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Host Conditioning with Total Lymphoid Irradiation and Antithymocyte Globulin Prevents Graft-versus-Host Disease: The Role of CD1-Reactive Natural Killer T Cells

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ABSTRACT

Our previous studies in mice showed that the nonmyeloablative conditioning regimen of fractionated irradiation of the lymphoid tissues (total lymphoid irradiation; TLI) and depletive anti-T–cell antibodies (anti-thymocyte serum) markedly increased the percentage of regulatory DX5$^+$ and natural killer 1.1$^+$ T cells in the mouse spleen, and prevented acute lethal graft-versus-host disease (GVHD) in BALB/c mice (H-2^d) following the transplantation of bone marrow (BM) and peripheral blood mononuclear cells (PBMC) from C57BL/6 (H-2^b) donors. The object of the current study was to determine whether the TLI and anti-thymocyte serum regimen protected natural killer T-cell deficient CD1$^{-/-}$ BALB/c mice against GVHD after BM and PBMC transplantation from C57BL/6 donors, and whether a similar conditioning regimen of TLI and anti-thymocyte globulin (ATG) can prevent GVHD in Lewis rat (RT1^l) hosts after BM and PBMC transplantation from ACI rat (RT1^a) donors. The experimental results in mice showed that, although wild-type BALB/c hosts are protected in association with a marked increase in CD1- reactive T cells expressing the invariant TCR identified with a CD1 tetramer reagent; CD1$^{-/-}$ BALB/c hosts are not. Studies of chimeric donor cells in mice protected from GVHD showed donor T-cell polarization to a Th2 cytokine pattern. Results in rats showed that approximately 1000 fold more donor PBMC cells were required to induce a similar incidence of lethal GVHD in TLI and ATG conditioned hosts as compared with hosts conditioned with single-dose total-body irradiation or total-body irradiation and ATG. Surviving TLI and ATG conditioned rat hosts were complete chimeras. In conclusion, the TLI and ATG/anti-thymocyte serum conditioning regimen protects against GVHD in rats and mice, and regulatory natural killer T cells are required for protection.

KEY WORDS

Bone marrow transplantation • Regulatory T cells • Graft-versus-host disease • Total lymphoid irradiation

INTRODUCTION

Nonmyeloablative host conditioning regimens have decreased the early toxicity associated with allogeneic bone marrow (BM) and blood stem cell transplantation in humans [1-4]. However, graft-versus-host disease (GVHD) continues to be an important problem, and 10% to 40% of hosts develop at least grade II GVHD even with reduced toxicity regimens [1-4]. Several approaches have been reported to ameliorate GVHD including manipulation or elimination of donor T cells [5-7], and manipulation of the host to eliminate antigen presenting cell targets of GVHD [8].

Our previous studies have shown that GVHD can also be prevented by manipulating the host to alter the balance of residual T cells to favor the presence of regulatory T cells (T_{reg}) cells [9]. Two types of mouse T_{reg} cells have been reported to suppress GVHD,

natural killer (NK) T cells, and $CD4^+CD25^+$ T cells [9-12]. Whereas the $CD4^+CD25^+$ T cells of donor origin can protect against GVHD induced by conventional donor T cells, NK T cells of either host or donor-type are protective [9,10,13]. The latter T_{reg} cells recognize CD1, a nonpolymorphic MHC class I-like molecule expressed by both host and donor-type cells regardless of the mouse strains used [14].

Conditioning mice with a combination of total lymphoid irradiation (TLI) and depletive anti-T–cell antibodies (anti-thymocyte serum; ATS) alters the balance of residual T cells such that the percentage of NK T cells among all T cells increases from about 1% to 2% before treatment to more than 90% after 17 doses of TLI (240 cGy each) and 3 doses of ATS given over a 3-week period [9]. The conditioned mice accept combined peripheral blood mononuclear cell (PBMC) and bone marrow transplants (BMT) from MHC-mismatched donors and become stable mixed chimeras [9]. The hosts do not develop acute lethal GVHD, as do all hosts conditioned with TBI or TBI and ATS that are given the same donor cell transplants [9]. Protection against GVHD can be transferred by T cells from the spleen of TLI-treated mice [9].

In the current study, we determined whether the TLI and depletive anti-T–cell antibody conditioning regimen could protect Lewis rats from GVHD after transplantation of MHC-mismatched PBMC and BM cells from ACI donors. Our previous studies showed that rats require fewer doses of TLI than mice (10 *v* 17 doses of 240cGy each) for engraftment of MHC-mismatched BMTs [15], and the TLI fields in rats are more similar to that used in humans. The results showed that the TLI regimen (10 × 24 cGy) used in rats is markedly protective as compared with TBI, and approximately 1000 fold more donor T cells are required to induce lethal GVHD using the former versus the latter conditioning regimen. In addition, we determined whether the presence of NK T cells in host mice are required for protection against GVHD after TLI conditioning by comparing NK T-cell deficient $CD1^{-/-}$ hosts and wild-type hosts. We found that protection was lost in the NK T-cell deficient hosts.

MATERIALS AND METHODS

Animals

Adult male Lewis ($RT1^l$) and ACI ($RT1^a$) rats weighing 280 to 299 g and 181 to 195 g, respectively, were purchased from Harlan Sprague Dawley, Inc (Walkersville, MD). Male C57BL/6 ($H\text{-}2^b$) and BALB/c ($H\text{-}2^d$) wild-type mice 8 to 10 weeks old were purchased from the Department of Comparative Medicine, Stanford University (Stanford, CA). $CD1^{-/-}$ BALB/c mice were kindly provided by Drs M.A. Exley and S.P. Balk (Harvard University, Boston, MA), and were backcrossed more than 10 generations on the BALB/c background.

Irradation

Total lymphoid irradiation was delivered to the abdomen, lymph nodes, thymus, and spleen with shielding of the skull, lungs, limbs, pelvis, and tail as described previously. Total lymphoid irradiation in Lewis recipients was started on day −14 before BMT and 10 doses of 240 cGy were administered as 5 doses per week. Total lymphoid irradiation in BALB/c mice was started on day −24 before transplantation, and 17 doses of 240 cGy each were administered. Total body irradiation was delivered to control Lewis recipients (1050 cGy) or BALB/c recipients (800 cGy) 24 hours before cell infusions. The irradiation was performed with a Philips x-ray unit (200 kV, 10 mA; Philips Electronic Instruments, Inc, Rahway, NJ) at a rate of 84 cGy/min with a 0.5 mm Cu filter.

Rabbit Anti-Thymocyte Globulin or Serum

New Zealand rabbit ATG was prepared as described previously [15]. Rabbit ATS was purchased from ACCURATE Laboratories (New York, NY). Lewis rats were injected intraperitoneally with 20 mg/kg ATG on days −14 and −12, and 10 mg/kg on days −10, −8, and −6. BALB/c mice were injected intraperitoneally with 0.05 mL ATS in 0.5 mL saline on days −12, −10, and −8.

Cell Preparation

Peripheral blood mononuclear cells were isolated on density gradients (Lympholyte-Rat or Lympholyte-M Cedarlane Laboratories, Ontario, Canada), and washed twice in ice-cold RPMI-1640 (Gibco, BRL Laboratories, Grand Island, NY). Femoral and tibial bones taken from donor ACI rats or C57BL/6 mice were rinsed, and residual muscle on the bones were carefully removed. Bone marrow cells were prepared by flushing the bones with RPMI-1640, and the cell suspension was filtered through nylon mesh to remove aggregates, and washed once before transfusion.

Flow Cytometry

Blood samples for rat and mouse chimerism analyses were hemolyzed with ammonium chloride potassium carbonate to remove red cells. The white cell pellets were washed twice with 0.05% sodium azide staining buffer, and incubated on ice for 15 minutes with saturation concentrations of monoclonal antibody mixtures as described previously [9,15]. Biotinylated anti-$RT1A^{a,b}$ (C3), APC conjugated anti-CD3 (G41.8) from Pharmingen, San Diego, CA and fluorescein isothiocyanate conjugated anti-granulocyte

and macrophage (R2-1A6a), and phycoerythrin conjugated anti-Pan–B (RLN-9D3) monoclonal antibodies from Caltag (Burlingame, CA) were used for rat chimerism analysis. Biotinylated anti-Gr–1 (RB6-8C5) and anti-Mac–1(M1/70.15), and APC conjugated anti-B220 (RA3-6B2) from Caltag, as well as fluorescein isothiocyanate conjugated anti-H–2K^b (AF6-88.5) and phycoerythrin conjugated anti-Thy1.2 (53-2.1) monoclonal antibodies from Pharmingen were used for mouse chimerism analyses. After incubation, cells were washed twice and followed by streptavidin-Texas Red (Caltag) staining on ice. Background staining for donor-type cells in normal control Lewis rats and BALB/c mice was ≤0.5%. For detection of CD1-reactive NK T cells in the spleen of BALB/c mice, splenocytes were incubated with a mixture of phycoerythrin conjugated CD1 tetramers loaded with α galactosyl ceramide [16] and APC conjugated anti-TCRαβ (H57-597) from Pharmingen. All mouse cells were incubated with CD16/32 (2.4G2) from Pharmingen to block the FcR-γ II/III receptors, and propidium iodide was added to exclude dead cells. Chimerism analysis used a lymphoid gate set by forward and orthogonal light scatter [9,15].

Cytokine Assays

Details of in vitro stimulation of sorted cell populations with phorbol myristate acetate and ionomycin and analysis of IL-4 and IFN-γ in the 48-hour supernatants by enzyme-linked immunosorbent assay (ELISA) assays are described elsewhere [9].

Histopathology of Liver, Skin, and Intestines

Tissues were fixed in formalin, embedded in paraffin blocks, and sections were stained with hematoxylin and eosin.

Statistical Analysis

Different groups of hosts given BMTs were compared for statistical differences in survival using the log rank test. Difference in mean levels of in vitro cytokine secretion were analyzed using the 2-tailed student *t* test of independent means.

RESULTS

Protection against Graft-versus-Host Disease in MHC-Mismatched Rats

Adult Lewis (RT1^l) rats given a single dose of TBI (1050 cGy) and an intravenous injection of BM cells (100×10^6) from MHC-mismatched ACI (RT1^a) donors survived at least 100 days without clinical signs of GVHD (Figure 1A). However, addition of 25×10^6 ACI PBMC to the BM cell infusion resulted in the death of all hosts by 20 days which was associated with

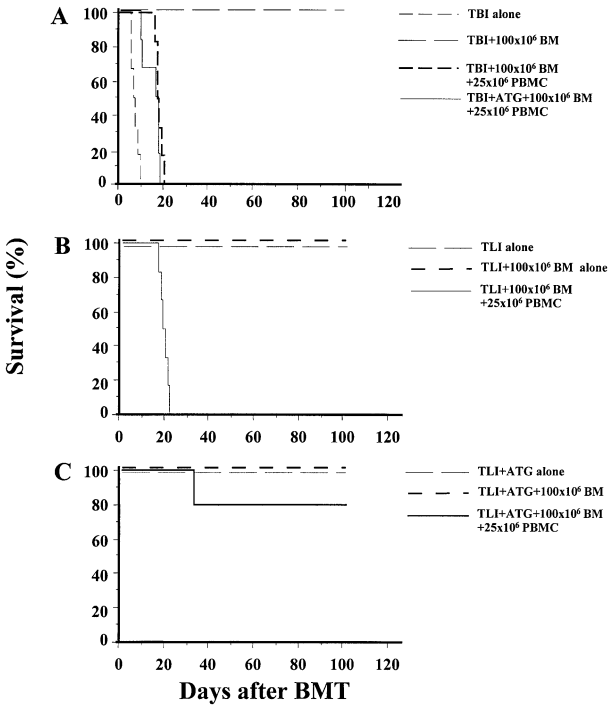

Figure 1. Survival of Lewis rat hosts given BMTs from ACI rat donors. A, Hosts were given a single dose of 1050 cGy TBI without a cell infusion or with an intravenous injection of 100×10^6 ACI BM cells alone, ACI BM cells with 25×10^6ACI PBMC, or TBI combined with rabbit ATG and an injection of 100×10^6ACI BM cells and 25×10^6 PBMC (n = 6 in all groups). B, Hosts were given 2400 cGy TLI without a cell infusion, TLI with BM cells, or TLI with 100×10^6 BM cells and 25×10^6 PBMC (n = 6 in all groups). C, Hosts were given TLI and ATG without a cell infusion, TLI and ATG with 100×10^6 BM cells, or TLI and ATG with 100×10^6 BM cells and 25×10^6 PBMC (n = 5 in all groups). Data from 2 replicate experiments were combined and groups were set up concurrently for each experiment.

typical features of GVHD including diarrhea, weight loss, hunched back, and hair loss (Figure 1A) ($P < .01$; log rank test). Control hosts given irradiation without cells all died by day 10 (Figure 1A).

Lewis hosts given TLI in 10 doses of 240 cGy each (total dose, 2400 cGy) targeted to the lymph nodes, spleen, and thymus with shielding of the BM, lungs, and other radiosensitive tissues during a 2-week period before the infusion of 100×10^6 ACI BM cells all survived for at least 100 days without clinical signs of GVHD (Figure 1B). However, injection of 25×10^6 PBMC together with the marrow cells resulted in uniform death by 24 days (Figure 1B) ($P < .001$). The radiation regimen was nonmyeloablative, and all irradiated hosts survived at least 100 days without a cell infusion (Figure 1B). In contrast, when Lewis hosts were given TLI and 5 intraperitoneal injections of rabbit ATG (a potent T-cell depletive reagent) during the first week of irradiation (days −14, −12, −10, −8, −6), the subsequent injection of ACI BM and PBMC

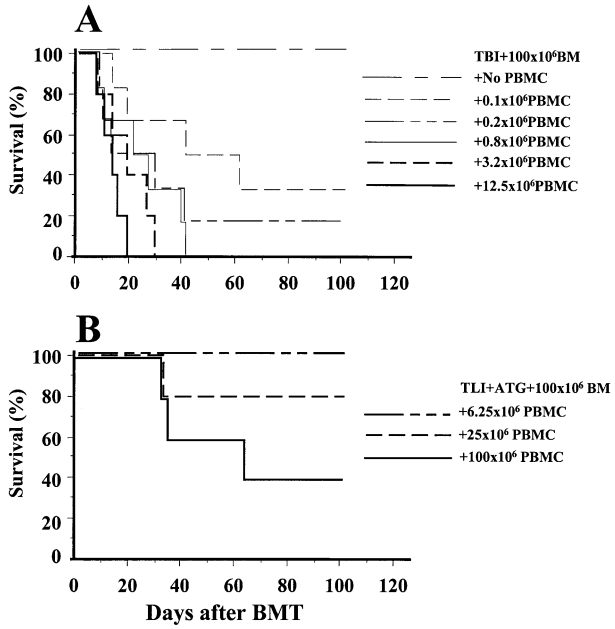

Figure 2. Survival of Lewis rat hosts given BMT from ACI donors. A, Hosts were given TBI and100 $\times$ 10^6 BM cells without PBMC or with graded doses of PBMC (n = 5, 6 in all groups). B, Hosts were given TLI, ATG, and 100 $\times$ 10^6 BM cells with graded doses of PBMC (n = 5 for all groups). Data from 2 replicate experiments were combined. Experiments in Figures 1 and 2 were performed in the same time interval.

failed to induce uniform acute lethal GVHD, and 4 of 5 hosts survived at least 100 days (Figure 1C). Intraperitoneal injections of ATG on days −14, −12, −10, −8, and −6 before TBI instead of TLI failed to protect Lewis hosts from GVHD induced by ACI BM and blood mononuclear cells, and all died by day 18 (Figure 1A). Thus, the TLI and ATG regimen resulted in markedly improved survival as compared to the TBI and ATG regimen ($P < .001$).

To determine the magnitude of protection against GVHD afforded by the TLI and ATG regimen as compared with conventional single-dose TBI, groups of Lewis hosts were given either of the 2 preparatory regimens and a subsequent infusion of a constant number (100 $\times$ 10^6) of ACI marrow cells and graded numbers of PBMC. In groups given TBI, injection of as few as 0.1 $\times$ 10^6 PBMC resulted in the death of two thirds of hosts within 60 days, and 0.8 $\times$ 10^6 cells induced uniform death by 42 days ($P < .01$ as compared with marrow cells alone) (Figure 2A). Injection of 12.5 $\times$ 10^6cells killed all hosts by 20 days. In contrast, injection of marrow and 6.25 $\times$ 10^6 PBMC into groups prepared with TLI and ATG resulted in uniform survival of hosts for at least 100 days ($P < .01$ as compared with TBI with 0.8 $\times$ 10^6 PBMC)(Figure 2B). Injection of marrow and 100 $\times$ 10^6 PBMC induced death in 60% of hosts by 60 days (Figure 2B). Thus, survival curves were similar when the dose of PBMC was about 1000-fold greater (100 $\times$ 10^6 versus 0.1 $\times$ 10^6) using TLI and ATG as compared with TBI ($P > .05$).

Histopathologic analysis of the tissues of 6 host rats that received the protective preparatory regimen and a combined infusion of BM cells and PBMC were examined when the surviving hosts were sacrificed within 1 week after the 100-day observation period. In all cases, microscopic analysis of the liver, small and large intestines, and skin was performed using hematoxylin and eosin staining to look for evidence of GVHD. Positive control tissues were those from 4 Lewis rat hosts that had survived at least 40 days after treatment with TBI, 100 $\times$ 10^6 BM cells, and low doses of PBMC (Figures 3A and B). Typical microscopic changes of GVHD in the skin included epidermal hyperplasia, a dermal inflammatory infiltrate, subepidermal blistering, and necrotic keratinocytes (Figure 3A). In the large intestine, changes included crypt apoptosis and inflammation and atrophy of mucin containing glandular cells (Figure 3B). These changes were not observed after 100 days in 6 of 6 Lewis rat hosts given the protective regimen as shown in representative tissue sections from a host in Figures 3C and D. The epidermis was 1 to 2 cells thick (Figure 3C) and plump mucin-containing cells lined the intestinal crypts (Figure 3D). Additional hosts given the TLI and ATG regimen and BM cells with PBMC were sacrificed at day 40. These hosts showed mild crypt inflammation in the colon, and a mild dermal inflammatory infiltrate in the skin despite the lack of obvious macroscopic skin lesions or diarrhea (data not shown). Thus, hosts given the protective regimen had evidence of transient mild microscopic lesions of GVHD that resolved by 100 days.

Chimerism in Protected Hosts

The percentage of ACI donor-type cells in the peripheral blood of surviving Lewis hosts injected with BM cells with or without PBMC was determined at 100 days by immunofluorescent staining for the donor-type (RT1A^{a,b}) marker. Lewis hosts prepared with TLI and ATG and injected with only donor marrow cells developed mixed chimerism (mean 43% donor-type cells; Table 1). All Lewis hosts prepared with TBI and injected with only donor marrow cells developed complete chimerism (Table 1). Lewis hosts treated with TLI, ATG, and an injection of donor BM and 25 $\times$ 10^6 PBMC showed complete chimerism also (Table 1). Representative stainings of nucleated blood cells from the 3 groups of chimeras for the RT1A^{a,b} marker versus T cell, B cell, granulocyte and macrophage markers, and subsequent 2-color flow cytometric analyses are shown in Figure 4. Complete chimerism was observed in all lineages in hosts given TLI, ATG, BM cells, and PBMC (Figure 4C), and mixed

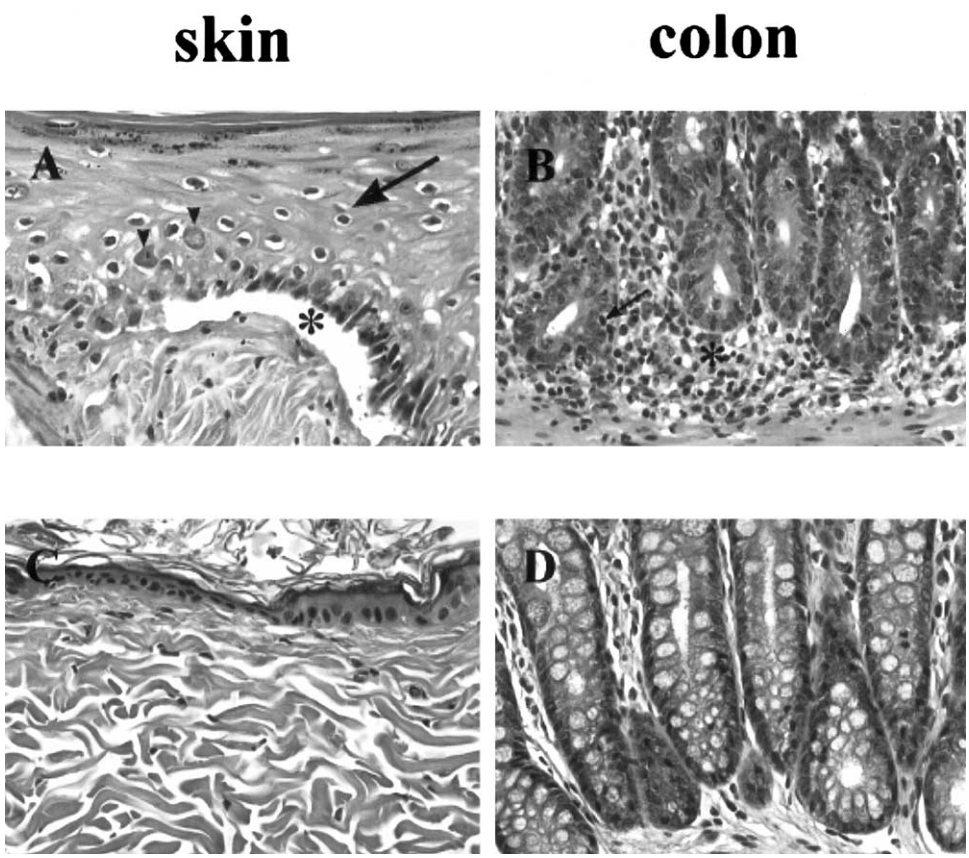

Figure 3. Histopathologic examination of Lewis rat hosts given BMT from ACI donors. A, Microscopic section (× 400) of skin from a Lewis rat host given TBI, 100×10^6 BM cells and 0.1×10^6 PBMC. Skin samples were harvested 40 days after BMT. Arrow shows hyperplastic epidermis, arrow heads show necrotic keratinocytes, and asterisk shows subdermal blister. B, Microscopic section of large intestine of host in part A. Asterisk shows lymphocyte infiltrate surrounding crypts; arrow indicates lymphocyte infiltration into crypt. Plump mucin-containing glandular cells are rare. C, Microscopic section of skin 100 days after BMT from host given TLI, ATG, 100×10^6 BM cells, and 6.25×10^6 PBMC. Epidermis is 1 to 3 cells thick, and no dermal infiltrate is seen. D, Microscopic section of large intestine from host in part C. Plump mucin-containing glandular cells are abundant, and no lymphocytic infiltrate is observed.

chimerism was observed amongst T cells and B cells when marrow cells were given alone (Figure 4A).

Protection against GVHD in Mice is Lost in CD1$^{-/-}$ Hosts

Our previous studies showed that BALB/c (H-2^d) mice conditioned with TLI (17 doses of 240 cGy each; total dose of 4080 cGy) and 6 doses of ATS were protected against GVHD after the combined transplantation of 3×10^6 C57BL/6 (H-2^b) donor BM cell and 0.5×10^6 donor PBMC [9]. Protection could be transferred to TBI-conditional hosts with enriched populations of splenic NK T cells [9]. To determine whether protection against GVHD after TLI and ATS conditioning was dependent on the presence of host NK T cells, we compared the ability of BM and PBMC mixtures from C57BL/6 donors to induce GVHD in either wild-type or CD1$^{-/-}$ BALB/c hosts. The latter hosts are deficient in CD1-reactive NK T cells because of the lack of positive selection by CD1-expressing cells in the thymus [14]. The percentage of CD1-reactive NK T cells among all gated TCR$\alpha\beta^+$ T cells in the spleen of BALB/c mice increased from 2.5% before TLI and ATS treatment to 58.5% after treatment in wild-type mice, as judged by staining with CD1d tetramers loaded with α galactosyl ceramide. The latter reagent stains only NK T cells expressing the invariant $V_\alpha 14$ TCR [16]. However,

Table 1. *Chimerism in Lewis Hosts 100 Days after ACI Bone Marrow Transplantation*

Host Treatment	Infused Donor Cells	% Donor Cells in Blood	Mean ± SE
TBI	100×10^6 BM	>99 × 6	>99
TLI + ATG	100×10^6 BM	1, 87, 4, 67, 82, 18	43 ± 40
TLI + ATG	100×10^6 BM+25×10^6 PBMC	>99 × 4	>99

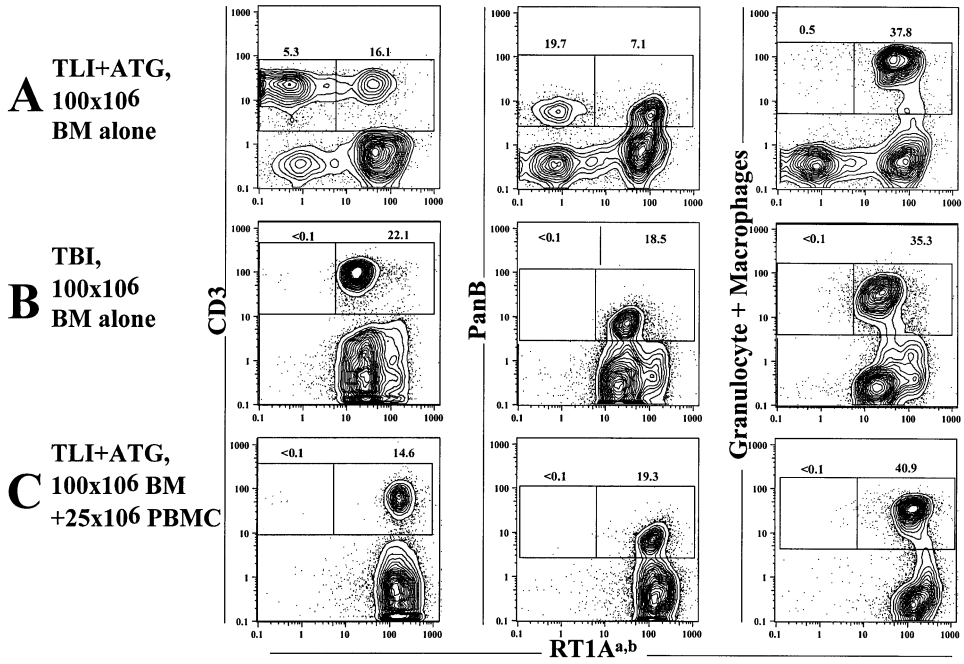

Figure 4. Chimerism in Lewis hosts given BMT from ACI donors: Two-color flow cytometric analysis of PBMC from a Lewis host given either TBI and 100×10^6 ACI BM cells or TLI, ATG, and 100×10^6 ACI BM cells with or without 25×10^6 ACI PBMC. Host cells were obtained at 100 days, and stained for CD3, pan B, or granulocyte and macrophage markers, and versus RT1A $^{(a,b)}$ (donor type) markers. Boxes on left enclose host cells, and boxes on right enclose donor cells. Percentages in each box are shown.

this considerable increase was not observed in CD1$^{-/-}$ BALB/c mice after treatment, and only 3.8% of tetramer$^+$ cells were observed (Figure 5A).

Figure 5B shows that wild-type BALB/c hosts given 800 cGy TBI and a mixture of 3×10^6 C57BL/6 BM cells and 0.5×10^6 C57BL/6 PBMC all died by day 32. These hosts had typical clinical signs of GVHD. Addition of 3 doses of ATS on days -12, -10, and -8 failed to protect the hosts and all died by day 52 (Figure 5B). In contrast, 5 out of 6 wild-type BALB/c hosts given 17 doses of TLI and 3 doses of ATS followed by infusion of the same mixture of C57BL/6 BM and PBMC survived for at least 100 days (Figure 5B) ($P < .01$ TBI versus TLI and ATG). When CD1$^{-/-}$ BALB/c hosts were given the same TLI and ATS conditioning regimen followed by the infusion of C57BL/6 BM cells and PBMC, the hosts developed clinical signs of GVHD, and all died by day 53. There was a significant difference ($P < .01$) in survival between the wild-type and CD1$^{-/-}$ groups of hosts. Wild-type BALB/c hosts that were conditioned with TLI and ATS, and that survived for at least 100 days were all mixed chimeras amongst T cells, B cells, and granulocytes and monocytes in the peripheral blood (data not shown).

Protected BALB/c Chimeras Show a Th2 Bias

In vivo activation of NK T cells has been reported to induce a Th2 bias among conventional T cells that protects mice against autoimmune diabetes and encephalomyelitis [17-20]. In addition, Th2 polarized donor T cells are weak inducers of GVHD as compared with Th1-polarized donor T cells [21,22]. Accordingly, we assayed the cytokine secretion pattern of both host and donor T cells in mixed chimeras that had been protected by the TLI and ATS conditioning regimen. Wild-type BALB/c hosts that survived at least 100 days after conditioning with TLI and ATS and transplantation of a mixture of C57BL/6 BM cells and PBMC were sacrificed and spleen cells were harvested. The latter cells were stained with anti-TCRαβ, anti-H–2K^b, and anti-H–2K^d monoclonal antibodies. Donor T cells (TCRαβ$^+$H-2K^{b+}) and host T cells (TCRαβ$^+$ H-2K^{d+}) were sorted using 2-color flow cytometry as shown in Figure 6. TCRαβ$^+$ T cells accounted for 24.6% of PBMC, and sorted donor T cells represented 90% of the gated TCRαβ$^+$ cells, and sorted host T cells represented 8%. Equal numbers (0.2×10^6) of the host and donor sorted T cells were stimulated in vitro with calcium ionophore and phorbol myristate acetate, and supernatants were collected at 48 hours. Control-sorted splenic T cells from untreated C57BL/6 mice were stimulated using the same procedures, and supernatants were harvested at 48 hours. All supernatants were assayed for the concentration of IFN-γ and IL-4 as shown in Figure 6. Whereas, the control C57BL/6 splenic T cells showed a Th1 biased pattern with the concentration of IFN-γ at least 10 fold higher than the concentration of IL-4. The sorted chimeric T cells

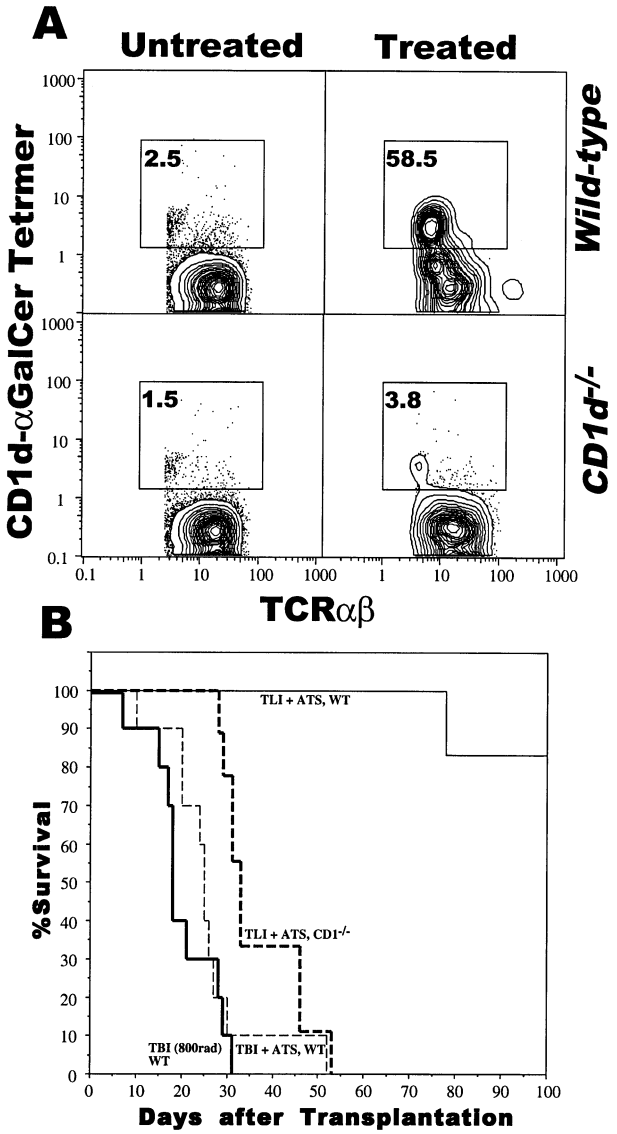

Figure 5. Wild-type and NK T-cell deficient $CD1^{-/-}$ BALB/c hosts compared for splenic T cell subsets and for survival after BMT from C57 BL/6 donors. A, Wild-type or $CD1^{-/-}$ BALB/c mice were given TLI and 3 injections of ATS or no treatment. Immediately after TLI/ATS conditioning, spleen cells were stained with anti-TCRαβ monoclonal antibodies versus CD1 tetramers loaded with α galactosyl ceramide. Panels show tetramer versus TCRαβ staining of gated $TCR\alpha\beta^+$ T cells. Boxes enclose $TCR\alpha\beta^+$ $tetramer^+$ cells. Background staining of TCRαβ cells was approximately 1.5%. B, Control wild-type BALB/c hosts were conditioned with either TBI (800 cGy) alone or TBI and 3 injections of ATS (days −12, −8, −6). Experimental wild-type or $CD1^{-/-}$ hosts were conditioned with TLI (17 doses of 240 cGy each) and ATS (days −12, −8, −6). All hosts were given 3×10^6 C57BL/6 BM cells and 0.5×10^6C57BL/6 PBMC within 24 hours after the completion of TLI or TBI. Survival is shown for 6 to 10 hosts in each group.

from both donor and host showed a marked increase in the mean concentration of IL-4 as compared with that of untreated C57BL/6 and BALB/c mice ($P < .05$ and $P < .01$ respectively; student t test). There was a significant decrease in the concentration of IFN-γ as compared with untreated C57BL/6 mice ($P < .01$) such that the ratio of IFN-γ:IL-4 was reduced to less than 2 fold (Figure 6). Differences in the IFN-γ concentrations were not significant as compared with untreated BALB/c mice ($P > .05$).

DISCUSSION

In the current study, we determined whether the TLI and ATS regimen that was successful in preventing GVHD in BALB/c mice could be used to protect Lewis rat hosts from GVHD after combined BM and PBMC transplantation from MHC-mismatched ACI donors. Some modifications of the regimen were made such that the number of TLI treatments of 240 cGy each was reduced from 17 in the mouse studies to 10 treatments (total dose 2400 cGy) in the rat studies, and 5 doses of ATG were used in the latter study instead of 3 doses of ATS used in the former.

As in the mouse studies, the combined TLI and ATG conditioning regimen protected the Lewis hosts from acute lethal GVHD observed in TBI-conditioned hosts given a combined inoculum of 100×10^6 ACI BM and 25×10^6 ACI PBMC. The addition of ATG to the TBI conditioning regimen failed to improve the rapid early mortality from GVHD. In further studies, TBI- and TLI/ATG-conditioned hosts were given a constant inoculum of 100×10^6 ACI BM cells, and graded doses of ACI PBMC to determine the number of PBMC required to induce lethal GVHD in about 50% of both types of hosts. Whereas 0.1×10^6 donor PBMC induced death in about 60% of TBI-conditioned hosts by 100 days, the dose of PBMC required to induce similar mortality in TLI/ATG-conditioned hosts was 1000 fold higher (100×10^6 PBMC).

Lewis hosts conditioned with TLI and ATG and given ACI 100×10^6 BM and 25×10^6 PBMC were complete chimeras in all cell types in the peripheral blood (T cells, B cells, monocytes, and granulocytes) when assayed more than 100 days after transplantation. As expected from the results of previous studies [9], host conditioned with TLI and ATG that were given only ACI BM cells were stable mixed chimeras at the same time point. Thus, the PBMC facilitated the transition from mixed to complete chimerism despite the lack of GVHD. This transition has been reported previously in murine studies in which stable mixed chimeras given donor lymphocyte infusions became complete chimeras without the development of clinical GVHD [23].

Histopathologic analysis of Lewis host chimeras conditioned with TLI and ATG and given combined ACI BM and PBMC transplants showed no evidence of microscopic tissue injury in the colon and skin more

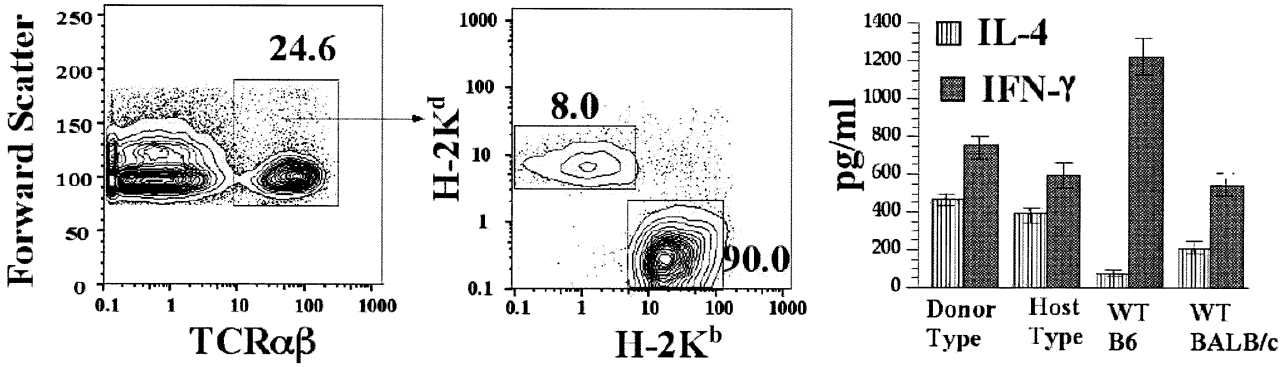

Figure 6. Cytokine secretion patterns of donor and host T cells in BALB/c mixed chimeras. Wild-type BALB/c hosts were given TLI, ATS, C57BL/6 BMT (3×10^6 BM cells and 0.5×10^6 PBMC), and observed for at least 100 days as in Figure 5. Spleen cells from 3 surviving hosts were pooled, stained for TCRαβ, H-2K^b (donor-type) and H-2K^d (host type) surface markers, and analyzed using 3-color flow cytometric analysis. Gated TCRαβ$^+$ T cells (enclosed in box in left panel) were analyzed for H-2K^b versus H-2K^d subsets (enclosed in boxes in middle panel). H-2K^b and H-2K^d–positive cells were sorted, and 0.2×10^6 cells per well were stimulated in vitro with phorbol myristate acetate and calcium ionophore. After 48 hours, culture supernatants were assayed for the concentration of IL-4 and IFN-γ by the ELISA technique (right panel). Control cultures used sorted splenic TCRαβ$^+$ T cells from wild-type C57BL/6 (WT B6) or BALB/c (WT BALB/c) untreated mice. Bars show the mean concentrations of 5 individual cultures and brackets show the standard errors. One of 2 replicate experiments is shown in which pooled cells from a total of 6 mice were analyzed.

than 100 days after transplantation. In contrast, Lewis hosts conditioned with TBI showed the typical GVHD-associated changes of crypt cell death and inflammatory cell infiltration in the colon and epidermal hyperplasia and dermal infiltrates in the skin.

To study the role of regulatory NK T cells in the protection against GVHD afforded by the TLI-based conditioning regimen, additional studies were performed using NK T-cell deficient CD1$^{-/-}$ BALB/c mice and wild-type BALB/c mice as hosts because such gene-deficient rats are not available. The TLI and ATS regimen markedly increased the percentage of CD1-reactive T cells among all T cells as judged by staining with CD1 tetramers that bind specifically to the invariant $V_\alpha 14$ TCR expressed by NK T cells [16]. This increase was not observed in CD1$^{-/-}$ BALB/c mice. As in the rat studies, wild-type BALB/c mice conditioned with TBI or TBI and ATS developed lethal GVHD after the transplantation of a mixture of MHC-mismatched PBMC and BM cells. Although the TLI and ATS conditioning regimen protected almost all wild-type hosts from severe GVHD and death during the 100-day observation period, the same conditioning regimen failed to protect the BALB/c CD1$^{-/-}$ hosts, and all succumbed by about 50 days. This result is consistent with the loss of protection against GVHD in TLI-conditioned hosts that were treated with anti-asialo–GM1 antibodies [24]. In vivo administration of the latter antibodies to TLI-treated hosts has been shown to deplete NK T cells [9]. Taken together, the failure of protection of the TLI conditioning regimen in NK T-cell deficient hosts, and the ability of enriched populations of splenic NK T cells to transfer protection to TBI-conditioned hosts [9], shows that the NK T cells play a critical role in suppressing GVHD. T cells found in the spleen of mice given TLI that are capable of suppressing GVHD were identified previously as "natural suppressor" cells [25].

Activation of NK T cells in vivo has been shown to ameliorate autoimmune diabetes and experimental allergic encephalomyelitis by polarizing conventional non-NK T cells from a Th1 to a Th2 type of cytokine secretion pattern [17-20]. We hypothesized that the suppressive activity of host NK T cells may be because of the prolonged polarization of donor conventional T cells toward a Th2 cytokine pattern as reported in the autoimmune disease models [17-20]. Polarization toward a Th2 pattern in hosts conditioned with TLI has been previously reported in donor T cells after BMT [26]. In addition, Th2-polarized donor T cells are considerably weaker than Th1-polarized donor T cells in inducing severe GVHD [21-22]. Accordingly, we sorted both host and donor T cells from the spleen of chimeric BALB/c mice conditioned with TLI and ATS more than 100 days after the transplantation of the donor PBMC and BM cells. The sorted cell populations were stimulated in vitro with calcium ionophore and phorbol myristate acetate, and both showed a Th2 biased cytokine secretion pattern. There were higher levels of IL-4 and lower levels of IFN-γ as compared with that observed with sorted T cells from the untreated donor spleen.

In conclusion, the TLI and ATG/ATS conditioning regimen protected both mice and rats against GVHD. Protection was dependent on the presence of host NK T cells and was associated with a Th2 bias of host and donor T cells in the surviving stable chimeras.

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