



## Research article

IL-23 production in human macrophages is regulated negatively by tumor necrosis factor  $\alpha$ -induced protein 3 and positively by specificity protein 1 after stimulation of the toll-like receptor 7/8 signaling pathwayRui Yamaguchi<sup>a</sup>, Arisa Sakamoto<sup>a</sup>, Reona Yamaguchi<sup>b</sup>, Misa Haraguchi<sup>a</sup>, Shinji Narahara<sup>a</sup>, Hiroyuki Sugiuchi<sup>a</sup>, Yasuo Yamaguchi<sup>a,\*</sup><sup>a</sup> Graduate School of Medical Science, Kumamoto Health Science University, Kitaku Izumi-machi 325, Kumamoto 861-5598, Japan<sup>b</sup> Department of Neuroscience, Graduate School of Medicine and Faculty of Medicine, Kyoto University, Yoshida-konoe-cho Sakyo-ku Kyoto 606-8501, Japan

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## ABSTRACT

The IL-23/IL-17 axis plays an important role in the development of autoimmune diseases, but the mechanism regulating IL-23 production is mainly unknown. We investigated how TNFAIP3 and Sp1 affect IL-23 production by human macrophages after exposure to resiquimod, a TLR7/8 agonist.

IL-23 production was significantly upregulated by resiquimod but only slightly by LPS (a TLR4 agonist). Interestingly, IL-23 levels were significantly attenuated after sequential stimulation with LPS and resiquimod, but IL-12p40 and IL-18 levels were not. TLR4-related factors induced by LPS may regulate IL-23 expression via TLR7/8 signaling. LPS significantly enhanced TNFAIP3 and IRAK-M levels but reduced Sp1 levels. After exposure to resiquimod, RNA interference of TNFAIP3 upregulated IL-23 significantly more than siRNA transfection of IRAK-M did. In contrast, knockdown of Sp1 by RNA interference significantly attenuated IL-23 production. Transfection with siRNA for TNFAIP3 enhanced IL-23 expression significantly. After stimulation with resiquimod, GW7647—an agonist for PPAR $\alpha$  (an inducer of NADPH oxidase)—and siRNA for UCP2 (a negative regulator of mitochondrial ROS generation) enhanced TNFAIP3 and reduced IL-23. siRNA for p22phox and gp91phox slightly increased Sp1 levels. However, after exposure to resiquimod siRNA-mediated knockout of DUOX1/2 significantly enhanced Sp1 and IL-23 levels, and decreased TNF $\alpha$ -dependent COX-2 expression. Concomitantly, TNFAIP3 levels was attenuated by DUOX1/2 siRNA. TNFAIP3 and Sp1 levels are reciprocally regulated through ROS generation.

In conclusion, after stimulation of the TLR7/8 signaling pathway IL-23 production in human macrophages is regulated negatively by TNFAIP3.

## 1. Introduction

Cytokines play a pivotal role in the pathogenesis of autoimmune diseases. IL-23 is a unique heterodimeric cytokine composed of IL-12p40 and IL-23p19 subunits [1]. T helper type 17 (Th17) cells also have been suggested to be closely associated with the development of autoimmune diseases [2], and IL-23 is required to differentiate and maintain Th17 cells [3]. Innate immunity response through activation of toll-like receptors (TLRs) is involved in the initiation and progression of autoimmune diseases.

Pattern recognition receptors (PRRs) expressed on macrophages include membrane receptor (TLR2/4/5), endosomal receptor (TLR3/7/8/9), and cytosolic receptor (nucleotide-binding oligomerization

domain-containing protein 1/2, NOD1/2). Viral infection, as an environmental trigger, may play a major role in the development of autoimmune diseases [4]. Lipopolysaccharide (LPS) is recognized through cell surface TLR4, and endosomal TLR7/8 reacts with nucleic acid ligands. Previously, we reported that IL-23 production in human macrophages was increased only slightly by LPS (a TLR4 agonist) but significantly by resiquimod (a TLR7/8 agonist) [5]. This difference in effect may be due to unknown TLR4-related factors induced by LPS that regulate IL-23 expression after TLR7/8 stimulation. To determine whether this is the case, studies first need to clarify whether sequential stimulation of human macrophages with LPS and resiquimod attenuates IL-23 levels significantly more than exposure to resiquimod alone. Indeed, crosstalk between TLR signaling pathways was reported to be

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important for innate immune responses [6]. Therefore, the present studies aimed to demonstrate that TLR-4-related factors induced by LPS are a key regulator for IL-23 production after TLR7/8 stimulation.

Exposure to LPS induced the expression of several negative regulators for TLR signaling in macrophages, including tumor necrosis factor  $\alpha$ -induced protein 3 (TNFAIP3) [7], interleukin-1 receptor-associated kinase (IRAK)-M [8], programmed cell death 1 (PDCD-1) [9], hypoxia inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) [10], and triggering receptor expressed on myeloid cells 1 (TREM-1) [11]. Moreover, LPS reduced the expression of transcription factors, including specificity protein 1 (Sp1) [12]. We hypothesized that LPS-associated proteins or transcription factors regulate IL-23 expression by human macrophages through TLR7/8-mediated signaling.

## 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. 17046).

### 2.2. Chemicals and reagents

Recombinant human GM-CSF and Escherichia coli 0111:B4 lipopolysaccharide (LPS) were purchased from Tocris Bioscience (Bristol, UK) or Sigma-Aldrich (St. Louis, MO; catalog #L2630-10MG), respectively, and Pam3CSK4 (catalog #4633/1) was purchased from R&D Systems (Minneapolis, MN). TLR7/8 agonist, resiquimod, was obtained from ChemScene Chemicals, Monmouth Junction, NJ. Mithramycin (Abcam, Cambridge, UK) was employed to investigate the intracellular signaling pathways involved in IL-23 production. The lipophilic agents resiquimod and mithramycin were dissolved in dimethyl sulfoxide (DMSO, Gaylord Chemical Company, Slidell, LA) according to the manufacturer's instructions. GM-CSF and LPS are hydrophilic.

### 2.3. Induction of M1 macrophages

Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized blood samples [13]. PBMCs collected using Lymphoprep gradients (Axis-Shield PoC As, Norway) were suspended with Lymphocyte medium for thawing (BBL YMPH1, 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  $87.04 \pm 1.29\%$  (mean  $\pm$  SE,  $n = 60$ ,  $83.9$ – $89.3$ ). Macrophages were obtained after monocytes stimulated with recombinant human GM-CSF on days 1, 3, and 6 of culture [14]. Macrophages (on day 9 of culture) were utilized in this study.

### 2.4. Preparation of whole-cell lysates from cell culture

Human macrophages (on day 9 of culture) were stimulated with LPS (10 ng/mL) or resiquimod (5 $\mu$ M) for 6 h and culture medium was carefully removed. Mammalian protein extraction reagent (100  $\mu$ L; M-PER, Thermo Fisher Scientific Inc., Waltham, MA) was pipetted into each well, after which the culture plate was gently shaken for 5 min. The lysate was collected and transferred to a microcentrifuge tube for centrifugation at 12,000 g for 10 min. The supernatants were used as a whole-cell lysates in this study.

### 2.5. Analysis of IL-23p19 mRNA expression by real-time reverse transcription polymerase chain reaction

IL-23p19 mRNA expression was determined by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). On day 9 of

culture, macrophages treated with or without Lipofectamine were stimulated with resiquimod (0 or 5  $\mu$ M) and LPS (10 ng/mL) for 6 h. Total RNA was extracted with Isogen (Nippon Gene, Tokyo, Japan), and equal amounts of RNA were reverse-transcribed to obtain cDNA by using a PrimeScript RT kit (TaKaRa Bio Inc., Shiga, Japan). The primer sequences for real-time RT-PCR were as follows:

5'-AGTGGCAGCAGCTTTCACAGA-3' (forward for IL-23p19),  
5'-AATCAGACCCTGGTGGATCCTT-3' (reverse for IL-23p19),  
5'-CTTCGCTCTGCTCCTCCTGTTCG-3' (forward for GAPDH), and  
5'-ACCAGGCGCCCAATACGACCAAAT-3' (reverse for GAPDH). These

primers and TB Green real-time PCR master mix were obtained from Takara Bio Inc. (Shiga, Japan). qRT-PCR was performed with a Light-Cycler<sup>®</sup> (Nippon Genetics Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions, and the level of IL-23p19 mRNA was normalized to that of GAPDH.

### 2.6. RNA interferences with TNFAIP3, IRAK-M, Sp1, PDCD-1, HIF1 $\alpha$ , TREM-1, UCP2, TGF $\beta$ 1/2/3, TAK-1, p22phox, gp91phox, DUOX1, DUOX2, IRF5, TRAF6, TNF $\alpha$ , OAS-1, p53, and cryopyrin

All siRNAs were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. On day 7–8 of cell culture, macrophages were transfected with siRNAs for tumor necrosis factor  $\alpha$ -induced protein 3 (TNFAIP3; catalog#sc-37655) (50nM), interleukin-1 receptor-associated kinase-M (IRAK-M; catalog#sc-39098) (50nM), specificity protein 1 (Sp1; catalog#sc-29487) (50nM), programmed cell death 1 (PDCD-1; catalog#sc-44002) (50nM), hypoxia-inducible factor 1 $\alpha$  (HIF1 $\alpha$ ; catalog#sc-35561) (50nM), triggering receptor expressed on myeloid cells 1 (TREM-1; catalog#sc-42999) (50nM), uncoupling protein 2 (UCP2; catalog#sc-42682) (50nM), transforming growth Factor $\beta$ 1/2/3 (TGF $\beta$ 1/2/3; catalog#sc-44146) (50nM), TGF $\beta$ -activated kinase 1 (TAK-1; catalog#sc-36606) (50nM), p22phox; catalog#sc-36149 (50nM), gp91phox; catalog#sc-35503 (50nM), dual oxidase 1 (DUOX1; catalog#sc-60550) (50nM), dual oxidase 2 (DUOX2; catalog#sc-60552) (50nM), TNF $\alpha$  (catalog#sc-37216) (50nM), p53 (catalog#sc-60550) (50nM), IRF5 (catalog#sc-72044) (50nM), TRAF6 (catalog#sc-36717) (50nM), OAS-1 (catalog#sc-61241) (50nM), and cryopyrin (catalog#sc-45469) (50nM) or control siRNA-A (catalog#sc-44236) (Santa Cruz Biotechnology, Santa Cruz, CA); Lipofectamine (Life Technologies, Carlsbad, CA) was used as the transfection reagent. We used 1  $\mu$ L of Lipofectamine to minimize p53 expression, according to the manufacturer's recommendations.

Transfected macrophages were stimulated with resiquimod (5 $\mu$ M) for 6 h, then IL-23 protein levels in whole-cell lysates were measured by enzyme-linked immunosorbent assay (ELISA; Abcam) and Western blotting.

Western blotting for IL-23, TNFAIP3, Sp1, TGF $\beta$ 1, cyclooxygenase-2 and OAS-1 After macrophages were exposed to LPS (10 ng/mL), or resiquimod (5 $\mu$ M) for 6 h, production of the proteins for IL-23p19, TNFAIP3, Sp1, TGF $\beta$ 1, and cyclooxygenase-2 (COX-2) were assessed by Western blotting with the following antibodies: IL-23p19 (catalog#sc-271279, Santa Cruz Biotechnology, Santa Cruz, CA), TNFAIP3 (catalog#sc-166692, Santa Cruz Biotechnology), Sp1 (catalog#sc-17824, Santa Cruz Biotechnology), TGF $\beta$ 1 (catalog#sc-130348, Santa Cruz Biotechnology), and COX-2 (catalog#sc-376861, Santa Cruz Biotechnology). After macrophages were treated with or without Lipofectamine for 6 h, OAS-1 protein levels were measured by Western blotting with the following antibody: OAS-1 (catalog#sc-374656, Santa Cruz Biotechnology).

The proteins in the whole-cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (ATTO corporation, Tokyo, Japan) and transferred onto polyvinylidene fluoride membranes (Thermo Fisher Scientific) for immunoblotting. The membranes were incubated with  $0.2 \times 10^3$   $\mu$ g/L mouse anti-human IL-23p19, TNFAIP3, Sp1, TGF $\beta$ 1, and COX-2 IgG (Santa Cruz Biotechnology) for 1 h at room temperature, washed and incubated with alkaline phosphatase-conjugated anti-mouse IgG (Santa Cruz Biotechnology) diluted to

1:5000. Then, the membranes were incubated with chemiluminescence enhancer (Immun-Star, Bio-Rad Laboratories, Hercules, CA) and exposed to XAR film (Kodak, Rochester, NY). After the film was developed, bands were quantified with a densitometer and ImageQuant software (Molecular Dynamics, Sunnydale, CA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also detected by Western blotting with an anti-GAPDH antibody (Santa Cruz Biotechnology), and the levels of IL-23, TNFAIP3, Sp1, TGF $\beta$ 1, and COX-2 were normalized to GAPDH.

### 2.7. Effect of Lipofectamine on IL-23 and p53 expression

We determined the effectiveness of the knockdown approach with Lipofectamine on IL-23p19 expression by real-time RT-PCR and Western blotting. On day 7 of culture, macrophages were treated with or without Lipofectamine (1  $\mu$ L). On day 9 of culture, the cells were stimulated with LPS (10 ng/mL) or resiquimod (5  $\mu$ M) for 6 h and IL-23p19 mRNA and protein levels were determined by real-time RT-PCR. p53 levels also were measured by Western blotting to assess the effect of Lipofectamine on its expression. Lipofectamine induces type 1 interferon (IFN), which is upregulated by the TNF receptor-associated factor 6 (TRAF6)/IFN regulatory factor 5 (IRF5) signaling pathway. Therefore, we investigated the effect of silencing for IRF5 and TRAF6 on IL-23 expression after exposure to resiquimod determined by ELISA. 2', 5'-oligoadenylate synthetase 1 (OAS-1) is upregulated by type I IFN induced with Lipofectamine. Cryopyrin belongs to the NLR family pyrin domain containing 3 (NLRP3). To investigate the effect of type 1 IFN induced by Lipofectamine or NLRP3 inflammasome activation on IL-23 expression, we determined protein levels of IL-23p19 in macrophages transfected with siRNA for OAS-1 or cryopyrin after exposure to resiquimod by ELISA.

### 2.8. ELISA for IL-12p40, IL-18, IL-23, TNFAIP3, IRAK-M, and Sp1

The levels of IL-12p40, IL-18, IL-23, TNFAIP3, IRAK-M, and Sp1 in whole-cell lysates of human macrophages were determined by ELISA (IL-12p40: Abcam, Cambridge, UK, IL-18: MBL life science, Woburn, MA, IL-23: Abcam; TNFAIP3: MYBioSource, San Diego, CA; IRAK-M, LifeSpan Biosciences, Seattle, WA; Sp1: Aviva Systems Biology Corporation, San Diego, CA) 6 h after exposure to LPS (10 ng/mL) or resiquimod (5  $\mu$ M) for 6 h. The levels of IL-23, IL-12p40, and IL-18 in whole-cell lysates prepared from human macrophages were determined by ELISA after sequential stimulation with LPS (0.1 ng/mL, 1 ng/mL, and 10 ng/mL for 6 h), Pam3Csk4 (1  $\mu$ g/mL for 6 h) and then resiquimod (6 h). The sensitivity of ELISA for the various proteins was as follows: IL-12p40, 12.29 pg/mL; IL-18, 8.3 pg/mL; IL-23, 16.3 pg/mL; TNFAIP3, 23.5 pg/mL; IRAK-M, 0.78 ng/mL; and Sp1, 0.15 ng/mL.

### 2.9. Effect of siRNA for UCP2, TGF $\beta$ 1/2/3, and TAK-1 on IL-23 production after exposure to resiquimod

Human macrophages (on day 9 of culture) transfected with siRNA for UCP2, TGF $\beta$ 1/2/3, or TGF- $\beta$ -activated kinase 1(TAK-1) were stimulated with resiquimod (5  $\mu$ M) for 6 h, after which IL-23 levels of whole-cell lysates were measured by ELISA (Abcam).

### 2.10. Effect of a PPAR $\alpha$ agonist (GW7647) and UCP2 siRNA on TNFAIP3 production after exposure to resiquimod

A peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) agonist (GW7647) and UCP2 siRNA were used to investigate the intracellular signaling pathways involved in TNFAIP3 production. Human macrophages (on day 9 of culture) treated with GW7647 (6  $\mu$ M) for 1 h and transfected or not with UCP2 siRNA were stimulated with resiquimod (5  $\mu$ M) for 6 h, after which TNFAIP3 levels were detected by Western blotting. Furthermore, macrophages treated with GW7647 (6  $\mu$ M) for 1 h and transfected or not with UCP-2 siRNA or transfected with TNFAIP3

siRNA were stimulated with resiquimod (5  $\mu$ M) for 6 h, after which IL-23 levels were measured by ELISA (Abcam).

### 2.11. Effect of small interfering RNA for p22phox, gp91phox, DUOX1, and DUOX2 on Sp1 and IL-23 production after exposure to resiquimod

Human macrophages (on day 9 of culture) transfected with siRNA for p22phox, gp91phox, DUOX1, and DUOX2 were stimulated with resiquimod (5  $\mu$ M) for 6 h, after which Sp1 levels were measured by ELISA (Aviva Systems Biology Corporation). Furthermore, IL-23 levels were detected through Western blotting of whole-cell lysates with the appropriate anti-mouse monoclonal antibody of IL-23 (Santa Cruz Biotechnology). Whole-cell lysates of untreated macrophages were used as the control. GAPDH was also detected by Western blotting with an anti-GAPDH antibody (Santa Cruz Biotechnology), and IL-23 protein levels were normalized for GAPDH.

### 2.12. Effect of small interfering RNA for PDCD-1, HIF1 $\alpha$ , TREM-1, and TNFAIP3 on Sp1 and IL-23 production after exposure to resiquimod

Human macrophages (on day 9 of culture) transfected with siRNA for PDCD-1, HIF1 $\alpha$ , TREM-1, and TNFAIP3 were stimulated with resiquimod (5  $\mu$ M) for 6 h, after which Sp1 levels were measured by ELISA.

### 2.13. Effect of small interfering RNA for TNFAIP3 and DUOX2 on IL-23 production after sequential administration of LPS and resiquimod

Human macrophages (on day 9 of culture) transfected with siRNA for TNFAIP3 or DUOX2 were stimulated by sequential administration of LPS (10 ng/mL) for 6 h and resiquimod (5  $\mu$ M) for 6 h, after which IL-23 levels were measured by ELISA.

### 2.14. Statistical analysis

Results are expressed as the mean (SE). Differences between two groups were analyzed using a *t*-test for independent means, and differences between more than two groups were compared by analysis of variance. When the *F* ratio was found to be significant, mean values were compared using a post hoc Bonferroni test. *P* < 0.05 was considered to indicate significance in all analyses.

## 3. Results

### 3.1. Real-time RT-PCR for IL-23p19 mRNA

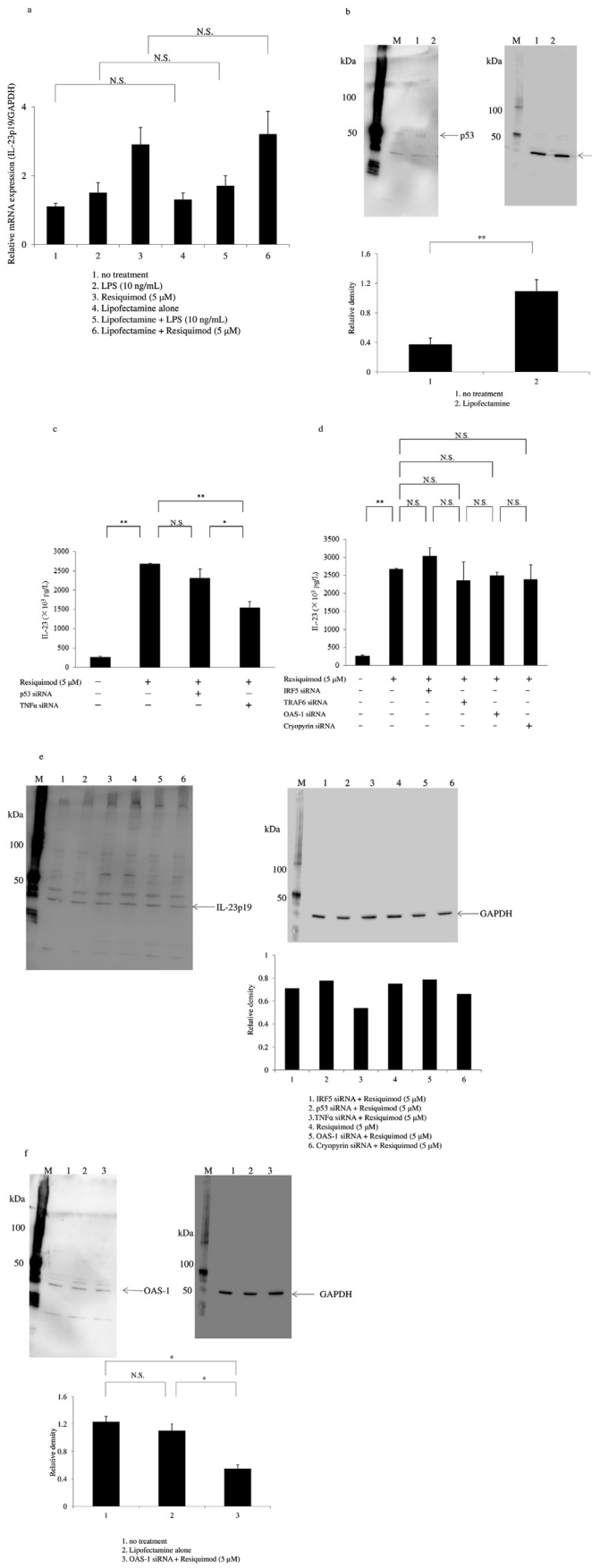
Real-time RT-PCR showed no significant difference in IL-23p19 mRNA levels of macrophages treated with or without Lipofectamine after exposure to LPS or resiquimod (Figure 1a).

### 3.2. Effect of p53 and TNF $\alpha$ on IL-23 levels after exposure to resiquimod

Lipofectamine induced a slight increase in p53 protein levels (Figure 1b). Silencing for TNF $\alpha$ , but not p53, showed a slight decrease in IL-23 levels after exposure to resiquimod, as determined by ELISA (Figure 1c).

### 3.3. Effect of IRF5, TRAF6, OAS-1, and cryopyrin on IL-23 levels

Furthermore, siRNA for IRF5, TRAF6, OAS-1, or cryopyrin had no effect on IL-23 levels determined by ELISA (Figure 1d). Furthermore, silencing for TNF $\alpha$  or cryopyrin, but not IRF5, p53 and OAS-1, induced a slight decrease in IL-23p19 protein levels after exposure to resiquimod, as determined by Western blotting (Figure 1e). Lipofectamine had no effect on OAS-1 expression (Figure 1f), as determined by Western blotting.



**Figure 1.** Effect of Lipofectamine on IL-23 expression. IL-23p19 mRNA levels were determined in macrophages treated with or without Lipofectamine by real-time reverse transcription polymerase chain reaction (RT-PCR) (a). p53 protein levels in macrophages treated with or without Lipofectamine for 6 h were measured by Western blotting. A representative blot is shown. Data were obtained by using samples from 3 individuals in each group. Results are shown as the mean (SE).  $**P < .01$  (with Bonferroni correction) (b). Furthermore, IL-23 protein levels in macrophages transfected with siRNA for p53, TNF $\alpha$ , IRF5, TRAF6, OAS-1, or cryopyrin after exposure to resiquimod were determined by enzyme-linked immunosorbent assay (c and d). Data were obtained by using samples from 3 individuals in each group. Results are shown as the mean (SE).  $**P < .01$ ;  $*P < .05$  (with Bonferroni correction); N.S., not significant IL-23p19 protein levels were measured by Western blotting in macrophages transfected with siRNA for IRF5, p53, TNF $\alpha$ , OAS-1 or cryopyrin after stimulation with resiquimod. A representative blot is shown. Data were obtained by using samples from 3 individuals in each group. Results are shown as the mean (SE).  $*P < .05$  (with Bonferroni correction); N.S., not significant (e). Furthermore, OAS-1 protein levels were measured by Western blotting in macrophages treated with or without Lipofectamine. A representative blot is shown. Data were obtained by using samples from 3 individuals in each group. Results are shown as the mean (SE).  $*P < .05$  (with Bonferroni correction); N.S., not significant (f).

3.4. Cross-talk between TLR2 or TLR4 and TLR7/8 agonists on IL-23 production

Resiquimod significantly enhanced IL-23 expression by macrophages, whereas LPS induced only a slight increase of IL-23 production. Furthermore, sequential stimulation of human macrophages with LPS and resiquimod significantly reduced IL-23 levels, as determined by ELISA. LPS 0.1 ng/mL caused a slight decrease of IL-23 levels after exposure to resiquimod, whereas LPS 1 ng/mL and 10 ng/mL caused a significant reduction of IL-23 levels (Figure 2a). A similar response was obtained with the TLR2 agonist Pam3CsK4 (Figure 2b).

3.5. Differential effect of sequential stimulation with LPS and resiquimod on IL-18 and IL-12p40 production

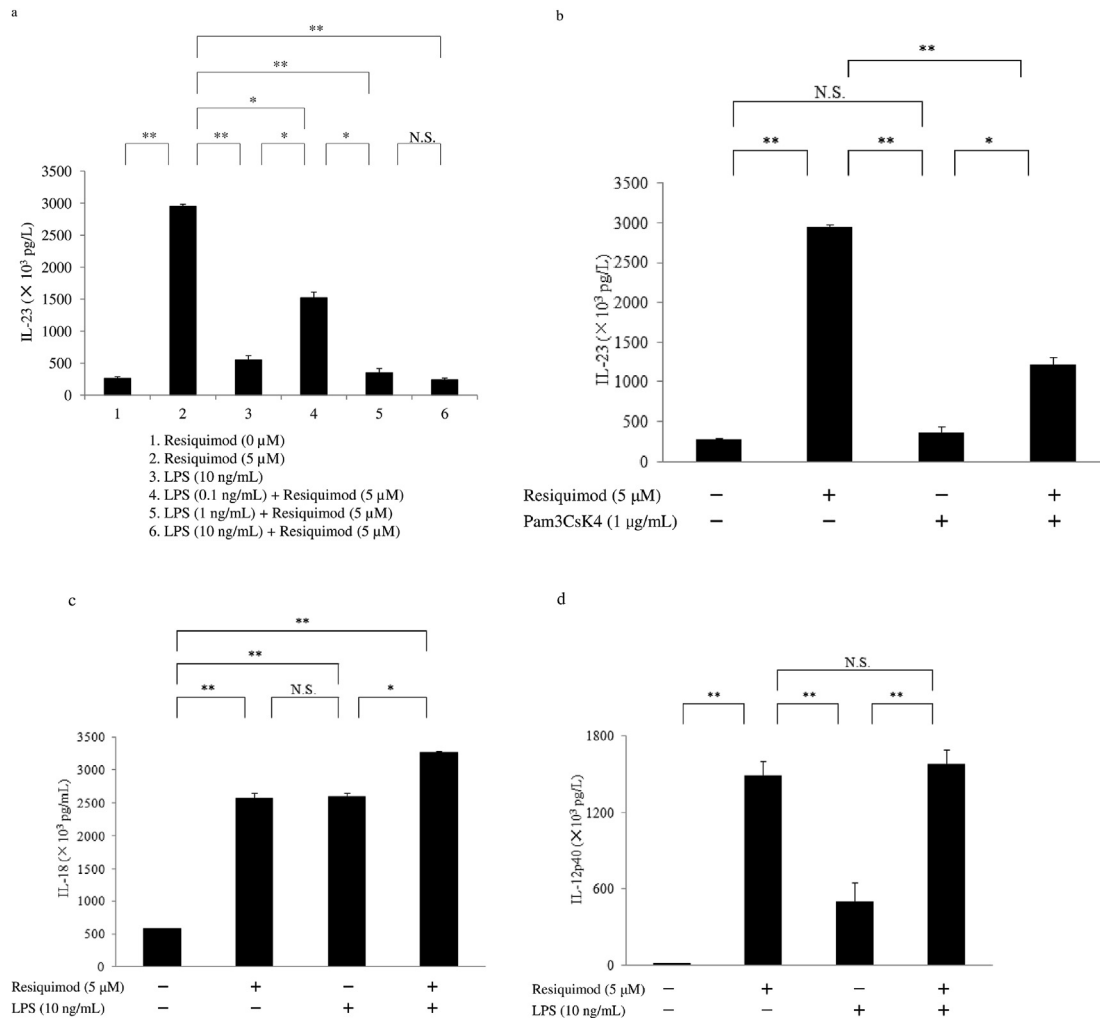
In contrast, both LPS and resiquimod significantly upregulated IL-18, and sequential stimulation with LPS and resiquimod did not reduce IL-18 levels (Figure 2c). Moreover, LPS reduced IL-12p40 levels, but sequential stimulation with LPS and resiquimod increased IL-12p40 levels (Figure 2d).

3.6. Production of TNFAIP3, IRAK-M, and Sp1 after stimulation with LPS or Pam3CsK4

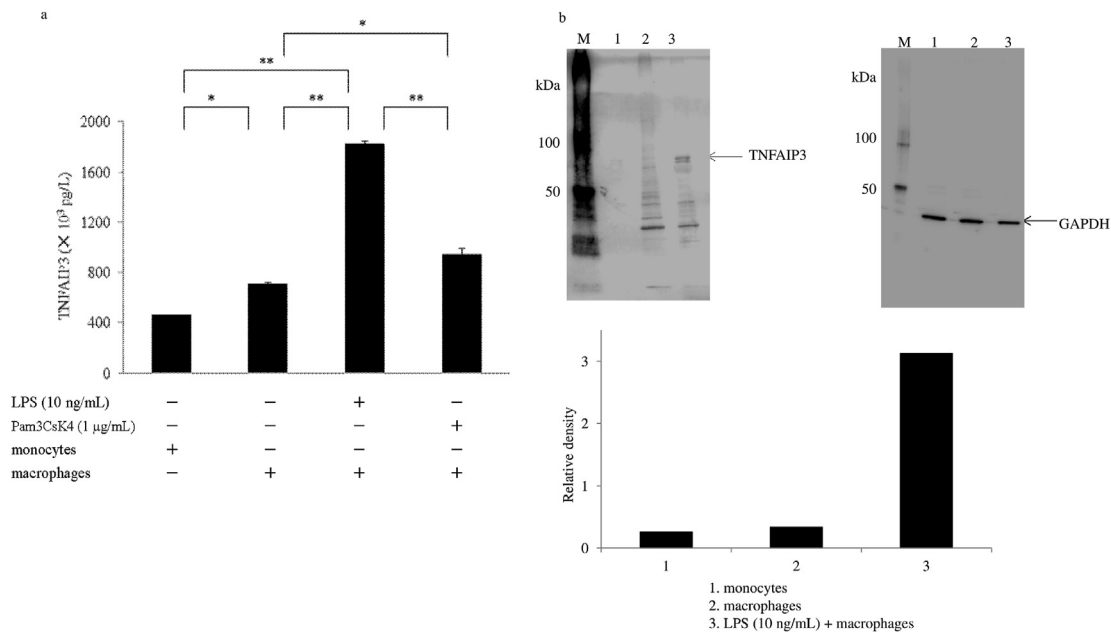
LPS upregulated TNFAIP3 expression, as determined by ELISA, significantly more than Pam3CsK4, which induced only a slight increase in TNFAIP3 levels (Figure 3a). Western blotting revealed increased TNFAIP3 levels in response to LPS (Figure 3b). IRAK-M levels were also enhanced after exposure to LPS, as determined by ELISA (Figure 4). In contrast, levels of Sp1 were attenuated after stimulation with LPS, as determined by ELISA (Figure 5a). Western blotting showed decreased Sp1 levels in response to LPS (Figure 5b).

3.7. Role of TGFβ1, TAK-1, and UCP2 in IL-23 production after exposure to resiquimod

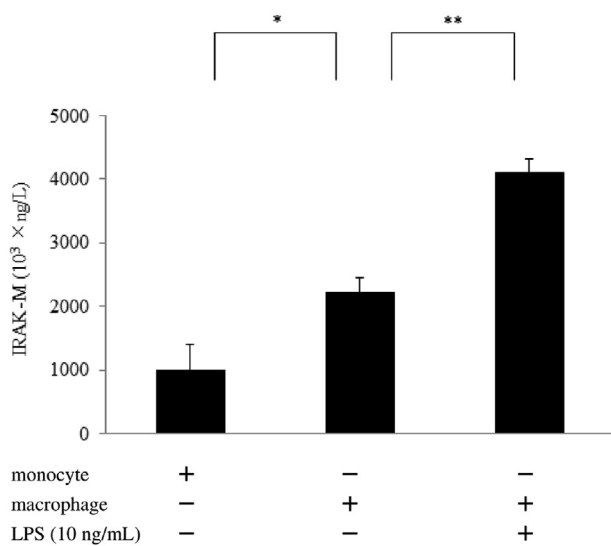
Resiquimod enhanced TGFβ1 production, as determined by Western blotting (Figure 6a). After exposure to resiquimod, interfering RNA for TGFβ1/2/3 or TAK-1 decreased IL-23 levels, as did silencing of RNA for UCP2 (Figure 6b).



**Figure 2.** IL-23 production by human macrophages after exposure to resiquimod and lipopolysaccharide (LPS) Human macrophages (on day 9 of culture) were stimulated with resiquimod (5μM) and LPS (10 ng/mL) or Pam3CsK4 (1 μg/mL) for 6 h. The cells also were exposed to sequential stimulation with LPS or Pam3CsK4 and resiquimod. IL-23 levels were measured by enzyme-linked immunosorbent assay (a and b). Data were obtained by using samples from 3 individuals in each group and represent the mean (SE). \*\*P < .01; \*P < .05 (with Bonferroni correction); N.S., not significant (a and b). Protein levels of IL-18 and IL-12p40 (d) were measured by enzyme-linked immunosorbent assay. Data were obtained by using samples from 3 individuals in each group. Results are shown as the mean (SE). \*\*P < .01; \*P < .05 (with Bonferroni correction); N.S., not significant.



**Figure 3.** Tumor necrosis factor  $\alpha$ -induced protein 3 (TNFAIP3) production by human monocytes and macrophages after exposure to lipopolysaccharide (LPS) or Pam3CsK4 Human monocytes and macrophages (on day 9 of culture) were stimulated with LPS (10 ng/mL) or Pam3CsK4 (1  $\mu$ g/mL) for 6 h TNFAIP3 levels were measured by enzyme-linked immunosorbent assay. Data were obtained by using samples from 3 individuals in each group. Results are shown as the mean (SE). \*\* $P < .01$ ; \* $P < .05$  (with Bonferroni correction) (a). Furthermore, the levels of TNFAIP3 were detected by Western blotting. The density of each band was normalized for that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). A representative result is shown. Data were obtained by using samples from 3 individuals in each group. Results are shown as the mean (SE). \*\* $P < .01$  (with Bonferroni correction); N.S., not significant (b).



**Figure 4.** Interleukin-1 receptor-associated kinase-M (IRAK-M) production by human monocytes and macrophages after exposure to lipopolysaccharide (LPS) Human monocytes and macrophages (on day 9 of culture) were stimulated with LPS (10 ng/mL) for 6 h. IRAK-M levels were measured by enzyme-linked immunosorbent assay. Data were obtained by using samples from 3 individuals in each group. Results are shown as the mean (SE). \*\* $P < .01$ ; \* $P < .05$  (with Bonferroni correction).

**3.8. Inhibitory effect of peroxisome PPAR $\alpha$  agonist (GW7647) and UCP2 siRNA on IL-23 levels**

Combined treatment with GW7647 and siRNA for UCP2 significantly attenuated IL-23 levels after exposure to resiquimod. In contrast, TNFAIP3 siRNA significantly upregulated IL-23 in response to resiquimod (Figure 7a). Treating macrophages with GW7647 or UCP2 siRNA

enhanced TNFAIP3 levels after exposure to resiquimod, as determined by Western blotting. The combined treatment with GW7647 and UCP2 siRNA upregulated TNFAIP3 levels more than treatment with either GW7647 or UCP2 siRNA alone (Figure 7b).

**3.9. Role of DUOX1/2 on Sp1 levels after exposure to resiquimod**

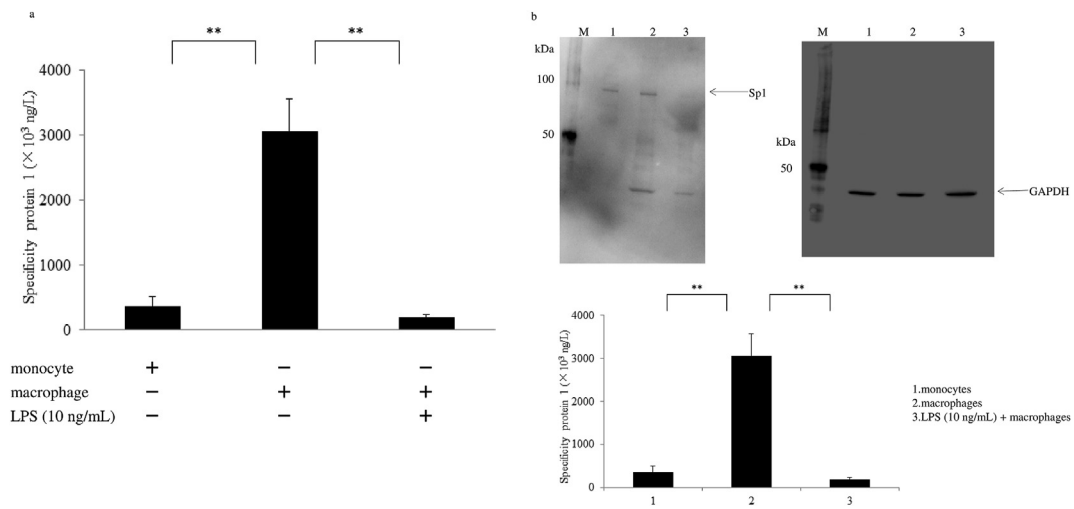
Resiquimod enhanced Sp1 levels, and after exposure to resiquimod siRNA for DUOX1/2 enhanced Sp1 levels significantly more than siRNA for p22phox and gp91phox did (Figure 8a). After exposure to resiquimod, siRNA-mediated knockout of DUOX1/2 upregulated IL-23 more than siRNA for p22phox and gp91phox did (Figure 8b) and also decreased COX-2 levels (Figure 8c), as determined by Western blotting. DUOX1 siRNA and DUOX2 siRNA significantly attenuated TNFAIP3 levels after exposure to resiquimod (Figure 8d), as determined by ELISA.

**3.10. Inhibitory effect of TNFAIP3 on Sp1 levels after exposure to resiquimod**

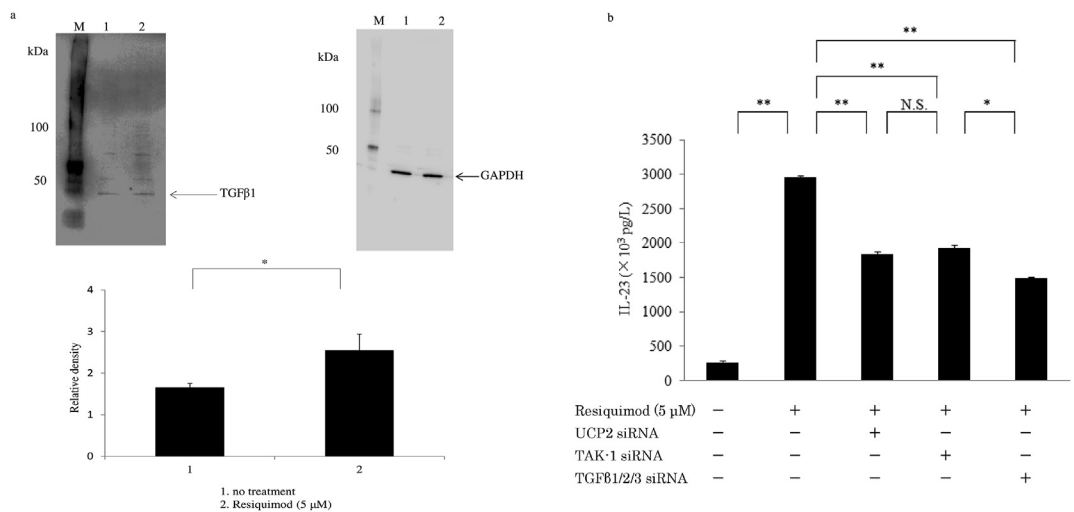
siRNA-mediated knockdown of TNFAIP3 significantly upregulated Sp1, as determined by ELISA, whereas siRNA for PDCD-1, HIF1 $\alpha$ , and TREM-1 did not enhance Sp1 (Figure 9).

**3.11. Effect of silencing for TNFAIP3 or DUOX2 on IL-23 production after sequential stimulation of human macrophages with LPS and resiquimod**

Transfection of macrophages with TNFAIP3 siRNA led to a significant increase in IL-23 levels after stimulation with resiquimod, whereas sequential stimulation with LPS (10 ng/mL) and resiquimod (5 $\mu$ M) led to a slight decrease in IL-23 levels. Similarly, transfection of macrophages with DUOX2 siRNA led to a significant increase in IL-23 production after exposure to resiquimod, whereas sequential stimulation of those macrophages with LPS and then resiquimod led to a slight decrease in IL-23 levels (Figure 10).



**Figure 5.** Specificity protein 1 (Sp1) production by human monocytes and macrophages after exposure to LPS Human monocytes and macrophages (on day 9 of culture) were stimulated with LPS (10 ng/mL) for 6 h Sp1 levels were measured by enzyme-linked immunosorbent assay (a). Data were obtained by using samples from 3 individuals in each group. Results are shown as the mean (SE). **\*\*P < .01** (with Bonferroni correction) Furthermore, the levels of Sp1 were detected by Western blotting. The density of each band was normalized for that of GAPDH. A representative result is shown as arbitrary density units. Data were obtained by using samples from 3 individuals in each group. Results are shown as the mean (SE). **\*\*P < .01**, **\*P < .05** (with Bonferroni correction) (b).



**Figure 6.** Effect of small interfering RNAs for transforming growth factorβ1/2/3 (TGFβ1/2/3), TGFβ-activated kinase 1 (TAK-1), and uncoupling protein 2 (UCP-2) on IL-23 production after exposure to resiquimod Human macrophages (on day 9 of culture) were stimulated with resiquimod (5μM) for 6 h, after which TGFβ1 levels were detected by Western blotting. The density of each band was normalized for that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). A representative result is shown as arbitrary density units. Data were obtained by using samples from 3 individuals in each group. Results are shown as the mean (SE). **\*P < .05** (with Bonferroni correction) (a). Macrophages transfected with small interfering RNA (siRNA) for TGFβ1/2/3, TAK-1, and UCP-2 were stimulated by resiquimod (5μM) for 6 h, after which IL-23 levels were measured by enzyme-linked immunosorbent assay (b). Results are shown as the mean (SE). **\*\*P < .01**; **\*P < .05** (with Bonferroni correction); N.S., not significant.

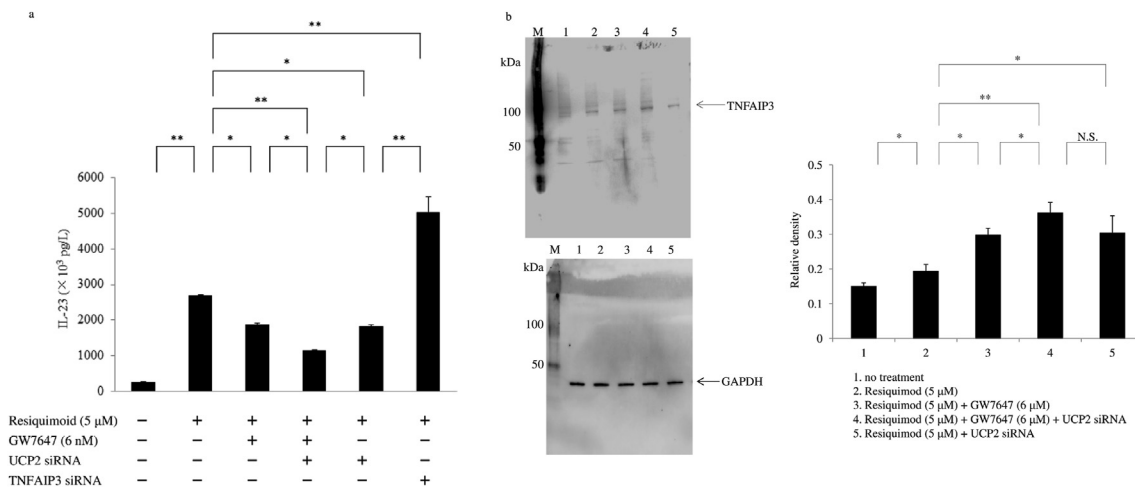
#### 4. Discussion

##### 4.1. Efficacy of transfection of siRNA with Lipofectamine

As regards quantitative knockdown of proteins by siRNA, we evaluated the silencing efficiency of each siRNA by Western blotting at 48 h after transfection in a previous study: After culture, the production of Sp1, TGFβ1, or TAK-1 protein by the transfected cells was assessed by Western blotting of whole-cell lysates with an anti-mouse monoclonal antibody for Sp1, TGFβ1, or TAK-1, respectively [15].

In the present study, we demonstrated the regulatory mechanism of IL-23 production by human macrophages after exposure to resiquimod by using a Lipofectamine-mediated knockdown approach. Lipofectamine, a cationic liposome, is used in non-viral gene delivery; however, this

application is limited by its cytotoxicity and apoptosis. First, we investigated the effectiveness of this knockdown approach at the protein and RNA levels. Real-time RT-PCR showed no statistical difference in IL-23p19 mRNA levels after exposure to LPS or resiquimod in macrophages treated with or without Lipofectamine. During apoptosis, phosphatidylserine (PS) was externalized and exposed to the cell membrane, which facilitated ingestion by phagocytic cells [16]. Then, macrophages phagocytose apoptotic cells and secrete IL-23 [17]. Externalization of PS is known to be activated by p53 [18]. Therefore, we investigated the effect of Lipofectamine on p53 expression by Western blotting. Use of Lipofectamine induced a slight increase in p53 levels. Next, we assessed the effect of p53 on IL-23 expression after exposure to resiquimod. TNFα also induces caspase activation [19], leading to development of apoptosis. We found that silencing for TNFα, but not p53, induced a slight



**Figure 7.** Effect of a peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) agonist (GW7647) and uncoupling protein 2 (UCP2) small interfering RNA (siRNA) on tumor necrosis factor  $\alpha$ -induced protein 3 (TNFAIP3) and IL-23 production after exposure to resiquimod Human macrophages (on day 9 of culture) transfected or not with UCP2 siRNA were pretreated with a PPAR $\alpha$  agonist (GW7647) for 1 h and then the cells were stimulated with resiquimod (5 $\mu$ M) for 6 h. Macrophages transfected with UCP2 siRNA and TNFAIP3 siRNA or pretreated with GW7647 for 1 h were stimulated with resiquimod (5 $\mu$ M) for 6 h, after which IL-23 levels were measured by enzyme-linked immunosorbent assay (a). Data were obtained by using samples from 3 individuals in each group; results are shown as the mean (SE). \*\* $P < .01$ ; \* $P < .05$  (with Bonferroni correction) (a). Furthermore, TNFAIP3 levels were detected by Western blotting. The density of each band was normalized for that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). A representative result is shown as arbitrary density units. Data were obtained by using samples from 3 individuals in each group; results are shown as the mean (SE). \*\* $P < .01$ ; \* $P < .05$  (with Bonferroni correction); N.S., not significant (b).

decrease in IL-23 levels after exposure to resiquimod, as determined by ELISA. The TLR7 agonist imiquimod, but not the TLR7/8 agonist resiquimod, was reported to enhance p53 expression and initiate p53-dependent apoptosis [20, 21]. Conversely, resiquimod induces the upregulation of TNF $\alpha$  [22], which promotes IL-23 production [23]. Lipofectamine also was reported to activate type 1 IFN signaling [24]. IFN- $\alpha$  was reported to enhance lipopolysaccharide-induced IL-23p19 transcription [25]. Interferon regulatory factor 5 (IRF5) induces type 1 IFN [26]. IRF5 is activated via myeloid differentiation primary response 88 (MyD88)/TNF receptor-associated factor 6 (TRAF6) signaling [27]. Therefore, we investigated the effect of siRNA for IRF5 and TRAF6 on IL-23 expression. We found that silencing for IRF5 and TRAF6 had no effect on IL-23 protein levels after exposure to resiquimod, as determined by ELISA. IRF5 is a direct target of p53. Silencing for p53 also had no effect on IL-23 production. OAS-1 is upregulated by type I IFNs and is also involved in the innate immune response to viral infection. Furthermore, we investigated the effect of OAS-1 on IL-23 expression after exposure to resiquimod. Silencing for OAS-1 did not have an effect on IL-23 protein levels, as determined by ELISA.

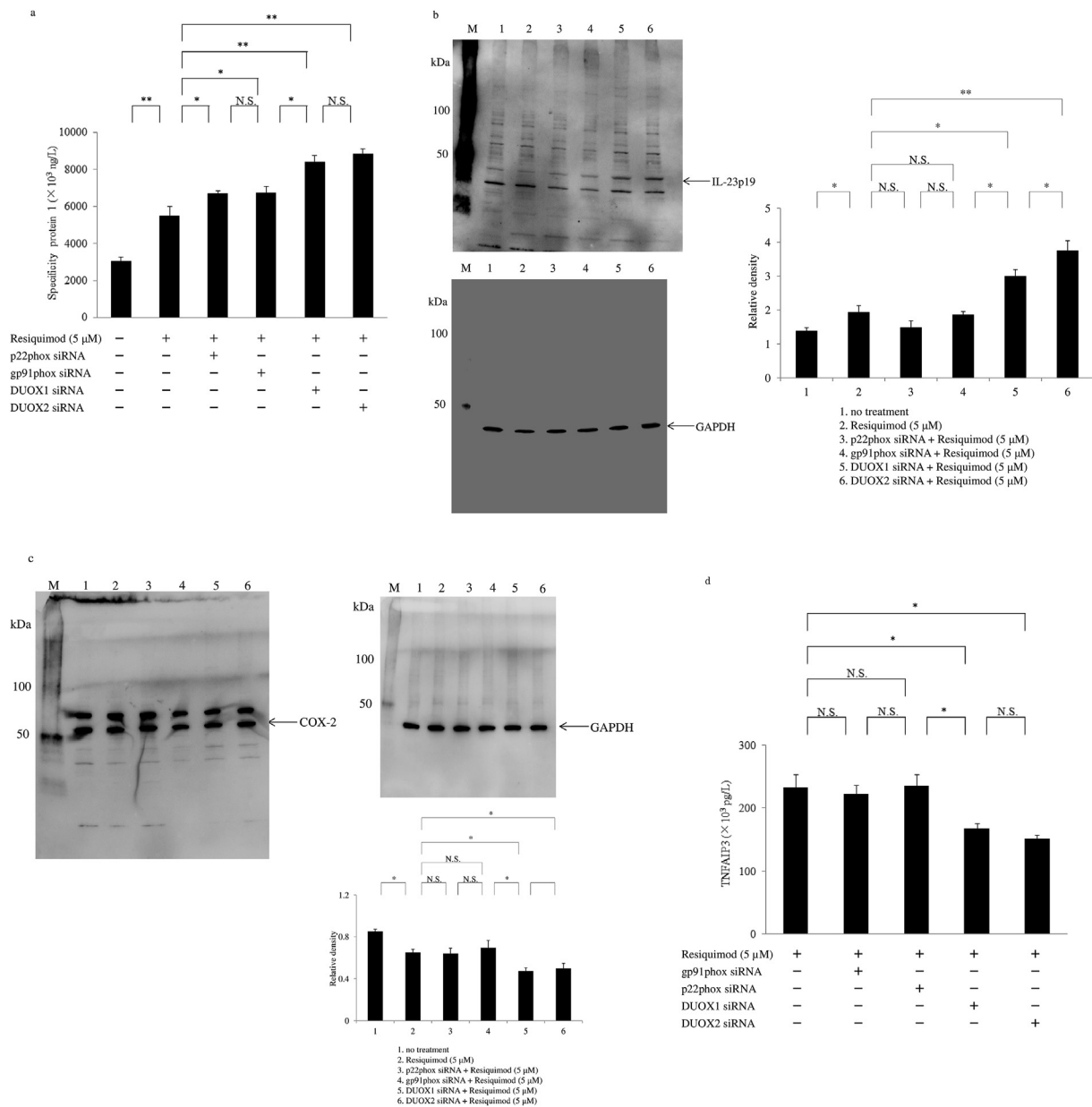
#### 4.2. Effect of LPS-related factor on IL-23p19 production via TLR7/8 signaling

We were able to confirm our hypothesis that LPS-associated proteins or transcription factors regulate IL-23 expression by human macrophages through TLR7/8-mediated signaling by showing that the TLR7/8 agonist resiquimod upregulated IL-23. Interestingly, sequential stimulation of macrophages with LPS and then resiquimod dramatically attenuated IL-23 levels but did not have the same effect on IL-12p40 and IL-18. IL-23 is a heterodimeric cytokine composed of the subunits IL-12p40 and IL-23p19. Our findings indicate that TLR4-related factors induced by LPS regulate IL-23p19 expression via the TLR7/8 signaling pathway but do not affect IL-12p40 production. TLR7 signaling triggers inflammasome activation, and caspase-1—which is activated by the inflammasome—cleaves pro-IL-18 to produce its active form, IL-18 [28, 29,30]. Inflammasome activation also was reported to be associated with IL-23 production [31]. However, on the basis of our results we assume that unknown, TLR4-related factors induced by LPS do not affect inflammasome activation or IL-23 expression because IL-18

expression was not affected by these factors. In this study, we further investigated the effect of the Nod-like receptor (NLR) family pyrin domain containing 3 (NLRP3)-inflammasome on IL-23 expression after exposure to resiquimod. Cryopyrin was reported to activate the inflammasome [32]. We found that silencing for cryopyrin had no effect on IL-23 production by human macrophages after exposure to resiquimod, as determined by ELISA, suggesting that cryopyrin/NLRP3 signaling is unlikely to be associated with IL-23 expression via TLR7/8 signaling. Therefore, we conclude that resiquimod-induced production of IL-23 is not dependent on inflammasome activation. This conclusion leads to the question which of the TLR4-related factors induced by LPS regulates IL-23 expression after exposure to resiquimod. LPS-associated proteins and transcription factors may be involved in mechanisms regulating IL-23 expression. Indeed, in our study LPS rapidly upregulated TNFAIP3 and IRAK-M, but it significantly reduced Sp1 levels. TNFAIP3 and IRAK-M are upregulated by TNF $\alpha$  stimulation [8], and the TLR7 ligand induces TNF $\alpha$  expression [33]. Therefore, we investigated the effect of TNFAIP3 and IRAK-M on IL-23 production via TLR7/8 signaling pathways. We found that siRNA-mediated knockdown of TNFAIP3 upregulated IL-23 after exposure to resiquimod significantly more than transfection with IRAK-M siRNA did. This difference may be due to the fact that TLR7/8 ligand inhibits TLR4-dependent activation of IRAK-M expression [34]. Therefore, TNFAIP3 may be one of the negative regulators of IL-23 expression after exposure to resiquimod. LPS binds to the CD14/TLR4/MD2 complex. A soluble form of CD14 (sCD14) is present in human serum [35], and LPS signaling through TLR2 is dependent on sCD14 [36]. Besides stimulating TLR4, LPS stimulates also TLR2 [37]. The TLR2 agonist Pam3Csk4 was reported to upregulate TNFAIP3 [38,39]. We found that LPS induced a significant increase in TNFAIP3 but that Pam3Csk4 induced only a slight increase. Therefore, we concluded that IL-23 production stimulated by resiquimod was reduced mainly by LPS (a TLR4 agonist) and only partially by Pam3Csk4 (a TLR2 agonist).

As mentioned above, the IL-23/IL-17 axis plays an important role in the development of autoimmune diseases. TGF $\beta$ 1 was reported to induce the differentiation of IL-17-producing Th17 cells [40], and our study showed that resiquimod enhanced TGF $\beta$ 1 levels. Therefore, we investigated whether, after exposure to resiquimod, TGF $\beta$ 1 signaling affects Th17 cells via IL-23 expression rather than directly. Both

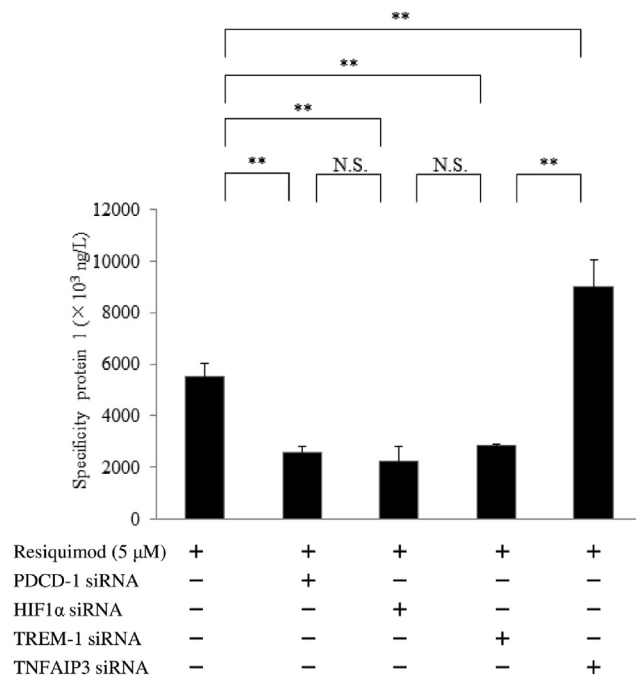




**Figure 8.** Effect of small interfering RNAs (siRNAs) for p22phox, gp91phox, dual oxidase 1 (DUOX1), and dual oxidase 2 (DUOX2) on specificity protein 1 (Sp1), IL-23, cyclooxygenase-2 (COX-2), and tumor necrosis factor  $\alpha$ -induced protein 3 (TNFAIP3) after exposure to resiquimod Human macrophages (on day 9 of culture) transfected with siRNA for p22phox, gp91phox, DUOX1, and DUOX2 were stimulated with resiquimod (5 $\mu$ M) for 6 h, after which Sp1 and TNFAIP3 levels were measured by enzyme-linked immunosorbent assay (a and d). Data were obtained by using samples from 3 individuals in each group, and results are shown as the mean (SE). \*\* $P < .01$ ; \* $P < .05$  (with Bonferroni correction); N.S., not significant. Furthermore, levels of IL-23 (b) and COX-2 (c) were detected by Western blotting. The density of each band was normalized for that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). A representative result is shown as arbitrary density units. Data were obtained by using samples from 3 individuals in each group, and results are shown as the mean (SE). \*\* $P < .01$ ; \* $P < .05$  (with Bonferroni correction); N.S., not significant.

Smad-dependent and -independent mechanisms have been proposed for the TGF $\beta$ -induced phosphorylation and activation of p38MAPK [41, 42], and p38MAPK (and the NF- $\kappa$ B signaling pathway) have been reported to induce IL-23p19 expression [43]. We found that siRNA for TGF $\beta$ 1/2/3 or TAK-1 reduced IL-23 levels after exposure to resiquimod; TGF $\beta$ 1 induces UCP2 expression [44]. We also found that UCP2 siRNA attenuated IL-23 levels after exposure to resiquimod. This led us to ask how a lack of UCP2 (as a result of UCP2 siRNA) attenuated IL-23 levels after exposure to resiquimod. Production of mitochondrial reactive oxygen species (ROS) is negatively regulated by UCP2 [45], and ROS has been shown to enhance TNFAIP3 expression [46]. When we investigated the signaling

pathway for TNFAIP3 production after exposure to resiquimod, we found that UCP2 siRNA increased TNFAIP3 production after exposure to resiquimod, leading to a decrease of IL-23 expression. ROS is generated mainly by mitochondria and NADPH oxidase. The NADPH oxidase subunits, such as p47phox and p67phox, and membrane p47phox protein are activated by PPAR $\alpha$  to generate ROS [47]. We found that PPAR $\alpha$  increased TNFAIP3 expression and that stimulation of UCP2 siRNA-transfected macrophages with the PPAR $\alpha$  agonist GW7647 additively enhanced TNFAIP3 levels. These findings indicate that both mitochondrial and non-mitochondrial ROS contributes to the production of TNFAIP3.

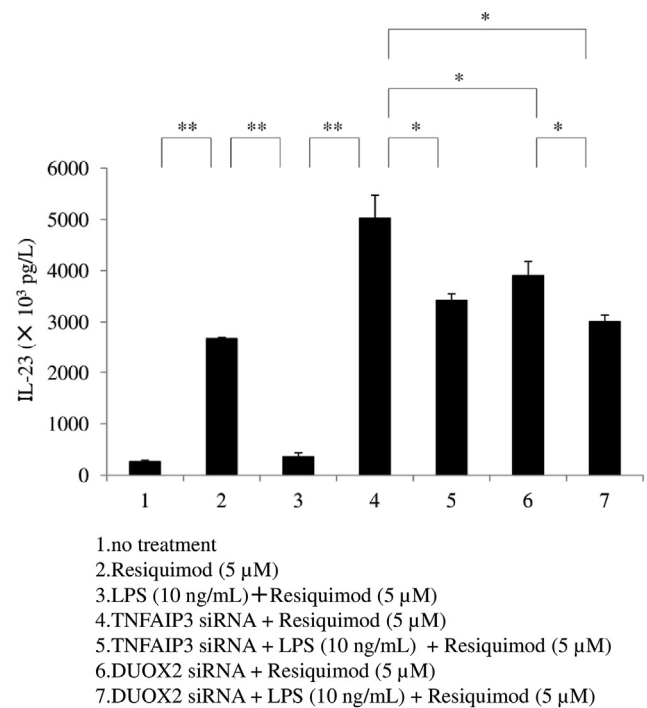


**Figure 9.** Effect of small interfering RNAs (siRNAs) for programmed cell death-1 (PDCD-1), hypoxia inducible factor 1α (HIF1α), triggering receptor expressed on myeloid cells 1 (TREM-1), and tumor necrosis factor α-induced protein 3 (TNFAIP3) on specificity protein 1 (Sp1) and IL-23 production after exposure to resiquimod Human macrophages (on day 9 of culture) transfected with small interfering RNA (siRNA) for PDCD-1, HIF1α, TREM-1, and TNFAIP3 were stimulated with resiquimod (5μM) for 6 h, after which Sp1 levels were measured by enzyme-linked immunosorbent assay. Data were obtained by using samples from 3 individuals in each group, and results are shown as the mean (SE). \*\**P* < .01; (with Bonferroni correction); N.S., not significant.

#### 4.3. Important role of ROS in TNFAIP3 and Sp1 production

LPS is known to enhance ROS production [48], and interleukin-1 receptor-associated kinase 1 (IRAK-1) is closely associated with LPS-induced generation of ROS [49]. In addition, the ROS-inducer piperlongumine was reported to decrease the expression of Sp1 [50], indicating that ROS negatively regulates Sp1 production. Therefore, to investigate the effect of upregulated Sp1 on IL-23 expression after exposure of macrophages to resiquimod we attempted to upregulate Sp1 by reducing the generation of ROS. Interestingly, this experiment showed that RNA interference of the NADPH subunit p22phox or gp91phox enhanced Sp1 protein production after exposure to resiquimod. Dual oxidases DUOX1/2 contain NADPH oxidase and peroxidase. Furthermore, RNA silencing for DUOX1 or DUOX2 upregulated Sp1 significantly more than RNA silencing for p22phox siRNA or gp91phox did. Indeed, increased Sp1 expression was reported to be associated with reduced ROS accumulation [51]. Most importantly, we found that upregulated Sp1 in macrophages transfected with DUOX1 siRNA or DUOX2 siRNA induced a significant increase in IL-23 levels after exposure to resiquimod. ROS also was reported to induce COX-2 expression through the TAK-1 signaling pathway [52, 53], and TNFα was reported to markedly enhance COX-2 expression in a dose-dependent manner [54]. TNFAIP3 also is induced by TNFα. We found that siRNA for DUOX1 or DUOX2 decreased COX-2 levels after exposure to resiquimod and also decreased TNFAIP3 levels. Sp1 upregulation is reciprocally associated with TNFAIP3 downregulation, resulting in enhanced IL-23 levels.

Our study of the effect of TLR4-related factors, including PDCD-1, HIF1α, TREM-1, and TNFAIP3, on Sp1 expression after exposure to resiquimod found that RNA interference of TNFAIP3 significantly enhanced Sp1 levels. However, RNA silencing for PDCD-1, HIF1α, or



**Figure 10.** Effect of TNFAIP3 and DUOX2 on IL-23 production after sequential exposure to lipopolysaccharide and resiquimod Human macrophages (on day 9 of culture) transfected with small interfering RNA (siRNA) for TNFAIP3 and DUOX2 were stimulated by sequential administration of lipopolysaccharide (LPS, 10 ng/mL) and resiquimod (5μM), after which IL-23 levels were measured by enzyme-linked immunosorbent assay. Data were obtained by using samples from 3 individuals in each group, and results are shown as the mean (SE). \*\**P* < .01; \**P* < .05 (with Bonferroni correction).

TREM-1 reduced Sp1 expression. Signal transducer and activator of transcription 6 (STAT6) activation upregulates Sp1 [55], and PDCD-1 increases STAT6 phosphorylation [56]. Accordingly, we found that transfection with PDCD-1 siRNA reduced Sp1 expression. HIF1α enhances transcription of Sp1 by binding to the promoter region, and RNA silencing for HIF1α decreases Sp1 mRNA levels [57]. This study also found that HIF1α siRNA reduced Sp1 expression. In our study, resiquimod was found to enhance Sp1 expression via the TLR7/8 signaling pathway. TREM-1 amplifies an inflammatory response in synergy with not only TLR4-signaling, but also TLR7/8-signaling [58, 59, 60], and we found that siRNA for TREM-1 reduced Sp1 levels after exposure to resiquimod.

## 5. Conclusion

IL-23 production in human macrophages is negatively regulated by TNFAIP3. Reciprocal expression of TNFAIP3 and Sp1 is dependent upon ROS generation via the DUOX1/2 signaling pathway.

## Declarations

### Author contribution statement

Rui Yamaguchi: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Arisa Sakamoto, Misa Haraguchi: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Reona Yamaguchi: Conceived and designed the experiments; Analyzed and interpreted the data.

Shinji Narahara, Hiroyuki Sugiuchi: Performed the experiments.

Yasuo Yamaguchi: Conceived and designed the experiments; Wrote the paper.

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#### Data availability statement

The data that has been used is confidential.

#### Declaration of interests statement

The authors declare no conflict of interest.

#### Additional information

No additional information is available for this paper.

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