Tolerance induction of alloreactive T cells via ex vivo blockade of the CD40:CD40L costimulatory pathway results in the generation of a potent immune regulatory cell

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We previously reported that ex vivo blockade of the CD40:CD40L costimulatory pathway in primary mixed lymphocyte reaction cultures resulted in profound in vitro secondary hyporesponsiveness and 30-fold or greater protection from graft-versus-host-disease (GVHD) lethality. Present studies demonstrate that tolerance induction via costimulatory blockade also results in the generation of a potent immunoregulatory cell that inhibits both naive and primed allosponses. The immunoregulatory capacity was dependent upon cell-to-cell contact that prevented the full activation of the naive or primed cells. The inhibitory effect of tolerized cells did not preclude the response of naive T cells to nominal protein antigen if antigen was present at high concentration. However, under suboptimal antigen concentration, nonspecific inhibition of responses occurred. The tolerized regulatory cell population maintained a polyclonal T-cell receptor Vβ repertoire that was broader than in control primed cultures. These data, the first to demonstrate that tolerance induction via CD40:CD40L costimulatory blockade results in potent regulatory function, are relevant to bone-marrow and solid-organ transplantation. The generation of potent immunoregulatory capacity during tolerization via CD40:CD40L blockade provides a fail-safe mechanism to control alloreactive T cells that may have escaped tolerization. These potent regulatory cells may be clinically exploitable for the treatment and prevention of GVHD or autoimmunity. (Blood. 2002;99:4601-4609)

Introduction

Bone-marrow transplantation is increasingly used for the treatment of a number of malignant and nonmalignant disorders of both hematologic and nonhematologic origin. Graft-versus-host disease (GVHD) remains a major cause of morbidity and mortality after transplantation. Although GVHD can be prevented by rigorous ex vivo T-cell depletion of the donor graft or prolonged global immunosuppression of the recipient, these strategies increase the rates of leukemic relapse, infections, and graft failure. A tolerization strategy that would selectively target only that small fraction of potentially alloreactive T cells but preserve beneficial T cells would be desirable. An ex vivo costimulatory blockade strategy is a potentially attractive candidate for clinical application for the prevention of GVHD.

We reported previously that ex vivo blockade of the CD40:CD40L costimulatory pathway in murine CD4+ T cells in mixed lymphocyte reaction (MLR) culture results in profound primary and secondary hyporesponsiveness and 30-fold or greater protection from GVHD lethality. These results fit well with the accepted 2-signal model of T-cell activation. Productive T-cell activation and proliferation require 2 signaling events. Signal 1 is the engagement of the T-cell receptor (TCR) with the MHC-peptide ligand complex on the surface of the antigen-presenting cell (APC). Additional costimulatory signals (signal 2) are required for the full activation of the intracellular signaling cascade, interleukin-2 (IL-2) production, and T-cell proliferation. Signal 1 in the absence of signal 2 renders a T-cell anergic or hyporesponsive upon antigen restimulation.

The novel finding of these studies is that T cells tolerized to alloantigenic stimulators via ex vivo CD40:CD40L blockade acquire potent regulatory function capable of inhibiting both naive and antigen-experienced T cells. While other investigators have reported that anergic T cells can acquire regulatory capacity, the anergy-inducing strategies in those studies did not involve costimulatory blockade and this would not necessarily be expected to result in the same phenomenon. Moreover, the acquisition of regulatory capacity as a result of ex vivo costimulatory blockade is not readily predictable from the 2-signal model of T-cell activation. The regulatory function of the tolerized cells requires cell-to-cell contact that prevents the full activation of the naive or primed cells. Tolerized cells do not require their obligate antigen for their suppressor function. The regulatory capacity of tolerized cells induced by CD40:CD40L costimulatory pathway blockade is not completely specific, but vigorous T-cell responses to neoprotein antigens can occur under optimal antigen conditions.

These studies implicate both tolerance induction and the generation of regulatory function as separate factors contributing to the profound degree of GVHD protection observed after the in vivo adoptive transfer of tolerized cells into irradiated recipients. These data further suggest that tolerized cells may be useful both for GVHD prevention and therapy.
Materials and methods

Mice

B6.C-H2b(129)/KbIeg/(bm12) and B10.BR (H2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), Congenic C57BL/6 (B6)–CD45.1, C57BL/6 (B6)–CD45.2 (H2b), BALB/c (H2d), and C57BL/6 × BALB/c (CB6) F1 mice were purchased from the National Institutes of Health (Bethesda, MD). B6 and bm12 (both H2b) mice differ at 3 amino acids owing to mutations in the class II IA region. DO11.10 (H2d) mice, TCR transgenic mice specific for chicken ovalbumin (OVA) in the context of IA2, backcrossed more than 10 generations on a BALB/c background, were a kind gift of Dr Marc Jenkins (University of Minnesota, Minneapolis). Mice were used at 8 to 12 weeks of age and housed in a specific pathogen-free facility in microisolator cages.

In vitro MLR cultures

To purify CD4+ T cells, peripheral lymph node cells were depleted of natural killer (NK) cells (hybridoma PK136, mouse immunoglobulin-G2a [IgG2a]) and CD8+ T cells (hybridoma 2.43, rat IgG2b) by coating with monoclonal antibody (mAb), followed by passage through a goat antimouse and goat antirat immunoglobulin-coated column (Cytovax, Edmonton, Alberta, Canada). The final composition of purified T cells was determined by flow-cytometric analysis as 94% or more CD4+ T cells. Where indicated, MLR responder CD4+ cells, depleted of CD25+ immune regulatory cells by incubation with anti-CD25 mAb (hybridoma 3C7, rat IgG2b) (BD PharMingen, San Diego, CA) and sheep antirat Dynabeads (Dynal, Lake Success, NY), were 99% CD4+CD25−. To generate tolerized cells, responder CD4+ T cells were mixed with irradiated (30 Gy), anti-Thy-1.2 mAb (hybridoma 30H-12, rat IgG2b) and anti-NK1.1 mAb plus complement–depleted (Nienfennegger, Woodland, CA) splenic stimulators. Responder and stimulator cells were suspended at a final concentration of 0.5 × 10^6/ml in 24-well plates (Costar, Acton, MA) containing Dulbecco modified Eagle medium (DMEM) (BioWhittaker, Walkersville, MD) with 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 50 mM 2-ME (Sigma, St Louis, MO), 10 mM HEPES buffer, 1 mM sodium pyruvate (Life Technologies, Grand Island, NY), and amino acid supplements (1.5 mM t-glutamine, l-arginine, and l-asparagine) (Sigma) and antibiotics (100 U/mL penicillin; 100 mg/mL streptomycin) (Sigma). Anti-CD40L mAb (hybridoma MR1, hamster IgG) was obtained by culturing the hybridoma in 10% FBS/DMEM in a hollow fiber bioreactor (Accucyst Jr; Cellex Biosciences, Minneapolis, MN). Supernatant was purified by ammonium-sulfate precipitation. Anti-CD40L mAb was added at a final concentration of 50 µg/mL on day 0 to MLR cultures for generation of tolerized cells. On days 8 to 10 of culture, cultures were harvested and washed to remove Ab and cytokines. The tolerized cells used in regulatory experiments were verified as being profoundly hyporesponsive to alloanti-T cells and activation antigens, including CD25, and L selectin (CD62L). Congenic B6 naive and tolerized cells from coculture were distinguished by CD45.1, and activation antigens, including CD25, and L selectin (CD62L). Congenic B6 naive and tolerized cells from coculture were distinguished by CD45.1, and L selectin (CD62L). Congenic B6 naive and tolerized cells from coculture were distinguished by CD45.1, and L selectin (CD62L). Congenic B6 naive and tolerized cells from coculture were distinguished by CD45.1, and L selectin (CD62L).

Flow cytometry

Cells were assessed for evidence of blastogenesis and activation by forward scatter (FSC) and side scatter (SSC) profiles and the coexpression of CD4 and activation antigens, including CD25, and L selectin (CD62L). Congenic B6 naive and tolerized cells from coculture were distinguished by CD45.1, and CD45.2. KJ1-26 (anti-clonotypic mAb) was used to distinguish DO11.10.10 CD4+ cells. All studies were performed with 3-color flow cytometry with the use of fluorescein- and phycoerythrin-conjugated mAb (PharMingen). Fewer than 5% of cells in the live gate were identified as apoptotic by 7-aminoactinomycin (7AAD) (Sigma). Results were obtained by means of CellQuest software on a FACSCalibur (Becton Dickinson, San Jose, CA). FSC and SSC settings were gated to exclude debris and dead cells. A total of 10^4 cells were analyzed for each determination.

Vβ spectratyping analysis

Total cellular RNA was prepared from freshly purified CD4+ cells or 10-day cultured primed or anti-CD40L–tolerized CD4+ cells and converted to complementary DNA (cDNA) as previously described. Polymerase chain reaction (PCR) was performed by means of a fluorescent-labeled constant primer (Cβ), purchased from Applied Biosystems (Foster City, CA), and 17 different Vβ family–specific primers (Vβ1 through Vβ16 and Vβ18). The fluorescently labeled PCR products were run on a sequencing gel and analyzed by Genotyper Genescan software program (PE–Applied Biosystems).

Quantitation of cytokine levels by enzyme-linked immunosorbent assay

Murine Tα1 (IL-2 and interferon-γ [IFN-γ]) and Tα2 (IL-4, IL-10, and IL-13) cytokine levels in the supernatant of MLR cultures were quantitated by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). Sensitivity of the assays was 4 pg/mL or less for each assay, except for IL-13 which was 22 pg/mL. A standard curve using recombinant protein was generated with each assay.

Statistics

Survival data were analyzed by life-table methods, and actuarial survival rates are shown. Group comparisons were made by log-rank test statistics. For other data, group comparisons were made by Student t test. P ≤ .05 was considered significant.

Results

Anti-CD40L mAb-tolerized CD4+ T cells down-regulate a naive alloreponse both in vitro and in vivo

A 10-day blockade of the CD40:CD40L costimulatory pathway in murine CD4+ T cells results in profound in vitro hyporesponsiveness and GVHD prevention. We reported that cells tolerized via ex vivo blockade of the CD40:CD40L pathway had evidence of activation as determined by flow cytometry and analysis of intracellular biochemical pathways. These data suggested that tolerized cells might have gained, rather than strictly lost, function. Additionally, other investigators have reported the acquisition of
regulatory function in tolerized cells. Therefore, we evaluated the anti-CD40L mAb-tolerized cultures for regulatory potential. To determine the effect of tolerized cells on a naive alloresponse, B6 CD4+ cells, previously tolerated to bm12 alloantigen by exposure of a 10-day MLR culture to anti-CD40L mAb, were washed to remove Ab and cytokines and were added to a naive B6 CD4+ MLR culture containing bm12 splenic stimulators. Figure 1 illustrates the proliferative responses in the culture. By day 3 of culture, the addition of 1 tolerized cell to 3 naive CD4+ T cells reduced the naive alloresponse by over 80%. At the time of peak response in the control culture on day 4, the addition of tolerized cells inhibited the response by 94%. As few as 1 tolerized cell to 10 naive CD4+ T cells down-regulated the naive alloresponse equally potently (data not shown).

To determine whether this profound in vitro regulatory effect would translate into protection from GVHD lethality in vivo, a uniformly lethal dose of naive B6 CD4+ cells was infused into sublethally irradiated bm12 recipients. The infusion of 10^5 naive B6 CD4+ T cells resulted in GVHD-induced bone marrow aplasia, killing all control mice by 3 weeks after infusion of cells (Figure 2A). In contrast, 75% of mice receiving 10^5 naive CD4+ T cells and 10^4 tolerized cells administered by separate injections survived the 2-month observation period (P < .001). As an indicator of donor CD4+ T-cell–mediated GVHD-induced bone marrow aplasia, HCT values were assessed in all mice on day 14 after transfer of cells. In one representative experiment, the HCT was 16.2% ± 5.6% in mice receiving naive CD4+ cells versus 32.1% ± 2.4% in mice receiving both naive and tolerized CD4+ cells (P < .001). At the time that mice were electively killed, at 2 months after transfer of cells, all survivors of naive and tolerized cells had recovered HCTs to normal values of 40% or higher. Clinically, survivors appeared GVHD-free and had weights exceeding their pretransplantation weight (data not shown).

The in vitro effect of tolerized cells on a primed alloresponse was difficult to evaluate since primed cells exhibited a vigorous and rapid proliferative response upon alloantigen restimulation (data not shown). Therefore, an in vivo model was used to evaluate whether tolerized cells could down-regulate a primed alloresponse. To determine the effect of tolerized cells on a primed response in vivo, 10^5 primed cells with or without a second injection containing 10^4 tolerized cells were injected into sublethally irradiated bm12 recipients. All mice receiving only primed cells died of bone marrow aplasia by day 20 (Figure 2B). In contrast, mice receiving both primed and tolerized cells had a delayed onset of GVHD mortality with approximately 20% long-term survivors (P < .001). In one representative experiment, mice receiving only primed cells had an average day-14 HCT of 13.5% ± 6.0% versus 33.5% ± 5.0% in recipients of both primed and tolerized cells (P < .001). The mice surviving the 2-month observation period normalized their HCT at the time they were electively killed. Collectively, these data indicate that cells tolerized by a 10-day ex vivo culture with anti-CD40L mAb have potent regulatory capabilities to inhibit both a naive and a primed alloresponse.

Conditions required for the down-regulatory effect of tolerized cells on naive cells

To determine if a soluble factor was implicated in the regulatory activity or if cell-to-cell contact was a requirement for the down-regulation induced by anti-CD40L mAb-tolerized cells, transwell culture inserts were used. Washed tolerized cells plated with fresh stimulators in the transwell chambers allowed for the diffusion of potentially suppressive soluble factors across the membrane into the culture containing the naive CD4+ T cells and
addition of 10-day ex vivo tolerized B6 CD45.2 CD4 bm12 splenic stimulators was established with and without the 10-day primary tolerization procedure. Supernatants from the tolerized cultures examined throughout the H9252 partially immunosuppressive cytokines, IL-4, IL-10, transforming growth factor-β (TGF-β), and IL-13 were undetectable in the supernatants of the tolerized cultures examined throughout the 10-day primary tolerization procedure. Supernatants from the transwell cultures were evaluated for IL-2 levels. The production of IL-2, known to be an indicator of tolerance induction, in the B6 anti-bm12 MLR was completely abrogated by day 4 of culture by the addition of tolerized cells if cell-to-cell contact was present (Table 1). Consistent with the proliferation data, if cell contact was denied, the diffusion of soluble factors from the tolerized culture had no effect on the levels of IL-2 detected in the supernatant.

To investigate the effects of tolerized T cells on the activation of naive T cells, a primary MLR of B6 CD45.1 CD4+ T cells and bm12 splenic stimulators was established with and without the addition of 10-day ex vivo tolerized B6 CD45.2 CD4+ T cells. On day 5, the cells were phenotyped, and recovery and the degree of activation were evaluated. Approximately the same percentage of naive CD45.1+ T cells was recovered whether they had been cocultured with tolerized cells or not (29% versus 35%). Although recovery of naive cells was similar for the 2 groups, the naive cells cocultured with tolerized cells did not exhibit the same degree of blastogenesis and activation as those in the control culture (Figure 4). The FSC and SSC of the naive cells cocultured with tolerized cells indicated reduced blastogenesis as compared with the control group. CD25 expression was also reduced in the naive cells cocultured with tolerized cells. Additionally, although L selectin (CD62L) had significantly down-regulated in the naive cells in the control group, expression remained high in those naive cells in the coculture, suggesting they had a different activation profile, as we have previously reported in tolerized versus naive cells. Perhaps surprisingly, 137% of the input number of the tolerized cells in the coculture group was recovered. As well as having high FSC and SSC, 90% of the recovered tolerized cells were CD25+ (Figure 4). Although the tolerized cells had several parameters of highly activated cells, they remained high for L selectin. We conclude that tolerized cells inhibit the proliferation of naive cells in response to alloantigen by a mechanism that requires cell-to-cell contact that prevents the full activation of the naive cells and inhibits their IL-2 production. It is unknown whether this inhibition of a naive alloresponse is due to suppression or killing of the small number of potentially alloreactive naive T cells in the bulk population.

**Table 1. Cell-to-cell contact is required for the inhibition of IL-2 production**

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<th>Culture day</th>
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<td>245</td>
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<tr>
<td>4</td>
<td>Naive + tolerized anti-bm12</td>
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<tr>
<td></td>
<td>Cell-to-cell contact</td>
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<td>Transwell</td>
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Primary mixed lymphocyte reaction (MLR) cultures were established with naive B6 CD4+ T cells as responders and irradiated, T-cell-depleted bm12 splenic stimulators. Tolerized cells were directly added to some wells of the primary MLR, permitting cell-to-cell contact, or were added with stimuli to transwells suspended above the MLR, permitting the diffusion of soluble factors but denying direct cell-to-cell contact. A ratio of 1 tolerized cell to 10 naive B6 CD4+ T cells was used. Supernatants were harvested on days 4 and 6 of culture. These data were reproduced in a second experiment. Final concentration of naive B6 CD4+ T cells was 0.5 x 10^6 cells per milliliter. Values are in pg per milliliter, and sensitivity of the enzyme-linked immunosorbent assay is 3.0 pg/mL.

Although tolerized cells can exert their down-regulatory effect on naive T cells in an antigen-nonspecific manner, this inhibition can be overcome if high-affinity T cells receive sufficient TCR signals.

To determine the specificity of the down-regulatory effect of the tolerized cells, a different model was used that would allow evaluation of the effects of tolerized cells on naive transgenic T-cell responses to their obligate peptide antigen. BALB/c CD4+ T cells were tolerized to B6 alloantigen via an 8-day culture with anti-CD40L mAb. Tolerized cells were washed to remove antibody and cytokines and added to a primary MLR of naive BALB/c CD4+ T cells and T-cell-depleted, irradiated B6 splenic stimulators. As expected, tolerized cells inhibited the naive alloresponse by day 3 of culture (Figure 5A). A ratio of 1 tolerized cell to 3 to 10 naive cells proved optimal for the down-regulation. Impressively, as few as 1 tolerized cell to 100 naive cells resulted in an approximately 50% inhibition of the peak naive alloresponse. To determine the specificity of the regulatory effect, BALB/c CD4+ cells tolerized to B6 alloantigen were added to naive DO11.10 CD4+ cells (1:1 ratio) and mixed with irradiated BALB/c APCs. OVA peptide was added at an optimal concentration of 5.0 μg/mL. Under these conditions, the alloantigen-tolerized BALB/c CD4+ cells did not inhibit the naive DO11.10 OVA response (Figure 5B). As further evidence of the specificity of the inhibitory effects of alloantigen-tolerized cells, the levels of both TGF-β and IL-2 cytokines in the supernatants of the culture were analyzed on day 3 and day 5 of culture. Tolerized cells profoundly inhibited the production of IL-2 and IFN-γ by naive BALB/c CD4+ cells in response to alloantigen (Table 2). Low to undetectable levels of IL-2 cytokines were detected in the allo-MLR. In contrast, the addition of tolerized cells had little effect on either TGF-β or IL-2 cytokine production by naive DO11.10 CD4+ cells in response to an optimal OVA peptide concentration.

To rule out the possibility that the OVA peptide response was too vigorous to uncover a nonspecific regulatory effect by the
tolerized cells, the model was modified to use CB6 F1 APCs. The use of CB6 F1 APCs to present OVA peptide resulted in a more delayed and lower peak proliferative response as compared with BALB/c APCs. The peak response with F1 APCs was on day 6 with approximately 20,000 cpm as compared with BALB/c APCs, which resulted in a day-4 or day-5 peak response of 80,000 cpm (Figure 5B-C). Importantly, the F1 APCs also provided relevant alloantigen to the tolerized cells in the event that this was a requirement for their regulatory capacity. These conditions should maximize the likelihood that a nonspecific down-regulation would be detected. Under these conditions and with the use of F1 APCs, the tolerized cells did not inhibit a naive DO11.10 OVA response if OVA was present at a high concentration of 5.0 μg/mL (Figure 5C).

It is important to note that DO11.10 mice have a high precursor frequency of high-affinity T cells for their obligate antigen, which generates responses that are probably difficult to suppress under high peptide concentration. However, if the concentration of OVA was reduced 10-fold to a suboptimal concentration of 0.5 μg/mL, the tolerized cells nonspecifically inhibited the naive DO11.10 response by 62% on day 5 and 87% on day 6 of culture (Figure 5D).

In a different nontransgenic model, nonspecific inhibition of a naive alloreaction was also noted. B6 CD4+ cells tolerized to bm12 alloantigen potently inhibited a naive B6 CD4+ response to B10.BR (H2k) stimulators (Figure 6). It is important to note that unlike the DO11.10 transgenic model, a B6 anti-B10.BR response is a polyclonal T-cell response composed of low-, intermediate-, and high-affinity alloresponsive T cells of a relatively low precursor frequency and, therefore, might be expected to be more easily suppressed. Together, these data suggest that if the net TCR signal of the bulk T-cell population is sufficiently low, owing to either a low frequency of antigen-reactive T cells or a low concentration of neoantigen antigen, then tolerized cells may nonspecifically inhibit a naive or primed T-cell response. In the case of high concentrations of neoantigen antigen and a high frequency of antigen-reactive T cells, nonspecific down-regulation may be less likely to occur.

Tolerized cells retain a relatively broad TCR Vβ repertoire

To determine whether the regulatory capacity of the tolerized bulk population was associated with a restricted Vβ repertoire, PCR-based TCR Vβ CDR3-size spectratyping was performed on 10-day cultured primed and tolerized cells and compared with that of freshly purified CD4+ cells. Vβ spectratype analysis of 17 different Vβ families indicated that anti-CD40L–tolerized cells retained a...
relatively broad Vβ repertoire similar to control CD4+ cells (Figure 7). In contrast, primed cells exhibited Vβ skewing and increased oligoclonality within Vβ families. These data indicate that tolerization prevents the restricted Vβ repertoire observed in control primed cultures, potentially providing a broader array of T cells that can respond to foreign pathogens and injured cells in vivo.

**CD4+CD25+ cells are required for the induction of tolerance and generation of regulatory capacity**

We have previously published that immune regulatory CD4+CD25+ T cells are required for tolerance induction via ex vivo costimulatory blockade. 10 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 lethal GVHD generation in vivo. To determine whether CD25+ cells were also required for the acquisition of regulatory capacity, whole B6 CD4+ T cells or B6 CD4+CD25+ T cells and bm12 splenic stimulators were cultured in the presence of anti-CD40L mAb. At the end of the 9-day MLR culture, cells were washed and coinjected with a lethal dose of naive CD4+ T cells (Figure 8). Recipients of 10^5 naive CD4+ T cells died by day 23 after infusion of cells. Of the mice receiving 10^5 naive CD4+ T cells and 10^4 anti-CD40L–cultured whole CD4+ T cells, 63% survived the 2-month observation period. In contrast, recipients of naive CD4+ T cells and anti-CD40L–cultured CD4+CD25+ T cells died by 20 days after infusion of cells. Thus, CD4+CD25+ immune regulatory cells are required for the acquisition of the regulatory capacity of anti-CD40L mAb-treated CD4+ T cells in MLR cultures.

**Discussion**

The novel finding of this study is that tolerance induction via ex vivo blockade of the CD40:CD40L costimulatory pathway in allo-MLR cultures results in the acquisition of potent suppressor function that inhibits naive and primed allore sponses both in vitro and in vivo. This immunoregulatory capacity requires cell-to-cell contact that prevents the full activation of the naive (or primed) cells and inhibits their production of IL-2 and IFN-γ. The regulatory capacity of the tolerized cells is not entirely antigen specific but does not preclude a vigorous response by naive T cells.

![Figure 5. Relationship of antigen to the effect of tolerized cells on naive T cells. Tolerized cells exert their down-regulatory effect on naive T cells in an antigen-nonspecific manner but do not inhibit naive T-cell responses to third-party antigen if antigen is present at optimal concentration and the responding T cells are present in high frequency. (A) BALB/c CD4+ T cells were tolerized to B6 alloantigen by an 8-day culture with anti-CD40L mAb. The tolerized cells were washed and added to a primary MLR culture containing naive BALB/c CD4+ T cells and B6 stimulators at the indicated ratios of tolerized-to-naive cells. (B) BALB/c CD4+ T cells tolerized to B6 alloantigen were plated with naive DO11.10 CD4+ T cells, BALB/c APCs, and OVA peptide (5.0 μg/mL). (C) BALB/c CD4+ T cells tolerized to B6 alloantigen were plated with naive DO11.10 CD4+ T cells, CB6 F1 APCs, and OVA peptide (2.5 μg/mL). (D) BALB/c CD4+ T cells tolerized to B6 alloantigen were plated with naive DO11.10 CD4+ T cells (3 × 10^5 per well each), CB6 F1 APCs, and OVA peptide (0.5 μg/mL). These data were reproduced in a second experiment. The y-axis presents the mean cpm ± 1 SD. On the x-axis are days in primary culture.]()
The generation of regulatory cells occurred without substantial repertoire restriction. B6 CD4+ T cells tolerized to B10.A alloantigen nonspecifically down-regulate a naive response to a different alloantigen. B6 CD4+ T cells tolerized to B10.BR alloantigen (1 tolerized cell to 10 naive cells). The y-axis presents the mean cpm ± 1 SD of the mean. On the x-axis are days in primary culture.

to a neoprotein antigen if the antigen is present at high concentrations. The tolerized regulatory cell population maintains a relatively broad TCR Vβ repertoire. CD4+CD25+ immune regulatory responder cells are required for the generation of the regulatory/suppressor function.

The immunoregulatory nature of anergic T cells has been reported by several investigators. Taams et al6 reported that autoreactive rat CD4+ T-cell clones for experimental autoimmune encephalomyelitis and adjuvant arthritis could be induced to become anergic via T-cell presentation of the antigen. Anergic encephalitogenic cells specifically inhibited the in vitro peptide response of nonanergic cells of the same clone although they did not inhibit peptide responses of a different arthritogenic T-cell clone.6 However, anergic T-cell clones were able to nonspecifically suppress proliferative third-party responses of polyclonal lymph-node T cells provided that peptide recognized by the anergic T cells was present.6 Another group rendered human T-cell clones, specific for influenza hemagglutinin peptide, anergic either by incubation with antigen in the absence of APCs or by incubation with immobilized anti-CD3 mAb.4 Anergic T cells added to cultures containing potentially reactive T cells, APCs, and antigen inhibited proliferation in a titratable fashion. Their model also exhibited some, but not complete, specificity. Chai et al5 found that an alloreactive T-cell clone anergized in vitro by immobilized anti-CD3 mAb and then transferred in vivo into recipients of allogeneic skin grafts led to prolonged skin survival. Other investigators used both in vivo transplantation tolerance by transplanting skin from minor histocompatibility antigen mismatched donors to recipient mice (n = 8 per group). On the x-axis are days after transfer of T cells. The y-axis presents the proportion of recipients surviving after transfer.

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Despite the well-documented role of IL-4, IL-10, TGF-β, and IL-13 in immune regulation and in the induction of tolerance,13-19 our studies did not implicate these immunosuppressive cytokines or other soluble factors as having a significant role in the mechanism of immune regulation. This is in general agreement with other studies on the immunoregulatory nature of anergic cells. It is important to note, however, that the cell-to-cell contact requirement does not necessarily preclude a role for either a soluble...
factor or cytokines in the mechanism of regulation. A recent study indicates that CD4+CD25+ immune regulatory T cells mediate their cell contact–dependent immune suppression by cell surface–bound TGF-β.20 It is unclear at this time whether the suppression mediated by the anergic T-cells is through modulation of the APC’s or by a direct T-cell–to–T-cell interaction.

Analysis of CD45 congenic naive and tolerized cells cocultured with allosimulators for 5 days revealed similar recovery of the naive cells regardless of whether tolerized cells were added to the culture despite an almost complete inhibition of proliferation as measured by tritiated thymidine uptake. The naive cells in the coculture phenotypically appear to be less activated compared with those cultures in which the tolerized cells have not been added. Additionally, the production of IL-2 and IFN-γ was profoundly reduced. Taken together, these data suggest that the similar recovery of naive cells in both cultures may have occurred for different reasons. In the culture containing no tolerized cells, proliferation of naive cells was probably accompanied by cell death. In contrast, naive cells present in cultures containing tolerized cells had a low proliferation index and a low degree of cell death. Thus, the net recovery of naive T cells in both cultures was comparable.

Previous data indicate that tolerized cells are not only hyporesponsive in terms of proliferation and cytokine production upon allogeneic restimulation but also have greatly reduced cytolytic effector function, at least for their relevant target alloantigen.9 These data do not rule out the possibility that tolerized cells are mediating their regulatory function by killing the relatively small number of allosreactive T cells (a likely source of Th1 cytokines) and leaving the other T cells intact. Interestingly, tolerized cells added to the naive cells had undergone at least some proliferation since more tolerized cells were recovered than were originally added to the culture. Additionally, the tolerized cells had evidence of increased activation status, as suggested by a significant increase in FSC and SSC and the up-regulation of CD25 expression from the absence of naive cells (data not shown). In contrast, tolerized cells showed no signs of activation upon allogeneic restimulation in secondary MLR in the absence of naive cells (data not shown). Thus, the activation of tolerized cells was increased by the presence of naive cells.

CD4+ T cells tolerized to allogeneic inhibition the response of a naive T cell to a neoprotein antigen under suboptimal, but not optimal, TCR signaling conditions regardless of whether the relevant stimulators were present in the culture or not (Figure 6 and data not shown). This may have important clinical implications if one assumes the same is true in vivo. Although the nonspecific down-regulation of third-party T-cell responses associated with tolerization may seem to be clinically undesirable, we hypothesize that some degree of nonspecificity will not preclude the in vivo generation of beneficial T-cell responses in settings where TCR signaling is high and the organism needs to respond. Consistent with this hypothesis, various heterogeneous populations of suppressor or regulatory cells occur normally in vivo and are essential to T-cell homeostasis and maintenance of peripheral tolerance but do not abrogate the ability of an animal to mount vigorous T-cell responses to antigen. The maintenance of a relatively broad Vβ repertoire is clinically encouraging as it also may indicate that a bulk tolerized population can retain beneficial T-cell responses.

We have previously published that CD4+CD25+ immune regulatory cells are required for tolerance induction via ex vivo costimulatory blockade.10 These studies extend those findings by demonstrating that tolerance induction results in the generation of regulatory function. The mechanism by which this CD4+CD25+ T-cell subset would positively influence the induction of tolerance and the generation of regulatory function is unknown. It is possible that the extended ex vivo incubation with anti-CD40L mAb might preferentially enrich and activate the CD4+CD25+ immune regulatory population while deleting or blocking the activation and proliferation of allogeneic CD25+ T cells. In this case, CD4+CD25+ immune regulatory T cells would be responsible for the suppressor capacity seen in the tolerized cultures. Alternatively, although CD4+CD25+ cells may be required for tolerance induction, these cells may not be the final mediators of regulatory capacity but rather may be necessary to confer regulatory capacity on a CD4+CD25− population. Future experiments will address these questions.

In summary, we have shown that costimulatory blockade of an allo-MLR culture does not simply induce hyporesponsiveness to allogeneic restimulation but also actively induces cells with a potent regulatory capacity. We hypothesize that the acquisition of this suppressor function during tolerance induction may be harnessed to contribute to GVHD protection observed after the in vivo adoptive transfer of tolerized cells into irradiated transplant recipients. In the event that some transferred cells escape their tolerant state and become alloresponsive, other regulatory cells may be able to inhibit their clonal expansion and capacity to mediate GVHD. The infusion of tolerized cells as a means of preventing and treating GVHD warrants further investigation.

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References


Tolerance induction of alloreactive T cells via ex vivo blockade of the CD40:CD40L costimulatory pathway results in the generation of a potent immune regulatory cell

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