Biol Blood Marrow Transplant 23 (2017) 1622–1630

Biology of Blood and Marrow Transplantation

journal homepage: www.bbmt.org

ASBMT American Society for Blood and Marrow Transplantation

Biology

STAT3 Expression in Host Myeloid Cells Controls Graft-versus-Host Disease Severity

Evelyn C. Nieves [1,†], Tomomi Toubai [1,†], Daniel C. Peltier [2], Katherine Oravecz-Wilson [1], Chen Liu [3], Hiroya Tamaki [4], Yaping Sun [1], Pavan Reddy [1,*]

[1] Division of Hematology and Oncology, Department of Internal Medicine, University of Michigan Comprehensive Cancer Center, Ann Arbor, Michigan
[2] Division of Hematology and Oncology, Department of Pediatrics, University of Michigan, Ann Arbor, Michigan
[3] Department of Pathology and Laboratory Medicine, Rutgers-Robert Wood Johnson Medical School, New Brunswick, New Jersey
[4] Division of Hematology, Department of Internal Medicine, Hyogo College of Medicine, Hyogo, Japan

Article history:
Received 13 February 2017
Accepted 27 June 2017

Key Words:
Bone marrow transplantation
Graft-versus-host disease
STAT-3
Myeloid cells

A B S T R A C T

Professional antigen-presenting cells (APCs) are important modulators of acute graft-versus-host disease (GVHD). Although dendritic cells (DCs) are the most potent APC subset, other myeloid cells, especially macrophages (MFs) and neutrophils, recently have been shown to play a role in the severity of GVHD. The critical molecular mechanisms that determine the functions of myeloid cells in GVHD are unclear, however. Signal transducer and activator of transcription 3 (STAT3) is a master transcription factor that plays a crucial role in regulating immunity, but its role in MF biology and in acute GVHD remains unknown. To determine the impact of myeloid cell-specific expression of STAT3 on the severity of acute GVHD, we used myeloid cell-specific STAT3-deficient LysM-Cre/STAT3$^{fl/-}$ animals as recipients and donors in well-characterized experimental models of acute GVHD. We found that reduced expression of STAT3 in myeloid cells from the hosts, but not the donors, increased inflammation, increased donor T cell activation, and exacerbated GVHD. Our data demonstrate that STAT3 in host myeloid cells, such as MFs, dampens acute GVHD.

INTRODUCTION

Graft-versus-host disease (GVHD) is a major life-threatening complication of allogeneic hematopoietic cell transplantation (allo-HCT). Approximately 35% to 50% of patients experience acute GVHD despite prophylaxis with calcineurin inhibitors, which suppress allogeneic T cells, the main effector cells of GVHD pathogenesis [1,2]. Therefore, new GVHD prophylaxis and treatment strategies are needed.

The biology of GVHD is complex. Antigen-presenting cells (APCs), derived from both donor and host, play important roles in the development of acute GVHD by producing proinflammatory cytokines, such as TNF-α and IL-6, as well as by directly stimulating donor T cells [3-9]. The complex role of dendritic cells (DCs), a potent subset of APCs, in GVHD pathogenesis is being increasingly appreciated [9]; however, the effect of macrophages (MFs), another type of APC that infiltrate GVHD target organs [10,11], is less well understood. In support of a role for MFs influencing GVHD, one study found that a reduction in acute GVHD when macrophage recruitment to GVHD target organs was inhibited by CYM-5442, a sphingosine 1-phosphate 1(S1P1) receptor agonist [12], and another study suggested that the anti-GVHD properties of corticosteroids are likely related in part to inhibition of MF functions [13]. However, the foregoing studies did not distinguish the role of donor-derived versus recipient-derived MFs in acute GVHD, which is important because both host and donor MFs are present early after allo-HCT owing to the resistance of host MFs to transplantation conditioning regimens [14]. In an effort to clarify this, Hashimoto et al. [15] showed that deleting host MFs increases donor T cell expansion and exacerbates GVHD, whereas colony-stimulating factor (CSF)-1 expanded host MFs reduced acute GVHD. In contrast to host-derived MFs, CSF-1-dependent donor MFs exacerbate chronic GVHD [16]; however, exactly how host versus donor MFs modulate GVHD, what macrophage molecular and signaling pathways are required for GVHD modulation, and how MFs affect allogeneic T cell responses remain unclear.

Signal transducer and activator of transcription 3 (STAT3) plays a central role in regulating innate and adaptive immune

Financial disclosure: See Acknowledgments on page 1629.

* Correspondence and reprint requests: Pavan Reddy, MD, Division of Hematology and Oncology, Department of Internal Medicine, University of Michigan Comprehensive Cancer Center, 7215 CCGC, 1500 E. Medical Center Drive, Ann Arbor, MI 48105-1942.
E-mail address: reddypr@med.umich.edu (P. Reddy).
† These authors are equally contributed.

http://dx.doi.org/10.1016/j.bbmt.2017.06.018
1083-8791/

responses [17,18]. STAT3 signaling in T cells exacerbates both acute and chronic GVHD, possibly owing to its essential role in donor T cell activation and inflammatory Th17 cell differentiation [19-26]. In addition, STAT3 signaling exacerbates GVHD by reducing the stability of naïve regulatory T (Treg) cells and by limiting the expansion of induced donor Treg cells [27]. In contrast, the absence of STAT3 in donor T cells ameliorates chronic GVHD by reducing the number of follicular helper T (Tfh) cells and increasing the number of T follicular regulatory (Tfr) cells [28].

STAT3 activation is required for the immune regulatory functions and survival of MFs [29,30]. Interestingly, IL-10 secretion by MFs driven by STAT3 signaling is crucial for the inhibition of inflammatory responses [31,32], whereas a deficiency of STAT3 in MFs increases proinflammatory cytokine production in response to lipopolysaccharide (LPS) and helps create a Th1-promoting milieu [33]. In DCs, STAT3 signaling is crucial for both DC activation and induction of immune tolerance [34,35]. Increased STAT3 expression in donor-derived plasmacytoid DCs (pDCs) reduces acute GVHD [36]. STAT3 acetylation negatively regulates DC function by promoting indoleamine 2,3-dioxygenase expression and ameliorates acute GVHD [37-40]. Taken together, these previously reported findings suggest that STAT3 signaling influences inflammation in a cell-intrinsic and presumably context-dependent manner.

To determine whether STAT3 contributes to the role of myeloid cells such as MFs in regulating GVHD, we tested the influence of STAT3-deficient myeloid lineage cells generated from LysM-Cre/STAT3$^{fl/-}$ animals in experimental models of acute GVHD [33]. We found that the absence of STAT3 in host-derived, but not donor-derived, MFs exacerbates GVHD. These data suggest that STAT3 signaling in host myeloid cells, such as MFs and polymorphonuclear leukocytes, mitigates the severity of acute GVHD.

MATERIALS AND METHODS

Mice

Female BALB/c (H-2^d, CD45.2$^+$) and C57BL/6 (B6, H-2^b, CD45.2$^+$) mice were purchased from Charles River Laboratories (Wilmington, MA). B6Ly5.2 (H-2^b, CD45.1$^+$) mice were purchased from National Cancer Institute (Frederick, MD). C3H.SW (H-2^b, CD45.2$^+$) mice were purchased from Jackson Laboratory (Bar Harbor, ME). B6-background Stat3$^{fl/+}$ animals were provided by S. Akira (Osaka University) and have been described previously [55]. All animals were cared for in accordance with regulations reviewed and approved by the University of Michigan's Committee on Use and Care of Animals, based on the university's laboratory animal medicine guidelines.

STAT3 Genotyping

Stat3$^{fl/+}$ females and *Stat3$^{fl/+}$* males were crossbred and genotyped to obtain *Stat3$^{fl/-}$* mice as described previously [33]. The mice were inbred and maintained by the University of Michigan breeding colony.

BMDCs

To obtain BMDCs, BM cells from B6-WT or LysM-Cre/Stat3$^{fl/-}$ animals were cultured with murine recombinant granulocyte-macrophage colony stimulating factor (20 ng/mL; PeproTech, Rocky Mill, NJ) for 7 days and then harvested as described previously. BMDCs were isolated using CD11c (N418) MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) on an autoMACS cell separator (Miltenyi Biotec).

STAT3 Detection

Peritoneal macrophages (MFs, 1×10^6), purified BMDCs (1×10^6), and whole splenic cells (1×10^6) from naïve B6 or LysM-Cre/Stat3$^{fl/-}$ animals were washed with PBS and lysed with RIPA buffer. Cell lysates were subjected to Western blot analysis using the antibodies STAT3 (Abcam, Cambridge, UK), β-actin-HRP (Abcam), and Cre-lox (Novus Biologicals, Littleton, CO) in accordance with the manufacturer's instructions.

STAT3 Inhibitor Study

WT Balb/c animals were irradiated (^{137}Cs source) with 800 cGy of total body irradiation. Immediately after irradiation, the mice were treated with a single i.p. injection of 20 mg/kg WP1066 STAT3 inhibitor (Sigma-Aldrich, St. Louis, MO) dissolved in DMSO/polyethylene glycol (PEG) 300 (1:4, vol/vol). Control mice were treated with only diluent. At 24 and 48 hours postinjection, the mice were euthanized and peritoneal (20-mL wash) and whole splenic CD11b$^+$ cells were analyzed with an Attune NxT acoustic focusing flow cytometer (Life Technologies, Carlsbad, CA).

Quantitative Real-Time PCR

Total RNA was obtained by phenol/chloroform separation. *Gapdh* (forward, 5′-GACATGCCGCCTGGAGAAAC-3′; reverse, 5′-AGCCCAGGATGCCCTTTAGT-3′) served as the control housekeeping gene. The primers for Stat3$^{fl/fl}$ detection were forward, 5′-GGGGTGAGAGTTACCGTGAA-3′ and reverse, 5′-CACACA CACACAAGCCATCA-3′. Real-time PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA) in an MasterCycler Realplex real-time PCR instrument (Eppendorf, Hamburg, Germany).

FACS Analysis

Flow cytometry was performed as described previously. In brief, to analyze phenotypes of peritoneal MFs, isolated cells were suspended in FACS wash buffer (2% BSA in PBS) and stained with FITC-, PE-, or allophycocyanin-conjugated mAbs. Anti-mouse mAbs for CD11b (M1/70), F4/80(BM8), I-Ab (AF6-120.1), CD40 (3/23), CD80 (16-10A1), Gr-1 (RB6-8C5), PD-L2 (TY25), and B220 (RA3-6B2) were purchased from Biolegend (San Diego, CA). PE-conjugated anti-PD-L1 mAb (M1H5) was purchased from eBioscience (San Diego, CA). After staining, cells were washed with FACS wash buffer and fixed with FACS lysing solution (BD Biosciences, San Jose, CA). Samples were analyzed with a flow cytometer (Accuri C6; BD Biosciences). To analyze donor T cell expansion after allo-BMT, isolated spleen cells were processed as above and then stained with CD4 (RM4-4), CD8a (53-6.7), CD90.2 (53-2.1), CD229.1 (30C7), CD45.1 (A20), and CD45.2 (104) mAbs purchased from Biolegend. For intracellular staining of Foxp3, cells were washed with permeabilization buffer (eBioscience) and then stained with PE-conjugated anti-Foxp3 mAb (FJK-16s; eBioscience) for 30 minutes at 4°C. The cells were then washed with FACS wash buffer and analyzed on a Accuri C6 cytometer (BD Biosciences).

BMT

Host animals were irradiated (^{137}Cs source) with 800-1100 cGy of total body irradiation on day -1 before allo-BMT, and donor BM cells were harvested from the femur and tibia. Where indicated, T cells were magnetically depleted from the BM (TCD-BM) using mouse CD90.2 microbeads and MACS LS columns (Miltenyi Biotec). Splenic T cells were magnetically isolated also using mouse CD90.2-microbeads and MACS LS columns. T cell purity was checked by flow cytometry and adjusted accordingly. Syngeneic or allogeneic BM (either whole or TCD-BM) and T cells were infused through the tail vein. Host mice were housed in sterilized microisolator cages and maintained on acidified water (pH <3) for 3 weeks after allo-BMT, as described previously [56]. Survival was monitored daily, and clinical GVHD was assessed weekly. All animal studies were performed in accordance with guidelines of the University of Michigan's Institutional Animal Care and Use Committee.

MLR

In an MLR, splenic T cells from B6-WT or BALB/c-WT mice (magnetically separated by MACS using CD90.2 microbeads) were used as responders, and B6-WT or B6-LysM-Cre/Stat3$^{fl/-}$ derived BMDCs or peritoneal MFs were used as stimulators. A total of 1×10^5 T cells and 2.5×10^3 irradiated (20 Gy) BMDCs or 1×10^5 irradiated (20 Gy) MFs were cocultured in 96-well U-bottom plates for 72 hours. The incorporation of ^{3}H-thymidine (1 μCi/well) by proliferating T cells during the final 16 hours of coculture was measured by a Betaplate reader (Wallad, Turku, Finland).

ELISA

IFN-γ, TNF-α, IL-6, Il-17A, and IL-10 were measured in culture supernatants or mouse serum by ELISA with specific anti-mouse mAbs for capture and detection. The appropriate standards were purchased from BD Biosciences (San Jose, CA). Assays were performed according to the manufacturer's protocol and read at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

Histology

Formalin-preserved livers and GI tracts were embedded in paraffin, cut into 5-mm-thick sections, and stained with hematoxylin and eosin for histological examination. Slides were scored in a blind fashion by C.L. using a semiquantitative scoring system that assesss the abnormalities known to be associated with GVHD.

RESULTS

LysM-Cre/STAT3$^{fl/-}$ Animals Show Reduced Expression of STAT3 in Macrophages

Host MFs regulate the severity of acute GVHD [9,15]. STAT3 is a master regulator of MF-dependent innate immune responses; however its role in the regulation of the allostimulatory functions of professional APCs, specifically MFs, is not known. To evaluate the role of STAT3 in MFs on allogeneic immune responses, we generated STAT3-deficient MFs by crossing B6 STAT3 hemizygous mice in which one STAT3 allele was floxed and the other was already ablated (STAT3$^{fl/-}$), with B6 mice expressing