LPAM (α4β7 integrin) is an important homing integrin on alloreactive T cells in the development of intestinal graft-versus-host disease

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Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is an important therapeutic modality for malignancies of hematopoietic origin, some metastatic solid tumors, as well as for a variety of nonmalignant diseases. Donor T lymphocytes are primarily responsible for both the beneficial graft-versus-tumor (GVT) effect following HSCT, as well as graft-versus-host disease (GVHD), which remains an important cause of posttransplantation morbidity and mortality. Although potential alloantigens are expressed on all host tissues, acute GVHD develops only in certain organs (skin, intestine, liver, and thymus), whereas other organs remain unaffected (eg, heart and kidney).1,2 The reasons for this GVHD target organ specificity remain largely unknown but could be related to organ specific differences in (1) susceptibility to tissue damage by the conditioning regimen and GVHD-associated cytokines and effector cells; (2) the inflammatory cytokine response; (3) numbers, types, and activation of antigen-presenting cells, and (4) activation, proliferation, and infiltration of T cells (and other effector cells).

The expression of specific adhesion molecules and chemokine receptors on T cells in combination with a spatial and temporal expression pattern of the ligands for these receptors by cells in the tissues is responsible for the tissue tropism of T-cell migration.3-6 Recirculation begins with a tethering and rolling phase, during which T cells in the blood transiently and reversibly interact with vascular adhesion receptors (including selectins, selectin ligands, and integrins) and sample the endothelium for activating factors (often chemokines). On activation, a combination of additional adhesion molecules, chemokines, and other signals will lead to an arrest of the T cell, followed by transmigration across the endothelium and further localization directed by tissue-associated chemokine gradients.3

Naive T cells express receptors, such as CCR7 (a receptor for CCL19 and 21), which allow them to recirculate through secondary lymphoid organs (spleen, lymph nodes, and Peyer patches).7 On encountering antigen presented by antigen-presenting cells in the secondary lymphoid organs, T cells become activated, begin to proliferate and differentiate, and reprogram their homing receptors so that they can migrate to specific extra-lymphoid tissues.

The α4β7, or LPAM (lymphocyte Peyer patch adhesion molecule) integrin is expressed on T (and B) cells and acts as an intestinal homing receptor. α4β7 interacts with mucosal addressin cell adhesion molecule-1 (MAdCAM-1) on high endothelial venules in the Peyer patches and intestinal lamina propria.8,9 Lymphocytes in spleen and mesenteric lymph nodes (MLNs) have low levels of α4β7 expression but can up-regulate their α4β7 expression on activation.10,11

Previous studies have demonstrated that inhibition of L-selectin, MAdCAM-1, or α4 and α6 integrin can ameliorate the development of acute GVHD.12-14 Therefore, we hypothesized that α4β7 expression on donor T cells would be important for the...
development of intestinal GVHD. In this study, we analyzed the ability of αβ⁺ and αβ⁻ selected donor T cells to infiltrate the intestinal mucosa of the recipient, cause systemic and organ-specific GVHD, and exert GVT activity.

Materials and methods

Cell line and antibodies

EL-4 is a murine T-cell leukemia/lymphoma derived originally from the C57BL/6 mouse and was obtained from ATCC (Manassas, VA). Cell culture medium consisted of Dulbecco modified essential medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine.

Antimurine CD16/CD32 Fc block (2.4G2) and fluorochrome-labeled antimurine antibodies against CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD62L (MEL-14), Ly-9.1 (30C7), and αβ⁺ (DATK32) were all obtained from Pharmingen (San Diego, CA).

Mice and BMT

Female CBA, B10.BR (H2k), C57BL/6 (B6, H-2b), and C3FeB6F1 (H2b/k) mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were used for the experiments between 8 and 12 weeks of age. BMT protocols were approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee. Briefly, BM cells were removed aseptically from the femurs and tibias and were T-cell depleted with anti-Thy-1.2 and low-TOX-M rabbit complement (Cedarlane Laboratories, Hornby, ON, Canada). Purified splenic T cells were obtained in 2 different ways. The first method consisted of passage of splenocytes over a nylon wool column (to remove monocytes/macrophages and B cells), followed by RBC lysis and staining with anti-CD3–fluorescein isothiocyanate (FITC) and anti-αβ⁺—phycoerythrin (PE) antibodies. These cells were then sorted into αβ⁺ and αβ⁻ CD3+ populations with the use of FACSVantage (BD Biosciences, San Jose, CA) or MoFlo (DakoCytomation, Fort Collins, CO) cell sorters in the Flow Cytometry Core Facility of the Memorial Sloan-Kettering Cancer Center. We opted for gating windows close to each other, so that we would capture sufficient αβ⁺ and αβ⁻ cells for in vivo experiments. We considered our highest priority to obtain sufficient numbers of αβ⁻ cells with as few as possible αβ⁺ T cells. Our αβ⁻ fraction purity after sorting was 98% ± 1.6% and our αβ⁺ fraction purity after sorting was 86.7% ± 4.9%. The second method of T-cell purification consisted of natural killer (NK) cell, B-cell, monocyte, granulocyte, and dendritic cell depletion of whole splenocytes by negative magnetic cell separation (Miltenyi Biotech, Auburn, CA), followed by anti-αβ⁺—PE antibody labeling and cell sorting into αβ⁻ and αβ⁺ populations. The allograft consisted of 5 × 10⁶ T-cell-depleted BM cells with or without 0.5 to 1 × 10⁶ unselected or αβ⁺ selected T cells. These cells were resuspended in DMEM and infused into the tail vein of lethally irradiated recipients on day 0. Prior to transplantation, recipients were killed on day 0 total body irradiation (137Cs source) of 1300 cGy (CBA) or 1100 to 1200 cGy (C57BL6, B10BR) as a split dose with 3 hours between doses. Mice were killed, and small intestine was dissected from the gastric-duodenal junction to the ileocecal junction. Intestines were washed/flushed by using a solution containing 10% HEPES (N-2-hydroxyethylpiperazine-N₂-ethanesulfonic acid), 10% HBSS (Hanks balanced salt solution), and 80% ddH₂O, cut into 1-cm-long pieces with luminal side exposed and placed in 50-ml conical tubes. Intestinal pieces were then incubated with DTE (dithioerythritol) for 20 minutes at 37°C, being continuously stirred. Following the incubation, the intestinal pieces were vortexed for 15 seconds, and the supernatant was collected. Incubation and collection of supernatant was repeated 2 more times, and then the supernatants were pooled and centrifuged at 1500 rpm for 5 minutes. The pellet was resuspended in 1 to 2 mL RPMI (without additives) and subsequently passed through a 0.3-μ nylon wool column to enrich the T-cell population.

Flow cytometric analysis

Splenocytes and intraepithelial lymphocytes (IELs), lamina propria lymphocytes (LPLs), and Peyer patch lymphocytes were washed, incubated with anti-CD16/CD32 Fc block, and subsequently incubated with fluorochrome-labeled primary antibodies at saturating concentrations for 20 minutes at 4°C. Flow cytometric analysis of these cells was performed on a FACSCalibur (Becton Dickinson) with CellQuest software. Appropriate isotype controls were used with each experiment.

Intracellular staining

Cells were incubated for 5 hours with phorbol myristate acetate (PMA); 10 ng/mL + ionomycin (2 μM). Brefeldin A (10 μg/mL) was added after 2 hours of incubation. The cells were then harvested, washed, and stained with fluorochrome-conjugated antibodies against cell surface antigens. Subsequently, cells were fixed and permeabilized with Cytofix/Cytoperm Kit reagents (Pharmingen) and stained with PE-conjugated anti-interferon γ (IFNγ; XMG1.2) antibodies from Pharmingen.

Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling

Cells were labeled with CFSE as described previously. Briefly, cells (thymocytes or splenocytes) were incubated with CFSE at a final concentration of 2.5 μM in HBSS at 37°C for 10 minutes. Cells were then washed 3 times with HBSS before intravenous injection.

Statistics

Histopathologic scores and thymocyte counts were compared between groups using the Wilcoxon rank sum statistics. The log-rank statistic was applied for comparison of survival data between groups. The area under the curve (AUC) was used to summarize the GVHD trajectory of each mouse under study. The statistic used to test whether a differential GVHD change occurred between treatment groups was the pairwise difference in the AUC between groups, using the Wilcoxon rank sum statistics. The log-rank statistic was applied for informative dropouts. The AUCs were calculated up to the minimum follow-up time for each pairwise difference in the double sum above. A permutation distribution was used to compute the achieved significance level.
Results

Recipients of $\alpha\beta^+$ donor T cells develop significantly less GVHD morbidity and mortality compared with recipients of LPAM$^-$ donor T cells

To study the role of $\alpha\beta^+$ expression on donor T cells in the development of intestinal GVHD, we used well-described clinically relevant major histocompatibility complex (MHC)-matched and -mismatched murine alllogeneic BMT models. We chose as an MHC-matched model with a disparity in minor histocompatibility antigens B10.BR → CBA.20 As an MHC-mismatched model, we used the B10.BR → B6 strain combination.21 In both models, lethally irradiated recipients were infused with $5 \times 10^6$ T-cell-depleted donor bone marrow (TCD-BM) cells, and GVHD was induced by the addition of donor splenic T cells to the allograft.

To determine the GVHD activity of $\alpha\beta^+$ and $\alpha\beta^-$ donor T cells, we obtained purified populations of $\alpha\beta^+$ or $\alpha\beta^-$ splenic donor T cells by flow cytometric cell sorting. We first analyzed if these $\alpha\beta^+$ or $\alpha\beta^-$ selected cells differed in their expression of CD62L, which plays an important role in the initial interactions of naive T cells with the endothelium in secondary lymphoid organs, and we found no differences ($\alpha\beta^+$ CD4$^+$CD62L$^+$, 78%; $\alpha\beta^-$ CD4$^+$CD62L$^+$, 66%; $\alpha\beta^+$ CD8$^+$CD62L$^+$, 89%; $\alpha\beta^-$ CD8$^+$CD62L$^+$, 86%; Figure 1A). We then assessed the alloreactive proliferation in vivo of the $\alpha\beta^+$ or $\alpha\beta^-$ selected T cells by adoptive transfer after labeling with CFSE into irradiated alllogeneic recipients and found no differences in the percentage of dividing cells and the number of divisions between the 2 selected populations (Figure 1B).

To detect potential differences in cytokine expression on allografting in vivo, we transferred $\alpha\beta^+$ and $\alpha\beta^-$ sorted B10.BR T cells and B10.BR TCD-BM into irradiated (750 cGy) CBA recipients and 3 days later determined intracellular IFN-γ expression in the splenocytes from these recipients (Figure 1C). We could not detect any profound differences in IFN-γ expression of donor CD4$^+$ or CD8$^+$ T cells in these recipients.

Finally, we determined that alloreactive donor T cells up-regulate their $\alpha\beta^+$ expression on allografting by adoptive transfer of unselected CFSE-labeled donor T cells into irradiated alllogeneic recipients (Figure 1D).

We then performed GVHD experiments by adding $0.5 \times 10^6$ (B10.BR → CBA) or $0.5 \times 10^6$ (B10.BR → B6) selected $\alpha\beta^+$ or $\alpha\beta^-$ T cells to the TCD-BM containing allograft. Allografts with only TCD-BM or TCD-BM + unsorted T cells were injected as control groups. CBA recipients of $\alpha\beta^-$ donor T cells had a significant delay and decrease in GVHD mortality and morbidity (Figure 2A) compared with recipients of $\alpha\beta^+$ or unsorted T cells. A similar delay in GVHD mortality and morbidity (Figure 2B) was observed when B6 recipients of $\alpha\beta^+$ donor T cells were compared with recipients of $\alpha\beta^-$ T cells. From these results we conclude that $\alpha\beta^-$ donor T cells have significantly less potential to induce GVHD than $\alpha\beta^+$ or unsorted donor T cells.

Recipients of $\alpha\beta^+$ donor T cells have higher numbers of $\alpha\beta^+$ donor T cells in their intestinal mucosa

To analyze whether $\alpha\beta^+$ or $\alpha\beta^-$ donor T cells differ in their capability to infiltrate into the intestinal mucosa of the recipient, we determined the numbers of B10.BR donor CD4$^+$ or CD8$^+$ $\alpha\beta^+$ T cells at 14 and 22 days after transplantation in the intestinal mucosa of the gut, spleen, peripheral lymph nodes, mesenteric lymph nodes, and liver in CBA recipients of $\alpha\beta^+$ or $\alpha\beta^-$ donor T cells (Figure 3). As expected, we found significantly higher numbers of $\alpha\beta^+$ T cells (both CD4$^+$ and CD8$^+$) in the mesenteric lymph nodes and gut of recipients of $\alpha\beta^+$ T cells at both time points. Interestingly, we also observed more $\alpha\beta^+$ T cells in the spleens of recipients of $\alpha\beta^+$ T cells on day 22, whereas $\alpha\beta^+$ T cells were increased in the peripheral lymph nodes of recipients of $\alpha\beta^-$ T cells on day 22.

Recipients of $\alpha\beta^+$ T cells develop intestinal and liver GVHD more rapidly than recipients of $\alpha\beta^-$ T cells

Mortality in our mouse models is primarily determined by intestinal GVHD and mucositis-associated infections related to intestinal GVHD. To analyze whether recipients of $\alpha\beta^+$ T cells incurred more severe intestinal GVHD, we analyzed GVHD-associated organ damage in terminal ileum, colon, liver, skin (ear and tongue), and thymus. We performed semiquantitative histopathologic analysis in a blinded fashion on the terminal ileum, colon, and liver. Dyskeratotic indices were calculated to determine the number of apoptotic keratinocytes per millimeter of epithelium. Thymic GVHD was assessed by calculating thymic cellularity as we described previously.22

We found significantly higher histopathologic scores of terminal ileum, colon, and liver in recipients of $\alpha\beta^+$ T cells on days 14 and 22 after transplantation compared with recipients of $\alpha\beta^-$ T cells (Figure 4A). The development of intestinal and liver GVHD
in recipients of $\alpha_\beta_7^+$ cells seemed to be slower than in $\alpha_\beta_7^+$ T-cell recipients, but histopathologic scores had increased to comparable levels by day 22 after transplantation.

In contrast, we observed no differences in skin and thymic GVHD in allogeneic or syngeneic recipients (Figure 4B) at 14 and 22 days after transplantation. These data suggest that intestinal and liver GVHD develops more rapidly in recipients of $\alpha_\beta_7^+$ T cells, which is consistent with the selective homing tropism of alloreactive $\alpha_\beta_7^+$ T cells. As expected, the development of skin and thymic GVHD is not affected by the expression of $\alpha_\beta_7$ by alloreactive T cells.

**Discussion**

Few studies have examined the role of integrin receptors in the development of GVHD. Tanaka et al. found in a parent-into-F1 allogeneic HSCT model with a full MHC class I and II disparity that the treatment of lethally irradiated recipients with anti-$\alpha_4$, -$\alpha_5$, or $\beta_3$ antibodies resulted in a moderate amelioration of intestinal GVHD (as measured by histopathologic criteria: reduction of the villous-crypt ratio and mononuclear infiltrate), whereas the combination treatment with anti-$\alpha_4$ and -$\alpha_5$ antibodies completely prevented intestinal GVHD by histopathologic criteria. These results suggested that these 2 integrins, which both can associate with $\beta_3$, are required for lymphocyte infiltration into the inflamed intestine during the development of GVHD. However, no data were provided regarding the effects of anti-$\alpha_4$ and -$\alpha_5$ antibody treatment on GVHD morbidity and mortality.

Li et al. used an acute GVH reaction (a-GVHR) model and transferred B6 splenocytes into nonirradiated C.B-17 severe combined immunodeficient (SCID) recipients. Preincubation of donor splenocytes with anti-$\alpha_4$ and anti-CD62L antibodies resulted in a delay in a-GVHR mortality with 3 of 10 long-term survivors.

Murai et al. recently demonstrated, using a-GVHR models, that the Peyer patches in the gut are required for the activation of donor antihost T cells. Their findings suggested that host dendritic cells in the subepithelial dome of Peyer patches express regulatory pathways that modulate T-cell activation and function during GVHD.

**Figure 2. Recipients of $\alpha_\beta_7^+$ T cells have increased GVHD mortality and morbidity.** Lethally irradiated (1300 cGy) CBA (A) and (1100-1200 cGy) C57BL/6 (B) recipients received transplants with B10.BR TCD-BM (5 × 10^7) and splenic T cells (0.5-1 × 10^7). T cells were included in the allograft as unsorted or $\alpha_\beta_7^+$-CD3+ and $\alpha_\beta_7^+$-CD3+ sorted populations from B10.BR donors. (A-B) Kaplan-Meier and clinical GVHD score (+ SEM) curves are shown that represent 4 (BM only), 8 to 11 (BM + T cells), and 13 to 16 (BM + $\alpha_\beta_7^+$ or $\alpha_\beta_7^+$ T cells) recipients per group from 2 combined experiments. Statistical analysis is as follows: (A) left, ○ versus $\square$ $P = .007$, $\square$ versus $\square$ $P < .0001$; right, ○ versus $\square$ $P < .01$, $\square$ versus $\square$ $P < .01$. (B) Left, ○ versus $\square$ $P = .0178$; right, ○ versus $\square$ $P < .02$.

**Figure 3. Recipients of $\alpha_\beta_7^+$ T cells have significantly higher numbers of $\alpha_\beta_7^+$ donor T cells in their intestinal mucosa and mesenteric lymph nodes.** CBA mice received transplants as described in Figure 2. Recipients of $\alpha_\beta_7^+$ and $\alpha_\beta_7^+$ T cells were killed on days 14 and 22 after BMT. Infiltrating $\alpha_\beta_7^+$ T cells of donor origin were determined by multicolor flow cytometry in multiple organs. Top panels represent averages of absolute cell numbers (+ SEM) of $\alpha_\beta_7^+$-CD4+ donor cells in spleen (day +14, n = 8; day +22, n = 9), peripheral lymph nodes (PLNs; day +14, n = 4; day +22, n = 4), mesenteric lymph nodes (MLNs; day +14, n = 3; day +22, n = 3), small intestine (gut, day +14, n = 3-4; day +22, n = 4-5) and liver (day +14, n = 6-7; day +22, n = 8-9). Bottom panels represent averages of absolute cell numbers of $\alpha_\beta_7^+$-CD8+ donor cells in the above mentioned organs. *Signifies statistically significant P values.*
in the attraction of CCR5+CD8+ donor T cells. The infiltration of donor splenocytes into the Peyer patches of the recipient and the development of a-GVHD could be prevented by disruption of CCR5 in donor T cells, by inhibition of the interaction between αβ7 and MadCAM-1 with neutralizing anti-MadCAM-1 antibodies, or by using recipients, which lack Peyer patches.

These studies established the importance of αβ7 in the development of intestinal GVHD and demonstrated that the inhibition of the α7 integrin with neutralizing antibodies can ameliorate intestinal GVHD. Our experiments using true GVHD (not a-GVHR) models confirm that αβ7 is important for the development of intestinal GVHD and demonstrate that intestinal GVHD can be delayed by depleting αβ7+ T cells from the allograft. This delay in intestinal GVHD is associated with a significant decrease in GVHD morbidity and mortality. The association between a delay in the invasion of an epithelial target tissue by alloreactive T cells and a decrease in GVHD morbidity and mortality is reminiscent of the well-documented decrease in the GVHD alloresponse seen with delayed posttransplantation infusion of donor lymphocytes.23,24 This relationship between the timing of donor lymphocyte infusion and the severity of the GVHD alloresponse could be related to the degree of inflammation, tissue damage, and levels of inflammatory cytokines (cytokine storm) early after transplantation,25 as well as the turnover of host-derived antigen-presenting cells.26

Naive T cells have a uniform intermediate cell surface expression of αβ7, whereas the expression of αβ7 on CD4+ activated/memory T cells follows a bimodal pattern with a negative and a positive population (Williams and Butler6 and A.P., M.R.M.v.d.B., our unpublished observations, February 27, 2003). For practical reasons, we selected in our experiments donor T cells only on their αβ7 expression. Therefore, both αβ7+ and αβ7– populations contained naive and activated/memory T cells, and we found equal percentages of CD62L expression in both populations. Several investigators have proposed that naive T cells express a variety of homing molecules, which allow them to recirculate through secondary lymphoid organs (spleen, lymph nodes, and Peyer patches).7,27 In support of this theory, Williams and Butler found that naive CD4+ splenic T cells, which had a uniform intermediate expression level of αβ7, homed equally well to spleen, Peyer patch, peripheral or mesenteric lymph nodes, when injected into congenic mice.7

On encountering antigen presented by antigen-presenting cells in the secondary lymphoid organs, T cells become activated, begin to proliferate and differentiate, and reprogram their homing receptors so that they can migrate to specific extra-lymphoid tissues. Therefore, we hypothesize that the naive T cells in our αβ7– selected population of donor T cells can become activated after encountering alloantigens in the mesenteric lymph nodes of the host resulting in a delayed (and decreased) infiltration of αβ7+ donor T cells in recipients of αβ7– selected T cells (Figure 3).

Table 1. Cause of death (GVHD versus tumor) for all recipients that died during the course of the experiment

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A similar delay between recipients of $\alpha_\beta^+$ and $\alpha_\beta^-$ T cells was observed in the development of intestinal and liver GVHD. Recipients of $\alpha_\beta^+$ T cells had developed extensive GVHD in liver and intestines at day +14 after transplantation, whereas recipients of $\alpha_\beta^-$ T cells developed maximal liver and intestinal GVHD by day +22 (Figure 4). Interestingly, the severity and kinetics of skin and thymic GVHD did not differ between recipients of $\alpha_\beta^+$ and $\alpha_\beta^-$ T cells, which argues against a general decrease in alloreactivity of $\alpha_\beta^-$ T cells compared with $\alpha_\beta^+$ T cells as an explanation for the differences in GVHD morbidity and mortality between recipients of $\alpha_\beta^+$ and $\alpha_\beta^-$ T cells.

The higher numbers of $\alpha_\beta^+$ donor T cells in the spleens of recipients of $\alpha_\beta^+$ donor T cells at day +22 could be due to greater allostimulation at this later time point, when the recipients of $\alpha_\beta^+$ donor T cells have developed more severe GVHD (see GVHD morbidity data in Figure 2A-B). This difference in overall GVHD was observed in the development of intestinal and liver GVHD. This could also be due to greater alloactivation in recipients of donor T cells, resulting in more rapid trafficking of alloactivated T cells from the peripheral lymph nodes into the target organs.$\alpha_\beta^+$ T cells have developed more severe GVHD in recipients treated with a sphingosine-1-phosphate receptor agonist (FTY720).10 FTY720 binds to T-cell G protein–coupled receptors, which affects migration of lymphocytes and traps them in secondary lymphoid tissues.

In conclusion, this study demonstrates the importance of the expression of $\alpha_\beta^+$ by alloreactive donor T cells in the development of intestinal GVHD and overall GVHD morbidity and mortality. It supports the notion that strategies aimed at the inhibition of homing of alloreactive T cells to gut, including neutralizing antibodies against $\alpha_\beta^+$, could result in significant improvement in the prevention and/or treatment of intestinal GVHD and overall morbidity and mortality from GVHD.

References
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