Immunology Letters 143 (2012) 177–183

Contents lists available at SciVerse ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

G-CSF-induced myeloid cells stimulated by TLR2 enhance engraftment after allogeneic hematopoietic stem cell transplantation

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ARTICLE INFO

Article history:
Received 24 December 2011
Received in revised form 7 February 2012
Accepted 16 February 2012
Available online 22 February 2012

Keywords:
G-CSF
Peripheral blood stem cell transplantation
Engraftment
Myeloid cells
TLR2

ABSTRACT

A high frequency of G-CSF-mobilized myeloid cells (gMCs) in a donor graft accelerates hematopoietic recovery after peripheral blood stem cell transplantation (PBSCT). However, because of the limited functional efficacy of gMCs, repeated transfusions of gMCs are frequently required. In this study, we investigated a strategy to improve the functional capacity of gMCs during hematopoietic engraftment after allogeneic transplantation. We found that toll-like receptor 2 (TLR2) is constitutively expressed on gMCs. Treating gMCs with the synthetic TLR2 ligand Pam_3CSK_4 (PAM) dramatically enhanced IL-10 and TNF-α production. However, PAM treatment does not induce substantial cellular maturation. Moreover, PAM treatment significantly improved gMC survival. PAM treated gMCs significantly promoted myeloid differentiation of donor hematopoietic stem cells (HSCs), resulting in accelerated engraftment after allogeneic transplantation. Our data suggest that TLR2-stimulated gMCs may be a novel cellular therapeutic for increasing the efficiency of allogeneic hematopoietic stem cell transplantation (HSCT) by reducing infectious complications associated with delayed engraftment.

1. Introduction

Strategies that enhance engraftment after allogeneic hematopoietic stem cell transplantation (HSCT) could decrease infectious complications and disease relapse and, improve the survival of HSCT recipients. Granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood stem cell transplantation (PBSCT) has been performed more frequently than bone marrow transplantation (BMT) in allogeneic HSCT. PBSCT is used preferentially because it leads to more rapid engraftment and immune reconstitution [1–5]. However, the risk of developing infectious complications after PBSCT is almost 25% [6]. Therefore, additional strategies to improve early engraftment must be identified.

G-CSF-mobilized myeloid cell types, including granulocytes and monocytes, represent a high proportion of PBSCT donor grafts. The functional significance of these cell types in PBSCT have been mainly reported in acute graft-versus-host disease (GVHD) [7]. These cell types possess immune suppressive activity against alloantigen reactive T cells through IL-10 secretion and regulatory T cell (Treg) induction [8,9]. We previously reported the characterization of G-CSF-mobilized myeloid cell (gMC) types in mice. gMCs are identified by $CD11b^+Gr\text{-}1^+$ and have an immature phenotype. The suppressive activity of gMCs is independent of indoleamine 2,3-dioxygenase (IDO) production [10]. In addition to their immune modulation activity, G-CSF-mobilized myeloid cell types have been reported to contribute to accelerated hematopoietic recovery after transplantation. However, the ability of gMCs to increase engraftment is transient, and repeated transfusions are required [11].

Recently, toll-like receptors (TLRs), which are innate immune sensors, have been also shown to be involved in hematopoiesis [12]. Under inflammatory conditions, HSCs were shown to differentiate into myeloid cells by TLR2 and TLR4 ligation even in the absence of hematopoietic growth factors [13]. Our previous results showed that G-CSF-mobilized PBSCs express high levels of TLR2 and that TLR2 signaling promoted the myeloid differentiation of PBSCs. This differentiation resulted in rapid cell engraftment [14]. Moreover, G-CSF is known to enhance TLR2 expression on certain types of immune cells [15–17]. This suggests that TLR2 can be

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doi:10.1016/j.imlet.2012.02.001

up-regulated on multiple hematopoietic cell types in G-CSF-mobilized donor grafts.

In this study, we investigated a strategy to improve the ability of gMCs to enhance hematopoietic engraftment after allogeneic transplantation. We found that gMCs constitutively expressed TLR2 and that the ligation of TLR2 with PAM enhances the ability of gMCs promote HSC differentiation. Our data demonstrate that TLR2 stimulated-gMCs promote engraftment after allogenic HSCT.

2. Materials and methods

2.1. Mice

Female C57BL/6 (B6, H-2^b, Ly5.2) and B6D2F1 (H-$2^{b/d}$, Ly5.2) mice were purchased from Orient Biotech Inc. (Seoul, Korea). B6.SJL-Ptprca Pep3b/BoyJ (B6, Ly5.1) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). TLR2$^{-/-}$ mice were provided by Akira (Osaka University, Osaka, Japan). All mice were used for the experiments at the age of 7–10 weeks. Mice were housed in microisolator cages at the Inje University College of Medicine. Animal protocols were approved by the Institutional Animal Care and Use Committee at Inje University.

2.2. Antibodies and reagents

The following antibodies were purchased from e-Bioscience (San Diego, USA) for flow cytometry: PE-Cy7-conjugated anti-TLR2 (T2.5), -TLR4 (MTS510), FITC-conjugated anti-MHC class I (28-14-8), MHC class II (M5/114.15.2), CD80 (16-10A1), CD86 (GL1), Gr-1 (RB6-8C5), PE-Cy5-conjugated anti-Ly5.1 (A20), PE-conjugated anti-CD11b/Mac-1 (M1/70), PerCP-Cy5.5-conjugated anti-CD14 (Sa2-8), anti-CD16/32 (2.4G2, 93). 7-AAD and Annexin-V were purchased from BD Bioscience (San Jose, CA). Pam_3CSK_4 (PAM) was purchased from InvivoGen (San Diego, CA). 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DiR) was purchased from Invitrogen (Carlsbad, CA). Recombinant human G-CSF was provided by Jeil-Kirin Pharm. Inc. (Grasin; Tokyo, Japan).

2.3. Isolation of cells, microarray, and in vitro stimulation

gMCs were isolated using previously described methods [10]. Briefly, B6 donor mice were subcutaneously (s.c) injected with recombinant human G-CSF (10 μg) daily for 5 days. The spleen was harvested 3 h after the final G-CSF injection and cut into small pieces and incubated collagenase type II (1 mg/ml; Sigma) and DNase I (15 μg/ml) at 37 °C for 40 min. A single cell suspension was obtained using a cell strainer (Falcon). Cells were incubated with anti-CD90, -CD19, and -c-kit magnetic beads (Miltenyi Biotec, Auburn, CA), and additional selection was carried out on MACS columns to deplete T cells, B cells, and HSCs. Negatively selected cells were incubated with biotin conjugated anti-Ly6G and added to anti-biotin magnetic beads to separate out CD11b$^+$Gr-1$^+$(Ly6G$^+$ Ly6c$^+$) cells. Bone marrow myeloid cells (bmMCs) were prepared from the tibia and femur of naïve B6 mice using the same procedure. The purity of isolated cells was 95–97%. For isolation of HSCs, total cells were prepared from tibia and femur of naïve B6 mice. Cells were incubated with antibody to biotin-conjugated anti-lineage markers (CD5, CD11b, B220, Gr-1, 7-4, and Ter-119) followed by negative selection with the auto-MACS cell separation system (Miltenyi Biotec, Auburn, CA). Subsequently, negative selected cells were incubated with anti-c-kit magnetic bead to separate out lin-c$^-$kit$^+$ cells. The purity of isolated cells was 95–97%. For gene profiling analysis with microarray, total RNAs were prepared from gMCs and bmMCs. The cDNA microarray analysis was carried out using the Mouse OpArray (Operon Biotechnologies GmbH) microarray containing whole 35k. For TLR2 stimulation, purely isolated gMCs were suspended in complete culture medium (RPMI1640+ 10% FBS) contained in G-CSF (50 ng/ml) and cultured in the absence or presence of PAM for 6–12 h.

2.4. Cytokine assay

Culture supernatants were collected from PAM- or not treated culture condition. IL-6, IL-10, and TNF-α were assayed using cytometric bead array (CBA) kit (BD Bioscience, San Diego) according to the manufacturers' protocols. G-CSF was assayed using ELISA kit (R&D System, Minneapolis).

2.5. Flow cytometry

For the expression of TLRs, cells were pre-incubated with anti-CD16/32 (2.4G2) to block non-specific antibody binding to Fc receptors and stained for FITC-anti-Gr-1, PE-anti-CD11b, and PE-Cy7-anti-TLR2, -TLR4, or PerCP-Cy5.5-anti-CD14 and analyzed by flow cytometry. To analyze the phenotype of gMCs, cultured cells were harvested and stained with FITC-anti-MHC class I, -anti-MHC class II, -anti-CD80, and -anti-CD86. To measure the cell survival, gMCs were isolated from WT and TLR2$^{-/-}$ mice. The cells were stimulated with or without PAM (0.25 μg/ml) in the presence of G-CSF for 6 h. The cells were harvested and washed with HBSS. The cells were suspended in complete culture media (RPMI1640 + 10% FBS) and re-cultured in 96 well plate. The apoptosis was measured by Annexin V + 7-AAD staining at the indicated time points. Fluorescence was measured using a FACScalibur (BD Biosciences), and data analysis was performed using Cellquest Pro software (BD Biosciences).

2.6. In vivo imaging

gMCs were incubated with DiR (1×10^7 cells in 10 ml PBS containing 3.5 μg/ml dye and 0.5% ethanol) for 30 min at 37 °C. The cells were washed twice with PBS. Labeled cells (3×10^6) were then adoptively transferred into irradiated BDF1 (850 cGy) via tail vein injection. In vivo imaging was performed with an IVIS Lumina imaging system (Xenogen, Alameda, CA) to monitor DiR intensity. Imaging was performed on days 1 and 3 after cell transfer. An ICG filter was used for detection. Images were acquired and analyzed using Image 3.0 software (Xenogen, Alameda, CA).

2.7. Colony forming assay

Lin$^-$c-kit$^+$ HSCs (1×10^2) were suspended without or with gMCs (3×10^4) or TLR2-stimulated gMCs (3×10^4) in methylcellulose-based medium supplemented with recombinant cytokines. Cell were plated into 35 mm culture dish and cultured at 5% CO_2 incubator. Colonies included in colony-formation of burst-forming unit-erythroid (BFU-Es), colony-forming unit-granulocyte (CFU-G), colony-forming unit-macrophage (CFU-M), colony-forming unit-granulocyte/macrophage (CFU-GM), and CFU-granulocyte/erythrocyte/monocyte/macrophage (CFU-GEMM) were counted at day 10.

2.8. In vivo engraftment assay

HSCs (3×10^4) were isolated from B6 BoyJ (Ly5.1) mice and transplanted with alone, gMCs (2×10^6), or TLR2-stimulated gMCs (2×10^6) into lethally irradiated (850 cGy) B6D2F1 mice via tail vein injection. Spleen cells were isolated on day 14 after transplantation and analyzed by flow cytometry for Ly5.1$^+$Gr-1$^+$CD11b$^+$, Ly5.1$^+$Gr$^-$1$^+$CD11b$^-$, or Ly5.1$^+$Gr-1$^-$CD11b$^+$ cells. For the spleen colony assay, spleen cells (1×10^5) were suspended in 1 ml of