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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

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Abstract

A major goal of the transplant field is to tolerize donor T cells to prevent graft-versus-host disease (GVHD) (1). We describe an ex vivo approach in which the blockade of CD40 ligand (CD40L:CD154):CD40 interactions, a pathway required for optimal T cell expansion, induces donor CD4⁺ T cells to become tolerant to host alloantigens (2). High doses of tolerized cells did not cause GVHD lethality in vivo. T cells had intact responses to antigens not present during tolerization. Tolerance was long lived and not readily reversible in vivo. These data have significant implications for the use of tolerization approaches to prevent human GVHD. (*J. Clin. Invest.* 1998. 102:473–482.) Key words: transplantation immunology • transplantation • allogeneic • T lymphocytes • immune tolerance • animal models

Introduction

Graft-versus-host disease (GVHD)¹ is a major cause of morbidity and mortality after bone marrow transplantation. Options for GVHD prevention have included rigorous T cell depletion ex vivo or prolonged in vivo immunosuppression (1). Both predispose the recipient to an extended period of poor immune reconstitution. Because a small fraction of donor T cells respond to host alloantigens and cause GVHD, a strategy in which GVHD-causing T cells are selectively inhibited is desirable (3).

Although T cells that receive sufficient signals via the T cell

receptor (TCR) are induced to proliferate, a sustained response requires a second (costimulatory) signal (4). Jenkins and Schwartz demonstrated that antigen-specific T cell clones combined with paraformaldehyde-fixed antigen-presenting cells and antigen become incapable of responding to antigen but are fully responsive to IL-2, a state termed anergy (4). Paraformaldehyde-fixation prevents the cell surface expression of molecules that provide a costimulatory signal to responding T cells.

Several APC surface determinants have been shown to be capable of providing T cell costimulation. CD40, a 50-kD member of the nerve growth factor/tumor necrosis factor receptor family, is expressed on classical nonprofessional and professional antigen-presenting cells (5). The T cell counter-receptor for CD40 is CD40L, a type II integral membrane glycoprotein that is transiently expressed on antigen-activated CD4⁺ T cells and a small fraction of activated CD8⁺ T cells (6). CD40L transduces a signal to CD40-expressing B cells to upregulate molecules involved in T cell costimulation (7). T cells are tolerized when exposed to antigen in a situation whereby APC are incapable of receiving a CD40 signal either due to anti-CD40L mAb or a genetic deficiency of CD40 (8, 9). In vivo blockade of CD40L:CD40 interaction in rodents has been shown to be effective in inhibiting the humoral and cellular inflammatory responses in autoimmune mice and recipients of solid organ or lymphohematopoietic allografts (7–12).

Despite the ability of investigators to tolerize T cell clones, there are no reported studies in which permanent alloantigen-specific hyporesponsiveness has been achieved by an ex vivo approach culminating in the prevention of GVHD in vivo in the absence of supplemental immune suppression. The present study was undertaken to determine whether ex vivo blockade of CD4⁺ T cell costimulation via disrupting the binding of CD40L to CD40 would induce a state of alloantigen-specific tolerization, resulting in the lack of GVHD generation in vivo.

Methods

Animals. B6.C-H2^{bm12}/KhEg (termed bm12) and C57BL/6-scid/scid (SCID: severe combined immunodeficient) (H2^b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 (H2^b) and BALB/c (H2^d) mice were purchased from the National Institutes of Health (Bethesda, MD). D011.10 transgenic mice, backcrossed > 10 generations onto a BALB/c background, were generated as described and provided by Marc Jenkins (University of Minnesota, Minneapolis, MN) (13, 14). Donors and recipients were 8–10 wk of age at the time of BMT. All mice were housed in a specific pathogen-free facility in microisolator cages.

B.R. Blazar and P.A. Taylor contributed equally to this work.

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1. Abbreviations used in this paper: CD40L, CD40 ligand; GVHD, graft-versus-host disease; MLR, mixed lymphocyte reaction; OVA, chicken egg ovalbumin; SCID, severe combined immunodeficient; TCR, T cell receptor.

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MLR cultures and monitoring of proliferation. To purify CD4⁺ T cells, single cell suspensions of axillary, mesenteric, and inguinal lymph node cells were mashed, passed through a wire mesh, and collected into phosphate-buffered saline supplemented with 2% fetal calf sera. Cell preparations were depleted of NK cells (hybridoma PK136, rat IgG2a, provided by Gloria Koo, Rahway, NJ) and CD8⁺ T cells by coating with anti-CD8 mAb (hybridoma 2.43, rat IgG2b, provided by David Sachs, Cambridge, MA), followed by passage through a goat anti-mouse and goat anti-rat Ig coated column (Biotex, Edmonton, Canada). The goat anti-mouse Ig coated column also removes most Fc receptor bearing cells including B cells, macrophages, and dendritic cells. The final composition of T cells in the donor graft was determined by flow cytometry and was always found to be $\geq 94\%$ CD4⁺ T cells. Responders were mixed with irradiated (30 Gray) anti-Thy1.2 mAb (hybridoma 30H-12, rat IgG2b, provided by D. Sachs), and anti-NK1.1 (hybridoma PK136, mouse IgG2a, provided by G. Koo) plus complement-treated splenocyte stimulators. Two types of cultures were established. In the first, C57BL/6 CD4⁺ T cells were mixed with bm12 splenic stimulators. In the second, non-transgenic BALB/c CD4⁺ T cells were mixed with D011.10 BALB/c TCR transgenic CD4⁺ T cells at a ratio of 3:1 and then mixed with C57BL/6 splenic stimulator cells. Responders and stimulator cells were suspended at a final concentration of $0.5 \times 10^6/\text{ml}$ in 24-well plates (Costar, Acton, MA) containing DME (Bio Whittaker, Walkersville, MD) with 10% fetal calf serum (Hyclone, Logan, UT), 2-mercaptoethanol (5×10^{-5} M) (Sigma Chemical Co., St. Louis, MO), 10 mM Hepes buffer, 1 mM sodium pyruvate (GIBCO BRL, Grand Island, NY), and amino acid supplements (1.5 mM L-glutamine, L-arginine, and L-asparagine) (Sigma Chemical Co.), antibiotics (penicillin: 100 U/ml, streptomycin: 100 mg/ml) (Sigma Chemical Co.). Anti-CD40L mAb (hybridoma MR1, hamster IgG [6]) was obtained by culturing the hybridoma in media with 10% fetal bovine serum in a hollow-fiber bioreactor (Accucyst[®], Cellex Biosciences, Minneapolis, MN), supernatant purified by ammonium sulfate precipitation. Anti-CD40L mAb was added at a final concentration of 50 $\mu\text{g}/\text{ml}$ to primary MLR cultures. Plates were incubated at 37°C and 10% CO₂ for 8 or 10 d. On day 5, the culture was fed 1:1 with new medium including mAb. In some experiments, human recombinant IL-7 (R & D Systems, Minneapolis, MN) was added at 0.1 ng/ml to support cell recovery. To monitor primary MLR proliferation, 96-well round bottom microtiter plates (Costar) were setup to contain 10^5 responders and 10^5 stimulator cells per well in the presence or absence of exogenous IL-2 (50 IU/ml).

To monitor secondary MLR proliferation, 3×10^4 B6 or 7.5×10^4 BALB/c responders and 10^5 irradiated (30 Gray) non-T cell depleted splenic stimulators were plated in the presence or absence of IL-2 (50 IU/ml). To assess OVA responses in secondary MLR cultures, 2.5×10^4 KJ1-261 CD4⁺ T cells were mixed with 10^5 BALB/c irradiated splenic feeder cells for plating in 96-well microtiter plates. Chicken egg ovalbumin (OVA) peptide 323–339 (ISQAVHAAHAEINEAGR) was added at a final concentration of 5.0 $\mu\text{g}/\text{ml}$ (14). Microtiter wells were pulsed with tritiated thymidine (1 μCi) for 16–18 h before harvesting and counted in the absence of scintillation fluid on a betaplate reader. Three to six wells were analyzed for each data point.

GVH induction and IL-2 administration. bm12 recipients were sublethally irradiated by exposing mice to 6.0 Gray total body irradiation from a ¹³⁷Cesium source at a dose rate of 85 cGy/min. Day 10 MLR-cultured cells were injected intravenously at the doses indicated. Hematocrit values were obtained at periodic intervals as an indicator of the possible bone marrow destructive effects of infused T cells (12, 15). Where indicated, exogenous IL-2 (AMGEN, Thousand Oaks, CA) was administered intraperitoneally once daily at a dose of 50,000 IU on Monday through Friday from days 0–14 of T cell transfer.

T cell transfer into syngeneic SCID recipients and thoracic duct cannulation. Control or anti-CD40L mAb-treated MLR bulk-cultured cells were injected intravenously at a dose of 2×10^6 CD4⁺ T

cells into non-irradiated B6-SCID recipients as a means of examining the persistence of CD4⁺ T cells in vivo in the absence of alloantigen. For thoracic duct lymphocyte isolation, cannulae were inserted in the thoracic duct of recipients on day 40 after transfer and cells were collected over a period of 18 h (12, 16). The number and phenotype of thoracic duct lymphocytes were quantified. Thoracic duct lymphocytes were adoptively transferred into sublethally irradiated secondary bm12 recipients at a dose of 10^5 CD4⁺ T cells per mouse via intravenous injection as described above.

Flow cytometry. T cells from bulk MLR cultures and thoracic duct lymphocytes were assessed for evidence of activation by forward- and side-scatter profiles and for the co-expression of CD4 and activation antigens including IL2R alpha chain (CD25). To distinguish BALB/c non-transgenic from D011.10 TCR transgenic CD4⁺ T cells, KJ1-26 mAb which demarcates a CD4⁺ T cell population specific for chicken OVA peptide 323–339 bound to IA^d was biotinylated (14). KJ1-26 mAb does not detect normal BALB/c T cells. All studies were performed with two or three color flow cytometry using fluorescein isothiocyanate, phycoerythrin, or biotin-conjugated mAb (PharMingen, San Diego, CA) along with SA-PerCP (Becton Dickinson, Mountain View, CA). Irrelevant mAb control values were subtracted from values obtained with relevant mAbs. Cultured cells were also examined for the incorporation of 7-AAD (7-amino-actinomycin D) (Sigma Chemical Co.). Cells with intact membranes exclude 7-AAD, which binds to DNA; thus, 7-AAD is an early marker for apoptosis (17). All results were obtained using a FACScalibur (Becton Dickinson). Forward- and side-scatter settings were gated to exclude debris. 10–20,000 cells were analyzed for each determination.

Statistics. Group comparisons were made by Student's *t* test. Survival data were analyzed by lifetable methods and actuarial survival rates are shown. Probability (*P*) values < 0.05 were considered significant.

Results

CD4⁺ T cells are induced into a state of alloantigen hyporesponsiveness by prolonged ex vivo CD40L:CD40 blockade. To determine whether blockade of CD40L:CD40 interaction during a mixed lymphocyte reaction (MLR) culture would induce alloantigen hyporesponsiveness, highly purified donor CD4⁺ lymph node T cells were reacted with irradiated, T cell depleted MHC class II disparate splenic stimulators in bulk cultures in the presence or absence of anti-CD40L mAb for a period of 10 d. MLR cultures were established with B6 responders and bm12 stimulators, which differ from responders only at three amino acids due to mutations in the IA region. T cells in the mAb treated had modestly reduced early (day 2) proliferative responses as compared to the control group (Fig. 1 A). However, proliferation in the control group continued to increase, peaking on day 5–6 of culture, in contrast to T cells in the mAb-treated group, which decreased throughout the evaluation period.

On day 10 of primary culture, cell recovery in the control group was 90% and in the anti-CD40L mAb-treated group 11%, of input T cell number. T cells in the control and mAb-treated groups had evidence of activation as denoted by forward- and side-scatter profiles and high levels of CD25 (IL-2 α chain receptor; Fig. 2). As compared to the control group, forward- and side-scatter profiles in the mAb-treated group were consistently lower, albeit in both groups, an increased forward- and side-scatter profile was observed as compared to day 0 responder cells. In contrast, CD25 levels are typically comparable in the mAb-treated and control groups. L-selectin downregulation was more pronounced in the control group, a

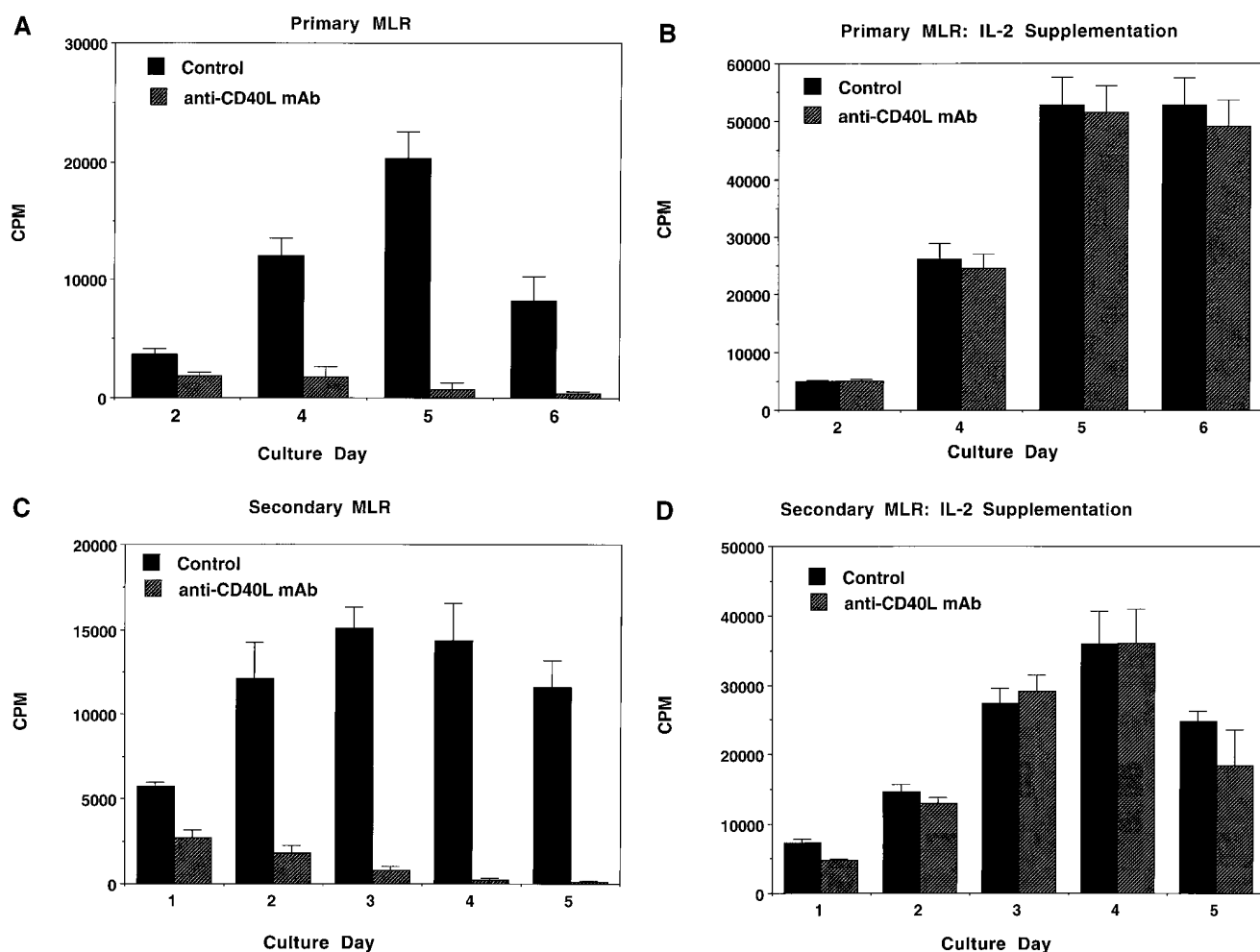


Figure 1. Induction of alloantigen hyporesponsiveness in anti-CD40L mAb-treated cultures (A and C) and restoration of alloantigen responses by exogenous IL-2 (B and D). Primary MLR cultures (A and B) consisted of C57BL/6 CD4⁺ lymph node T cell responders and bm12 splenic stimulators. After 10 d of primary culture, secondary MLR cultures were established (C and D). On the y-axis are mean cpm \pm 1 SD of the mean. On the x-axis are days in primary or secondary culture. A representative experiment is shown. Findings were entirely reproduced in five additional experiments.

consistent finding in all experiments analyzed to date. To determine whether T cells in anti-CD40L mAb-treated cultures had evidence of early apoptosis, we quantified the frequency of 7-AAD⁺ cells. 7-AAD⁺ T cells from control or mAb-treated cultures were present at a frequency of $\leq 3\%$. The addition of exogenous IL-2 prevented the induction of anti-host hyporesponsiveness (Fig. 1 B). Collectively, these data indicate that the alloantigen hyporesponsiveness induced by anti-CD40L mAb did not preclude the activation of donor CD4⁺ T cells in this culture system.

To assess the response of control or mAb-treated T cells to alloantigen restimulation, day 10 MLR cells were washed to remove mAb, and secondary MLR cultures were established. Anti-CD40L mAb-treated T cells continued to be hyporesponsive to host alloantigen (Fig. 1 C). Responses of tolerized T cells to exogenous IL-2 were comparable to control T cells (Fig. 1 D).

Ex vivo tolerized CD4⁺ T cells have a markedly impaired capacity to mediate GVHD lethality even with exogenous IL-2 supplementation in vivo. To determine whether ex vivo tolerized T cells were impaired in inducing GVHD in vivo, a series

of experiments were performed in which various doses of control or an equal number of anti-CD40L mAb-treated CD4⁺ T cells were infused into sublethally irradiated recipients bearing the same alloantigen used as stimulator cells in MLR cultures. As few as 3×10^4 control cultured T cells were lethal to 97% of recipients (Fig. 3). A 33-fold higher dose of T cells (10^6) was unable to cause lethality in any of the recipients of anti-CD40L mAb-treated cells.

Because IL-2 can prevent anergy induction and anergic cells will respond to alloantigen in the presence of exogenous IL-2, cohorts of recipients of control or anti-CD40L mAb were given IL-2 at a dose of 50,000 IU on Monday through Friday from days 0–14 after transfer. Recipients of control cultured cells, including those receiving IL-2 injections, all died by day 22 after transfer (Fig. 4). In contrast, 100% of recipients of anti-CD40L mAb-treated cells and 80% of IL-2-treated recipients of tolerized cells survived. As an indicator of CD4⁺ T cell-mediated GVHD-induced aplasia, hematocrit values were assessed in all mice on day 14 (15) (Table I). Day 14 mean hematocrit values were significantly lower in recipients of control cells than recipients of ex vivo tolerized cells,

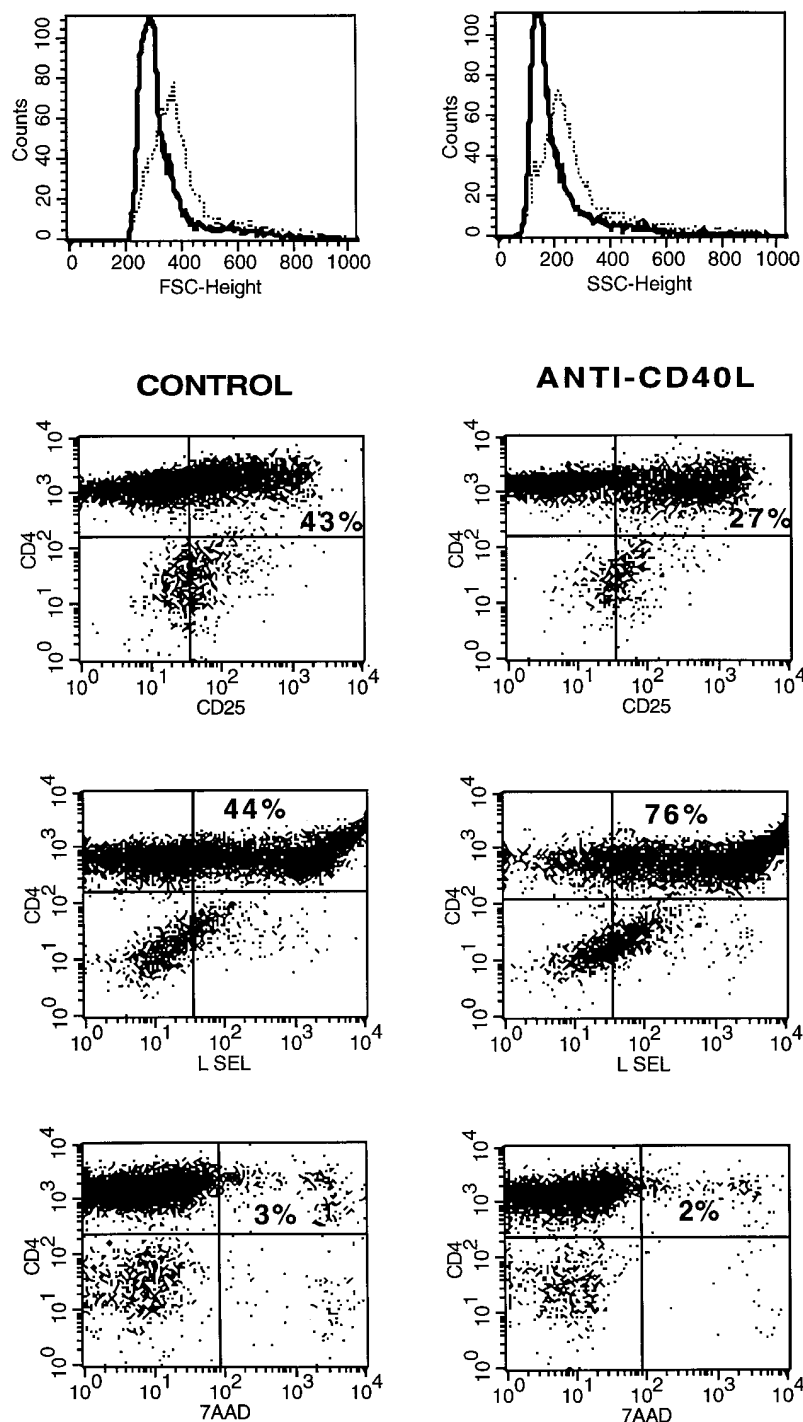


Figure 2. Flow cytometry analysis of day 10 MLR cultured cells. Primary MLR cultures from Fig. 1 A were analyzed on day 10 for evidence of T cell activation. Flow cytometry was performed to determine forward- (FSC) and side- (SSC) scatter contours as a measurement of activation according to size and granularity characteristics. Overlay histograms are shown with the dotted line representing T cells from control cultures and the bold line, CD4⁺ T cells from the anti-CD40L mAb-treated culture. Three color flow cytometry was performed to determine the proportion of CD4⁺ T cells that coexpressed CD25 or L-selectin (*L-sel*) and 7-AAD (a marker of early apoptosis). The percent of CD4⁺ T cells coexpressing the indicated determinant is listed in the upper right hand quadrant.

whether or not recipients were also given exogenous IL-2. Recipients of anti-CD40L mAb had mean hematocrit values of $\geq 30\%$ on day 28 and at time periods > 2 mo after transfer, regardless of whether or not mice also received IL-2. All mice that received control cultured cells died before day 28 and, therefore, were not analyzable for hematocrit values. These data indicate that the tolerant state of anti-CD40L mAb-treated cells is not readily reversed *in vivo* by exogenous IL-2.

Ex vivo tolerized CD4⁺ T cells persist in vivo. As a rigorous test for persistence of anti-CD40L mAb-treated donor CD4⁺ T cells, T cells from control, and anti-CD40L mAb-

treated 10-d MLR cultures were parked in SCID mice that were syngeneic to the donor (responder) strain. Because SCID mice do not produce their own T cells, all T cells isolated from these recipients are of donor origin. After 40 d, thoracic duct lymphocytes from both groups of recipients were collected overnight from the recirculating pool and the number of lymphocytes quantified (12, 15). As compared to the input number, there was a modest expansion (1.8-fold) of CD4⁺ T cells retrieved from recipients of control cultured cells. In recipients of anti-CD40L mAb-treated cells, 45% of the input cells were recovered (Table II). CD4⁺ T cells from recipients of control

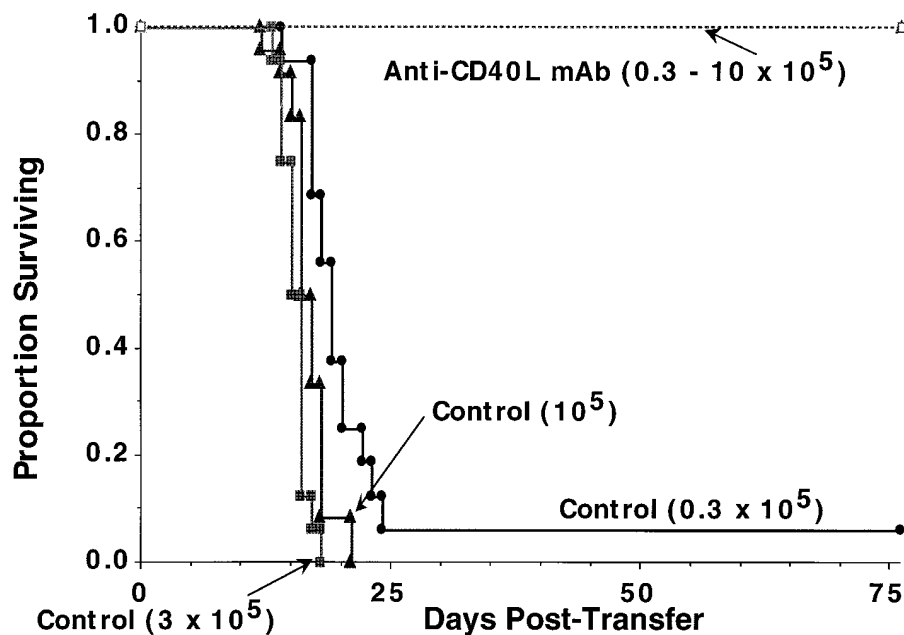


Figure 3. B6 anti-bm12-d 10 MLR cells cultured in the presence of anti-CD40L mAb do not cause GVHD lethality in sublethally irradiated bm12 recipients. Control or anti-CD40L mAb-treated CD4⁺ T cells were injected into bm12 recipients. CD4⁺ T cell doses for recipients of control cells were: 3×10^5 ($n = 16$), 10^5 ($n = 24$), or 0.3×10^5 ($n = 16$); doses for anti-CD40L mAb-treated cultures were: 10×10^5 ($n = 3$), 3×10^5 ($n = 16$), 10^5 ($n = 22$), or 0.3×10^5 ($n = 16$). Data are pooled from four replicate experiments. On the x-axis are days post-transfer. On the y-axis is the proportion of recipients surviving post-transfer. Recipients of anti-CD40L mAb treatment had a significantly higher survival rate as compared to recipients of a comparable number of control cultured cells at each cell dose tested.

and anti-CD40L mAb-treated cells had similar flow cytometric characteristics. As compared to B6 controls, CD4⁺ thoracic duct lymphocytes from B6-SCID recipients of control or anti-CD40L mAb-treated day 10 MLR cells had evidence of prior activation as determined by forward- and side-scatter profiles, downregulation of L-selectin, and upregulation of CD44 antigen density, although CD25 was not upregulated (Table II). These data are consistent with the generation of a memory cell phenotype and the *in vivo* persistence of T cells from an *ex vivo* tolerized culture.

The GVHD lethality capacity of CD4⁺ T cells from both groups was assessed by adoptive transfer into sublethally irradiated secondary recipients (Fig. 5). All recipients of control T cells uniformly succumbed to GVHD-induced lethality, whereas none of the recipients of an equal number of anti-CD40L mAb-treated T cells died. Taken together, these data

indicate that CD4⁺ T cells exposed *ex vivo* to anti-CD40L mAb can survive long term in SCID recipients, and the tolerance induced by anti-CD40L mAb was long lived *in vivo* in the absence of alloantigen.

CD4⁺ T cell responses to nominal antigen responses are preserved during *ex vivo* tolerization to alloantigen. A culture system was established in which T cell responses to an antigen not present during the tolerization process could be accurately quantified. To optimize quantification, we physically tracked the responses of D011.10 TCR transgenic CD4⁺ T cells that have the appropriate TCR for a vigorous response to the nominal antigen, OVA (13). CD4⁺ T cell cultures contained 25% D011.10 TCR transgenic T cells and 75% non-transgenic CD4⁺ T cells of the same genetic (BALB/c) background. B6 stimulator cells differed from the responders at MHC class I plus II loci, as well as several minor histocompatibility loci.

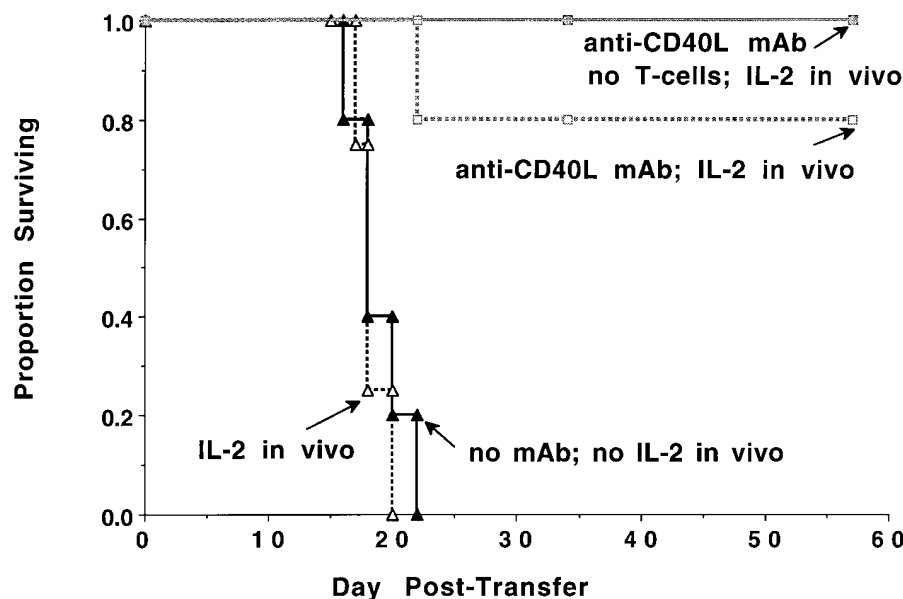


Figure 4. IL-2 administration *in vivo* does not readily break the impaired GVHD capacity of anti-CD40L mAb-treated day 10 MLR cells. Control or anti-CD40L mAb-treated primary MLR cultured cells were injected into sublethally irradiated recipients ($n = 5$ per group). Cohorts of mice, as indicated, received IL-2 injections at a dose of 50,000 IU on Monday through Friday from days 0–14 post-transfer. On the x-axis are days post-transfer. On the y-axis is the proportion of recipients surviving post-transfer. As compared to controls receiving the same numbers of CD4⁺ T cells, recipients of anti-CD40L mAb had a significantly higher actuarial survival rate.

Table I. Effect of Exogenous IL-2 Administration or GVH-induced Aplasia of Control or Anti-CD40L mAb-treated Day 10 MLR Cultured cells Transferred into Sublethally Irradiated Recipients*

Day 10 MLR cells	IL-2	Day	Hematocrit
Control	None	14	24±1
Control	Yes	14	21±3
Anti-CD40L mAb	None	14	42±2 [‡]
Anti-CD40L mAb	Yes	14	37±1 [‡]
None	Yes	14	44±1 [‡]

*Sublethally irradiated bm12 recipients ($n = 5/\text{group}$) were given day 10 MLR cultured cells that were control cultured or treated with anti-CD40L mAb. 10^5 cells were infused per group. Cohorts of mice were given exogenous IL-2 at a dose of 50,000 IU i.p. on days 0–4 and days 7–11 after transfer. [‡] $P < 0.001$, as compared to recipients of control cultured cells.

Anti-CD40L mAb treatment induced alloantigen hyporesponsiveness in primary (Fig. 6A) MLR cultures. Anti-CD40L mAb-induced alloantigen hyporesponsiveness was prevented by the addition of exogenous IL-2 (data not shown). To determine whether inhibition of alloantigen responses occurred at the expense of nominal antigen responses, secondary cultures were established to monitor alloantigen and nominal antigen responses. Because the proportion of TCR transgenic CD4⁺ T cells was higher in the control as compared to the mAb-treated group (36% versus 16%, respectively), the culture was adjusted such that the control and anti-CD40L mAb-treated cultures contained the same number of non-transgenic CD4⁺ T cells per well for measuring alloresponses and the same number of transgenic CD4⁺ T cells for measuring OVA responses. Secondary MLR cultures in this strain combination were performed on day 8 rather than day 10 to ensure that sufficient numbers of transgenic and non-transgenic T cells were available for analysis. At the end of 8 d, cell recovery in the control group was 158% and, in the anti-CD40L mAb-treated group, 28% of input T cell number. Anti-CD40L mAb treatment lead to secondary alloantigen hyporesponsiveness (Fig. 6B) as previously noted. Due to the earlier time period of primary cul-

ture, secondary responses to alloantigen-bearing stimulator cells peaked on day 1 of secondary culture, a reproducible finding in this and the B6 anti-bm12 system when cultures are analyzed on day 8 rather than day 10 (our unpublished data). Although 8 d are sufficient to tolerize alloreactive T cells to host alloantigen-bearing stimulators ex vivo and to markedly inhibit GVHD lethality in vivo, the longer 10-d culture system results in a secondary MLR response that peaks later than observed with 8-d cultures (unpublished data). The addition of exogenous IL-2 facilitated effective T cell responses to alloantigen in secondary cultures (data not shown). Importantly, anti-CD40L mAb did not impair responses to OVA challenge in secondary culture under conditions that were sufficient for inducing alloantigen hyporesponsiveness after primary culture (Fig. 6C).

Discussion

In this study, we have shown that an ex vivo tolerization approach can be used to prevent GVHD lethality mediated by MHC class II alloantigen-responsive CD4⁺ T cells. Donor CD4⁺ T cells exposed ex vivo to host alloantigens in the presence of anti-CD40L mAb were rendered hyporesponsive to host alloantigen as assessed in both primary and secondary MLR culture. Ex vivo tolerized T cells were incapable of causing GVHD in vivo. Despite effective alloantigen tolerization, nominal antigen responses remained intact. Tolerance was long lived and not readily reversible by exogenous IL-2.

An interesting aspect of the present studies is the high degree of GVHD protection afforded by ex vivo as compared to in vivo anti-CD40L mAb exposure in this same strain combination. In previous studies, we have shown that sublethally irradiated bm12 recipients of 10^5 CD4⁺ T cells given anti-CD40L mAb in vivo for up to 1 mo after transfer had a 55% survival rate, corresponding to an approximate threefold reduction in GVHD-induced lethality. In contrast, our ex vivo results described in the present report show a > 30-fold reduction in GVHD capacity of CD4⁺ T cells exposed ex vivo to bm12 alloantigen-bearing stimulator cells in the presence of anti-CD40L mAb. The high degree of GVHD protection conferred by ex vivo anti-CD40L mAb treatment cannot be accounted for by a decreased GVHD lethality capacity of cul-

Table II. Recovery and Flow Cytometry Analysis of Thoracic Duct Lymphocytes Isolated 40 d after Parking Control or Anti-CD40L mAb-treated 10 d MLR Cultured Cells into B6-SCID Recipients*

Group	% CD4	CD4 ⁺ T cells	CD4 ⁺					
		per mouse $\times 10^6$	FSC	SSC	% CD25	% L-selectin	% CD44	7-AAD
Control cultured	79	3.7	364	286	4	11	77 (734)	1
Anti-CD40L mAb	63	0.9	354	268	5	16	62 (629)	1
B6 controls	31	12.4	307	186	3	32	24 (154)	0

*Primary MLR cultures were established using highly purified lymph node CD4⁺ T cells from B6 mice as responders and irradiated, T cell depleted bm12 splenic stimulators. At the end of 10 d, cells were infused into sublethally irradiated bm12 recipients ($0.3\text{--}10 \times 10^5/\text{mouse}$) (see Fig. 2) or B6-SCID mice ($2 \times 10^6/\text{mouse}$). After 40 d, thoracic duct lymphocytes were isolated from B6-SCID mice or non-BMT B6 controls ($n = 5/\text{group}$) during an overnight collection period. The number of thoracic duct lymphocytes was quantified and data normalized for a 24-h period. Flow cytometry was performed to determine CD4⁺ T cell content and values used to calculate the total number of CD4⁺ T cells produced in 24 h. Forward- (FSC) and side- (SSC) scatter contour plots of CD4⁺ T cells were acquired as a measurement of activation according to size and granularity. Three color flow cytometry was performed to determine the percent of cells that coexpressed CD4 and CD25 (activation marker), memory cell markers (L-selectin^{lo}, CD44^{hi}) or 7-AAD (a marker of early apoptosis). The numbers shown in () are the mean fluorescent channel for quantifying CD44 antigen density.

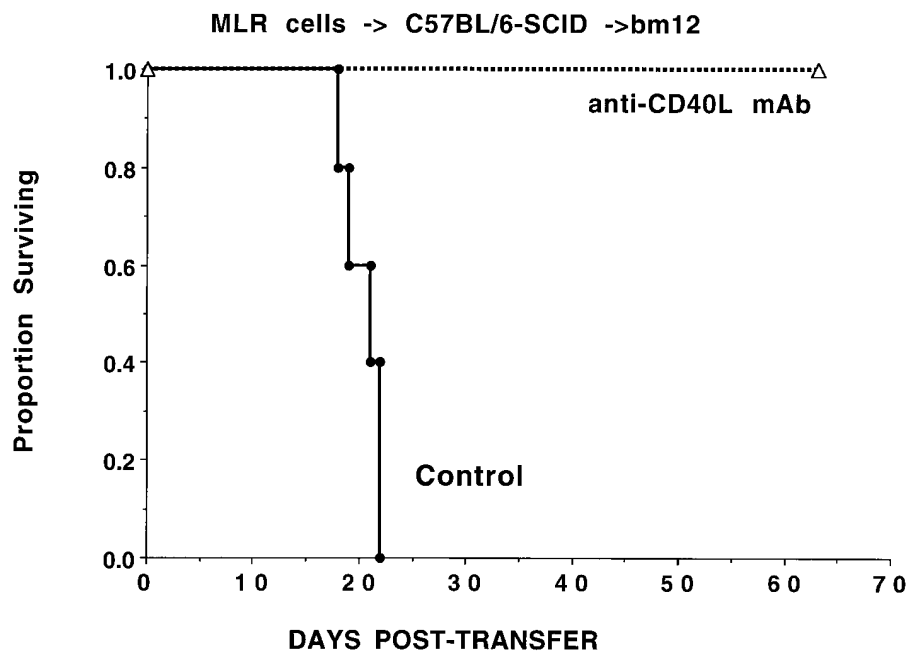


Figure 5. Ex vivo anti-CD40L mAb-treated day 10 MLR cultured cells have a long-lasting impairment in their capacity to mediate GVHD lethality. Control or anti-CD40L mAb-treated CD4⁺ T cells were injected into non-irradiated C57BL/6-SCID mice 40 d before thoracic duct cannulation. Thoracic duct lymphocytes from recipients of control or anti-CD40L mAb-treated primary MLR cultured cells were injected at a dose of 10^5 CD4⁺ T cells per mouse into sublethally irradiated secondary recipients ($n = 5$ per group). On the x-axis are days post-transfer. On the y-axis is the proportion of recipients surviving post-transfer. Recipients of anti-CD40L mAb-treated cells parked for 40 d had a significantly higher actuarial survival rate as compared to recipients of control cultured cells parked for 40 d.

tured cells, as few as 0.3×10^5 CD4⁺ T cells resulted in 95% lethality when transferred into sublethally irradiated recipients, similar to results we have observed with fresh CD4⁺ T cells in this system.

It is possible that the superior survival results obtained with ex vivo as compared to in vivo anti-CD40L mAb are due to a prolonged continuous exposure of CD4⁺ T cells to mAb ensured by ex vivo culture. In support of this hypothesis, we have observed that the tolerization of CD4⁺ T cells to MHC class II alloantigens is not complete by 4 d of culture (our unpublished data). Because CD40L is only transiently expressed on activated CD4⁺ T cells (6), the availability of saturating concentrations of anti-CD40L mAb for a prolonged period of time ex vivo may increase the probability that all T cells that can respond to alloantigen do so in the presence of blocking concentration of anti-CD40L mAb. In vivo, mAb may not be immediately available at the time and site of T cell activation to downregulate the response.

The quality of the in vitro alloresponse in the bulk culture may differ from that observed in vivo. For example, donor T cells might recognize host MHC antigens that are processed by donor APC and presented in the context of donor MHC molecules, a process known as indirect allorecognition (18–22; reviewed in 23). Alternatively, donor T cells may directly recognize host alloantigen-bearing cells, a process known as direct allorecognition. The predominant APC in vivo would by necessity be host-derived, as purified donor T cells containing few donor APC were infused in vivo in our initial experiments in which anti-CD40L mAb was infused in vivo and in the present studies using ex vivo tolerized T cells. Because of the paucity of donor APC that would be available to encounter donor T cells in vivo, the in vivo GVHD response in this sublethal TBI system likely would involve direct and not indirect allorecognition. Although purified donor T cells are used as responders, the ex vivo bulk culture system theoretically would offer a greater opportunity for indirect allorecognition because

the few remaining APC present in the purified donor T cell population could permit indirect allorecognition to occur. Conceivably, the ease to which tolerization to host alloantigens is achieved could be due to the type of allorecognition such that tolerization might be more readily obtained via an indirect rather than a direct allorecognition pathway. Tolerance to the dominant antigenic determinants presented via the indirect allorecognition pathway in fact has been shown to be required to prevent amplification and perpetuation of organ graft rejection (22). However, the predominant APC in the ex vivo cultures would be of host origin and few, if any, functional donor APC would be present using these highly purified T cell responders. Moreover, the requirement for the indirect allorecognition pathway in the ex vivo tolerization culture system is unclear and the frequency of T cells capable of using the indirect pathway is lower (~ 100 -fold lower to HLA-DR) (19). Although it is formally possible that the superior GVHD protective effects obtained with ex vivo as contrasted to in vivo anti-CD40L mAb could be due to a greater dependency of the indirect rather than the direct pathway for allorecognition, this hypothesis seems unlikely.

It is also possible that anti-CD40L mAb may have skewed CD4⁺ T cells away from a Th-1 phenotype. Data from several investigators have shown that the ex vivo generation of skewing of CD4⁺ T cells away from a T helper type 1 (Th-1: IL-2, interferon gamma producing) cytokine phenotype is an effective means of preventing GVHD lethality (e.g., 24, 25). Although control cultured cells have a Th-1 phenotype, anti-CD40L mAb-treated CD4⁺ T cells produce markedly lower amounts of Th-1 cytokines (our unpublished data). These data are consistent with our previous findings that in vivo anti-CD40L mAb treatment of allogeneic BMT recipients inhibits the generation of CD4⁺ T cells with a Th-1 phenotype (12). The protective effect of anti-CD40L mAb ex vivo also does not appear to be due to skewing toward a T helper type 2 (Th-2: IL-4 producing) cytokine production since CD4⁺ T cells

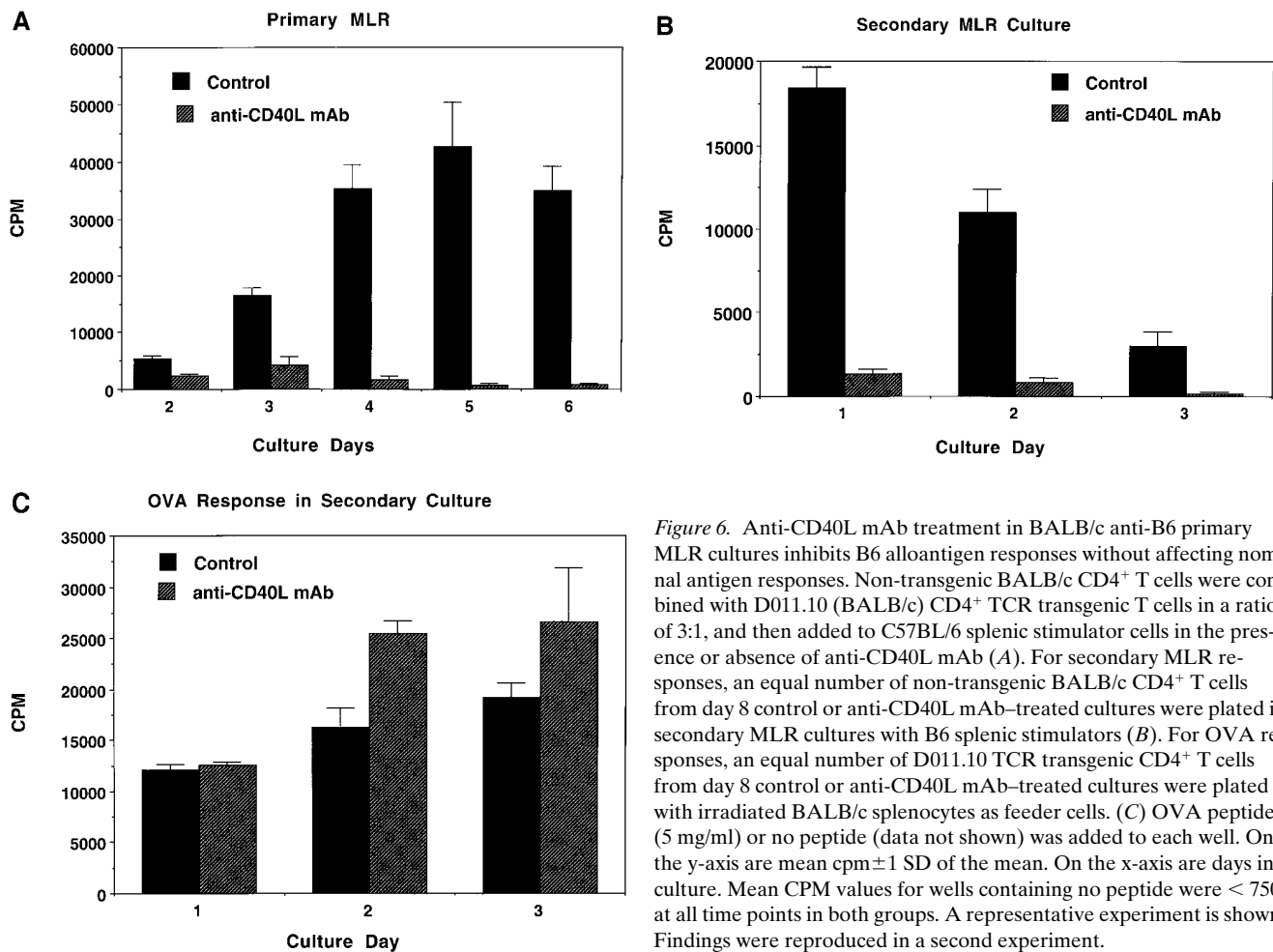


Figure 6. Anti-CD40L mAb treatment in BALB/c anti-B6 primary MLR cultures inhibits B6 alloantigen responses without affecting nominal antigen responses. Non-transgenic BALB/c CD4⁺ T cells were combined with D011.10 (BALB/c) CD4⁺ TCR transgenic T cells in a ratio of 3:1, and then added to C57BL/6 splenic stimulator cells in the presence or absence of anti-CD40L mAb (A). For secondary MLR responses, an equal number of non-transgenic BALB/c CD4⁺ T cells from day 8 control or anti-CD40L mAb-treated cultures were plated in secondary MLR cultures with B6 splenic stimulators (B). For OVA responses, an equal number of D011.10 TCR transgenic CD4⁺ T cells from day 8 control or anti-CD40L mAb-treated cultures were plated with irradiated BALB/c splenocytes as feeder cells. (C) OVA peptide (5 mg/ml) or no peptide (data not shown) was added to each well. On the y-axis are mean cpm \pm 1 SD of the mean. On the x-axis are days in culture. Mean CPM values for wells containing no peptide were < 750 at all time points in both groups. A representative experiment is shown. Findings were reproduced in a second experiment.

from IL-4 knockout (Th-2 deficient) mice were susceptible to ex vivo anti-CD40L mAb induced tolerization (our unpublished data).

Although anti-CD40L mAb may have altered the capacity of T cells to respond to alloantigen, it is possible that anti-CD40L mAb treatment resulted in a high degree of cell death. Cell recovery was consistently lower in anti-CD40L mAb-treated cultures. However, anti-CD40L mAb treatment did not lead to an increased frequency of cells undergoing early apoptosis as measured by 7-AAD staining (17) either at the end of the culture or at intervals during the culture period. Anti-CD40L mAb treatment of MLR cultures ex vivo did not preclude the in vivo maintenance of the majority of CD4⁺ T cells infused into syngeneic SCID recipients as one-half of CD4⁺ T cells exposed to anti-CD40L mAb ex vivo and rested (parked) in B6-SCID mice were retrievable later by thoracic duct cannulation. Sprent et al. have observed that approximately equal numbers of naive T cells parked in syngeneic SCID mice are retrievable after a period of > 1 mo (26). Future experiments designed to track TCR transgenic alloreactive T cells will be required to determine whether deletion/cell death, failure of clonal expansion, or a functional alteration (tolerization) of T cells is the primary mechanism responsible for the lack of GVHD lethality observed after the ex vivo exposure to anti-CD40L mAb.

An important aspect of our studies was the demonstration that anti-CD40L mAb-induced tolerance was long lasting. After 40 d of parking in vivo, control but not tolerized cells were capable of causing GVHD. Thus, a long-lived state of tolerance was maintained. Although IL-2 in high concentrations (50 U/ml) that cannot be sustained in vivo post-BMT (27) could prevent or reverse the generation of tolerized cells, IL-2 administration even at dose of 50,000 IU/dose given intraperitoneally in vivo could not readily induce GVHD by tolerized cells. Collectively, these data would suggest that TCR triggering by environmental antigens, infectious agents, or tumor antigens even early after transfer are not likely to cause GVHD by the provision of exogenous IL-2.

It was important to determine whether immune responses of T cells not involved in the GVHD response were intact. GVHD prevention by ex vivo anti-CD40L mAb was not due to the global immune suppression of both relevant alloantigen-responsive cells and bystander (no anti-host alloreactive potential) cells. By seeding TCR transgenic T cells in a MLR culture containing wild-type CD4⁺ T cells and alloantigen, we have shown that anti-host hyporesponsiveness can be achieved as assessed in both primary and secondary MLR cultures without causing bystander cell inhibition as assessed by quantifying the response of OVA-responsive TCR transgenic cells to OVA nominal antigen. The fact that nominal antigen re-

sponses remain intact during the tolerization process suggests that the anti-viral or anti-leukemia responses of alloantigen nonresponsive cell populations may be preserved. In that event, ex vivo tolerization would have an advantage compared to ex vivo approaches, which rigorously deplete T cells or in vivo approaches, which globally immunosuppress the recipient.

Although D011.10 CD4⁺ thymic T cells are capable of responding to B6 alloantigens in vivo because these cells are negatively selected in B6 mice (28), we do not favor the explanation that the B6 alloresponse in the primary culture decreased the threshold for OVA responses of the tolerized T cells in secondary culture for several reasons. First, D011.10 CD4⁺ T cells do not appear to have been activated in primary culture in the anti-CD40L mAb-treated group based upon low CD25 expression in the mAb-treated versus control group (7.5% versus 38% of CD4⁺ KJ1-261 cells, respectively) and lower forward- (335 versus 460) and side- (156 versus 280) scatter contours. D011.10 CD4⁺ T cells in the mAb-treated group were not entirely quiescent as CD62L (L-selectin) was down-regulated on the transgenic T cells to a similar extent in both the control and mAb-treated groups (20% versus 16%). However, even if the D011.10 CD4⁺ T cells in the control cultures were activated directly rather than indirectly by B6 alloantigens, the mAb-treated cells would have been significantly less activated, such that a lower OVA response using tolerized cells might have been expected. Second, in the absence of OVA in secondary cultures, the proliferative response of D011.10 CD4⁺ T cells is low using T cells obtained from either group such that the stimulation index to OVA (response to OVA divided by response in absence of OVA) was > 30 at all time points. Thus, although we formally cannot exclude the possibility that a small subset of D011.10 that have responded to B6 alloantigens has not been tolerized and that this subset contributes to the OVA response when using T cells obtained from the anti-CD40L mAb-treated cultures, B6 alloantigen responsiveness by D011.10 CD4⁺ T cells does not appear to be a major contributing factor to the observed preservation of OVA responses in secondary cultures.

In summary, the present experiments provide the first formal demonstration that T cells cultured ex vivo with host alloantigen-bearing stimulator cells can enter a state of long-lived antigen-specific tolerance that is not readily reversible in vivo and that does not impair nominal antigen responses. These data suggest that beneficial CD4⁺ T cell responses that support the generation of anti-leukemia (29) or anti-viral effector cells may be preserved. Because CD4⁺ T cell number and function return slowly after bone marrow transplantation (30, 31) especially in adults, the repetitive infusion of large numbers of CD4⁺ T cells incapable of causing GVHD may be useful in supporting the recipient until T cell reconstitution occurs. If shown to be the case in humans, this strategy would offer a clear advantage to currently available approaches that are associated with prolonged periods of immune reconstitution, especially in the adult recipient.

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