Brief Definitive Report

$CD4^+CD25^+$ Immune Regulatory Cells Are Required for Induction of Tolerance to Alloantigen via Costimulatory Blockade

By Patricia A. Taylor,* Randolph J. Noelle,‡ and Bruce R. Blazar*

*From the *University of Minnesota Cancer Center and Department of Pediatrics, Division of Bone Marrow Transplantation, Minneapolis, Minnesota 55455; and the ‡Department of Microbiology, Dartmouth Medical College, Hanover, New Hampshire 03756*

Abstract

Immune regulatory $CD4^+CD25^+$ cells play a vital role in the induction and maintenance of self-tolerance and are essential for T cell homeostasis and the prevention of autoimmunity. Induction of tolerance to allogeneic donor grafts is a clinically desirable goal in bone marrow and solid organ transplantation. To determine whether $CD4^+CD25^+$ cells regulate T cell responses to alloantigen and are critical for tolerance induction, murine $CD4^+$ T cells were tolerized to alloantigen via ex vivo CD40 ligand (CD40L)/CD40 or CD28/cytotoxic T lymphocyte–associated antigen 4/B7 blockade resulting in secondary mixed leukocyte reaction hyporesponsiveness and tolerance to alloantigen in vivo. $CD4^+CD25^+$ T cells were found to be potent regulators of alloresponses. Depletion of $CD4^+CD25^+$ T cells from the $CD4^+$ responder population completely abrogated ex vivo tolerance induction to alloantigen as measured by intact responses to alloantigen restimulation in vitro and in vivo. Addback of $CD4^+CD25^+$ T cells to $CD4^+CD25^-$ cultures restored tolerance induction. These data are the first to indicate that $CD4^+CD25^+$ cells are essential for the induction of tolerance to alloantigen and have important implications for tolerance-inducing strategies targeted at T cell costimulatory pathways.

Key words: regulatory T cell • IL-2 receptor α chain (CD25) • tolerance • transplantation • in vivo animal models

Introduction

Immunoregulatory $CD4^+CD25^+$ cells play a vital role in the induction and maintenance of peripheral self-tolerance. These professional regulatory cells prevent the activation and proliferation of potentially autoreactive T cells that have escaped thymic deletion or recognize extrathymic antigens. Sakaguchi et al. (1) found that the transfer of $CD4^+CD25^-$ T cells into nude mice led to the development of organ-specific and systemic autoimmune disorders which could be prevented by the cotransfer of $CD4^+$ $CD25^+$ T cells. Other studies demonstrated that transfer of $CD4^+CD25^+$ T cells prevented autoimmunity resulting from neonatal thymectomy and inhibited the effector function of autoantigen-specific T cell clones (2–4). CD25-deficient mice develop a profound peripheral lymphadenopathy associated with autoimmune disorders (5). Although these data indicate that $CD4^+CD25^+$ cells are essential for self-tolerance, their role in tolerance to alloantigen has not been studied.

Productive T cell activation and proliferation require two signaling events. The first signal, antigen recognition, is the engagement of the TCR with the MHC–peptide ligand complex on the surface of the APCs. Costimulatory signals are required for the full activation of the intracellular signaling cascade, IL-2 production, T cell proliferation, and effector function (6). In vivo blockade of the CD28/cytotoxic T lymphocyte–associated antigen (CTLA)-4/B7 and CD40 ligand (CD40L)/CD40 costimulatory pathways has been shown to induce tolerance to allografts (7–11). We have reported previously that ex vivo blockade of the CD40L/CD40 pathway results in tolerance induction of murine $CD4^+$ T cells to alloantigen (12, 13). Because of the central role of $CD4^+CD25^+$ cells in self-tolerance, we investigated the potential role of these professional suppressor cells in regulating T cell responses to alloantigen and in the induction of tolerance to alloantigen.

To determine whether $CD4^+CD25^+$ cells were required for tolerance induction to alloantigen, we used an in vitro

Address correspondence to Bruce R. Blazar, Department of Pediatrics, BMT Division, University of Minnesota Cancer Center, MMC 109, 420 SE Delaware St., Minneapolis, MN 55455. Phone: 612-626-2734; Fax: 612-624-3913; E-mail: blaza001@tc.umn.edu

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/2001/06/1311/07 $5.00
Volume 193, Number 11, June 4, 2001 1311–1317
http://www.jem.org/cgi/content/full/193/11/1311

culture in which $CD4^+$ T cells or $CD4^+CD25^-$ T cells were incubated with alloantigen in the presence of anti-CD40L or anti-B7 mAbs. Our data indicate that $CD4^+$ $CD25^+$ T cells (8–12% of $CD4^+$ cells in most strains of mice) are essential to achieve tolerance induction by costimulatory blockade as measured by antigen rechallenge in vitro and by adoptive T cell transfer into antigen-bearing hosts. These studies reveal a fundamental role for $CD4^+$ $CD25^+$ cells in ex vivo tolerance induction and suggest that $CD4^+CD25^+$ cells may be vital to tolerance induction to alloantigen in vivo.

Materials and Methods

Mice. B6.C-$H2^{bm12}$/KhEg (bm12) mice were purchased from The Jackson Laboratory. C57BL/6 (B6) mice were purchased from the National Institutes of Health. B6 and bm12 (both $H2^b$) mice differ at three amino acids due to mutations in the class II I^A region.

In Vitro Mixed Leukocyte Reaction Cultures. B6 lymph node cells were depleted of NK cells (hybridoma PK136, rat IgG2a) and $CD8^+$ T cells (hybridoma 2.43, rat IgG2b) by incubation with mAb, followed by passage through a goat anti–mouse and goat anti–rat Ig-coated column (Cytovax). Purified cells were ≥94% $CD4^+$ T cells. Cells, depleted of $CD25^+$ cells by incubation with anti-CD25 mAb (hybridoma 3C7, rat IgG2b; BD PharMingen) and sheep anti–rat Dynabeads (Dynal), were 99% $CD4^+CD25^-$. Purified $CD4^+CD25^+$ and $CD4^+CD25^-$ cells, obtained by incubating $CD4^+$ cells with anti-CD25 PE (hybridoma PC61, rat IgG1; BD PharMingen) and sorting into positive and negative fractions (>90 and 99% pure, respectively) (FACS Vantage™; Becton Dickinson), were combined at the indicated ratios. Splenic bm12 stimulators were prepared by incubation with anti–Thy-1.2 mAb (hybridoma 30H-12, rat IgG2b) and anti-NK1.1 mAb plus baby rabbit complement (Nieffenegger). The phenotype of splenic stimulators was ~90% B cells, 1–3% T cells, and ~7% macrophages. Bulk cultures of responder $CD4^+$ or $CD4^+CD25^-$ T cells mixed with irradiated (30 Gy) stimulators at a 1:1 ratio at a final concentration of 0.5×10^6 cells per milliliter were plated in 24-well plates (Costar) in DMEM (BioWhittaker) supplemented with 10% fetal bovine serum (HyClone), 50 mM 2-Me (Sigma Aldrich), 10 mM Hepes buffer, 1 mM sodium pyruvate (Life Technologies), amino acids (1.5 mM L-glutamine, L-arginine, and L-asparagine; Sigma Aldrich), and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin; Sigma Aldrich). Anti-CD40L mAb (hybridoma MR1, hamster IgG) or a mixture of anti-B7.1 mAb (hybridoma 16-10A1, hamster IgG or 1G10, rat IgG) and anti-B7.2 mAb (hybridoma 2D10 or GL1, both rat IgG) was added to the mixed leukocyte reaction (MLR) at 50 μg/ml. To determine stability of anti-CD40L mAb in cultures, supernatants were obtained from MLR cultures after 2 and 5 d of incubation. Anti-CD40L mAb was detected at >80% of added concentration on both days by an anti–hamster IgG ELISA assay (data not shown). Anti-CD40L mAb was found to be functional as assessed by binding/blocking assays with a CD40L-transfected cell line (data not shown). MLR cultures were incubated at 37°C, 10% CO_2. On day 9, cells were washed, counted, and either plated with fresh stimulator cells in the absence of mAb or infused in vivo as described below. To monitor primary and secondary MLR proliferation, responder and stimulator cells were plated in replicates of six at numbers indicated in 96-well round-bottomed microtiter plates (Costar). Wells were pulsed with tritiated thymidine (1 μCi/well; Amersham Life Science) on the indicated days for 20–24 h before harvesting and counted in the absence of scintillant amplication on a β-plate reader (Packard Instrument Co.).

Adoptive T Cell Transfer and GVHD Lethality. To determine whether T cells tolerized to alloantigen in vitro could mount a response to alloantigen in vivo, bm12 recipients were sublethally irradiated with 6.0 Gy (^{137}Cesium) total body irradiation 4 h before cell infusion. Control-cultured or mAb-cultured MLR cells (10^5) were infused into antigen-bearing bm12 recipients via tail-vein injection. In a separate experiment, freshly purified, naive whole $CD4^+$, $CD4^+CD25^+$, whole $CD4^+$ and $CD4^+CD25^-$, or whole $CD4^+$ and $CD4^+CD25^+$ cells were infused into bm12 recipients. Mice were monitored daily for GVHD lethality.

Flow Cytometric Analysis. To determine the expression of CD40L on $CD4^+CD25^-$ versus $CD4^+CD25^+$ cells, purified cell populations were incubated with allostimulators for 4 d in the presence of 1 μg/ml anti-CD40L biotin (BD PharMingen). The addition of a biotin-labeled antibody has been shown to stabilize cell surface expression of CD40L (14). This concentration of antibody is insufficient to inhibit alloresponses (unpublished data). Cells

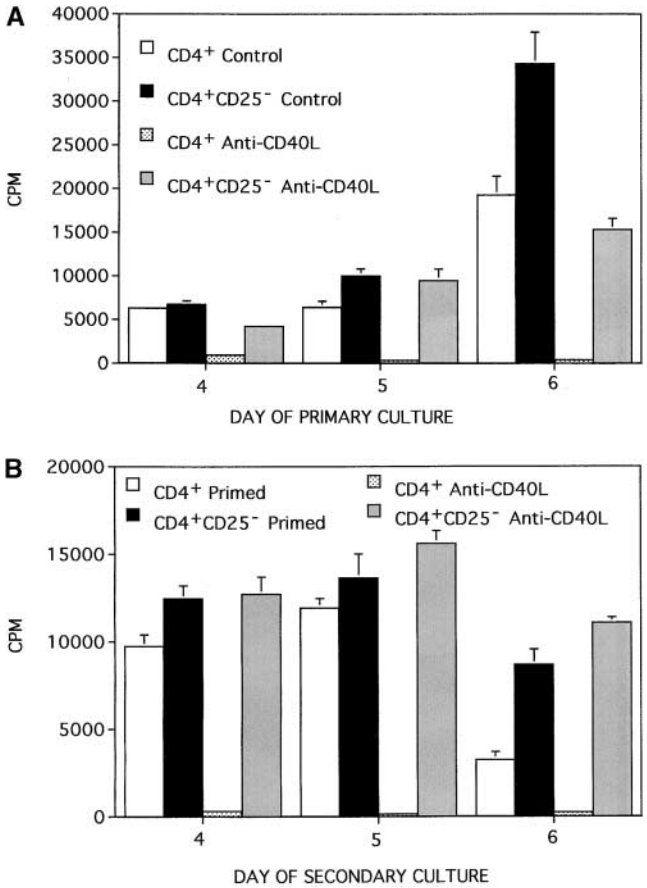

Figure 1. $CD4^+CD25^+$ T cells are required for tolerance induction by CD40L/CD40 costimulatory blockade. (A) Primary MLR culture consisted of 10^5 cells per well whole $CD4^+$ or $CD4^+CD25^-$ T cell responders and 10^5 cells per well bm12 splenic stimulators and/or anti-CD40L mAb. (B) Secondary MLR culture consisted of 10^4 cells per well responder cells from 9-d bulk cultures as described in (A) and 10^5 cells per well fresh bm12 stimulators. Tritiated thymidine was added 20 h before harvesting. On the y-axis are mean cpm ± 1 SEM. On the x-axis are days in culture. One of five representative experiments is shown.

were incubated with anti-CD4 FITC and SA-perCP (BD PharMingen) and the level of CD40L expression evaluated on $CD4^+$ cells (FACScalibur™, CELLQuest™ software; Becton Dickinson).

Results and Discussion

$CD4^+CD25^+$ Cells Are Required for the Inhibition of Primary Proliferation by Anti-CD40L mAb. Although data indicate that $CD4^+CD25^+$ cells are essential for self-tolerance, their role in the induction of tolerance to alloantigen has not been studied. We have published previously that murine whole $CD4^+$ T cells incubated with allostimulator cells in the presence of anti-CD40L mAb results in an inhibition of primary MLR, hyporesponsiveness in secondary MLR, and tolerance to alloantigen in vivo (12, 13). To determine whether $CD4^+CD25^+$ cells were required for the induction of tolerance to alloantigen via ex vivo blockade of the CD40L/CD40 costimulatory pathway, MLR cultures were established with purified whole B6 $CD4^+$ cells or B6 $CD4^+CD25^-$ cells as responders and bm12 splenic allostimulators. As reported previously, the addition of anti-CD40L mAb inhibited peak proliferative responses in the whole $CD4^+$ cell population by 98% (Fig. 1 A). In contrast, peak responses in the CD25-depleted $CD4^+$ population were inhibited by only 55% (Fig. 1 A). A threefold increase in the concentration of anti-CD40L mAb did not result in greater inhibition of primary MLR (data not shown). Moreover, the CD25-depleted $CD4^+$ cells reproducibly had a higher and more sustained peak alloresponse than did whole $CD4^+$ control cells (5/5 experiments, Fig. 1 A, and data not shown).

Depletion of $CD4^+CD25^+$ Cells Abrogated the Induction of Hyporesponsiveness to Antigen Rechallenge In Vitro and In Vivo. To determine whether hyporesponsiveness to antigen rechallenge achieved in anti-CD40L mAb-treated primary MLR cultures required $CD4^+CD25^+$ cells, day-9 MLR cultures were washed and plated with fresh bm12 splenic stimulators in the absence of antibody. Both the whole $CD4^+$ and the CD25-depleted $CD4^+$ control-primed cells responded vigorously to alloantigen restimulation (Fig. 1 B). As expected, anti-CD40L–tolerized whole $CD4^+$ cells were profoundly hyporesponsive to alloantigen restimulation. In striking contrast, CD25-depleted $CD4^+$ cells that had been cultured with anti-CD40L mAb had secondary proliferative responses identical to ($n = 2$ experiments) or in excess of ($n = 3$ experiments) the control-primed cells

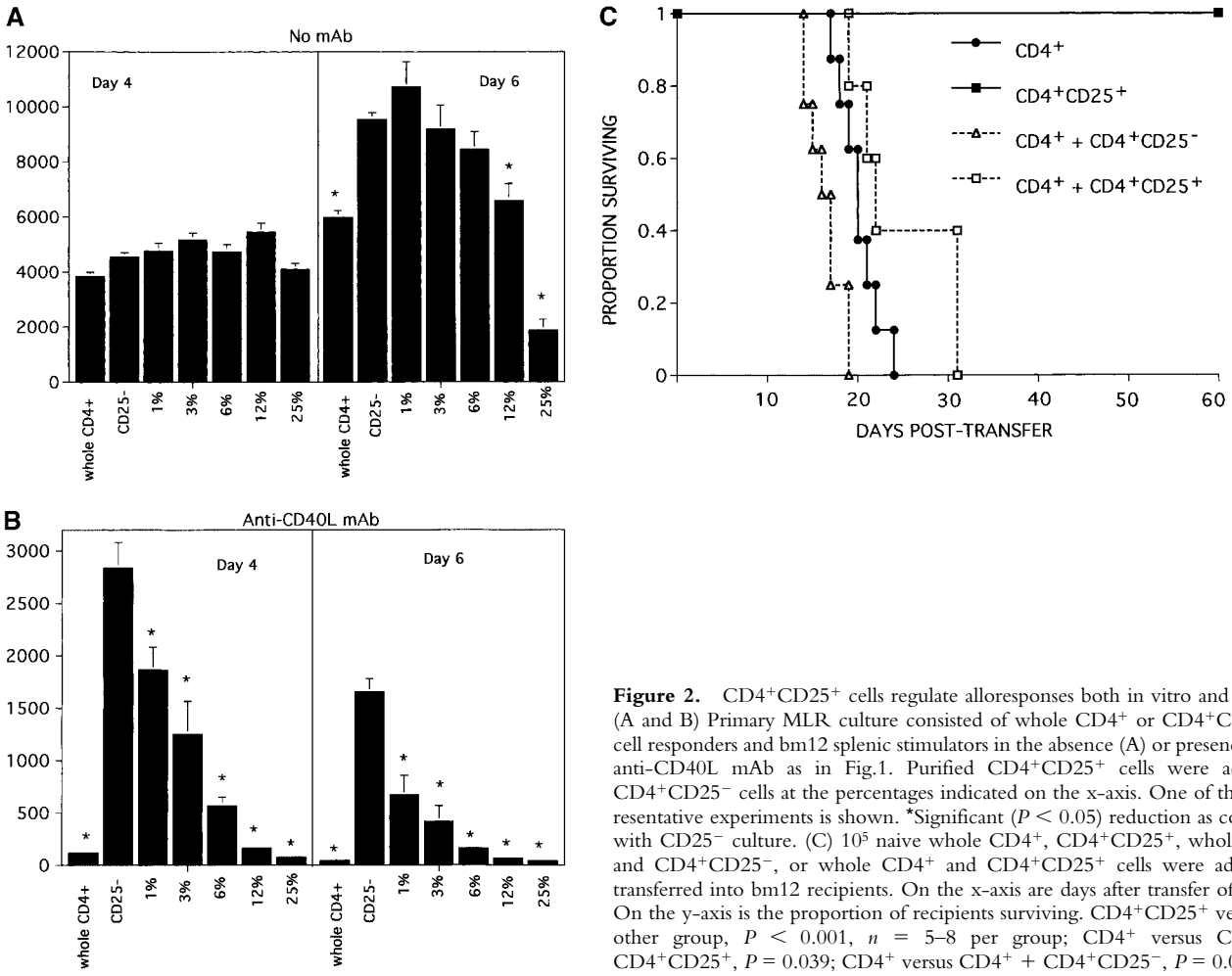

Figure 2. $CD4^+CD25^+$ cells regulate alloresponses both in vitro and in vivo. (A and B) Primary MLR culture consisted of whole $CD4^+$ or $CD4^+CD25^-$ T cell responders and bm12 splenic stimulators in the absence (A) or presence (B) of anti-CD40L mAb as in Fig.1. Purified $CD4^+CD25^+$ cells were added to $CD4^+CD25^-$ cells at the percentages indicated on the x-axis. One of three representative experiments is shown. *Significant ($P < 0.05$) reduction as compared with $CD25^-$ culture. (C) 10^5 naive whole $CD4^+$, $CD4^+CD25^+$, whole $CD4^+$ and $CD4^+CD25^-$, or whole $CD4^+$ and $CD4^+CD25^+$ cells were adoptively transferred into bm12 recipients. On the x-axis are days after transfer of T cells. On the y-axis is the proportion of recipients surviving. $CD4^+CD25^+$ versus any other group, $P < 0.001$, $n = 5$–8 per group; $CD4^+$ versus $CD4^+ + CD4^+CD25^+$, $P = 0.039$; $CD4^+$ versus $CD4^+ + CD4^+CD25^-$, $P = 0.007$.

(Fig. 1 B, and data not shown). These data indicate that $CD4^+CD25^+$ cells were essential for the tolerization of $CD4^+$ T cells induced via ex vivo CD40L/CD40 blockade.

To further investigate the role of $CD25^+$ cells on in vitro alloresponses, various numbers of purified $CD4^+CD25^+$ cells were added to $CD4^+CD25^-$ responder cells in both the absence and the presence of anti-CD40L mAb and proliferation was monitored (Fig. 2). Anti-CD40L mAb inhibited alloresponses of whole $CD4^+$ cells (containing 12% $CD4^+CD25^+$ cells) by 97% on day 4 and 99% on day 6 of culture. In contrast, anti-CD40L mAb inhibited alloresponses of $CD4^+CD25^-$ cells by only 25% on day 4 and 72% on day 6 as compared with whole $CD4^+$ cells (Fig. 2, A and B). The addition of $CD4^+CD25^+$ cells in the presence of anti-CD40L mAb resulted in increasingly profound inhibition of proliferation in a dose-dependent fashion (Fig. 2 B). The addition of as few as 1% $CD4^+CD25^+$ inhibited alloresponses of $CD25^-$ cells in the presence of anti-CD40L mAb. Because the degree of inhibition of proliferation by $CD4^+CD25^+$ cells was more pronounced on day 6 than on day 4, these data suggest that $CD4^+CD25^+$ cells may become more potent regulatory cells with increased culture duration. On day 6, in the absence of antibody, the addition of 12% $CD4^+CD25^+$ cells prevented the hyperproliferative alloresponse of $CD4^+CD25^-$ cells. The addition of 25% $CD4^+CD25^+$ cells had a pronounced suppressive effect even in the absence of anti-CD40L mAb (Fig. 2 A). These data indicate that $CD4^+CD25^+$ cells are required for the maximal inhibition of primary responses to alloantigen by the addition of anti-CD40L mAb and that $CD4^+CD25^+$ cells have a potent capacity to regulate $CD4^+$ T cell alloresponses.

The adoptive transfer of alloreactive T cells results in GVHD lethality, a clinically relevant in vivo model of alloresponsiveness (12, 13). To investigate the role of $CD4^+CD25^+$ cells on alloresponses in vivo, 10^5 freshly isolated, naive whole $CD4^+$, $CD4^+CD25^+$, whole $CD4^+$ and $CD4^+CD25^-$, or whole $CD4^+$ and $CD4^+CD25^+$ cells were infused into bm12 recipients (Fig. 2 C). $CD4^+CD25^+$ cells did not mediate GVHD lethality in any recipients in contrast to the uniform lethality mediated by naive whole $CD4^+$ cells 23 d after transfer. Mortality was delayed by 8 d in mice receiving equal numbers of purified $CD4^+CD25^+$ cells and whole $CD4^+$ cells as compared with mice receiving only whole $CD4^+$ cells ($P = 0.039$). In contrast, the coinjection of $CD4^+CD25^-$ cells with whole $CD4^+$ cells accelerated GVHD mortality by 1 wk as compared with recipients receiving only whole $CD4^+$ cells ($P = 0.007$). These data provide further evidence that naive $CD4^+CD25^+$ T cells have a modest capacity to downregulate alloreactive T cells in vivo.

To determine whether CD40L was expressed on $CD4^+CD25^+$ cells, we examined cell surface expression of

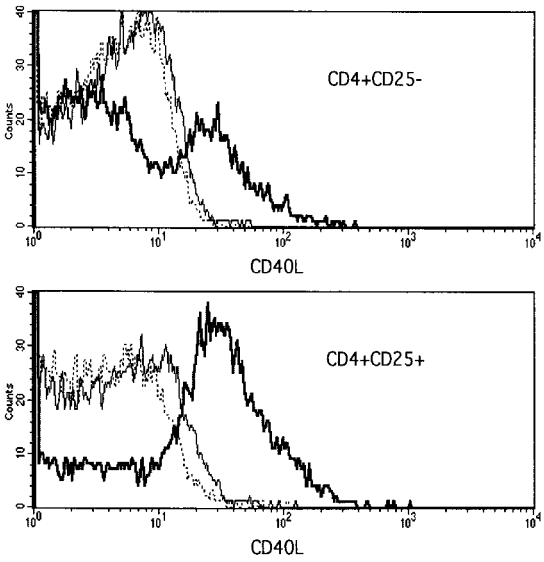

Figure 3. $CD4^+CD25^+$ cells upregulate CD40L on their cell surface during incubation with allostimulators. $CD4^+CD25^-$ (top) and $CD4^+CD25^+$ (bottom) cells were phenotyped for constitutive and inducible expression of CD40L. Dotted line indicates negative control. The thin solid line indicates the constitutive expression of freshly purified naive cells. The bold line indicates CD40L expression after 4-d incubation with allostimulators.

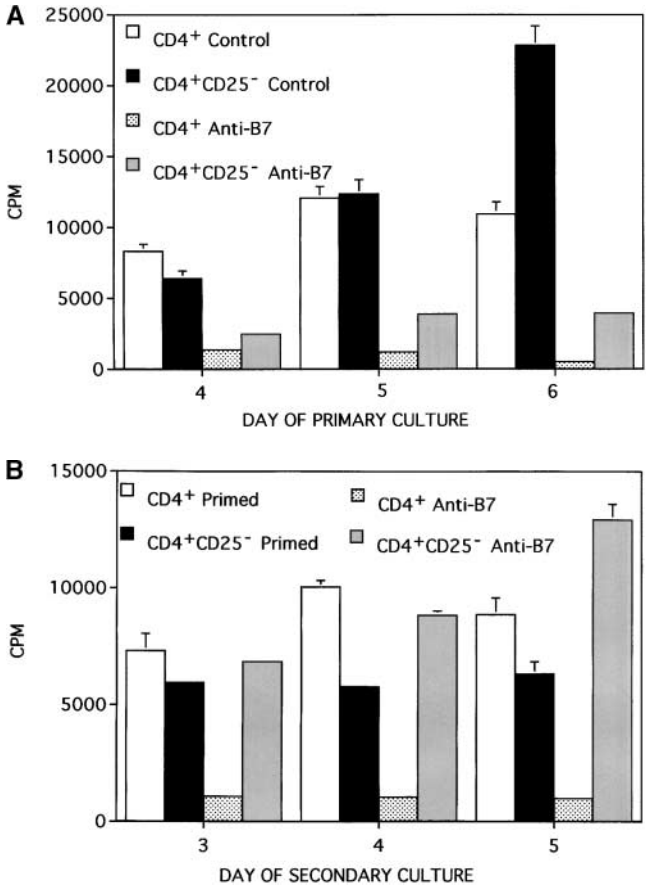

Figure 4. $CD4^+CD25^+$ T cells are required for tolerance induction by CD28/CTLA-4/B7 costimulatory blockade. (A) Primary MLR culture consisted of whole $CD4^+$ or $CD4^+CD25^-$ T cell responders and bm12 splenic stimulators and/or anti-B7 mAbs as in Fig. 1. (B) Secondary MLR culture consisted of 10^4 cells per well responder cells from 9-d bulk cultures as described in (A) and fresh bm12 stimulators. One of four representative experiments is shown.

CD40L on fresh, naive $CD4^+CD25^+$ cells and $CD4^+CD25^-$ cells (Fig. 3). Although there was only a very modest increase in constitutive expression of CD40L on $CD25^+$ cells as compared with $CD25^-$ cells, by day 4 of culture with allostimulators 69% of purified $CD4^+CD25^+$ cells expressed CD40L. In contrast, a lower proportion (30%) of $CD4^+CD25^-$ cells expressed CD40L. These data demonstrate that CD40L is expressed on a high proportion of $CD4^+CD25^+$ cells after alloantigen stimulation and suggest that the CD40L/CD40 pathway may play an important role in the regulation of alloresponses in $CD4^+CD25^+$ cells.

To determine if the requirement of $CD4^+CD25^+$ T cells for tolerance induction was unique to blockade of the CD40L/CD40 costimulatory pathway, experiments were performed using anti-B7.1 and anti-B7.2 mAbs to block the CD28/CTLA-4/B7 pathway. Although inhibition of primary MLR of $CD4^+CD25^-$ cells was more profound with anti-B7 mAbs than with anti-CD40L mAb (compare Figs. 4 A with 1 A), the degree of inhibition was not nearly as profound as that achieved with whole $CD4^+$ cells. More importantly, $CD4^+CD25^-$ cells that had been cultured with anti-B7 mAbs had intact or increased proliferative responses upon restimulation with alloantigen (Fig. 4 B). These data indicate that $CD4^+CD25^+$ cells are required for the ex vivo induction of tolerance to alloantigen via costimulatory blockade of either the CD40L/CD40 or the CD28/CTLA-4/B7 pathway.

An advantage of our ex vivo tolerization model is that it allows a clinically relevant, in vivo readout of alloresponsiveness. To assess alloreactivity in vivo, aliquots of cells used to establish secondary MLRs were adoptively transferred into bm12 recipients. All recipients of either whole $CD4^+$ or $CD4^+CD25^-$ control-primed cells died of GVHD 20–25 d after transfer (Fig. 5 A). Whole $CD4^+$ cells tolerized to alloantigen by a 9-d incubation in the presence of anti-CD40L mAb did not mediate GVHD lethality providing definitive in vivo evidence that tolerance induction had been successful. In contrast, all recipients of anti-CD40L mAb-cultured $CD4^+CD25^-$ T cells died of GVHD 18 d after transfer (Fig. 5 A). Similar results were seen with cultures incubated with anti-B7 mAbs (Fig. 5 B). No recipients of anti-B7–tolerized whole $CD4^+$ cells died of GVHD. In contrast, all recipients of anti-B7–cultured CD25-depleted $CD4^+$ cells died of GVHD by 25 d. These data indicate an essential requirement for $CD4^+CD25^+$ cells for tolerance induction as measured by protection from GVHD lethality.

Collectively, our data indicate that $CD4^+CD25^+$ cells are required for the ex vivo induction of tolerance to alloantigen via costimulatory blockade and that $CD4^+CD25^+$ cells regulate responses to alloantigen. Previous studies have described $CD4^+CD25^+$ cells as important immunoregulatory cells essential for T cell homeostasis and for the prevention of autoimmunity. Mice deficient in this population have a high incidence of various autoimmune disorders (1–4, 15, 16). $CD4^+CD25^+$ cells have been shown to suppress diseases induced by autoantigen-specific T cell clones (17). Shimizu et al. (18) found that depletion

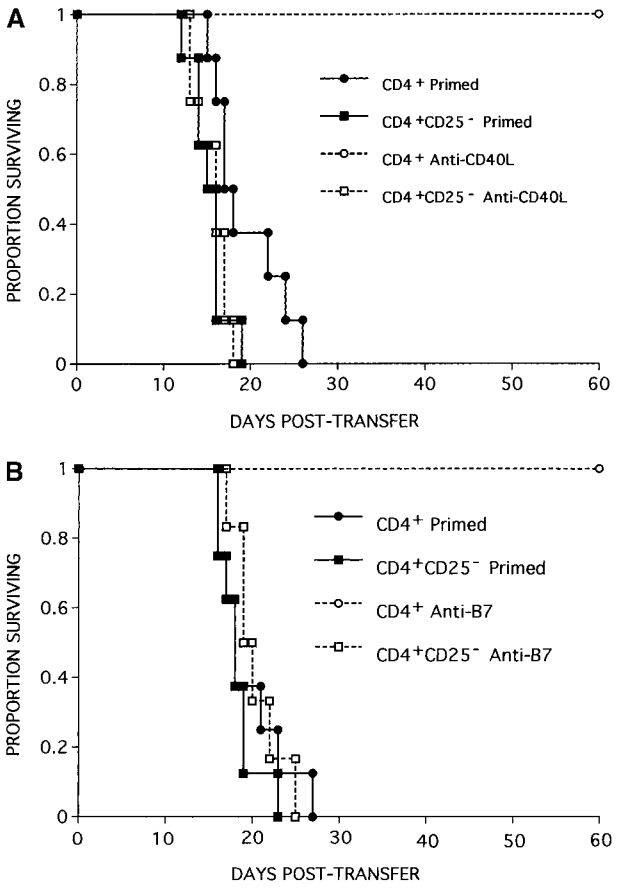

Figure 5. Depletion of $CD4^+CD25^+$ cells abrogated protection from GVHD lethality induced by tolerization via costimulatory blockade. 10^5 cells from washed, 9-d bulk primary MLR cultures as described in Figs. 1 and 4 were injected into bm12 recipients. (A) Anti-CD40L–tolerization. Two replicate experiments with similar results were pooled; $n = 16$. $CD4^+$ Anti-CD40L versus any other group, $P < 0.001$; $CD4^+$ Primed versus $CD4^+CD25^-$ Primed, $P = 0.056$. (B) Anti-B7–tolerization. $n = 8$ per group. $CD4^+$ Anti-B7 versus any other group, $P < 0.001$.

of $CD4^+CD25^+$ cells resulted in the breaking of immunological tolerance to autologous tumors and evoked tumor immunity. These studies have implicated $CD4^+CD25^+$ cells as an active mechanism for the induction and maintenance of immunologic tolerance to self. Fewer studies have addressed the role $CD4^+CD25^+$ T cells may play in response to nonself antigen. Sakaguchi et al. (1) found that nude mice rejected allogeneic skin grafts faster if transferred lymphocytes were first depleted of $CD25^+$ cells suggesting that these immunoregulatory cells downregulate responses to alloantigen. This is consistent with our finding that CD25-depleted $CD4^+$ cells had a heightened proliferative response to alloantigen and that the addition of $CD4^+CD25^+$ cells to either whole $CD4^+$ cells or CD25-depleted $CD4^+$ cells had a suppressive effect on in vitro and in vivo alloresponses. It is necessary to note that our culture system involves direct recognition to a single MHC class II disparity and therefore, may not be fully extrapolatable to other allogeneic systems. Nonetheless, our data extend the field by demonstrating an essential requirement of

the $CD4^+CD25^+$ regulatory population in allotolerance induced by costimulatory blockade. We hypothesize that these data implicating a professional suppressor cell population in the regulation of immune responses to nonself antigens may have important ramifications in many areas of clinical immunology.

The mechanism for the requirement of $CD4^+CD25^+$ cells in tolerance induction remains to be elucidated. Studies are in progress to determine whether $CD4^+CD25^+$ cells are the direct targets in costimulatory blockade tolerization strategies or if $CD4^+CD25^+$ cells provide indirect but essential help for tolerization of $CD4^+CD25^-$ cells. Thornton and Shevach (19, 20) have shown that $CD4^+CD25^+$ T cells block the induction of IL-2 production by the $CD4^+CD25^-$ cells at the level of RNA transcription and that activation enhances the immunosuppressive function of these cells. Activated $CD4^+CD25^+$ cells induced to become potent inhibitors of IL-2 transcription could facilitate anergy induction. Consistent with this hypothesis, IL-2 production is reduced by >90% in primary and secondary cultures of tolerized cells (13). Moreover, the addition of exogenous IL-2 precludes anergy induction in anti-CD40L, mAb-treated primary culture (12). Our data indicate that the activation of $CD4^+CD25^+$ cells is important in tolerance induction. The suppressive effect of the addition of 25% $CD4^+CD25^+$ cells to $CD4^+CD25^-$ cultures, not evident on day 4, was profound on day 6 of culture. Interestingly, we have found that a 4-d ex vivo incubation with anti-CD40L mAb was insufficient to induce tolerance in whole $CD4^+$ cells (13) suggesting a link between the activation of $CD4^+CD25^+$ cells and tolerance induction. Takahashi et al. (21) offers the intriguing suggestion that CTLA-4 and CD28 may have unique roles in $CD4^+CD25^+$ cells with costimulation through CTLA-4 or CD28 resulting in the activation or attenuation, respectively, of suppressor function (21).

However, these findings do not address the more provocative issue of why $CD4^+CD25^-$ cells are resistant to tolerance induction via ex vivo costimulatory blockade. One potential explanation may relate to the mechanisms by which $CD4^+CD25^+$ cells influence $CD4^+CD25^-$ cells. It has been reported recently that CTLA-4, the inhibitory T cell receptor for B7 molecules, is constitutively expressed on $CD25^+CD4^+$ regulatory cells but not $CD4^+CD25^-$ cells (21, 22). Since CTLA-4/B7 engagement has been reported to be required for the induction of peripheral T cell tolerance in vivo (23), constitutive CTLA-4 on $CD4^+CD25^+$ cells may facilitate ex vivo tolerance induction. CTLA-4/B7 engagement also has been shown to result in the secretion of the immunosuppressive cytokine, TGF-β, by $CD4^+CD25^+$ cells (22) which might facilitate tolerance induction. Although other studies have indicated that cell–cell contact appears to be required for the downregulatory capacity of $CD4^+CD25^+$ cells, it is possible that unidentified, labile, soluble factor(s) may be involved (15, 19, 20, 24). However, we have shown that exogenously added TGF-β alone (1 ng/ml) did not induce tolerance in our model (25) and further, that reduced alloreactivity is not achieved in our model via a Th2 skewing or by the production of the immunoregulatory cytokine, IL-10 (13).

Antibodies blocking costimulation administered in vivo can induce tolerance to donor bone marrow and organ allografts (7, 26–28). It is unknown whether depletion of host $CD4^+CD25^+$ cells would preclude tolerance to donor allografts, but it is tempting to speculate that they may also be essential for in vivo tolerance induction to alloantigen, at least in a strategy involving costimulatory blockade. Future studies will address this question. Although a $CD4^+CD25^+$ T cell with suppressor function has not been published in humans, there is evidence that professional immune regulatory/suppressor cells with similar function, if not phenotype, exist in humans and mice. A conditioning regime for allotransplant that preserves these cells may be clinically desirable.

The authors would like to thank Ms. Brigit G. Durell for excellent technical assistance and Dr. Laurence Turka for helpful discussions and suggestions.

This work was supported by National Institutes of Health grants R01 AI34495, 2R37 HL56067, R01 HL63452, and P01 AI35225, and a grant from the National Marrow Donor Program (to B.R. Blazar).

Submitted: 30 November 2000
Revised: 9 March 2001
Accepted: 20 March 2001

References

1. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155:1151–1164.
2. Suri-Payer, E., P.J. Kehn, A.W. Cheever, and E.M. Shevach. 1996. Pathogenesis of post-thymectomy autoimmune gastritis. Identification of anti-H/K adenosine triphosphatase-reactive T cells. *J. Immunol.* 157:1799–1805.
3. Asano, M., M. Toda, N. Sakaguchi, and S. Sakaguchi. 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J. Exp. Med.* 184:387–396.
4. Bonomo, A., P.J. Kehn, E. Payer, L. Rizzo, A.W. Cheever, and E.M. Shevach. 1995. Pathogenesis of post-thymectomy autoimmunity. Role of syngeneic MLR-reactive T cells. *J. Immunol.* 154:6602–6611.
5. Willerford, D.M., J. Chen, J.A. Ferry, L. Davidson, A. Ma, and F.W. Alt. 1995. Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. *Immunity*. 3:521–530.
6. Schwartz, R.H. 1990. A cell culture model for T lymphocyte clonal anergy. *Science*. 248:1349–1356.
7. Blazar, B.R., P.A. Taylor, A. Panoskaltsis-Mortari, J. Buhlman, J. Xu, R.A. Flavell, R. Korngold, R. Noelle, and D.A. Vallera. 1997. Blockade of CD40 ligand-CD40 interaction impairs $CD4^+$ T cell-mediated alloreactivity by inhibiting mature donor T cell expansion and function after bone marrow transplantation. *J. Immunol.* 158:29–39.
8. Seung, E., N. Iwakoshi, B.A. Woda, T.G. Markees, J.P. Mordes, A.A. Rossini, and D.L. Greiner. 2000. Allogeneic

hematopoietic chimerism in mice treated with sublethal myeloablation and anti-CD154 antibody: absence of graft-versus-host disease, induction of skin allograft tolerance, and prevention of recurrent autoimmunity in islet-allografted NOD/Lt mice. *Blood*. 5:2175–2182.

9. Durham, M.M., A.W. Bingaman, A.B. Adams, J. Ha, S.Y. Waitze, T.C. Pearson, and C.P. Larsen. 2000. Cutting edge: administration of anti-CD40 ligand and donor bone marrow leads to hemopoietic chimerism and donor-specific tolerance without cytoreductive conditioning. *J. Immunol.* 165:1–4.
10. Kirk, A.D., L.C. Burkly, D.S. Batty, R.E. Baumgartner, J.D. Berning, K. Buchanan, J.H. Fechner, Jr., R.L. Germond, R.L. Kampen, N.B. Patterson, et al. 1999. Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. *Nat. Med*. 5:686–693.
11. Parker, D.C., D.L. Greiner, N.E. Phillips, M.C. Appel, A.W. Steele, F.H. Durie, R.J. Noelle, J.P. Mordes, and A.A. Rossini. 1995. Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. *Proc. Natl. Acad. Sci. USA*. 92:9560–9564.
12. Blazar, B.R., P.A. Taylor, R.J. Noelle, and D.A. Vallera. 1998. $CD4^+$ T cells tolerized ex vivo to host alloantigen by anti-CD40 ligand (CD40L:CD154) antibody lose their graft-versus-host disease lethality capacity but retain nominal antigen responses. *J. Clin. Invest*. 102:473–482.
13. Taylor, P.A., A. Panoskaltsis-Mortari, R.J. Noelle, and B.R. Blazar. 2000. Analysis of the requirements for the induction of $CD4^+$ T cell alloantigen hyporesponsiveness by ex vivo anti-CD40 ligand antibody. *J. Immunol.* 164:612–622.
14. Roy, M., A. Aruffo, J. Ledbetter, P. Linsley, M. Kehry, and R. Noelle. 1995. Studies on the interdependence of gp39 and B7 expression and function during antigen-specific immune responses. *Eur. J. Immunol.* 25:596–603.
15. Takahashi, T., Y. Kuniyasu, M. Toda, N. Sakaguchi, M. Itoh, M. Iwata, J. Shimizu, and S. Sakaguchi. 1998. Immunologic self-tolerance maintained by $CD25^+CD4^+$ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int. Immunol.* 10:1969–1980.
16. Shevach, E.M. 2000. Regulatory T cells in autoimmmunity. *Annu. Rev. Immunol.* 18:423–449.
17. Suri-Payer, E., A.Z. Amar, A.M. Thornton, and E.M. Shevach. 1998. $CD4^+CD25^+$ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. *J. Immunol.* 160:1212–1218.
18. Shimizu, J., S. Yamazaki, and S. Sakaguchi. 1999. Induction of tumor immunity by removing $CD25^+CD4^+$ T cells: a common basis between tumor immunity and autoimmunity. *J. Immunol.* 163:5211–5218.
19. Thornton, A.M., and E.M. Shevach. 1998. $CD4^+CD25^+$ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med.* 188:287–296.
20. Thornton, A.M., and E.M. Shevach. 2000. Suppressor effector function of $CD4^+CD25^+$ immunoregulatory T cells is antigen nonspecific. *J. Immunol.* 164:183–190.
21. Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T.W. Mak, and S. Sakaguchi. 2000. Immunologic self-tolerance maintained by $CD25^+CD4^+$ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J. Exp. Med.* 192:303–310.
22. Read, S., V. Malmstrom, and F. Powrie. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of $CD25^+CD4^+$ regulatory cells that control intestinal inflammation. *J. Exp. Med.* 192:295–302.
23. Perez, V.L., L. Van Parijs, A. Biuckians, X.X. Zheng, T.B. Strom, and A.K. Abbas. 1997. Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. *Immunity*. 6:411–417.
24. Cederbom, L., H. Hall, and F. Ivars. 2000. $CD4^+CD25^+$ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. *Eur. J. Immunol.* 30:1538–1543.
25. Zeller, J.C., A. Panoskaltsis-Mortari, W.J. Murphy, F.W. Ruscetti, S. Narula, M.G. Roncarolo, and B.R. Blazar. 1999. Induction of $CD4^+$ T cell alloantigen-specific hyporesponsiveness by IL-10 and TGF-β. *J. Immunol.* 163:3684–3691.
26. Wekerle, T., J. Kurtz, H. Ito, J.V. Ronquillo, V. Dong, G. Zhao, J. Shaffer, M.H. Sayegh, and M. Sykes. 2000. Allogeneic bone marrow transplantation with co-stimulatory blockade induces macrochimerism and tolerance without cytoreductive host treatment. *Nat. Med.* 6:464–469.
27. Zheng, X.X., M.H. Sayegh, X.G. Zheng, Y. Li, P.S. Linsley, R. Peach, F. Borriello, T.B. Strom, A.H. Sharpe, and L.A. Turka. 1997. The role of donor and recipient B7-1 (CD80) in allograft rejection. *J. Immunol.* 159:1169–1173.
28. Kirk, A.D., D.M. Harlan, N.N. Armstrong, T.A. Davis, Y. Dong, G.S. Gray, X. Hong, D. Thomas, J.H. Fechner, Jr., and S.J. Knechtle. 1997. CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. *Proc. Natl. Acad. Sci. USA*. 94:8789–8794.