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Upregulation of TLR2 expression on G-CSF-mobilized peripheral blood stem cells is responsible for their rapid engraftment after allogeneic hematopoietic stem cell transplantation

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ABSTRACT

Granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood stem cells (PBSCs) are more frequently used as the cellular source in allogeneic hematopoietic stem cell transplantation (HSCT) than bone marrow stem cells (BMSCs) because they promote more rapid engraftment and immune reconstitution. However, the underlying mechanism for this is not fully understood. Here, we investigated the role of Toll-like receptor 2 (TLR2) on PBSCs in promoting rapid engraftment after allogeneic HSCT. We found that PBSCs highly expressed TLR2 in comparison to BMSCs, and TLR2 was directly induced by G-CSF signaling. Treatment with the TLR2 ligand, Pam_3CSK_4 (PAM), more efficiently induced myeloid differentiation of PBSCs than BMSCs. Similarly, endogenous TLR2 ligands from the serum of recipients of allogeneic transplantation more rapidly stimulated myeloid differentiation of PBSCs compared with BMSCs. PAM treatment of $TLR2^{-/-}$ syngeneic recipient mice transplanted with PBSCs resulted in significantly elevated numbers of PBSC-derived myeloid cells and spleen colony formation compared with controls. Our results demonstrate that TLR2 signaling in PBSCs correlates with their ability to rapidly differentiate into myeloid cells, resulting in improved engraftment. Thus, TLR2 may be a novel target for increasing the efficiency of allogeneic HSCT by overcoming engraftment failure or delayed engraftment.

1. Introduction

Hematopoietic stem cell transplantation (HSCT) is the most effective therapy for several hematological disorders. After transplantation, donor stem cells migrate to the bone marrow and begin to differentiate into myeloid blood cells, a process referred to as engraftment. If engraftment is delayed, it significantly limits the success of transplantation and increases the risk of infection in the recipient [1,2].

Peripheral blood stem cells (PBSCs) obtained from granulocyte colony-stimulating factor (G-CSF) mobilized donors have been used more frequently than bone marrow stem cells (BMSCs) in allogeneic HSCT. The clinical advantages of using PBSCs are more rapid engraftment and immune reconstitution, which serve to reduce the risk of infection and disease relapse [3–7]. Studies have shown that there are higher numbers of HSCs and more differentiated precursor cells in PBSC grafts than in BMSC grafts [8–11]; however, the mechanism responsible for the rapid hematopoietic repopulation by PBSCs remains to be defined. In particular, little is known about the molecules that are induced by G-CSF on HSCs and enhance hematopoietic repopulation in the host after allo-HSCT.

Toll-like receptors (TLRs) are primary sensor molecules that play an integral role in innate immunity because of their capacity to recognize pathogen-associated molecular patterns, thus allowing for the detection of infection and inflammation [12,13]. In addition to their role as innate immune sensors, TLRs have been shown to be involved in the function of variety cell types [14,15]. Recent research has shown that TLRs may play a role in hematopoiesis. TLR2 and TLR4 are expressed by HSCs and hematopoietic progenitor cells (HPCs), and these cells were shown to differentiate into myeloid cells by TLR ligation even in the absence of hematopoietic growth factors [16]. This suggests that TLR signaling involved in the myeloid differentiation pathway may offer a means for the rapid replenishment of the innate immune system upon inflammatory stimuli.

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It is well known that recipients of allo-HSCT develop systemic inflammation due to the allo-responses of donor T cells against host intestinal bacterial products, a process that may promote engraftment [17–19]. It is also known that G-CSF enhances TLR2 expression on certain types of immune cells [20]. Based on these studies, we investigated whether TLR expression levels were associated with the ability of PBSCs and BMSCs to differentiate into myeloid cells. We found that PBSCs expressed higher levels of TLR2 than BMSCs, and TLR2 signaling promoted the differentiation of PBSCs into myeloid cells both in vitro and in vivo. Our results demonstrate that TLR2 signaling in PBSCs correlates with their differentiation, resulting in improved engraftment after allo-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-$Ptprc^a$ $Pep3^b$/BoyJ (B6, Ly5.1) mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). $TLR2^{-/-}$ mice (B6; H-2^b) were kindly provided by Akira (Osaka University, Osaka, Japan). All mice used for the experiments were 7–10 weeks old. 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, CA) for flow cytometry: biotinylated anti-TLR2 (6C2), biotinylated anti-TLR4 (MTS510), FITC-conjugated anti-F4/80 (BM8), FITC-conjugated anti-CD3 (145-2C11), FITC-conjugated anti-CD45R/B220 (RA3-6B2), FITC-conjugated anti-Gr-1 (RB6-8C5), FITC- or PE-conjugated anti-CD11b/Mac-1 (M1/70), PE-conjugated anti-CD11c (N418), PE-conjugated anti-c-kit (ACK2), PE-Cy5-conjugated anti-Ly5.1 (A20), and purified anti-CD16/32 (2.4G2). Flt3-ligand (FL) was obtained from Strathmann Biotec (Hamburg, Germany). Stem cell factor (SCF) was obtained from R&D Systems (Minneapolis, MN). Pam_3CSK_4 (PAM) was purchased from InvivoGen (San Diego, CA). Recombinant human G-CSF was provided by Jeil-Kirin Pharm., Inc. (Grasin; Tokyo, Japan).

2.3. Isolation of PBSCs and BMSCs

For preparation of PBSCs, WT and $TLR2^{-/-}$ B6 mice were subcutaneously (s.c.) injected with 10 μg of recombinant human G-CSF daily for 5 days. Spleens were harvested 3 h after the final injection, cut into small pieces, and incubated with collagenase type II (1 mg/ml; Sigma) and DNase I (15 μg/ml; Sigma) at 37 °C for 40 min. A single cell suspension was obtained using a cell strainer (Falcon). Cells were incubated with antibodies to biotin-conjugated anti-lineage markers followed by negative selection with the auto-MACS cell separation system (Miltenyi Biotec, Auburn, CA). The lineage negative cells were then incubated with anti-c-kit magnetic beads to separate out $lin^-c\text{-}kit^+$ cells. BMSCs were prepared from the tibias and femurs of naïve B6 mice using the same procedure. The purity of isolated cells was 95–97%.

2.4. In vitro assay

Isolated $lin^-c\text{-}kit^+$ cells (2×10^4) were suspended in RPMI 1640 medium (with 10% fetal bovine serum, 5×10^{-5} M 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin) and cultured in the presence of FL (100 ng/ml) and SCF (20 ng/ml) with or without PAM (0.5 μg/ml) in a flat-bottomed 96-well plate. To analyze the effects of serum, isolated cells were cultured in the presence of FL and SCF with serum at a concentration of 1.5% of the total volume. After 72 h in culture, cells were analyzed by flow cytometry for expression of F4/80, and cell yields were calculated based on total input cells.

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 with FITC-anti-lineage markers (CD3, CD45R/B220, CD11b, and Gr-1), PE-Cy5-anti-c-kit, and PE-anti-TLR2 or PE-anti-TLR4. To determine the frequency of $F4/80^+$ cells, cultured cells were harvested and stained with FITC-anti-F4/80. To analyze the donor HSC-derived myeloid cells, spleen cells were isolated from recipient mice and stained with PE-Cy5-anti-Ly5.1, FITC-anti-CD11b, and PE-anti-Gr-1 or PE-Cy5-anti-Ly5.1 and PE-anti-CD11c. Fluorescence was measured using a FACSCalibur (BD Biosciences), and data analysis was performed using CellQuest Pro software.

2.6. In vivo engraftment assay

PBSCs (1×10^4) were isolated from BoyJ (Ly5.1, H-2^b) mice treated with G-CSF for 5 days, suspended in PBS, and transferred into lethally irradiated (950 cGy) $TLR2^{-/-}$ (H-2^b) recipients via tail vein injection. The recipients were injected intraperitoneally (i.p.) with PAM (1 μg) or PBS on days 1, 3, and 5. To determine the number of donor PBSC-derived myeloid cells, spleen cells were isolated on day 10 after transplantation and analyzed by flow cytometry for $Ly5.1^+Mac\text{-}1^+Gr\text{-}1^+$ or $Ly5.1^+CD11c^+$ cells. For the colony-forming cell (CFC) assay, donor-derived cells were isolated from spleens using the biotin-anti-Ly5.1 and anti-biotin magnetic bead cell separation system. These cells (1×10^5) were suspended in 1 ml of methylcellulose medium with recombinant cytokines (MethoCult, Stem cell Biotech.), plated onto 35 mm culture dishes, and incubated at 5% CO_2. Colonies (CFU-GM and CFU-GEMM) were counted at day 10.

2.7. Detection of endogenous TLR2 ligands in serum

To obtain the blood serum samples, recipient (B6D2F1) mice were given two separate doses of total body irradiation (TBI, 850 cGy) within 3 h to minimize the degree of gastrointestinal toxicity. T cell-depleted bone marrow (TCDBM) and T cells were purified from donor naïve C57BL/6 mice using a microbead separation system (Miltenyi Biotec). Recipient mice were transplanted with T cells (3×10^6) plus TCDBM (5×10^6) or TCDBM alone via tail vein injection. Blood was obtained from each group of recipient mice on day 7 after transplantation. For detection of endogenous TLR2 ligands, HEK-293 cells stably transfected with either an empty plasmid (Null) or mouse TLR1/2 genes were purchased from InvivoGen (San Diego, CA) and maintained in DMEM culture medium. Cells were seeded into 24-well tissue culture plates at a concentration of 2×10^5 cells/well and cultured in the presence of serum at a concentration of 1.5% of the total volume. After 24 h in culture, total RNA was extracted from the cells and reverse transcribed into complementary DNA using a polymerase chain reaction (PCR) cDNA synthesis kit. The PCR was performed using sense/antisense primers. The PCR primer sequences were as follows: human IL-8 forward, 5′-ATGACTTCCAAGCTGGCCGTGGCT-3′ and reverse, 5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′; human GAPDH forward, 5′-TGATGACATCAAGAAGGTGG-3′ and reverse, 5′-TTACTCCTTGGAGGCCATGT-3′.