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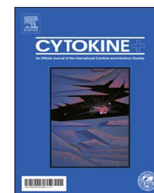

Differential regulation of IL-23 production in M1 macrophages by TIR8/SIGIRR through TLR4- or TLR7/8-mediated signaling

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ABSTRACT

Cross-talks between toll-like receptors (TLRs) including various negative regulatory mechanisms are many unknown. We investigated the differential mechanism of IL-23 production in M1 macrophages by single immunoglobulin interleukin-1 receptor-related (SIGIRR) molecule through TLR4 or TLR7/8.

IL-12p40 production by M1 macrophages pretreated with human neutrophil elastase (HNE) was synergistically enhanced IL-12p40, but not IL-23 production, after exposure to lipopolysaccharide (LPS). LPS (a TLR4 agonist) induced a slight increase of IL-23 production, while Resiquimod (a TLR7/8 agonist) significantly enhanced IL-23 production. Expression of SIGIRR protein, a negative regulator of TLR4, was higher in M1 macrophages than in monocytes. Interestingly, SIGIRR siRNA induced a slight increment of IL-23 production after exposure of macrophages to LPS, while IL-23 production in response to Resiquimod was significantly upregulated by SIGIRR siRNA. Silencing SIGIRR enhanced IRF4 protein level determined by western blotting or ELISA. IRF4 siRNA dramatically restored IL-23 production after exposure to Resiquimod in macrophages transfected with SIGIRR siRNA.

In conclusion, production of IL-23 is differentially regulated in M1 macrophages by SIGIRR through TLR4- or TLR7/8-mediated signaling. SIGIRR is both a negative regulator of TLR4 and a positive regulator of TLR7/8.

1. Introduction

The interleukin 12 (IL-12) family includes IL-12, IL-23, IL-27, and IL-35. Among the members of this family, IL-12 and IL-23 induce Th1 cells or Th17 cells, respectively [1-3]. IL-23 is composed of two subunits, which are IL-12p40 and IL-23p19. Toll-like receptors (TLRs) influence the balance between production of different IL-12 family cytokines [1]. TLRs stimulated with various pathogens induce the production of different IL-12 family members [4].

Inflammatory cytokines are produced by stimulation with all TLRs through myeloid differentiation primary response gene 88 (MyD88) signaling. TRAF6 exerts TLR2- and TLR4-mediated proinflammatory cytokine production. Activation of TRAF6-dependent signaling is associated with induction of IL-12 [5]. In addition, the interferon regulatory factor (IRF) family is activated by MyD88 -dependent and/or -independent TLR signaling pathways. IRF5 is elevated in M1 macrophages and activates IL-23p19 transcription [6]. One of critical downstream mediators of TLR7/8 signaling is IRF5. Therefore, we investigated the mechanisms regulating IL-23 production by M1 macrophages after exposure to TLR7/8 agonists.

Both innate and adaptive immunity are regulated by the TLR family. A balance between stimulatory and inhibitory immune response needs to maintain homeostatic immunity. Thus, there are many negative regulatory immune systems for TLRs [7]. Transmembrane protein regulators include TRAILR, SIGIRR, ST2, and RP105. Among them, SIGIRR is known to negatively regulate TLR4-mediated signaling. The specificity protein 1 (SP1) binds to SIGIRR proximal promoter and enhances its transcription [8]. GM-CSF also induces the activation of transcription factor SP1 [9].

IRFs are master regulators of TLR signaling. IRF4 interacts with MyD88 to negatively regulate TLR signaling and GM-CSF upregulates IRF4 expression [10]. Transcription factor Kruppel-like factor 2 (KLF2)

Abbreviations: ELISA, enzyme-linked immunosorbent assay; GM − CSF, granulocytemacrophage colony-stimulating factor; HNE, human neutrophil elastase; IL, interleukin; IRF, interferon regulatory factor; KLF2, kruppel-like factor 2; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; PAR, protease-activated receptor; SIGIRR, single immunoglobulin interleukin-1 receptor-related molecule; siRNA, small interfering RNA; SP1, specificity protein 1; TIR, toll/interleukin-1 receptor; TLR, toll-like receptor; TRAF6, tumor necrosis factor receptor-associated factor 6

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is a negative regulator of pro-inflammatory activity [11]. It has been reported that IRF4 is activated by KLF2 and that KLF2 is reciprocally upregulated by IRF4 [12].

Activation of TLR4 also induces IRF4 expression in macrophages [13], and proinflammatory cytokine production after TLR stimulation is negatively regulated by IRF4 [14]. It was reported that IRF4 interferes with MyD88/IRF5 signaling [15]. TLR7/8 signaling is mediated by IRF5 [16], and IL-12 is down-regulated by TLR7/8 while IL-23 is upregulating [17]. Cross-talk between TLRs is still not well understood. The pattern of cytokine production is dependent on engagement of different TLR with various pathogens [18]. Cellular signaling by various combinations of TLR2-9 agonists show inert, stimulatory, synergistic, or inhibitory activities. Among them, the interaction of TLR4-meadiated with TLR7/8-mediated signaling [19], which may be caused by cross-talk with these TLRs. We reported synergistic elevation of IL-12p40 by M1 macrophages pretreated with human neutrophil elastase after lipopolysaccharide (LPS) exposure. Neutrophil elastase activated AR-2 and then transactivated EGFR/TLT4 signaling [20]. This time, we examined the influence of inter-relationships among TLR4 and TLR7/8 agonists on IL-23 production by M1 macrophages.

2. Materials and methods

2.1. Ethics statement

The Board of Ethics in Kumamoto Health Science University approved to obtain blood from volunteers in conformity with the declaration of Helsinki after obtaining their informed consent (No. 26–20).

2.2. Chemicals and reagents

Recombinant human GM-CSF, human neutrophil elastase (HNE) and Escherichia coli 0111:B4 lipopolysaccharide (LPS) were purchased from SERVA Electrophoresis (Heidelberg, Germany), Tocris Bioscience (Bristol, UK), or Sigma-Aldrich (St. Louis, MO) respectively. Protease-activated receptor (PAR)-2 agonists (AC264613), Tocris Bioscience and PAR-2 antagonist (GB83), Axon Medchem, Reston, VA were purchased to study the induction of IRF5. TLR7/8 agonist, Resiquimod, was obtained from ChemScene Chemicals, Monmouth Junction, NJ.

2.3. Induction of M1 macrophages

Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized blood samples [21]. PBMCs collected using Lymphoprep gradients (Axis-Shield PoC As, Norway) were suspended with Lymphocyte medium for thawing (BBLYMPH1, Zen-Bio, Inc. Research Triangle Park, NC). The monocytes were stained with CD14-phycoerythrin (PE) mouse anti-human monoclonal antibody (Life technologies, Staley Road Grand Island, NY). The purity of monocytes was determined by Fluorescence Activated Cell Sorting (FACS), showing 86.28 ± 0.14% (mean ± SE, n = 64, 83.6–89.1). M1 macrophages were obtained after monocytes stimulated with recombinant human GM-CSF on days 1, 3, and 6 of culture [22]. Macrophages (on day 9 of culture) were utilized as M1 macrophages in this study.

2.4. ELISA for IL-12p40, IL-23, IRF5, and SIGIRR

M1 macrophages were incubated with HNE (50 μM) for 6 h and then treated with LPS (10 ng) for 6 h. The IL-12p40 and IL-23 protein levels in whole-cell lysates were determined by ELISA with an anti-IL-12p40 antibody or anti-IL23 antibody, respectively (Abcam, Cambridge, UK). Additionally, after M1 macrophages treated with Resiquimod (5 μM) or LPS (10 ng) for 6hr, IL-23 levels were determined by ELISA.

M1 macrophages were treated with HNE (50 μM) or AC264613 (10 μM) for 6 h. The protein levels of IRF5 in whole-cell lysates was determined by ELISA. In addition, HNE (50 μM)- or AC264613 (10 μM)-treated M1 macrophages with or without GB83 (4 μM) were stimulated with Resiquimod (5 μM) for 6hr and IL-23 levels were determined by ELISA. Moreover, SIGIRR protein levels in whole-cell lysates of monocytes or M1 macrophages were measured by ELISA (RayBiotech, Norcross, GA) with an anti-SIGIRR monoclonal antibody.

2.5. RNA interferences with SP1, SIGIRR, or IRF4 siRNA

Transfection of M1 macrophages with siRNAs for SP1 (50 nM), SIGIRR (50 nM), IRF4 (50 nM) or control siRNA-A (Santa Cruz Biotechnology, Santa Cruz, CA) was performed day 7–8 of cell culture using Lipofectamine (Life Technologies, Carlsbad, CA). IL-23 protein levels in whole-cell lysates were measured by ELISA.

2.6. Effect of siRNA for SIGIRR, SP1, or IRF4 on IL-23 production by M1 macrophages stimulated with Resiquimod

After transfection of M1 macrophages with siRNA for SIGIRR, SP1, or IRF4, the cells were stimulated with LPS (10 ng) and/or Resiquimod (5 μM) for 6 h and IL-23 protein was measured by ELISA.

2.7. Western blotting or ELISA for IRF4 and SIGIRR

M1 macrophages were transfected with siRNA for SIGIRR, KLF2 or SP1. Then IRF4 or SIGIRR protein production by the transfected cells was detected by western blotting of whole-cell lysates with an anti-mouse monoclonal antibody for IRF4 or SIGIRR, respectively (Santa Cruz Biotechnology, Santa Cruz, CA). Whole-cell lysates of M1 macrophages stimulated with LPS (10 ng) for 6 h were utilized as the positive control. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also detected by western blotting with an anti-GAPDH antibody (Santa Cruz Biotechnology). Furthermore, IRF4 and SIGIRR protein levels in whole-cell lysates were measured by ELISA (RayBiotech or MyBioSource, respectively).

3. Results

Stimulation of M1 macrophages with LPS enhanced IL-12p40 levels, whereas HNE (50 μM) alone did not. Synergistic elevation of IL-12p40 was observed after exposure to HNE and LPS (Fig. 1a). LPS (a TLR4 agonist) induced a slight increment of IL-23 levels. Unexpectedly, synergistic upregulation of IL-23 levels was not observed by treatment with HNE and LPS, unlike production of IL-12p40 (Fig. 1b). Resiquimod significantly upregulated IL-23 production (Fig. 2).

Next, we elvaluated the roles of HNE and LPS on IL-23 production by M1 macrophages. A PAR-2 agonists, HNE or AC264613 attenuated IRF5 production by M1 macrophages, while GB83 (a PAR-2 antagonist) restored it (Fig. 3). Pretreatment of M1 macrophages with HNE or AC264613 reduced IL-23 production by these cells after stimulation with Resiquimod, whereas GB83 restored it (Fig. 4). The level of SIGIRR protein was significantly higher in M1 macrophages than in monocytes (Fig. 5). Interestingly, transfection of macrophages with SIGIRR siRNA led to a slight increment of IL-23 production after stimulation with LPS. Unexpectedly, SIGIRR siRNA dramatically blunted IL-23 production by M1 macrophages in response to Resiquimod. Importantly, silencing of IRF4 restored Resiquimod-stimulated IL-23 production by macrophages transfected with SIGIRR siRNA. Surprisingly, IL-23 levels in M1 macrophages in response to Resiquimod stimulation was diminished by adding LPS. SP1 siRNA also significantly attenuated IL-23 levels in response to Resiquimod (Fig. 6). Western blotting and ELISA showed that transfection of macrophages with SIGIRR siRNA or SP1 siRNA led to increased expression of IRF4 compared to that in untreated M1 macrophages, while transfection with KLF2 siRNA reduced IRF4 expression. Transfection with SIGIRR or SP1 siRNA reduced the expression of

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