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Graft-versus-Host Disease Causes Broad Suppression of Hematopoietic Primitive Cells and Blocks Megakaryocyte Differentiation in a Murine Model

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Cytopenia and delayed immune reconstitution with acute graft-versus-host disease (aGVHD) indicate a poor prognosis. However, how donor-derived cell hematopoiesis is impaired in aGVHD is not well understood. We addressed this issue by studying the kinetics of hematopoiesis and the functions of hematopoietic stem and progenitor cells in an aGVHD model with haplo-MHC–matched murine bone marrow transplantation. Although hematopoiesis was progressively suppressed during aGVHD, the hematopoietic regenerative potential of donor-derived hematopoietic stem cells remains intact. There was a dramatic reduction in primitive hematopoietic cells and a defect in the ability of these cells to generate common myeloid progenitors (CMPs) and megakaryocyte/erythrocyte progenitors (MEPs). These effects were observed along with a concomitant increase in granulocyte/macrophage progenitors, suggesting that differentiation into MEPs is blocked during aGVHD. Interestingly, cyclosporine A was able to partially reverse the hematopoietic suppression as well as the differentiation blockage of CMPs. These data provide new insights into the pathogenesis of aGVHD and may improve the clinical management of aGVHD.

INTRODUCTION

In patients with graft-versus-host disease (GVHD), cytopenia is considered one of the most consistent and strongest predictive factors for poor survival [1]. Prolonged thrombocytopenia may require platelet transfusion for the management and prevention of severe bleeding. This condition may also impact treatment outcomes and prognosis [2-5]. However, the exact mechanisms responsible for cytopenia in patients with GVHD are poorly defined.

Hematopoietic stem cells (HSCs) are traditionally defined as cells that are able to produce all types of blood cells in a specific bone marrow (BM) microenvironment. Some key molecules that regulate the self-renewal, proliferation, and differentiation of HSCs during steady-state hematopoiesis have been identified using knock-out mouse models and bone marrow transplantation (BMT)-induced stress [6,7]. However, how the HSC compartment responds to pathological conditions is still poorly understood. In a lipopolysaccharide-induced sepsis model, the expansion of the HSC pool and neutrophil depletion were observed by Rodriguez et al. [8]. The self-renewal ability of HSCs was significantly impaired, which led to insufficient myeloid cell generation to fight sepsis. With chronic infection, Baldridge et al. [9] found that an increased proportion of proliferative long-term repopulating HSCs was caused by IFN-γ. Our previous study showed that the effects of the leukemic environment on HSCs and hematopoietic progenitor cells (HPCs) were distinct in that the repopulation potential of HSCs is preserved, whereas HPCs were exhausted [10]. The results of several mouse studies have shown that the number of donor-derived blood cells is reduced and that Fas-mediated apoptosis is involved in the process [11-14]. However, a systemic investigation of the kinetics of donor-derived hematopoietic stem and progenitor cells (HSPCs) in acute GVHD (aGVHD) hosts is lacking.

In the current study, using a haplo-MHC–matched murine BMT model that recapitulates the BM failure process in hosts with aGVHD, we documented the kinetics of donor-derived HSPCs during aGVHD and explored the underlying mechanisms. Furthermore, based on the current management of aGVHD in the clinic, we used cyclosporine A (CsA) to partially

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reverse the hematopoietic depression and the differentiation blockage of common myeloid progenitors (CMPs).

METHODS

Mice

C57BL/6J (CD45.2$^+$) and BALB/C (CD45.2$^+$) mice were purchased from the Institution of Zoology of the Chinese Scientific Academy (Shanghai, China). B6.SJL-Ptprc^{a}Pepc^b/BoyJ mice (B6.SJL, CD45.1$^+$) were obtained from Dr. Tao Cheng (State Key Laboratory of Experimental Hematology, Tianjin, China) and maintained in our animal facility. CB6F1 mice (CD45.2$^+$) were the F1 generation of male C57BL/6J mated to female BALB/C mice. All mice were maintained in a specific pathogen-free condition. All animal protocols were approved by the Institutional Animal Care and Use Committees of all institutions participating in this study.

BMT Procedures and the Induction of aGVHD

Lethally irradiated (8.0 Gy) female CB6F1 recipients (CD45.2$^+$, 6 to 8 weeks old) were intravenously injected with 5×10^6 BM nucleated cells (BMNCs) from male B6.SJL mice (CD45.1$^+$, 6 to 8 weeks old) along with 6×10^7 spleen cells from either female C57BL/6 mice (CD45.2$^+$, 6 to 8 weeks old, the aGVHD group) or female CB6F1 mice (CD45.2$^+$, 6 to 8 weeks old, the control group) 4 to 6 hours after irradiation, as indicated in Figure 1A. We tested various amounts of splenocytes for transplantation (1 to 8×10^7) and found 6×10^7 to be the ideal amount for the induction of aGVHD.

The recipient mice were killed every week unless otherwise specified. Hematopoietic reconstitution by the donor-derived cells was monitored. The severity of GVHD was assessed with a clinical GVHD scoring system as first described by Cooke et al. [15]. The following 5 clinical parameters were scored 3 times a week on a scale from 0 to 2: weight loss, posture, activity, fur texture, and skin integrity. The clinical GVHD index was generated by summing the 5 criteria scores (0 to 10). Survival was monitored on a daily basis.

Flow Cytometry

Mouse BM cells were obtained by flushing ilia, femurs, and tibias as previously described [16]. The immunophenotypes of murine long-term HSCs (LT-HSCs; CD34$^-$Flk2$^-$Lin$^-$c-Kit$^+$Sca-1$^+$, CD34$^-$Flk2$^-$LKS), short-term HSCs (ST-HSCs; CD34$^+$Flk2$^-$Lin$^-$c-Kit$^+$Sca-1$^+$, CD34$^+$Flk2$^-$LKS), multipotential progenitors (MPPs; CD34$^+$Flk2$^+$Lin$^-$c-Kit$^+$Sca-1$^+$, CD34$^+$Flk2$^+$LKS), and HPCs (Lin$^-$c-Kit$^+$Sca-1$^-$, LKS$^-$, including CMPs [CD34$^+$CD16/32^{lo}Lin$^-$c-Kit$^+$Sca-1$^-$,CD34$^+$CD16/32^{lo}LKS$^-$], common lymphoid progenitors [CLPs; CD34$^+$IL-7α^+Lin$^-$c-Kit^{lo}Sca-1^{lo}], granulocyte/macrophage progenitors [GMPs; CD34$^+$CD16/32^{hi}Lin$^-$c-Kit$^+$Sca-1$^-$, CD34$^+$CD16/32^{hi}LKS$^-$], and megakaryocyte/erythrocyte progenitors [MEPs; CD34$^-$CD16/32^{lo}Lin$^-$c-Kit$^+$Sca-1$^-$, CD34$^-$CD16/32^{lo}LKS$^-$]) [17-19] were used to quantify these different cell types within the donor-derived cell populations. All antibodies were purchased from eBioscience (San Diego, CA) unless otherwise noted.

For the detection of donor-derived HSPCs, the following antibodies were used: a mixture of lineage-specific antibodies (biotin anti-CD3, CD4, CD8, B220, Gr-1, Mac-1, and Ter-119), PE-Cy7 anti-Sca-1, PE anti-Flk2 or PE anti-CD16/32, APC anti-c-Kit, PE-Cy5.5 anti-CD45.1, FITCanti-CD34, and streptavidin-conjugated APC-Cy7. For the detection of T cells, B cells, monocytes, and granulocytes, PE anti-CD3, FITC anti-B220, APC anti-Mac-1, PE-Cy7 anti-Gr-1, and PE-Cy5.5 anti-CD45.1 were used.

For HSPCs isolation, c-Kit$^+$ cells were enriched using CD117-conjugated magnetic microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The enriched cells were stained with antibodies as described above, and then the CD45.1$^+$ CMPs were directly sorted into different tubes and lysed for gene expression analysis. For donor-derived hematopoietic cell (CD45.1$^+$) sorting, BM cells from mice in the aGVHD or control groups were pooled and stained with FITC anti-CD45.1 and PE anti-CD45.2 antibodies (eBioscience, San Diego, CA). A FACS AriaII (BD Biosciences) was used for sorting.

Cell Cycle and Apoptosis Analyses

In this study, we defined murine HSCs as Lin$^-$Sca1$^+$CD150$^+$cells, MPPs as Lin$^-$Sca1$^+$cells, and HPCs as Lin cells. We did not include c-kit in the immunophenotypes because it is undetectable shortly after BMT [20]. For cell cycle analysis, prestained cells were stained with 10 μg/mL Hoechst-33342 (Sigma Aldrich, St. Louis, MO) and FITC anti-Ki67 (BD Biosciences). The level of apoptosis was measured by labeling BM cells with Annexin-V and the DNA dye 7-aminoactinomycin D (7-AAD, BD Biosciences) in combination with cell surface markers.

In Vitro Clonal Assay

Two weeks after BMT, CD45.1$^+$ cells were sorted for an in vitro clonal assay and placed in M3434 methylcellulose medium (Stem Cell Technologies, Vancouver, BC, Canada) in 24-well plates with a volume of .5 mL at a density of 2×10^4 cells/mL with 4 replicate wells. At day 10, the colonies were counted under an inverted microscope and recorded as the number of colonies for the specific lineage.

Competitive BMT

A competitive BMT model was used as described previously [10,16]. A total of 2×10^6 sorted CD45.1$^+$ cells along with an equal number of CD45.2$^+$ BMNCs isolated from healthy nontransplanted donor (competitive cells) were cotransplanted into lethally irradiated (8.0 Gy) female CB6F1 recipients ($n = 20$ per group, 6 to 8 weeks old). The chimerism of the peripheral blood (PB) was monitored monthly for up to 6 months. The relative contributions of the donor-derived cells (CD45.1$^+$) and the competitive cells (CD45.2$^+$) to hematopoietic reconstitution were measured by flow cytometry using FITC anti-CD45.1 and PE anti-CD45.2 antibodies.

Cytokine Profiling

At the early stage after BMT (16 hours and day 3), serum samples were collected from at least 4 mice per group. A Milliplex MAP immunoassay (Merck Millipore, Billerica, MA) was used to determine the cytokine profile according to the manufacturer's instructions. The levels of mouse IL-1α, IL-2, and IFN-γ were examined and reported in units of pg/mL.

CsA Administration

CsA (Novartis PharmaSchweiz AG, Switzerland) diluted in 200 μL PBS was administered *intraperitoneally* daily to aGVHD mice from day 0 to day 28 at a dose of 10 mg/kg/day.

Quantitative Reverse Transcriptase PCR

At 2 weeks after BMT, CD45.1$^+$ LKS cells were sorted directly into RNeasy Lysis Buffer (Buffer RLT, Qiagen, Denmark) containing β-mercaptoethanol. Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Denmark). cDNA was synthesized using Improm-II reverse transcriptase (Promega, Madison, WI). Real-time PCR was conducted with SYBR Green Master Mix (Roche, Indianapolis, IN). All the primer sequences are listed in Supplemental Table 1.

Statistical Analysis

GraphPad Prism 5.0 (San Diego, CA) was used to analyze the results and create graphs. All comparisons represent 2-tailed unpaired *t*-test analyses unless otherwise specified. The flow cytometry data were analyzed with FlowJo Version 7.6.1 software (TreeStar, Costa Mesa, CA).

RESULTS

Donor-Derived Hematopoiesis Is Suppressed in aGVHD Hosts

A haplo-MHC–matched BMT mouse model was used in the present study to recapitulate aGVHD after allogeneic (allo)-BMT in humans [21]. CD3$^+$ cells accounted for approximately 30% of transplanted spleen cells (data not shown). All mice in the aGVHD group developed typical aGVHD symptoms and died within 28 days after allo-BMT (Figure 1B). The body weight began to decrease at day 3 in both groups. From day 13 to day 27, the body weight in the aGVHD group was significantly lower than that in the BMT control group (Figure 1C). Histological examinations of the liver, intestines, lungs, and spleen revealed lymphocyte infiltration and inflammation in the aGVHD group but not in the control group (Figure 1D). The average aGVHD score was 2.7 at day 14 after allo-BMT (data not shown). By day 21, all surviving recipients in the aGVHD group developed severe cytopenia (Figure 1E-G), with a 4.4-fold reduction in the WBC count ($P < .0001$), a 1.5-fold reduction in hemoglobin ($P = .0008$), and a 2.3-fold reduction in the platelet count ($P = .0377$) compared with the control group.

To quantify the contribution of donor-derived hematopoietic cells, we monitored the kinetics of the hematopoiesis of donor-derived hematopoietic cells (CD45.1$^+$) in both the PB and BM weekly after BMT. Both the total number and the frequency of CD45.1$^+$ cells in the aGVHD BM were lower than those in the controls (Figure 2A-C). At day 14 after BMT, the total number of BM cells in the aGVHD mice was approximately 44% of that in the control mice. The average