Established T Cell-Driven Germinal Center B Cell Proliferation Is Independent of CD28 Signaling but Is Tightly Regulated Through CTLA-4

Lucy S. K. Walker, Helen E. Wiggett, Fabrina M. C. Gaspal, Chandra R. Raykundalia, Margaret D. Goodall, Kai-Michael Toellner and Peter J. L. Lane

*J Immunol* 2003; 170:91-98; doi: 10.4049/jimmunol.170.1.91
http://www.jimmunol.org/content/170/1/91

**References**

This article cites 33 articles, 18 of which you can access for free at:
http://www.jimmunol.org/content/170/1/91.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Established T Cell-Driven Germinal Center B Cell Proliferation Is Independent of CD28 Signaling but Is Tightly Regulated Through CTLA-4

Lucy S. K. Walker, Helen E. Wiggett, Fabrina M. C. Gaspal, Chandra R. Raykundalia, Margaret D. Goodall, Kai-Michael Toellner, and Peter J. L. Lane

CD4 T cell activation is positively (CD28) and negatively (CTLA-4) regulated by the costimulatory ligands CD80 and CD86. A central question is how the balance between these two opposing forces is controlled as T cells differentiate. We have previously shown that CD28 signaling is absolutely required to prime naive CD4 T cells to differentiate into effectors that provide help for germinal centers and class-switched Ab responses. In this study, we show that the requirement for CD28 signaling is transient and effector CD4 T cells do not require CD28 signals to sustain their function. The CD28 independence of effector T cells within germinal centers suggested that a key function for CD80/CD86 under these circumstances might be to provide negative regulatory signals via the CD28 homologue CTLA-4. By examining germinal center responses in mice where the ability to signal through T cell CTLA-4 was compromised, we provide data that supports a critical role for CTLA-4 in down-regulating T cell help for germinal center B cells. The Journal of Immunology, 2003, 170: 91-98.

The clustered genes CD28, CTLA-4, and inducible costimulator (ICOS) play a critical role in regulating the development of T cell help for B cells. Although all three genes are homologous and represent tandem duplications of an ancestral gene, their functions and ligands are distinct. CD28 and CTLA-4 share common ligands (CD80 and CD86) (1) but the ligand for ICOS is B7RP1/B7h (2, 3). Although CD28 is constitutively expressed on CD4 T cells, the expression of both ICOS (4) and CTLA-4 (5) is induced following primary costimulation through CD28. The CD28 signal plays a critical role in optimally expanding naive CD4 T cells during priming and, in particular, it is required for their recruitment to B follicles to foster germinal center (GC) development (6, 7). We have previously shown that this reflects a requirement for CD28 signaling to optimally induce the chemokine receptor CXCR-5, which directs CD4 T cell migration into B follicles to drive GC B cell proliferation (8). The CD28 homologue ICOS is also involved in GC development (9–11). In contrast to CD28 and ICOS, the inducible CD28 homologue CTLA-4 is implicated in negatively regulating CD28-driven CD4 T cell responses (12).

There are several unresolved issues. Although both CD28 and ICOS participate in normal GC development, it is not clear whether both pathways are required once CD28 signals have primed the initial expansion and migration of CD4 T cells to B follicles. Since CTLA-4 is strongly expressed within the GC T cell population (13), how is the balance between CD28 and CTLA-4 signals struck in effector T cells within GCs? Because the ligands CD80 and CD86 can independently activate (CD28) and inhibit (CTLA-4) CD4 T cells (14), it is unlikely that the major control of T cell responsiveness is by differential expression of CD80 and CD86.

The studies shown here provide insight into this paradox. We have developed an in vivo experimental model that dissects the relative contribution of CD28 and CTLA-4 signals within effector GC CD4 T cells. This model depends on transient restoration of CD28 signaling by injection of agonistic CD28 mAb into mice that transgenically express soluble CTLA-4-Ig at a level that effectively blocks CD80 and CD86. Injection of anti-CD28 mAb into these CTLA-4-Ig-transgenic (tg) mice can be used to selectively restore CD28 but not CTLA-4 signaling. We show that effector CD4 T cells survive and provide GC B cell help long after the CD28 mAb is cleared from the serum and the CD28 blockade is restored. These T cell-driven CD28-independent GCs are much larger than in normal mice, suggesting a critical role for intact signaling through CTLA-4 to negatively regulate effector GC T cells. This conclusion is independently supported by experiments designed to selectively interfere with CTLA-4 signaling either by injecting blocking mAbs to CTLA-4, or by comparing immune responses between normal mice and mice with only one functional CTLA-4 allele. Although GCs were much larger in mice with deficient CTLA-4 signaling, the ratio of GC B:T cells was not distorted. These studies demonstrate that the survival and effector function of CD4 T cells within GCs is independent of CD28 but is tightly regulated by CTLA-4.

Materials and Methods

Preparation of Abs for injection

Anti-CD28 mAb (clone 37.51) and anti-CTLA-4 mAb (clone 9H10) were a kind gift from J. Allison (University of California, Berkeley, CA). Purified mAbs were prepared by protein G purification of cell supernatants and sterilized by filtration through 0.2-μm filters, and frozen in aliquots.

Copyright © 2003 by The American Association of Immunologists, Inc.

0022-1767/03/$02.00
before use. Hamster IgG was prepared from hamster serum and filtered and stored before use as above.

**Mice**

CTLA-4-Ig (6) and dendritic cell (DC)-OX40L (15)-tg experimental animals were bred and maintained in accordance with animal house guidelines. Mice heterozygous for deficiency in CTLA-4 (CTLA-4+/− BALB/c) mice and their littermates (16) were kindly provided by A. Sharpe (Harvard University, Boston, MA) and A. Abbas (University of California, San Francisco, CA) and housed in the University of California, San Francisco animal facility.

**Immunization protocol**

Groups of mice were immunized i.p. with 50 μg of protein Ags resuspended in 200 μl of saline (either chicken γ-globulin [Cyg; Sigma-Aldrich, Poole, U.K.], keyhole limpet hemocyanin [KLH] [Sigma-Aldrich], or 4-hydroxy-3-nitrophenyl acetyl (NP)-KLH which was haptenated using the succinimide ester of NP [Biosearch Technologies, Novato, CA]). For primary but not secondary immunizations, proteins were alum precipitated. Anti-CD28 mAb (100 μg) or control hamster Ig (100 μg) was injected i.p. where indicated 24 h following Ag administration. One hundred microliters of blocking anti-CTLA4 mAb or control hamster Ig was injected i.p. on days 1, 4, and 7 where indicated. Mice were bled at the indicated times for assessment of specific Ab titers. Groups of mice were culled by CO₂ asphyxiation, and their spleens were snap frozen in liquid nitrogen and stored at −70°C before analysis.

**Immunohistology and quantitative analysis**

Cryostat sections (5 μm) from frozen spleen were mounted onto four-spot glass slides that were air dried for 1 h, fixed in acetone at 4°C for 20 min, dried again, and stored in sealed polythene bags at −20°C. Serial sections for analysis of gene expression were stored in RNase-free polypropylene tubes at −70°C.

Tissue sections were stained for IgG (sheep anti-mouse IgG; Binding Site, Birmingham, U.K.), CD3 (KST; Sertoli, Kidlington, Oxford, U.K.), peanut agglutinin (Vector Laboratories, Burlingame, CA), and biotinylated anti-mouse ICOS (clone 15F9; eBioscience, Newington, NH). Slides were stained as described elsewhere (17). In brief, second-step reagents were donkey anti-sheep peroxidase (Binding Site) and biotinylated rabbit anti-rat (DAKO, High Wycombe, U.K.) followed by StreptABComplex alkaline phosphatase (DAKO). Substrate for peroxidase was 3,3′-diaminobenzidine (Sigma-Aldrich) and for alkaline phosphatase the substrate Naphthol AS-MX phosphate and chromogen Fast Blue BB salt (Sigma-Aldrich) were used. Levamisole (Sigma-Aldrich) was used to block endogenous alkaline phosphatase activity.

For quantitative analysis, GC size was assessed by counting the number of graticule intercepts (units) at ×400 magnification of IgG-negative B cell areas within B follicles (18). The numbers of CD3-positive T cells were counted in each GC. Twenty consecutive GCs were counted in each spleen section, and the results are expressed as the mean size of GC and mean numbers of T cells per GC. The ratio of these two numbers gives T cell number per unit GC area. In Fig. 4, GC size was assessed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) and the GC area shown as a percentage of the total section area imaged. Nonparametric Mann-Whitney U methods were used to calculate p values.

**ELISA**

Plates were coated with 5 μg/ml Cyg, KLH, or anti-CD28 mAb diluted in 0.05 M carbonate buffer (pH 9.6). Serum samples were titrated on ELISA plates blocked with 1% BSA in PBS (pH 7.4), bound IgG was detected using alkaline phosphatase-conjugated rat anti-IgG (Southern Biotechnology Associates, Birmingham, AL) and developed with p-nitrophenyl phosphate substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and absorbance read at 405 nm.

For assessment of Ab affinity, sera from mice immunized with NP-KLH were titrated on ELISA plates coated with low (NP2) and high (NP15) haptenated NP-KLH (Sigma-Aldrich). The titer (cts) were measured at the end of each APC cycle and many ICOS-expressing cells (Fig. 1b) and many ICOS-expressing cells (Fig. 1c) were also shown that these mice have increased numbers of GC T cells (Fig. 1d). To restore GC formation in CTLA-4-Ig-tg mice, agonistic CD28 mAb was routinely injected 24 h after administration of protein Ag. However, CD28 mAb was also successful at restoring formation of Ag-specific GCs if added 2 days, but not 10 days, before immunization with protein Ag (Fig. 1e). Associated with the restoration of GC formation in CTLA-4-Ig-tg mice, CD28 mAb restored switching to IgG in Ab responses to protein Ags, and affinity maturation was comparable to normal mice (Fig. 2a and bi). The IgG responses persisted until the animals were sacrificed 14 wk later (data not shown). The capacity to generate T-dependent IgG Ab responses to new protein Ags was temporary. This was because the mice made an anti-hamster Ab response against the injected hamster anti-CD28 mAb that was detectable by day 10 and persisted for at least 6 wk (Fig. 2bi). As a result of clearance of the anti-CD28 mAb, injection of a new protein, KLH, 6 wk after the start of the experiment elicited a weak nonswitched Ab response that was indistinguishable from untreated CTLA-4-Ig-tg mice (Fig. 2bii). In contrast, wild-type littermates responded normally to the KLH Ag. These results suggested that the injected anti-CD28 mAb became the target of an immune response that rapidly cleared it from the serum. This was consistent with our observation that injection of anti-CD28 mAb was unable to stimulate the induction of Ag-specific GCs in CTLA-4-Ig-tg mice if it was injected 10 days before immunization with the relevant protein Ag (Fig. 1e) and the fact that hamster Ab could not be detected in the serum at this time point (data not shown).
GC T cells do not require the CD28 pathway to provide effector help to B cells.

**CTLA-4 regulates GC size, GC T cell numbers**

Having shown that CD28 signals were not essential to sustain T cell help for B cells, our model allowed us to then dissect the role of CTLA-4 signals within GCs since CTLA-4 signals are blocked in CTLA-4-Ig-tg mice. A striking feature of the CD28 mAb-induced GCs in CTLA-4-Ig-tg mice is that they are huge compared with nontransgenic littermate controls (Fig. 3a). The median GC area in CD28 mAb-treated CTLA-4-Ig-tg mice was ~9-fold larger than that of normal nontransgenic littermates. Since GCs are three-dimensional structures, this underestimates the true difference in GC size between these two groups of animals and in volumetric terms the difference is ~27-fold. The large GC size was not due to the CD28 mAb alone, as GC size in nontransgenic mice that received CD28 mAb was, if anything, slightly smaller than in a control group of animals that received hamster IgG (Fig. 3b).

CTLA-4 mRNA was strongly up-regulated in spleen tissue from CTLA-4-Ig-tg mice injected with mAb to CD28 at a time when there were large GCs (Fig. 4a). This suggests that T cells from CTLA-4-Ig-tg mice up-regulate CTLA-4 normally, but fail to receive inhibitory signals from the CD80 and CD86 ligands which are blocked by CD28 blockade. One consequence of CTLA-4 signaling in T cells is the inhibition of ICOS expression, and this occurs primarily through regulation of ICOS mRNA levels (21). CTLA-4-Ig mice in which CD28 (but not CTLA-4) signaling had been restored exhibited increased levels of ICOS mRNA, consistent with a failure to receive an inhibitory signal through CTLA-4 (Fig. 4a). Consistent with the quantitative PCR data, we also observed positive staining for ICOS protein in the GCs of anti-CD28-treated CTLA-4-Ig mice (Fig. 1c). No ICOS staining was observed in control hamster IgG-injected CTLA-4-Ig mice or with isotype-matched control mAb (data not shown).

To directly test the link between impaired CTLA-4 signaling and GC size, we performed two separate additional experiments. First, we compared protein Ag-induced GC responses in mice injected with either anti-CTLA-4 mAb (22) or control hamster IgG. The anti-CTLA-4 mAb (9H10) used is believed to function as a blocking Ab since both intact Ab and Fab have been shown to similarly augment T cell expansion in vivo in response to the superantigen staphylococcal enterotoxin B (23). Examination of spleens taken 10 days after primary immunization showed that CTLA-4 mAb-injected mice had larger GCs compared with controls that received the hamster IgG (p < 0.05, Fig. 4b). The differences in observed GC size were not as striking as in CD28 mAb-injected CTLA-4-Ig-tg mice, probably because the CTLA-4 mAb is less efficient at blocking CTLA-4 interactions than CTLA-4-Ig which blocks the ligands. Second, we compared GC responses between littermates bearing either one or two functional CTLA-4 alleles. Mice with one functional CTLA-4 allele, and therefore reduced CTLA-4 protein expression (24), showed a similar increase in GC size compared with normal littermates (p < 0.02, Fig. 4c).

The degree of signaling through CTLA-4 does not alter the ratio of GC B:T cells

Effective affinity maturation within GCs depends on T cell-driven B cell proliferation followed by selection of rare B cells expressing mutant Ig receptors with improved affinity for the immunizing Ag. Since selection is highly dependent on T-B interactions, we compared T:B cell ratios in the GCs of normal mice and CTLA-4-Ig-tg mice injected with CD28 mAb (Fig. 3a, i and ii). This showed that the ratio of T cells per unit GC area in the CD28 mAb-treated
CTLA-4-Ig-tg mice was not statistically different from that of normal mice ($p > 0.5$, Fig. 3aiii). In other words, the increased number of GC T cells in anti-CD28-treated CTLA-4-Ig mice compared with normal mice was accompanied by a similar increase in GC size between these two groups of mice. This is compatible with the idea that homeostatic mechanisms exist to ensure that the number

FIGURE 2. Ab responses in CD28 mAb and control Ab-injected CTLA-4-Ig-tg mice. a, Mice were immunized with alum-precipitated NP-KLH (50 μg), boosted with 50 μg of soluble NP-KLH at day 17, and bled at day 27. Panels show titers on ELISA plates coated with NP$_2$ or NP$_{15}$ BSA and the ratio of the titers of NP$_2$/NP$_{15}$, which gives a measure of Ab affinity. Normal littermates (○), CTLA-4-Ig-tg mice injected with 100 μg of hamster IgG (△), and CTLA-4-Ig-tg mice injected with 100 μg of CD28 mAb (▲) are shown. Hamster IgG or CD28 mAbs were injected 24 h following immunization with protein Ag. Data are representative of three experiments. b, Ag-specific IgG titers for normal littermates (○), CTLA-4-Ig-tg mice injected with 100 μg of hamster IgG (△), and CTLA-4-Ig-tg mice injected with 100 μg of CD28 mAb (▲). Mice were immunized on day 0 with 50 μg of alum-precipitated CyG, then 24 h later were treated with hamster IgG or CD28 mAb as indicated. Serum Ab levels were measured 24 days later (i). Mice were boosted with CyG or KLH on day 42 and serum Ab levels were measured 7 days later (ii and iii). iv, Serum levels of Abs raised against the hamster mAb to mouse CD28 on day 42. No anti-hamster response was detected in CTLA-4-Ig mice not injected with anti-CD28 mAb (data not shown). Data are representative of six (i and ii) or two (iii and iv) experiments. c, Relative GC size after immunization in normal (○), i) or CTLA-4-Ig-tg mice treated with CD28 mAb (▲, ii). Mice were immunized with 50 μg of alum-precipitated CyG and 24 h later CTLA-4-Ig-tg mice received 100 μg of anti-CD28 mAb.
of T cells recruited to GCs is proportional to the number of B cell mutants requiring selection.

Alternatively, the large GCs observed in mice with reduced CTLA-4 signaling might simply have been a consequence of the increased T cell recruitment to GCs. To examine this possibility, we took advantage of our previous observation that mice expressing OX40L constitutively on DCs (DC-OX40L) have greatly increased numbers of T cells in B follicles and GCs (15). These mice

FIGURE 3. Quantitative immunohistology from spleen sections of mice immunized i.p. 5 wk previously with 50 μg of alum-precipitated protein Ag. Results are shown for (a) normal littermates (○), CTLA-4-Ig-tg mice treated with 100 μg of control hamster Ab (△), CTLA-4-Ig-tg mice treated with 100 μg of anti-CD28 mAb (●); (b) normal mice treated with 100 μg of control hamster Ab (○), normal mice treated with 100 μg of anti-CD28 mAb (●), and (c) normal littermates (○) and DC-OX40L-tg mice (●). Control hamster IgG or CD28 mAb was injected 24 h after immunization with protein Ag. i, GC area; ii, number of T cells per GC; and iii, number of T cells per unit area GC. Quantitation was performed on spleen sections stained for CD3 and IgD. Each point shows the average value from 20 consecutive GCs from a spleen section of an individual mouse. Data are representative of four experiments. The p value in a refers to the comparison between normal mice and anti-CD28-treated CTLA-4-Ig mice.
allowed us to examine independently the effect of increased T cell recruitment to B follicles on GC size in a situation where CTLA4 signaling was intact. Although there were clearly higher numbers of T cells within the GCs of DC-OX40L-tg mice (Fig. 3cii), GC size in these mice was not increased compared with nontransgenic littermates (Fig. 3ci). Consequently, the ratio of T cells per unit GC area was significantly increased ($p<0.01$, Fig. 3ciii), contrasting with the situation in anti-CD28 mAb-injected CTLA-4-Ig mice. GC T cell numbers and GC size were also uncoupled in anti-CD28-treated normal mice, leading to an increase in the ratio of T cells per unit GC area ($p<0.01$, Fig. 3biii). Collectively, these data indicate that GC size is not controlled solely by T cell numbers, but also by how effectively individual T cells stimulate B cell proliferation, and that both of these parameters are regulated by CTLA-4.

**Discussion**

**Effector CD4 T cells in vivo are not dependent on CD28 signals**

The first point to emerge from these studies is that in vivo there are fundamental differences between naive and effector CD4 T cells in their CD28 dependence. This is the first model in which the CD28 dependence of primary and effector CD4 responses can be dissected independently in an intact animal, since by blocking (rather than genetically deleting) the CD28 pathway, the defect can be overcome by agonistic CD28 mAb. Whereas naive CD4 T cells require CD28 engagement to differentiate into effector GC T cells (6–8), effector T cell help for GC B cell proliferation is not dependent on continued CD28 signaling. Ongoing GC responses could not be explained by failure of CTLA-4-Ig blockade in the experiments described here, as serum CTLA-4-Ig levels remained high and mice could not make de novo Ab responses to foreign protein Ags once the agonistic CD28 mAb had been cleared from the serum. CTLA-4-Ig has been used therapeutically to block unwanted immune responses. This study explains why the timing of CD28 blockade might be critical as it shows that effector T cells can function in the absence of intact CD28 signaling.

The likely explanation for the CD28 independence of effector GC T cells is that other costimulatory interactions control their fate. We have previously shown that CD28 signaling regulates expression of OX40 (CD134) (8). Furthermore, mice deficient in
OX40 signaling (25, 26) have impaired CD4 T cell memory responses, and recent studies have confirmed a role for OX40 in promoting the survival of effector/memory T cells (27). However, the most obvious candidate for regulating effector T cell help for GC B cells is the CD28 homologue ICOS (2) that has been shown to regulate T-dependent help in secondary but not primary Ab responses (28). The striking absence of GCs in ICOS−/− mice following secondary Ag challenge is strongly suggestive of a role for ICOS in maintaining GC T cells (9–11), and we show that high levels of ICOS mRNA and protein are expressed in mice with established GCs in our study. Furthermore, like CD28, the ICOS pathway is subject to regulation by CTLA-4 (21). Since CD28 is indispensable for GC initiation (6, 7) while the most dramatic effect of ICOS deficiency is in response to secondary challenge (29), the data are consistent with a sequential signaling model whereby CD28 signals are crucial during T cell priming and ICOS plays the dominant role in effector T cells within GCs. The expression of CD40L on GC T cells (13) may be critical to maintaining B cell expression of the ligand for ICOS (B7RP1/B7h) (30). Distinct costimulatory requirements for effector, memory, and naïve CD4 T cells could provide an elegant mechanism for maintaining homeostasis within different T cell pools in the peripheral lymphoid compartment.

**CTLA-4 signaling negatively regulates GC size and duration**

The fact that CD28 signals were not required for T cell help in established GCs focused our attention on the role of CTLA-4 in regulating the GC T cell population. Addressing this question is problematic in CTLA-4 KO animals due to uncontrolled T cell activation. Our model allowed us to study the effect of selectively disabling CTLA-4-Ig, once GCs were initiated, was associated with improved affinity maturation and cell switching despite normal priming of CD4+ T cells. J. Exp. Med. 179:819.

**Acknowledgments**

We thank Ian MacLennan, Abul Abbas, and David Sansom for reading this manuscript and making many helpful suggestions. We also thank Jim Allison for providing the hybridomas producing anti-CTLA-4 and anti-CD28 mAbs. These experiments could not have been done without the expert help of the staff of the Birmingham University animal facility.

**References**


The Journal of Immunology