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# Microglia express a functional receptor for interleukin-23

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ABSTRACT

Interleukin (IL)-23 plays a predominant role in the development of autoimmune diseases by inducing IL-17-producing helper T (Th17) cells. The receptor for IL-23 consists of a heterodimer composed of the IL-12 receptor β1 (IL-12Rβ1) and the IL-23 receptor (IL-23R), which is mainly expressed on Th17 cells. A recent study showed that macrophages express IL-23R mRNA and can be distinguished from microglia by IL-23R expression. However, in this study, we show by RT-PCR and immunocytochemistry that microglia express IL-23R and IL-12Rβ1 mRNA and protein, respectively. Additionally, microglia expressed a functional receptor for IL-23, as IL-23 enhanced the Interferon (IFN)-γ-induced signal transducer and activator of transcription (STAT)1 phosphorylation and chemokine production. Thus, IL-23R expression does not discriminate peripheral macrophages from microglia. Moreover, since microglia produce IL-23, it may function in an autocrine manner to recruit inflammatory cells by inducing chemokine production.



Microglia are resident macrophages in the central nervous system (CNS) that resemble peripheral macrophages in many aspects including cytokine and chemokine production, antigen presentation, and CD11b and Iba1 expression [1]. Discrimination between microglia and peripheral macrophages in the CNS during normal conditions, and during inflammatory diseases (such as experimental autoimmune encephalomyelitis (EAE), bacterial infection, and human immunodeficiency virus encephalitis) has been attempted based on CD45 surface expression levels [2,3]. However, the ability of CD45 to distinguish microglia from invading peripheral macrophages is limited, and thus, a new marker is needed to identify these cell types in the brain. Recently, it was reported that peripheral macrophages express interleukin (IL)-23 receptor (IL-23R) mRNA, but microglia do not [4]. Thus, IL-23R expression has been proposed as a marker for discriminating peripheral macrophages from microglia.

IL-23 is a heterodimeric cytokine composed of the proteins p19 and p40. It was originally discovered as an inducer of T helper type 1 (Th1) responses [5] and was recently recognized as a critical inducer of Th17 differentiation [6,7]. IL-23 is produced by antigen presenting cells such as macrophages, dendritic cells, and microglia [5,8,9]. IL-23 binds to a heterodimeric receptor composed of the IL-12 receptor β1 (IL-12Rβ1) and IL-23R and this subsequently activates Jak2, Tyk2, and signal transducers and activators of transcription (STATs)1, 3, 4, and 5 [10]. Mice lacking the p19 subunit are resistant to EAE [4]. Similarly, IL-12Rβ1-deficient mice are also completely resistant to myelin oligodendrocyte glycoprotein-induced EAE [11]. IL-23 reportedly plays an important role in the induction phase of EAE [12]. Furthermore, administration of anti-IL-23 antibody effectively inhibits EAE by suppressing not only IL-17 production, but also the expression of chemokine mRNAs in the CNS [13]. These findings suggest that IL-23 is critical for autoimmune inflammation of the brain.

In the CNS diseases such as neurodegenerative disorders or inflammatory diseases, microglia produce various chemokines such as Interferon (IFN)-γ-inducible protein (IP)-10/CXCL-10, monocyte chemoattractant protein (MCP)-1/CCL-2, and RANTES/CCL-5 [14,15]. In EAE, it has been shown that the chemokines produced by microglia recruit leukocytes to the sites of axonal injury in the CNS [16]. For example, when IL-23p19 deficient mice are infected with *Cryptococcus neoformans*, which causes meningioencephalitis in immunodeficient hosts, activation of microglia and expression of MCP-1/CCL-2 in the brain are lower than wild type mice [17]. These findings suggest that IL-23 might act on microglia in an autocrine fashion to induce chemokine production and recruit inflammatory T cells.

In this study, we examined microglial IL-23R expression to confirm whether it is a useful marker for distinguishing microglia from macrophages. For the first time, we show that microglia express IL-23R mRNA and have surface IL-23R protein expression. Moreover, signal transduction analyses confirm that the receptor for IL-23 is functional. These data indicate that IL-23R expression does not discriminate between microglia and peripheral macrophages. We also

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show that IL-23 enhances IFN-γ-induced chemokine production by microglia at the mRNA and protein levels, suggesting that IL-23 might play a role in chemokine production by these resident inflammatory cells in the CNS.

## Materials and methods

*Isolation of microglia.* Microglia were isolated from the primary mixed glial cell cultures from newborn C57BL/6 mice (Japan SLC, Hamamatsu, Shizuoka, Japan) on the 14th day using the "shaking off" method as described previously [18]. Adult mouse microglia were purified from naïve C57BL/6 mice by the method described by Li et al. [8].

*RNA isolation and reverse transcription (RT).* Microglia were plated in 24-well culture plates (BD Biosciences, San Jose, CA, USA) at a density of $2 \times 10^5$/well and incubated for 6 h with or without various stimuli including lipopolysaccharide (LPS) (Sigma, St. Louis, MO, USA), IFN-γ (R&D systems, Minneapolis, MN, USA), tumor necrosis factor (TNF)-α (R&D systems), and IFN-β (R&D systems). Total RNA was extracted from the adherent cells using the RNeasy Mini kit according to the manufacturer's protocol (Qiagen, Valencia, CA, USA). After 0.2 μg of total RNA was denatured for 5 min at 65 °C, the RT reaction was performed as described previously [9].

*Real-time PCR.* Real-time PCR was performed using an ABI Prism® as described previously [9]. In brief, the primers were designed on different exons using Primer Express® software version 2.0 (Applied Biosystems, Lincoln Centre Drive Foster City, CA, USA) and each PCR product was confirmed to be a single band by agarose gel electrophoresis analysis. Then, 2 μl of cDNA from the RT reaction was added to a mixture of 1 × SYBR®Green PCR Master Mix (Applied Biosystems), 500 nM forward primer and reverse primers (Table 1), and deionized water (25 μl total volume). The optimized parameters for the thermal cycler were as follows: activation at 95 °C for 10 min followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. The amount of mRNA in each sample was normalized against GAPDH mRNA.

*Flow cytometry.* Microglia were resuspended in fluorescence activated cell sorting (FACS) buffer (PBS supplemented with 2% FBS and 0.01% Sodium azide), blocked with rat anti-Fc receptor antibodies (Abs, anti-CD16/32) (BD Biosciences) for 30 min at 4 °C, and then incubated with isotype control or PE-conjugated anti-IL-12Rβ1 monoclonal Abs (BD Biosciences) for 30 min at 4 °C. The samples were examined with a Cytomics FC500 (Beckman Coulter, Fullerton, CA, USA) and analyzed with CXP Software Ver.2.0 (Beckman Coulter).

**Table 1**
Primer sets for real-time PCR

| Molecule | Forward primer | Reverse primer |
|---|---|---|
| IL-23R | TCAGTGCTACAATCTTCAGAGGACAT | GATGGCCAAGAAGACCATTCC |
| IL-12Rβ1 | GGACCAGCAAACACATCACCTT | CAACGCAGCAGCCATCAC |
| MCP-1/CCL-2 | GGCTCAGCCAGATGCAGTTAA | CCTACTCATTGGGATCATCTTGCT |
| RANTES/CCL-5 | GCAAGTGCTCCAATCTTGCA | CTTCTCTGGGTTGGCACACA |
| IP-10/CXCL-10 | GGCCATAGGGAAGCTTGAAAT | CTTTTTCATCGTGGCAATGATC |

*Immunocytochemistry.* Microglia were plated on 8-well chamber slides at a density of $1 \times 10^5$ cells/well. After stimulation with 1 ng/ml IFN-γ for 24 h, microglia were fixed with 2% paraformaldehyde for 30 min and incubated with PE-conjugated mouse anti-mouse-IL-12Rβ1 monoclonal Abs (BD Biosciences), goat anti-mouse-IL-23R polyclonal Abs (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or their isotype controls. Microglia were then incubated with Alexa 488-conjugated anti-goat IgG Abs (Invitrogen, Carlsbad, CA, USA) for 30 min. Cells were examined with a deconvolution fluorescence microscope system (Bio Zero, Keyence, Osaka, Japan).

*Western blotting.* Microglia were first incubated with IFN-γ for 24 h. After starvation (DMEM without FCS for 18 h), microglia were stimulated with 50 ng/ml IL-23. Cells were lysed in TNES buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP-40, 2 mM EDTA, 0.1% sodium dodecyl sulfate) containing protease (Roche Diagnostics, Basel, Switzerland) and phosphatase inhibitor cocktails (Sigma). Cell lysates were separated using SDS–PAGE and blotted to Hybond™-P membranes (GE Healthcare, Buckinghamshire, UK) as described previously [19]. Membranes were incubated at 4 °C with primary Abs against phosphorylated STAT1 (p-STAT1) (Cell Signaling Technology, Danvers, MA, USA), or STAT1 (Santa Cruz biotechnology), then incubated with a secondary Abs against horse radish peroxidase-conjugated anti-rabbit IgG (GE Healthcare) or anti-goat IgG (Santa Cruz biotechnology) Abs at room temperature for 90 min. Blots were visualized using the ECL Plus Western Blotting Detection System (GE Healthcare). Signal intensities were quantified by densitometry using Image J software.

*Chemokine production.* Microglia were incubated with IFN-γ and graded doses of IL-23 for 24 h. The supernatants were collected and concentrations of IP-10/CXCL-10, MCP-1/CCL-2, RANTES/CCL-5, and IP-10/CXCL-10 were evaluated using Quantikine ELISA kits (R&D systems).

*Statistical analysis.* Data are presented as means ± SD. Statistical significance was assessed with a one-way ANOVA followed by Tukey's post hoc test.

## Results

### *Microglia express IL-23R and IL-12Rβ1*

The purity of isolated microglia was almost 100% as determined by immunostaining with anti-CD11b antibody (Fig. S1). Using RT-PCR, we analyzed IL-23R and IL-12Rβ1 mRNA expression in unstimulated or LPS, IFN-γ, TNF-α, or IFN-β stimulated microglia. Unstimulated microglia did not express either IL-23R or IL-12Rβ1 mRNA. LPS induced IL-23R mRNA the most strongly (Fig. 1A), but LPS had only a minimal effect on IL-12Rβ1 induction. In contrast, IFN-γ induced IL-12Rβ1 the most strongly, but only moderately induced IL-23R mRNA. The induction of both IL-12Rβ1 and IL-23R mRNA by IFN-γ was dose-dependent (Fig. 1B), but TNF-α did not induce either and IFN-β induced both at very low level (Fig. 1A). IFN-γ also induced IL-23R mRNA, whereas LPS did not in adult mouse microglia (Fig. S2). These results suggested that IFN-γ was

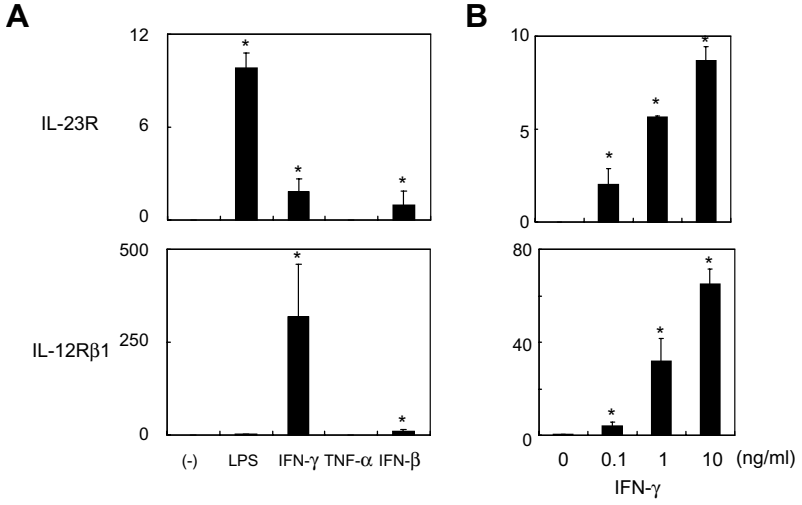


**Fig. 1.** Expression of IL-23R and IL-12Rβ1 mRNA. (A,B) Microglia were left untreated or were incubated with (A) 1 μg/ml LPS, 1 ng/ml IFN-γ, 20 ng/ml TNF-α, or 1000 U/ml IFN-β or (B) 0.1–10 ng/ml IFN-γ for 6 h. Data show the average ratio of IL-23R or IL-12Rβ1 mRNA to GAPDH mRNA, as determined using real-time RT-PCR (±SD, $n = 3$). $p$ values refer to comparison between untreated and treated cells. $^*p < 0.05$.

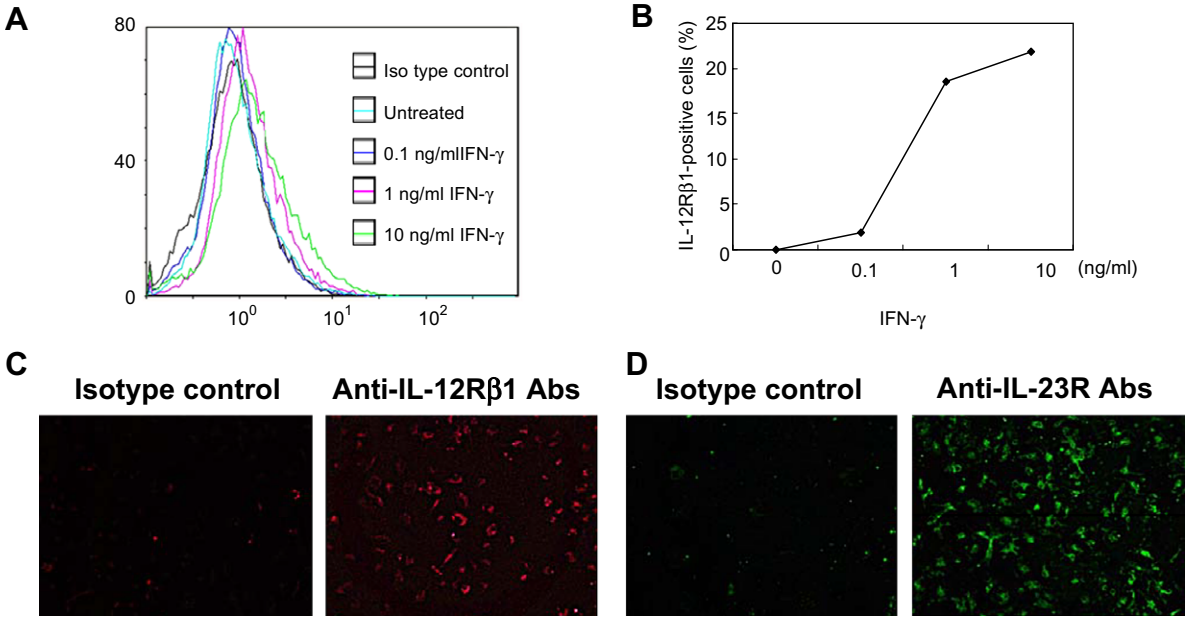


**Fig. 2.** Microglia express surface IL-23R and IL-12Rβ1. (A,B) Microglia were incubated with or without 0.1–10 ng/ml IFN-γ for 24 h. (A) The histogram and (B) the line graph show the expression of IL-12Rβ1, as determined by flow cytometry. (C,D) Microglia were incubated with or without 1 ng/ml IFN-γ for 24 h. After fixation, the surface expressions of IL-23R (C) and IL-12Rβ1 (D), or their isotype controls, were analyzed by immunocytochemistry.

more suitable to induce a functional receptor for IL-23 than the other cytokines.

Next, we analyzed microglial IL-12Rβ1 surface expression in response to IFN-γ stimulation using flow cytometry. IFN-γ induced surface expression of IL-12Rβ1 in a dose-dependent manner (Fig. 2A) and only 1 ng/ml IFN-γ was required to induce this expression (Fig. 2B). Similarly, immunocytochemical analysis demonstrated that IFN-γ stimulated microglia to express IL-12Rβ1 and IL-23R (Fig. 2C).

To assess whether the receptor for IL-23 was functional, we evaluated whether IL-23 affected IFN-γ-induced phosphorylation of STAT1. Western blot analysis showed that IL-23 enhanced IFN-γ-induced STAT1 phosphorylation (Fig. 3). However, IL-23 alone did not induce STAT1 phosphorylation (data not shown).

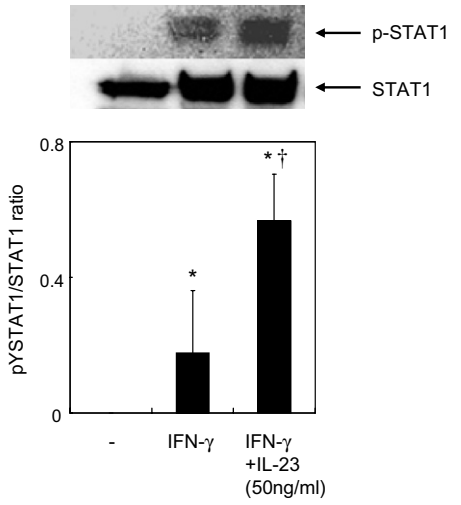


**Fig. 3.** IL-23 enhances IFN-γ-induced STAT1 phosphorylation. (A,B) Microglia were incubated with or without 1 ng/ml IFN-γ for 24 h. After serum starvation for 18 h, microglia were incubated with or without 50 ng/ml IL-23. (A) Western blots were performed on whole cell lysates by using anti-p-STAT1 or anti-STAT1 Abs. (B) Graph shows the average ratio of p-STAT1/STAT1 (±SD, $n = 3$). $^{*}p < 0.05$, untreated vs IFN-γ-treated. $^{\dagger}p < 0.05$, IFN-γ-treated vs IFN-γ + IL-23-treated.

*IL-23 increases IFN-γ-induced chemokine production by microglia*

To further assess whether IL-23 affected microglial function, we evaluated their IFN-γ-induced chemokine production with or without IL-23 stimulation. IL-23 stimulation dose-dependently enhanced the IFN-γ-induced mRNA expression and secretion of MCP-1/CCL-2, RANTES/CCL-5, and IP-10/CXCL-10 in microglia (Fig. 4 and Fig. S3).

## Discussion

Despite several attempts to discriminate microglia and invading peripheral macrophages in the CNS [2,3], it is still difficult to distinguish these cells under pathological conditions. Cua et al. showed that in normal and EAE-induced mice, macrophages expressed IL-23R mRNA but $CD4^{-}CD11b^{+}CD45^{lo}$ resident microglia did not [4]. In addition, Li et al. showed that macrophages were induced to express IL-23R mRNA by stimulation with LPS, whereas primary microglia from adult naïve mice did not, even after stimulation [8]. Based on these reports, we expected that the surface expression of IL-23R on surface would be a marker to distinguish peripheral macrophages from microglia. However, we found that microglia expressed IL-23R. Furthermore, IL-23R mRNA was induced by IFN-γ alone, suggesting that microglia in inflammatory or infectious lesions might express IL-23R. The expression of the components of the IL-23 receptor complex was confirmed by immunostaining with antibodies against IL-23R and IL-12Rβ1, indicating that microglia expressed the functional heterodimeric receptor. Moreover, STAT1 phosphorylation was increased by IL-23, further confirming that microglia express a functional IL-23 receptor. Although the reason for the discrepancy between previous reports and our mRNA expression data remain unknown, the protein expression and signal transduction data strongly support the notion that microglia express a functional IL-23 receptor.

Th17 cells, which produce IL-17, IL-21, IL-22, and express ROR-γt, mainly express IL-23R, and currently its expression is believed to be a specific marker for these cells [20]. IFN-γ, a Th1 cytokine, suppresses Th17 differentiation and downregulates IL-23R mRNA in

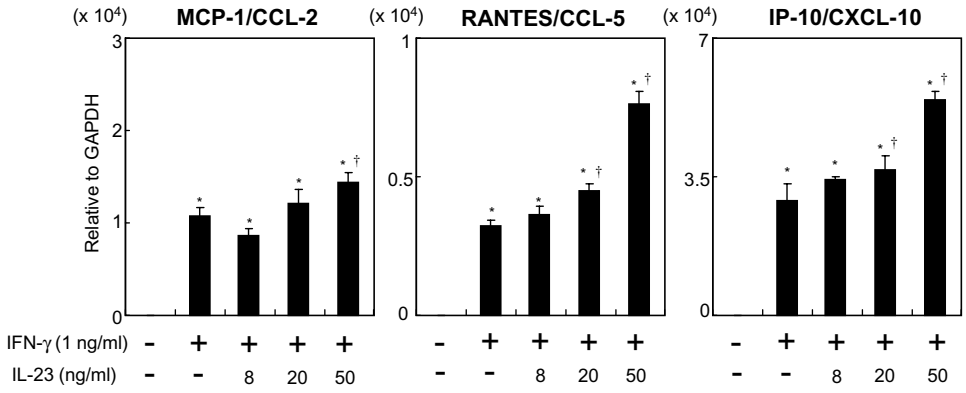


**Fig. 4.** IL-23 enhances IFN-γ-induced chemokine mRNA expression. Microglia were incubated with or without 1 ng/ml IFN-γ in the presence or absence of graded doses of IL-23 for 6 h. Data show the average ratio of the indicated mRNA to GAPDH mRNA, as determined using real-time RT-PCR (±SD, $n = 3$). $^{*}p < 0.05$, untreated vs IFN-γ-treated. $^{\dagger}p < 0.05$, IFN-γ-treated vs IFN-γ + IL-23-treated.

CD4$^+$ T cells [6,7,21–23]. However, in our study, IFN-γ induced IL-23R expression by microglia. Thus, regulatory mechanisms of IL-23R expression in microglia may be different from those of T cells. In addition, since IFN-γ induces production of the Th17-inducing cytokine IL-6 in microglia [24], microglia in the CNS may play an important role in the regulation of the Th1/Th17 balance.

Previously, we and another group showed that microglia in the CNS produce IL-23 [8,9]. IL-23 production by CNS cells seems to be critical for the development of EAE by regulating Th17 differentiation [25]. Furthermore, the Th17 cytokines IL-17 and IL-22 disrupt the tight junction of blood–brain barrier [26]. Interestingly, once encephalitogenic T cells have been generated, subsequent EAE development is unaffected by the absence of IL-23, suggesting that IL-23 is critical during the induction phase of EAE [12]. Our previous work suggested that IL-17-producing CD4$^+$ T cells already exist in the CNS during the induction phase of EAE [27]. Collectively, these findings suggest that the IL-23–IL-17 axis is important during the induction phase of EAE. In addition, we have shown here that IL-23 enhances IFN-γ-induced chemokine production by microglia. Since chemokine production is necessary for the infiltration of inflammatory lymphocytes [16,28], IL-23 may play a critical role during the induction phase of EAE by regulating the generation of Th17 cells and microglial chemokine production. Consistent with this, IL-23p19 mRNA in CD11b$^+$ microglia/macrophages is induced during the early phase of EAE [8]. Thus, the expression of a functional IL-23 receptor on microglia may be important for the induction phase of CNS pathology during autoimmune processes.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.03.059.

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