Effects of Donor T Cell Trafficking and Priming Site on GVHD

Induction by Naïve and Memory Phenotype CD4 T Cells

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Running title: T cell trafficking in GVHD induction
Abstract

Graft-versus-host disease (GVHD) remains a major cause of morbidity and mortality in allogeneic stem cell transplantation (alloSCT). Effector memory T cells (TEM) do not cause GVHD but engraft and mount immune responses, including graft-vs.-tumor effects. One potential explanation for the inability of TEM to cause GVHD is that TEM lack CD62L and CCR7, which are instrumental in directing naïve T cells (TN) to lymph nodes (LN) and Peyers patches (PP), putative sites of GVHD initiation. Thus TEM should be relatively excluded from LN and PP, possibly explaining their inability to cause GVHD. We tested this hypothesis using T cells deficient in CD62L or CCR7, transplant recipients lacking PNAd ligands for CD62L, and recipients without LN, PP or LN, PP and spleen. Surprisingly, CD62L and CCR7 were not required for TN-mediated GVHD. Moreover, in multiple strain pairings GVHD developed in recipients that lacked LN and PP. Mild GVHD could even be induced in mice lacking all major secondary lymphoid tissues (SLT). Conversely, enforced constitutive expression of CD62L on TEM did not endow them with the ability to cause GVHD. Taken together, these data argue against the hypothesis that TEM fail to induce GVHD because of inefficient trafficking to LN and PP.
Introduction

The application of allogeneic stem cell transplantation (alloSCT) is limited by graft-versus-host disease (GVHD). GVHD can be prevented by purging T cells from the graft; however, donor T cells are important for facilitating stem cell engraftment, providing immediate post-transplant immunity and eliminating residual leukemic cells\(^1\). A key challenge is to balance the positive and negative effects of donor T cells to optimize outcome.

We showed that purified CD4\(^+\) T\(_N\) (CD62L\(^+\)CD44\(^-\)CD25\(^-\)) initiated severe GVHD, whereas T\(_{EM}\) (CD62L\(^-\)CD44\(^+\)CD25\(^-\)) did not, but mounted a strong recall responses to a vaccination antigen\(^2\). Chen and colleagues demonstrated that total T\(_{EM}\) (CD4 and CD8 cells) did not induce GVHD but if primed \textit{in vivo} against leukemia, these cells induced graft-versus-leukemia responses\(^3\). These studies and others\(^2-5\) suggested that transplanting memory T cells may provide some of the benefits of donor T cells without the risk of GVHD.

However, the reason for reduced GHVD-induction by T\(_{EM}\) is unclear. One fundamental difference between T\(_N\) and T\(_{EM}\) is their trafficking patterns. T\(_N\) primarily circulate through the blood, spleen and LN and are excluded from non-lymphoid tissues. In contrast, T\(_{EM}\) generally bypass LN but can enter parenchymal tissues as well as spleen\(^6\). These circulation patterns are largely determined by the expression of CD62L, CCR7 and CD44. T\(_N\) express CD62L and CCR7 while T\(_{EM}\) express neither but upregulate CD44,
which promotes entry to inflamed non-lymphoid tissues\textsuperscript{7,8}. CD62L binds to PNAd ligands on high endothelial venules, is essential for T cell entry into peripheral lymph nodes (pLN), and promotes but is not required for T cell migration to mesenteric LN (mLN) and Peyer’s patches (PP)\textsuperscript{9,10}. CCR7 is essential for optimal homing of T\textsubscript{N} to all LN and to a lesser extent governs entry to PP\textsuperscript{11,12}.

In GVHD donor T\textsubscript{N} and T\textsubscript{EM} encounter host antigens regardless of where they traffic. Secondary lymphoid organs, including LN and PP, contain high concentrations of “professional” antigen-presenting cells that display host antigens and are required to activate donor T cells\textsuperscript{13-15}. Therefore, it is reasonable to hypothesize that donor T cells will be effectively primed to recipient antigens in LN and PP. Thus, the inability of T\textsubscript{EM} to induce GVHD could be because they lack CD62L and CCR7 and, relative to T\textsubscript{N}, they are restricted from LN and PP. A corollary of this idea is that T\textsubscript{N} must be primed in LN and PP to induce GVHD.

In support of this hypothesis, in GVHD models, donor T cells homed to secondary lymphoid organs within 24 hours after infusion but did not infiltrate target organs until days later\textsuperscript{16,17}. Moreover, when T\textsubscript{N} and T\textsubscript{EM} were transplanted separately, T\textsubscript{N} accumulated in LN and PP whereas T\textsubscript{EM} did not\textsuperscript{16}. Also consistent with an important role for LN priming, interrupting the engagement of LN-homing receptors CD62L and \(\alpha 4\beta 7\) on donor T cells can ameliorate GVHD. Incubating donor T cells with antibodies against CD62L and CD49d (\(\alpha 4\) integrin) prior to their infusion reduced homing of T cells to recipient LN by half and significantly delayed or prevented GVHD\textsuperscript{18,19} and GVHD.
mediated by CD62L⁻/⁻β7⁻/⁻ donor T cells was significantly delayed²⁰. However, in the latter studies, GVHD was not completely prevented, suggesting that LN-priming of donor T cells may not be essential for GVHD. Thus, data conflict regarding the hypothesis that TEM do not cause GVHD because they do not traffic to LN.

Here we use several approaches to test the hypothesis that TEM do not induce GVHD because they are restricted from LN and PP and the related hypothesis that TN must access these SLT to cause GVHD. Contrary to our expectations, blocking homing of TN to LN—thereby rendering them more like TEM—had little impact on GVHD, whereas TEM genetically modified to improve access to LN still failed to induce GVHD. Overall, these results suggest that differential ability to traffic to LN/PP plays a limited role in explaining why TN cause GVHD and TEM do not.

Materials and methods

Mice

BALB/c, C57Bl/6 (B6), 129/Sv and CD45.1 (B6) mice were from the National Cancer Institute (Frederick, MD). B6.C mice (B6-H2d congenics) were from Jackson Labs (Bar Harbor, ME). B6bm12 and IAb⁻/⁻ (B6 MHC II-deficient) were from Taconic Farms (Hudson, NY). BALB.B mice were from Harlan (Indianapolis, IN). CD62L⁻/⁻ (B6)⁹ and GST2/3⁻/⁻ (B6)²¹ mice were gifts from Tom Tedder and Steve Rosen, respectively. CCR7⁻/⁻ (BALB/c)¹¹ were a gift from Martin Lipp and Uta Hopken. Aymphoplasia (B6) (aly/aly)²² and heterozygous littermates (aly/+) were obtained from Fadi Lakiss. Mice transgenic for CD62L with a mutated protease site (LΔP) and control transgenic mice
with a normal protease site were described\textsuperscript{23,24}. All mice were bred at Yale University or at the University of Minnesota under specific pathogen free conditions. Recipients were 8-12 weeks at the time of initial transplant.

**Splenectomy**

Mice were anesthetized with ketamine (100mg/kg)/xylazine (10mg/kg) solution via i.p. injection and received 0.3mg/kg meloxicam i.p. prior to surgery for pain relief. Splenectomies were performed as described\textsuperscript{25}. Postoperative treatment included ibuprofen in the drinking water (30mg/kg/day) for 3 days after surgery and antibiotic (trimethoprin/sulfamethoxizole)-supplemented water for 7 days after surgery. Mice were allowed to recover for 3-5 weeks prior to undergoing a bone marrow transplant (BMT).

**Bone Marrow Transplant**

Transplants were performed according to protocols approved the by Yale University or University of Minnesota IACUC. Recipients received total body irradiation as indicated from a \textsuperscript{137}Cs source (Yale) or x-irradiator (Minnesota). Three to five hours later all recipients received T cell depleted bone marrow (BM) with or without donor T cells via tail vein injection. Unfractionated spleen cells, spleen cells enriched for CD4\textsuperscript{+} or CD4\textsuperscript{+}CD25\textsuperscript{+} cells were transplanted to induce GVHD depending on the GVHD model. CD25\textsuperscript{+} cells were depleted in some experiments to reduce the GVHD-modulating effects of regulatory T cells\textsuperscript{26-28}. Transplanted T cell type and doses are indicated in the figure legends. For studies performed at Yale University, animals were fed moistened chow and water supplemented with trimethoprin/sulfamethoxizole for two weeks following
BMT. For wild type (WT) or MHC II^{−}→aly/aly Spl- chimeras, splenectomized recipients received two doses of 500cGy separated by 3 hours. Two hours later, mice received 8×10^{6} CD45.1 (B6) or 10.5×10^{6} MHC II^{−}CD45.1 (B6) non-T cell depleted BM cells. Mice were allowed to recover for 7 weeks and then 3 mice of each group were sacrificed and analyzed for full donor chimerism and antigen presenting cell (APC) turnover by FACS analysis of cells isolated from BM, liver and intestine, and tissue staining of skin and intestine. For BALB/c→C57BL/6 aly/+ or aly/aly transplants at the University of Minnesota, mice were given 800 cGy total body irradiation day -1 followed by pan-T-cell depleted BM (day 0) with or without supplemental BALB/c splenocytes, as described^{28}.

**Cell separations**

BM cells were isolated and prepared as previously described^{2}. Remaining Thy1.2-positive cells were routinely less than 0.5% of BM cells as determined by flow cytometry.

CD4^{+}CD25^{−} cells were enriched from RBC-depleted splenocytes by negative selection using streptavidin-coated MACS beads and an AutoMACS® (Miltenyi Biotech) after incubating with blocking antibody (anti-FcR, clone 2.4G2), biotinylated anti-CD8 (TIB105) and biotinylated anti-CD25 (PC61). Resulting cells had <1% CD4^{+}CD25^{+} or CD8^{+} cells as determined by flow cytometry.
CD4\(^+\) cells were positively-selected using streptavidin-coated MACS beads and biotinylated anti-CD4 (GK1.5) on an AutoMACS. Resulting cells were >95% CD4\(^+\) as determined by flow cytometry. The type of CD4 purification used and whether CD25\(^+\) cells were depleted is stated in the text legend of each figure; for simplicity groups receiving any type of CD4\(^+\) cell are designated “CD4” on the graphs themselves.

T\(_N\) and T\(_{EM}\) CD4\(^+\)CD25\(^-\) subsets were isolated from L\(\Delta P\) transgenic mice by staining positively-selected CD4\(^+\) cells with streptavidin-Alexa488 (Invitrogen, Eugene, OR), anti-CD45RB-PE (C363.16A, BD Biosciences, San Jose, CA), CD25-PE/Cy7 (PC61, BD Biosciences) and CD44-APC (IM7, BD Biosciences). Cells were sorted into T\(_N\) (CD45RB\(^{high}\)CD44\(^-\)) and T\(_{EM}\) (CD45RB\(^{low}\)CD44\(^+\)) subsets using a FACSARia (BD Biosciences). We validated this approach by analysis of B6 splenocytes stained with anti-CD4, anti-CD25, anti-CD45RB, anti-CD44 and anti-CD62L. Back-gating showed the CD4\(^+\)CD25\(^-\)CD45RB\(^{high}\)CD44\(^-\) T\(_N\) population to be approximately 80% CD62L\(^+\) and the CD45RB\(^{low}\)CD44\(^+\) T\(_{EM}\) population to be approximately 90% CD62L\(^-\).

**Antibodies and Fusion Proteins**

Mel-14 protein was purified from serum-free tissue culture medium as described\(^{29}\). LT\(\beta\)RIg and TNFRIg fusion proteins were kind gifts of Drs. Jeff Browning and Evangelia Notidis (BiogenIdec) and were used exactly as described\(^{30}\). Rat IgG control protein was obtained from Sigma-Aldrich (St. Louis).
GVHD Analysis

Recipients were weighed on day +1 or day -1 after BMT and every 3-4 days thereafter. Beginning on day +16 mice were monitored for clinical signs of GVHD: diarrhea, skin disease and hunched posture. Mice that died of radiation toxicity early after transplant (days 0-10) prior to the onset of GVHD were censored with the exception of experiments shown in Figure 3. Mice with evidence of severe GVHD (very hunched and/or lethargic) or having lost >20% of their original weight (following recovery from irradiation) or considered clinically moribund (University of Minnesota) were sacrificed and scored as dead. The last weight recorded for sacrificed animals remained in the data set.

Throughout this work, unless otherwise indicated, BM-only controls among various types of recipients in the same experiment were indistinguishable and were thus pooled for the purposes of graphing and statistical analysis.

Pathological Analysis

Tissues were fixed in 10% phosphate buffered formalin, paraffin embedded, sectioned and stained with hematoxylin and eosin. Slides were read by pathologists expert in liver (A.D.), gastrointestinal (A.D, A.P-M., and D.J.), and skin disease (J.M.) without knowledge of experimental groups. Liver scoring criteria: portal inflammation (0-3), bile duct injury (0-3), central perivenulitis (0-3), lobular necroinflammatory activity (0-4). Colon slides in Figure 3B were scored by D.J. Scoring criteria: inflammation (0-3), apoptosis (0-3), crypt loss (0-3). Colon slides in Figure 3D were scored by APM using published criteria31. Colon slides in Figure 4B and 5D were scored by A.D. Scoring
criteria: inflammation (0-3), apoptosis (0-4), neutrophilic abscesses (0-3), crypt loss (0-3), ulceration (0-3).2.

**Statistical Methods**

Significance for differences in weight loss was calculated by an unpaired t-test. P-values for survival curves were calculated by log rank test. P-values for histology comparisons were calculated by Mann-Whitney or Fisher’s exact test [Figure 3F, BM ctrls vs. CD4→alyaly Spl- only; and Figure 3G, BM vs. CD4→(MHC IIalyaly Spl-) and BM vs. CD4→(WTalyaly Spl-) only].

**Results**

**Functional CD62L on donor T cells is not required for GVHD**

To test whether CD62L-mediated homing to recipient peripheral LN was required for GVHD, we transplanted BALB/c recipient mice with CD4+CD25 T cells from B6 wild type (WT) or CD62L-/- donors9 (Figure 1A). Recipients of WT T cells were also treated on days -1, +2 and +5 with control rat IgG or an effective dose32 of blocking antibody to CD62L (Mel-1433), which markedly reduces T cell migration to peripheral LNs in vivo33. Mel-14 treatment resulted in a significant, but slight delay in the kinetics of GVHD death as compared to control treatment though the final mortalities were identical (Figure 1A).

CD62L-/- CD4+CD25- (Figure 1A) or CD4+ T cells (Figure 1B) caused GVHD-mediated mortality similar to that induced by WT T cells (Figure 1A and B, P>0.3), confirming
recently published results\textsuperscript{20}. GVHD is maximal with both CD4 and CD8 cells in the MHC-matched B6\textrightarrow{}BALB.B model\textsuperscript{34} and total CD62L\textsuperscript{−/−} T cells caused indistinguishable GVHD-related mortality to WT T cells (Figure 1C). Taken together, these experiments indicate that T\textsubscript{N} need not express CD62L in order to cause GVHD. While Mel-14 treatment slightly reduced GVHD, given the results with CD62L-deficient T cells, this may be attributable to depletion of antibody-coated T cells and/or a partial blockade of neutrophil entry to inflamed sites\textsuperscript{35,36}.

To test whether CD62L\textsuperscript{−/−} T cells are primed the spleen, we transferred B6 CD62L\textsuperscript{−/−} CD4\textsuperscript{+} T cells into splenectomized BALB/c recipients (Figure 1B). Unexpectedly, GVHD mortality was paradoxically accelerated in splenectomized recipients of CD62L\textsuperscript{−/−} CD4\textsuperscript{+} T cells as compared to spleen-intact recipients (Figure 1B). Thus, splenic priming is not required and presumably donor T cells can be primed in other locations less-dependent on CD62L for T cell entry, such as mLN and PP.

**Impairing CD62L ligands on recipient LN does not ameliorate GVHD**

We also tested GVHD induction by WT T cells in recipients that lack glucosyl transferases 2 and 3 (GST2/3\textsuperscript{−/−}) which are necessary to generate ligands for CD62L on HEV\textsuperscript{21}. There is greatly reduced homing of T cells to pLN and a substantial reduction in homing to mLN in these mice\textsuperscript{21}. We used GST2/3\textsuperscript{−/−} mice as recipients in the MHC-matched 129/Sv\textrightarrow{}B6 nonlethal GVHD model, which typically demonstrates moderate weight loss and significant liver pathology. Similar to the results with CD62L\textsuperscript{−/−} T cells,
GST2/3−/− recipients of WT CD4+CD25− T cells developed GVHD as severe, if not more so, than WT recipients (Figures 2A-B).

While the prior experiments with CD62L−/− T cells and GST2/3−/− recipients exclude an essential role for the engagement of CD62L on donor T cells with PNAd ligands in the host, T N also use CCR7 to enter LN and PP 11,12 and residual homing to LN in GST2/3−/− mice is likely mediated by CCR721. To impair both CD62L and CCR7-mediated T cell homing, we transplanted GST2/3−/− recipients with CCR7−/− donor CD4 T cells in the BALB/c→B6 strain pairing. At day 37 after transplant, there was equivalent death and liver pathology in GST2/3−/− recipients of either T cell type (Figures 2C-D). Therefore reducing LN homing mediated by both CD62L and CCR7 did not significantly ameliorate GVHD.

**LN, PP and/or spleen deficient recipients develop GVHD**

That CD62L−/− T cells induced GVHD in splenectomized recipients raised the possibility that even without CD62L or CCR7 donor T cells could gain sufficient access to LN and PP to cause GVHD. Although trafficking studies that used large number of transferred cells have clearly shown important roles for these molecules in LN entry one or a few hours after transfer, this has been less-well studied in the context of GVHD, with studies only visualizing T cells from day 2 and beyond16,17,20. In our experience it was technically difficult to track the smaller numbers of cells typically used in a GVHD experiment at very early time points (day 1 and earlier) that reflect direct homing potential. It was not
possible to sort enough TEM cells to clearly visualize them post-transplant (unpublished observations).

We therefore took a more definitive approach by using recipient mice that lack LN and PP altogether. aly/aly mice are devoid of most lymphoid tissue\(^2\)\(^2\), although they retain nasal associated lymphoid tissue (NALT) and cryptopatches\(^2\)\(^3\)\(^7\)\(^9\). aly/+ (with WT phenotype) or aly/aly mice were splenectomized or left intact to create the following types of recipients: WT (aly/+); LN/PP present, no spleen (aly/+ Spl-); no LN/PP, spleen present (aly/aly); no LN/PP or spleen (aly/aly Spl-). We initially used these recipients in the B6\(^{bml2}\)→B6 GVHD model along with minimally lethal transplant conditions so that histologic GVHD could be more readily and comparably assessed. As expected, aly/+ recipients of CD4\(^+\)CD25\(^-\) cells had significant weight loss compared to BM controls. Most importantly, GVHD occurred in the absence of LN/PP or LN/PP and spleen, as aly/aly and aly/aly Spl- recipients of CD4\(^+\)CD25\(^-\) cells lost weight, albeit significantly less than aly/+ recipients. aly/aly Spl- recipients had further reduced weight loss compared with aly/aly recipients, suggesting in this context that lack of spleen led to amelioration of disease. Unexpectedly, aly/+ Spl- T cell recipients developed accelerated and severe GVHD (Figure 3A). Thus, in this model, spleen or LN/PP alone are sufficient for moderate to severe clinical GVHD to develop. Conversely, despite the absence of nearly all organized SLT, even aly/aly Spl- recipients developed significant weight loss as compared to BM controls, although weight loss was reduced as compared to the other T cell recipient groups.
T cell infiltration and target tissue damage occurred in the majority of T cell recipients compared to BM controls, regardless of *aly* genotype and spleen presence (Figure 3B). The severity of histologic GVHD paralleled the weight loss data. In both the colon and skin, *aly/+* mice had the highest average pathology scores, followed by *aly/aly* and then *aly/aly* Spl- recipients. It is difficult to make comparisons with *aly/+* Spl- T cell recipients as these organs were harvested between days 8 and 10 post BMT as compared to day 23 for the other recipients. We could not evaluate the liver for GVHD as *aly/aly* mice develop spontaneous hepatic inflammation that is difficult to distinguish from GVHD. Taken together, these data show that GVHD can develop in the complete absence of LN and PP, as well as in recipients without LN, PP and spleen. However, that GVHD is markedly reduced in *aly/aly* Spl- recipients relative to mice that have either LN, PP or spleen indicates that these are important sites for donor T cell priming that are redundant among themselves.

We extended the investigation of the roles of LN/PP to a second MHC-mismatched strain pairing, BALB/c→B6. B6 *aly/+* recipients of BALB/c spleen cells died of rapidly lethal GVHD whereas the majority of *aly/aly* recipients survived for the duration of the experiment (Figure 3C). Although in this strain pairing the absence of LN/PP was protective, *aly/aly* spleen cell recipients still had clear histologic evidence of colonic GVHD, even at day 9 post-transplant (Figure 3D). Therefore, although the clinical disease was reduced in the absence of LN/PP, donor T cells could still be primed.
Since the majority of human alloSCTs are MHC-matched, we investigated requirements for CD4 T cell priming in the MHC-matched 129/Sv→B6 model. This is generally a nonlethal model with little clinical skin disease (unpublished data). We were therefore surprised that severe clinical skin disease developed in nearly all aly/aly (19/22) and a quarter of the aly/aly Spl- (5/20) CD4 cell recipients (Figure 3E). In contrast, 0/35 BM control mice, 1/23 aly/+ CD4 cell recipients and 1/19 aly/+ Spl- CD4 cell recipients had clinical skin disease. Pathologic analysis of skin sections confirmed that aly/aly T cell recipients had typical cutaneous GVHD (Figure 3F). While several individuals in the aly/aly Spl- cohort had very high skin pathology scores, statistical significance (by Mann-Whitney test) was not reached when compared to the BM control group, most likely due to fluctuation of scores in the mildly affected range. A Fisher’s exact test on the number of unaffected (score <5) and affected (score >5) mice demonstrated a significant difference (P<0.01) between the groups. Thus, CD4 cells need not be primed in LN, PP or spleen to induce cutaneous GVHD—even in MHC-matched alloBMT. Furthermore, in the absence of LN/PP, priming of T_N donor T cells in the spleen and elsewhere may generate T cells that preferentially induce skin GVHD.

aly/aly mice have defects in NFκB signaling, which could have contributed to our findings, independent of the absence of LN/PP^{22}. Although one would have anticipated that host APCs defective in NF-κB signaling would induce blunted T cell responses, to confirm our findings in mice rendered LN/PP-deficient due to the aly/aly mutation, we created LN/PP-deficient mice by treating wild type BALB/c pregnant mothers with both LTβR Ig and TNFRIg, which prevents the development of LN and PP in pups^{30}. The
absence of LN/PP was confirmed in littermates prior to transplantation and at the time of sacrifice post-transplant. Both WT and LN/PP-deficient (LTβR1g/TNFRIg) BALB/c recipients of B6.C CD4+CD25− cells developed similar GVHD as revealed by survival and histologic score (Figures 4A-B).

Thus in four different strain pairings CD4+ T N did not require priming in LN and PP to induce GVHD. Interestingly, depending on the model studied, manifestations of GVHD were ameliorated, equivalent or more severe in the absence of recipient LN/PP suggesting that the relative importance of LN/PP to the GVHD syndrome is model-dependent.

MHCII expression on hematopoietic cells is required for T cell priming in aly/aly Spl- recipients

Occurrence of GVHD in aly/aly Spl- recipients suggested that parenchymal tissues alone could activate T N donor T cells when priming in SLT is prevented. To address this we created B6/MHC II−/− → B6/aly/aly Spl- and control WT → aly/aly Spl- BM chimeric recipients in which host APCs were MHC Class II-deficient or intact, and used these mice as recipients in a second GVHD-inducing transplant (B6bm12 → B6 model). We observed histologic GVHD in the colons of CD4→ (WT → aly/aly Spl-) recipients, which paralleled the disease seen in the CD4→ aly/aly Spl- (non-chimeric) recipients shown in Figure 3B. However, we observed no histologic disease in the CD4→ (MHC II−/−
Memory cells constitutively expressing CD62L do not cause GVHD

We addressed the hypothesis that TEM would induce GVHD if more efficiently recruited into LN by taking advantage of transgenic mice we created in which T cells constitutively express CD62L. These transgenic mice (LΔP) express CD62L driven by the CD2 promoter and have been backcrossed to B6 CD62L+/− mice. Additionally, the metalloprotease site in CD62L was mutated such that CD62L cannot be shed. A control transgenic with an intact metalloproteinase site expresses CD62L constitutively that can be shed after activation. Activated LΔP T cells enter pLN more efficiently than activated WT T cells, although somewhat less efficiently than unactivated, naive WT or LΔP T cells.

Because we could not use CD62L as a marker to sort LΔP TEM, CD4+CD25- cells from WT B6 or LΔP mice were sorted into T_N and T_EM subsets based on CD45RB and CD44 expression (Figure 5A and Materials and Methods) and transplanted separately into BALB/c recipients. Recipients of T_N from either WT or LΔP donors developed severe GVHD as demonstrated by extensive weight loss and death and high pathology scores (Figure 5B-D). Hence, CD62L downregulation, which does not occur in LΔP mice, is not required for T cells to exit LN and infiltrate tissues during GVHD. In contrast, all recipients of T_EM from either WT or LΔP donors survived and returned to approximately
100% of their original weight (Figure 5B and C). Weight gain and pathologic scores for recipients of LΔP T_{EM} versus WT T_{EM} were not statistically different, indicating that CD62L-expressing LΔP T_{EM} did not have an enhanced ability to cause GVHD. Similar studies using control transgenic (instead of WT) donor T cells versus LΔP donor T cells also showed minimal GVHD caused by both transgenic T_{EM} (Figure 6). Overall, these data demonstrate that constitutive CD62L expression on T_{EM} does not endow them with the ability to cause severe GVHD similar to that caused by T_{N}.

**Discussion**

Compared to T_{N}, T_{EM} cells are much less effective at causing GVHD\(^2\,^5\). We tested two related hypotheses to explain why T_{N} cause GVHD and T_{EM} do not: 1) donor T cells need to be primed in recipient LN in order to cause GVHD; and 2) donor T_{EM} cells lack the expression of LN-homing molecules, in particular CD62L and CCR7, which reduces their entry to and priming in recipient LN and dampens their ability to cause GVHD.

Our results argue against both of these hypotheses. With regard to the first, though CD62L and CCR7 are critically important for T cell entry into LN, T_{N} lacking CD62L or CCR7 caused severe GVHD. Furthermore, recipients with defective or completely absent LN still developed GVHD. Thus, restricting T_{N} homing to sites accessible by T_{EM} did not impair their ability to cause GVHD. With respect to the second hypothesis, we found that T_{EM} with constitutive expression of CD62L still had very limited capacity to cause GVHD. These data formally exclude the hypothesis that priming in LN is required for GVHD induction and suggest that, at least as a consequence of reduced CD62L-
expression, reduced LN-homing by $T_{EM}$ does not by itself account for why $T_N$ cells cause GVHD but $T_{EM}$ do not.

We took multiple approaches to suppress $T_N$ cell entry into LN. CD62L-deficiency had no effect on GVHD, while inhibiting WT donor T cells with CD62L blocking antibody delayed GVHD only slightly. We further inhibited T cell migration to LN by infusing CCR7$^{-/}$ T cells into GST2/3$^{-/}$ mice, resulting in simultaneous inhibition of both chemokine and CD62L-mediated T cell entry, yet GVHD was essentially unaffected and in some cases even exacerbated (Figure 2). Given the lack of impact of these pathways on GVHD-induction by $T_N$, we reason that the absence of CCR7 and CD62L on $T_{EM}$ in turn could not solely explain their inability to cause GVHD.

Dutt et al.$^{20}$ also found that CD62L$^{-/}$ and WT T cells initiated comparable GVHD, but concluded that the ability to traffic to mLN was nonetheless critical for GVHD because recipients of CD62L$^{-/}$β7$^{-/}$ T cells had improved survival and in particular little gut GVHD. Yet, in several of the models we tested, gut GVHD was evident in the absence of LN/PP. To reconcile these results, we first note that even in the CD62L$^{-/}$β7$^{-/}$ mice, there was weight loss and histologic changes consistent with GVHD. In addition, the apparent difference in the conclusions from the two studies could be ascribed to the interpretation of the effect of β7 deficiency. We suggest that genetic deletion of β7 on donor T cells ameliorated GVHD by reducing the entry of activated T cells into not just mLN but also intestinal tissue. Thus, if priming can occur alternatively in the spleen, as our data would indicate, the $\alpha4\beta7^{hi}$ cells generated in mice lacking LN/PP could still enter target tissues,
causing GVHD, as we observed. Whereas β7-deficient cells would be impaired in ability
to enter target tissues thus leading to reduced GVHD. Indeed, α4β7hi but not α4β7low cells
enter intestinal sites41. This explanation is supported by the finding that after
transplantation donor T cells did not highly upregulate α4β7 until 5 rounds of cell
division in recipient mLN16, suggesting that high levels of this molecule were not
necessary for SLT entry and priming. Consistent with this view, Petrovic et al.42 found
that α4β7-deficient T cells caused reduced intestinal but similar skin and thymic GVHD
compared to WT cells.

Experiments exploiting genetic deficiency of CD62L, CCR7, and GST2/3 were limited
by how well the particular gene deficiencies restrict T cell trafficking as well as their
potential to affect T cell entry into target tissues. To eliminate T cell priming in LN/PP
completely, we induced GVHD in recipient animals lacking all LN/PP. In all strain pairs
tested, recipients deficient in all LN/PP, but with an intact spleen, still developed GVHD.
GVHD was ameliorated (but present) in two models, exacerbated in one model and
equivalent in a third. Taken together, these data suggest that priming in LN/PP is not
required to induce GVHD but may be required for maximal GVHD in certain genetic
mismatches or experimental conditions.

Somewhat surprisingly, we found that GVHD developed in splenectomized aly/aly
recipients that lacked essentially all SLT, although the disease was reduced compared to
intact controls. BM chimera experiments proved that BM-derived APCs were still
required to initiate GVHD in the absence of spleen, LN and PP. These experiments did
not address where T cells were primed. Potential sites include the liver\textsuperscript{43}, bone marrow\textsuperscript{44}, NALT or cryptopatches, or other inducible gut or lung associated lymphoid tissue\textsuperscript{45}. Indeed, recent studies showed that CD8 responses to influenza infection do not require secondary lymphoid organs\textsuperscript{45,46}. Although GVHD initiation is not accompanied by the same infection-induced inflammatory signals, secondary lymphoid architecture may not be absolutely required when antigen is ubiquitous, as is the case of GVHD.

We unexpectedly observed that altering the trafficking and priming site of donor T cells affected the nature of GVHD. In three MHC-mismatched models, LN/PP-intact but splenectomized recipients developed more rapidly lethal GVHD than normal recipients. There are several nonexclusive explanations for this finding. The spleen may be a source of radiation-resistant recipient regulatory cells, which can suppress GVHD\textsuperscript{47}. If the spleen traps alloreactive cells\textsuperscript{48,49}, in its absence donor T cells may have enhanced ability to migrate to target organs and LN. Finally, T cells activated in the spleen vs. LN may develop differently. For example, there may be altered cytokine skewing or expression of alternate homing receptors\textsuperscript{6,50-52}, which in some contexts could affect GVHD induction.

These results are relevant to a related paper that was submitted and published while our manuscript was under review\textsuperscript{53}. In parallel with our studies, though using a single MHC-mismatched model of GVHD, Beilhack et al. also used a variety of strategies to restrict trafficking and priming sites of GVHD-inducing lymphocytes. These workers found that preventing priming in the LN/PP had no effect, from which they concluded that priming
site did not alter GVHD. In contrast, we clearly found effects of absence of LN/PP alone on GVHD in three models—decreased GVHD in 2 and augmented skin GVHD in the third. Mice lacking spleen, leading therefore to priming in LN/PP and elsewhere also had more severe and different GVHD. Thus, we conclude that priming site does affect the character of GVHD. The different conclusions could be attributable to different methodologies, as we documented GVHD by clinical assessment, weight loss, and quantitative pathologic assessment whereas Beilhack et al. relied on bioluminescence imaging and in some cases survival curves. Alternatively or in addition, these outcomes are model-dependent and by studying more and different models we uncovered the dependence of GVHD on priming site that was not found in the model used by Beilhack et al. These workers also concluded that there was no GVHD in mice lacking LN/PP (B6.LTa KO mice) that had also been splenectomized. However, we found, in both MHC-matched and MHC-mismatched models, that mice completely lacking SLT do get GVHD. Again, differences between our positive result and their negative result could be attributable to methodology, model systems or both.

Though our studies restricting the trafficking and priming venues of T_N cells suggested that the reason T_EM are unable to mediate GVHD is not because of inability to traffic to particular sites, a complementary aspect of the work used the opposite approach—to restore homing capacity to T_EM cells using our L∆P transgenic system in which CD62L cannot be downregulated or shed. Activated L∆P cells can enter LN more efficiently than activated or memory WT cells. Therefore L∆P memory cells should have better
trafficking to recipient LN than WT memory cells. Nevertheless, ΔP TEM cells were no different from WT TEM in promoting minimal or no GVHD.

Considering our results in context\textsuperscript{16,20,54-56}, it is no longer reasonable to think that any one site, or even SLT per se, is required or critical for the induction of GVHD. However, as altering the priming site modulates disease quality or severity, we suggest that the priming site does qualitatively affect the nature of GVHD. This for example would account for the predominant effect on gut GVHD of α4β7 integrin expression and presumptive T cell priming in mLN\textsuperscript{20,42}. By the same token, GVHD induction in the absence of all SLT, as well as the inability of constitutive CD62L expression by TEM to restore the ability to mediate GVHD, argue that trafficking differences alone do not account for the markedly greater GVHD-inducing potency of T\textsubscript{N} over TEM cells, as had been proposed\textsuperscript{16,20}. Rather, additional explanations for this clinically relevant phenomenon remain to be uncovered.

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Authorship

Contribution: B.E.A. designed and executed experiments, analyzed data, and wrote the paper; A.A. contributed unique reagents and insights; P.A.T. and B.R.B designed and carried out experiments; J.M.M., D.J., A.J.D. and A.P.-M. interpreted and scored histopathologic sections; W.D.S. and M.J.S. designed experiments, analyzed data, and wrote the paper.

W.D.S. and M.J.S. contributed equally to this study.

Conflict of Interest Disclosure: the authors declare no competing interests.

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References


Figure Legends

Figure 1. Impairing CD62L on donor T cells has minimal impact on GVHD.
Survival curves are shown. (A) On day 0 BALB/c recipients received 800 cGy and \(10^7\) BM cells from WT B6 donors with or without \(3 \times 10^5\) CD4\(^+\)CD25\(^-\) cells from WT B6 or CD62L\(^{-/-}\) (B6) donors. Mice that received WT CD4\(^+\)CD25\(^-\) cells were injected on days -1 (intravenous), +2 (intraperitoneal) and +5 (intraperitoneal) with 250µg Mel-14 or control Rat IgG. \(\ast\) \(P<0.05\) for CD4 \(\rightarrow\) Mel-14 vs. CD4 \(\rightarrow\) Rat IgG. \(P=0.3334\) for WT CD4 \(\rightarrow\) Rat IgG vs. CD62L\(^{-/-}\) CD4. (B) BALB/c recipients were splenectomized (Spl-) or left intact (Spl intact) 3 weeks prior to BMT. On day 0 recipients received 800 cGy and \(10^7\) BM cells from WT B6 donors with or without \(3 \times 10^5\) CD4\(^+\) cells from WT B6 or CD62L\(^{-/-}\) (B6) donors. \(\ast\) \(P<0.05\) for Spl intact vs. Spl\(^-\) recipients of CD4 T cells. (C) On day 0 BALB.B recipients received 850 cGy and \(10^7\) BM cells from WT B6 donors with or without \(3 \times 10^6\) total T cells from WT B6 or CD62L\(^{-/-}\) (B6) donors. NS = not significant for recipients of WT vs. CD62L\(^{-/-}\) T cells.

Figure 2. Impairing CD62L ligands on recipient LN has minimal impact on GVHD.
(A and B) On day 0 WT B6 or GST2/GST3\(^{-/-}\) (B6) (GST) recipients received 1000 cGy and \(7 \times 10^6\) BM cells with or without \(4 \times 10^6\) CD4\(^+\)CD25\(^-\) cells from 129/Sv donors. Mice were sacrificed on days 38 and 41 to collect tissues for histopathological analyses. (A) Weight curve. \(\dagger\) \(P<0.05\) for BM ctrls vs. CD4\(\rightarrow\)WT or vs. CD4\(\rightarrow\)GST for all days 24 and later; \(\ast\) \(P<0.05\) for CD4\(\rightarrow\)WT vs. CD4\(\rightarrow\)GST for all days 14 and later. (B) Liver pathology. NS: not significant for CD4\(\rightarrow\)WT vs. CD4\(\rightarrow\)GST; \(P<0.001\) for BM ctrls vs. CD4\(\rightarrow\)WT or vs. CD4\(\rightarrow\)GST. (C and D) On day 0 GST recipients received 1000cGy
and 1.3x10^7 BM cells from BALB/c donors with or without 3x10^5 CD4^+ cells from BALB/c or CCR7^-/-(BALB/c) donors.  (C) Survival curve. NS: not significant for WT CD4→GST vs. CCR7^- CD4→GST; P<0.05 for BM ctrls vs. WT CD4→GST or vs. CCR7^- CD4→GST.  (D) Liver pathology. NS: not significant for WT CD4→GST vs. CCR7^- CD4→GST; P<0.01 for BM ctrls vs. CD4→WT or vs. CD4→GST.

**Figure 3. LN, PP (aly/aly) and/or spleen deficient mice develop GVHD.** (A and B) aly/+ or aly/aly recipients were splenectomized (Spl-) or left intact 3-5 weeks prior to BMT. On day 0 recipients received 1000 cGy and 8-10x10^6 BM cells with or without 10^6 CD4^+CD25^- cells from B6^bm12 donors. All CD4→aly/+ Spl- animals died by day 10 or were sacrificed in a premorbid state for pathologic examination. Shown are combined data from two experiments. (A) Weight curve. † P<0.01 for BM ctrls vs. all CD4 groups for all days 6 and later; ✝ P<0.01 for CD4→aly/+ vs. CD4→aly/aly or vs. CD4→aly/aly Spl- for all days 10 and later, @ P<0.05 for CD4→aly/aly vs. CD4→aly/aly Spl- for all days 17 and later. (B) Skin and colon pathology. † P<0.05 for BM ctrls vs. all CD4 groups; other P values are indicated on the graphs. (C and D) On day 0 aly/+ or aly/aly recipients received 800 cGy and 10^7 T cell depleted BM cells with or without 5x10^5 spleen cells from BALB/c donors. (C) Survival curve. (D) Colon pathology at day 9 post-BMT. † P<0.05 for BM ctrls vs. Spl→aly/+ or vs. Spl→aly/aly. (E and F) aly/+ or aly/aly recipients were splenectomized (Spl-) or left intact 3-5 weeks prior to BMT. On day 0 recipients received 1000 cGy and 10^7 BM cells with or without 5x10^6 CD4^+CD25^- cells from 129/Sv donors. Shown are combined data from two experiments. (E) Incidence of clinical skin disease. † P<0.05 for BM ctrls vs. CD4→aly/aly or vs.
CD4→aly/aly Spl-. (F) Skin pathology. † P<0.0001 for BM ctrls vs. CD4→aly/aly. (G) aly/aly recipients were splenectomized, allowed to rest for 3-5 weeks and then transplanted with WT B6 BM (WT→aly/aly Spl-) or MHC II⁻/⁻ (B6) BM (MHC II⁻/⁻→aly/aly Spl-). Eight weeks later these mice received two doses of 450 cGy (separated by three hours) and 10⁷ BM cells with or without 10⁶ CD4⁺CD25⁻ cells from B6bml2 donors. On day 27 post transplant, mice were sacrificed and colon tissue collected for histologic analysis. Colon pathology; note that BM control groups are shown separately. NS: not significant for BM vs. CD4→(MHC II⁻/⁻→aly/aly Spl-); P=0.0005 for CD4→(WT→aly/aly Spl-) vs. CD4→(MHC II⁻/⁻→aly/aly Spl-); P<0.001 for BM vs. CD4→(WT→aly/aly Spl-).

**Figure 4. LN and PP deficient LTβRIg/TNFRIg-treated recipients develop GVHD.**

LN and PP-deficient BALB/c recipients were created by treating pregnant mothers with LTβRIg and TNFRIg. On day 0 WT or LN/PP-deficient (LTβRIg/TNFRIg) recipients received 750 cGy and 9x10⁶ BM cells with or without 1.3x10⁶ CD4⁺CD25⁻ cells from B6.C donors. (A) Survival curve. † P<0.05 for BM ctrls vs. CD4→WT or vs. CD4→LTβRIg/TNFRIg. Two BM control mice were sacrificed at day 17 due to extensive weight loss (>30% of original weight) but there was no histologic evidence of GVHD in these animals. (B) Liver, colon and skin pathology. P<0.05 for BM ctrls vs. CD4→WT or vs. CD4→LTβRIg/TNFRIg for all analyses; * P<0.05 for CD4→WT vs. CD4→LTβRIg/TNFRIg; NS: not significant for CD4→WT vs. CD4→LTβRIg/TNFRIg.
Figure 5. Memory cells constitutively expressing CD62L do not cause severe GVHD. (A) Sorting strategy. MACs-purified CD4⁺CD25⁻ cells (left panel) were subsequently sorted into T<sub>N</sub> and T<sub>EM</sub> fractions based on CD45RB and CD44 expression according to the indicated gates; post-sort analysis in rightmost panels. Sort of LΔP cells is shown; sorting parameters for WT and LΔP cells were identical. (B-D) On day 0 BALB/c recipients received 800 cGy and 10<sup>7</sup> BM cells from CD45.1 B6 donors with or without 3x10<sup>5</sup> naïve or memory cells from WT B6 or LΔP (B6) donors. (B) Weight curve. † P<0.05 for BM ctrls vs. WT naïve or vs. LΔP naïve for all days 10 and later; @ P<0.05 for BM ctrls vs. LΔP memory for all days 20 and later. * P<0.05 for BM ctrls vs. WT memory at day 23 only. (C) Survival curve. † P<0.01 for BM ctrls vs. WT naïve or vs. LΔP naïve. (D) Liver, colon and skin pathology. P<0.05 for BM ctrls vs. WT naïve or vs. LΔP naïve for all analyses; NS: not significant for WT memory vs. LΔP memory.

Figure 6. Memory cells constitutively expressing CD62L do not cause severe GVHD. On day 0, BALB/c recipients received 800 cGy and 10<sup>7</sup> BM cells from CD45.1 B6 donors with or without 3x10<sup>5</sup> naïve or memory cells from control transgenic (metalloproteinase site intact, Ctrl Tgic) or LΔP donors. Shown are combined data from three experiments with sort-purified cells as described in the Figure 5 legend. (A) Weight curve. † P<0.05 for BM ctrls vs. Ctrl Tgic naïve or vs. LΔP naïve for all days 6 and later; @ P<0.05 for BM ctrls vs. Ctrl Tgic memory or vs. LΔP memory for all days 17 and later. (B) Survival curve. NS: not significant for BM ctrls vs. WT memory or vs. LΔP memory; NS: not significant for WT memory vs. LΔP memory; † P<0.0001 for BM ctrls vs. Ctrl Tgic naïve or vs. LΔP naïve.
Figure 1

A  B6 → BALB/c

- — BM ctrl (n=4)
- — WT CD4 + Rat IgG (n=7)
- — WT CD4 + Mel14 (n=10)
- — CD62L−/− CD4 (n=8)

B  B6 → BALB/c

- — all BM ctrl (n=10)
- — CD62L−/− CD4 + Spl intact (n=15)
- — CD62L−/− CD4 + Spl− (n=15)

C  B6 → BALB.B

- — BM ctrl (n=5)
- — WT total T cells (n=12)
- — CD62L−/− total T cells (n=12)
Figure 2

A weight curve

B liver pathology

BALB/c → B6

C survival curve

D liver pathology

Figure 2
Figure 4
Figure 5
Figure 6

B6 → BALB/c

A weight curve

B survival

Figure 6
Effects of donor T cell trafficking and priming site on GVHD induction by naive and memory phenotype CD4 T cells