Discussion

FTY720 is a promising pharmacological agent to prolong allograft survival in solid organ transplantation [19, 20, 28–30]. Kim and colleagues [21] showed that the long-term administration of FTY720 (1–3 mg/kg) between days 0 and 29 markedly reduces GVHD in mouse models of haploidentical BMT. In contrast, other studies failed to demonstrate any such suppressive effect when the treatment of FTY720 started on either day 2 post-BMT or after the onset of clinical GVHD [25, 31]. We herein showed that the brief administration of FTY720 (0.3 mg/kg) on days 0–10 significantly reduced GVHD in MHC-mismatched experimental BMT. Taken together, FTY720 appears to be more effective in preventing rather than in treating GVHD when administered immediately after BMT.

The chief mechanism by which FTY720 exerts immunosuppression has been assumed to be altered lymphocyte trafficking. However, this does not seem to be the sole mechanism for the immunosuppression by FTY720 because the cessation of FTY720 did not result in an exacerbation of GVHD in either our current or a previous study [21], despite the fact that the inhibition of lymphocyte egression was reversible by the cessation of FTY720 administration [18, 32].

We herein demonstrate a novel mechanism by which FTY720 inhibits allogeneic T cell responses: FTY720 blunts the allogeneic T cell responses by the induction of apoptosis of alloreactive T cells in LN in addition to a well-described mechanism; namely, the inhibition of donor T cell migration to target organs. Our results indicate that the former mechanism alone may play a significant role in the reduction of GVHD. First, the symptoms of GVHD were not exacerbated after the discontinuation of FTY720 on day 10. In contrast, the inhibition of GVHD became apparent after the discontinuation of FTY720 as shown in Fig. 1E. Second, adoptive transfer experiments have demonstrated the alloreactivity of donor T cells to be already impaired in the LN of FTY720-treated recipients, thus resulting in the reduced GVHD in secondary recipients. The apoptosis of alloreactive T cells is involved in the mechanisms of FTY720-mediated inhibition of donor T cells alloreactivity, since such inhibitory effects of FTY720 were abolished when a pan-caspase inhibitor was given to the recipients.

The sphingosine-1-phosphatase/S1P1 axis is important for controlling the events of T cell entry and egress from the thymus and SLO [33–36]. The activation-induced down-regulation of S1P1 leads to a transient retention of T cells in SLO, leading to a further activation and proliferation of T cells [33, 34]. FTY720 induces a prolonged down-regulation of S1P1, resulting in a prolonged sequestration of activated effector T cells within SLO. We showed that FTY720 allowed the initial activation of alloreactive donor T cells in SLO after allogeneic BMT. In fact, FTY720 did not affect the kinetics of disappearance of host DC that are essential for activation of host-reactive T cells [1, 37]. However, several days after BMT, numbers of donor T cells decreased in association with the enhanced apoptosis in FTY720-treated mice.

Since the majority of donor T cells early after allogeneic BMT have been shown to be host-reactive T cells [38], the reduction of whole donor T cells is likely due to enhanced apoptosis of host-reactive T cells. Furthermore, we confirmed that apoptosis was only observed in dividing cells in LN after allogeneic BMT and that FTY720 enhanced apoptosis of solely dividing cells, suggesting that FTY720 selectively enhances the deletion of host-reactive T cells. Using TCR-Tg mice, we also confirmed that FTY720 enhanced the apoptosis of clonal host-reactive T cells. Caspase-dependent apoptosis was involved in this mechanism because FTY720-induced protection was abrogated when a pan-caspase inhibitor was given. As a consequence, the residual T cells that responded to CD3 stimulation promptly survived, thus also indicating the selective elimination of alloreactive T cells within LN. The low alloresponse by T cells from FTY720-treated mice was not overcome by the addition of exogenous IL-2 into the culture, thus ruling out the possibility that host-reactive T cells become anergized.

There are several possibilities to explain why administration of FTY720 enhances apoptosis of activated T cells in SLO. It is possible that FTY720 may enhance apoptosis of activated T cells due to the inhibition of egress from LN, or stimulation of T cells or APC to secrete IL-2, IL-12, or IL-18 that augment AICD of alloreactive T cells [7, 9, 39–41]. In contrast, accumulation of donor T cells in overcrowded LN without enough growth factors may also induce apoptosis [12, 42]. The precise mechanism by which FTY720 enhances donor T cell apoptosis will be explored in future studies.

A similar contraction of antigen-specific T cells in LN by the administration of FTY720 has been shown in a local antigen challenge mouse model [24]. However, our results are contrary to those of a previous report in which FTY720 sequestered alloreactive T cells in draining LN without any augmentation of apoptosis in a murine skin transplantation model [43]. AICD in particular pertains to the recipients of intensified conditioning, which induces a greater T cell activation and therefore, the intensified conditioning used in our study may facilitate AICD [44].

The selective elimination of antigen-specific T cells represents a promising strategy to maintain T cell responses against pathogens and tumor-specific antigens. The induction of AICD in alloreactive T cells by

repetitive or prolonged stimulation with alloantigens may enable the selective elimination of host-reactive T cells while leaving other T cell specificities which recognize pathogens and tumors unaffected [45], although significant bystander cell death may occur in non-host-specific donor T cells [46]. The strategies for a selective elimination of alloantigen-specific T cells include a depletion of the alloreactive T cells based on CFSE dye dilution, based on CD25 and CD69 expression, by using agonistic mAb to Fas, or using the protease inhibitor bortezomib, after *in vitro* culture with host alloantigens [45, 47–50]. The administrations of Th1 or IFN-γ-inducing cytokines such as IL-2, IFN-γ, IL-12, and IL-18, as well as the administration of anti-CD28 mAb, anti-CD137 mAb, and anti-CD162 mAb also potentially enhance antigen-specific AICD [7, 9, 39–41, 51–54]. Visilizumab, a humanized anti-CD3 mAb which induces apoptosis selectively in activated T cells, has been tested in a phase II study [55].

The administration of FTY720 represents an alternative to induce antigen-specific hyporesponsiveness of alloreactive T cells *in vivo*. In clinical kidney transplantation, the adverse effects of FTY720 are mild [56]. Furthermore, the protective effects of FTY720 against renal toxicity caused by calcineurin inhibitors facilitate the co-administration of these drugs [57]. The finding that FTY720 could support the CXCR4-dependent BM homing of human $CD34^+$ progenitor cells may therefore be useful for engraftment after HSCT [58]. S1P1 is therefore considered to be an attractive molecular target for improving the outcome of allogeneic HSCT.

Materials and methods

Mice

Female B6 (H-2^b, CD45.2^+), B6D2F1 (H-$2^{b/d}$, CD45.2^+), and B6C3F1 (H-$2^{b/k}$, CD45.2^+) mice were purchased from Charles River Japan (Yokohama, Japan). B6-Ly5a (H-2^b, CD45.1^+) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). The MHC class I molecule H2-L^d-specific 2C TCR-Tg mice produced by Dr. Dennis Loh were kindly provided by Dr. Paul Martin at the Fred Hutchinson Cancer Research Center. Mice, between 8 and 16 wk of age, were maintained in a specific pathogen-free environment and received normal chow and hyper-chlorinated drinking water for the first 3 wk post-BMT. All experiments involving animals were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources at Okayama University.

BMT and adoptive transfer

The mice were transplanted according to a standard protocol described previously [59]. Briefly, the recipient mice received lethal total body irradiation (X-ray), split into two doses separated by 3 h to minimize gastrointestinal toxicity. The recipient mice were injected with 1×10^6 or 4×10^6 T cells plus 4×10^6 TCD BM cells from the donors. The purification of T cells from the spleens or MLN and T cell depletion of BM cells were performed using autoMACS (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. In adoptive transfer experiments, B6D2F1 mice were lethally irradiated and injected with 1×10^6 LN T cells harvested from the recipients of primary BMT 6 days after BMT together with 2×10^6 naive TCD BM cells. Donor cells were resuspended in 0.25 mL of HBSS (Invitrogen, Carlsbad, CA) and injected i.v. into recipients on day 0. The survival was monitored daily and the degree of systemic acute GVHD was assessed weekly by a scoring system incorporating five clinical parameters, weight loss, posture, activity, fur texture, and skin integrity, as described previously [60, 61].

Reagents

FTY720 (2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol hydrochloride) was synthesized and provided by Novartis Pharma AG (Basel, Switzerland). FTY720 was dissolved in sterile distilled water at a concentration of 24 μg/mL, and administered to recipients by daily oral gavage at a dose of 0.3 mg/kg from day 0 until day 10 after BMT. The control mice received distilled water only. ZVAD-fmk was purchased from Enzyme Systems products (Livemore, CA), dissolved in 100% DMSO, and diluted in 0.15 M saline at a concentration of 1 mg/mL. ZVAD-fmk was injected s.c. at a dose of 10 mg/kg per day from 24 h after BMT until day 5 [62].

CFSE labeling and analysis of *in vivo* proliferation of donor T cells

Purified donor T cells were resuspended in PBS at a concentration of 10^6 cells/mL and an equal volume of 2 μM CFSE (Molecular Probes Inc., Eugene, OR) was added in PBS. The cells were incubated at 37°C for 15 min and unbound CFSE was washed and quenched by the addition of an equal volume of 10% FBS/DMEM. These CFSE-labeled cells were combined with unlabeled TCD BM cells and then were infused into the recipient mice *via* the tail vein. MLN from recipients of each experimental group were harvested and combined 4 days after BMT, and T cell division was assessed as a dilution of CFSE in the donor T cells as determined by flow cytometry [2].

Flow cytometric analysis

mAb used were FITC-, PE-, or allophycocyanin-conjugated anti-mouse CD4, CD8, CD45.1, CD45.2, TCRβ, H-$2K^b$, H-$2K^d$, and H2-K^k (BD Bioscience, San Diego, CA). Anti-2C TCR (1B2) mAb were kindly provided by Dr. Kazuya Iwabuchi at Hokkaido University. The cells were stained and analyzed as previously described [63]. Dead cells were determined based on the positivity of DAPI (Molecular Probes Inc.) or 7-AAD (BD Bioscience). The cells were analyzed using a FACSCalibur or a FACSAria flow cytometer (BD Bioscience), and the data were analyzed using FlowJo software (Tree Star, San Carlos, CA). For analysis of the donor T cell apoptosis, the cells were first stained with CD45.1-PE, washed, and then stained with FITC-

or allophycocyanin-labeled Annexin-V (MBL Co. Ltd., Nagoya, Japan) and 7-AAD in the dark for 15 min at room temperature.

Cell cultures

MLN were removed from the recipients on the indicated days. The MLN from each experimental group were combined. The numbers of cells were normalized for T cells and cells were cultured in completed DMEM in 96-well plates, at a concentration of 1×10^5 T cells/well with 1×10^5 irradiated (20 Gy) peritoneal cells harvested from naive B6D2F1 (allogeneic) animals or at a concentration of 1.5×10^4 T cells/well with 5 μg/mL of plate-bound anti-CD3ε mAb (BD Pharmingen) and 2 μg/mL of anti-CD28 mAb (BD Pharmingen). Recombinant human IL-2 (PeproTec, Rocky Hill, NJ) at a concentration of 10 U/mL was added to MLR. Forty-eight hours after the initiation of the culture, the cells were pulsed with [^{3}H]thymidine (1 μCi per well; Amersham Biosciences, Piscataway, NJ) and the proliferation was determined 20 h later on a TOPCOUNT Microplate Scintillator (Packard Instrument, Meriden, CT).

ELISA

ELISA was performed according to the manufacturer's protocol to measure the IFN-γ (BD Bioscience) and TNF-α levels (R&D Systems, Minneapolis, MN) levels, as described previously [63]. Samples were obtained by retro-orbital plexus bleeding, diluted appropriately, and run in duplicate. The plates were read at 450 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA). The sensitivity of the assays was 156.3 pg/mL for IFN-γ and 23.4 pg/mL for TNF-α.

Statistical analysis

The Mann–Whitney U-test was applied for the analysis of the cytokine data and clinical scores. We used the Kaplan–Meier product limit method to obtain the survival curves and the log-rank test was applied for comparing the survival curves. We defined $p<0.05$ as statistically significant.

Acknowledgements: This study was supported by research funds from the Ministry of Education, Culture, Sports, Science and Technology, Grant 17390280 (T.T.), the Health and Labor Science Research Grants for Clinical Research for Evidence Based Medicine (T.T.), and Advanced Clinical Research Organization (T.T.). We thank Novartis Pharma Co. Ltd. for providing the FTY720.

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EXPERIMENTAL HEMATOLOGY

Experimental Hematology 35 (2007) 274–286

Lymphopenia-induced proliferation of donor T cells reduces their capacity for causing acute graft-versus-host disease

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(Received 17 August 2006; revised 16 October 2006; accepted 17 October 2006)

Objective. **T cells that undergo lymphopenia-induced proliferation (LIP) are characterized by greater effector and anti-tumor function than naïve T cells. But the ability of these T cells in causing graft-versus-host disease (GVHD) is not known.**

Methods. **We tested the hypothesis that donor T cells that had undergone LIP would cause more severe GVHD than naïve T cells by utilizing well-characterized murine experimental models of allogeneic bone marrow transplantation (BMT).**

Results. **Contrary to our hypothesis, LIP of donor T cells under either noninflammatory or irradiated conditions caused significantly reduced GVHD as determined by survival, clinical, pathologic, and biochemical parameters than naïve T cells. Compared to naïve donor T cells, LIP T cells demonstrated reduced expansion in vivo and in vitro after allogeneic BMT. The reduction in GVHD mortality and severity was observed across multiple strains after allogeneic BMT. In vivo mechanistic studies by cell depletion demonstrated an increase in the $CD44^{hi}$ "memory" phenotype T cells and not the $CD4^{+}CD25^{+}$ T cell subset to be critical for the reduction in GVHD.**

Conclusions. **These data demonstrate that LIP of T cells regulates acute GVHD severity in contrast to their ability to cause increased allograft rejection, autoimmunity, or anti-tumor immunity.**

Despite thymic atrophy with increasing age and the ability of the antigen-specific lymphocytes to rapidly expand and contract following an immune response [1,2], the numbers of T cells in the periphery are kept fairly constant through the lifespan of most humans and mice [3]. Lymphopenia-induced proliferation (LIP) is among the key peripheral mechanisms for the maintenance of T-cell homeostasis [4,5]. LIP refers to the process by which, in a lymphodeficient host, T cells proliferate in response to self peptide – self MHC and nonself peptides (such as normal flora in the gastrointestinal [GI] tract–gut commensals) – self MHC complexes with the help of cytokines [6–11]. Homeostatic pressures during LIP cause changes in T-cell differentiation and function. LIP T cells acquire memory phenotype, display enhanced effector functions (cytokine secretion and cytolysis), and have an altered repertoire compared to that of naïve T cells [6,7,9,10,12–14]. The phenotypic and functional changes during LIP have recently been demonstrated to have in vivo functional consequences. Specifically, LIP of T cells can induce greater anti-tumor immunity [15–19] and also trigger autoimmunity [20,21]. Furthermore, in a murine solid organ allograft model, it has been recently demonstrated that partial T-cell depletion during peritransplant period caused LIP of the residual nondepleted lymphocytes that resulted in prevention of tolerance to the allograft [14,22–24]. Together all of these data suggest that LIP induces changes in the differentiation and function of T cells that promote their in vivo reactivity in auto- and allo-immune responses.

The utility of allogeneic bone marrow transplantation (BMT), a potentially curative therapy for a number of

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0301-472X/07 $–see front matter.
doi: 10.1016/j.exphem.2006.10.010

hematological malignant and nonmalignant diseases [25], has unfortunately been limited by its major complication, graft-versus-host disease (GVHD) [25]. Donor T-cell recognition of host allo-antigens on either host or donor antigen preventing cells is critical for induction and maintenance of GVHD [26]. The phenotype and repertoire of the donor T-cell subsets prior to BMT is therefore crucial for the severity of GVHD [26].

Prior to BMT, donor T cells are subject to transient LIP within the donors as a consequence of natural lymphopenia in the very young [4,27] and old [28,29] or secondary to infections [1,2], deficiency in dietary minerals such as zinc [30], or medications [31–33,5,34,35]. In addition, donor T cells also encounter a lymphopenic environment in the host (from conditioning with chemo-radiotherapy) after BMT [11]. But the importance of LIP of donor T cells in the induction and severity of GVHD is not known. Given that LIP of T cells increased the T-cell anti-tumor [36], autoimmune [20], and allo-rejection [24] reactivity, we tested the hypothesis that LIP of donor T cells would aggravate GVHD severity and surprisingly found that LIP reduced their capacity for induction of acute GVHD.

Materials and methods

Induction of lymphopenia-induced proliferation and BMT

Female C57BL/6 (B6, H-2^b), B6.Ly-5a (H-2^b, CD45.1^+), BALB/c (H-2^d), B6.Ly-5.1 (H-2^b, CD45.2^+), B6.C-$H2^{bm12}$ (bm12), and C57BL/6 SCID (B6 H-2^b -SCID) mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). LIP was induced as follows; B6Ly-5.2 splenocytes were FACS (Vantage SE cell sorter, Becton-Dickinson, San Jose, CA, USA) sorted $CD3^+CD62L^+$ and $CD3^+44^{hi}$ $62L^+$ were separated by nylon wool or on automacs. A total of 2×10^6 of these T cells were intravenously injected into syngeneic nonirradiated B6-SCID or irradiated (11 Gy) B6 recipients to induce LIP. Fourteen to 17 days later, splenic T cells that had undergone LIP were harvested from these mice and used as donor T cells in BMT. Recipient BALB/c mice were irradiated with 8 to 9 Gy total-body irradiation (TBI) and injected with 1×10^6 LIP T cells from [B6→B6] or [B6→SCID] and naïve B6 donors along with 5×10^6 T cell–depleted (TCD) B6 BM cells from naïve B6 donors [37]. For cell depletion and/or enrichment studies, $CD4^+25^+$ T cells were depleted by automacs (purity of 85 to 90%) and for some $CD44^{hi}64L^-$ and $CD44^{low}62L^+$ T cells were fractionated by FACS sorter (95 to 99% purity). Mice were housed in sterilized microisolator cages and received normal chow and autoclaved hyperchlorinated water for the first 3 weeks after BMT and filtered water thereafter. Survival was monitored daily, clinical GVHD was assessed weekly, and detailed histopathologic analyses of liver and intestine were performed as described previously [38]. All animal studies were performed per the institutional IACUC guidelines.

Flow cytometric analysis

Flow cytometric analysis was performed using FITC-, PE-, PE-Cy5, or allophycocyanin-conjugated monoclonal antibody (mAbs) to mouse annexin, CFSE, CD3, CD4, CD8, CD45.1, CD44, CD25, CD122, and $H2D^d$ and *CD45.1 were obtained from* BD Pharmingen (San Jose, CA, USA). Cells were stained, analyzed, and/or sorted on a FACS Vantage SE cell sorter as described before (Becton-Dickinson, San Jose, CA, USA) [37].

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISAs) for TNF-α (BD Pharmingen) and Limulus Amebocyte Lysate (LAL) assay (Bio Whittaker, Walkersville, MD, USA) for lipopolysaccharide (LPS) were performed as described previously and per manufacturer's protocol [38].

In vitro proliferation assay

In vitro MLR assays were performed as described before [39]. Briefly, B6 naïve or B6→B6 LIP T cells were magnetically separated from the spleen by AutoMACS using CD90 microbeads and 2×10^5 of these were incubated with irradiated (^{137}Cs source) BALB/c peritoneal exudative cells (1×10^5) for 72 hours. Incorporation of ^3H-thymidine (1 μCi/well) by proliferating cells was measured during the last 6 hours of culture.

Cell-mediated cytotoxicity assay

Spleen cells (3×10^7) from naïve B6 or *[B6→B6]* mice were cultured with irradiated (^{137}Cs source) BALB/c spleen cells (2×10^7) at 37°C in a 5% CO_2 atmosphere for 7 days and used as effector cells after making $CD8^+$ T cell number the same. B6 or BALB/c spleen cells (2×10^7) were cultured with Con A (Sigma) at a concentration of 5 μg/mL for 3 days to generate Con A blasts [39]. P815 tumor cells and the Con A blasts were labeled by incubating 2×10^6 cells with 2 MBq of $Na_2^{51}CrO_4$ (PerkinElmer Life, Boston, MA, USA) for 1.5 hours at 37°C in a 5% CO_2 atmosphere and used as target cells. After washing, 2.5×10^3 labeled targets were resuspended and the ^{51}Cr-release assay was performed as described [39]. These preparations were added to triplicate wells at varying effector-to-target ratios and incubated for 4 hours. Maximal and background release was determined by the addition of Triton-X 100 (Sigma) or media alone to targets, respectively. ^{51}Cr activity in supernatants taken 4 hours later was determined in an autogamma counter (Packard, Meridian, CT, USA) as described [39].

Histology

Formalin-preserved small and large bowel were embedded in paraffin, cut into 5-μm-thick sections, and stained with hematoxylin and eosin for histologic examination. Slides were coded without reference to prior treatment and examined in a blinded fashion by a pathologist (C. Liu). A semiquantitative scoring system was used to assess the following abnormalities known to be associated with GVHD [40]. Briefly, for small intestine: villous blunting, crypt regeneration, loss of enterocyte brush border, luminal sloughing of cellular debris, crypt cell apoptosis, crypt destruction, and lamina propria lymphocytic infiltrate; for colon: crypt regeneration, surface colonocytes, colonocyte vacuolization, surface colonocyte attenuation, crypt cell apoptosis, crypt destruction, and lamina propria lymphocytic infiltrate. The scoring system denoted 0 as normal, 0.5 as focal and rare, 1.0 as focal and mild, 2.0 as diffuse and mild, 3.0 as diffuse and moderate, and 4.0 as diffuse and severe. Scores were added to provide a total score for each specimen. After scoring the codes were broken and data compiled.

In vitro analysis of donor T-cell expansion and apoptosis

Spleens from naïve and from [B6 Ly5.2→B6] animals 2 weeks after BMT were harvested and enriched for T cells by autoMACS. Aliquots of enriched T cells were stained with PE-anti-CD45.1 mAb and APC-CD3e mAb (BD Bioscience) and checked for T-cell purity, which was greater than 90%. The cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) and analyzed for expansion and also for apoptosis of the divided cells by annexin staining [40]. Briefly, T cells were washed and resuspended at 5×10^6 cells/mL concentration in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA). A 5-mM CFSE (Molecular Probes, Inc, Eugene, OR, USA) in dimethylsulfoxide (DMSO) was added to T cell suspension to a final concentration of 10 μM. Cells were gently mixed and incubated at 37°C for 10 minutes. The staining was quenched by the addition of 10% fetal calf serum (FCS) containing Dulbecco's modified Eagle's medium (DMEM), and the cells were then washed three times. Analysis of cells immediately following CFSE labeling indicated a labeling efficiency that exceeded 99%. These CFSE-labeled cells were then resuspended and cultivated with or without 1 μg/mL of anti-CD3e mAb (145-2C11, BD Biosciences) in 48-well plates. After 3 days, the cells were stained with PE-conjugated anti-annexin-V mAb (BD Biosciences) in the dark for 15 minutes at room temperature in labeling buffer, and analyzed by FACS-Vantage SE (BD Biosciences). Cell apoptosis was identified based on staining for annexin-V.

Statistical analysis

The Mann-Whitney *U*-test was used for the statistical analysis of in vitro data while the Wilcoxon rank test was used to analyze survival data. $p < 0.05$ was considered statistically significant.

Results

GVHD induction by LIP T cells

Because LIP enhances T-cell effector function and causes greater anti-tumor immunity [36], triggers autoimmunity, and prevents induction of allograft tolerance [21,22,24], we explored the hypothesis that naïve donor T cells that have undergone LIP would cause more severe acute GVHD than naïve T cells. We injected 2×10^6 $CD3^+CD62L^+$ T cells from naïve B6 animals into lethally irradiated (11 Gy) B6 recipients to induce LIP for 2 weeks [6–9]. T cells were harvested from these [B6→B6] animals and used as donor T cells in a well-characterized allogeneic BMT model of GVHD, B6 ($H2^b$) → BALB/c ($H2^d$) [38]. BALB/c mice were lethally irradiated and transplanted with TCD BM from naïve B6 animals along with splenic T cells from either naïve B6 or from the [B6→ B6] animals after LIP as described in Materials and methods. Mice receiving syngeneic BMT and allogeneic recipients that received TCD BM alone showed 100% survival. Contrary to our hypothesis, allogeneic recipients that received LIP donor T cells showed significantly better survival (50% vs 5%, $p < 0.05$, Fig. 1A) and less severe clinical GVHD ($p < 0.05$, Fig. 1B) than those that received allogeneic naïve T cells. To rule out the confounding effects of the inflammatory milieu caused by irradiation on the function and generation of LIP of T cells, we also transferred B6 T cells into unirradiated, lymphopenic B6 SCID ($H2^b$) animals. T cells that had undergone LIP for 2 weeks in the [B6→SCID] donors also caused significantly less mortality (30% vs 90%, $p < 0.01$, Fig. 1A) and clinical GVHD than naïve B6 T cells ($p < 0.05$, Fig. 1B). It is possible that the reduced GVHD potential of the $CD3^+$ $CD62L^+$ T cells after LIP is due to the initial absence of $CD3^+$ $CD62L^-$ T cells during homeostatic expansion. To rule out this possibility we first transferred unfractionated B6 Ly5.2 $CD3^+$ splenic T cells (thus containing both $CD62L^-$ and $CD62L^+$ subsets) into lethally irradiated B6 animals. After 2 weeks of LIP, the infused unfractionated LIP $CD3^+$ T cells from these [B6→B6] animals or the unfractionated nonhomeostatically proliferated $CD3^+$ T cells from naïve B6 were injected along with B6 TCD BM into irradiated allogeneic BALB/c animals. Additionally, to rule any potential confounding effects on the T-cell function due to antibody-dependent cell purification, we also infused naïve splenic T cells from B6 animals following nylon wool separation into allogeneic BALB/c as controls. As shown in Figure 1C, the allogeneic animals that received unfractionated [B6→B6] $CD3^+$ T cells following LIP induced significantly less GVHD mortality than the allogeneic recipients of unfractionated FACS-sorted $CD3^+$ T cells or the nylon wool–separated T cells from naïve B6 animals (60% vs 0%, $p < 0.05$). Together these data demonstrate that LIP of donor $CD3^+CD62L^+$ T cells in noninflammatory, the unfractionated ($CD62L^+$ and $CD62L^-$) T cells, or $CD3^+CD62L^+$ T cells in irradiated inflammatory conditions reduces their ability to induce GVHD.

LIP T cells reduce GVHD target organ damage

We next evaluated the effect of LIP of donor T cells on the principal target organ of acute GVHD, the GI tract (small bowel and colon). Samples were taken from animals (n = 4/group) on day 7 after transplantation and scored in a coded fashion as described in Materials and methods. Animals that received T cells from [B6→SCID] donors demonstrated significantly less severe GI GVHD than the allogeneic controls as determined by a semi-quantitative GVHD-specific histopathologic changes (Fig. 2A,B, $p < 0.05$) [38].

We next determined the levels of TNF-α and LPS, the serum biochemical markers that correlate with the severity of intestinal and systemic acute GVHD [38,41,42]. As shown in Figure 2C and D, the serum levels of both LPS (5.4 ± 1.1 U/mL vs 12.4 ± 2.3 U/mL;) and TNF-α (6 ± 1 pg/mL vs 46 ± 11 pg/mL) in animals receiving allogeneic LIP T cells from [B6→SCID] donors were significantly lower compared with allogeneic recipients from B6 controls ($p < 0.05$). Thus, LIP of donor T cells in noninflammatory or irradiated-inflammatory lymphopenic

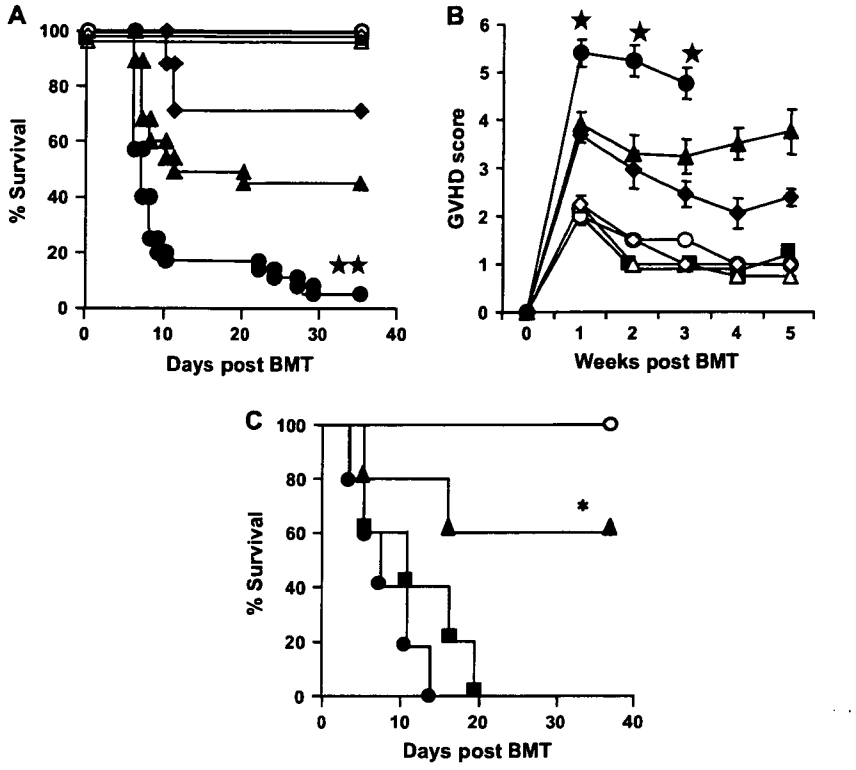

Figure 1. Allogeneic animals that received LIP of donor T cells reduced GVHD. Naïve B6 spleen $CD3^+$ T cells (2×10^6) were intravenously injected into nonirradiated syngeneic [B6→B6 SCID] or irradiated (11 Gy) B6 [B6→irrad. B6] recipients. Two weeks later, splenic T cells that had undergone LIP were harvested from these mice and used for BMT. BALB/c (closed symbols) or B6 (open symbols) were irradiated with 8 Gy TBI and injected with 5×10^6 TCD BM cells from B6 donors alone (■, n = 4) or with 1×10^6 LIP [B6→B6 SCID] (◆, n = 17; ◇, n = 12), [B6→irrad. B6] (▲, n = 35; △, n = 12) or naïve B6 T cells (•, n = 35; ○, n = 12) and evaluated as in Methods. **(A)** Survival: ▲ vs • $*p < 0.05$ and ◆ vs •, $**p < 0.01$. **(B)** Clinical GVHD score: ▲or ◆ vs •, $*p < 0.05$. Data from four similar experiments are combined. **(C)** Allogeneic BALB/c animals were injected with T cells from LIP [B6→B6] (▲, n = 5) or naïve B6 donors that were isolated either by Automacs (•, n = 5) or nylon wool (■, n = 5). The recipient animals were conditioned and transplanted and evaluated for survival as above. ▲ vs • or ■, $*p < 0.05$.

conditions, prior to allogeneic BMT, reduces their ability to induce GVHD as determined by survival, clinical, pathologic, and biochemical parameters.

Reduction in GVHD by LIP is due to expansion of "memory-like" T cells

To explore the mechanisms of the reduction in GVHD we performed phenotypic analyses of the splenic T cells from naïve B6 Ly5.2 animal and the LIP in the [B6→SCID] animals. As shown in Figure 3, the ratio of $CD4^+$ and $CD8^+$ and consistent with previous reports [6,7,9] T cells that have undergone LIP demonstrated the conversion of cells from naïve → memory phenotype for both $CD4^+$ and $CD8^+$ (Fig. 3A and B, respectively) as determined by the increased expression of "memory" markers such as $CD44^+$ and $CD122^+$.

We next determined whether the increase in "memory-like" T cells is critical for the reduction in GVHD induced by the LIP-T cells. We first evaluated the ability of naïve and "memory-like" T cells from B6 donors to induce GVHD without expansion in lymphopenic hosts. We injected 2×10^6 naïve $CD3^+CD44^{low}CD62L^+$ or the $CD3^+CD44^{hi}CD62L^-$ "memory-like" T cells without prior homeostatic expansion along with TCD BM from naïve B6 animals into lethally irradiated BALB/c recipients to induce GVHD. We found that 80% of the allogeneic animals that received $CD3^+CD44^{low}CD62L^+$ naïve T cells died with signs of GVHD. By contrast, consistent with recent data [43–45], the vast majority of the allogeneic animals that received $CD3^+CD44^{hi}CD62L^-$ "memory-like" T cells were alive at the end of the observation period with minimal signs of GVHD (Fig. 4A, 80% vs 20%, $p < 0.02$). All of the syngeneic B6 animals that received unfractionated $CD3^+CD44^{low}CD62L^+$ survived, thus ruling out nonallospecific toxicity. These data suggested that the conversion of naïve T cells into "memory-like" T cells by LIP might be critical for the reduction in the induction of GVHD.

To directly test this possibility, we FACS sorted the [B6→B6 SCID] T cells after and obtained cellular fractions enriched for the$CD44^{low}CD3^+$ and $CD44^{hi}CD3^+$ (memory) subsets. As shown in Figure 4B, 100% of the allogeneic animals injected with 1×10^6 $CD44^{low}CD3^+$

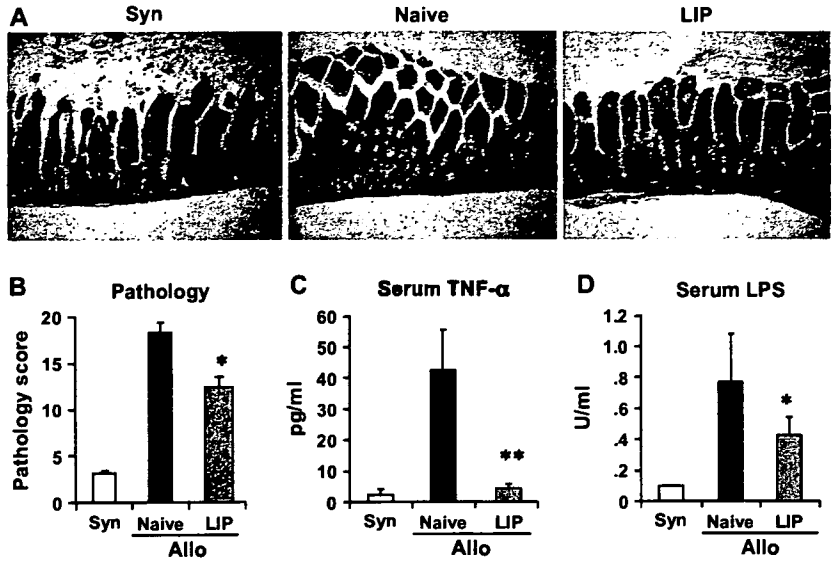

Figure 2. GVHD target organ (GI) damage. Syngeneic B6 (n = 5) and allogeneic BALB/c animals were transplanted with naïve B6 T cells (n = 6) or [B6→B6 SCID] LIP T cells (n = 4). On day 7 after BMT, gastrointestinal (GI) tract was analyzed. **(A)** Damage to syngeneic bowel was minimal (left), whereas naïve T cell recipients exhibited severe villous blunting, crypt destruction changes and atrophy, and increased lymphocytic infiltrates (middle). Small bowel of LIP T cell recipients showed significantly less damage. Original magnification: ×100. **(B)** Coded slides in A were scored for pathologic damage as described in Materials and methods. GI pathologic score of B6 (white bar) and BALB/c animals that were transplanted with naïve B6 T cells (black bar) or [B6→B6 SCID] LIP T cells (gray bar) are represented. Naïve T recipients (black bar) vs LIP T recipients (gray bar), $*p < 0.05$. Serum was obtained on day 7 after BMT and analyzed for TNF-α **(C)** and LPS **(D)**. Each graph represents one of three similar experiments. Naïve T recipients (black bar) vs LIP T recipients (gray bar), $*p < 0.05$; $**p < 0.02$.

T cells from B6→B6 SCID died with signs of GVHD. All of the syngeneic B6 animals were alive, while only 20% of the recipients of allogeneic T cells from naïve donors survived. By contrast, 70% of the allogeneic animals that received [B6→B6 SCID] T cells enriched for the $CD44^{hi}CD3^{+}$ "memory" fraction were alive at the end of the observation period, thus demonstrating that the reduction in GVHD mortality after LIP was due to the expansion of the $CD44^{hi}$ T cells ($p < 0.01$).

Effect of extended LIP of donor T cells on GVHD

We next determined whether prolonged LIP of donor T cells further reduced their ability to induce GVHD. We induced LIP of unfractionated B6 Ly5.2 $CD3^{+}$ T cells as above, but allowed the homeostatic expansion to occur for 9 weeks. $CD3^{+}$ T cells from these [B6→B6] and those from naïve B6 T cells were harvested and used as donor T cells along with B6 TCD BM into allogeneic BALB/c and syngeneic B6 animals. In contrast to the reduction in GVHD after 2 weeks of LIP, prolonged (9 weeks) homeostatic proliferation of donor T cells prior to allogeneic BMT failed to reduce the severity of GVHD mortality compared to T cells from a naïve donor (Fig. 5A, 30% vs 20%, p = NS). To determine the cause for this lack of benefit after prolonged LIP, we hypothesized that extended homeostatic proliferation might lead to the loss of the increase in the numbers of the "memory-like" T cells and thus show a phenotype of T cells that is similar to a normal B6 animal. As shown in Figure 5B, Table 1 consistent with the hypothesis we found that phenotype of both $CD4^{+}$ and $CD8^{+}$ T cells from the [B6→B6] animals after 9 weeks of LIP was not significantly different from the phenotype of $CD4^{+}$ and $CD8^{+}$ T cells from a naïve B6 animal as determined by the expression of CD25 and CD122. Together these data show that the conversion of the naïve T cells to "memory-like" phenotype after LIP is transient and that the loss of this conversion after extended LIP is associated with the reversal of their potential for reducing GVHD.

LIP T cells reduce GVHD in a strain- and $CD4^{+}CD25^{+}$ T-cell-independent manner

It is possible that the LIP T expanded memory T cells that do not cross react with a specific allo-antigen and the results are a result of strain-dependent artifact. To rule out this possibility we first tested the ability of LIP of donor $CD4^{+}$ cells to mediate GVHD after a MHC class II mismatched allogeneic BMT. Naïve T cells from wild-type B6 animals and LIP T cells from [B6→B6] donors were transferred into lethally irradiated MHC class II–mismatched B6.C-$H2^{bm12}$ (bm12) recipients. Consistent with the previous data, the mortality (Fig. 6A, 50% vs 100%, $p < 0.05$) and clinical severity (Fig. 6B) from $CD4^{+}$-mediated GVHD in class II–mismatched recipients was also significantly reduced by LIP of donor T cells, thus ruling out strain-dependent artifact. We also found reduced GVHD

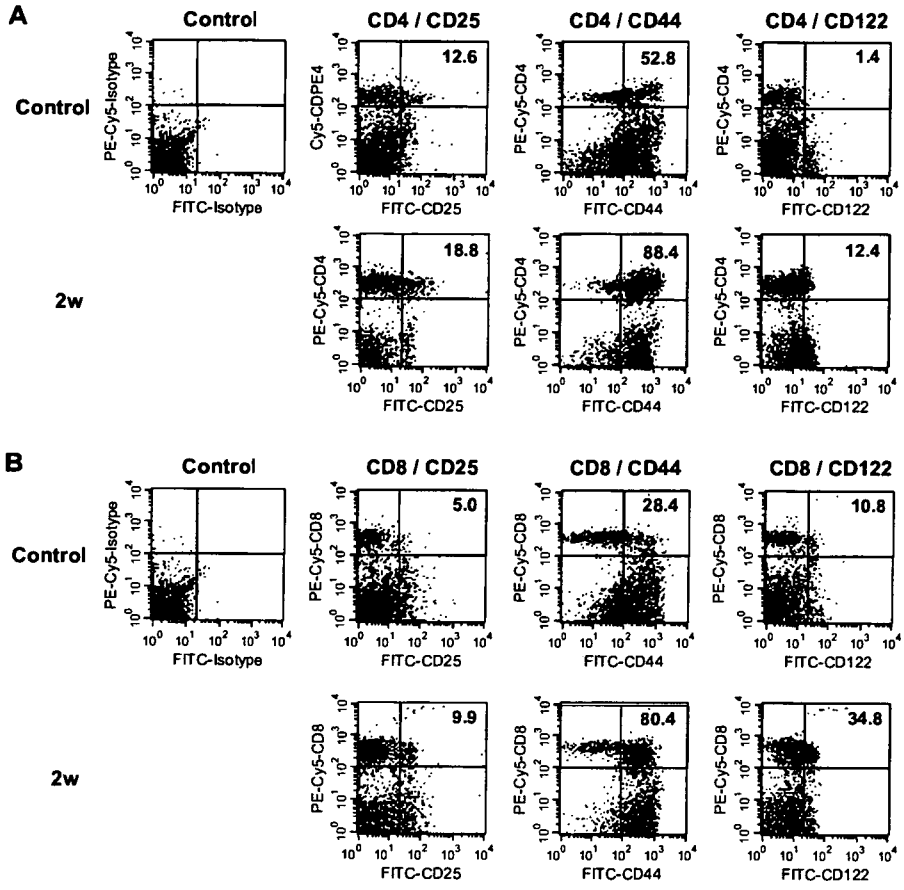

Figure 3. LIP of naïve T cells induces memory phenotype. Naïve B6 spleen $CD3^+$ T cells (2×10^6) were intravenously injected into nonirradiated syngeneic [B6→B6 SCID] recipients as in Figure 1. Two weeks later, splenic T cells that had undergone lymphopenia-induced proliferation were harvested from these mice and expression of CD25, CD44, and CD122 on $CD4^+$ T cell (**A**) and $CD8^+$ T cell (**B**) were determined by FACS analysis as described in Methods. Data are from one of three similar experiments.

after LIP of donor T cells in $CD8^+$-mediated GVHD after MHC-matched multiple miHA-mismatched barriers, B6→C3H.SW, allogeneic BMT (unpublished preliminary observations). Thus, taken together these data demonstrate that LIP of donor T cells reduces GVHD across multiple strain combinations.

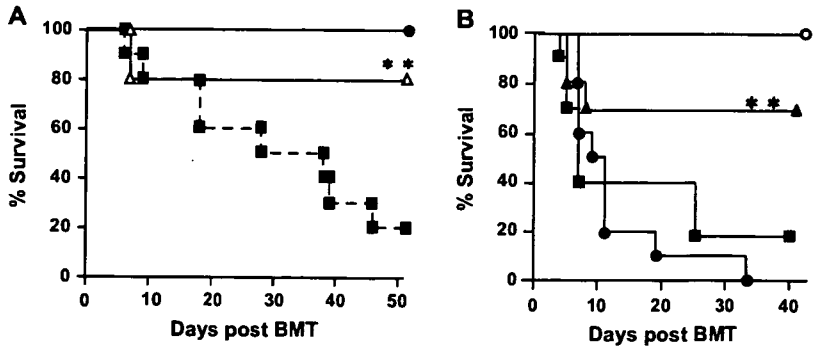

Figure 4. Reduction in GVHD is due to an increase in $CD44^{hi}$ "memory-like" T cells. (**A**) BALB/c animals were transplanted with enriched $CD44^{hi}62L^{low}$ "memory-like" $CD3^+$ T cells (Δ, n = 10) or the $CD44^{low}62L^{hi}$ naïve $CD3^+$ T cells (■, n = 10) from naïve B6 donors and evaluated for survival as in Figure 1. ■ vs Δ, $^{**}p < 0.02$. (**B**) T cells that had undergone LIP in [B6→B6 SCID] were enriched for $CD44^{low}CD3^+$ (•, n = 10; ○, n = 5) and $CD44^{high}$ $CD3^+$ (▲, n = 10) subsets. Splenic T cells enriched for $CD44^{low}CD3^+$ were obtained from naïve B6 animals (■, n = 10). These cells were transplanted along with BM from naïve B6 donors into either allogeneic BALB/c (closed symbols) or syngeneic B6 (open symbols). Data from two similar experiments are combined. ▲ vs • or ■, $^{**}p < 0.01$.

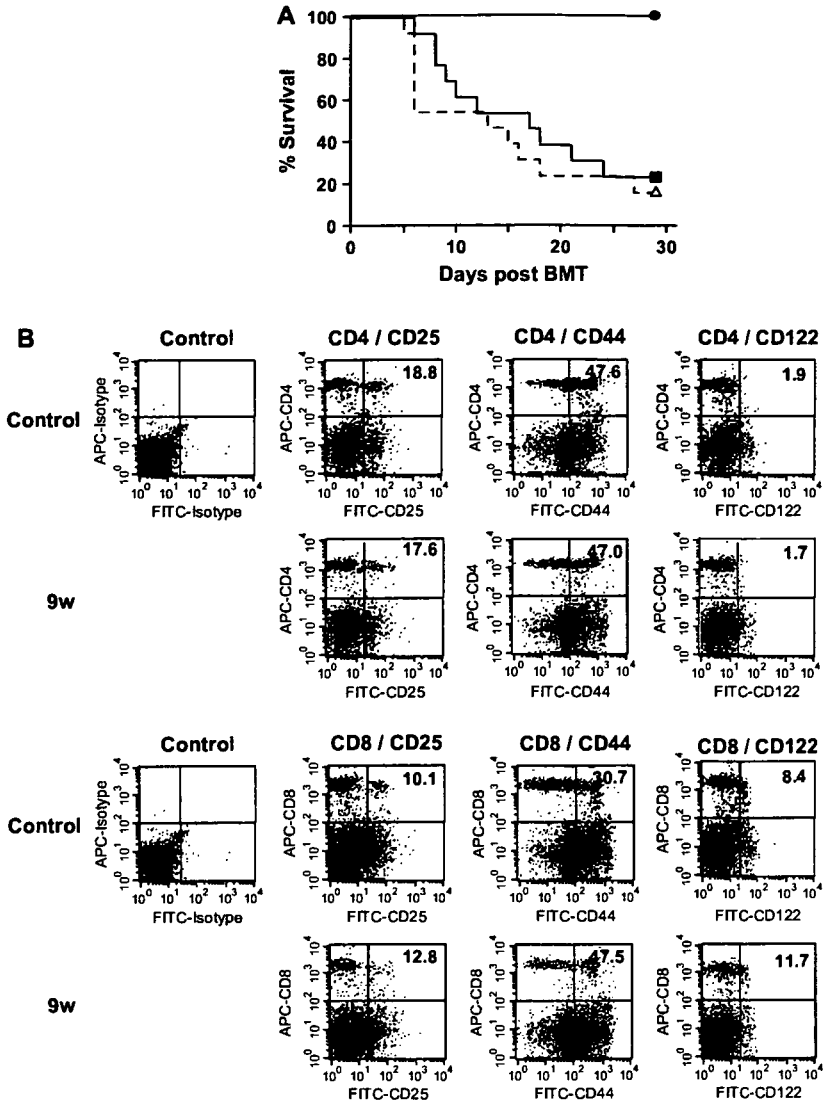

Figure 5. Effect of prolonged LIP on GVHD. **(A)** T cells from naïve donors (Δ, n = 10) or after LIP in the irradiated [B6→B6] donors for prolonged duration of 9 to 10 weeks (■, n = 10) were used to induce GVHD in BALB/c recipients and were monitored for survival as in Figure 1. ■ vs Δ, p = NS. **(B)** Naïve B6 spleen $CD3^+$ T cells (2×10^6) were intravenously injected into irradiated syngeneic [B6→B6] recipients as in Figure 1. Nine weeks later, splenic T cells that had undergone LIP were harvested and evaluated for the expression of CD25, CD44, and CD122 on $CD4^+$ and $CD8^+$ by FACS analysis as described in Methods. Data are from one of two individual experiments with three mice/group.

$CD4^+CD25^+$ regulatory T cells (Tregs) suppress GVHD [46,47]. Therefore we determined whether the reduced severity of GVHD mediated by the LIP of $CD44^{hi}$ T cells was due to expansion of $CD4^+CD25^+$ or due to an intrinsic property of the $CD44^{hi}$ "memory" T cells. Consistent with previous observations [6,9], we found a small but not significant increase in the percentage of $CD4^+$ expressing $CD25^+$ in the [B6→B6 SCID] and naïve B6 animals (Fig. 2). We next depleted $CD25^+$ cells from the [B6→B6 SCID] donor T cells [37] to determine the contribution of this cellular subset to the reduction of GVHD after LIP. Depletion of $CD25^+$ did not alter the ability of LIP T cells for causing a reduction in GVHD mortality compared to an equal number of $CD3^+$ T cells from a naïve B6 donor (40% vs 0% survival, $p < 0.05$, Fig. 7A). These data demonstrate that the reduction in GVHD caused by LIP of donor T cells is not because of the increased numbers of $CD4^+CD25^+$ T cells. Experiments are in progress to determine if LIP T cells express greater amounts of Foxp3, CTLA-4, and IL-10.

In vivo proliferation and function of LIP T cells

To further determine the mechanism for the reduced GVHD potential of LIP T cells, we next determined the amplitude of donor T cell expansion on day +7 after BMT. As shown

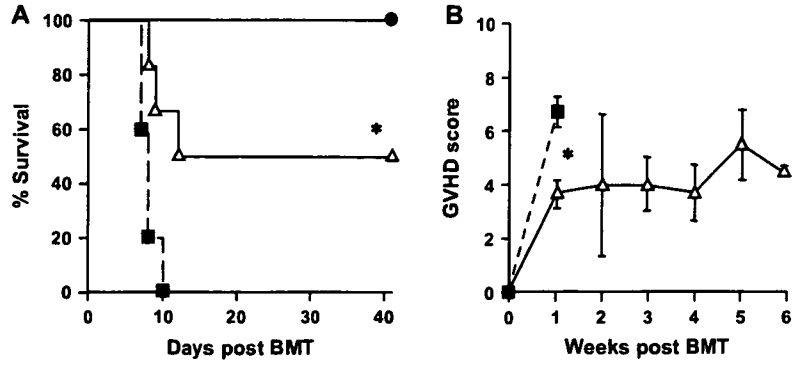

Figure 6. LIP of donor T cells reduces $CD4^+$-mediated GVHD. 3×10^6 TCD BM cells alone (•, n = 5) or along with naïve T cells (■, n = 5) from B6 animals or LIP [B6→B6] (△, n = 5) were injected intravenously and assessed for survival (**A**), clinical score (**B**). ■ vs △,*$p < 0.05$.

in Figure 7B, a significant reduction in the numbers of donor T cells, in both $CD4^+$ and $CD8^+$ subsets, was observed in the animals that received LIP T cells from [B6 Ly 5.2→B6] donors compared to the naïve T cells from control B6 Ly5.2 donor animals ($p < 0.01$). These data are consistent with previous reports that "memory-like" T cells from naïve donors show reduced expansion after allogeneic BMT [43,45]. To further analyze the mechanisms for reduction, we performed in vitro MLR assay to determine the allo-proliferative responses of the LIP T cells. T cells from wild-type B6 animals and from the [B6→B6] animals after 2 weeks of LIP were cultured with irradiated allogeneic BALB/c peritoneal cells as in Materials and methods. Consistent with reduced in vivo proliferation, LIP T cells showed significantly reduced proliferation (Fig. 7C) compared to T cells from naïve donors ($p < 0.01$). We next analyzed whether LIP of T cells modulated the allo-specific cytotoxicity of $CD8^+$ T cells. In contrast to the diminished proliferative responses, LIP T cells demonstrated equivalent CTL of two different allo-specific targets, BALB/c con A blasts and P815 tumor cells ($H2^d$), when compared with naïve B6 T cells (Fig. 7D).

We next analyzed whether in vivo and in vitro the reduction in LIP T cells compared to non-LIP T cells is due to enhanced proliferation and/or apoptosis. We FACS sorted $CD3^+$ cells from [B6Ly5.2→B6] following LIP for 2 weeks or from naïve B6 animals. The Automacs-separated cells were checked for T cell purity, stained with CFSE, and stimulated with anti-CD3 as in Materials and methods. As shown in Figure 8 and Table 2, LIP T cells showed a consistent trend towards fewer cell divisions compared to the control T cells. To determine the extent of activation-induced cell death (AICD), we next stained for annexin on the proliferating cellular fraction of the LIP and control T cells. As shown in Figure 8 and Table 2, LIP T cells showed a significantly greater AICD compared to the naïve T cells ($p < 0.05$). Together these data show that LIP of T cells show greater AICD following stimulation but their allo-specific CTL responses are preserved.

Discussion

Together our data demonstrate that LIP of donor T cells reduces their ability to cause GVHD in a strain- and $CD4^+CD25^+$ T cell–independent manner because of an increase in the numbers of $CD44^{hi}$ "memory"-type T cells. We also found that the reduced GVHD potential of the LIP T cells is lost after prolonged LIP and this correlated with the transient nature of the increase in the numbers of $CD44^{hi}$ "memory"-type T cells with LIP. These results are consistent with several recent observations that "memory"-type $CD44^{hi}$ donor T cells do not cause GVHD [43–45], LIP is not associated with an expansion of the $CD25^+$ T cell fraction [6,9], and the increase in "memory-like" phenotype after LIP is a transient phenomenon [6,48,49]. However, it is possible that prolonged LIP caused T cells to revert back to naïve cells not as a direct consequence of LIP but due to the de novo naive T-cell development from the infused hematopoietic stem cells in the thymus of irradiated B6 donors [50,51]. It also remains unknown whether LIP generates non-$CD4^+CD25^+$ T cells that are capable of regulating naïve T-cell responses. Future studies will explore these aspects by utilizing thymectomized LIP donors. In any event, our data clearly demonstrate that short-term expanded LIP T cells reduce GVHD. Recent data suggest that LIP can be either fast or slow [10,11,52]. Our data show that both slow (as in the acutely T-deficient irradiated [B6→B6] donors) and rapid (in genetically lymphopenic hosts, as in [B6→B6 SCID]) LIP of donor T cells (Fig. 1A) reduces their capacity for inducing GVHD.

The antigens to which the LIP generates a memory response in the donor prior to BMT are not known, and it is important to distinguish these "memory" cells from the bona fide allo-specific "memory" cells that are generated in the host after BMT [53] because there are differences between phenotypic alterations and survival between these cellular subsets [6,7,9]. Moreover, basal proliferation of the T cells, i.e., the non–antigen-specific proliferative responses of the mature T cells in a nonlymphopenic (full) environment, also plays a role in the regulation of

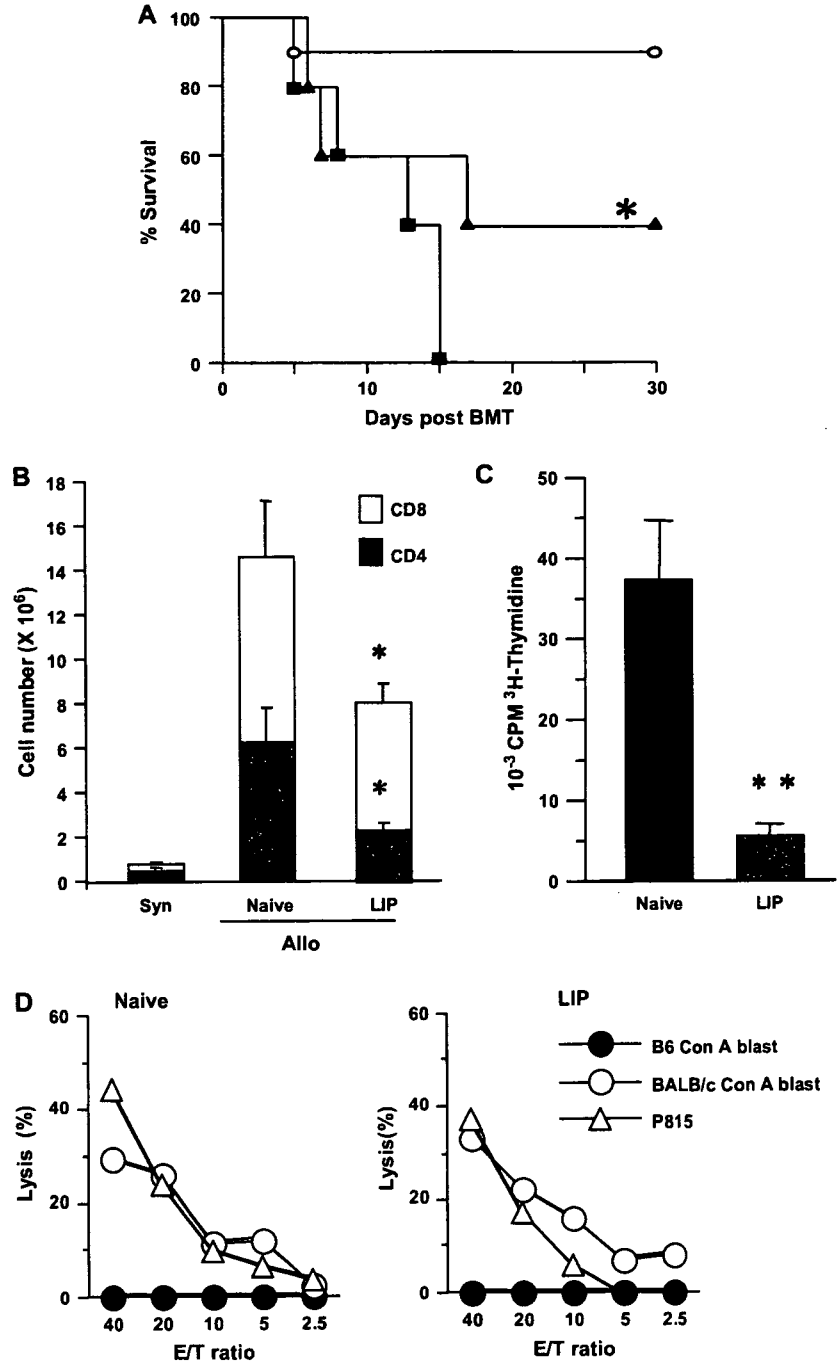

Figure 7. (A) $CD4^+CD25^+$ T cells are not critical for the reduction in GVHD. Syngeneic B6 (○, n = 5) or allogeneic BALB/c (▲, n = 5) were transplanted with $CD3^+$ T cells that had undergone LIP in [B6→B6 SCID] and were then depleted of $CD25^+$ T cells ($CD3^+CD25^-$). Some of the allogeneic BALB/c recipients (■, n = 5) received $CD3^+$ T cells from naïve B6 donors as in Figure 1 and evaluated for survival. Data are from one of two similar experiments. ■ vs ▲, $*p < 0.05$. (**B,C**) T-cell expansion and cytotoxicity. (**B**) In vivo T-cell expansion: B6 (white bar) and BALB/c recipients were transplanted as above with TCD BM from B6 and T cells from naïve B6 Ly5.2 (black bar) and LIP T from [B6 Ly 5.2→B6] animals (gray bar). Black bar vs gray bar, $**p < 0.01$. (**C**) In vitro MLR: Naïve (black bar) and LIP B6 T cells (gray bar) were cultured with irradiated BALB/c peritoneal exudative cells for 72 hours, and proliferative activities were measured by ^{3}H-thymidine uptake. Black bar vs gray bar, $**p < 0.01$. (**D**) Cytotoxicity assay: Spleen cells (3×10^7) from naïve or [B6→B6] mouse were cultured with irradiated BALB/c spleen cells (2×10^7) for 7 days, and cell-mediated cytotoxicities were measured by ^{51}Cr-release assay against syngeneic B6 Con A blasts, allogeneic BALB/c Con A blasts, and P815 tumor cells.

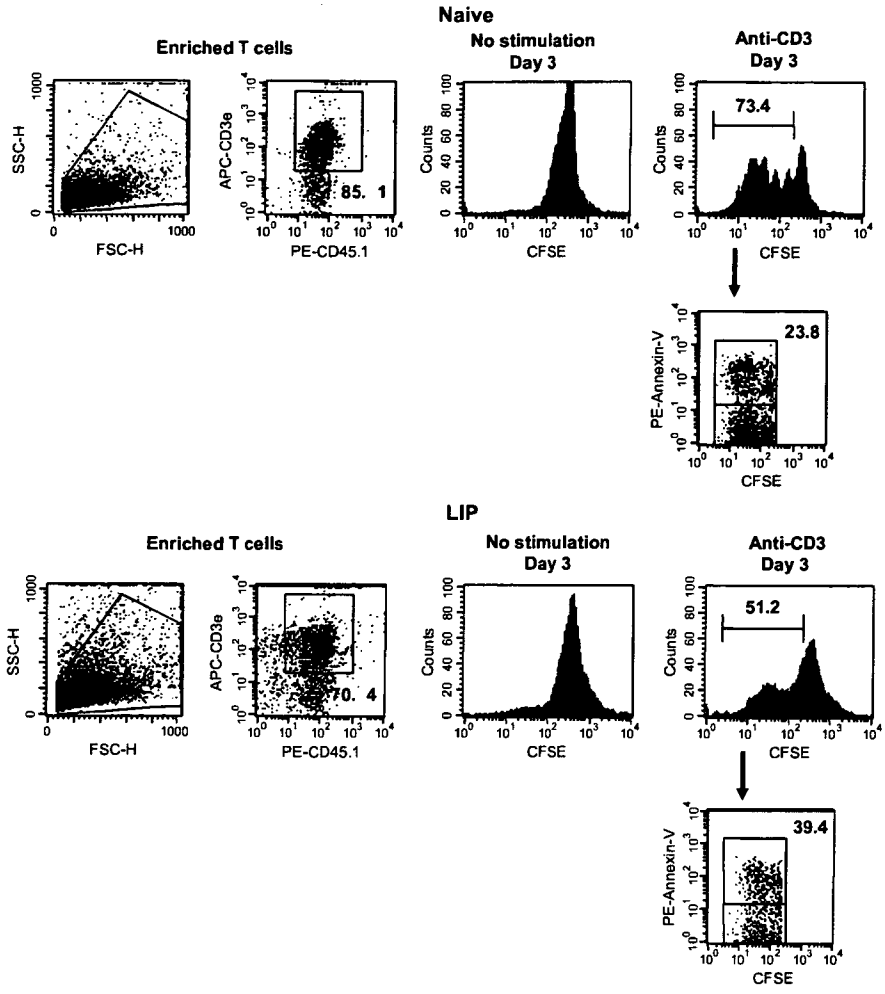

Figure 8. LIP T-cell proliferation and apoptosis. Splenic $CD3^+$ T cells were enriched from naïve B6 or following LIP for 2 weeks in irradiated [B6Ly5.2 → B6] animals. They were stained with CFSE and stimulated with anti-CD3 mAb for 3 days and T-cell proliferation was analyzed as in Materials and methods. Apoptosis of the T cells was analyzed after gating on the proliferating cells based on the CFSE dilution. Data are from three individual mice/group.

peripheral T-cell homeostasis [54,55]. Our results are therefore clearly not applicable to the T cells undergoing basal proliferation.

The barriers posed by LIP in induction of tolerance to solid organ allo-transplantation [22–24] are in contrast to our and others observations of reduced alloreactivity of these cells after experimental allogeneic BMT [43–45]. The reduced alloreactivity in GVHD in experimental models could be due to absence of cross reactivity with the host allo-antigens because of restricted donor memory T cell repertoire in the pathogen-free inbred mice. Furthermore, it has been suggested that LIP of polyclonal T cells alters their repertoire [5,11,52]. However this clearly was not the case in organ rejection studies utilizing similar mouse strains. But in contrast to solid organ allograft rejection studies, the donor T cells in our study were twice subjected to LIP, in the donors

Table 1. Effect of prolonged LIP on T cell phenotypes

	Naïve	LIP (9 w)
CD4/CD25	12.6 ± 2.2	11.0 ± 4.5
CD4/CD122	1.2 ± 0.3	0.8 ± 0.4
CD8/CD25	4.6 ± 2.0	6.6 ± 2.0
CD8/CD122	6.3 ± 2.0	12.9 ± 2.8

Table 2. LIP T-cell proliferation and apoptosis

	Mouse #	1	2	3	
Naïve	Divided cells (%)	73.4	39.6	55.4	55.4 ± 9.8
	Annexin-V^+/divided cells (%)	23.8	31.8	28.1	27.9 ± 2.3*
	Mouse #	1	2	3	
LIP (2 w)	Divided cells (%)	51.2	43.5	52.5	49.1 ± 2.8
	Annexin-V^+/divided cells (%)	39.4	48.9	43.4	43.9 ± 2.8

*$p < 0.05$.

before and in the hosts after BMT. This would suggest that greater rounds of lymphopenic expansion might reduce their allo-reactive potential. Studies are in progress to evaluate whether naïve T cells that are present after LIP might have impaired survival. Nonetheless, it is also possible that alloresponses after allogeneic BMT might be altered by the greater inflammatory milieu, interaction with host (non-self MHC) antigen-presenting cells, the target organ immunogenicity, and the antigen density that could distinctly modify the function of the LIP T cells.

Understanding the effect of LIP of donor T cells might have important clinical ramifications. For example, increasing age of the donors (with reduction in thymic output) and/or exposure to a number of infections and/or medications that cause lymphopenia of variable severity with concomitant homeostatic expansion will result in the alterations of their TCR repertoire [5,11] and could modulate their alloreactive potential [56,57]. The less severe acute GVHD from cord blood transplantation compared to allogeneic unrelated donor BMT might in part be due to their lymphopenic expansion in newborns [58,59]. More importantly, our observations that the conversion of donor T cells to "memory-like" phenotype after LIP is a transient but significant enough phenomenon to reduce their GVHD potential would suggest that deliberate induction of mild lymphopenia in the donors prior to harvesting their cells for BMT might reduce GVHD in the hosts without causing any long-term changes in the donor immune system. Moreover, LIP of donor T cells might occur not only in the donors before BMT but also in the hosts after BMT. It is conceivable that interactions between donor-derived APC and the donor T cells in the context of lymphopenia in the hosts cause nonalloreactive LIP that could modulate GVHD and/or immune reconstitution after BMT. However, T-cell division during immune reconstitution after transplantation could be associated with clinical events such as episodes of infections and GVHD, and therefore the extent and impact of LIP on GVHD might be difficult to distinguish from the effects of donor T-cell interactions with host APCs after MHC-mismatched BMT [60,61]. Nonetheless, our data with the induction of LIP of donor T cells prior to BMT might explain the recent observations that appropriately dosed and timed injections of IL-7 increased the proliferation of nonalloreactive donor T cells and enhanced immune reconstitution without enhancing GVHD [62]. Because LIP increases T cell–mediated anti-tumor effect [15–18,36] and preserves CTL function, it is tempting to speculate that induction of LIP of naïve donor T cells might reduce GVHD but preserve graft-versus-leukemia responses. However it should be noted that the anti-tumor potential of the LIP T cells has not been tested in an allogeneic context and studies are in progress to determine whether the strategy of LIP of donor T cells will retain anti-tumor response despite their reduced potential for causing GVHD.

Acknowledgments

Supported by grants from the National Institutes of Health (K08 AI052863-01) (P.R.); Japanese Ministry of Education, Culture, Sports, Science and Technology (17390280) (T.T.); the Health and Labor Science Research Grants for Clinical Research for Evidence Based Medicine (T.T.); and Japan Leukemia Research Fund (T.T.).

P.R. is the recipient of the Alaina J. Enlow Scholar Award from Amy Strelzer Manasevit—National Marrow Donor Program and the New Investigator Award from American Society of Blood and Marrow Transplantation.

The authors have no conflicting financial interests.

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European Journal of Haematology ISSN 0902-4441

ORIGINAL ARTICLE

Distinctive expression of myelomonocytic markers and down-regulation of CD34 in acute myelogenous leukaemia with FLT3 tandem duplication and nucleophosmin mutation

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Abstract

Objective: Patients with acute myelogenous leukaemia (AML) show co-existing frequently internal tandem duplications of FLT3 (FLT3-ITD) and mutations of nucleophosmin (NPM1-Mt). We investigated the biological and clinical significance of FLT3-ITD and/or NPM1-Mt in this context. *Methods:* We analysed 89 AML patients according to whether NPM1 and FLT3-ITD were single mutants, double mutants, or wild type for both. *Results:* FLT3-ITD was detected in 19 of 89 patients (21.3%), while NPM1-Mt was detected in 19 of 89 patients (21.3%); eight of 89 patients (9.0%) carried both FLT3-ITD and NPM1-Mt. By multivariate analysis, white blood cell count and peripheral blood blast cell count at diagnosis were significantly higher in patients with FLT3-ITD but not in those with only NPM1-Mt. NPM1-Mt was significantly related to female gender, normal karyotype, and M4 or M5 disease according to French–American–British criteria. In addition, leukaemic blast cells with NPM1-Mt, FLT3-ITD, or both expressed CD34 less frequently than wild-type blasts ($P < 0.0001$ and $P = 0.005$ respectively), while myelomonocytic markers such as CD11b and CD14 were expressed more frequently in patients with NPM1-Mt. *Conclusion:* FLT3-ITD may increase potential for cell proliferation to produce a leukaemic population; NPM1-Mt may cause cells to develop along the myelomonocytic lineage. Extensive analyses and detailed experiments will be required to clarify how NPM1 and FLT3 mutations interact in leukaemogenesis.

Key words acute myelogenous leukaemia; FLT3 internal tandem duplication; nucleophosmin mutation; myelomonocytic markers; CD34

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Accepted for publication 20 March 2007

doi:10.1111/j.1600-0609.2007.00866.x

Acute myelogenous leukaemia (AML) is a heterogeneous haematologic malignant disease that can be distinguished by morphology, immunophenotyping or cytogenetic analyses. Approximately 50% of patients with AML carry chromosomal abnormalities that identify categories with significant clinical and prognostic features: patients with t(8;21), t(15;17) and inv(16) are considered to have 'good-risk' cytogenetics, while those with 5q-, 17q-, t(9:22), 11q23, and complex karyotypes are at greater risk for poor outcomes. On the other hand, conventional karyotypic analysis shows no chromosomal abnormalities in about half of patients with AML; while these patients with a normal karyotype have been considered an 'intermediate-risk' group (1), they are biologically and clinically the least well understood. Recent investigations have suggested involvement of several mutations of genes encoding transcription factors (AML1, CEBP/alpha) (2,3), receptor tyrosine kinases (FLT3, KIT) (4,5) and nucleophosmin (NPM1) (6), overexpression of the *BAALC* (brain and acute leukaemia, cytoplasmic) gene,

or overexpression of ETS-related gene in the pathogenesis of AML (7,8). One therefore needs to understand mechanisms by which these mutated genes are involved in leukaemogenesis and how then influence prognosis in AML.

FLT3, a member of the class 3 receptor tyrosine kinase family, is expressed on haematopoietic progenitor cells and is important for the survival and proliferation of early haematopoietic progenitors (9). An internal tandem duplication (ITD) of the FLT3 gene (FLT3-ITD) has been reported in 20–30% of adult patients with AML, especially in normal-karyotype and t(15;17)-positive patients (4,10,11). FLT3-ITD encodes an abnormal protein that can cause ligand-independent receptor dimerisation, autophosphorylation, and constitutive activation of downstream signalling pathways involved in cell proliferation, differentiation, and survival (12). Recent studies have shown that an activating FLT3 mutation is associated with higher white blood cell (WBC) counts and relapse rates, and may carry a worse prognosis than that for intermediate-risk AML patients in general (4,11,13). Accordingly, these patients can be assigned to a distinct subgroup to be considered for risk-adapted treatment options such as allogeneic and autologous stem cell transplantation (14).

Another novel mutation, the NPM1 gene mutation (NPM1-Mt), may be present in 25–45% of patients with AML (6,15–18). In normal haematopoiesis, wild-type nucleophosmin protein plays a key role in regulating protein synthesis, cell growth and cell proliferation, involving access to maturing ribosomes (19,20) and regulation of oncosuppressors such as ARF and p53 (6). In AML, the most common NPM1-Mt appears to be a four-base-pair insertion leading to a frameshift causing replacement of amino acids in the C-terminal portion of NPM1 (6). This mutation can result in loss of tryptophan residues 288 and 290 (or 290 only), and ultimately an aberrant cytoplasmic location of the protein product that may contribute to leukaemogenesis. NPM1-Mt has been reported to occur predominantly in patients with a normal karyotype, and to be associated with little or no CD34 expression and higher WBC and blast cell counts (18,21). Further, patients with NPM1-Mt harbour FLT3-ITD approximately twice as often as those with wild-type NPM1 (NPM1-Wt) (6,18). To investigate the consequences of FLT3-ITD and NPM1-Mt, we investigated pathologic and clinical features of AML subgroups with or without FLT3-Wt or NPM1-Wt among 89 AML cases. Multivariate analysis showed occurrence of double mutations three times more frequently than single mutations. A significant increase in WBC and blast cell counts was associated more closely with FLT3-ITD, while increased expression of monocytic markers and down-regulation of CD34 expression were noted in patients with NPM1-Mt. These results suggested that FLT3-ITD increased proliferation potential, while NPM1-Mt might favour leukaemic cell maturation along the myelomonocytic lineage. Together, these two mutations could bring about a leukaemia with specific myelomonocytic features.

Patients, materials and methods

Patients

We retrospectively analysed 89 AML patients with cell samples from the time of initial diagnosis up to December 2005 available for gene analyses. Patients with acute promyelocytic leukaemia, which very often is associated with FLT3-ITD (4,11), were excluded from the study. Patients studied included 56 men and 33 women, with a median age of 48 yr (Table 1). Chromosomal G-banding was carried out using a standard method. In each case, according to the International System for Human Cytogenetic Nomenclature guidelines, at least 20 mitotic events were analysed to exclude clonal abnormalities. Patient data including WBC and peripheral blood (PB) blast cell counts at the time of the initial diagnosis, morphologic types according to the French–American–British (FAB) classification and cytogenetic findings are shown in Table 1.

Patients were treated with a conventional remission-induction regimen consisting of idarubicin or daunorubicin for 3 d and cytosine arabinoside for 7 d. After achieving complete remission, patients were assigned to one of several types of postremission therapy including conventional consolidation chemotherapy, autologous PB stem cell transplantation (auto-PBSCT) and allogeneic haematopoietic stem cell transplantation (allo-SCT), based on prognostic factors such as chromosomal abnormalities. All patients or their guardians gave written informed consent in accordance with the requirements of the local Institutional Review Board.

Screening for FLT3-ITD and NPM1 mutations

Genomic DNA was isolated from bone marrow slides prepared at initial diagnosis, as described previously (14). Cells were dissociated from the slides, with contents then dissolved in phosphate-buffered saline. Genomic DNA was extracted using QIAamp DNA mini-kits (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations.

For FLT3-ITD, a polymerase chain reaction (PCR)-based amplification of genomic DNA was carried out as previously described (12), using primers 11F and 12R located in FLT3 exon 14 and 15. PCR products were separated by electrophoresis using a 3% Nusieve GTG agarose gel (Cambrex Bio Science Rockland, Rockland, ME, USA). FLT3-ITDs were detected as abnormally