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Allogeneic Th1 Cells Home to Host Bone Marrow and Spleen and Mediate IFNγ-Dependent Aplasia

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Bone marrow graft failure and poor graft function are frequent complications after hematopoietic stem cell transplantation and result in significant morbidity and mortality. Both conditions are associated with graft-versus-host disease (GVHD), although the mechanism remains undefined. Here we show, in 2 distinct murine models of GVHD (complete MHC- and class II-disparate) that mimic human peripheral blood stem cell transplantation, that Th1 $CD4^+$ cells induce bone marrow failure in allogeneic recipients. Bone marrow failure after transplantation of allogeneic naïve $CD4^+$ T cells was associated with increased $CD4^+$ Th1 cell development within bone marrow and lymphoid tissues. Using IFNγ-reporter mice, we found that Th1 cells generated during GVHD induced bone marrow failure after transfers into secondary recipients. Homing studies demonstrated that transferred Th1 cells express CXCR4, which was associated with accumulation within bone marrow and spleen. Allogeneic Th1 cells were activated by radiation-resistant host bone marrow cells and induced bone marrow failure through an IFNγ-dependent mechanism. Thus, allogeneic Th1 $CD4^+$ cells generated during GVHD traffic to hematopoietic sites and induce bone marrow failure via IFNγ-mediated toxicity. These results have important implications for prevention and treatment of bone marrow graft failure after hematopoietic stem cell transplantation.

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is an increasingly utilized therapy for treatment of malignant and nonmalignant disorders. Although outcomes continue to improve, significant morbidity and mortality continue to limit this treatment for many patients. Bone marrow (BM) graft failure and poor graft function occur in up to 25% of patients undergoing HSCT and both are associated with an increased risk of infection and death [1,2]. Risk factors for development of graft failure and poor graft function include infection, medication side effects, and graft-versus-host disease (GVHD) [1]. The mechanistic basis for the relationship between GVHD and BM failure remains poorly defined.

Previous adoptive transfer studies have demonstrated that allogeneic Th17 cells, produced in vitro, induce an atypical form of GVHD manifested primarily by skin and lung disease [3]. Similar studies with Th2 cell transfers indicated a decreased pathogenicity of these cells in HSCT mouse models [4,5]. Transfer studies using in vitro-generated Th1 cells have been limited by previous isolation methods, and no studies have conclusively determined the role of committed Th1 cells in GVHD using adoptive transfer methodology [6,7]. Here, using a previously reported IFNγ-reporter mouse model [8], we describe GVHD mediated by purified, committed Th1 cells in clinically relevant murine models.

Th1 development is under control of the transcription factor, T-bet, which can be upregulated by IL-12 and other signals [9]. Th1 cells produce the signature cytokine, IFNγ, which acts to further promote Th1 development and suppress the development of other lineages. T-bet is elevated in T cells from aplastic anemia patients with BM failure [10]. Previous studies have also demonstrated an important role for IFNγ in BM suppression and failure [11-16]. In addition, a direct negative effect of IFNγ on $CD34^+$ cord blood hematopoietic stem cells has been demonstrated [17]. Elegant studies using IFNγ-receptor-deficient recipients revealed increased levels of IFNγ present in recipient blood and tissues, which was associated with hematopoietic failure and lymphoid aplasia. Disease in these mice was dependent on both IFNγ and Fas-FasL [18]. IFNγ is a ubiquitous cytokine produced by multiple cell lineages within the immune system, including Th1 cells. $CD8^+$ cells, in particular, are an important source of IFNγ, and several studies have indicated that $CD8^+$ cells are critical for inducing BM disease [11,16]. Previous work using polyclonal allogeneic $CD4^+$ cells indicated that IFNγ was important for BM disease in the setting of sublethal conditioning, but not in lethal conditioning [13]. Other studies exploring $CD4^+$ mediated BM suppression have implicated IFNγ-independent mechanisms. Fas-FasL interactions, in particular, seemed to be important in mediating the BM manifestations in these studies [19,20]. It remains uncertain, therefore, whether allogeneic Th1 cells directly mediate suppression of recipient BM function, and, if so, the mechanism(s) of this suppression.

This study significantly extends previous work by definitely demonstrating that allogeneic Th1 cells directly mediate host hematopoietic failure. In addition, we have

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performed novel studies, through the use of transgenic reporter mouse systems, determining allogeneic Th1 cell homing, and detailed analyses, including mechanism, of Th1-mediated suppression of host hematopoiesis.

MATERIAL AND METHODS

Mice

The following mice were purchased from Jackson Laboratory and/or bred at our facility: BALB/cJ (BALB/c), B6.C-H-2bm12 (bm12), C57BL/6J (B6), C57BL/6.Ly5.2 (CD45.1-homozygous), B6.MRL-*Fas*^{lpr}/J (Fas deficient), and B6.129S7-*Ifngr1*^{tm1Agt}/J (IFNγ receptor deficient). The *Ifng*-reporter BAC-In transgenic mice were previously described [8]. The 3BBM74 transgenic TCR mice were a kind gift from E. Palmer (University Hospital, Basel, Switzerland). All animals were bred and maintained in accordance with the University of Alabama at Birmingham (UAB) Institutional Animal Care and Use Committee regulations.

Cell Preparation

CD4$^+$ cells were purified from pooled spleen and/or lymph nodes by magnetic bead positive selection (Dynal beads, Invitrogen, Life Technologies, Grand Island, NY). CD25-depletion was performed using PE-labeled anti-CD25 antibody (eBiosciences, San Diego, CA) followed by anti-PE magnetic bead depletion (Miltenyi Biotech, Auburn, CA). Post-sort purity was confirmed by flow cytometry.

Th1 cell purification was performed by first isolating lymphocytes from pooled spleens. Splenocytes were then treated with PE-labeled anti-Thy1.1 (eBiosciences) followed by anti-PE magnetic bead selection (Miltenyi Biotech).

Donor BM cells were prepared by collecting cells from both femurs and tibias of donor mice. In all experiments, BM was obtained from CD45.1- or CD45.2-homozygous donor mice to differentiate from coadministered donor CD4$^+$ cells. T cell depletion was performed using PE-labeled Thy1.2 antibody (BD Biosciences) followed by anti-PE magnetic bead depletion (Miltenyi Biotech, San Jose, CA).

Cell Culture

Purified wild-type B6 CD4$^+$ cells were cultured in vitro with irradiated B6 splenocytes in Th1 conditions with 1 ng/mL rmIL12 (R&D Systems, Minneapolis, MN) and 10 μg/mL anti-IL4 antibody (clone 11B11), along with 2.5 μg/mL anti-CD3 (clone 145-11) stimulation. Cells were cultured for 3 days and purified for transfer.

Transplant Procedure

Donor and recipient mice were 4 to 8 weeks of age at time of transplantation. Transplants were performed according to UAB Institutional Animal Care and Use Committee-approved protocols. Recipient mice received 900 cGy of total body irradiation in 2 split fractions 3 hours apart from an x-irradiator (X-RAD 320, Precision X-ray Inc., North Branford, CT). At least one hour later, T cell-depleted BM, with or without purified CD4$^+$ cells, was administered to anesthetized mice via intravenous injection. Cell doses and experimental groups specified in text and legends.

All animals were given water supplemented with Trimethoprim and Sulfamethoxazole for 4 weeks after transplantation.

Mice were weighed at least twice weekly, and mice exhibiting severe disease, evidenced by lethargy, severe skin disease, hunching, or weight loss >20% original weight, were euthanized and scored as dead.

To simplify the manuscript nomenclature, the authors refer to suppression of BM in the mouse recipients of allogeneic cells as *suppression of recipient bone marrow*. However, these recipient mice are lethally irradiated and reconstituted with BM from donor mice.

Flow Cytometric Analysis

Intracellular staining was performed as previously described [21]. Live cells were identified using LIVE/DEAD Fixable Far Red Dead Cell Stain (Invitrogen). All fluorescent antibodies for analysis purchased from eBiosciences, unless otherwise specified. Data were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Bone Marrow Progenitor Analysis

Red blood cells were lysed using ammonium chloride, and BM cells were stained for 30 minutes in HBSS, 2% FBS containing the following antibody combinations. Both stains included anti-c-Kit (2B8; BD Pharmingen) and Sca-1 (E13-161.7; Biolegend, San Diego, CA) antibodies conjugated to APC and Pacific Blue, respectively, and, for the lineage stains, PE-Cy7 conjugated antibodies against CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), B220 (RA3-6B2), CD19 (1D3), Mac-1 (M1/70), Gr-1 (RB6-8C5), and TER119 (TER119). PE-conjugated anti-IL7-Ra (SB/199: BD Pharmingen) was included in the HSC/lymphoid progenitor stain. Anti-CD34 (RAM34) and CD16/32 (2.4G2; BD Pharmingen) antibodies conjugated to FITC and PE, respectively, were included in the myeloid progenitor stain.

Histology

Tissue samples were fixed in 10% phosphate-buffered formalin solution and labeled without experimental details. Slides were embedded in paraffin, sectioned, and stained by the UAB Animal Resources Program Comparative Pathology Laboratory, and then read by a pathologist (T. Schoeb) blinded to experimental details. Tissues were scored according to the extent of disease and the severity of disease, and the product of these scores was reported.

Laboratory Analysis

Complete blood counts were performed on heparinized whole blood samples obtained by tail bleeding, and analysis was performed by the UAB Animal Resources Program Comparative Pathology Laboratory using Abaxis VetScan HMII7 (Abaxis VetScan, Union City, CA).

Statistical Analysis

Statistical significance was determined using either unpaired or paired Student *t*-test, as indicated in text. *P* values are reported in text. Survival analysis was performed using the Cox-Mantel test.

RESULTS

GVHD Induced by Naïve CD4$^+$ cells is Associated with BM and Spleen Hypocellularity and Th1 Cell Predominance

GVHD occurs in the setting of allo-disparity between hematopoietic stem cell donor and recipient, and can be mediated by both CD4$^+$ and CD8$^+$ T cells. To investigate effector CD4$^+$ T cell development in the setting of GVHD and BM failure, we used the MHC class II-disparate C57BL/6 (B6) to B6.C-H-2bm12 (bm12) model, in which GVHD is mediated solely by CD4$^+$ T cells [22,23].

Bm12 recipients of allogeneic naïve B6 CD4$^+$ T cells succumb to GVHD within 3 weeks of transfer of 10^5 or 10^6 cells (Figure 1A). BALB/c recipients of naïve B6 CD4$^+$ T cells (complete MHC mismatch) develop GVHD with a similar time course (Figure 1B). In the absence of GVHD, irradiated murine recipients of BM alone are usually well engrafted within 3 weeks of transplantation (not shown). Irradiated CD45.2 bm12 recipients of 10^4 allogeneic CD4$^+$CD25$^-$CD45.1 T cells were monitored for at least 6 weeks posttransplantation. Death from GVHD generally occurred between weeks 2 to 4 posttransplantation for those mice experiencing severe disease (Figure 1A). To ensure adequate time for engraftment of donor BM cells, mice dying after 3 weeks were selected for histological evaluation. GVHD target organs (lung, skin, liver, and gastrointestinal tract) were evaluated histologically, as were hematopoietic sites (BM and spleen). In addition to disease in typical GVHD target organs, the BM and spleen demonstrated loss of normal architecture and moderate to severe aplasia, whereas those tissues from the negative control groups had absent or mild disease (Figure 1C).

Donor T cells recovered from spleen and lymph nodes of bm12 recipients were distinguished by the CD45.1/45.2 allotype and phenotyped by flow cytometry. In agreement with previous studies [24-26], the majority of donor CD4$^+$ T cells produced IFNγ, consistent with a Th1 allo-response (Figure 2A, B). TNFα levels were slightly decreased in donor CD4$^+$ T cells (Figure 2C). There was no significant increase in IL-17, IL-4, or IL-13 in donor CD4$^+$ T cells (Figure 2A, not shown) suggesting that the IFNγ-positive T cells in the BM developed via the Th1 pathway. Studies performed using BALB/c recipients gave similar results (Figures 1B, 2A).

Because of the presence of severe BM disease in allogeneic recipients of naïve CD4$^+$ T cells (Figure 1C), we isolated