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Polarized Type 2 Alloreactive CD4$^+$ and CD8$^+$ Donor T Cells Fail to Induce Experimental Acute Graft-Versus-Host Disease[1]

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Acute graft-vs-host disease (GVHD) is thought to be mediated by alloreactive T cells with a type 1 cytokine phenotype. To prevent the development of acute GVHD, we have successfully polarized mature donor T cells toward a type 2 cytokine phenotype ex-vivo by incubating them with murine rIL-4 in a primary MLC. Polarized type 2 T cells were then transplanted with T cell-depleted bone marrow cells into irradiated recipients across either MHC class II (bm12→C57BL/6) or class I (bm1→C57BL/6) barriers, and the intensity of GVHD was measured by assessment of several in vitro and in vivo parameters. The injection of polarized type 2 T cells abrogated the mitogen-induced production of IFN-γ by splenocytes from transplanted hosts on day 13 after bone marrow transplantation (BMT). Injection of polarized type 2 T cells failed to induce secretion of the effector phase cytokine TNF-α by splenocytes stimulated with LPS both in vitro and in vivo, and survival of transplanted mice after i.v. injection with LPS was significantly improved. Furthermore, cell-mixing experiments revealed that polarized type 2 T cells were able to inhibit type 1 cytokine responses induced by naive T cells after BMT. These data demonstrate that both polarized CD4$^+$ and CD8$^+$ type 2 alloreactive donor T cells can be generated in vitro from mature T cell populations. These cells function in vivo to inhibit type 1 T cell responses, and such inhibition attenuates the systemic morbidity of GVHD after BMT across both MHC class II or class I barriers in mice. *The Journal of Immunology,* 1995, 155: 585–593.

Graft-vs-host disease (GVHD)[3] is the major complication after allogeneic bone marrow transplantation (BMT) and develops in a multi-step process that includes the activation of alloreactive donor T cells (afferent phase) and the subsequent process of tissue destruction (efferent phase) (1). Recent evidence suggests that the efferent phases of both experimental and clinical acute GVHD are mediated in large part by proinflammatory cytokines such as TNF-α and IL-1 (2–5). To limit the considerable morbidity and mortality after BMT, some current transplantation protocols include the depletion of T cells before infusion into the host (6, 7). Although this maneuver reduces the risk of subsequent development of GVHD, it also increases the risk of relapse of malignancy and impairs engraftment of donor cells (8, 9). Clinical protocols, therefore, require further modification to improve the long-term outcomes of BMT.

Several studies have demonstrated that mature CD4$^+$ T cells, and recently also CD8$^+$ T cells, polarize into two subsets with different cytokine production profiles (type 1: IL-2, IFN-γ; type 2: IL-4, IL-10) (10–13). Differential activation of these two subsets determines the character of an ensuing immune response (14, 15). Polarization of T cell function to a type 2 cytokine response by treatment of mice with IL-4 (16) or by injection of Th2 clones (17) can modulate inflammatory responses and prevent induction of experimental autoimmune encephalomyelitis, a prototypic Th1 cell-mediated disease. Recently, it has been suggested that polarization of T cell subsets also plays an important role in the development of GVHD (18–24). Acute GVHD has been linked to the activation of alloreactive T cells with a type 1 cytokine phenotype because IFN-γ is important in several aspects of this form of GVHD, including immunodeficiency and mortality (1, 25–27). IFN-γ acts to prime mononuclear cells to secrete TNF-α in response to LPS (28), and TNF-α then mediates tissue destruction and

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[3] Abbreviations used in this paper: GVHD, graft-vs-host disease; BMT, bone marrow transplantation; TBI, total body irradiation; TCD-BM, T cell-depleted bone marrow cells; Con A, concanavalin A.

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systemic morbidity and mortality during the effector phase of GVHD (29).

A recent study by Fowler and co-workers has demonstrated that skewing the cytokine profiles of donor T cells has the potential to diminish acute GVHD (30). These authors treated donor mice in vivo with a combination of human rIL-2 and murine rIL-4 and generated CD4$^+$-enriched splenic populations with a Th2-like cytokine phenotype. Transplantation of these cells into nonirradiated F1 recipients resulted in a significant decrease in mortality induced by LPS. We initiated these studies to evaluate whether polarized mature alloreactive donor T cells with a type 2 cytokine phenotype could be generated from naive donor T cells in vitro. We analyzed further whether the polarization of these cells would suffice to prevent the development of acute GVHD when mediated either by CD4$^+$ or CD8$^+$ T cells. We used two well described murine GVHD models of BMT across isolated MHC differences in which GVHD is mediated entirely by CD4$^+$ T cells in response to a MHC class II difference (bm12→C57BL/6) or in which GVHD is mediated by CD8$^+$ T cells in response to a MHC class I difference (bm1→C57BL/6) (31). Our data clearly demonstrate that alloreactive donor T cells can be polarized toward a type 2 cytokine phenotype in vitro, that cells modified by this approach are able to modulate type 1 T cell responses in vivo, and they fail to mediate acute GVHD in both CD4$^+$ and CD8$^+$ T cell-mediated murine BMT models.

Materials and Methods

Mice

Female B6.C-H2^{bm12} (bm12, K^{b}D^{b}I-A^{bm12}I-E^b), B6.C-H2^{bm1} (bm1, K^{bm1}D^{b}I-A^{b}I-E^b), C57BL/6 (B6, H-2^b), and B6.PL-Thy1a (Thy1.1$^+$) mice were purchased from The Jackson Laboratories (Bar Harbor, ME). The donors and recipients in this study were between the ages of 8 and 12 wk and were housed in sterilized microisolator cages in which they received autoclaved food and autoclaved acidified water for 2 wk after BMT.

Reagents

The following mAbs were used: for detection of murine IFN-γ, purified R46A2 (32) and biotin-conjugated XMG1.2 (33); for detection of murine IL-4, purified 11B11 (34) and biotin-conjugated BVD6–24G2 (35); for detection of murine TNF-α, purified MP6-XT22 and biotin-conjugated rabbit anti-mouse TNF-α (36) (PharMingen, San Diego, CA). For cell surface analysis, the following mAbs were used: phycoerythrin-conjugated anti-CD3ϵ and anti-CD8 mAbs; fluorescein-conjugated anti-CD4 mAb (PharMingen), fluorescein-conjugated anti-Thy1.2 (Boehringer Mannheim, Indianapolis, IN). Murine rIL-4 with a specific activity of 7×10^7 U/mg by FDCP-2 assay was obtained from Immunex Corp. (Seattle, WA). Murine rIFN-γ was purchased from Amgen (Thousand Oaks, CA), and human rIL-2 was purchased from Pharmacia Diagnostics Inc. (Silver Spring, MD). The anti-Thy1.2 mAb used for T cell depletion was isolated from tissue culture supernatants from hybridoma clone HO-13-4 (American Type Culture Collection, Rockville, MD) (37). Low-Tox-M rabbit complement was obtained from Accurate Corp. (Westbury, NY). LPS (specific activity 5×10^4 endotoxin units/mg), concanavalin A (Con A), and the alkaline phosphatase substrate-104 for detection in ELISA were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell culture

For the generation of alloreactive T cells with a type 2 cytokine phenotype in vitro, we adapted the methods initially described by LeGros et al. (38) and Swain et al. (39). In brief, cells were cultured in DMEM supplemented with 1% normal mouse serum (Pel-Freez, Rogers, AR), 2×10^{-5} M 2-ME, and additional amino acids (Life Technologies, Grand Island, NY). Nylon-wool-purified responder T cells from bm12 or bm1 mice (3×10^7) were cocultured in a MLC with 4.5×10^7 irradiated (2000 rad) splenocytes from B6 mice in the presence of 10^3 U/ml murine rIL-4 for 7 days in upright flasks. Controls were cultured in the presence of 25 U/ml human rIL-2. After this culture, cells were collected and washed extensively to minimize carryover of cytokines. The percentage of CD4$^+$ or CD8$^+$ cells within the cell preparations was determined by FACS analysis, and numbers of CD4$^+$ (bm12) or CD8$^+$ (bm1) cells in these unseparated populations were normalized for further experiments. For analysis of cytokine secretion patterns in a secondary MLC, primed cells (10^5 cells/well) were restimulated in flat-bottom 96-well plates with T cell-depleted (anti-Thy1.2 and rabbit complement-treated), irradiated splenocytes from B6 mice (2×10^5 cells/well) without any additional cytokines. A syngeneic MLC was included in each experiment as a control. Supernatants for cytokine analysis were taken after 48 h of the secondary stimulation.

For the cell-mixing experiments, nylon-wool-purified bm12 CD4$^+$ splenocytes were cultured with B6 stimulators in a primary MLC, washed, and then restimulated with B6 stimulator cells in a secondary MLC as described above. At the initiation of these secondary cultures, bm12 T cells previously cultured with B6 stimulators in the presence of murine rIL-4 (10^3 U/ml) were added in graded numbers ($0.8–50 \times 10^3$ cells/well). This created ratios of 0.008 to 0.5 of polarized type 2 cells to nonpolarized cells. IFN-γ concentrations in the supernatants after 48 h of culture were analyzed as described below, and the proliferative response was measured after 72 h by [^{3}H]thymidine incorporation (1 μCi/well) for the last 20 h.

Bone marrow transplantation

For BMT, 5×10^5 cultured CD4$^+$ or CD8$^+$ T cells from bm12 or bm1 mice were added to 5×10^6 T cell-depleted bone marrow cells (TCD-BM) from the appropriate mouse strains. This cell mixture was then injected i.v. (in 0.25 ml of Leibovitz's L-15 media, Life Technologies) into sex-matched B6 recipient mice that had been given a lethal radiation dose of 1100 cGy (^{137}Cs source) in two doses separated by 3 h. For positive GVHD controls, 5×10^5 CD4$^+$ or CD8$^+$ cells from nylon-wool-purified naive T cells were injected together with TCD-BM. Non-GVHD controls received TCD-BM only. For the co-injection experiments, B6 recipient mice were transplanted with TCD-BM containing 5×10^5 naive bm12 CD4$^+$ T cells alone or TCD-BM containing 5×10^5 naive cells with additions of either 5×10^4 polarized type 2 CD4$^+$ cells or the same number of cultured but nonpolarized CD4$^+$ cells.

Assessment of graft-vs-host disease

To measure T cell responsiveness in GVHD, splenocytes were isolated from transplanted mice 13 days after BMT and cultured at 4×10^5 cells/well in flat-bottom 96-well plates in the presence of 2.5 μg/ml of the T cell mitogen Con A as described previously (40). Supernatants were taken after 48 h of in vitro stimulation and analyzed for IFN-γ and IL-4 production. In addition, splenocytes from recipient mice were stimulated with LPS (1.0 μg/ml) and supernatants were taken after 4 h to measure TNF-α levels. To analyze GVHD-associated T cell expansion, we injected naive or polarized type 2 T cells from donor bm12 or bm1 mice (Thy1.2$^+$) into irradiated B6.PL-Thy1a (Thy1.1$^+$) congenic mice. The origin of expanding splenic T cells was determined on day 13 post BMT by FACS analysis as described using triple staining with fluorescein-conjugated anti-Thy1.2 mAb and phycoerythrin-conjugated anti-CD4 and anti-CD8 mAbs. To measure GVHD severity in vivo, we used the method described by Nestel et al. (29). In brief, BMT mice were challenged with LPS (2.5–25 μg i.v. in 0.2 ml of PBS) on day 13 post BMT. Ninety minutes after injections, mice were anesthetized and blood samples taken from the orbital sinuses to determine the serum levels of TNF-α. Mortality induced by LPS was assessed twice daily within the first 7 days after injection.

Cell surface phenotype analysis

Cells from a primary MLC or from spleens of BMT-recipient mice (day 13 post BMT) were resuspended in PBS for flow cytometric analysis. Cells (5×10^5) were incubated for 20 min at 4°C with mAb 2.4G2 (41) to block nonspecific staining via FcγR, then incubated with fluorescein- or phycoerythrin-conjugated mAbs for 30 min at 4°C. The cells were

then washed twice with PBS before fixing in 1% *p*-formaldehyde. Two-color flow cytometric analysis of 10^4 stained cells was performed using a FACScan (Becton Dickinson, Mountain View, CA). The FACScan was calibrated using PE- and FITC-conjugated, nonspecific IgG Abs. Live cells were gated by forward and side scatter parameters.

Cytokine assays

Levels of IL-4, IFN-γ, and TNF-α in tissue culture supernatants or sera were determined by sandwich ELISA using specific anti-murine mAbs for capture and detection (PharMingen). An alkaline phosphatase kit (Vector Laboratories, Burlingame, CA) was used for detection, and a standard curve was generated to determine the cytokine concentration in the sample. Detection limits of assays were 10 pg/ml for TNF-α and 2 U/ml for IFN-γ and IL-4.

Statistical analysis

Values are mean ± SEM. For statistical analysis, the two-tailed Student's *t*-test was used except for the mortality data, where *p* values were determined using the nonparametric Wilcoxon rank sum test.

Results

Addition of rIL-4 to a primary mixed lymphocyte culture generates polarized alloreactive T cell populations with a type 2 cytokine phenotype

We first asked whether alloreactive $CD4^+$ T cells with a Th2 cytokine phenotype could be generated in vitro from mature splenic T cells. We chose a murine MLC system with only a single MHC class II alloantigenic difference between donor and host strains (bm12 anti-B6) because the concept of the Th1/Th2 dichotomy of $CD4^+$ cells is well described (42, 43) and the methodology to produce Th2 $CD4^+$ T cell subsets is established (38, 39). We cocultured nylon-wool-purified responder T cells from bm12 donor mice with stimulator cells from B6 mice in a primary MLC for 7 days in the presence or absence of murine rIL-4 (10^3 U/ml). These responder cells were then collected and restimulated with B6 stimulator cells in a secondary MLC without any additional cytokines. As demonstrated in Figure 1*A*, $CD4^+$ responder T cells from bm12 mice cultured in the presence of low levels of rIL-2 but no rIL-4 during the primary MLC (black bars) were of a Th1 cytokine phenotype in the secondary MLC. The presence of murine rIL-4 during the primary MLC (white bars) changed the cytokine phenotype: IFN-γ production by cells in response to B6 alloantigens was about 15-fold less when compared with cells that had been cultured without rIL-4 during the first MLC. By contrast, the production of IL-4 was more than an order of magnitude higher, confirming that they were of a Th2 cytokine phenotype. We next tested whether the same approach would generate alloreactive type 2 $CD8^+$ T cells in response to MHC class I alloantigens (bm1 anti-B6). When rIL-4 was added to primary cultures, a polarization of the responder population toward a type 2 function was observed, even though a large majority (85%) of responder bm1 cells were $CD8^+$ (Fig. 1*B*). The production of IL-4 by these bm1 responders was much lower than the levels produced by bm12 cells (38 ± 12 vs 254 ± 57 U/ml), a finding similar to the results of Croft and co-workers, where type 2 $CD8^+$ T cells secreted more than 100-fold less IL-4 than $CD4^+$ T cells

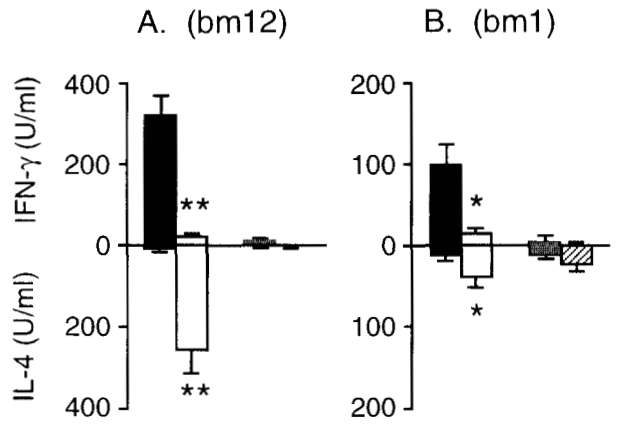

FIGURE 1. Polarization of alloreactive T cells toward a type 2 cytokine phenotype in a secondary MLC. T cells from bm12 (*A*) or bm1 (*B*) donor mice were cocultured with allogeneic stimulator cells from B6 mice in a primary MLC with or without addition of murine rIL-4. Cells were then restimulated with B6 stimulators or with syngeneic cells, and cytokine concentrations were determined in tissue culture supernatants taken after 48 h of the secondary MLC. Responder T cell populations were generated during a primary MLC against B6 alloantigens in the absence (■) or presence (□) of murine rIL-4 (10^3 U/ml). As controls, responder T cells previously cultured without (▩) or with (▨) rIL-4 were cocultured with syngeneic stimulator cells in the secondary MLC. Data are expressed as mean ± SEM from at least three separate experiments. Statistical analysis was performed using the two-tailed Student's *t*-test. The indicated groups were significantly different ($*p < 0.05$, $**p < 0.01$) from primary cultures containing no IL-4.

(44). However, this production was still significant when compared with syngeneic cultures (checked bars).

Transplantation of in vitro polarized type 2 T cells prevents type 1 cytokine responses during the afferent phase of graft-vs-host disease

We then examined the potential of these type 2 donor T cells to modulate responses to host alloantigens and to prevent GVHD. We used well described BMT models across either MHC II barriers (bm12→B6) where $CD4^+$ T cells mediate GVHD, or across MHC I barriers (bm1→B6) where $CD8^+$ T cells are the exclusive mediators of disease (31). We added 5×10^5 naive or cultured $CD4^+$ (bm12) or $CD8^+$ T cells (bm1) to 5×10^6 freshly isolated TCD-BM cells from the same donor strains and transplanted the mixture into irradiated (1100 cGy) B6 hosts. The recipient mice were then killed on day 13 post BMT, and splenocytes were stimulated with Con A for 48 h. As expected, splenocytes from transplanted B6 mice with acute GVHD after injection of naive bm12 T cells produced high levels of IFN-γ when restimulated with Con A in vitro (Fig. 2*A*). Increased production of this type 1 cytokine has been shown to be characteristic in the afferent phase of acute GVHD (1, 45). Likewise, recipients of T

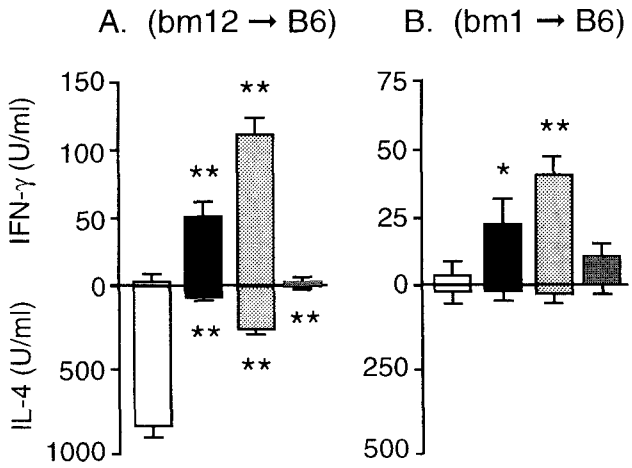

FIGURE 2. Transplantation of type 2 allogeneic donor cells prevents the induction of type 1 cytokine responses after BMT. B6 recipient mice were transplanted with TCD-BM containing T cells cultured with (□) or without (■) murine rIL-4, naive T cells (▩) or no T cells (▩) from bm12 (*A*) or bm1 (*B*) donor mice as described in *Materials and Methods*. Splenocytes from B6 recipient mice were removed on day 13 post BMT and cultured for 48 h with Con A. Cytokine concentrations (U/ml) in tissue culture supernatants are expressed as mean ± SEM from two experiments for each strain combination (n = 3–6 mice/group). Statistical analysis was performed using the two-tailed Student's t-test. The indicated groups were significantly different ($*p < 0.05$, $**p < 0.01$) from groups receiving polarized type 2 T cells.

cells that were cultured in the presence of rIL-2 but in the absence of rIL-4 produced high amounts of IFN-γ. These levels were ~50% lower than those produced by splenocytes of recipients of naive T cells. This may be due to specific culture conditions and, therefore, these cultured T cells may be less effective than naive donor T cells in mediating allorecognition. However, the injection of T cells cultured in the presence of rIL-4 (polarized toward type 2) resulted in a complete (>97%) abolition of IFN-γ production by B6 recipient mice when compared with the control group (112.0 ± 11.9 vs 3.5 ± 2.9 U/ml). These levels were similar to that in mice without GVHD (TCD-BM only). A similar pattern of IFN-γ production was seen in the bm1→B6 model (Fig. 2*B*). Although Con A-induced secretion was lower in animals with GVHD in this BMT model, there was a further four- to eightfold decrease in IFN-γ production when polarized type 2 T cells were injected. The production of IL-4 was increased about fourfold in recipients of type 2 T cells in $CD4^+$ T cell-mediated GVHD, although no enhanced IL-4 secretion was found in $CD8^+$ T cell-mediated GVHD. It is possible that these cells produced IL-4 at a level not detectable with our cytokine assay (i.e., <2 U/ml). Taken together, these data demonstrate that the donor T cell response to host alloantigens in vivo is identical to their response in vitro in a secondary MLC.

Table I. *T cell expansion in bm12→B6 and bm1→B6 BMT models*[a]

Donor Strain	T Cell Preparation[b]	Positive Cells per Spleen (×10⁻⁶)[c] $CD4^+$	$CD8^+$
bm12	10^3 U/ml rIL-4 in primary MLC	8.0 ± 1.3	0.4 ± 0.1
	No rIL-4 in primary MLC	12.5 ± 1.4	1.8 ± 0.4**
	Naive T cells	7.3 ± 0.9	2.5 ± 0.4**
	None	1.2 ± 0.2**	0.4 ± 0.1
bm1	rIL-4 in primary MLC	0.4 ± 0.1	6.2 ± 1.1
	No rIL-4 in primary MLC	0.5 ± 0.1	13.4 ± 2.0*
	Naive T cells	1.4 ± 0.3**	13.0 ± 2.3*
	None	0.7 ± 0.2	0.5 ± 0.1*

[a] Splenocytes from transplanted B6 mice were analyzed on day 13 post BMT.

[b] Transplanted B6 mice received TCD-BM (5×10^6) containing cultured or naive donor T cells (5×10^5) as described in *Materials and Methods*.

[c] FACS analysis values are expressed as total positive cells × 10^{-6} per spleen (mean ± SEM) and represent pooled data from at least two experiments for each strain combination (n = 6–9 mice/group). Statistical analysis was performed using the two-tailed Student's t-test. The indicated groups were significantly different ($*p < 0.05$, $**p < 0.01$) from groups receiving polarized type 2 T cells.

Polarized type 2 donor $CD4^+$ or $CD8^+$ T cells expand in hosts by day 13 post bone marrow transplantation

We next analyzed the cell surface phenotype of splenic T cells on day 13 post BMT to determine whether lack of IFN-γ production was due to a lack of expansion of donor T cells. In B6 mice with GVHD that received naive bm12 T cells, $CD4^+$ splenocytes increased significantly above TCD-BM levels, as expected ($1.2–7.3 \times 10^6$). We also found a statistically significant expansion of $CD8^+$ cells, albeit at a lower level ($0.4–2.5 \times 10^6$), probably due to a paracrine stimulation by cytokines released from $CD4^+$ cells (Table I, *top*). The absolute number of T cells per spleen in transplanted mice receiving polarized type 2 T cells was almost identical to the number in recipients of naive T cells, confirming that lack of IFN-γ secretion was not due to the absence of donor T cells. In the second BMT model (bm1→B6), the predominant expanded T cell population was $CD8^+$ (Table I, *bottom*). Culture of donor T cells with rIL-4 before BMT significantly reduced this expansion by approximately twofold, but this reduction was not sufficient to explain the observed eightfold decrease in IFN-γ production. Furthermore, experiments using congenic B6.PL-Thy1a ($Thy1.1^+$) mice as hosts showed that the expanded T cell populations were ≥97% donor origin ($Thy1.2^+$) in both BMT models (data not shown).

Polarized type 2 T cells fail to induce TNF-α secretion by lipopolysaccharide-stimulated splenocytes from recipient mice

The above results demonstrate that the afferent arm of GVHD is effectively modulated away from a type 1 cytokine profile by transplanting alloreactive T cells that have been polarized first to a type 2 phenotype. We next analyzed whether the effector mechanisms of GVHD were

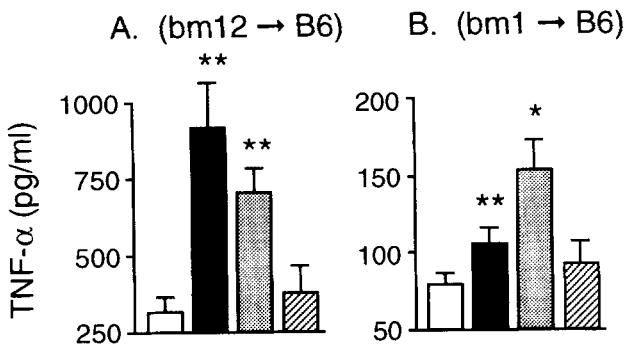

FIGURE 3. The LPS-induced production of TNF-α is diminished in recipients of type 2 alloreactive T cells. B6 recipient mice were transplanted with TCD-BM containing T cells cultured with (□) or without (■) murine rIL-4, naive T cells (▩), or no T cells (▨) from bm12 (*A*) or bm1 (*B*) donor mice as described in *Materials and Methods*. Splenocytes from B6 recipient mice were removed on day 13 post BMT and cultured for 4 h with LPS (1.0 μg/ml). TNF-α concentrations (pg/ml) in tissue culture supernatants are expressed as mean ± SEM from two experiments for each strain combination (n = 3–8 mice/group). Statistical analysis was performed using the two-tailed Student's t-test. The indicated groups were significantly different ($^*p < 0.05$, $^{**}p < 0.01$) from groups receiving polarized type 2 T cells.

also affected by this functional difference. The proinflammatory cytokine TNF-α has been shown to be an important mediator of acute GVHD, and the production of TNF-α in response to injections of LPS is directly related to the severity of GVHD (29, 46). We first examined whether injection of polarized type 2 T cells would result in a reduced production of TNF-α in vitro by splenocytes from mice with GVHD. In both $CD4^+$ and $CD8^+$ T cell-mediated GVHD, splenocytes isolated from transplanted B6 mice receiving naive T cells or T cells cultured without rIL-4 secreted large amounts of TNF-α after stimulation with LPS (Fig. 3). By contrast, splenocytes from transplanted mice receiving polarized type 2 T cells produced significantly less TNF-α to LPS in both BMT models at a level equivalent to the TNF-α generated by non-GVHD controls. The pattern of spontaneous secretion of TNF-α by these groups was similar to that observed after LPS stimulation, albeit at significantly lower levels (results not shown). It should be noted that the scale of TNF-α production by LPS-activated day 13 splenocytes was higher in the bm12→B6 combination after transplantation of TCD-BM. It is likely that this difference reflects a mild subclinical GVH reaction caused by the few remaining T cells in the treated BM inoculum. We have demonstrated previously that such reactions can occur to minor histocompatibility Ags after TCD-BMT, particularly when large doses of total body irradiation (TBI) were used to condition BMT recipients (47).

We next analyzed the production of TNF-α in vivo in response to LPS injection. BMT hosts received 2.5 μg of LPS i.v. via tail vein injection on day 13 post BMT. This

Table II. *Transplantation with type 2 alloreactive donor T cells results in decreased TNF-α serum levels after in vivo challenge with LPS*[a]

T Cell Preparation[b]	Donor:	Serum TNF-α (ng/ml)[c] bm12	bm1
10^3 U/ml rIL-4 in primary MLC		0.65 ± 0.16	1.17 ± 0.41
No rIL-4 in primary MLC		2.30 ± 0.44**	2.38 ± 0.79*
Naive T cells		1.63 ± 0.25**	5.87 ± 2.18*
None		0.55 ± 0.14	0.24 ± 0.09*

[a] On day 13 post BMT, transplanted B6 mice were injected with 2.5 μg of LPS (in 0.2 ml of PBS) via tail vein. Ninety minutes later, mice were bled from the orbital sinuses and serum was collected.

[b] Transplanted B6 mice received TCD-BM (5×10^6) containing cultured or naive donor T cells (5×10^6) as described in *Materials and Methods*.

[c] Concentrations of circulating TNF-α were determined by ELISA and are expressed as ng/ml serum (mean ± SEM). Values represent pooled data from two separate experiments for each strain combination (n = 3–6 mice/group). Statistical analysis was performed using the two-tailed Student's t-test. The indicated groups were significantly different ($^*p < 0.05$, $^{**}p < 0.01$) from groups receiving polarized type 2 T cells.

quantity is insufficient to induce the production of TNF-α from normal, unprimed mononuclear cells (results not shown). Serum TNF-α levels were assessed 90 min after LPS injections. As expected, elevated serum levels developed during this short time in GVHD mice that had received naive T cells from either bm12 or bm1 mice (Table II). However, serum TNF-α levels were markedly reduced in recipients of polarized type 2 T cells in both BMT models, and in recipients of bm12 T cells were equivalent to levels found in TCD-BM controls. Thus, type 2 T cells did not prime mononuclear cells to secrete TNF-α, confirming that type 1 cytokines are critical to the induction of acute GVHD.

Transplantation with type 2 alloreactive donor T cells results in improved survival after in vivo challenge with lipopolysaccharide

Injection of 25 μg of LPS on day 13 post BMT resulted in a rapid development of septic shock, cachexia, and death within 7 days in 100% of hosts receiving naive bm12 T cells (Fig. 4*A*). However, transplanted B6 mice receiving polarized type 2 T cells showed only 40% mortality. This was significantly reduced from GVHD controls but still higher than the TCD-BM group (15% mortality). In the bm1→B6 strain combination, all recipients of type 2 T cells survived (Fig. 4*B*). In this model, there was no difference between recipients of TCD-BM and polarized type 2 T cells, but it should be noted that the mortality rate of the positive GVHD control group was not as pronounced in this model (40 vs 100% in bm12→B6 BMT). Thus, the transplantation of type 2 donor T cells had profound and long lasting effects on systemic manifestations of GVHD in both of these strain combinations.

In vitro polarized type 2 T cells regulate type 1 T cell responses in vivo

Despite the fact that type 2 T cells expanded in vivo on day 13 post transplant (Table I) and produced IL-4 in the

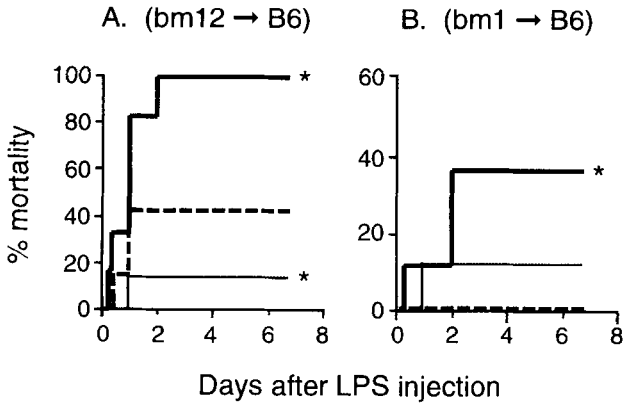

FIGURE 4. Recipients of type 2 alloreactive T cells show improved survival after LPS challenge. B6 recipient mice were transplanted with TCD-BM containing T cells cultured with murine rIL-4 (– – – –), naive T cells (——) or no T cells (——) from bm12 (*A*) or bm1 (*B*) donor mice as described in *Materials and Methods*. On day 13 post BMT, B6 recipient mice were injected with 25 μg of LPS. The mortality was assessed every 12 h for 7 days post injection. The figure represents pooled data from two experiments for each strain combination (n = 6–8 mice/group). Statistical analysis was performed using the nonparametric Wilcoxon rank sum test. The indicated groups were significantly different ($*p < 0.03$) from groups receiving polarized type 2 T cells.

bm12→B6 model (Fig. 2), it was still possible that these cells were functionally impaired after BMT, thus accounting for the lack of cytokine production and resistance to challenge with endotoxin. To evaluate further the function of polarized type 2 T cells in vivo, we performed cell-mixing experiments and determined whether these cells could modulate normal type 1 responses after BMT. A titration experiment demonstrated that addition of IL-4-polarized type 2 cells to nonpolarized bm12 cells inhibited 90% of the production of IFN-γ to B6 alloantigen (Fig. 5*A*, closed symbols). As few as 5×10^4 type 2 cells were sufficient to block production of this cytokine by 5×10^5 nonpolarized alloreactive cells. In contrast, the proliferative response to B6 alloantigen was not affected by addition of type 2 cells (open symbols), indicating the functional specificity of this effect.

Based on these results, we performed BMT where 5×10^4 polarized type 2 CD4+ T cells were co-transplanted together with 5×10^5 naive CD4+ T cells (and TCD-BM), i.e., at a ratio of 1:10 of polarized vs naive T cells. We then analyzed whether the type 1 GVH reaction was modified in vivo by this treatment as described earlier. Figure 5*B* demonstrates that the Con A-stimulated production of IFN-γ by splenocytes harvested on day 13 post BMT was reduced by 90% in mice receiving this 1:10 cell mixture (white bar) compared with recipients of naive T cells alone (gray bar) or to recipients of a mixture of 5×10^4 cultured but nonpolarized cells and 5×10^5 naive T cells (black bar). This potent regulatory effect of the polarized type 2 cells, therefore, confirms their viability and functional capacity in vivo and demonstrates their active modulatory role during GVHD induction in this model.

Discussion

We have polarized in vitro alloreactive T cells toward a type 2 cytokine phenotype and demonstrated that both CD4+ and CD8+ donor T cells failed to mediate the induction of acute GVHD upon transplantation into irradiated hosts across both MHC class II or class I Ag differences, respectively. We assessed the development of GVHD in both BMT models on day 13 post BMT by several parameters both in vitro and in vivo. First, the production of IFN-γ in response to mitogen, a hallmark of the afferent phase of acute GVHD, was abrogated. Second, the secretion of the effector phase cytokine TNF-α in response to LPS both in vitro and in vivo was reduced to levels of TCD-BM controls. Third, the down-regulation of these effector events correlated with a significant protection from lethality after in vivo challenge with LPS. Fourth, cell mixing experiments confirmed the viability and functional specificity of polarized type 2 cells in vivo and demonstrated their capacity to modulate type 1 responses after the induction of GVHD.

Recent studies have shown that the development of acute GVHD in a nonirradiated P→F1 murine model could be prevented by injecting recipients with a Th2 cell-enriched CD4+ population (30). We have confirmed these results and extended them in three important areas. First, we have shown that GVHD mediated by CD8+ T cells can also be prevented effectively using our technique. We demonstrated that the same in vitro protocol used for polarization of CD4+ T cells (bm12 anti-B6) was able to skew the cytokine profile of CD8+ T cells to a type 2 phenotype in a strain combination driven by MHC I alloantigens (bm1 anti-B6). Importantly, the amount of IL-4 produced by CD8+ T cells in response to a single MHC class I difference was about 10-fold less than that produced by the same number of CD4+ T cells in response to a single MHC II difference. This unusual aspect of type 2 CD8+ T cells has been demonstrated recently in other systems (44). A large majority (85%) of bm1 responders were CD8+ after primary cultures, but because unseparated cell populations were used for the secondary MLC, contributions of other cell types to the production of IL-4, such as CD4+ T cells responding to indirect presentation of MHC class I Ags (48), cannot be ruled out completely. It should be noted, however, that earlier reports have demonstrated that allorecognition in the bm1→B6 system depends exclusively on CD8+ cells (31).

Upon transplantation of these cultured type 2 T cells into irradiated B6 mice, the initial T cell response remained polarized toward a type 2 profile in both CD4+ and CD8+ T cell-mediated GVHD on day 13 post BMT. The resultant abrogation of IFN-γ production by these T cells influenced the effector phase of GVHD in both BMT

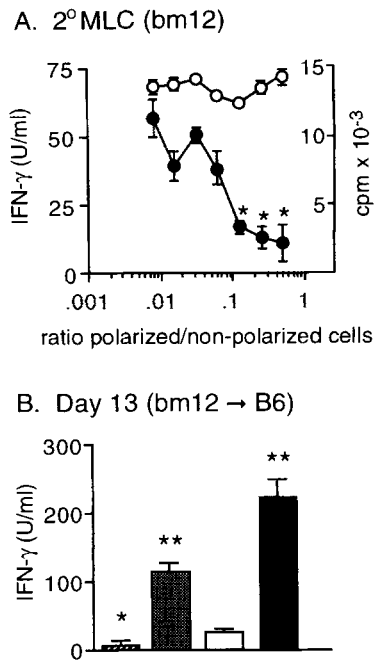

FIGURE 5. Regulation of T cell responses in vitro and in vivo by IL-4 polarized T cells. *A*, Proliferation and IFN-γ production to alloantigen in a secondary MLC. Nylon-wool purified bm12 $CD4^+$ splenocytes were cultured with B6 alloantigens in a primary MLC, washed, and then restimulated (10^5 $CD4^+$ cells/well) with B6 stimulator cells (2×10^5 cells/well) in a secondary MLC as described in *Materials and Methods*. At the initiation of these secondary cultures, bm12 T cells previously cultured with B6 stimulators in the presence of murine rIL-4 (10^3 U/ml) were added in graded numbers ($0.8–50 \times 10^3$ cells/well), creating ratios of 0.008 to 0.5 of IL-4-polarized to nonpolarized cells). The concentrations of IFN-γ (●) were determined in tissue culture supernatants taken after 48 h, and the proliferative response was assessed after 72 h by [^{3}H]thymidine incorporation for the last 20 h (○). Data are expressed as mean ± SEM. Statistical analysis was performed using the two-tailed Student's *t*-test. The indicated levels of IFN-γ were significantly different ($*p < 0.05$) when compared to levels obtained without polarized type 2 cells added to culture. *B*, Co-injection of type 2 T cells and naive T cells prevents IFN-γ production during GVHD. B6 recipient mice were transplanted with TCD-BM alone (▨), TCD-BM containing 5×10^5 naive bm12 $CD4^+$ T cells alone (■), or TCD-BM containing 5×10^5 naive cells with additions of either 5×10^4 IL-4 polarized type 2 $CD4^+$ cells (□) or the same number of cultured, but nonpolarized $CD4^+$ cells (■). Splenocytes from transplanted B6 mice were removed on day 13 post BMT, cultured for 48 h in the presence of Con A, and tested for IFN-γ levels (U/ml) in tissue culture supernatants as in Figure 2. Statistical analysis was performed using the two-tailed Student's *t*-test. The indicated groups were significantly different ($*p < 0.01$, $**p < 0.001$) from groups receiving polarized type 2 T cells.

models, where we demonstrated a dramatic decrease in the production of the proinflammatory cytokine TNF-α both in vitro and in vivo (Fig. 3 and Table II).

The ability of rIL-4 to affect both T cell subsets is significant because $CD4^+$ and $CD8^+$ T cell subsets each are capable of causing GVHD (49, 50). GVHD directed at MHC class I and minor histocompatibility Ag differences is largely independent of $CD4^+$ T cells (51). By contrast, fully MHC-mismatched GVHD is largely dependent on $CD4^+$ T cells, which play an essential role in regulating $CD8^+$ alloreactive T cells (52). Recent data from another experimental BMT system demonstrated that administration of IL-2 can inhibit GVHD to full MHC mismatches (53). Although the mechanisms for this observation are still under investigation, it appears that exogenous IL-2 down-regulates the early production of IFN-γ by $CD4^+$ T cells after BMT (54) in a similar way as polarized type 2 T cells. However, IL-2 could prevent mortality only when GVHD was mediated by $CD4^+$ T cells (55), whereas our approach allows for the inhibition of $CD8^+$-mediated GVHD as well. These findings have important implications for clinical allogeneic BMT, because BMT across single HLA differences is undertaken frequently (56) due to the limited availability of HLA-matched siblings. Because the risk of significant GVHD increases with greater HLA disparity between donor and host, the ability to polarize both $CD4^+$ and $CD8^+$ T cells is of particular interest.

A second important aspect of these studies is the demonstration that polarized type 2 T cells do not mediate GVHD after BMT even when the hosts have been conditioned with TBI. TBI is often used as part of the conditioning regimen in clinical BMT, and it is an important variable in the pathogenesis of acute GVHD because it causes the release of the inflammatory cytokines TNF-α and IL-1 from host tissues (57). The cytokine milieu in which donor T cells encounter host alloantigens clearly can affect the development of T cell cytokine profiles. The presence of inflammatory cytokines during this phase may direct the T cell response toward a type 1 cytokine phenotype. Our data demonstrate that despite host conditioning with TBI, polarization of donor T cells ex-vivo with rIL-4 could prevent the type 1 cytokine response of those cells as assessed on day 13 after allogeneic BMT.

Third, we have simplified and streamlined the polarization of T cell responses by performing the protocol in vitro. Incubation of T cells from bm12 or bm1 mice with 10^3 U/ml murine rIL-4 ex-vivo during the initial culture with MHC class II or class I alloantigens was sufficient to polarize these T cells toward a type 2 cytokine phenotype. In the study by Fowler and co-workers (30), the parental donor cells were generated by in vivo treatment of normal B6 mice 3 times a day over 5 days with a combination of human rIL-2 and murine rIL-4. This complicated protocol would have potential difficulties in the clinical setting because of the risks of cytokine injections in healthy donors and because of logistical considerations. These potential variations would be effectively eliminated with an in vitro protocol. In this regard, recent experiments have shown that in vivo administration of the type 2 cytokines IL-4 or IL-10 after BMT were not sufficient in reducing the systemic effects of acute GVHD (40, 58). Finally, the generation of type 2 T cells in vitro permits

additional flexibility in terms of cell mixtures. Our results clearly demonstrate that polarized type 2 T cells can exert a regulatory role on T cells activated by host alloantigens (Fig. 5) and may offer new approaches to the prevention and treatment of acute GVHD.

This approach to the prevention of GVHD has attractive features, but several issues have to be addressed. First, the protocol needs to be tested for its ability to preserve a graft-vs-leukemia effect after transplantation of polarized type 2 T cells. Clinical allogeneic BMT trials have shown that the advantages of T cell depletion of donor bone marrow for the prevention of acute GVHD are usually offset by an increase in graft failure and leukemic relapse (59, 60). Future studies must determine whether anti-leukemic efficacy can be maintained after injection of type 2 donor T cells. In this respect, recent data demonstrating that a subset of IL-4-producing $CD8^+$ T cells could maintain its cytotoxic activity are very encouraging (61). Furthermore, the effects of type 2 T cells on engraftment must be evaluated. A recent paper by Fowler et al. (62) suggested that graft failure could be avoided if cells were given within an appropriate time window. Additional experiments will be needed to determine whether the effector mechanisms of acute GVHD can be controlled significantly by our approach while preserving the beneficial effects of allogeneic BMT.

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