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Homing in on Acute Graft vs. Host Disease: Tissue-Specific T Regulatory and Th17 Cells

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Abstract

Acute graft vs. host disease (aGVHD) is a major limitation of hematopoietic stem cell transplantation (HSCT), and it causes significant morbidity and mortality for this patient population. This immune-mediated injury occurs unpredictably and is caused by donor-derived T cells reacting to recipient alloantigens. Although donor Th1 cells play a critical role in aGVHD generation, numerous arms of both the innate and the adaptive immune systems along with determinants of lymphocyte trafficking are likely involved in the multifaceted cascade of immunological events that culminates in clinical aGVHD. T regulatory and Th17 cells are T cell subsets distinct from Th1 cells that are likely involved with aGVHD. Regulatory T cells (Tregs) have been implicated in the prevention of aGVHD in both mouse and man, while Th17 cells may modulate early inflammatory responses associated with aGVHD, especially those involving the skin and the lungs. Interestingly, these two lymphocyte subsets appear to be reciprocally regulated in part through retinoic acid, through cytokines such as IL-6, and via interactions with dendritic cells. Another area under tight regulation appears to be the homing of lymphocytes to lymph nodes, skin, and gut. Adhesion molecules including chemokine receptors, selectins, and integrins may identify specific T cell subsets with unique migratory functional properties during HSCT. Controlling the migration patterns of Th17 cells and Tregs represents a potential therapeutic target. A major goal of HSCT research will be to develop approaches to pharmacologically manipulate T cell subsets *in vivo* or to select, expand, and infuse T cell subsets that will maximize the targeted graft vs. tumor effect while minimizing the potentially fatal side effects of aGVHD. A better understanding of Tregs and their tissue specificity should lead to improvement in the success of HSCT.

1 Introduction

1.1 Acute Graft vs. Host Disease

Allogeneic HSCT is a curative therapy for fatal hematological disorders and hereditary immunodeficiency syndromes. In the context of treatment for hematological malignancies, HSCT is capable of eradicating residual malignant cells escaping chemotherapy and radiation via immune surveillance mechanisms known as graft vs. tumor effect (GVT). GVT is essential for the success of the transplant and for controlling disease relapse post-HSCT.

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Conversely, the donor immune system can also recognize recipient alloantigens as foreign, resulting in immune-mediated tissue injury known as graft vs. host disease (GVHD). GVHD is thought to be primarily a T cell mediated process. GVHD is the most important medical limitation of this procedure. GVHD is divided into two broad categories, acute and chronic, based on the phenotype of the disease (Filipovich et al. 2005). Classical acute GVHD (aGVHD) occurs in the first 100 days following HSCT and is characterized by the triad of dermatitis, gastroenteritis, and cholestatic hepatitis. aGVHD usually begins with a maculopapular rash involving the palms of the hands or soles of the feet. It can quickly spread to become a generalized erythroderma. The gastrointestinal tract also can be involved, leading to nausea, vomiting, anorexia, diarrhea, and even ileus or bloody diarrhea. When severe, aGVHD can be associated with bullous skin lesions and desquamation of cutaneous tissues and intestinal mucosa. Hepatic dysfunction usually is marked by a rise in serum bilirubin and occasionally the transaminases. aGVHD is a significant risk factor for the development of chronic GVHD (cGVHD), which in turn dictates long-term morbidity, quality of life, and nonrelapse mortality following HSCT (Arai and Vogelsang 2000; Przepiorka et al. 2001). Prophylaxis strategies with calcineurin inhibitors and either methotrexate or mycophenolate mofetil have reduced the incidence of aGVHD; however, aGVHD still affects 40–50% of patients undergoing a matched related donor (MRD) HSCT (Arai and Vogelsang 2000; Nash et al. 1992; Weisdorf et al. 1991) and 50–80% of patients receiving human leukocyte antigen (HLA)-mismatched or unrelated donor (URD) transplants (Beatty et al. 1985, 1991). Although the triad of organ involvement is characteristic, aGVHD occurs unpredictably with regards to actual tissue involvement and severity following HSCT. When moderate to severe aGVHD occurs, it requires additional treatment with potent immunosuppressive agents, usually high-dose corticosteroids, which further increases the morbidity and infectious risk associated with transplantation. Unfortunately, only 50–60% of patients receiving treatment for aGVHD will have a durable response (Martin et al. 1990; Alousi et al. 2009). Most patients with severe aGVHD and many patients failing initial therapy die eventually from complications of aGVHD or its therapy (Martin et al. 1990, 1991). Thus, much effort has been placed on trying to understand the immunology of aGVHD as a means to improve patient outcomes.

1.2 T Cells and aGVHD

Recently, much interest has focused on T cell subsets and lymphocyte homing as a way to explain the clinical heterogeneity and the organ tropism of aGVHD. The chemokines, chemokine receptors (CCRs), and other adhesion molecules necessary for lymphocyte trafficking to lymph nodes, skin, gut, or to areas of inflammation have been established in the mouse and increasingly so in the human. These avenues are now being actively explored in HSCT recipients as a way to explain the organ involvement by aGVHD.

Differences in T cell differentiation and subtype could also be playing a role in the pathophysiology of aGVHD. Recently, a suppressive subset of CD4+ T cells has been identified. These regulatory T cells (Tregs) are characterized by high expression of the interleukin (IL)-2 receptor α chain (CD25) and intracellular expression of the transcription factor forkhead box protein P3 (Foxp3) (Sakaguchi et al. 1995; Shevach 2002; Fontenot et al. 2003; Hori et al. 2003). Physiologically, these cells have been implicated in the prevention of autoimmune diseases (Sakaguchi et al. 1995), host tolerance to chronic infections (Belkaid et al. 2002), and escape of immune surveillance by malignant cells (Curiel et al. 2004). There is also increasing evidence that high Treg frequencies post-HSCT are associated with reduced incidence or severity of aGVHD.

Another lineage of CD4+ T cells distinct from Tregs and Th1/Th2 cells, designated Th17 cells, has been identified. These Th17 cells are characterized by secretion of the pro-inflammatory cytokines, IL-17, IL-17F, IL-21, and IL-22. In contrast to the suppressive role

of Tregs, Th17 cells appear to be associated with inflammation, the elimination of extracellular pathogens, autoimmunity, and solid organ allograft rejection. Their role in aGVHD is now being explored.

By combining increasing knowledge about specific T cell subsets and their patterns of homing, we can gain better insight into the immunology of aGVHD, which may improve treatment options and outcomes for patients undergoing HSCT. In this review, we focus on recent progress in our understanding of naturally occurring Tregs with varying patterns of expression of chemokine receptors (CCRs) and other homing molecules and their relationship to the development of aGVHD. We also will describe briefly the emerging role of Th17 cells and their chemokine receptor expression in the pathophysiology of aGVHD.

2 Immunology of aGVHD

2.1 Model of Interactions

Based on data obtained from preclinical animal models of transplantation, a three-phase model for the development of aGVHD has been proposed. The first stage occurs prior to the infusion of the hematopoietic stem cell graft. During this initial stage, high doses of chemotherapy and radiation damage recipient tissues, causing the release of inflammatory cytokines IL-1 and TNF-α (Xun et al. 1994). In response to TNF-α secretion, dendritic cells increase expression of MHC antigens and co-stimulatory molecules, while lymphoid and peripheral tissues upregulate integrins and chemokines necessary for the migration of immune cells (Norton and Sloane 1991; Thornhill et al. 1991; Leeuwenberg et al. 1988). The graft, which contains hematopoietic stem cells along with donor lymphocytes, is then infused into the recipient, setting the stage for the second phase of aGVHD characterized by donor T cell activation. Recipient dendritic cells primed by inflammatory cytokines are thought to play a major role in the activation of donor CD4+ T cells via the presentation of disparate major and minor histocompatibility antigens (Shlomchik et al. 1999). The clonal expansion and differentiation of Th1 type CD4+ T cells are thought to drive the aGVHD reaction (Via and Finkelman 1993; Allen et al. 1993). These cells secrete Th1 cytokines including IL-2 and IFN-γ, leading to the third phase of aGVHD, the effector stage. Macrophages, NK cells, and CD8+ cytotoxic T cells stimulated by Th1 cytokines can exert end-organ damage via reactive oxygen species, TNF-α, perforin/granzyme, and Fas/Fas-ligand (CD95/CD95L), further perpetuating the above cycle (Shresta et al. 1998; Piguet et al. 1987; Graubert et al. 1997; Via et al. 1996). The culmination of these immunological events leads to the clinical syndrome that we recognize as aGVHD. Although the immunology of alloreactive T cells and the role of host dendritic cells in the inception of aGVHD has been reviewed extensively (Ferrara et al. 1999; Reddy 2003; Shlomchik 2007; Welniak et al. 2007; Socie and Blazar 2009), this model provides a useful framework in which to explore the relationship between antigen presentation, chemokine expression, and lymphocyte compartmentalization with the generation of organ-specific aGVHD.

2.2 Lymph Node Physiology

As implicated previously, secondary lymphoid organs play a critical role in the generation of aGVHD. In vivo bio-luminescence imaging of the mouse with transplanted luciferase-labeled allogeneic splenocytes demonstrated that naive but not memory donor T cells first localize to the lymph nodes and spleen within hours of infusion. During the next 2 days, activated T cells expand within these secondary lymphoid organs followed by migration over the next 3–6 days to the intestines, liver, and skin (Beilhack et al. 2005). Inhibiting lymphocyte entry into (or exit from) lymphoid tissues by either blocking antibodies/drugs, genetic manipulation, or surgical removal of organs greatly reduced the incidence and severity of aGVHD in murine models of transplantation (Kim et al. 2003; Murai et al. 2003;

Beilhack et al. 2008). In human HSCT, preparative regimens containing total lymphoid irradiation followed by T cell depletion with anti-thymocyte globulin decreased the incidence of aGVHD to almost undetectable levels in patients with hematological malignancies (Lowsky et al. 2005).

The migration of donor T cells from the vascular compartment to the lymph node followed by lymph node egress and migration to the peripheral tissues requires a multi-step adhesion cascade involving CCRs, selectins, and integrins (von Andrian and Mempel 2003; Agace 2006; Sigmundsdottir and Butcher 2008). Naïve and central memory T cells express high levels of CD62L (L-selectin) and CCR7, which facilitate their migration to lymph nodes. CD62L and CCR7 can interact with peripheral node addressin (PNAD) and chemokine ligand (CCL)21, respectively, which are constituitively expressed on high endothelial venules (HEVs) and allow entry of the lymphocyte into the lymph node (von Andrian and Mempel 2003; Berg et al. 1991; Gunn et al. 1998). Interactions between the integrin $\alpha_4\beta_7$ with mucosal vascular addressin cell-adhesion molecule 1 (MADCAM1) also may play a role in mesenteric lymph node localization (Berlin et al. 1993; Arbones et al. 1994). The importance of lymph node compartmentalization in HSCT is illustrated further by the fact that naive ($CD44^{lo}CD62L^{hi}$) but not memory ($CD44^{hi}CD62^{lo}$) T cells are able to cause GVHD (Zhang et al. 2005). However, $CD44^{hi}CD62^{lo}$ T cells previously sensitized to recipient alloantigens can initiate GVHD, presumably by bypassing the initial activation step occurring in the lymph node.

2.3 Lymphocyte Compartmentalization

The lymph node environment and dendritic cells also play important roles in polarizing lymphocytes for homing phenotypes towards specific tissues (Agace 2006; Sigmundsdottir and Butcher 2008). During T cell activation, dendritic cells cause the upregulation of chemokine receptors and other adhesion molecules on lymphoyctes. Remarkably, these homing receptors allow the lymphocytes to migrate back to the tissues where the antigen was first encountered by the dendritic cell. In Peyer's patches and mesenteric lymph nodes, antigen-experienced T cells up-regulate gut-homing markers including $\alpha_4\beta_7$ and CCR9 and reciprocally downregulate skin-homing adhesion molecules via signaling by all-trans-retinoic acid (ATRA) produced by dendritic cells (Iwata et al. 2004; Mora et al. 2005; Dudda et al. 2005; Kim et al. 2008). Enzymes important for metabolizing retinol (vitamin A) to retinoic acid are not expressed by dendritic cells from peripheral tissues, which may contribute to the maintenance of lymphocyte tissue specificity (Sigmundsdottir and Butcher 2008; Iwata et al. 2004). When released back into the circulation by way of the efferent lymphatics and thoracic duct, these activated T cells migrate towards gastrointestinal tissues via $\alpha_4\beta_7$-MADCAM1 and CCR9-CCL25 interactions.

In an analogous situation, dendritic cells from the skin migrate toward peripheral lymph nodes where they are able to induce a skin-homing phenotype in T cells with subsequent downregulation of $\alpha_4\beta_7$ and CCR9 (Iwata et al. 2004; Mora et al. 2005; Dudda et al. 2005; Kim et al. 2008). T cells activated in peripheral nodes upregulate cutaneous lymphocyte antigen (CLA) and CCR4. CLA binds to endothelial-cell selectin (E-selectin) and platelet selectin (P-selectin), which are constitutively expressed on cutaneous tissues, while CCR4 interacts with CCL17 expressed on skin venules (von Andrian and Mempel 2003; Agace 2006; Sigmundsdottir and Butcher 2008). During inflammation, both the selectins and the CCL17 are upregulated by skin, thus facilitating lymphocyte entry into peripheral tissues (Agace 2006). CCR10 and its ligand CCL27 also may function in directing lymphocytes from the dermis to the epidermal junction (Agace 2006). The mechanism by which lymphocytes are polarized towards a skin-homing phenotype has been elucidated less clearly, but is thought to be related to vitamin D metabolites and possibly IL-12 (Sigmundsdottir et al. 2007; Picker et al. 1993). Additionally, skin-homing may occur by

way of a default mechanism during T cell–dendritic cell interactions in the absence of retinoic acid signaling (Agace 2006).

The importance of the lymph node and the stereotypical involvement of some organs but not others strongly suggests that lymphocyte homing might play a role in aGVHD generation. The expression of specific homing molecules and CCRs can be used to define unique populations of effector and suppressor T cells. As noted earlier, two nonoverlapping antigen-experienced T cell populations have been well characterized in mice and in humans. One subset is characterized by $\alpha_4\beta_7$/CCR9 expression (gut-homing) and the other by CLA/CCR4 expression (skin-homing). Interestingly, these homing patterns correspond to the two most commonly involved tissues during aGVHD, skin, and gut. These populations of cells may have unique functions and, when perturbed, may result in specific pathological outcomes in HSCT. Homing of particular functional subsets of T cells such as Tregs and Th17 cells may explain the organ-specific nature of clinical aGVHD. Here we will consider the evidence for homing of these cells in control or induction of aGVHD.

3 Tregs, aGVHD, and Adhesion Molecules

3.1 History of Suppressor Cells

Suppressor T cells were first described over 30 years ago by Kondo and Gershon (Gershon and Kondo 1970). These cells were thought to regulate the immune system by secretion of antigen-specific factors. The failure to clone these factors in the 1980s led to widespread skepticism about suppressor T cells. This field lay dormant for several decades until suppressor T cells were “rediscovered” as Tregs in the 1990s. In a seminal paper, Sakaguchi et al. demonstrated that the depletion of CD4+CD25+ cells in mice led to the development of autoimmune-induced diabetes mellitus, thyroiditis, gastritis, and other disorders (Sakaguchi et al. 1995). Autoantibodies and immune-mediated end organ damage were significantly increased in BALB/c nu/nu mice receiving T cell suspensions obtained from BALB/c nu/+ mice depleted of CD4+CD25+ Tregs. This paper was the first report demonstrating that CD25 could be used as a marker to define a population of immunoregulatory T cells. Since this initial report, much work has been done on Tregs pertaining to their identification, development, mechanisms of action, homing characteristics, and their relationship to various human diseases and conditions.

3.2 Natural Treg Phenotype and Characterization

Tregs are a naturally occurring subset of T lymphocytes that make up about 5–10% of normal circulating CD4+ cells (Sakaguchi et al. 1995; Shevach 2002). Tregs develop in the thymus (“natural Treg”) or they can be generated in the periphery from naive T cells (“induced Treg”) (Chen et al. 2003; Izcue et al. 2006). Tregs suppress the proliferation of activated T cells via a cell-contact-dependent mechanism and in an antigen nonspecific manner. They are further characterized by relative hyporesponsiveness to stimulation by pan T cell activators (Shevach 2002). In vivo, they are thought to function in maintaining immunological self-tolerance. Tregs were identified initially by the expression of high levels of the IL-2 receptor α chain, CD25. Numerous other markers have been attributed to Treg phenotype and function including cytotoxic T-lymphocyteassociated antigen 4 (Takahashi et al. 2000), glucocorticoid tumor necrosis factor receptor (Shimizu et al. 2002), CD62L (Ermann et al. 2005; Taylor et al. 2004), CCR4 (Oswald-Richter et al. 2004), folate receptor 4 (FR4) (Yamaguchi et al. 2007), and low expression of the IL-7 receptor α chain, CD127 (Liu et al. 2006; Seddiki et al. 2006). However, none of these molecules are uniquely expressed on Tregs, and many of them can be identified on T cells without suppressor activity.

3.3 Foxp3 Expression

Perhaps the best characterized and most reliable marker for Tregs is Foxp3, a member of the fork-head/winged-helix family of transcription factors (Fontenot et al. 2003; Hori et al. 2003). The identification of Foxp3 as a critical transcription factor necessary for Treg development and function was derived from a combination of basic science, mouse genetics, and clinical medicine studies. Foxp3 mutations were first identified in an inbred mouse line named *scurfy* and later in an analogous human genetic disorder called IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome). These syndromes are characterized by a wasting illness associated with immune dysfunction, lymphoproliferation, diarrhea, rash, and numerous autoimmune/endocrine abnormalities. The phenotype of these inherited disorders was very similar to that occurring in mice depleted of CD4+CD25+ Tregs. Similarities between IPEX and aGVHD were also noted. Through a series of elegant experiments it was shown that Foxp3 was upregulated in unmanipulated CD4+CD25+ Tregs as compared to CD4+CD25− T cells, CD8+ T cells, and CD19+ B cells. In addition, forced expression of Foxp3 by CD25− T cells via retroviral transduction led to the development of a T cell subset with both *in vivo* and *in vivo* suppressive properties (Fontenot et al. 2003; Hori et al. 2003).

Initially it was felt that Foxp3 was an unambiguous marker for Tregs; however, later it was shown that recently activated T cells without regulatory characteristics can up-regulate Foxp3 transiently (Morgan et al. 2005; Gavin et al. 2006; Wang et al. 2007). So it appears that an all-inclusive, highly specific single marker for Tregs remains elusive. Recently, some interest has focused on GARP, also known as LRRC32, a cell surface molecule important for both Foxp3 expression and the suppressive properties of Tregs (Wang et al. 2008). More research will need to be done to further elucidate the properties of GARP and other molecules that are used to identify Tregs. The identification of more specific and preferably extracellular molecules may help with the use of Tregs as a diagnostic and therapeutic tool in the treatment of human diseases. Because of the intracellular location of this transcription factor and the need for fixation and permeabilization of cells prior to detection with antibodies, viable Tregs cannot be isolated currently based on the expression of Foxp3.

3.4 Treg Mechanisms of Action

The mechanisms by which Tregs mediate suppression have already been reviewed extensively (Tang and Bluestone 2008; Vignali et al. 2008). Briefly, Tregs function by various manners, including secretion of the immunosuppressive cytokines IL-10, IL-35, and TGF-β (Tang and Bluestone 2008; Takahashi et al. 1998; Thornton and Shevach 1998), granzyme/perforin-induced cell lysis (Qin et al. 2006), metabolic disruption via cytokine (IL-2) deprivation (Pandiyan et al. 2007) or CD39/CD73-associated generation of adenosine metabolites, which suppresses T cells by binding to their adenosine receptor 2A (Deaglio et al. 2007), and modulation of dendritic cell function (Curti et al. 2009; Sharma et al. 2009; Chung et al. 2009). With regards to this last mechanism, special mention should be made of the upregulation of indoleamine 2,3-dioxygenase (IDO) on dendritic cells by Tregs and IFN-γ. Increased expression of IDO leads to depletion of tryptophan, which is an essential amino acid required by proliferating T cells. In addition, a byproduct of the tryptophan metabolism called kynurinine has potent immunosuppressive properties (Curti et al. 2009; Xu et al. 2008). Recent data suggests that IDO expression by dendritic cells could be an important mechanism of Treg-induced immune suppression following HSCT by increasing kynurinine (Jasperson et al. 2009). The proposed mechanism is through reduction of IL-6, an important cytokine for Th17 cell generation, thereby preventing aGVHD and linking Tregs and Th17 cells (Sharma et al. 2009; Chen et al. 2009). In the end, the exact mechanism by which Tregs induce immune regulation is not fully defined but likely involves numerous functions that vary depending on the clinical situation. The mechanisms of action may differ depending on

whether the Treg is involved with the prevention of autoimmunity, resolution of ongoing inflammation, maintenance of immune homeostasis, or regulating alloimmune responses.

3.5 Tregs and Murine aGVHD

Much of the early work in this area focused on natural Treg suppression of autoimmune phenomena. However, mixed lymphocyte reactions showed that natural Tregs also could efficiently control the proliferation of alloreactive T cells (Taylor et al. 2001). This finding indicated a potential role for natural Tregs in the prevention of allograft rejection during solid organ transplantation and during the inhibition of aGVHD following HSCT. In preclinical animal models, several groups demonstrated that aGVHD severity and lethality could be attenuated by the co-administration of freshly isolated Tregs with T cell effectors when compared to mice receiving only T effectors, in whom aGVHD was rapidly fatal (Taylor et al. 2001; Cohen et al. 2002; Hoffmann et al. 2002). In a similar experiment, when allogeneic bone marrow and T cell grafts were depleted of CD4+CD25+ Tregs, the mice quickly succumbed to the effects of aGVHD (Taylor et al. 2001; Cohen et al. 2002). Interestingly, in the experiments involving the co-transfer of Tregs and T effector cells, supraphysiologic ratios of Tregs to T effectors (i.e., 1:2 or 1:1) were needed to induce this suppression, as physiologic ratios of 1:10 did not show a protective effect.

Further studies demonstrated that only the $CD62L^{hi}$ Treg subset could decrease the incidence and severity of aGVHD in murine models of transplantation (Ermann et al. 2005; Taylor et al. 2004). Similarly, in an autoimmune diabetes model, only the $CD62L^{hi}$ Tregs that also expressed high levels of CCR7 were able to prevent the induction of diabetes (Szanya et al. 2002). These data were important as they suggested that Treg subsets could be defined by the expression of adhesion molecules and CCRs, with each subset possessing unique *in vivo* functional properties. The importance of lymph node homing in aGVHD generation again was illustrated, and the experiments also suggested that the lymph node was a possible *in vivo* site for Treg-induced immune suppression. Indeed the $CD62L^{hi}$ Tregs homed more efficiently to secondary lymphoid organs including the spleen, mesenteric, and peripheral LN, and were better able to prevent the proliferation of alloreactive T cells at these sites when compared to animals receiving the $CD62^{lo}$ Treg infusions (Ermann et al. 2005; Taylor et al. 2004). This hypothesis was further supported by bio-luminescence imaging studies that showed early co-localization of T effector cells with Tregs in secondary lymphoid organs after transplant, followed by egress of Tregs from lymph nodes and migration to peripheral tissues (Nguyen et al. 2007). Although this model would explain how Tregs prevent the induction of aGVHD, it does not necessarily elucidate how Tregs can suppress established aGVHD (Jones et al. 2003). Alternatively, lymph node localization may function to polarize Tregs towards homing patterns for specific tissues, so that, when released back into circulation, they are able to mediate organ-specific immune suppression. Only preliminary data is available to support this hypothesis in HSCT (Engelhardt et al. 2008).

As noted earlier, the prevention of aGVHD by adoptive transfer requires the infusion of large numbers of Tregs, which may not be feasible in clinical practice due to the low frequency of natural Tregs in circulation (i.e., 5–10% of CD4+ T cells). To deal with this problem, various groups developed protocols to expand Tregs ex vivo through stimulation with anti-CD3 antibodies or allogeneic APCs and exogenous high-dose IL-2. In addition, activated Tregs appear to suppress immune responses more efficiently than resting cells, suggesting additional clinical benefit from the expansion process (Cohen et al. 2002; Taylor et al. 2002; Hoffmann et al. 2004). Along these lines, two groups independently showed that the infusion of ex vivo expanded Tregs could improve survival in murine models of aGVHD (Cohen et al. 2002; Taylor et al. 2002). Although both freshly isolated and expanded Tregs could prevent the induction of aGVHD when co-transferred with effector T cells, their

activity in treating established aGVHD is less clear. Jones et al. showed that the infusion of Tregs up to 10 days after the initial infusion of CD8+ T cells could prevent aGVHD lethality in a MHC-matched model. Delayed administration of Tregs (>2 days after CD4+ infusion); however, could not prevent aGVHD mortality in a haploidentical model of transplant (Jones et al. 2003). These data suggest that Tregs can treat evolving aGVHD; however, optimal suppression seems to occur early in the disease process and prior to profound immune activation as in the setting of MHC mismatch. Taken as a whole, these observations imply that human Tregs could be expanded ex *vivo* and infused with the stem cell graft to prevent aGVHD or possibly to treat early aGVHD in HSCT.

3.6 Graft vs. Tumor Concerns

The immune mechanisms associated with GVT appear to be closely related to GVHD. This apparent association raised concern that the therapeutic use of Tregs to prevent aGVHD could lead to an increased risk of cancer relapse following HSCT. In murine models where animals were challenged with conventional T cells, Tregs, and either leukemia or lymphoma cell lines, Tregs could control aGVHD without disrupting GVT following MHC-matched or -mismatched transplants (Jones et al. 2003; Edinger et al. 2003; Trenado et al. 2003). This finding suggested that the immunological mechanism of GVT could be separated from GVHD, which would further support the therapeutic use of Tregs in human HSCT. However, some conflicting results have been obtained depending on the malignant cell line used (Trenado et al. 2003), and one human study has shown that higher Treg frequencies post-transplant were associated with an increased risk of relapse of chronic myelogenous leukemia (Nadal et al. 2007). Further research is needed to decipher the specific role of Tregs in aGVHD and GVT.

Prevention of stem cell graft rejection and immune reconstitution following transplant are key characteristics necessary for the long-term survival of patients undergoing HSCT. Interestingly, both donor and recipient Treg infusions facilitate donor hematopoietic progenitor cell engraftment, perhaps by suppressing recipient anti-donor immune responses (Taylor et al. 2004; Hanash and Levy 2005). Immune reconstitution also is improved with higher lymphocyte counts and increased frequencies of CD4+ and CD8 T+ cells post-transplant, indicating that normal immune development and function requires appropriate regulation by Tregs (Trenado et al. 2003). In summary, there is substantial evidence that naturally occurring Tregs are associated with a decrease in the incidence and severity of aGVHD in animal models of transplantation, improvement of immune reconstitution, and preservation of the beneficial effects of GVT.

3.7 Tregs and Human aGVHD

In spite of the overwhelming data supporting Treg prevention of aGVHD in murine models of transplantation, the role of Tregs in human aGVHD is less clear. Human HSCT is complex. Differing chemotherapy and immunosuppression regimens are used based on patient age, overall health, and disease status. Donors can be related, unrelated, HLA-identical, or HLA-mismatched. Stem cell grafts can be derived from bone marrow, peripheral blood, or cord blood. In addition, the graft can be manipulated with the removal or addition of specific lymphocyte subsets to facilitate engraftment, to prevent aGVHD, or to decrease relapse rates. All of these variations could potentially confound analysis of the role of Tregs in GVHD in humans. Numerous observational and retrospective studies have been performed using different patient populations and transplant techniques. Not surprisingly, heterogeneous results have been obtained.

In one of the first human studies examining CD4+CD25+ Tregs in patients undergoing HLA-identical sibling transplantation, the frequency of CD4+ cells co-expressing CD25+ in

the peripheral blood stem cell graft was significantly higher in those individuals who developed aGVHD (Stanzani et al. 2004). The *in-vitro* suppressive properties of these isolated Tregs were not analyzed in this study. The authors suggested that CD25 alone maybe insufficient to adequately identify human Tregs in the transplant setting. In a similar study, CD4+CD25+ Tregs were enumerated during the first 100 days following transplant in a series of patients primarily undergoing matched related sibling transplants. Here, there was no significant difference in the relative or absolute number of CD4+CD25+ Tregs in patients with or without aGVHD (Sanchez et al. 2004). Tregs were identified only by the expression of CD25 in both of these studies. It is difficult to reliably quantify Tregs in peripheral blood using only CD25, especially in HSCT patients who may have increased numbers of activated CD25-expressing T cells.

The later identification of Foxp3 as a more specific marker for Tregs has greatly facilitated Treg analysis in human transplantation. Initially, Foxp3 expression by peripheral blood mononuclear cells was analyzed by real-time quantitative polymerase chain reaction (PCR). The patient population studied was heterogeneous, consisting of patients with both HLA-matched and -mismatched, related or unrelated donors. Bone marrow was the stem cell source for all patients. Blood samples were obtained from the recipient at the time of aGVHD occurrence. Foxp3 mRNA expression was decreased significantly in patients with any aGVHD compared to patients without aGVHD or healthy controls. In addition, Foxp3 expression was inversely related to the severity of aGVHD, confirming the importance of Foxp3 for Treg analysis/identification and supporting the data previously obtained in murine studies (Miura et al. 2004).

In addition to PCR analysis, Tregs can also be enumerated using specific antibodies to Foxp3. Much of the early work analyzing the frequency and absolute numbers of human Foxp3+ Tregs was performed at the National Institutes of Health (NIH) using patients undergoing T cell depleted, HLA-identical sibling transplants. The frequency and absolute numbers of Foxp3+ Tregs were analyzed in these donors, stem cell grafts, and recipients both pre- and post-transplant. In patients undergoing myeloablative conditioning at the NIH, high absolute numbers of CD4 +Foxp3+ cells in the stem cell graft or in the recipient at day +30–45 was associated with a reduced risk of grade II–IV (moderate to severe) aGVHD. The proportion of CD4+CD25+ T cells expressing Foxp3 at day +30 was also lower in patients developing aGVHD (Rezvani et al. 2006). In another study from the NIH, this time examining patients undergoing reduced intensity chemotherapy (RIC) transplantation, moderate-to-severe aGVHD was more likely to occur in patients whose donors had fewer Tregs. The absolute and relative frequencies of Tregs were increased in the donors of patients who did not develop aGVHD (Mielke et al. 2007). Tregs in the stem cell graft or in the patient before and early after transplant were not associated with the development of aGVHD in this study.

The above data were obtained from patients receiving T cell-depleted transplants. In patients receiving more conventional HSCT with cells from either a related or an unrelated donor, the infusion of stem cell grafts containing higher absolute numbers of Foxp3+ cells was associated with a significantly lower cumulative incidence of aGVHD (Pabst et al. 2007; Wolf et al. 2007). The association of Tregs with aGVHD incidence appeared to be strongest in patients undergoing myeloablative MRD, as the significance was lost by RIC patients when the cohort was stratified based on the intensity of the conditioning regimen (i.e., myeloablative vs. RIC) (Wolf et al. 2007). Additionally, improved survival was found in patients receiving both myeloablative conditioning and grafts from sibling donors with high Treg numbers (Wolf et al. 2007). The risk of relapse was not affected by the Treg content of the graft. These data suggest that Treg infusions or naturally occurring high Treg numbers could improve survival by decreasing treatment-related mortality associated with aGVHD.

Alternatively, Tregs could improve post-transplant immune reconstitution, thereby leading to less frequent or severe infectious complications.

Initially it appeared that data from Foxp3 studies might explain why earlier studies were unable to demonstrate a relationship between human Tregs and the prevention of aGVHD. However, not all studies have shown a clear association between the number of Foxp3+ cells and incidence of aGVHD. In a series of pediatric patients undergoing either matched-related or unrelated transplants, Foxp3 expression by CD4+CD25+ cells was determined by real time PCR analysis. Post-transplant Foxp3 expression in patients was similar to that in healthy controls, irrespective of the presence or the absence of aGVHD (Seidel et al. 2006). Furthermore, these investigators demonstrated that Foxp3 expression was closely linked to the CD4+CD25+ T cell population/phenotype regardless of their suppressor potential. These data imply that recently activated naïve T cells can upregulate CD25 and express abundant amounts of Foxp3 mRNA, independent of the immunoregulatory function of the cells. Once again, these studies cast doubt on the use of any single marker to exclusively identify Tregs in the setting of human HSCT.

To date, conflicting results have been obtained in human studies examining the relationship between the suppressor activity of Tregs and the incidence of aGVHD. These discrepancies likely are due to numerous factors, including the clinical heterogeneity of human transplantation, timing of Treg analysis, and the origin of the sample in which Tregs were enumerated (i.e., recipient, donor, or stem cell graft). In addition, the differences in human studies could result from the difficulties with identifying and isolating pure Treg subsets. Although imperfect, the identification of Tregs by Foxp3 expression has given great insight into the role of Tregs in human transplantation. Currently, there is growing evidence that supports a role for human Tregs in the prevention of aGVHD. These data suggests that donor-derived Tregs influence transplant outcomes and Treg frequencies post-transplant. With growing acceptance of this concept, a logical next step is to explore the unique features and functions of Treg subsets as they relate to HSCT.

3.8 Adhesion Molecules and Tissue-Specific Tregs

aGVHD primarily involves the skin, gut, liver, secondary lymphoid organs, and possibly the lungs. The stereotypical involvement of specific organs by aGVHD strongly suggests that dysregulation of lymphocyte trafficking is important for the pathogenesis of aGVHD. The role of lymphocyte homing and expression of important adhesion molecules including selectins, CCRs, and integrins in aGVHD has been reviewed (Sackstein 2006; Wysocki et al. 2005a). Similar to conventional T cells, there is significant evidence that Treg localization after HSCT is of importance. The expression of adhesion molecules including selectins, CCRs, and integrins may serve to define Treg subsets with specific migratory patterns and suppressor properties (Huehn and Hamann 2005; Kim 2006; Wei et al. 2006).

Based on homing patterns, Tregs can be divided into two general populations: (1) lymphoid-homing (i.e., naïve-like), which express CCR7, CXCR4, CD62L and (2) nonlymphoid-homing (i.e., effector/memory-like), which variably express CCR2, CCR4, CCR5, CCR6, CCR8, CXCR3, CXCR6, CLA, and CD103 (Kim 2006; Huehn et al. 2004; Lee et al. 2007; Lim et al. 2006). As previously outlined, only the $CD62L^{hi}$ Treg population could decrease the lethality of aGVHD in animal models (Ermann et al. 2005; Taylor et al. 2004). Presumably this principle also will hold true for the CCR7+ subset of Tregs, since this molecule is often co-expressed with CD62L (Szanya et al. 2002). However, Tregs express diverse homing molecules and are present in both lymphoid and nonlymphoid tissues, suggesting that Tregs maintain immunologic tolerance at various sites. In addition, Tregs can suppress the initiation of aGVHD or established aGHVD, further supporting the idea of dual sites of immune regulation (i.e., suppression of allo-responses in secondary lymphoid

organs during the priming phase and in target tissues during the effector phase of aGVHD. This latter function likely is facilitated by the expression of integrins, CCRs, and selectins.

In murine models of transplantation, Treg expression of CCR5 and CCR6 has been shown to be of critical importance in preventing the development of aGVHD (Varona et al. 2006; Wysocki et al. 2005b). Normally, CCR5 and CCR6 are present on various types of leukocytes, including subsets of T cells and dendritic cells, and serve to mediate chemoattraction of these cells to areas of inflammation. The ligands for CCR5 (CCL3, CCL4, and CCL5) and CCR6 (CCL20) are present in aGVHD target tissues and are increased during inflammation (Wysocki et al. 2005a; Varona et al. 2006). In these Treg experiments, the severity and mortality of aGVHD induced by wild-type T cells was increased when CCR5- or CCR6- deficient Tregs were infused into either an unconditioned GVHD animal model (Varona et al. 2006) or an irradiated murine model of transplantation (Wysocki et al. 2005b), respectively. The *in-vitro* suppressive properties of both of these Treg subsets (i.e., CCR5−/− and CCR6−/−) were maintained, suggesting that lack of CCRs did not result in loss of suppressor phenotype. In addition, Varona et al. demonstrated that unmanipulated CCR6+ Tregs exhibit decreased expression of CD62L but upregulate other homing molecules including CCR4, CCR8, CD29, CD11a, and CLA (P-selectin ligand) (Varona et al. 2006). Similarly, the absence of CCR5 on Tregs resulted in normal *in-vivo* localization of Tregs in secondary lymphoid organs during the first week of transplantation; however, later homing of Tregs to specific target organs of aGVHD was inhibited (Wysocki et al. 2005b). Thus, it appears that Treg expression of CCR5 or CCR6 is not specific for cells pertinent to a single aGVHD target tissue, but instead is necessary for Treg migration to areas of inflammation following HSCT. In these models, the inhibition of aGVHD severity and mortality appears to be related to Treg-mediated suppression at peripheral sites as opposed to the lymph node.

To date, Treg expression of adhesion molecules and CCRs have been incompletely explored in human HSCT. Currently, there is direct evidence that tissue localization is important for Treg-mediated prevention of aGVHD in human HSCT, which in turn indirectly suggests that Treg expression of homing molecules is necessary and critical for *in vivo* function following transplant. In patients undergoing allogeneic HSCT, the frequency of mucosal Foxp3+ Tregs in intestinal biopsies as determined by double immunoenzymatic labeling was significantly higher in those individuals without gastrointestinal aGVHD when compared to either healthy controls or patients with symptomatic gut aGVHD (Rieger et al. 2006). In a similar study, the frequency of Foxp3+ Tregs in skin biopsies was related inversely to the severity of skin aGVHD and correlated with a positive response to treatment (Fondi et al. 2009). These data support the importance of Treg compartmentalization; however, Treg expression of homing receptors was not analyzed in either study. Therefore, the direct association between circulating tissue-specific Tregs with eventual tissue infiltration or the prevention of organ-specific aGVHD could not be assessed from this work.

3.9 Recent Work on Tissue-Specific Tregs and aGVHD

Our work has focused on identifying unique subsets of tissue-specific Tregs as they relate to the pathogenesis of organ-specific aGVHD in human HSCT.

As previously noted, Tregs can be generally divided into lymphoid- and non-lymphoid-homing subsets. Similar to other T cells, the nonlymphoid-homing Tregs can be further subdivided into mutually exclusive groups characterized by expression of either $\alpha_4\beta_7$/CCR9 (gut-homing) or CLA/CCR4 (skin-homing). The same principles and mechanisms that govern the regulation of selectins, integrins, and CCRs on other T cell subsets also seem to operate in Tregs (Kang et al. 2007; Siewert et al. 2007). Consistent with the known reciprocal regulation of these homing receptors, we have found an inverse relationship

between $\alpha_4\beta_7$ and CLA expression by human Tregs early after HSCT. Furthermore, we found that increased frequencies of circulating CLA+ Tregs early after transplant was associated with the prevention of initial skin aGVHD, and that higher percentages of CLA+ Tregs and $\alpha_4\beta_7$+ Tregs were related inversely to the severity of skin or gut aGVHD, respectively (Engelhardt et al. 2008). These studies suggest that circulating tissue-homing subsets of Tregs may regulate organ-specific risk and severity of aGVHD in human HSCT.

3.10 Summary of Tregs and aGVHD

In summary, Treg-mediated prevention of aGVHD morbidity and mortality may occur by several mechanisms of action that occur in diverse anatomical sites. Evidence seems to support immune regulation in both the lymph node and more peripherally in the target tissues of aGVHD. Furthermore, lymph node localization appears to be critical for appropriate tissue compartmentalization of lymphocytes. We suggest that early after HSCT Tregs may function to suppress initial activation of alloreactive T cells in secondary lymphoid organs. Then antigen-activated Tregs may upregulate adhesion molecules and leave the lymph node to exert their suppressor functions at distal sites. The induction of certain CCRs, such as CCR5 and CCR6, may direct Tregs to areas of ongoing epidermal and mucosal inflammation or to other activated lymph nodes. In addition, the lymph node may function to polarize Tregs for homing to specific tissues via induced expression of $\alpha_4\beta_7$/CCR9 or CLA/CCR4, thereby allowing Tregs to migrate to and concentrate in the tissues where the alloantigen was originally encountered. Many of the ligands for the above adhesion molecules are expressed constitutively by aGVHD target tissues and expression is increased during periods of inflammation induced by chemotherapy or established aGVHD. This inflammatory environment therefore supports Treg migration to these tissues to help suppress alloreactive responses and to reestablish immune homeostasis. Thus, a well orchestrated suppression of immune responses in both the lymph node and the peripheral tissues likely allows Tregs to prevent aGVHD.

4 Th17 Cells and aGVHD

4.1 Biology of Th17 Cells

Th17 cells are a newly identified lineage of T cells with distinct characteristics that separate them from the previously described Th1/2 subsets and Tregs [reviewed in Bettelli et al. (2007), Miossec et al. (2009) and Ouyang et al. (2008)]. Th17 cells are characterized by the production of proinflammatory cytokines including IL-17 (also called interleukin-17A), IL-17F, IL-21, and IL-22. Normally, these cells help to induce peripheral inflammation and function to coordinate host defenses against extracellular pathogens (Aujla et al. 2008). Th17 cells also have been implicated in several pathological states, including induction of autoimmunity and the rejection of solid organ allografts (Bettelli et al. 2007; Miossec et al. 2009; Ouyang et al. 2008).

Normally, IL-6 in the presence of TGF-β promotes the differentiation of naïve T cells into Th17 cells via a STAT3 pathway (Miossec et al. 2009; Bettelli et al. 2006; Mangan et al. 2006). IL-21 and IL-23 further support Th17 expansion and survival (Miossec et al. 2009; Mangan et al. 2006), while the Th1 cytokine, IFN-γ, acts as a negative regulator. Murine retinoid-related orphan receptor (ROR)t (or its human counterpart RORc) is the key transcription factor necessary for Th17 differentiation (Miossec et al. 2009; Bettelli et al. 2006; Ivanov et al. 2007). IL-6, IL-21, and IL-23 in the appropriate setting help to induce expression of RORt (Bettelli et al. 2006; Ivanov et al. 2007). Interestingly, IL-6, a critical cytokine for Th17 commitment, has been shown to interfere with peripheral (i.e., induced) Treg cell generation through up-regulation of the TGF-β pathway inhibitor SMAD7 (Dominitzki et al. 2007). Thus, these two T cell subsets, one with an inflammatory

phenotype and the other with a suppressor phenotype, appear to be related and reciprocally regulated in part by IL-6. Furthermore, retinoic acid, a mediator of induction of gut homing phenotype that has been shown to increase Foxp3 expression and decrease Th17 lineage commitment by enhancing TGF-β -induced SMAD3 signaling, simultaneously inhibits IL-6 and IL-23 pathways by decreasing RORt expression (Schambach et al. 2007; Elias et al. 2008; Kim2008; Xiao et al. 2008; Mucida et al. 2007). Physiologically, this process may function to protect the gut from unwanted inflammation resulting from constant antigen exposure by skewing this mucosal milieu towards tolerance. These studies also suggest that the expression of gut-homing markers will be under-represented on Th17 cells; however, this concept needs to be explored further.

4.2 Th17 Cells and CCRs

It has been reported that circulating Th17 cells are characterized by the CCR profile of either CCR6+CCR4+ (Acosta-Rodriguez et al. 2007) or CCR2+CCR5– (Sato et al. 2007). Specifically, CCR6 expression appears to be upregulated on CD4+ T cells capable of producing IL-17 (Singh et al. 2008). However, more extensive analysis of Th17 cells isolated from adult peripheral blood, umbilical cord blood, or tonsillar lymphoid tissue has demonstrated that these cells are capable of expressing a diverse range of CCRs. Similar to Tregs, Th17 cells also seem to express either secondary lymphoid tissue homing receptors (CCR7, CXCR4, CD62L) or nonlymphoid homing molecules (CCR2, CCR4, CCR5, CCR6, CXCR3, and CXCR6) (Lim et al. 2008; Kim 2009). Although CCR6 is highly expressed by Th17 cells, this CCR is also found on approximately 50% of all circulating CD4+ memory T cells (Singh et al. 2008). In the end, there is likely no single CCR expression pattern that will universally and specifically identify all Th17 cells.

4.3 Th17 Cells and the aGVHD Disease Process

Because of their association with inflammation and autoimmunity, the relationship between Th17 cells and aGVHD is now being explored. Th17 cells have been indentified in secondary lymphoid organs and in aGVHD target tissues of animals undergoing allogeneic bone marrow transplant (Carlson et al. 2009; Kappel et al. 2009). In one preclinical model of transplant, the transfer of murine IL-17−/− CD4+ T cells led to delayed aGVHD development when compared to animals receiving wild-type CD4+ T cells. aGVHD still occurred and the mortality rate was unchanged in this setting (Kappel et al. 2009). In a similar set of experiments using a MHC-mismatched HSCT model, transfer of IL-17−/− T cells paradoxically caused increased aGVHD mortality and was associated with a skewed Th1 differentiation pattern in the donor T cells with associated liver and gut injury (Yi et al. 2008). Another group showed that the infusion of naïve CD4+ T cells that were polarized towards a Th17 phenotype resulted in significant aGVHD primarily involving the skin and the lung (Carlson et al. 2009). Overall, the data suggest that Th17 cells may modulate Th1 donor T cell differentiation, which in turn may affect end organ damage. Further evidence supporting this model was derived from animal models where the infusion IFN-γ −/− CD4+ T cells led to preferential differentiation of cells with a Th2 and Th17 phenotype, with subsequent increase in skin and pulmonary aGVHD and decrease in T cell expression of gut-homing associated adhesion molecules (Yi et al. 2009). Many questions remain with regards to Th17 cells as they relate to aGVHD in both murine models of transplantation and in human HSCT. The preferential involvement of skin and lung over gut tissues by Th17 cells does suggest that Th17 cell compartmentalization and expression of adhesion molecules may play a role in aGVHD development. Of particular interest in future studies will be the role of CCR6. This CCR is highly expressed on Th17 cells (Singh et al. 2008) and when absent on Tregs was shown previously to be associated with accelerated aGVHD lethality (Varona et al. 2006).

5 Summary

GVHD is one of the principal complications following HSCT that limit success. There is a large amount of data now in both animal models and humans after transplant to suggest that complex dynamics in the T cell compartment regulate the clinical expression of disease. Most studies suggest that the suppressive function of Tregs is essential for maintaining successful outcomes after transplantation. Recent data suggests that the location of Tregs and their ability to migrate to organs such as skin and gut significantly affect the expression of disease in target tissues. These findings may allow early stratification of clinical risk of skin or gut aGVHD following transplantation based on numbers and phenotype of circulating Tregs. These studies also suggest that induction of Tregs with an appropriate phenotype or adoptive transfer of such cells could be considered in the future as a prophylactic or therapeutic intervention in transplantation. However, the biology of these cells *in vivo* is complex and still incompletely understood. Many practical questions remain as to the number and phenotype of cells that would be needed in clinical intervention, and the exact mechanisms by which Tregs maintain immune homeostasis in the setting of human HSCT.

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Abbreviations

aGVHD	Acute graft vs. host disease
ATG	Antithymocyte globulin
ATRA	All-trans-retinoic acid
CCL	Chemokine ligand
CCRs	Chemokine receptors
cGVHD	Chronic graft vs. host disease
CLA	Cutaneous lymphocyte antigen
CTLA4	Cytotoxic T-lymphocyte associated antigen 4
E-selectin	Endothelial-cell selectin
Foxp3	Forkhead box protein P3
GITR	Glucocorticoid tumor necrosis factor receptor
GVT	Graft vs. tumor effect
HEVs	High endothelial venules
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
IDO	Indoleamine 2,3-dioxygenase
IL	Interleukin
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome

MADCAM1	Mucosal vascular address in cell-adhesion molecule 1
MRD	Matched related donor
P-selectin	Platelet selectin
RIC	Reduced intensity chemotherapy
ROR	Retinoid-related orphan receptor
Tregs	Regulatory T cells
URD	Unrelated donor

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