags in normal nonlymphopenic hosts, the initial studies on the role of costimulation in LIP have been somewhat inconsistent. The previous studies and the current report found no evidence for a role of B7:CD28 signals in driving LIP of CD8 T cells. However, this is clearly not true for CD4 T cells. CD28 costimulation accelerates the accumulation of a fraction of highly divided CD4 T cells that emerges in the course of LIP and is characterized by the ability to make IFN-\(\gamma\). This fraction becomes most evident when the number of responder T cells is relatively limited, and consequently, there is a greater amount of space that needs to be filled. Furthermore, the contribution of CD28 costimulation may be best revealed in a direct competitive race between CD28-sufficient and CD28-deficient CD4 T cells within the same hosts. Although our data suggest that blockade of B7:CD28 interaction was the dominant effect of CTLA-4-Ig treatment in these experiments, the data also show that CTLA-4-Ig has additional effects, because it also restrains LIP of CD28-deficient CD4 T cells. The nature of B7 blockade-independent mechanisms of CTLA-4-Ig treatment remains unclear and deserves further investigation. One consideration is induction of the immunomodulatory enzyme IDO following ligation of B7 on dendritic cells.
by CTLA-4 (16). In addition, it has been suggested that there may be another B7 ligand besides CD28 capable of providing a positive signal to T cells (17).

Although we documented that CD28 costimulation significantly accelerates the accumulation of highly divided CD4 T cells during LIP, we found that commitment to the first several cell cycles is relatively CD28 independent. Similarly to previous studies (7), we observed that TCR Tg CD4 T cells undergo only limited proliferation if initially present in relatively high numbers. We and others (11, 18, 19) have shown that LIP can be limited by competition for self-ligands among T cells. Thus, it is likely that T cells experience greater strength of TCR signaling by self-ligands when there are fewer competitors. We hypothesize that CD28 costimulation comes into play only after a certain minimal threshold of TCR stimulation is achieved. Indeed, this is plausible, because CD28 engagement is facilitated by immunological synapse formation initiated by TCR signaling (20).

The biological significance of costimulatory signals in LIP requires further rigorous testing. It is possible that the only effect of costimulation is merely acceleration of LIP. In fact, our data show that CD4 T cells within the highly divided fraction can become independent of costimulation in their ability to produce IL-2 and IFN-γ in response to Ag stimulation in vivo. Wild-type DO11.10 RAG−/− cells and CD28−/− DO11.10 cells were CFSE labeled and transferred into RAG−/− recipients. Two weeks after the adoptive transfer, the recipient mice were injected with 250 μg of the OVA peptide. Spleens and lymph nodes were taken for cytokine staining 1 h later. The bar graphs below show the fractions of highly divided cells for each donor population and the fractions of cytokine-positive cells among the highly divided cells. This result may be explained by the dependence of allograft rejection on both CD4 and CD8 T cells. Furthermore, preferential expansion of alloreactive T cells may be of relatively little importance, because their precursor frequency is high from the outset.

The observation of greater LIP of wild-type responder CD4 T cells in irradiated CD28-deficient hosts suggested a possible role for regulatory CD25+ CD4 T cells, which depend on B7:CD28 signals for their own homeostasis. Indeed, CD25+ CD4 T cells have been shown to restrain expansion of polyclonal CD45RBhigh T cells in RAG−/− hosts that can lead to colitis and limit its population size (24). In this study, we show that CD25+ CD4 T cells can effectively inhibit LIP of TCR Tg CD4 T cells (DO11.10 and HA) that do not have known cross-reactivities with exogenous Ags and do not cause inflammatory diseases (data not shown), which in turn could affect the course of LIP. Interestingly, the pattern of LIP inhibition exhibited by CD25+ CD4 T cells was quite similar to that of B7 blockade—the highly divided fraction was affected the most. It is tempting to speculate that the mechanism of suppression by CD25+ CD4 T cells involves ligation of B7 by CTLA-4, which CD25+ CD4 T cells express at high levels. However, we were not able to abrogate their suppressive ability by administration of anti-CTLA-4 mAb (data not shown).

The success of the vertebrate adaptive immune system depends on the maintenance of a large and diverse population of T lymphocytes, each bearing a unique Ag receptor, enabling it to recognize multitude of potential foreign Ags. However, the T cells also must avoid mounting inflammatory responses toward self-Ags, or risk autoimmunity. Clearly, random recombination of TCR
genes and negative selection in the thymus are critically important mechanisms that help to achieve these goals. However, a reasonable state of T cell homeostasis can also be maintained by peripheral mechanisms, although multiple animal models (25–27) and certain clinical scenarios (28–30) suggest that LIP may increase the risk of autoimmunity. In fact, T cells with the greatest affinity for self-peptide/MHC complexes have a competitive advantage during T cell repopulation in the course of LIP (18, 19, 31), and may fail to become functionally inactivated by normal tolerance mechanisms (23). It is reasonable to ask what peripheral mechanisms can maximize TCR diversity during LIP? Our experiments suggest that regulatory CD25+ CD4 T cells and B7 blockade may serve to restrain the expansion of most autoreactive clones and allow for the emergence of a more diversified T cell population.

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