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

Karin A. Hagen,*† Christina T. Moses,*† Erin F. Drasler,*† Kelly M. Podetz-Pedersen,*† Stephen C. Jameson,*‡ and Alexander Khoruts2*†

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.

The size of the peripheral T lymphocyte pool is tightly regulated and remains relatively constant throughout the adult life. Lymphopenic states induced by viral infections or radiation and chemotherapy are typically followed by recovery that is at least in part achieved by proliferation of residual peripheral T cells. The positive signals driving this lymphopenia-induced proliferation (LIP) include stimulation by self-peptide/MHC ligands and certain cytokines, such as IL-7, IL-15, and IL-12 (1, 2). However, the role of costimulatory signals, such as those provided by CD28, which are critical for optimal Ag-induced expansion of mature T cells in nonlymphopenic animals, has been less well defined.

A functional role for B7:CD28/CTLA-4 in T cell homeostasis was suggested by analysis of animals that lack or overexpress B7 molecules (3). Although not lymphopenic, B7-deficient animals have an increased CD4/CD8 T cell ratio, whereas transgenic (Tg) mice overexpressing B7 molecules have the reciprocal increase in the relative numbers of CD8 T cells. Interestingly, there has been little evidence to suggest any requirement for CD28 signaling for LIP of naive CD8 T cells in various models of lymphopenia, including RAG-deficient, irradiated, and neonatal animals (4–6). However, the results with naive CD4 T cells have not been entirely consistent. Although CD28-deficient CD4 T cells appeared to proliferate at least as well as wild-type CD4 T cells in irradiated and RAG-deficient recipients (5), they were noted to proliferate poorly in neonates (6). Furthermore, treatment of RAG-deficient mice with CTLA-4-Ig inhibited proliferation of a subset of CD4 T cells characterized normally by a high degree of proliferation and differentiation into IFN-γ-producing cells (7).

The apparent inconsistency between results obtained using CD28-deficient CD4 T cells and using CTLA-4-Ig to block B7 signals prompted us to wonder whether CTLA-4-Ig treatment has additional inhibitory effects. In this study, we tested the role of CD28 costimulation in LIP using CD28-deficient and CD28-sufficient responders in wild-type and B7-deficient animals, as well as animals treated with CTLA-4-Ig using a variety of naive responder CD4 and CD8 T cells. As expected, little role for CD28 in LIP was found for CD8 T cells. However, in all experimental systems, CD4 T cells benefited from CD28 costimulation. Interestingly, treatment with CTLA-4-Ig was inhibitory for LIP of wild-type CD4 T cells and to a significantly lesser degree of CD28-deficient CD4 T cells. In addition, we examined the potential role of regulatory CD25+ CD4 T cells in controlling LIP, a population of cells that has been shown to be suppressive to virtually every kind of immune response examined, and itself quite dependent on B7:CD28 costimulation for proliferation and survival (8).

Materials and Methods

Mice

The recombine-deficient (RAG-2−/−) DO11.10 (9) and hemagglutinin (HA) TCR Tg mice were bred and maintained under specific pathogen-free conditions as previously described (11). In addition, in some of the experiments, we used RAG-2−/− DO11.10 and RAG-2−/− HA TCR Tg mice bred onto the Thy1.1 BALB/c congenic background, which in turn were originally generously provided by Dr. L. Turka (University of Pennsylvania, Philadelphia, PA). In some of the experiments, we used DO11.10 mice bred onto the CD28−/− BALB/c background (The Jackson Laboratory, Bar Harbor, ME). CD28−/−, B7−/− (B7-1 and B7-2 double-knockout) mice on C57BL/6 background were purchased from The Jackson Laboratory and bred in our facility. In some experiments, we used CD28−/− mice bred onto the Thy1.1 C57BL6 congenic background. CD45.1 C57BL/6 mice were purchased from the National Cancer Institute (Fredrick, MD). RAG-2−/− BALB/c mice were purchased from Taconic Farms (Germantown, NY), and RAG-1−/− C57BL/6 mice were purchased from The Jackson Laboratory. All mice used were generally 6–12 wk of age. All animals were maintained in a specific pathogen-free facility in microisolator cages with filtered air according to National Institutes of Health guidelines. In some experiments, mice received 700 cGy of sublethal irradiation from a cesium source.

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2 Address correspondence and reprint requests to Dr. Alexander Khoruts, Center for Immunology, University of Minnesota, 6-134 Basic Science and Biomedical Engineering Building, 312 Church Street SE, Minneapolis, MN 55455. E-mail address: khorut01@umn.edu

3 Abbreviations used in this paper: LIP, lymphopenia-induced proliferation; Tg, transgenic; HA, hemagglutinin.

4 Center for Immunology, and Departments of 5 Medicine, and 6 Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55455

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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 BioMag particles (Polysciences, Warrington, PA), followed by depletion of CD44high 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 10^6 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, 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 CD25+ CD4 T cells using i.v. injection of anti-CD25 mAb, PC61, 250–400 μg per dose per mouse, repeated three times every 4 days. Adoptive transfer of responder T cells was done 4 days after the last injection of PC61.

Detection of intracellular production of cytokines

Production of intracellular cytokines by polyclonal CD4 T cells was detected by flow cytometry following 4-h in vitro stimulation with PMA and ionomycin; brefeldin A was added during 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. Spleens and lymph nodes were taken from euthanized animals 1 h after they received an i.v. injection of the OVA25-33 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 cotransferred into RAG−/− recipients. Although the two

![FIGURE 1.](http://www.jimmunol.org/) CTLA-4-Ig treatment inhibits LIP of HA RAG−/− CD4 T cells transferred into RAG−/− mice. A, Indicated numbers of HA RAG−/− CD4 T cells were labeled with CFSE and adoptively transferred into RAG−/− mice. CFSE content of responder cells was measured 1 wk after the transfer. The experiment is representative of three independent experiments. B, A total of 1 × 10^6 CFSE-labeled HA RAG−/− CD4 T cells was transferred into RAG−/− recipients that were either left untreated (unshaded histograms) or treated with CTLA-4-Ig. CFSE content was measured at indicated times following transfer. Both panels shows responder HA T cells in spleen, and similar data were seen in the lymph nodes. The experiment is one of two independent experiments.

![FIGURE 2.](http://www.jimmunol.org/) CTLA-4-Ig treatments inhibits LIP of wild-type and CD28−/− DO11.10 CD4 T cells. CFSE-labeled wild-type DO11.10 RAG−/− CD4 T cells and CD28−/− DO11.10 CD4 T cells were transferred, 2.5 × 10^6 cells per mouse. Some animals were left untreated, whereas others were treated with CTLA-4-Ig. CFSE content was measured 2 wk after the transfer. The figure shows data from spleen, and similar data were seen in the lymph nodes. The data are representative of four independent experiments.
responder populations were injected in similar numbers, the wild-type CD4 T cells were found to dominate the CD28−/− CD4 T cells almost by a factor of 10 after 1 wk in untreated animals, whereas the cell ratio remained relatively unchanged in animals treated with CTLA-4-Ig (Fig. 3). Once again, CTLA-4-Ig treatment resulted in a small, but consistent decrease in the number of highly divided CD28−/− CD4 T cells (Fig. 3). Notably, the effects of CTLA-4-Ig required engagement of B7 molecules on the host cells, because it had absolutely no effect in sublethally irradiated B7−/− recipients (data not shown). Finally, we tested the effects of CTLA-4-Ig on LIP of wild-type and CD28−/− polyclonal CD44low CD8 T cells. Contrary to our results for CD4 T cells, CD28 deficiency had little impact on naive CD8 T cells (Fig. 4). In addition, the effect of CTLA-4-Ig treatment was relatively minimal (Fig. 4). Thus, the rest of the study focused on CD4 T cells.

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 CD44low 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 content of responder cells clearly defined two subpopulations of cells—the highly divided cells (greater than six to eight cell divisions) and cells that underwent relatively few divisions (zero to four) (Fig. 5). After 1 wk, the highly divided fraction was seen among the CD28-sufficient CD4 T cells in wild-type and CD28-deficient recipients, but not in B7-deficient recipients. This fraction increased further by the second week among the CD28-sufficient responders. Notably, the CD28-deficient CD4 T cells revealed the highly divided fraction later than CD28-sufficient CD4 T cells, and only in the B7-deficient hosts. This is most likely because B7-deficient hosts provided the only environment in which CD28-sufficient responders had no competitive advantage over CD28-deficient T cells, and could not occupy the available space before the latter had the chance. In fact, the lack of any differences between the CD28-sufficient and CD28-deficient CD4 T cell proliferation patterns in irradiated B7-deficient hosts argues against the existence of any compensatory mechanisms that may be expected to arise in CD28-deficient T cells.

CD28−/− 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−/− 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+ 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).

Potential role for regulatory CD25+ 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+ CD4 T cells, which are present in greatly reduced numbers in CD28-deficient animals (8, 13). However, depletion of CD25+ 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+ 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−/− CD4 T cells, equal numbers of CD45.1 CD25−/− CD4 T cells, or left untreated, before transfer of 2 × 10^6 responder naive polyclonal Thy1.1+ CD4 T cells. Indeed, CD25+ CD4 T cells were more potent inhibitors of LIP than CD25− CD4 T cells (Fig. 8, left panel), despite their own relatively limited ability to undergo LIP when compared with CD25− CD4 T cells (data not shown). In addition, we found that CD25+ CD4 T cells also dramatically inhibit LIP by CD28-sufficient CD4 T cells.

**FIGURE 3.** CTLA-4-Ig treatment inhibits LIP of polyclonal naive CD4 T cells. Purified CD44low CD4 T cells from wild-type (Thy1.1) and CD28−/− (Thy1.2) mice 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.
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/H11002 CD4 T cells had absolutely no effect. Finally, CD25/H11001 CD4 T cells modestly inhibited LIP of CD8 T cells, whereas CD25/H11002 CD4 T cells actually enhanced LIP of CD8 T cells (data not shown). Taken together, these results suggest that regulatory CD25/H11001 CD4 T cells certainly can inhibit LIP, although do not necessarily explain the greater degree of LIP in observed in CD28-decient 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/H11001 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
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 co-stimulation 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-γ 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-γ 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-γ 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-γ. 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

![Figure 6](image-url)  
**FIGURE 6.** CD28−/− CD4 T cells exhibit similar patterns of LIP in B7−/− deficient and B7−/− sufficient (CD28+/+) recipients. Purified CD44mCD4 T cells from Thy1.1 CD28−/− mice were labeled with CFSE and transferred into sublethally irradiated (700 cGy) B7−/− and CD28−/− (both Thy1.2) mice. Solid line shows a representative histogram showing CFSE content of CD28−/− T cells in spleens of B7−/− recipients, and hatched histogram shows CFSE content of CD28−/− T cells in spleens of CD28−/− recipients. Similar data were seen in the lymph nodes. The bar graphs show geometric mean fluorescence intensity of responder T cells in FL-1 and the average percentage of responder T cells in the most highly divided fraction ± SD. Each group contained three recipient animals.

![Figure 7](image-url)  
**FIGURE 7.** Depletion of recipient CD25+ cells does appear to enhance LIP of naive CD4 T cells, although the treatment also depletes some of the most highly divided responders. Flow cytometry dot plots show CFSE content and CD25 expression of polyclonal CD4 T cells transferred as purified CD44mCD4 T cells 1 wk prior to sublethally irradiated (700 cGy) recipients, some of which received a course of depleting anti-CD25 Ab before irradiation. The percentages in the left upper quadrant show the fraction of cells that underwent a limited number of cell divisions. The percentages in the right lower quadrant show the fraction of cells that exhibited a high degree of proliferation and expressed CD25. The plots show representative animals from one of three independent experiments.

![Figure 8](image-url)  
**FIGURE 8.** CD25+ CD4 T cells inhibit LIP of naive polyclonal and TCR Tg CD4 T cells. The left panels show the CFSE content of polyclonal CD44mCD4 T cells measured 1 wk after adoptive transfer (1 × 10⁶ per recipient) into sublethally irradiated CD28−/− mice (700 cGy), some of which also received 5 × 10⁶ purified CD25+ CD4 or CD25− CD4 T cells. The center panels show the CFSE content of HA RAG−/− CD4 T cells 1 wk after adoptive transfer (5 × 10⁶ per recipient) into RAG−/− mice, some of which also received 5 × 10⁶ purified CD25+ or CD25− CD4 T cells. The right panels show the CFSE content of DO11.10 RAG−/− CD4 T cells 2 wk after adoptive transfer (5 × 10⁶ per recipient) into RAG−/− mice, some of which also received 5 × 10⁶ purified CD25+ or CD25− CD4 T cells. Each panel is representative of two animals per group in these experiments, and each experiment is representative of three separate experiments. Pooled data showed greater potency of CD25+ CD4 T cells compared with CD25− CD4 T cells in the ability to inhibit accumulation of the highly divided polyclonal CD4 T cells in the course of LIP to be statistically significant (p = 0.0023; paired Student’s t test).
FIGURE 9. Highly divided CD28<sup>−/−</sup> CD4 T cells accumulate slower than wild-type CD4 T cells in the course of LIP, but also develop the potential to produce proinflammatory cytokines. Purified CD4<sup>4low</sup> CD4 T cells from wild-type and CD28<sup>−/−</sup> donors were CFSE labeled and adoptively transferred into separate RAG<sup>−/−</sup> recipients. The percentages in different quadrants show the fraction of donor cells that underwent a limited number of cell divisions. The percentages in the upper left quadrant of isotype-stained cells show the fraction of donor cells that underwent a limited number of cell divisions. The percentages in the lower right quadrant of isotype-stained cells show the fraction of donor cells undergoing greater strength of TCR signaling by self-ligands when there are fewer competitors. The plots show representative animals from one of two independent experiments.

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 following Ag stimulation. However, we propose that, for a polyclonal population of responders, costimulatory signals may facilitate preferential expansion of T cell clones with the greatest avidity for MHC signals. These clones will come to occupy the T cell space faster in the presence of costimulation and thus limit the expansion of their competitors. Conversely, blockade of costimulation during LIP may allow for the emergence of a more diversified population of CD4 T cells. This hypothesis may explain why polyclonal CD4<sup>4high</sup> CD4 T cells fail to induce colitis following transfer into RAG<sup>−/−</sup> mice in the absence of B7 signals (21). Notably, the emergence of disease in this model is dependent on transfer of limited numbers of responder T cells, which allows sufficient space for oligoclonal expansion of relatively rare pathogenic T cell clones reactive to enteric flora (22). In contrast, costimulatory blockade had marginal effects in the prevention of allograft rejection in partially reconstituted lymphopenic hosts (23). 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<sup>+</sup> CD4 T cells, which depend on B7:CD28 signals for their own homeostasis. Indeed, CD25<sup>+</sup> CD4 T cells have been shown to restrain expansion of polyclonal CD4<sup>4high</sup> T cells in RAG<sup>−/−</sup> hosts that can lead to colitis and limit its population size (24). In this study, we show that CD25<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> CD4 T cells involves ligation of B7 by CTLA-4, which CD25<sup>+</sup> 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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