Leukocyte migration and graft-versus-host disease

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Abstract

Graft-versus-host disease (GVHD) remains a significant complication of allogeneic bone marrow transplantation (allo-BMT). Acute GVHD is mediated by immunocompetent donor T cells, which migrate to lymphoid tissues soon after infusion, recognize host alloantigens, and become activated upon interaction with host antigen presenting cells (APCs). Recent work from our group and others suggests that activated effector T cells exit lymphoid tissues and traffic to mucosal sites and parenchymal target organs such as the GI tract, liver, lung, and skin where they cause tissue damage. The molecular interactions necessary for effector cell migration during GVHD have become the focus of a growing body of research, as these interactions represent potential therapeutic targets. In this review we will discuss chemokine/chemokine receptor interactions and adhesion molecules that have been shown to play roles in effector cell migration in experimental GVHD models, and discuss a potential model for the role of chemokines during the activation phase of GVHD.
Graft-versus-host disease (GVHD) remains a significant complication of allo-BMT, and limits the clinical applicability of transplantation. Three phases have been described in acute GVHD\textsuperscript{1,2}. In the conditioning phase, host tissues are damaged by the pre-transplant conditioning regimen. Conditioning regimens upregulate inflammatory mediators including IL-1 and TNF\textsubscript{α}, and adhesion molecules, and enhance the expression of MHC and costimulatory molecules by tissue APCs. In the activation phase, donor T cells interact with host APCs, leading to activation/differentiation toward a T\textsubscript{H}1/T\textsubscript{C}1 effector pathway, and migration of these cells to target tissues affected during acute GVHD. The effector phase involves target organ damage via cytolytic effector mechanisms such as TNF\textsubscript{α}, perforin/granzyme, Fas/FasL (CD95/CD95L), and reactive oxygen species. This review will focus on the migration of donor T cells during the activation phase of GVHD and the proteins that are important for this.

**Migration of Donor Cells During Experimental GVHD**

Our group tracked the migration of enhanced green fluorescent protein (eGFP) transgenic donor cells during the first week post-transplant in a fully MHC-mismatched murine allo-BMT model\textsuperscript{3}. Interestingly, donor T cells partitioned to lymphoid tissues within hours after transplantation, independently of recipient conditioning and allogeneic disparity. Within 2-3 days after transplantation, allogeneic T cells expanded in lymphoid tissues. Between 3 and 7 days post-transplant, allogeneic T cell numbers increased in GVHD target organs including the GI tract, liver, lung, skin and bone marrow, and tissues not considered to be common GVHD target organs such as the CNS, gingiva, and nasal.
mucosa. These data suggested that donor T cells had seeded target organs at a level below the detection limits of the imaging system, and required 3-7 days to expand to detectable levels in situ, or that donor T cells had been activated in lymphoid tissues during the first days after transplant, and subsequently migrated into target organs. The latter notion is supported by data described below demonstrating the presence, early post-transplant, of T cell effector cytokine expression in spleen but not target organs such as liver and skin\textsuperscript{4,5}. Additionally, the sphingosine-1-phosphate receptor inhibitor FTY720, which prevents lymphocyte egress from lymphoid organs\textsuperscript{6}, inhibited target organ infiltration and GVHD lethality when administered starting at the time of transplant, in a murine model\textsuperscript{7}. The efficacy of FTY720 in reducing target organ infiltration suggests that T cells do not arise de novo from direct donor cell expansion in target organs, but from the migration of donor cells previously activated in lymphoid tissues.

**Leukocyte Migration Paradigm**

Lymphocytes migrate into secondary lymphoid tissues via a well characterized, multi-step process\textsuperscript{8} (see Supplemental online video 1). The initial step involves reversible tethering and rolling of lymphocytes on the surface of specialized high endothelial venules (HEV), primarily via the interaction of selectins and their carbohydrate ligands. Next, the rolling lymphocyte encounters chemokines linked to the lumenal surface of the HEV via proteoglycans. Signaling through lymphocyte chemokine receptors leads to firm arrest of the lymphocyte on the HEV surface\textsuperscript{9}. Transmigration through the HEV wall and into the node is currently not well understood, but may involve interactions
between lymphocyte integrins and junctional adhesion molecules, as well as CD31, and CD99, which localize to the intercellular junctions between high endothelial cells (reviewed in 10).

While the migration of T cells via HEV has been well characterized, the migration of leukocytes into parenchymal organs during inflammation is less well understood. This process likely involves changes in vascular permeability, and in certain systems, has been shown to require specific selectin/ligand, chemokine/receptor, and integrin/ligand interactions.

Supplemental Online Video 1. T Cell Migration Paradigm. This animated sequence demonstrates the steps involved in the firm arrest and migration of a T cell across the vascular endothelium.

Introduction to Leukocyte Migration

Selectins

The selectins, L-, P-, and E-selectin, are a family of C-type lectins expressed primarily by leukocytes and endothelial cells, which bind specific carbohydrate epitopes (reviewed in 11). The interaction of selectins with their ligands is characterized by a rapid on-off rate, leading to low-affinity adhesion of leukocytes to endothelial cells, and rolling during
blood flow. L-selectin is constitutively expressed on myeloid cells, naïve lymphocytes, and central memory T cells. Carbohydrate ligands for L-selectin are the peripheral node addressins (PNAds). PNAds are constitutively present on HEV, and inducibly present at sites of chronic inflammation. E- and P-selectin are expressed on endothelial cells, (as well as platelets in the case of P-selectin). P-selectin was previously shown to be constitutively expressed on lung endothelium, and E-selectin on cutaneous endothelium. However, in response to inflammation, both are upregulated on endothelial cells regardless of tissue location. The carbohydrate ligands for E and P selectins are expressed on various leukocyte subsets, including activated T cells. P- and E-selectins have been shown to play crucial roles in T cell recruitment to inflamed skin and synovium, but their role in recruiting effector cells to GVHD target organs is not yet clear.

**Chemokines and their receptors**

Chemokines are a large family of predominantly 8-12 kD proteins which primarily function as leukocyte chemoattractants. These proteins have additional functions in processes such as angiogenesis (reviewed in), hematopoiesis (reviewed in), and immune cell activation (reviewed in). The family is subdivided based on the number and position of NH₂-terminal cysteine (C) residues. The majority of chemokines fall into the CC (CCL1-28) and CXC (CXCL1-16) subfamilies, while the C family contains only two (XCL1 and XCL2), and CX₃C only one member (CX₃CL1). These proteins exert their effects through interactions with a family of 7 transmembrane domain-containing G-
protein coupled receptors (GPCR). Signaling by chemokine receptors is mediated by heterotrimeric G-proteins containing $G_{\alpha_i}$. These activate protein and lipid kinases such as MAP, JAK-STAT, and phosphatidyl inositol-3-kinase (PI3K), which mediate actin cytoskeleton rearrangement, changes in integrin affinity/avidity, leukocyte proliferation, differentiation and apoptosis (reviewed in 26). There is significant redundancy in the chemokine system as shown by the binding of multiple chemokines to a particular receptor, and multiple receptors interacting with a particular chemokine. There are currently 10 identified CC chemokine receptors (CCR1-10), 6 CXC receptors (CXCR1-6) one C receptor (XCR1) and one CX$_3$C receptor (CX$\_3$CR1)$^{24,25}$.

Three functional classes of chemokines/receptors have been defined$^{19}$. The homeostatic family includes those molecules functioning specifically in the migration of leukocytes to and within lymphoid tissues, as well as those functioning in hematopoiesis, including chemokines such as CCL19 and 21, which bind to CCR7$^{27-33}$, CXCL12 and its receptor CXCR4$^{34-39}$, and CXCL13 and its receptor CXCR5$^{40-42}$. The inflammatory family includes a wide array of molecules involved in the migration of innate and adaptive immune effector cells to sites of inflammation (see Fig. 1). Chemokines in this group are expressed in response to a host of inflammatory stimuli including pathogen-associated molecular products, and inflammatory cytokines such as TNF$\alpha$, and type I and type II interferons$^{19}$. These chemokines are expressed by tissue cells, fibroblasts, endothelial cells, dendritic cells, monocytes, NK-cells and T cells. The dual function family includes CCL1, 17, 25 (and receptors CCR8, 4, and 9, respectively), CXCL9-11 (interacting with CXCR3), and CXCL16 (interacting with CXCR6) which function both in inflammation,
and in the migration of T cells within the thymus. Several chemokines within this group are involved in the recirculation of memory T cells restricted to specific tissues such as the small intestine (CCL25/CCR9) and skin (CCL17, 22/CCR4).

**Fig. 1. Inflammatory Chemokines and Receptors**

<table>
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<tr>
<th>Cells Producing</th>
<th>Chemokines</th>
<th>Receptors</th>
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<tbody>
<tr>
<td>APCs, endothelium, epithelial cells (various)</td>
<td>CXCL8 (IL-8)</td>
<td>CXCR1</td>
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<td>CXCL1 (GROα/KC)</td>
<td>CXCR2</td>
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<td>CXCL2 (GROβ)</td>
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<td>APCs, endothelium, epithelial cells, neutrophils, thymus, T cells</td>
<td>CXCL9 (Mig)</td>
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<td>CXCL11 (T-TAC)</td>
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<td>Endothelium, epithelial cells, fibroblasts, monocytes</td>
<td>CCL2 (MCP-1)</td>
<td>CCR2</td>
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<td>CCL7 (MCP-3)</td>
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<td>CCL25 (TECK)</td>
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<td>CX3CL1 (Fractalkine)</td>
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<td>CX4CR1</td>
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**Leukocyte Receptor Expression**

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<th>L</th>
<th>Ma</th>
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**Fig. 1. Inflammatory chemokines and receptors. Abbreviations:** APC (antigen presenting cell), B (B cell), D (dendritic cell), E (eosinophil), L (Langerhan’s cell), Ma (mast cell), Mφ (macrophage), N (neutrophil), NK (natural killer cell), P (platelet), T1 (T<sub>H1</sub>/T<sub>C1</sub> cell), T2 (T<sub>H2</sub>/T<sub>C2</sub> cell). (References 20,44-94).

The cellular makeup of infiltrates at a site of inflammation is determined both by the chemokines induced at the site, and the responsiveness of various cell types to those...
chemokines. After activation within secondary lymphoid tissues, T cells downregulate CCR7 and become less responsive to chemokines involved in trafficking of naïve cells to lymphoid tissues. Activated T effector cells upregulate different sets of receptors for inflammatory chemokines\textsuperscript{95}. \(T_H1/T_C1\) cells preferentially express CCR1, CCR2, CCR5, CXCR3 and CXCR6, whereas \(T_H2/T_C2\) cells preferentially upregulate CCR4, and some studies indicate CCR3 (Fig. 1).

Responsiveness to chemokines is further regulated by desensitization of receptors after chemokine binding. Homologous desensitization occurs in a ligand-dependent manner and involves phosphorylation of the receptor via specific G-protein-coupled receptor kinases (GRKs), followed by \(\beta\)-arrestin-mediated targeting for endocytosis\textsuperscript{96}. Ligand-independent desensitization also occurs, when stimulation of a heterologous GPCR leads to phosphorylation of others through second messenger-dependent kinases such as PKC, and subsequent G-protein de-coupling\textsuperscript{97}. In this manner, cross-talk between chemokine receptors has been demonstrated\textsuperscript{98,99}.

**Integrins**

The integrins are a family of heterodimeric transmembrane proteins functioning in cell-cell and cell-extracellular matrix (ECM) adhesion. There are currently 18 known \(\alpha\) subunits and 8 \(\beta\) subunits in mammals\textsuperscript{100}. The integrins relevant to the immune system include those in the \(\beta1\), \(\beta2\) and \(\beta7\) families (see Fig. 2). The \(\beta2\) family is restricted to leukocytes\textsuperscript{101} and includes \(\alpha_L\beta2\) (CD11a/CD18, LFA-1), \(\alpha_M\beta2\) (CD11b/CD18, Mac-1),
$\alpha_X\beta_2$ (CD11c/CD18) and $\alpha_D\beta_2$ (CD11d/CD18). $\alpha_4\beta_2$ is expressed constitutively on all leukocytes, whereas the other members of this family are restricted to various subsets. The $\beta_2$ integrins bind ligands in the immunoglobulin superfamily (IgSF), expressed by leukocytes, endothelial, and epithelial cells, called intracellular adhesion molecules, [ICAM-1-3 (CD54, CD102, CD50)]\textsuperscript{102}. The $\beta_1$ integrins are expressed on a wide array of cells, including immune and inflammatory cells, and primarily bind ECM-components such as collagen and fibronectin, as well as the vascular cell adhesion molecule, VCAM-1 (CD106)\textsuperscript{102,103}. $\beta_7$ integrins are expressed on lymphocytes residing in the intestinal mucosa including lamina propria and intraepithelial lymphocytes. These integrins bind ligands such as mucosal addressin cell adhesion molecule-1 (MadCAM-1) and E-cadherin, expressed on mucosal endothelium and intestinal epithelial cells, respectively\textsuperscript{8,102}.  

10
### Fig. 2. Integrins expressed on leukocytes, and ligands

<table>
<thead>
<tr>
<th>Integrin</th>
<th>Common Name</th>
<th>Ligands</th>
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<tbody>
<tr>
<td>α1</td>
<td>(VLA-1)</td>
<td>Collagen, Laminin</td>
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<tr>
<td>α2</td>
<td>(VLA-2)</td>
<td>Collagen, Laminin</td>
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<tr>
<td>α3</td>
<td>(VLA-3)</td>
<td>Collagen, Laminin, Fibronectin</td>
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<tr>
<td>α4</td>
<td>(VLA-4)</td>
<td>Collagen, Laminin, VCAM-1</td>
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<tr>
<td>α5</td>
<td>(VLA-5)</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>α6</td>
<td>(VLA-6)</td>
<td>Laminin</td>
</tr>
<tr>
<td>αV</td>
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<td>Fibronectin, Vitronectin</td>
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<tr>
<td>β1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α4</td>
<td>(LPAM)</td>
<td>MadCAM-1, VCAM-1, Fibronectin</td>
</tr>
<tr>
<td>αE</td>
<td>(HML-1)</td>
<td>E-Cadherin</td>
</tr>
<tr>
<td>β2</td>
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<td></td>
</tr>
<tr>
<td>αL</td>
<td>(LFA-1)</td>
<td>ICAM-1, 2, 3</td>
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<tr>
<td>αM</td>
<td>(Mac-1)</td>
<td>ICAM-1</td>
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<tr>
<td>αD</td>
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<td>ICAM-3</td>
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<td>αX</td>
<td>(CR4)</td>
<td>ICAM-1</td>
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<tr>
<td>β7</td>
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<td>α4</td>
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<td>αE</td>
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</table>

**Fig. 2. Integrins expressed on leukocytes, and ligands.** Leukocyte integrins separated into β1, β2 and β7 families. In parentheses are the common names for the given αβ heterodimer. (References 8,100,102).
Integrins function in both tethering and rolling of leukocytes on endothelium through low affinity and avidity interactions, and in the firm arrest and transmigration of leukocytes through the vascular wall\(^{104}\). Most integrins expressed on circulating leukocytes exist in low affinity states. Stimuli such as chemokine/receptor interactions, initiate intracellular signaling events (“inside-out” signaling), which rapidly and transiently induce both conformational changes and clustering of integrins at the site of cell-cell or cell-ECM contact, resulting in increased affinity and avidity\(^{105}\). This results in rapid and transient adhesion of rolling leukocytes to the ligand-expressing vascular endothelium\(^9\). Signaling leading to integrin clustering involves activation of PI3K\(^{105}\), the GTPase Rap-1\(^{106,107}\), the Rac-1 guanine nucleotide exchange factor Vav-1 (in T cells)\(^{108}\), and the Ca\(^{++}\)-sensitive protease, calpain\(^{105}\). The net effect of these molecular events is the release of integrins from their attachments to the cytoskeleton, and increased lateral mobility in the plasma membrane. Inside-out signaling events leading to conformational changes that enhance integrin affinity are not yet clear. Upon ligand binding, integrins activate phospholipase C\(\gamma\), and tyrosine kinases including Lck, FAK, Pyk2 and ZAP70, ultimately leading to activation of Rap1, Rho-family GTPases, and myosin light chain kinase, which play roles in cytoskeletal rearrangements important in cell adhesion and migration (reviewed in \(^{104}\)).

The involvement of specific integrins in cell migration is determined by their expression on specific cell types, the regulation of their ligand-affinity/avidity, and the site-specific expression/deposition of their ligands. \(\alpha_4\beta_2\), for example, is constitutively expressed on naïve and activated lymphocytes, and functions primarily in migration into lymphoid tissues via HEV, which express high levels of ICAM-1\(^{100}\). Certain \(\beta_1\) integrins (the
VLA, or very late activation antigens), are expressed after T cell activation and function in migration on ECM components such as collagen and fibronectin, which are deposited during inflammatory processes\textsuperscript{100,102}.

In addition to their role in leukocyte migration, integrins function in T cell activation by stabilizing the interaction of T cells with APCs. The interaction of $\alpha_4\beta_2$ integrin on T cells with ICAM-1 expressed on APCs is a critical component of the immunologic synapse (reviewed in \textsuperscript{109}). Furthermore, integrins can provide costimulatory signals to T cells during activation, facilitating proliferation and acquisition of effector functions\textsuperscript{100}.

**Molecular Interactions Directing the Migration of Effector Cells During GVHD**

*Chemokines and Chemokine Receptors*
Fig. 3 Chemokines/receptors in GVHD target organs.

**Lung:**
- Ligands: CXCL9-11, CCL2, 3, 5
- Receptors: CXCR3, CCR2, 5

**Liver:**
- Ligands: CXCL9-11, CCL2, 3
- Receptors: CXCR3, CCR2, 5

**GI-tract:**
- Ligands: CXCL9-11, CCL25 (?)
- Receptors: CXCR3, CCR9 (?)

**Secondary Lymphoid Organs:**
- Ligands: CXCL9-11, CCL2, 3, 5, 19, 21 (?)
- Receptors: CXCR3, CCR2, 5, 7 (?)

**Skin:**
- Ligands: CXCL9-11, CCL2, 5, 17
- Receptors: CXCR3, CCR2, 4, 5, 10

Fig. 3. Chemokines and chemokine receptors that may be important in recruitment of effector cells to target organs during GVHD. (References 4, 5, 44, 45, 48, 110-128).
Lymphoid tissues

Our group has shown that T cells infiltrate into lymphoid tissues within hours of administration. The proteins involved in this early phase of migration have not yet been determined. Within 3 days after transplant, however, several studies have demonstrated upregulation of pro-inflammatory chemokines such as CCL2-5, and CXCL9-11 in lymphoid tissues, suggesting that after activation, alloreactive T cells may respond to pro-inflammatory chemokines to recirculate to these sites during GVHD. Expression of CXCL9-11 preceded that of other chemokines. Expression of all chemokines increased earlier in spleen than in liver and lung, consistent with earlier expansion of donor cells at this site. Interestingly, production of CCL3 in spleen required expression by donor T cells themselves.

Specific pro-inflammatory chemokine/receptor interactions have been examined with regard to lymphoid homing during GVHD. CCR5 expression on donor T cells was shown to play a critical role in their accumulation in lymphoid tissues including the spleen and Peyer’s patch (PP) at day 14 during GVHD in non-conditioned recipients. Similarly, eliminating the expression of a CCR5 ligand, CCL3, from donor T cells resulted in reduced CD8+ T cell accumulation in spleen in a sublethally conditioned GVHD model. However, this was not the case in lethally conditioned recipients, as eliminating CCR5 expression on or CCL3 production by donor T cells did not significantly impact their accumulation in the PP, and actually modestly increased their accumulation in the spleen.
Elimination of CXCR3 expression from donor T cells in conditioned models resulted in increased accumulation of T cells in the spleen, with a concomitant reduction of accumulation in small bowel and lung\textsuperscript{117,118}. The overall GVH response was not different in recipients of wild type versus CXCR3\textsuperscript{3/-} T cells. Therefore, CXCR3 was not required for recruitment and expansion of T cells in lymphoid tissues, but was required for migration from those sites to these parenchymal target organs.

CCR2 expression did not affect the accumulation of CD4\textsuperscript{+} T cells in the spleen, as determined by the transfer of allogeneic CCR2-deficient CD4\textsuperscript{+} T cells to sublethally conditioned recipients in a GVHD model. However, when CCR2-deficient whole splenocytes were transferred, a significant increase in activated CD4\textsuperscript{+} T cells was observed in the spleen, suggesting that CCR2 impacts the recruitment or function of a regulatory population that does not express CD4\textsuperscript{4119}.

**Liver**

Several studies have demonstrated expression of the chemokine ligands CCL2–5, CXCL2, and CXCL9-11 in the liver during experimental GVHD\textsuperscript{4,110-112,120}. In studies using conditioned murine GVHD models, a consistent pattern in the kinetics of expression of these ligands was observed. Ligands including CCL2, and CXCL9-11 were expressed early after transplant. CXCL9-11 are interferon-\(\gamma\)-inducible, yet IFN-\(\gamma\) protein was not expressed in liver during the timeframe in which these ligands were
expressed. IFN-γ protein was detected in the spleen during this timeframe, suggesting that the expression of chemokines could be induced in the liver via endocrine effects of IFN-γ. The roles of CXCL9-11 in recruitment of alloreactive T cells to liver are not yet clear, although elimination of CXCR3, the receptor that binds these ligands, from donor T cells resulted in reduced liver pathology in a murine model of GVHD induced by minor histocompatibility antigen (mHA) mismatch. The roles of CCL2 and its primary receptor CCR2 in liver GVHD are similarly unclear. Elimination of CCR2 from donor T cells did not impact liver pathology, although pathology was assessed at a late timepoint after transplant. As CCL2 expression in liver was demonstrated early after transplant, its impact on T cell infiltration may occur prior to the timepoint assessed in this study.

CCL3, and its primary receptor on T H/T C T cells, CCR5, have been shown to play critical roles in liver GVHD in non-conditioned, and reduced-intensity conditioned murine GVHD models. CCL3 production in liver during GVHD was shown to be dependent on expression by donor T cells. Expression of CCL3 occurred later compared to the expression of CXCL9-11, and correlated with donor T cell infiltration. Studies using either antibody-mediated neutralization of CCL3, or transfer of CCL3−/− donor T cells, resulted in reduced CD8+ T cell infiltration into liver. This correlated with lower serum alanine aminotransferase (ALT), and GVHD-specific liver pathology, respectively, demonstrating that a feedback mechanism exists in which CCL3 production by liver-infiltrating T cells serves to recruit additional CD8+ cytotoxic T cells. Interestingly, CD4+ T cell infiltrates were not significantly impacted by treatment of recipient mice with anti-CCL3 mAb, and were increased when
CCL3−/− donor cells were used, indicating that CD4+ and CD8+ T cells may utilize different chemokines/receptors during recruitment to the liver.

The expression of several receptors found on T cells, NK cells and macrophages, including CXCR3, CCR1, 4 and 5 were shown in the liver during the first two weeks after donor cell transfer. The kinetics of CCR5 expression correlated with donor T cell infiltration in this model. Antibody-mediated blocking of CCR5 resulted in selective inhibition of CD8+ T cell infiltration in the non-conditioned model. This finding was confirmed using CCR5−/− (eGFP+) donor cells, although in the latter study differences in CD4+ and CD8+ T cell infiltrates were not addressed114. The clear role of CCR5 in directing the migration of donor T cells to the liver during GVHD in non-conditioned mice correlated with high-level expression of the CCR5 ligands CCL3 and CCL4 in the livers of these mice112. Interestingly, when recipients in this model were conditioned with a myeloablative dose of total body irradiation (TBI) prior to transplantation, mice receiving CCR5+/− donor T cells suffered exacerbated GVHD112,116 and had significantly increased donor CD4+ and CD8+ T cell infiltration in liver112. Therefore, in the conditioned host, CCR5 expression on alloreactive T cells may function to down-modulate accumulation of alloreactive T cells in the liver and other sites of inflammation. Importantly, expression of CCR5-ligands in conditioned recipients did not achieve the high levels observed in non-conditioned recipients112. Activated T cells from CCR5−/− mice had an increased migratory response as compared to wild-type cells, to CXCL10 (a CXCR3-ligand)112. These data suggested that stimulation of CCR5 may cause heterologous desensitization of the CXCR3 receptor, and eliminating this receptor cross-
talk could impact effector cell recruitment to GVHD target organs. Thus, current data suggest that in our model, migration of T cells to the liver is influenced early by the expression of the chemokine ligands CXCL9-11, which are produced by host cells, but dependent on the receipt of alloreactive donor T cells. More than one week post transplantation, the chemokine ligands CCL3-5, which are generated by donor T cells, appear to play a significant role in the recruitment of alloreactive T cells expressing CCR5.

**Gastrointestinal Tract**

A comprehensive analysis of chemokine expression in the small intestine and colon during GVHD has not yet been performed. A recent study identified an important role for CXCR3 in infiltration of the small intestine during GVHD in a conditioned, mHA mismatched GVHD model\textsuperscript{117}. Eliminating CXCR3 expression from donor T cells resulted in reduced pathology, and infiltration of CD8\textsuperscript{+} T cells into the lamina propria and epithelium at this site.

The cellular sources of chemokines in the GI tract may be different than in other target organs. For example, production of CCL3 was observed in the colon in murine allogeneic transplant recipients, but this was not dependent on expression by donor T cells\textsuperscript{111}. This suggests that the major sources of CCL3 in the colon during GVHD are the cells of the colonic mucosa itself, and that the CCL3-mediated feedback mechanism described above may not play a significant role in recruitment of donor T cells to this...
As high levels of CCL3 were produced in the colon after allogeneic T cell transfer, eliminating CCR5 expression from donor T cells was expected to significantly impair GI tract infiltration. In non-conditioned recipients, eliminating CCR5 expression from donor splenocytes did result in significantly reduced accumulation of donor cells in PPs and small intestinal epithelium\textsuperscript{114}. In conditioned recipients, eliminating CCR5 expression from donor T cells had no effect on the infiltration of donor T cells into the PPs or colon\textsuperscript{112}, again showing that the function of chemokines/chemokine receptors is different in conditioned compared to non-conditioned recipients. The expression of CXCL9-11 and recruitment of CXCR3-expressing T cells may compensate for the loss of CCR5 in migration of T cells to the GI tract.

Other chemokines expressed in the GI tract may also play important roles in conditioned recipients. The chemokine CCL25 is expressed by small intestinal epithelium\textsuperscript{44,45}. This molecule interacts with CCR9, expressed on T cells activated within mucosal tissues such as PPs and the mesenteric LN, to direct the specific migration of effector memory cells to the small intestine\textsuperscript{122,123}. Whether CCL25/CCR9 interaction plays a role in directing GI tract-specific T cell recruitment during GVHD is an area of current research, but a central role for PPs in initiating GVHD has been proposed\textsuperscript{114}.

**Skin**

A comprehensive analysis of chemokine and chemokine receptor expression in the skin in a lethally conditioned model of GVHD caused by mHA mismatch was recently
performed\textsuperscript{5}. As in the liver, the IFN-\(\gamma\)-inducible chemokines CXCL9 and 10 were expressed at high levels within the first week after allogeneic transplant, although IFN-\(\gamma\) itself was not. CCL2 expression was upregulated early after transplant, as were CCL6, 7, 9 and 11, and CXCL1. Also consistent with data in the liver, CCL5 was upregulated later post-transplant (week 2), and its expression correlated with two of its receptors, CCR1 and CCR5. CCL17, which binds to CCR4 and has been shown, in conjunction with CCL27/CCR10, to play an important role in the recruitment of memory T cells to inflamed skin, was significantly upregulated during the first week post transplant\textsuperscript{48}. This finding raises the possibility that interactions such as CCL17/CCR4 and CCL27/CCR10 may participate in the tissue-specific migration of alloreactive T cells during GVHD. No definitive roles have yet been identified for specific chemokine/receptor interactions in the recruitment of GVHD effector cells to skin, although a requirement for CCR6 in the recruitment of donor Langerhan’s cell precursors to the epidermis after allogeneic BMT was recently demonstrated\textsuperscript{124}.

**Lung**

Several studies have examined chemokine/receptor interactions involved in the recruitment of effector cells to the lung during GVHD and idiopathic pneumonia syndrome (IPS). Chemokines expressed in lung after allo-BMT include CXCL9-11, CCL2-5 and 11\textsuperscript{111-113}. TNF-\(\alpha\) production by infiltrating donor T cells was shown to be critical for pro-inflammatory chemokine expression in the lung after allo-BMT\textsuperscript{125}. The expression of CCL2, CXCL10 and CXCL11 were found prior to the expression of other chemokines such as CCL3 and 4\textsuperscript{112,113,125}. Antibody-mediated neutralization of CXCL9
or 10 reduced IPS severity and CD8\(^+\) T cell infiltrates. The effect of inhibiting these ligands was additive, and further inhibition was possible by eliminating expression of CXCR3 from donor T cells. Thus, as demonstrated in the liver, CXCR3 was critical for donor T cell recruitment to lung after allo-BMT\(^{118}\).

CCL3 production in the lung was dependent on expression by allogeneic donor T cells\(^{111}\). Significantly reduced recruitment of CCL3\(^+/\) CD8\(^+\) donor T cells to the lung was observed in sublethally conditioned recipients. Additionally, recruitment of CCR5\(^+/\) CD4\(^+\) and CD8\(^+\) donor T cells to lung was impaired in a non-conditioned model, demonstrating that lung-infiltrating donor T cells recruit additional donor T cells to this site through a CCL3/CCR5-mediated feedback mechanism\(^{111,112}\). As in the liver, however, recruitment of donor T cells to lung was enhanced in lethally conditioned transplant recipients, receiving CCL3- or CCR5-deficient donor T cells\(^{112,115}\). That this occurred only in conditioned recipients, likely related to differences in the relative expression of CXCR3 and CCR5 ligands in the lung after allo-BMT in conditioned versus non-conditioned recipients\(^{112}\). Interestingly, eliminating CCL5 expression from donor T cells impaired their recruitment to the lung at late timepoints (4-6 weeks) after transplant in a lethally conditioned model\(^{126}\). In this study, CCR5 expression was reduced at late timepoints, while CCR1, which also binds CCL5, remained high.

The pathogenesis of IPS involves recruitment of host macrophages to the lung during the peri-transplant period, which are believed to contribute to alloantigen presentation to donor T cells infiltrating the lung, and to lung damage through cytokine production\(^{127}\).
CCL2 production in lung correlated with the early influx of host macrophages\textsuperscript{113}. However, eliminating CCL2 did not significantly affect host macrophage influx into lung or IPS severity early post transplantation\textsuperscript{128}. Interestingly, another study assessed the role of CCR2, the receptor for CCL2, on donor T cells, and found that eliminating it reduced the severity of late onset IPS\textsuperscript{121}. IPS was assessed at weeks four and six, timepoints at which the authors had demonstrated full donor chimerism in lung lymphocytes and macrophages. IPS at these timepoints was significantly reduced in recipients of CCR2-deficient T cells, correlating with reduced donor T cell accumulation in the lung. Thus, although CCL2/CCR2 interaction does not play a role in early recruitment of host macrophages to lung after allo-BMT, this interaction has clear significance in the recruitment of donor T cells later. Thus, the roles of chemokines and chemokine receptors may differ depending on when they are evaluated after allo-BMT.

**Selectins and Adhesion Molecules**

**Lymphoid Tissues**

The complexity of the proteins involved in migration was underscored by Li et al. who evaluated the functions of L-selectin (CD62L) and α4-integrins during GVHD. They did not find an impact on the occurrence of GVHD by targeting CD62L or α4-integrins alone. However, treating donor cells with anti-CD62L and anti-α4-integrin mAbs prior to infusion delayed GVHD, predominantly by delaying the homing of donor T cells to the mesenteric LN\textsuperscript{129}. This work underscores the important role of donor T cell migration to
secondary lymphoid tissue in the initiation of GVHD.

**Liver**

Very early upregulation of ICAM-1 on endothelium was demonstrated in a mouse model of BMT across mHA barriers and was associated with increased numbers of infiltrating cells expressing $\alpha_4\beta_2$ integrin\textsuperscript{130}. Harning et al. demonstrated in a parent into F1 mouse model that a combination of anti-$\alpha_4\beta_2$ and anti-ICAM-1 antibodies prevented GVHD\textsuperscript{131}. This therapy decreased the number of T cells homing to the liver with a consequent decrease in the generation of IL-2 and IL-12 in the liver. Our group confirmed a role for $\alpha_4\beta_2$ using an anti-$\alpha_4\beta_2$ mAb, along with CTLA4-Ig, to prevent GVHD across fully mismatched MHC BMT in mice\textsuperscript{132}. Interestingly, our work using ICAM-1\textsuperscript{-/-} recipients in a lethally conditioned MHC-mismatched GVHD model indicates that there is not a specific requirement for ICAM-1 in liver inflammation\textsuperscript{133}. Thus, the interaction of $\alpha_4\beta_2$ integrin with other ligands, such as ICAM-2, or 3, may contribute to the homing of alloreactive effector cells to liver during GVHD. The interaction of $\alpha_4\beta_1$ integrin with VCAM-1 has also been shown to play a role in liver inflammation during GVHD\textsuperscript{134}.

**GI Tract**

The $\alpha_4\beta_7$ integrin (lymphocyte PP adhesion molecule, or LPAM) and it’s ligand, mucosal addressin cell adhesion molecule (MadCAM-1), expressed on HEVs in PPs and lamina propria, have been shown to be important in the homing of alloreactive T cells to
the intestine during GVHD. Treatment of lethally irradiated recipients with anti-α1 and α4 integrin mAbs, inhibited mucosal GVHD in a parent into-F1 model\textsuperscript{135}. When allogeneic recipient mice, mismatched at minor or major Ags, were given α4β7\textsuperscript{−} T cells, they suffered less GVHD morbidity and mortality than mice receiving α4β7\textsuperscript{+} T cells. This correlated with reduced GVHD histopathology in the intestine and liver. No effect on GVHD in the skin and thymus was observed, underscoring the role of α4β7 in selective migration of alloreactive T cells to the intestinal mucosa\textsuperscript{136}.

**Lung**

While the absence of ICAM-1 expression in recipient mice did not impact the incidence of GVHD, or the migration of alloreactive donor T cells to the liver, we did observe a marked decrease in IPS in ICAM-1\textsuperscript{−} recipients\textsuperscript{133}. The influx of T cells, macrophages and neutrophils, and the production of inflammatory cytokines, was dramatically decreased in the lungs of ICAM-1\textsuperscript{−} compared to wild type recipients. In contrast, systemic levels of these cytokines were unaffected, and GVHD-related mortality was accelerated in ICAM-1\textsuperscript{−} recipients. This implies that ICAM-1 plays a critical role in the generation of post-allogeneic BMT inflammation in the lung, but may be redundant in its function with other adhesion molecules in inducing GVHD in the liver and GI tract.

**Discussion and Conclusions**

Based on our demonstration of the early events in donor cell migration during GVHD,
and quantitative and kinetic analyses of inflammatory chemokine and adhesion molecule expression in target organs, we propose a general model for the migration of effector cells during GVHD (Fig. 4). Donor T cells initially migrate to spleen and peripheral lymphoid tissues within hours after transplant, where they encounter host APCs matured by the effects of the conditioning regimen (Fig. 4A). This initial migration may depend on homeostatic chemokines, and adhesion molecules such as L-selectin, and α4β2 integrin. Interaction with those APCs leads to activation of alloantigen-specific donor T cells, and their differentiation into Th1/TC1 effector cell types, expressing CCR2, CCR5, CXCR3 and CXCR6. During the first 2-3 days, activated, proliferating T cells produce inflammatory cytokines such as IFN-γ and TNFα within lymphoid tissues (Fig. 4B), which enter the circulation and induce the production of chemokines in target organs, by host cells. The inflammatory milieu that occurs after myeloablative conditioning appears to be quite important in early donor T cell migration. The interferon-inducible chemokines, CXCL9-11, produced in target organs such as liver, lung and skin (Fig. 4C), play primary roles in directing the early migration of activated, CXCR3 expressing T cells after egress from lymphoid tissues (Fig. 4D). These chemokines likely direct the migration of effector cells until the host APCs producing them are eliminated.

Following this initial phase of production of chemokine ligands, a second wave of production is induced from recruited donor T cells. These T cells produce CCR5 ligands such as CCL3 - CCL5, which serve to propagate target organ infiltration after CXCL9-11 production subsides (Fig. 4E, F). Initially however, production of chemokines such as CCR5-ligands by donor T cells appears to desensitize CXCR3, and limit inflammation by
limiting effector cell responsiveness to CXCL9-11. The reverse may also be true, and CXCL9-11 may at some point serve to limit responsiveness to chemokines such as the CCR5-ligands. The net effects may be dependent on the inflammatory conditions created by the conditioning regimen and the degree of antigen-mismatch, which likely influence the chemokines and receptors that dominate the GVHD response.

Entry of effector T cells into the lung involves interactions between $\alpha_4\beta_2$/ICAM-1 and into the liver involves $\alpha_4\beta_2$/ICAM-1, 2, 3 and $\alpha_4\beta_1$/VCAM-1. Entry into the intestinal mucosa requires $\alpha_4\beta_7$/MadCAM. As disease progresses in animals surviving the early post-transplant period, changes may occur in the magnitude and types of cytokines, chemokines, chemokine receptors and adhesion molecules expressed in target tissues and on effector cells. Thus, several weeks after transplant, the molecular interactions directing the recruitment of effector cells to target organs may be quite different from those playing primary roles during the first weeks post-transplant. This model is based on findings in a parent into F1 transplant model. Other chemokines and chemokine receptors may be critical for migration in other models.
Fig. 4. Early donor T cell migration during acute GVHD

A.
- Conditioning
- Lymph nodes
- Spleen
- Peyer’s Patch
- Lymph nodes

B.
- IFN-\(\gamma\), TNF\(\alpha\)
- Other cytokines

C.
- CXCL9-11;
- Other “early response” chemokines?

D.

E.
- CCL3-5;
- Other T cell-derived chemokines?

F.

Fig. 4. Model for early donor T cell migration during acute GVHD.  A) Recipients
of allo-BMT receive conditioning therapy, followed by i.v. infusion of bone marrow or peripheral blood stem cells (containing mature donor T cells). Within the first hours after transplantation, donor T cells (green) accumulate in peripheral lymphoid tissues such as LNs, Peyer’s Patches and spleen. **B)** Alloreactive donor T cells expand in peripheral lymphoid tissues, differentiating into $T_{el}^H/T_{el}^C$ effectors, and producing IFN-$\gamma$, TNF$\alpha$ and other cytokines (light blue arrows). **C)** Circulating IFN-$\gamma$ synergizes with inflammatory mediators and bacterial products released into circulation via the conditioning regimen to induce production of interferon-inducible chemokines (blue gradient arrows) by APCs, endothelial and epithelial cells in target organs. **D)** Activated effector T cells follow gradients of these chemokines during early target organ infiltration (green arrows). **E)** Amplification of T cell infiltrates: donor T cells having infiltrated target organs produce T cell-tropic chemokines CCL3-5 (and possibly others) (pink gradient arrows). **F)** Effector cells continue to follow gradients of these chemokines to infiltrate target organs (green arrows), leading to tissue pathology and clinical manifestations of GVHD, represented by green infiltrates in GI-tract, liver, lung and skin. References $^3, 4, 5, 111-113$. 

While there has been increasing interest in the roles of various proteins in the migration of T cells during GVHD, the results of animal studies have often been confusing and in specific instances have yielded conflicting data. We believe that this model begins to address some of these contradictions. Our work shows that chemokine receptors have different functions in both limiting and exacerbating GVHD depending on the use of conditioning therapy. Thus, comparisons between studies need to take into account the use and intensity of conditioning therapy. Additionally, in recipients of myeloablative
conditioning, simultaneous targeting of multiple different chemokine ligands and receptors may be required to ameliorate GVHD target organ infiltration in recipient animals. As a corollary to this, preferential roles for specific chemokine ligand/receptor interactions may only be found in the absence of conditioning, or in the setting of reduced-intensity conditioning. Additionally the degree of MHC-disparity may play a role in the production of chemokines by alloreactive donor T cells, confounding results found using different models. Finally, the current data suggest that certain chemokine/receptor and integrin/ligand interactions direct the recruitment of alloreactive donor T cells to a specific or selective set of target organs. Thus, the failure to find a broad impact on GVHD incidence in experimental systems does not preclude the involvement of a specific ligand or receptor in organ-specific T cell infiltration.

Small-molecule chemokine receptor inhibitors are currently available or in development for the treatment of inflammatory disorders, and as HIV co-receptor inhibitors (reviewed in 137). Anti-LFA-1 and α4 mAbs are or should be available in the next year for the treatment of psoriasis and multiple sclerosis. These therapies may be evaluated in clinical trials for the treatment of GVHD. However, our studies suggest that a thorough knowledge of chemokine/receptor and adhesion molecule/integrin interactions important in target organ inflammation in multiple specific transplantation settings is critical prior to initiating these clinical trials. For example, targeting receptors important in migration after reduced-intensity allo-BMT, may exacerbate GVHD after a fully myeloablative transplant. Thus, the tools with which to prevent target organ inflammation through inhibition of chemotactic and adhesion molecules may already be in place, but currently
await a more thorough understanding of the function of this complex system during GVHD.

**Figure Legends:**

**Fig. 1. Inflammatory chemokines and receptors.** *Abbreviations:* APC (antigen presenting cell), B (B cell), D (dendritic cell), E (eosinophil), L (Langerhan’s cell), Ma (mast cell), Mφ (macrophage), N (neutrophil), NK (natural killer cell), P (platelet), T1 (T\(^H\)\_1/T\(^C\)\_1 cell), T2 (T\(^H\)\_2/T\(^C\)\_2 cell). (References 20, 44-94).

**Fig. 2. Integrins expressed on leukocytes, and ligands.** Leukocyte integrins separated into \(\beta1\), \(\beta2\) and \(\beta7\) families. In parentheses are the common names for the given \(\alpha\beta\) heterodimer. (References 8, 100, 102).

**Fig. 3. Chemokines and chemokine receptors that may be important in recruitment of effector cells to target organs during GVHD.** (References 4, 5, 44, 45, 48, 110-128).

**Fig. 4. Model for early donor T cell migration during acute GVHD.**

A) Recipients of allo-BMT receive conditioning therapy, followed by i.v. infusion of bone marrow or peripheral blood stem cells (containing mature donor T cells). Within the first hours after transplantation, donor T cells (green) accumulate in peripheral lymphoid tissues such as LNs, Peyer’s Patches and spleen. B) Alloreactive donor T cells expand in peripheral lymphoid tissues, differentiating into T\(^H\)\_1/T\(^C\)\_1 effectors, and producing IFN-\(\gamma\), TNF\(\alpha\) and
other cytokines (light blue arrows).  

C) Circulating IFN-\(\gamma\) synergizes with inflammatory mediators and bacterial products released into circulation via the conditioning regimen to induce production of interferon-inducible chemokines (blue gradient arrows) by APCs, endothelial and epithelial cells in target organs.  

D) Activated effector T cells follow gradients of these chemokines during early target organ infiltration (green arrows).  

E) Amplification of T cell infiltrates: donor T cells having infiltrated target organs produce T cell-tropic chemokines CCL3-5 (and possibly others) (pink gradient arrows).  

F) Effector cells continue to follow gradients of these chemokines to infiltrate target organs (green arrows), leading to tissue pathology and clinical manifestations of GVHD, represented by green infiltrates in GI-tract, liver, lung and skin.  

References 3, 4, 5, 111-113.

SUPPLEMENTAL MATERIAL IS AVAILABLE ONLINE AT THE TIME OF FINAL PUBLICATION ONLY.

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Leukocyte migration and graft-versus-host disease

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