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Peptidoglycan accelerates granulopoiesis through a TLR2- and MyD88-dependent pathway

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ABSTRACT

Granulopoiesis is accelerated during Gram-negative bacterial infection through activation of toll-like receptor 4 (TLR4). In this study, we tested whether activation of TLR2 promotes granulopoiesis by using the well-known TLR2 agonist, peptidoglycan (PGN). Neutrophils in bone marrow and spleen, and plasma granulocyte colony-stimulating factor (G-CSF) were increased in mice that had received intraperitoneal PGN administration. Incorporation of BrdU into bone marrow neutrophils increased, demonstrating that PGN accelerated granulopoiesis. Treatment of bone marrow cells (BMCs) with PGN increased neutrophils *in vitro* and promoted the secretion of G-CSF from Ly-6G^{-}Ly-6C^{+} monocytes. The accelerated granulopoiesis caused by PGN was not seen in TLR2-deficient and MyD88-deficient BMCs. Additionally, PGN induced G-CSF production in human umbilical vein endothelial cells. These findings demonstrate that PGN promotes the secretion of G-CSF from monocytes and endothelial cells, leading to the acceleration of granulopoiesis. Our results illustrate that bacterial recognition by TLR2 facilitates granulopoiesis during Gram-positive bacterial infection.

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1. Introduction

Neutrophils play an important role in the innate immune system by phagocytosing and killing pathogenic bacteria [1]. During bacterial infection, granulopoiesis is accelerated to replenish neutrophils, which contribute to the elimination of bacteria. This so-called emergency granulopoiesis has been elucidated using mouse models with Gram-negative bacteria and their components [2].

Pattern recognition receptors, such as toll-like receptors (TLRs) and NOD-like receptors (NLRs), are responsible for the recognition of structural components of microorganisms [3,4]. Among TLR family members, TLR4 has been shown to recognize the Gram-negative bacterial endotoxin lipopolysaccharide (LPS) [5]. During Gram-negative bacterial infection, LPS stimulates the production of granulocyte colony-stimulating factor (G-CSF) leading to the acceleration of granulopoiesis, and this phenomenon depends on TRL4-expressing non-hematopoietic cells [6]. G-CSF is a glycoprotein that influences the proliferation, survival, and differentiation of neutrophils and their progenitor cells [7]. G-CSF-deficient mice exhibit chronic neutropenia, impaired ability to eliminate injected bacteria, and reduced infection-driven granulopoiesis [8]. Granulopoiesis is also impaired in G-CSF receptor-deficient mice [9]. Endothelial cells are the prime source of G-CSF after LPS or *Escherichia coli* stimulation, and they play a key role in sensing systemically spread pathogens [10]. The endothelial cell- and G-CSF-mediated emergency granulopoiesis process depends on TLR4 and MyD88 [10], which is an adaptor molecule contributing to TLR4 signaling [11]. Synergistic stimulation of TLR4 and NOD1 during systemic *E. coli* infection mobilizes CD150^{+}CD48^{-}Lineage$^{-/low-}$Sca1^{+}cKit^{+} hematopoietic stem cells (HSCs) to the spleen to give rise to neutrophils and monocytes, which also contributes to host defence [12]. Thus, granulopoiesis is precisely regulated to replenish neutrophils during Gram-negative bacterial infection. However, it is much less well understood whether Gram-positive bacterial infection accelerates granulopoiesis.

TLR2 has been identified as being pivotal for the recognition of Gram-positive bacteria [13], and its ligands include cell wall components such as peptidoglycan (PGN) [14,15]. Previously, it was reported that HSCs and progenitor cells, such as common myeloid

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progenitors (CMPs) and granulocyte-macrophage progenitors (GMPs), express TLR2, and the activation of TLR2 by a specific agonist, Pam_3CSK_4, was able to drive monocyte differentiation of HSCs and progenitors [16], indicating that the activation of TLR2 can modify myelopoiesis. However, it remains unclear whether TLR2 contributes to the regulation of granulopoiesis during Gram-positive bacterial infection.

Recently, synthetic peptide agonists against TLR2/TLR1, Pam_3-Cys, and TLR2/TLR6, fibroblast-stimulating lipopeptide (FSL-1), were reported to increase the expression of G-CSF in human umbilical vein endothelial cells (HUVECs) [17]. The results suggested that the activation of TLR2 signaling might play a role in granulopoiesis. In this study, to clarify whether activation of TLR2 modifies granulopoiesis, we evaluated the production of neutrophils in PGN-inoculated mice.

2. Materials and methods

2.1. Mice

For all experiments, mice aged more than 8 weeks old were used. Animal experiments were approved by the Animal Care and Use Committee of Tokushima Bunri University, and procedures were performed in accordance with institutional guidelines. The institutional guidelines conform to the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, 2006. Wild-type C57BL/6J mice were from Charles River Laboratories Japan, Inc. Femurs and tibias derived from $Tlr2^{-/-}$ (C57BL/6) [13], $Tlr4^{-/-}$ (C57BL/6) [18] and $Myd88^{-/-}$ (C57BL/6) [19] were purchased from Oriental Bio Service, Inc., Japan.

2.2. Reagents

Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated specific antibodies against mouse CD11b, Ly-6G/6C (Gr-1), Ly-6G, or Ly-6C, and purified rat anti-mouse CD16/CD32 (Fc Block) were purchased from BD Biosciences. A PE-conjugated specific antibody against mouse CD31 and an allophycocyanin (APC)-conjugated specific antibody against mouse CD45 were from BioLegend. Giemsa's azur eosin methylene blue solution was purchased from Merck. Mouse G-CSF was from Miltenyi Biotec. Peptidoglycan from *B. subtilis* and 5-Bromo-2'-deoxyuridine (BrdU) were purchased from Sigma. The cell-counting kit-8 was from Dojindo. All other chemicals were of the highest grade available from commercial sources.

2.3. Flow cytometry analysis

Antibodies described above were used to label cells after blocking Fc-receptors with purified rat anti-mouse CD16/CD32. Antibodies were diluted with phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS; AusGeneX). The labeled cells were analyzed using a Guava easyCyte (Millipore). FlowJo software (Tree Star) was used to analyze data.

2.4. PGN injection and BrdU injection

PGN was suspended in PBS. Mice received three intraperitoneal injections with 100 μg PGN at 0, 24, and 48 h, and were analyzed 24 h after the last administration as depicted in Fig. 1A. Control mice were injected with the same volume of PBS. For BrdU incorporation assay, mice were intraperitoneally administrated PBS or 100 μg PGN three times at 0, 24, and 48 h, and 2 mg BrdU at 48 h. The mice were analyzed 24 h after the last administration as depicted in Fig. 1E.

2.5. BrdU detection

BrdU incorporation was detected using a FITC BrdU Flow Kit (BD Pharmingen) in accordance with the manufacturer's protocol. Briefly, BMCs were stained with PE-conjugated specific antibody against mouse Ly-6G to label neutrophils after blocking Fc-receptors. The cells were fixed and permeabilized with BD Cytofix/Cytoperm buffer and BD Cytoperm Plus buffer followed by additional incubation with DNase to expose incorporated BrdU. The cells stained with an FITC-conjugated specific antibody against BrdU were analyzed using a Guava easyCyte.

2.6. Preparation of bone marrow cells and cell culture

To obtain BMCs, femurs and tibias were crushed in PBS supplemented with 2% heat-inactivated FBS. After the cells were filtered through a 40 μm mesh, red blood cells were hemolyzed with ACK lysing buffer (GIBCO). Isolated BMCs were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

2.7. Magnetic cell isolation

Gr-1^+ or Ly-$6G^+$ cell isolation was performed using an EasySep system (StemCell Technologies) in accordance with the manufacturer's protocol as described previously [20]. For isolation of Ly-$6G^-$Ly-$6C^+$ cells, we performed two-step separation. Firstly, Ly-$6G^+$ cells were labeled with the PE-conjugated specific antibody, separation was performed, and the negative fraction was obtained as Ly-$6G^-$ cells. Next, the Ly-$6G^-$ cells were labeled with an FITC-conjugated specific antibody against Ly-6C followed by antibody conjugation to magnetic nanoparticles using EasySep FITC Selection cocktail. The positive fraction was obtained as Ly-$6G^-$Ly-$6C^+$ cells.

2.8. ELISA

Using heparinized syringes, peripheral blood was obtained via the vena cava from mice administered PGN as described above. Isolated BMCs, Ly-$6G^+$ cells, Gr-1^+ cells, and Ly-$6G^-$Ly-$6C^+$ cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and the indicated amounts of PGN. The culture supernatants were harvested. Measurement of G-CSF levels was performed using a mouse G-CSF Quantikine ELISA kit (R&D Systems) in accordance with the manufacturer's instructions.

HUVECs were purchased from PromoCell, and the cells were cultured in Endothelial Cell Growth Medium 2 (PromoCell) in accordance with the manufacturer's instructions. After treatment of the cells with the indicated amounts of PGN, the culture supernatants were harvested, and G-CSF levels were measured using a human G-CSF Quantikine ELISA kit (R&D Systems).

2.9. Statistical analysis

One-way analysis of variance (ANOVA) followed by the Tukey's test was used to evaluate differences among three or more groups. Differences between two groups were evaluated using two-tailed Student's t-test. All statistical analyses were performed with Easy R [21] (Saitama Medical Center, Jichi Medical University). Differences were considered to be significant for values of $P < 0.05$.

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