ELSEVIER

EXPERIMENTAL HEMATOLOGY

Experimental Hematology 33 (2005) 1564–1573

T-cell P/E-selectin ligand α(1,3)fucosylation is not required for graft-vs-host disease induction

Hyeon-Seok Eom[a], Marie-Therese Rubio[a], Terry K. Means[b], Andrew D. Luster[b], and Megan Sykes[a]

[a]*Transplantation Biology Research Center, Bone Marrow Transplantation Section, Massachusetts General Hospital/Harvard Medical School, Boston, Mass., USA;* [b]*Center for Immunology and Inflammatory Diseases, Division of Rheumatology, Allergy and Immunology, Massachusetts General Hospital/Harvard Medical School, Boston, Mass., USA*

(Received 19 May 2005; revised 8 August 2005; accepted 11 August 2005)

Objective. **Recognition of E- and P-selectins on vascular endothelium by their leukocyte glycoprotein counterreceptor P-selectin glycoprotein ligand-1 (PSGL-1) initiates and sustains leukocyte rolling, culminating in extravasation of lymphocytes from blood into organs. PSGL-1 is rendered functional by terminal glycosylation steps, which occur mainly in activated Th1 but not Th2 cells. α(1,3)Fucosyltransferases IV and VII control this glycosylation pathway. Mice lacking these transferases (Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$) lack functional E- and P-selectin ligands. We hypothesized that Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ donor T cells might have reduced capacity to roll on vessels of inflamed target tissues and mediate graft-vs-host disease (GVHD).**

Materials and Methods. **We compared the ability of Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ and wild-type (WT) C57BL/6 (B6) spleen cells (SPCs) to produce GVHD in lethally irradiated major histocompatibility complex (MHC) haplotype-mismatched B6D2F1 recipients. Clinical GVHD, GVHD pathology in target organs, memory phenotype conversion, proliferation of donor T cells, and tissue and serum cytokine expression were examined.**

Results. **Surprisingly, clinical GVHD was not reduced in lethally irradiated mice receiving full haplotype MHC mismatched or matched Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ SPCs compared to those receiving WT SPCs. GVHD pathology in target organs, memory phenotype conversion, and proliferation of donor T cells were similar in both groups. However, reduced interferon-γ was detected in liver and lung, and serum levels of tumor necrosis factor-α were higher in mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ SPCs compared with WT SPCs.**

Conclusions. **These results suggest that donor T cells, including Th1, are capable of trafficking to GVHD target tissues independently of P- and E- selectin ligand in conditioned hosts.**

Graft-vs-host disease (GVHD) and graft-vs-leukemia can be separated by confining the GVH alloresponse to the lymphohematopoietic system, avoiding T-cell migration into epithelial GVHD target tissues [1–4]. Inflammation in the epithelial GVHD target tissues following conditioning induces GVH-reactive T cells to leave the lymphohematopoietic system and traffic to these target tissues (R. Chakraverty and M. Sykes, unpublished).

T-cell migration involves tethering, rolling, activation, arrest, and transmigration [5]. Blocking T-cell tethering and rolling might attenuate GVHD. P- and E-selectins are selectively displayed on the surface of endothelial cells at inflammatory sites and sustain leukocyte rolling, promoting leukocyte extravasation into organs [6]. P-selectin glycoprotein ligand-1 (PSGL-1), the leukocyte counterreceptors for P- and E-selectin, and peripheral-node addressin and mucosal addressin-cell adhesion molecule-1, the endothelial counterreceptors for L-selectin, only recognize appropriately glycosylated selectins [5,7]. The final reaction in this selectin glycosylation pathway is controlled by a family of α(1,3)fucosyltransferases (Fuc-T) IV and VII in leukocytes [8]. Mice doubly deficient for Fuc-TIV and Fuc-TVII are

Offprint requests to: Megan Sykes, M.D., Transplantation Biology Research Center, Bone Marrow Transplantation Section, Massachusetts General Hospital/Harvard Medical School, MGH-East Building 149-5102, 13th Street, Boston, MA 02129; E-mail: Megan.Sykes@tbrc.mgh.harvard.edu

0301-472X/05 $–see front matter.
doi: 10.1016/j.exphem.2005.08.003

characterized by deficiency of E-, P-, and L-selectin binding activity [8]. Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ double knockout mice demonstrate complete deficiency in leukocyte rolling and defective adaptive immune responses [9,10]. Th1 cells express functional PSGL-1, which undergoes glycosylation following activation, allowing migration to acutely inflamed tissues [11]. Polarizing cytokines lead to selective downregulation of Fuc-TVII mRNA in Th2 but not in Th1 cells [12]. Therefore, Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ mice exhibit a leukocyte-trafficking defect, especially of Th1 cells, into inflamed tissues. All three selectins can promote CD8$^+$ type 1 cytotoxic T as well as CD4$^+$ Th1 cell migration into the inflamed skin [13].

P- and E-selectins are upregulated on the microvasculature of inflamed tissues, and therefore might play a role in the trafficking of T cells into such tissues in proinflammatory conditions, such as those induced by conditioning for bone marrow transplantation (BMT), thereby promoting GVHD. Th1 activity correlates with GVHD [14,15], although Th2 cells can mediate liver and skin GVHD [16]. We hypothesized that Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ donor T cells, because of their functional defect in P- and E-selectin ligands, might induce less GVHD than wild-type (WT) T cells following BMT. The studies presented here address this hypothesis.

Materials and methods

Animals

Female donor C57BL/6 (H2^b: K^b, I^b, D^b) mice were purchased from Frederick Cancer Research Facility, National Cancer Institute (Frederick, MD, USA). Female recipient B6D2F1 (H2$^{b/d}$: K$^{b/d}$, I$^{b/d}$, D$^{b/d}$) and C3.SW-H2^b/Sn5 (H2^b: K^b, I^b, D^b) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). B6 mice deficient in α(1,3)fucosyltransferases IV and VII (Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$) were kindly provided by Dr. John B. Lowe [17]. Mice were used at 6 to 12 weeks of age, and all recipients were at least 12 weeks old. Mice were housed in autoclaved microisolator environments, and all manipulations were performed in a laminar flow hood. All animal protocols were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Bone marrow transplantation

B6D2F1 mice received 9.5 Gy total body irradiation (TBI) from a cesium^{137} irradiator followed by injection of 10^7 T-cell−depleted (TCD) bone marrow cells (BMCs) and either WT or Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 spleen cells (SPCs) containing 2.2 to 2.4 × 10^6 CD4$^+$ T cells and 1.3 to 1.5 × 10^6 CD8$^+$ T cells intravenously. C3.SW mice received 10.5 Gy TBI followed by injection of 10^7 TCD WT B6 BMCs and either WT or Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs containing 1.5 to 1.6 × 10^6 CD4$^+$ T cells and 0.5 to 0.6 × 10^6 CD8$^+$ T cells. In vitro depletion of CD4$^+$ and CD8$^+$ T cells from BMCs was performed using anti-CD4 and anti-CD8 magnetic beads (Miltenyi Biotec, Auburn, CA, USA) according to manufacturer's instructions. SPCs were harvested and gently teased in ACK-lysing buffer (BioWhittaker, Walkersville, MD, USA). Single-cell suspensions were filtered through nylon mesh. T cells were purified from SPCs was performed by negative selection using the Pan T cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. Flow cytometry (FCM) analysis showed that T-cell depletion of each subset from the BM inoculum was 99% complete and the purity of T cells isolated from SPCs was 94%. Following FCM analysis, the number of WT and Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs was adjusted to contain similar numbers of T cells. As a syngeneic control, lethally irradiated B6 mice received 10 × 10^6 syngeneic BMCs.

Assessment of T-cell proliferation and activation and FCM analysis

WT and Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs were labeled with carboxy-fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) prior to administration on day 0. Briefly, single-cell SPC suspensions were prepared and resuspended at 3 × 10^7 cells/mL in 10 mL phosphate-buffered saline with 1 μM CFSE, incubated at 37°C for 15 minutes, then washed with RPMI 1640 (Biowhittaker) with 10% fetal calf serum (Sigma, St Louis, MO, USA).

Expression of activation markers was assessed by FCM using a FACSCalibur (Becton Dickinson). For three-color staining, 10^6 cells were incubated in the presence of directly fluorescein-isothiocyanate (FITC)-, or phycoerythrin (PE)-, or biotin (Bio)-conjugated monoclonal antibodies (mAb) for 30 minutes at 4°C. To reduce nonspecific binding, 10 μL 2.4G2 (anti-Fcγ-RII receptor, CDw32) hybridoma supernatant [18] was added. The following antibodies were used for phenotypic analyses: anti-CD25-FITC, anti-CD44-FITC, anti-CD45RB-FITC, anti-CD62L-FITC, anti-CD4-PE, anti-CD8β-PE, and 34-2-12-Bio (anti-H2-D^d) plus streptavidin (SA)-allophycocyanin (APC) (all purchased from PharMingen, San Diego, CA, USA). Mouse IgG2a mAb HOPC1-FITC or HOPC1-Bio (HOPC1 has no reactivity to mouse leukocyte cell surface molecules) plus SA-APC and rat IgG2b-PE (PharMingen) were used as negative controls. Propidium iodide (PI) staining and live gating on PI-negative cells was performed.

Assessment of clinical GVHD and histopathology

Clinical GVHD was assessed by scoring five parameters, including hunching, ruffled fur, diarrhea, periorbital edema, and activity [3]. Clinical GVHD scores and weights were monitored twice weekly for the first 2 weeks and weekly thereafter. Histopathologic analysis of GVHD target organs was performed by scoring: skin (dermal and epidermal lymphocyte infiltration, dyskeratotic epidermal keratinocytes), lung (perivascular cell infiltration, peribronchiolar cell infiltration), liver (inflammatory cell infiltration in portal tract, inflammatory cell infiltration in bile ducts, apoptosis of bile duct epithelial cells, vascular endothelialitis, parenchymal apoptosis, parenchymal microabscesses, mitoses in parenchyma), and colon (crypt regeneration, apoptosis in crypt epithelial cells, crypt loss, surface colonocyte attenuation, inflammatory cell infiltration in lamina propria, mucosal ulceration, thickening of mucosa). A severity scale from 0 to 4 was used: 0 = normal, 0.5 = focal and rare, 1 = focal and mild, 2 = diffuse and mild, 3 = diffuse and moderate, and 4 = diffuse and severe.

Cytokine enzyme-linked immunosorbent assay

Mouse interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) immunoassay kits (R&D Systems Inc., Minneapolis, MN, USA)

were used with serum diluted 1:10 for IFN-γ and undiluted for TNF-α measurements. Microplates were read using a microplate reader (PerkinElmer, Wellesley, MA, USA) set to 450 nm. Minimum detectable concentrations of IFN-γ and TNF-α were 2 pg/mL and 5.1 pg/mL, respectively.

Quantitative real-time PCR of frozen tissues

Total RNA was extracted from frozen tissues using homogenization with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After isopropanol (Sigma, St Louis, MO, USA) precipitation, the RNA pellet was washed with 70% ethanol and resuspended in DEPC water (Sigma). Optical density at 260/280 nm was measured using an ultraviolet spectrophotometer (Beckman Coulter, Fullerton, CA, USA) to estimate RNA and protein content. After DNase I (Invitrogen) treatment, 1 μg total RNA from each sample was used as template for the reverse transcription reaction using oligo (dT)15, random hexamers, and multiscribe reverse transcriptase (Applied Biosystems, Foster City, CA, USA). Quantitative polymerase chain reaction (QPCR) was performed as described previously [19]. Interleukin (IL)-4, IL-5, IL-6, IL-10, IFN-γ, and TNF-α genes were examined and all oligonucleotide primers for QPCR were designed using Primer Express software 1.0 (PE Biosystems, Foster City, CA, USA) and synthesized by Invitrogen. Quantitative values for gene expression were generated by comparison of the fluorescence generated by each sample with standard curves of known quantities by using the adaptive baseline algorithm in the MX4000 analysis software (Stratagene, La Jolla, CA, USA). Calculated values for the gene of interest were normalized to the housekeeping gene glyceraldehyde phosphate dehydrogenase.

Statistical analysis

Values are shown as mean ± standard deviation. Mann-Whitney *U*-test or Student's *t*-test were used for statistical analysis of clinical GVHD scores, GVHD histopathologic scores, cell numbers, cell phenotypes, proliferation, cytokine expression, and serum cytokine levels. Survival data were analyzed using the log-rank test.

Results

GVHD is not diminished in mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ SPCs compared to those receiving WT SPCs following haplotype-mismatched BMT

We first compared GVHD in mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ versus WT B6 SPCs in the MHC haplomismatched B6 (H2^{b}) to B6D2F1 (H2$^{b/d}$) BMT model. In a preliminary experiment, lethally irradiated recipients of TCD Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 BM and SPCs died within 10 days, whereas mice receiving WT B6 BM and SPCs did not show early lethality (data not shown). We attributed the mortality to a potential homing defect of Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ hematopoietic stem cells [20,21]. Therefore, in subsequent experiments, lethally irradiated B6D2F1 mice received TCD WT B6 BM and either Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ or WT B6 SPCs. The number of SPCs was adjusted to ensure the administration of similar numbers of T cells from WT or Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ donors. The number of injected CD4^{+}CD25^{+} T cells, which might also influence GVHD [19,22,23], was similar in both groups (data not shown).

Surprisingly, mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs showed more weight loss (Fig. 1A, $p < 0.05$ on day 10; $p < 0.01$ on days 14 and 17) and increased clinical GVHD scores from days 8 to 29 (Fig. 1B, $p < 0.05$) compared to those receiving WT SPCs. Moreover, mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs showed increased GVHD mortality (median survival time [MST] 33 days) compared to mice receiving WT SPCs (Fig. 1C, MST > 100 days, $p < 0.01$).

To determine whether the observed effects of Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ deficiency reflected effects of T cells or other splenocyte populations (e.g., natural killer cells), lethally irradiated B6D2F1 mice received TCD WT B6 BM and 3.8×10^{6} purified T cells from either Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ or WT B6 mice. Mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ T cells again showed increased weight loss and GVHD scores (Fig. 2A and B) and should similar mortality compared to those receiving WT B6 T cells (Fig. 2C, $p > 0.05$). Thus, T cells from Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 mice induced more severe clinical GVHD than T cells from WT B6 mice in an MHC haplomismatched BMT model. T cells defective in functional P- and E-selectins readily induce GVHD.

GVHD pathology was evaluated in animals sacrificed on day 30 (data not shown). There was a trend toward increased GVHD pathology scores in the colons of mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ SPCs, but this did not attain statistical significance ($p = 0.08$). Liver, skin, and lung showed similar GVHD pathology scores between the groups.

WT and Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ donor T cells show similar activation and proliferation

Because mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ SPCs showed similar GVHD pathology but greater clinical illness compared to those receiving WT SPCs, we hypothesized that increased systemic illness might reflect enhanced activation and/or expansion of Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ T cells. Therefore, expression of CD44, CD45RB, and CD62L (L-selectin) was compared in lymph nodes (LNs) and SPCs from both groups at various times. The numbers of WT and Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ donor T cells in LNs and spleens were generally similar, except for an increase in the spleens of mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs on day 4 (Fig. 3A). WT and Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ donor T cells showed marked downregulation of CD62L and CD45RB on CD4^{+} and CD8^{+} T cells in LNs between days 4 and 8 to 19 (Fig. 3B), consistent with the conversion to the "memory" phenotype typically observed in our GVHD studies. Consistently, almost all donor CD4 and CD8 cells acquired the CD44^{high} phenotype in both groups. However, a slight but significant increase in

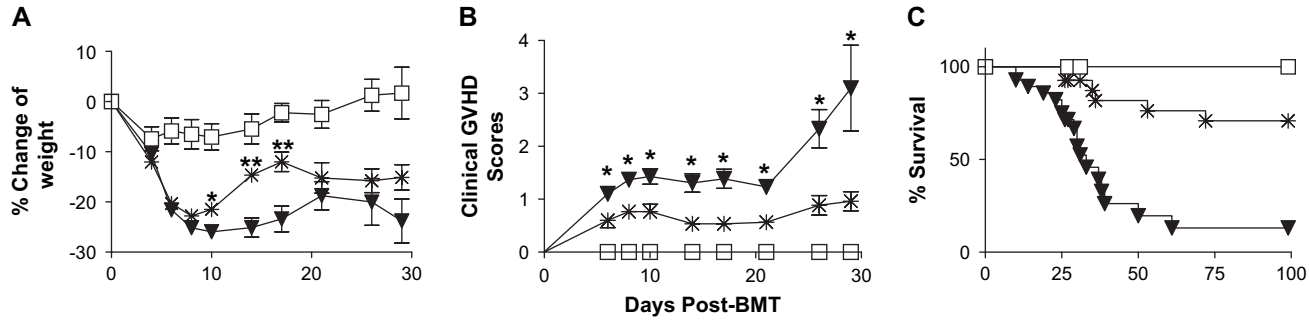

Figure 1. Mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs show increased clinical graft-vs-host disease (GVHD) scores and mortality compared to those receiving wild-type (WT) B6 spleen cells (SPCs). Lethally irradiated B6D2F1 mice received T-cell−depleted (TCD) WT B6 (10×10^6 bone marrow cells [BMCs]) and either Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ (13.2×10^6 SPCs including 2.4×10^6 CD4$^+$ and 1.3×10^6 CD8$^+$ T cells, ▼, n = 27) or WT B6 SPCs (12×10^6 SPCs including 2.2×10^6 CD4$^+$ and 1.5×10^6 CD8$^+$ T cells, *, n = 27). As a control, lethally irradiated B6D2F1 mice received B6D2F1 (10×10^6 BMCs, □, n = 7). (**A**) Mean percentage of weight change compared to baseline. Mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs showed more weight loss than those receiving WT B6 SPCs. (**B**) Clinical GVHD scores. Mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs had increased clinical GVHD scores compared to those receiving WT B6 SPCs. (**C**) Mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs showed accelerated and increased mortality compared to controls. Data from two of two experiments, which showed similar results, are combined. *$p < 0.05$; **$p < 0.01$.

the proportion of CD44^{high} donor CD4$^+$ and CD8$^+$ LNT cells was seen on day 7 ($p < 0.05$) in mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ compared to WT SPCs and a small difference was seen for donor CD8$^+$ T cells on day 12 ($p < 0.05$). In spleens, WT and Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ donor T cells showed similar conversion to the activated/memory phenotype on days 4, 7, 12, and 19 (data not shown).

To examine proliferation of WT and Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ donor T cells, we analyzed CFSE dye dilution (data not shown). WT and Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ donor CD4$^+$ and CD8$^+$ T cells in LNs and spleens showed similar proliferation on days 3, 5, and 7. Thus, Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ donor CD4$^+$ and CD8$^+$ T cells lacking functional P- and E-selectins are activated, expand, and accumulate in secondary lymphoid organs to similar levels as WT T cells in this model.

Th1 and Th2 cells can traffic to GVHD target tissues without functional P- and E-selectin activity

Fuc-TVII deficiency reduces the expression of functional P- and E-selectin ligands by Th1 cells [10]. Therefore, we compared cytokine expression in nonlymphoid GVHD target tissues and spleen by real-time QPCR (Fig. 4). We selected early and late time points to evaluate cytokine expression. Consistent with an impairment of Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ Th1 tissue migration, mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs expressed lower levels of IFN-γ mRNA in liver on day 7 and lung on day 30, but higher levels of IFN-γ mRNA in their spleens compared to those receiving WT B6 SPCs ($p < 0.05$) on day 7 post-BMT. Mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs also expressed higher levels of TNF-α mRNA in their livers compared to those receiving WT B6 SPCs ($p <$

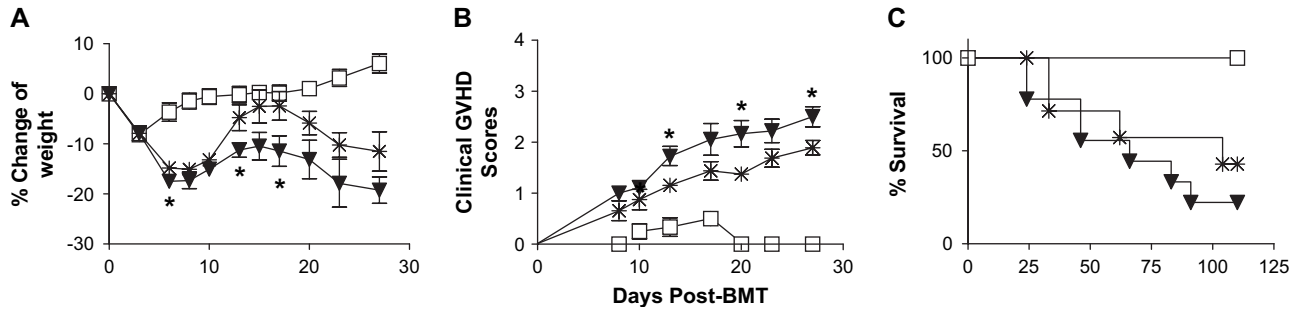

Figure 2. Mice receiving purified Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 T cells show increased clinical graft-vs-host disease (GVHD) scores and a trend toward increased mortality compared to those receiving wild-type (WT) B6 T cells. Lethally irradiated B6D2F1 mice received T-cell−depleted WT B6 (10×10^6 bone marrow cells) and either Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ (3.8×10^6 T cells including 2.4×10^6 CD4$^+$ and 1.4×10^6 CD8$^+$ T cells, ▼, n = 9) or WT B6 splenic T cells (3.8×10^6 T cells including 2.2×10^6 CD4$^+$ and 1.6×10^6 CD8$^+$ T cells, *, n = 8). As a control, lethally irradiated B6D2F1 mice received B6D2F1 (10×10^6 bone marrow cells, □, n = 3). (**A**) Mean percentage of weight change compared to baseline. Mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 T cells showed more weight loss than those receiving WT B6 T cells. (**B**) Clinical GVHD scores. Mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 T cells had increased clinical GVHD scores compared to those receiving WT B6 T cells. (**C**) Mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 T cells showed a trend toward increased mortality compared to controls. *$p < 0.05$. BMT = bone marrow transplantation.

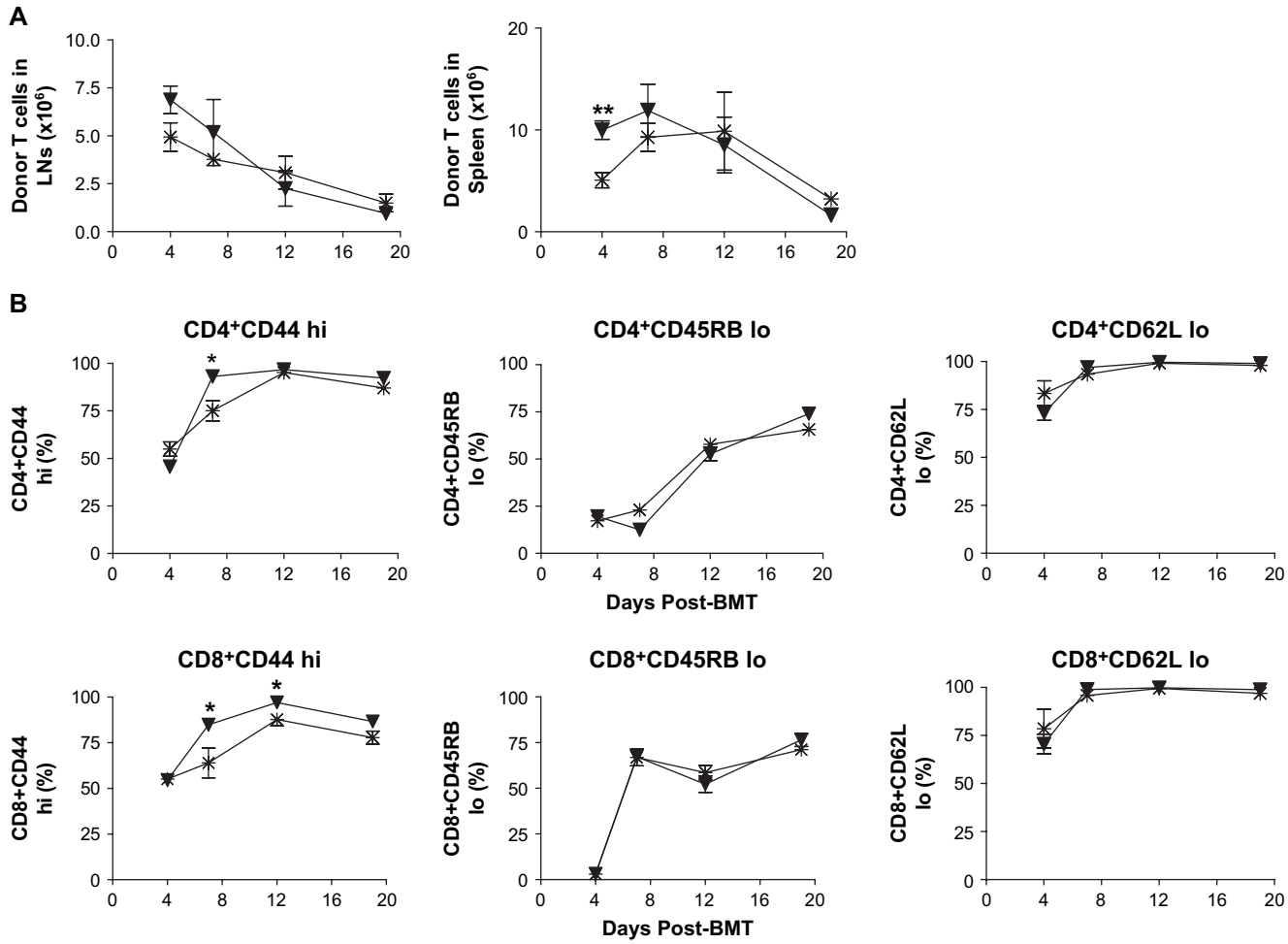

Figure 3. T-cell activation in mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$, or wild-type (WT) B6 spleen cells (SPCs). Lethally irradiated B6D2F1 mice received T-cell−depleted WT B6 bone marrow and either Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$, or WT B6 SPCs. (**A**) On days 4, 7, 12, and 19, SPCs and lymph node (LN) cells were harvested and counted. Gated donor (D^{d-}) T-cell subsets were analyzed by flow cytometry (FCM) to count donor T cells. (**B**) On days 4, 7, 12, and 19, LN cells and splenocytes (not shown) were harvested to evaluate expression of activation markers by three-color FCM. CD4$^+$ and CD8$^+$ T cells were labeled with anti-CD44, CD45RB, and CD62L monoclonal antibodies and 34-2-12 (anti-D^d). Gated donor (D^{d-}) T cell subsets were analyzed for expression of CD44, CD45RB, and CD62L. ▼, mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs, n = 5; *, mice receiving WT B6 SPCs, n = 5. *$p < 0.05$; **$p < 0.01$.

0.01). Levels of IL-10 in spleens of recipients of Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs were increased compared to those in recipients of WT SPCs on day 7. Lower levels of IL-6 mRNA were detected in the lungs on day 30 in mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs compared to recipients of WT SPCs ($p < 0.05$). Tissue cytokines in other GVHD target organs (skin and colon) were not significantly different between recipients of Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ or WT B6 SPCs on days 7 or 30, or in lung on day 7 (data not shown). These findings suggest that both Th1 and Th2 cells can traffic to the GVHD target tissues in the absence of any functional P- and E-selectin activity. However, the data also suggest reduced trafficking of Th1 cells to the liver and lung in mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs.

Increased serum TNF-α levels in mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ SPCs compared to those receiving WT B6 SPCs

Because we observed greater mortality and more clinical GVHD, but similar T-cell activation, proliferation and histopathologic features in mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ compared to WT B6 SPCs, we next compared serum cytokine levels in the two groups (Fig. 5). Serum IFN-γ levels were lower in mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs than in those receiving WT SPCs on day 7 (Fig. 5; 535.5 ± 200.14 pg/mL vs 1189 ± 283.41 pg/mL, $p < 0.01$). In contrast, serum levels of TNF-α were significantly higher in mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs than in those receiving WT SPCs on day 7 (101.1 ± 11.48 pg/mL vs

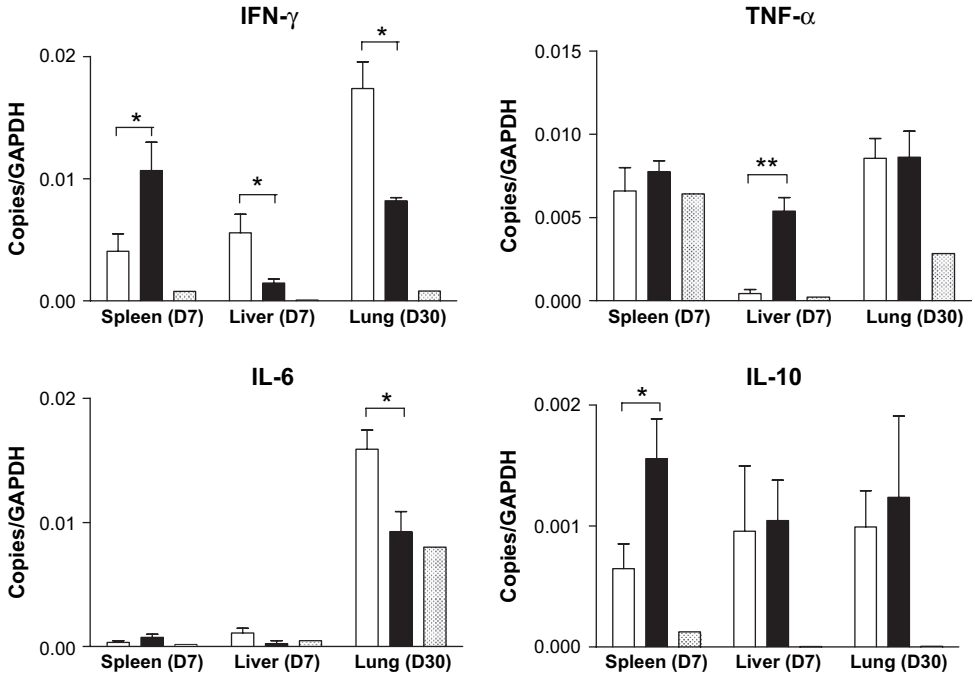

Figure 4. Altered cytokine expression in spleen and graft-vs-host disease (GVHD) target tissues of mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ spleen cells (SPCs). Lethally irradiated B6D2F1 mice received T-cell−depleted wild-type (WT) B6 bone marrow and either Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ or WT B6 SPCs. On days 7 and 30 post-bone marrow transplantation (BMT), GVHD target organs (colon, liver, skin, and lung) were harvested and tissue cytokine expression was evaluated by quantitative real-time polymerase chain reaction. The calculated values for the gene of interest were normalized to the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH). □, mice receiving WT B6 SPCs; ■, mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs; ▨, syngeneic BMT controls. *$p < 0.05$; **$p < 0.01$. IFN-γ = interferon-γ; IL = interleukin; TNF-α = tumor necrosis factor-α.

56.02 ± 6.28 pg/mL, $p < 0.01$) and on day 12 (64.5 ± 17.22 pg/mL vs 40.5 ± 7.55 pg/mL, $p < 0.05$). Elevated TNF-α levels might explain the greater mortality and increased clinical signs of GVHD in mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$/B6 SPCs.

Similar GVHD severity in mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ or WT SPCs following MHC-matched BMT

Although T cells from Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ donors induced increased GVHD mortality compared to WT B6 T cells in a full haplotype mismatched strain combination, it seemed possible that GVHD might be attenuated by the lack of functional donor T cell E- and P-selectin ligand in a less severe GVHD model. We therefore evaluated an MHC-matched, mHAg-mismatched model in which GVHD is primarily mediated by CD8$^+$ T cells [24]. Lethally irradiated C3.SW mice received Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ or WT MHC-matched mHAg-mismatched B6 SPCs containing 2.1 × 10^6 T cells, along with WT TCD B6 BMC. Similar weight loss, clinical GVHD scores, and mortality were observed in both groups (Fig. 6). In additional mice receiving a higher dose of SPCs containing 4.7 × 10^6 T cells, we observed similar results (data not shown). Therefore, T cells from Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 mice induce GVHD to the same extent as T cells from WT B6 mice following MHC-matched mHAg-mismatched BMT.

Discussion

P- and E-selectins mediate recruitment of T cells, especially Th1, into inflamed tissues [11]. PSGL-1 cross-linking on Th1 cells induces clustering of leukocyte function antigen-1 (LFA-1), and increased LFA-1 avidity may enhance binding to intercellular adhesion molecule 1-(ICAM-1) [25]. Moreover, dendritic cells from peripheral lymph nodes induce skin tropism of CD8$^+$ T cells by generation of functional PSGL-1. This effect on CD8 cells is not dependent on Th1 cytokines, which can upregulate expression of functional PSGL-1 on CD4$^+$ T cells [11,26]. Surprisingly, we have demonstrated that the inability to generate functional PSGL-1 does not impair the capacity of T cells to induce GVHD in any tissue, including skin. The trafficking of T cells lacking functional P- and E-selectin ligand to inflammatory sites most likely reflects the utilization of redundant pathways. Integrin family members such as $\alpha_4\beta_1$ and $\alpha_4\beta_7$ can mediate lymphocyte tethering and rolling [27,28], although this attachment is known to be less efficient than that mediated by selectins [28]. In the presence of ICAM-1, L-selectin can mediate leukocyte rolling in the absence

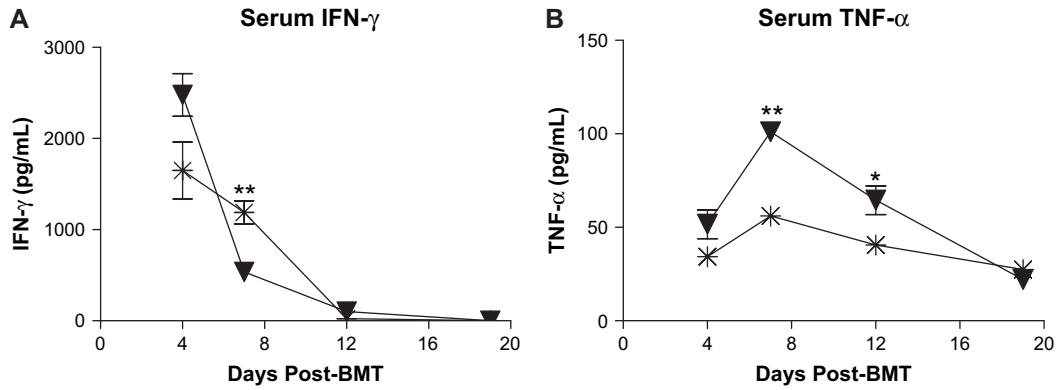

Figure 5. Serum levels of tumor necrosis factor-α (TNF-α) are increased, but interferon-γ (IFN-γ) levels are reduced in mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 spleen cells (SPCs) compared to those receiving wild-type (WT) B6 SPCs. Serum cytokines were measured on days 4, 7, 12, and 19 in B6D2F1 mice receiving T-cell−depleted WT B6 bone marrow and either Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ or WT B6 SPCs. (**A**) Serum IFN-γ levels were lower on day 7 in mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs than in those receiving WT SPCs. (**B**) Serum levels of TNF-α were higher on days 7 and 12 in mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs than in those receiving WT SPCs. ▼, mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs, n = 5; *, mice receiving WT B6 SPCs, n = 5. *$p < 0.05$; **$p < 0.01$.

of P-selectin [29], and L-selectin and ICAM-1 function synergistically to mediate optimal leukocyte rolling and entry into sites of inflammation [30]. Our results emphasize the considerable redundancy of T-cell trafficking pathways in the presence of conditioning-induced tissue injury.

We observed accelerated CD44 upregulation among CD4 and CD8 cells of Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ compared to WT T cells in the GVHD setting. While this observation is unexplained, upregulation of CD44 on Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ donor T cells might facilitate their extravasation synergistically with very late antigen-4 (VLA-4), as an association between CD44 and VLA-4 promotes firm adhesion and subsequent extravasation of T cells [31]. We have observed that blocking anti-CD44 mAb modestly delays GVHD mortality in a fully MHC-mismatched model, suggesting a significant role for CD44 in the migration of GVH-reactive T cells into the target tissues (G. Szot, D. Pearson, and M. Sykes, unpublished data). Furthermore, blockade of VLA-4−VCAM-1 interactions has been shown to inhibit GVHD in several models [32,33].

Another possible explanation for the robust GVHD observed in recipients of Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ T cells is that functional PSGL-1 is required for regulatory T-cell trafficking, and that reduced Th1 trafficking is counterbalanced by reduced trafficking of regulatory cells to the GVHD target tissues in recipients of Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ T cells.

WT and Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ SPCs in our studies showed slightly different CD4 to CD8 ratios (WT SPCs: 1.44, Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ SPCs 1.90). Therefore, mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ SPCs received 10% more CD4$^+$ T cells and 16% fewer CD8$^+$ T cells than

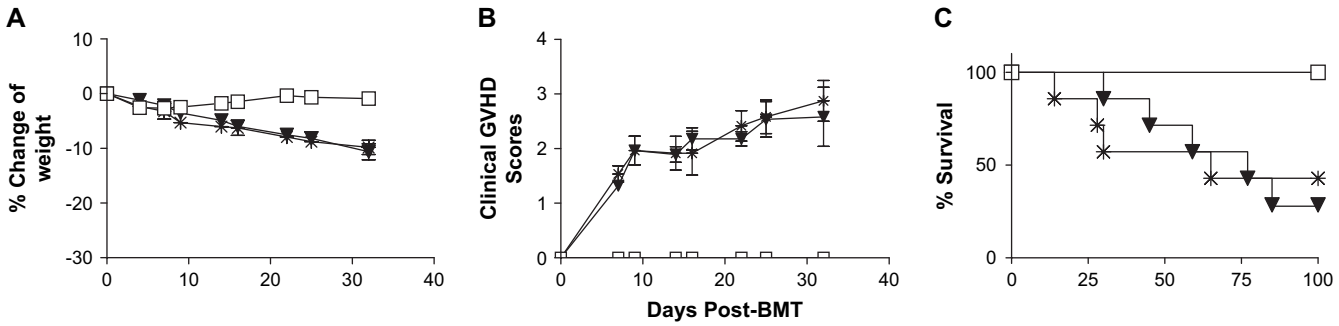

Figure 6. Similar graft-vs-host disease (GVHD) in recipients of Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$, or wild-type (WT) B6 spleen cells (SPCs) following major histocompatibility complex-matched mHAg-mismatched bone marrow transplantation. Lethally irradiated C3.SW mice received T-cell−depleted WT B6 (10×10^6 bone marrow cells [BMC]) and either Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ (1.6×10^6 CD4$^+$ and 0.5×10^6 CD8$^+$ T cells, ▼, n = 7) or WT B6 SPCs (1.5×10^6 CD4$^+$ and 0.6×10^6 CD8$^+$ T cells, *, n = 7). As a control, lethally irradiated C3.SW mice received C3.SW (10×10^6 BMCs, □, n = 3). (**A**) Mean percentage of weight change. C3.SW mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs show similar weight changes as those receiving WT B6 SPCs. (**B**) Clinical GVHD scores. C3.SW mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs had similar clinical GVHD scores as those receiving WT B6 SPCs. (**C**) C3.SW mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ and WT B6 SPCs showed similar mortality.

those receiving WT SPCs. While these differences might account for the increased GVHD severity in recipients of Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ SPCs in the mismatched model, this possibility does not detract from the clear-cut observation that severe GVHD and multiorgan tissue accumulation can be achieved by T cells that cannot generate functional PSGL-1.

Damage to intestinal mucosa induced by conditioning mediates translocation of lipopolysaccharide (LPS) from the intestinal lumen to the circulation, and stimulates the production of inflammatory cytokines such as TNF-α and IL-1, which promote GVHD [34]. However, the levels of serum LPS in both groups were similar, with the exception of a slight increase on day 4 in recipients of Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ SPCs compared to recipients of WT SPCs (data not shown). Therefore, increased translocation of LPS does not appear to explain the high level of mortality and clinical GVHD seen in the fully mismatched combination in recipients of Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ T cells. We also examined serum cytokine levels and tissue cytokine expression. TNF-α is an important mediator of tissue damage during GVHD, and its production is induced by conditioning, host tissue injury and allogeneic T-cell proliferation [14,35]. In our study, serum levels of TNF-α began to rise in the first week following transplantation, consistent with a clinical study [36]. On days 7 and 12, serum TNF-α levels were higher in mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ SPCs than in those receiving WT SPCs, possibly contributing to the accelerated mortality in mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ SPCs.

Analyses of cytokine expression in GVHD target tissues suggested that Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ Th1 cells as well as Th2 cells can traffic to the GVHD target tissues of irradiated recipients. However, in some organs, such as liver and lung, Th1 activity in the GVHD target tissues was reduced in recipients of the knockout T cells, as evidenced by lower levels of IFN-γ mRNA. At the same time, IFN-γ mRNA was increased in spleen, suggesting that Th1 may have been impaired in their ability to migrate out of the lymphohematopoietic system into GVHD target tissues. The results suggest that PSGL-1 plays a role, but is not absolutely required for tissue trafficking of Th1 cells in GVHD. The increased expression of IL-6 mRNA in lungs of Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ T-cell recipients on day 30 is consistent with Th2 cytokine deviation in some tissues of mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ SPCs, but other cytokines, including IFN-γ, show similar expression patterns in other GVHD target tissues. Therefore, both Th1 and Th2 cells derived from Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ mice appear to traffic to the GVHD target tissues.

Conflicting data have been obtained regarding the role of IFN-γ in acute GVHD. Increased levels of IFN-γ are observed in the serum of patients with acute GVHD [35,37–40]. Exogenous IFN-γ prevents GVHD [39], and is responsible for the protective effect of IL-12 against GVHD [41]. IFN-γ knockout donors produce increased or decreased GVHD, depending on the model examined [42,43]. Our study showed that the serum IFN-γ levels were lower on day 7 after a peak on day 4 in mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs compared to those receiving WT SPCs, but were similar at other time points. The lower level of serum IFN-γ after a peak in mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ SPCs might correlate with activity of alloreactive Th1 cells. On the other hand, the reduced IFN-γ expression in liver and lungs of mice receiving Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs might permit increased local expansion of GVH-reactive T cells, as IFN-γ promotes activation-induced cell death and limits T cell expansion [44].

In addition to the full haplotype mismatched strain combination, we also failed to observe resistance to GVHD in MHC-matched, mHAg-mismatched recipients of Fuc-TIV$^{-/-}$/Fuc-TVII$^{-/-}$ B6 SPCs. In the MHC-mismatched B6→B6D2F1 strain combination, CD4^{+} T cell-dependent acute GVHD can be mediated by inflammatory cytokines [45]. However, in the MHC-matched mHAg-mismatched BMT B6→C3.SW strain combination, acute GVHD is CD8^{+} T cell-dependent and involves both cytokines and cell-mediated cytotoxicity [45–47]. The lack of dependence on PSGL-1 function of GVHD in the MHC-matched strain combination suggests that CD8^{+} T cells do not absolutely require this molecule in order to migrate to GVHD target tissues.

Taken together, our results demonstrate that donor T cells can traffic to GVHD target tissues independently of PSGL-1 activity. They also suggest a possible role for P- and E-selectins in the regulation of Th1-cell−mediated GVHD in the liver and lungs, and possibly in the control of systemic cytokine production, especially IFN-γ and TNF-α. However, the redundancy of T-cell trafficking pathways in the highly inflamed environment of the conditioned recipient suggests that blockade of a single adhesion pathway is unlikely to have a major impact on GVHD across extensive MHC barriers.

Acknowledgments

We thank Mr. Orlando Moreno for technical assistance and expert oversight of animal care, Dr. Ronjon Chakraverty for thoughtful review the manuscript and Ms. Luisa Raleza for expert assistance with the article.

This work was supported by National Institutes of Health grant no. RO1 CA79989 and a Senior Research Award from the Multiple Myeloma Foundation.

References

1. Pelot MR, Pearson DA, Swenson K, et al. Lymphohematopoietic graft-vs-host reactions can be induced without graft-vs-host disease in murine mixed chimeras established with a cyclophosphamide-based non-myeloablative conditioning regimen. Biol Blood Marrow Transplant. 1999;5:133–143.

2. Mapara MY, Kim Y-M, Wang S-P, et al. Donor lymphocyte infusions mediate superior graft-versus-leukemia effects in mixed compared to fully allogeneic chimeras: a critical role for host antigen-presenting cells. Blood. 2002;100:1903–1909.
3. Kim Y-M, Sachs T, Asavaroengchai W, Bronson R, Sykes M. Graft-versus-host disease (GvHD) can be separated from graft-versus-lymphoma effects by controlling lymphocyte trafficking with FTY720. J Clin Invest. 2003;111:659–669.
4. Sykes M, Sheard MA, Sachs DH. Graft-versus-host-related immunosuppression is induced in mixed chimeras by alloresponses against either host or donor lymphohematopoietic cells. J Exp Med. 1988;168: 2391–2396.
5. von Andrian UH, Mackay CR. T-cell function and migration. Two sides of the same coin. N Engl J Med. 2000;343:1020–1034.
6. McEver RP. Selectins. Curr Opin Immunol. 1994;6:75–84.
7. McEver RP, Moore KL, Cummings RD. Leukocyte trafficking mediated by selectin-carbohydrate interactions. J Biol Chem. 1995;270: 11025–11028.
8. Maly P, Thall AD, Petryniak B, et al. The α(1, 3)Fucosyltransferase Fuc-TVII controls leukocyte trafficking through an essential role in L-, E-, and P-selectin ligand biosynthesis. Cell. 1996;86:643–653.
9. Weninger W, Ulfman LH, Cheng G, et al. Specialized contributions by alpha(1, 3)-fucosyltransferase-IV and FucT-VII during leukocyte rolling in dermal microvessels. Immunity. 2000;12:665–676.
10. Smithson G, Rogers CE, Smith PL, et al. Fuc-TVII is required for T helper 1 and T cytotoxic 1 lymphocyte selectin ligand expression and recruitment in inflammation, and together with Fuc-TIV regulates naive T cell trafficking to lymph nodes. J Exp Med. 2001;194:601–614.
11. Austrup F, Vestweber D, Borges E, et al. P- and E-selectin mediate recruitment of T-helper-1 but not T-helper-2 cells into inflamed tissues. Nature. 1997;385:81–83.
12. Blander JM, Visintin I, Janeway CA Jr, Medzhitov R. a(1, 3)-Fucosyltransferase VII and α(2, 3)-sialyltransferase IV are up-regulated in activated CD4 T cells and maintained after their differentiation into Th1 adn migration into inflammatory sites. J Immunol. 1999;163:3752.
13. Hirata T, Furie BC, Furie B. P-, E-, and L-selectin mediate migration of activated CD8+ T lymphocytes into inflamed skin. J Immunol. 2002;169:4307–4313.
14. Antin JH, Ferrara JLM. Cytokine dysregulation in acute graft-versus-host disease. Blood. 1992;80:2964–2968.
15. Blazar BR, Korngold R, Vallera DA. Recent advances in graft-versus-host disease (GVHD) protection. Immunol Rev. 1997;157:79–109.
16. Nikolic B, Lee S, Bronson RT, Grusby MJ, Sykes M. Th1 and Th2 cells both contribute to acute GVHD, and each subset has distinct end-organ targets. J Clin Invest. 2000;105:1289–1293.
17. Homeister JW, Thall AD, Petryniak B, et al. The alpha(1, 3)fucosyltransferases FucT-IV and FucT-VII exert collaborative control over selectin-dependent leukocyte recruitment and lymphocyte homing. Immunity. 2001;15:115–126.
18. Unkeless JC. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. J Exp Med. 1979;150:580–596.
19. Means TK, Hayashi F, Smith KD, Aderem A, Luster AD. The Toll-like receptor 5 stimulus bacterial flagellin induces maturation and chemokine production in human dendritic cells. J Immunol. 2003; 170:5165–5175.
20. Frenette PS, Subbarao S, Mazo IB, von Andrian UH, Wagner DD. Endothelial selectins and vascular cell adhesion molecule-1 promote hematopoietic progenitor homing to bone marrow. Proc Natl Acad Sci U S A. 1998;95:14423–14428.
21. Katayama Y, Hidalgo A, Furie BC, et al. PSGL-1 participates in E-selectin-mediated progenitor homing to bone marrow: evidence for cooperation between E-selectin ligands and alpha4 integrin. Blood. 2003;102:2060–2067.
22. Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft- versus-host disease after allogeneic bone marrow transplantation. J Exp Med. 2002;196:389–399.
23. Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. Blood. 2002;99:3493–3499.
24. Baker MB, Altman NH, Podack ER, Levy RB. The role of cell-mediated cytotxicity in acute GVHD after MHC-matched allogeneic bone marrow transplantation in mice. J Exp Med. 1996;183: 2645–2656.
25. Atarashi K, Hirata T, Matsumoto M, Kanemitsu N, Miyasaka M. Rolling of Th1 cells via P-selectin glycoprotein ligand-1 stimulates LFA-1-mediated cell binding to ICAM-1. J Immunol. 2005;174:1424–1432.
26. Mora JR, Cheng G, Picarella D, et al. Reciprocal and dynamic control of CD8 T cell homing by dendritic cells from skin- and gut-associated lymphoid tissues. J Exp Med. 2005;201:303–316.
27. Berlin C, Bargatze RF, Campbell JJ, et al. Alpha 4 integrins mediate lymphocyte attachment and rolling under physiologic flow. Cell. 1995;80:413–422.
28. Alon R, Kassner PD, Carr MW, et al. The integrin VLA-4 supports tethering and rolling in flow on VCAM-1. J Cell Biol. 1995;128: 1243–1253.
29. Kunkel EJ, Jung U, Bullard DC, et al. Absence of trauma-induced leukocyte rolling in mice deficient in both P-selectin and intercellular adhesion molecule 1. J Exp Med. 1996;183:57–65.
30. Steeber DA, Tang ML, Green NE, et al. Leukocyte entry into sites of inflammation requires overlapping interactions between the L-selectin and ICAM-1 pathways. J Immunol. 1999;163:2176–2186.
31. Nandi A, Estess P, Siegelman M. Bimolecular complex between rolling and firm adhesion receptors required for cell arrest; CD44 association with VLA-4 in T cell extravasation. Immunity. 2004;20: 455–465.
32. Itoh S, Matsuzaki Y, Kimura T, et al. Suppression of hepatic lesions in a murine graft-versus-host reaction by antibodies against adhesion molecules. J Hepatol. 2000;32:587–595.
33. Schlegel PG, Vaysburd M, Chen Y, Butcher EC, Chao NJ. Inhibition of T cell costimulation by VCAM-1 prevents murine graft-versus-host disease across minor histocompatibility barriers. J Immunol. 1995; 155:3856–3865.
34. Hill GR, Ferrara JLM. The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation. Blood. 2000; 95:2754–2759.
35. Imamura M, Hashino S, Kobayashi H, et al. Serum cytokine levels in bone marrow transplantation: synergistic interaction of interleukin-6, interferon-gamma, and tumor necrosis factor-alpha in graft-versus-host disease. Bone Marrow Transplant. 1994;13:745–751.
36. Visentainer JE, Lieber SR, Persoli LB, et al. Serum cytokine levels and acute graft-versus-host disease after HLA-identical hematopoietic stem cell transplantation. Exp Hematol. 2003;31:1044–1050.
37. Niederwieser D, Herold M, Woloszczuk W, et al. Endogenous IFN-gamma during human bone marrow transplantation. Analysis of serum levels of interferon and interferon-dependent secondary messages. Transplantation. 1990;50:620–625.
38. Allen RD, Staley TA, Sidman CL. Differential cytokine expression in acute and chronic murine graft-versus-host disease. Eur J Immunol. 1993;23:333–337.
39. Brok HPM, Heidt PJ, Van der Meide PH, Zurcher C, Vossen JM. Interferon-gamma prevents graft-versus-host disease after allogeneic bone marrow transplantation in mice. J Immunol. 1993;151:6451–6459.
40. Szebeni J, Wang M-G, Pearson DA, Szot GL, Sykes M. IL-2 inhibits early increases in serum gamma interferon levels associated with graft-vs-host disease. Transplantation. 1994;58:1385–1393.

41. Yang Y-G, Sergio JJ, Pearson DA, et al. Interleukin-12 preserves the graft-versus-leukemia effect of allogeneic CD8 T cells while inhibiting CD4-dependent graft-vs-host disease in mice. Blood. 1997;90: 4651–4660.
42. Murphy WJ, Welniak LA, Taub DD, et al. Differential effects of the absence of interferon-g and IL-4 in acute Graft-versus-host disease after allogeneic bone marrow transplantation in mice. J Clin Invest. 1998;102:1742–1748.
43. Welniak LA, Blazar BR, Anver MR, Wiltrout RH, Murphy WJ. Opposing roles of interferon-gamma on CD4+ T cell-mediated graft-versus- host disease: effects of conditioning. Biol Blood Marrow Transplant. 2000;6:604–612.
44. Refaeli Y, Van Parijs L, Alexander SI, Abbas AK. Interferon gamma is required for activation-induced death of T lymphocytes. J Exp Med. 2002;196:999–1005.
45. Teshima T, Ordemann R, Reddy P, et al. Acute graft-versus-host disease does not require alloantigen expression on host epithelium. Nat Med. 2002;8:575–581.
46. Duffner U, Lu B, Hildebrandt GC, et al. Role of CXCR3-induced donor T-cell migration in acute GVHD. Exp Hematol. 2003;31:897–902.
47. Baker MB, Altman NH, Podack ER, Levy RB. The role of cell-mediated cytotoxicity in acute GVHD after MHC-matched allogeneic bone marrow transplantation in mice. J Exp Med. 1996;183: 2645–2656.