Type II interleukin-4 receptor-mediated anti-inflammatory response in Mm1 and J774.1 macrophages

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Received on October 21, 2013, accepted on December 3, 2013

ABSTRACT

Introduction: Macrophages are involved in various inflammatory processes. Although the therapeutic potential of interleukin-4 (IL-4)-mediated immunomodulation has been proposed, it has not yet been established. The type I IL-4 receptor (IL-4RI) consists of IL-4Rα and a common β chain, whereas the type II IL-4R (IL-4RII) is a heterodimer of IL-4Rα and IL-13Rα1. In the present study, we assessed the selective activation of IL-4R isoforms in the mouse macrophage cell lines, Mm1 and J774.1, to investigate the role of receptor-specific signaling in inflammatory activation. Materials and Methods: The plasmid expressing murine IL-4/Q116E, which is analogous to the human IL-4RI-specific agonist IL-4/R121E, was transfected to assess IL-4RI activation. IL-4RII activation was induced by IL-13. Activation of IL-4R isoforms was confirmed by the activation of signal transducer and activator of transcription 6 (STAT6). Lipopolysaccharide (LPS)-induced tumor necrosis factor-α (TNF-α) induction was examined using real-time polymerase chain reaction. LPS plus interferon-γ (INF-γ)-stimulated inducible nitric oxide synthase induction was tested using western blotting. Results: IL-4/Q116E induced STAT6 activation in Mm1 cells but not in J774.1 cells. In Mm1 cells, IL-4/Q116E-induced STAT6 activation was inhibited by kaempferol, a specific inhibitor for Janus kinase 3 (JAK3). IL-4/Q116E did not exhibit anti-inflammatory activity in either macrophage cell line. However, IL-13 inhibited inflammatory activation of these cells. Conclusions: Our data suggest that Mm1 cells expressed both IL-4RI and IL-4RII, whereas J774.1 cells expressed only IL-4RII. Although IL-4R isoform expression pattern was diverse in these two macrophages, the IL-4-mediated anti-inflammatory effect was thought to be largely dependent on IL-4RII-mediated signaling. Thus, we could further conclude that IL-4 signaling plays a pivotal role in the regulation of macrophage inflammatory properties. Understanding IL-4-mediated anti-inflammatory signaling in macrophages may be beneficial for the development of therapeutics.

Key words: IL-4, IL-13, receptor, macrophage, inflammation
INTRODUCTION

Macrophages are innate and adaptive immune cells which are highly involved in various pathologic processes\textsuperscript{1,5}. Macrophages are phenotypically polarized by the pathophysiological microenvironment and are broadly classified into two main groups: classically activated macrophages (M1) and alternatively activated macrophages (M2)\textsuperscript{2,5,4}. Differentiation into M1 and M2 macrophages is linked to the T-cell immune responses because M1 macrophages are activated by interferon-\(\alpha\) (INF-\(\alpha\)), a representative Th1 cytokine, and M2 macrophages are activated by interleukin-4 (IL-4) and IL-13, representative Th2 cytokines\textsuperscript{1,2}. Thus, immunomodulation with certain cytokines may offer a therapeutic benefit against macrophage-related disease processes.

IL-4 has been recognized as a prototypic immunoregulatory cytokine, which plays a central role in T cell differentiation to Th2 subsets and suppresses Th1 lymphocyte induction\textsuperscript{3-5,9}. The receptors for IL-4 and IL-13 share an IL-4R\(\alpha\) chain that can be activated as an essential component in their heterodimeric structure\textsuperscript{4-30}. Type I IL-4R (IL-4RI) consists of IL-4R\(\alpha\) and a common \(\gamma\) chain (\(\gamma\)c). Type II IL-4R (IL-4RII) is a heterodimer of IL-4R\(\alpha\) and IL-13R\(\alpha\)\textsuperscript{5,30}. Lymphocytes require a \(\gamma\)c for IL-4 responses; in mouse macrophages, all of the observed IL-13 responses pass through IL-4RII\textsuperscript{11}. Engagement of the IL-4RI and IL-4RII complexes commonly leads to activation of the transcriptional factor STAT\textsuperscript{6,8,9}. IL-4RII responds to a different ligand, IL-4 or IL-13, but different signaling potencies and kinetics have been revealed based on the crystal structures\textsuperscript{9}: This IL-4 and IL-13 receptor system plays an important role in the process of allergic inflammation and immune responses to specific pathogens\textsuperscript{6,30}. Hematopoietic cells mostly express IL-4RI, which is mainly responsible for the expansion of Th2 cells, whereas nonhematopoietic cells predominantly express IL-4RII\textsuperscript{10}. IL-4 exerts a pro-inflammatory effect in endothelial cells\textsuperscript{11} and vascular smooth muscle cells (SMC)\textsuperscript{12} and a profibrotic effect in fibroblasts\textsuperscript{12}. Taken together, these data suggest that IL-4 would be a potential double-edged sword in inflammatory disease processes, based on the distinct expression patterns of the IL-4R complex and cytokine sensitivities in the various cell types\textsuperscript{10,11}.

Several IL-4 antagonists have been designed by mutating amino acids in the receptor subunit binding sites\textsuperscript{6,7,9}. Replacing three amino acids (R121, Y124, and S125) that are located at the human IL-4 with aspartate has a powerful effect, each resulting in the generation of a partial IL-4 agonist or antagonist\textsuperscript{17}. The double mutant IL-4/R121D/Y124D and its mouse analogue of IL-4/Q116D/Y119D have been shown to abolish detectable IL-4 biological activity without the loss of receptor binding affinity\textsuperscript{6,7,30}. Administration of a murine IL-4 mutein with a CI18 deletion, which is analogous to human IL-4/Y124D and to IL-4/Q116D/Y119D, prevents ovalbumin-induced airway hyperresponsiveness in-vivo\textsuperscript{30,31}. Intraperitoneal injection of the plasmid expressing IL-4/Q116D/Y119D also controls Th2-type chronic dermatitis in mice\textsuperscript{32}. Moreover, pitakrin, a human recombinant protein that acts as a dual IL-4/IL-13 antagonist has been developed as a potential medication for asthma and eczema\textsuperscript{33}. To avoid the potential pro-inflammatory property of IL-4RII-mediated signaling\textsuperscript{11,15}, an immune cell-selective IL-4 agonist has also been proposed\textsuperscript{34}. Human IL-4/R121E retains physical interaction with \(\gamma\)c but not with IL-13R\(\alpha\); thus this mutein exhibits complete activity for lymphocytes without the induction of vascular cell adhesion protein-1, monocyte chemotactic protein-1, and IL-6 in endothelial cells\textsuperscript{11}. Thus, it could be anticipated that the replacement of the Q116 residue of murine IL-4 with charged polar amino acids will generate an IL-4 mutein analogous to human IL-4/R121E, which would be expected to act as a selective agonist for IL-4R\textsuperscript{12,35}.

Taken together, it is worth to investigating the receptor-selective cell responses against IL-4 in macrophages and in various disease processes. In the present study, we selectively activated IL-4R isoforms in two different mouse macrophage cell lines, Mm1 and J774.1 using IL-4/Q116E, a promising IL-4RI-selective agonist and IL-13, an IL-4RII agonist. The immunomodulatory effects of IL-4/Q116E were also tested and compared to those of wild-type IL-4 (IL-4WT) or IL-4/Q116D/Y119D, a dominant negative mutein.
MATERIALS and METHODS

Reagents

Dulbecco’s modified Eagle’s medium (DMEM, 1 g/l glucose) containing sodium pyruvate (1 mM), L-glutamine (4 mM), and sodium bicarbonate was purchased from Nacalai Tesque (Kyoto, Japan). Gibco-brand penicillin and streptomycin, fungizone, and trypsin-EDTA were purchased from Life Technologies (Carlsbad, CA). Fetal calf serum (FCS) was obtained from Nichirei Bioscience Inc. (Tokyo, Japan). Recombinant murine IL-4 (rIL-4), human IL-4 (rhIL-4), human IL-13 (rIL-13) and murine INF-β were purchased from PeproTech Inc. (Rocky Hill, NJ). Kaempferol and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture

Murine Mm1, a macrophage cell line established from bone marrow-derived myeloid leukemia and J774.1, a macrophage cell line established from reticulum cell sarcoma ascites were obtained from JCRB (Japanese Collection of Research Bioresources, Osaka, Japan). Macrophages were maintained using DMEM (1 g/l of glucose) containing 10% FCS. All cells were cultured at 37 °C in a humidified 5% CO₂-95% air mixture.

Murine IL-4 muteins and in-vitro transfection

Murine IL-4s, (IL-4WT, IL-4/Q116E, and IL-4/Q116D/Y119D) sub-cloned into the mammalian expression vector, pcDNA3.1(+) (Life Technologies, Carlsbad, CA) containing a CMV promoter/enhancer were generous gift from Dr. Kazushige Sugama (Osteopharma Inc., Osaka, Japan). Transfection was performed with lipofectamine2000 (Life Technologies) according to the manufacturer’s instructions. IL-4 protein secretion was measured using enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN). In the present study, IL-4 levels in un-stimulated or empty-vector transfected controls were undetectable. In murine macrophages transfected with IL-4s, however, 1-3 ng/ml of secreted IL-4 was consistently detected in each in-vitro experiment (data not shown).

In-vitro analysis study protocol

Anchorage-dependent Mm1 and J774.1 cells were transfected with the plasmid expressing IL-4s and were then cultured for 48 hours with normal growth media. The conditioned media were harvested, centrifuged at 15,000 rpm for 5 minutes and stored at -80 °C for future use. Comparable IL-4s concentrations in the conditioned media were confirmed by ELISA. To test STAT6 phosphorylation, freshly seeded cells were stimulated with conditioned medium obtained from transfected cells of the same kind, and cell lysates were collected one hour after stimulation.

RNA isolation and conventional or real-time polymerase chain reaction

Total RNA was isolated using Trizol (Life Technologies) and 1 μg of extracted RNA was exposed to PrimeScript II reverse transcriptase (Takara Bio Inc., Otsu, Japan) in a total volume of 20 μl, according to the manufacturer’s instructions. One microliter of reverse transcriptase reaction (RT) mixture was subjected to conventional polymerase chain reaction (PCR) using a Program Temperature Control System (model PC818, ASTEC, Fukuoka, Japan). Complementary DNAs

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<th>Anti-sense primer (5’ to 3’)</th>
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were amplified using AmpliTaq Gold PCR Master Mix (Life Technologies). PCR product was applied to a 1% agarose gel, electrophoresed, and visualized using a UV transilluminator. Real-time PCR was performed using Fast SYBR-Green fluorescence dye (Applied Biosystems) and a StepOne real-time PCR system (Applied Biosystems). Amplification reactions were performed in duplicate and fluorescence curves were analyzed using the accompanying software. All PCR results were normalized to β-actin expression. PCR primer sets used in the present study are listed in Table 1.

**Western blotting**

Whole cell extracts were obtained in RIPA buffer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and were then subjected to the Quick Start Bradford Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Twenty micrograms of whole cell extract was subsequently resolved in 8% sodium dodeyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), then electronically transferred to PVDF membranes (ImmobilonTM, Millipore, Billerica, MA). The membranes were probed with a 1:200 dilution of a rabbit polyclonal anti-signal transducer and activator of transcription 6 (STAT6, Cell Signaling Technology, Danvers, MA), a rabbit polyclonal anti-phospho-STAT6 Tyr641 (Cell Signaling Technology) or a rabbit polyclonal anti-inducible nitric oxide synthase (iNOS) (Thermo Fisher Scientific Inc., Waltham, MA) followed by incubation with peroxidase-conjugated secondary antibodies. The proteins were subsequently developed using ImmunoStar LD reagents (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and visualized with a luminescent imager (EZ-Capture, ATTO Co., Tokyo, Japan). Alternatively, some blots were incubated with Restore PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific) and re-probed with a 1:2000 dilution of anti-β -actin antibody (AC-15, Sigma-Aldrich).

**Statistical analysis**

All data are presented as the mean ± SD. Mean values were compared using an ANOVA and unpaired Student’s t-test. Probabilities less than 0.05 were considered to be statistically significant.

**RESULTS**

**IL-4R-selective mulelin, IL-4/Q116E, induced STAT6 activation in Mm1 but not in J774.1 cells**

Macrophages are highly heterogeneous cells that represent diverse functions in disease processes28. and thus we tested two cell lines, Mm1 and J774.1, which were established from different tissue origins. In Mm1 cells, rIL-4 (1 ng/ml) and IL-4/WT as well as IL-4/Q116E-induced STAT6 activation (Fig. 1A). IL-4/Q116D/Y119D completely inhibited IL-4-induced STAT6 activation (Fig. 1B). In J774.1 cells, rIL-4 and IL-4/WT, but not IL-4/Q116E, induced STAT6 activation (Fig. 2A). IL-4/Q116D/Y119D also inhibited IL-4-induced STAT6 activation (Fig. 2B). Reportedly, kinase activity of the IL-4 receptor system is dependent on Janus kinases (JAKs) activation; IL-4Rα associates with JAK1 and JAK3, whereas IL-13Rα1 interacts with either JAK2 or tyrosine kinase 2 (Tyk2)29. To confirm the agonistic effect of IL-4/Q116E on JAK3-coupled IL-4R, we further tested the effect of kaempferol, a specific JAK3 inhibitor, on STAT6 activation in IL-4/Q116E-stimulated cells20. As shown in Fig. 3, pretreatment with 40 μM of kaempferol inhibited STAT6 activation induced by IL-4/Q116E in Mm1 cells.

**IL-4/Q116E did not induce an anti-inflammatory response in mouse macrophages**

LPS-induced tumor necrosis factor-α (TNF-α) induction was then examined using real-time PCR. In Mm1 cells, LPS-induced TNF-α induction was inhibited by rIL-4 and IL-4/WT, but neither IL-4/Q116E nor IL-4/Q116D/Y119D had an inhibitory effect on TNF-α induction (Fig. 4A). LPS plus INF-α stimulated iNOS induction in the Mm1 cells20, which was suppressed by rIL-4 and IL-4/WT. IL-4/Q116E or IL-4/Q116D/Y119D had no effect on iNOS induction (Fig. 4B). In J774.1 cells, rIL-4 and IL-4/WT inhibited LPS-induced TNF-α induction, but IL-4/Q116E had no effect, similar to that seen in Mm1 cells (Fig. 5A). LPS plus INF-α induced iNOS expression, which was also suppressed by rIL-4 and IL-4/WT but not by IL-4/Q116E or IL-4/Q116D/Y119D (Fig. 5B).
**Fig. 1** Effect of IL-4 muteins on STAT6 activation in Mm1 cells. The cells were transfected with pcDNA3 (empty vector) or the plasmid expressing IL-4/WT, IL-4/Q116E, or IL-4/Q116D/Y119D. Freshly seeded cells were stimulated for 1 hour with the supernatant of transfected cells, serum free medium (-), and/or rIL-4 (1 ng/ml). STAT6 activation was measured using western blotting. Hela cells (American Type Culture Collection) were also stimulated for 1 hour with rIL-4 (1 ng/ml) and the lysate was used as the positive control.

**Fig. 2** Effect of IL-4 muteins on STAT6 activation in J774.1 cells. The cells were transfected with pcDNA3 (empty vector) or the plasmid expressing IL-4/WT, IL-4/Q116E, or IL-4/Q116D/Y119D. Freshly seeded cells were stimulated for 1 hour with the supernatant of transfected cells, serum free medium (-), and/or rIL-4 (1 ng/ml). STAT6 activation was measured using western blotting. Hela cells (American Type Culture Collection) were also stimulated for 1 hour with rIL-4 (1 ng/ml) and the lysate was used as the positive control.

**Fig. 3** Effects of kaempferol on IL-4/Q116E-induced STAT6 activation. The cells (Mm1 and J774.1) were stimulated for 1 hour with the supernatant of IL-4/Q116E-transfected cells in the presence (-) or absence (-) of 40 μM kaempferol. STAT6 activation was measured using western blotting.

**Fig. 4** Effect of IL-4 muteins on LPS-induced TNF-α expression and LPS plus INF-γ-induced iNOS expression in Mm1 cells. Mm1 cells were stimulated for 1 hour with the supernatant of pcDNA3 (empty vector) or the plasmid expressing IL-4/WT, IL-4/Q116E, or IL-4/Q116D/Y119D-transfected cells and were then incubated for 2 hours with LPS (10 ng/ml) in the presence or absence of rIL-4 (1 ng/ml). Real-time PCR was performed and TNF-α mRNA expression levels were normalized to β-actin mRNA. Data are expressed as the mean ± SD (n=3). **P<0.01; ns: not statistically significant against the control (A). Mm1 cells were stimulated for 24 hours with LPS (10 ng/ml) plus INF-γ (2 ng/ml) in the presence or absence of rIL-4 (1 ng/ml). Western blotting for iNOS and control β-actin was performed (B; left panel). The cells were transfected with pcDNA3 (empty vector) or the plasmid expressing IL-4s and were then stimulated for 24 hours with LPS (10 ng/ml) plus INF-γ (2 ng/ml). Western blotting for iNOS and control β-actin was performed (B; right panel).
**IL-13 induced anti-inflammatory response in mouse macrophages**

IL-4RII-mediated anti-inflammatory responses in macrophages were further tested using rIL-13, an IL-4RII agonist. In both macrophage cell lines, rIL-13 inhibited LPS-induced TNF-α expression and LPS plus INF-α-induced iNOS expression (Fig. 6 and 7).

**DISCUSSION**

We initially used conventional PCR to screen the mRNA expression of IL-4R components such as c, IL-4R c, and IL-13R c in the macrophages used in the present study. We found that all possible components of IL-4Rs were expressed, at least at the mRNA level (data not shown). However, based on the pattern of STAT6 activation, Mm1 cells were found to express both IL-4RI and IL-4RII, whereas J774.1 cells were found to express only IL-4RII (Fig. 1 and 2). Similarly, in human macrophage cell lines, it is known that U937 cells, established from pleural effusions of histiocytic lymphoma express both IL-4RI and IL-4RII whereas THP1 cells established from peripheral blood of acute monocytic leukemia express only IL-4RI(10–12). The effect of IL-4/Q116E was confirmed using kaempferol, a JAK 3 selective inhibitor (Fig. 3), and we showed for the first time that a murine IL-4 mutein, IL-4/Q116E, was an IL-4RI-selective agonist similarly to human IL-4/R121E(14,25). The hypothetical pathway of IL-4RII, but not IL-4RI, mediated anti-inflammatory responses in macrophages tested in our study is illustrated in Fig. 8. Moreover, we also confirmed the successful induction of IL-4/WT and IL-4/Q116D/Y119D, which exerted activity as a wild type or a dominant-negative IL-4, respectively (Fig. 1, 2, 4 and 5). These IL-4 constructs may be promising tools to investigate IL-4 signaling in mouse models.

In Mm1 and J774.1 cells, the observed anti-inflammatory effects were thought to be mediated via IL-4RII because IL-4/Q116E did not inhibit inflammatory responses (Fig. 4, 5 and 8) and also IL-13 exerted effects similar to those of rIL-4 and IL-4/WT (Fig. 6 and 7). Irrespective of differences in the IL-4R expression pattern, IL-4/Q116E did not inhibit LPS-induced inflammatory responses in either cell type (Fig. 4 and 5). Consistently, IL-4 has been shown to up-regulate major histocompatibility class II molecules and to inhibit iNOS production in peritoneal macrophages isolated from c knockout mice(10). Reportedly, human IL-4/R121E, which is analogous to mouse IL-4/Q116E inhibits LPS-induced TNF-α secretion in freshly isolated peripheral blood mononuclear cells(19); however this finding may not conflict with our data because the IL-4 response in a mixed population of human blood cells is not expected to be identical to the response in murine macrophage cell lines. A study using IL-13R c knockout mice showed that IL-4RII plays essential roles in

**Fig. 5 Effect of IL-4 muteins on LPS-induced TNF-α expression and LPS plus INF-γ-induced iNOS expression in J774.1 cells.** J774.1 cells were stimulated for 1 hour with the supernatant of pcDNA3 (empty vector) or the plasmid expressing IL-4/WT, IL-4/Q116E, or IL-4/Q116D/Y119D-transfected cells and were then incubated for 2 hours with LPS (10ng/ml) in the presence or absence of rIL-4 (1 ng/ml). Real-time PCR was performed and TNF-α mRNA expression levels were normalized to β-actin mRNA. Data are expressed as the mean ± SD (n=3). *P<0.05; **P<0.01; ns: not statistically significant against the control (A). J774.1 cells were stimulated for 21 hours with LPS (10 ng/ml) plus INF-γ (2 ng/ml) in the presence or absence of rIL-4 (1 ng/ml). Western blotting for iNOS and control β-actin was performed (B; left panel). The cells were transfected with pcDNA3 (empty vector) or the plasmid expressing IL-4s and were then stimulated for 24 hours with LPS (10 ng/ml) plus INF-γ (2 ng/ml). Western blotting for iNOS and control β-actin was performed (B; right panel).
airway hypersensitivity and mucus hypersecretion but not in fibroblast or alternative macrophage activation, suggesting that IL-4RII-mediated signaling does not always exert anti-inflammatory activity. IL-4RI-mediated signaling is responsible for inducing arginase 1, FIZZ1, and Ym1 via selective activation of insulin receptor substrate-2 (IRS-2) in murine bone marrow-derived macrophages; this pathway has also been implicated in allergic inflammation. Thus further research is needed to clarify the distinct roles of IL-4RI- and IL-4RII-mediated signaling in the regulation of macrophage phenotypes and in various inflammatory processes.

In the present study, we used IL-13 as an IL-4RII agonist. However, IL-13 is also recognized with high affinity by the IL-13Rα2 receptor. IL-13Rα2 receptor does not have a functional intracellular domain and thus it acts as a decoy receptor for negative regulation. The role of the IL-13Rα2 receptor in the regulation of M1 and M2 macrophage inflammatory activation remains to be determined. Although IL-4/QI16E muatein, a selective IL-4RI agonist, did not show an anti-inflammatory effect in macrophages, this muatein is expected to stimulate Th2 deviation in lymphocytes via IL-4RI without IL-4RII-mediated inflammatory activation in some cell types. Further study using different cell types and also in vivo disease models are required to establish potential therapeutics based on IL-4 signal modulation.

In conclusion, the IL-4-mediated anti-inflammatory effect was thought to largely depend on the IL-4RII-mediated signaling in murine M1 and M2 macrophages irrespective of the presence or absence of IL-4RI. Thus we could further conclude that IL-4 signaling plays a pivotal role.
Fig. 8 Hypothetical pathway of IL-4RII, but not IL-4RI, mediated anti-inflammatory responses in tested macrophages. IL-4/Q116E acts as an IL-4RI-specific agonist without activation of IL-4RII. IL-13 was used as the IL-4RII agonist. The transcriptional factor STAT6 and kinases coupled with IL-4RI (JAK1 and JAK3) and IL-4RII (JAK1, JAK2 and Tyk2) are illustrated. Mm-1 cells expressed both IL-4RI and IL-4RII. J774.1 cells expressed only IL-4RII. An anti-inflammatory response was observed downstream of IL-4RII in both macrophages.

role in the regulation of macrophage inflammatory properties. Manipulating IL-4-mediated anti-inflammatory signaling in macrophages may be beneficial for understanding the disease process and for the development of therapeutics.

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