The Interleukin-13 Receptor- α 1 Chain Is Essential for Induction of the Alternative Macrophage Activation Pathway by IL-13 but Not IL-4

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Key Words

Alternative macrophage activation · Arginase · Interleukin-4 · Interleukin-13 · Interleukin-13 receptor- α 1 · STAT6

Abstract

Macrophages coexpress both the interleukin (IL)-2R γ chain (γ_c) and IL-13R α 1. These receptor chains can heterodimerize with IL-4R α to form type I or type II IL-4 receptor complexes, respectively. We used macrophages derived from *Il2rg* and *Il13ra1* knockout (KO) mice to evaluate the requirements for these receptor chains for induction of the alternative macrophage activation (AMA) pathway by IL-4 and IL-13. Absence of γ_c significantly decreased activation of STAT6 by IL-4 but not IL-13. However, although activation of STAT6 by IL-4 was markedly reduced in γ_c KO macrophages, it was not abolished, indicating that IL-4 can still signal through type II IL-4 receptors via the IL-13R α 1 chain. IL-13 failed to activate STAT6 in macrophages derived from *Il13ra1* KO mice; however, these cells remained fully responsive to IL-4. The inability of IL-13 but not IL-4 to signal in *Il13ra1*^{-/-} macrophages correlated with the inability of IL-13 but not IL-4 to induce expression of genes such as *Arg1*, *Retnla* and *Ccl11* that are characteristically expressed by alternatively activated macrophages. In addition, IL-13 but not IL-4 failed to induce membrane fusion and giant cell formation by *Il13ra1* KO

macrophages. These findings demonstrate that the IL-13R α 1 chain is essential for induction of the AMA pathway by IL-13 but not IL-4.

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Introduction

T cell-derived cytokines such as interferon- γ (IFN- γ) and interleukin (IL)-4 induce distinct gene expression profiles and distinct activation states in macrophages by differentially activating the transcription factors STAT1 and STAT6, respectively. The classical macrophage activation pathway is activated by the Th1-type cytokine, IFN- γ [1, 2]. Signaling through IFN- γ receptors on macrophages induces activation of STAT1 and expression of STAT1-responsive genes such as *Nos2* (inducible nitric oxide synthetase, iNOS), *Cxcl9* (Mig) and *Cxcl10* (IP-10) [3]. The alternative macrophage activation (AMA) pathway was originally defined to distinguish the phenotype of IL-4-treated macrophages from that of classically activated macrophages induced by treatment with IFN- γ [4]. Signaling through IL-4 receptors on macrophages induces activation of STAT6 and expression of STAT6-responsive genes such as *Arg1* (arginase I), *Retnla* (Fizz1) and *Ccl11* (eotaxin) [5–7]. Alternatively activated macro-

phages also exhibit elevated expression of certain cell surface markers, including mannose receptor C type 1 (CD206) and dectin-1 (*Clec7a*) [4, 8, 9]. In addition to IL-4, it was subsequently found that the AMA pathway can also be activated by IL-13 [10, 11].

IL-4 and IL-13 induce functional responses by binding to specific cell surface receptors and activating a signal transduction cascade that results in rapid activation of latent cytosolic STAT6 [12, 13]. Once activated, STAT6 translocates from the cytosol to the nucleus, and binds to STAT-binding elements (SBE) in the promoters of various STAT6-responsive genes. SBEs typically contain the core nucleotide sequence, TTCNNGAA, where NNN can be any nucleotide. However, unlike most SBEs that contain an N3-type spacer between the palindromic TTC and GAA nucleotides, STAT6-binding elements frequently contain N4-type spacers [14, 15]. As a consequence, the promoter regions of IL-4/IL-13-responsive genes usually contain N4-type SBEs that have a high affinity for STAT6. In contrast, the promoter regions of IFN- γ -responsive genes usually contain N3-type SBEs that have a much higher affinity for STAT1 than STAT6. This difference explains, at least in part, why the transcriptional signature induced by IL-4 and IL-13 is so distinct from the gene signature induced by IFN- γ .

Activation of STAT6 is critical for induction of the AMA pathway by both IL-4 and IL-13 [16–18]. Although both IL-4 and IL-13 can induce AMA, IL-4 is generally more potent than IL-13 in terms of its ability to induce activation of STAT6 and gene expression in both murine macrophages and human monocytes [19, 20]. The molecular basis for the greater potency of IL-4 versus IL-13 in macrophages is not completely understood, but likely results, at least in part, from the overall higher affinity of type I IL-4 receptors for IL-4 versus the lower affinity of type II IL-4 receptors for IL-13. In this study, we used bone marrow-derived macrophages from mice with targeted deletions of the genes for the IL-4R α chain (*Il4ra*), IL-2R γ (*Il2rg*) or IL-13R α 1 (*Il13ra1*) to define the requirements for these receptor chains in the activation of STAT6 and induction of alternatively activated macrophages by IL-4 and IL-13.

Materials and Methods

Culture Medium and Reagents

The complete medium used for culturing macrophages consisted of RPMI-1640 medium (Life Technologies, Grand Island, N.Y., USA) supplemented with 10% FBS (HyClone, Logan, Utah, USA), 2 mM L-glutamine and 50 μ g/ml gentamycin. Recombinant murine IL-4, IL-13 and IFN- γ were obtained from R&D Systems

Inc. (Minneapolis, Minn., USA). The rabbit polyclonal anti-phospho-(Tyr⁶⁴¹)-STAT6 antibody (catalog No. 9361) was obtained from Cell Signaling Technology (Danvers, Mass., USA), and the rabbit polyclonal anti-STAT6 antibody (catalog No. sc-621) was obtained from Santa Cruz Biotechnology Inc. (Dallas, Tex., USA).

Cells

Bone marrow-derived macrophage cultures were generated as described previously [21] from bone marrow aspirates extracted from the femurs of wild-type BALB/c or C57BL/6 mice (Taconic Farms, Germantown, N.Y., USA) or from gene knockout (KO) mice, including *Il4ra*^{-/-} [22], *Il2rg*^{-/-} [23] or *Il13ra1*^{-/-} [24] mice. The cells were cultured at 1×10^6 cells/ml in complete RPMI-1640 medium containing 50 ng/ml of recombinant murine M-CSF (R&D Systems Inc.) for 7–10 days at 37°C. After that time, the M-CSF was washed off the cultures and the cells were treated with recombinant murine IL-4, IL-13 and/or IFN- γ , as indicated in the text.

The methods used to generate primary mouse lung fibroblast cultures have been described previously [24]. Briefly, the lungs from wild-type or *Il13ra1* KO mice were harvested and then perfused with 10 ml of HBSS containing 2 mg/ml of collagenase D (Roche Diagnostics, Indianapolis, Ind., USA), excised, minced and incubated with 2 mg/ml of collagenase in DMEM for 45 min at 37°C. The enzymatically digested tissue was subsequently forced through a 100- μ m nylon sieve and the cells were plated on 100-mm petri dishes containing Iscove's media supplemented with 5% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin, and incubated for 2 weeks at 37°C. The resulting fibroblast monolayers were harvested and plated onto fresh 100-mm petri dishes, and allowed to expand for an additional 7 days prior to use.

Electrophoretic Mobility Shift Assays

Nuclear protein extracts were prepared from cells after treatment with IL-4 or IL-13 as previously described [25]. A double-stranded oligonucleotide, based on a DNA sequence in the promoter of the human IL-1 receptor antagonist gene *IL1RN*, was used as a probe for STAT6 in the gel shift assays [26]. This oligonucleotide contains SBE1, which has a high affinity for STAT6 complexes. The GAS probe used to measure STAT1 activity was derived from a sequence in the promoter of the human IgG Fc γ R1 gene, *FCGR1a* [27]. These probes were end-labeled with [γ -³²P]-ATP using T4 polynucleotide kinase, and binding reactions were performed as described previously [25]. A portion of each binding reaction mixture (8 μ l per sample) was electrophoresed on non-denaturing, 6% polyacrylamide gels (Invitrogen, Carlsbad, Calif., USA) using 0.25 $\times$ Tris-borate-ethylenediamine-N,N,N',N'-tetraacetic acid buffer (22 mM Tris-HCl, pH 8.0, 22 mM borate and 0.5 mM ethylenediamine-N,N,N',N'-tetraacetic acid). The gels were subsequently dried and visualized by autoradiography.

Western Blots

The levels of tyrosine-phosphorylated STAT6 (pY-STAT6) were measured by immunoblotting as described previously [28]. After treatment with recombinant IL-4, IL-13 or IFN- γ for 30 min at 37°C, the cells were washed three times with Dulbecco's PBS and whole cell protein lysates were prepared. Total STAT6 protein was immunoprecipitated with rabbit anti-STAT6 Ab (sc-621; Santa Cruz Biotechnology). Immunoprecipitated proteins were resolved by electrophoresis on 8% SDS-PAGE gels (Invitrogen) and then transferred to polyvinylidene difluoride membranes. The levels of

pY-STAT6 and total STAT6 were visualized by enhanced chemiluminescence using a rabbit antibody specific for Tyr⁶⁴¹-phosphorylated STAT6 (catalog No. 9361; Cell Signaling Technology) or rabbit anti-STAT6 Ab, respectively.

Quantitative Real-Time PCR

Changes in gene expression were measured by qRT-PCR analyses of individual ISGs. Total RNA was isolated from macrophages using RNazol B (Tel-Test, Friendswood, Tex., USA) by the acid/guanidinium thiocyanate/phenol/chloroform extraction method, as described previously [25]. The RNA samples were DNase treated to remove any residual genomic DNA using RT² qPCR-grade RNA isolation kits from Qiagen Inc. (Valencia, Calif., USA). One microgram of purified RNA from each treatment group was used as a template for synthesis of first-strand cDNAs using Reaction-Ready First Strand cDNA synthesis kits from Qiagen. Specific primer assays for selected ISGs were obtained from Qiagen and analyzed on the Mx3000P system (Agilent Technologies, Santa Clara, Calif., USA). PCR amplification was performed by thermal cycling using SuperArray RT² Real-Time SYBR Green PCR Master Mix with real-time detection by SYBR Green and 5-carboxy-X-rhodamine (ROX) dyes on the Mx3000P instrument according to the manufacturer's instructions.

Changes in gene expression levels were analyzed using MxPro software v.4.10 (Agilent), and the results are expressed as the mean fold increase relative to the control levels after normalization to the housekeeping gene GAPDH. Changes in the relative levels of gene expression in treated versus nontreated control cells were calculated using the $2^{-\Delta\Delta C_T}$ method as described previously [29]. Graphing and statistical analysis of qPCR results were performed using Prism 5.0 (Graph Pad Software, San Diego, Calif., USA). The data were analyzed using a two-tailed Student t test. Values represent the mean $\pm$ SD of triplicate determinations. p values <0.05 were considered statistically significant. All experiments were repeated at least three times with similar results.

Cell Fusion Assays

The cell fusion assays were performed as described previously [9]. Briefly, bone marrow-derived macrophages were resuspended in OptiMEM-10 medium and plated on Permanox plastic slides at 1×10^5 cells/well. IL-4 or IL-13 (100 ng/ml) were added and the cultures were incubated for 48 h. The slides were stained using the Hemacolor staining kit (EMD Millipore), and photographs were taken using a Nikon Coolscope slide scanner. Four to eight independent images per well were acquired, and the number of giant and single cell nuclei were counted. Percent fusion values were determined based on the number of giant cell nuclei (>2 nuclei) divided by the number of total nuclei.

Results

The IL-4R α Chain Is Required for Activation of STAT6 by both IL-4 and IL-13

To determine if the IL-4R α chain is required for signaling by both IL-4 and IL-13 in macrophages, we examined the ability of IL-4 and IL-13 to induce activation of STAT6 in macrophages derived from control (wild-type) and IL-

Fig. 1. The IL-4R α chain is required for induction of STAT6 activity by both IL-4 and IL-13. **a** Bone marrow-derived macrophages from control (wild-type) and IL-4R α chain KO (*Il4ra*^{-/-}) mice (BALB/c) background) were treated with IL-4, IL-13 or IFN- γ (20 ng/ml) for 30 min at 37°C. Nuclear protein extracts were then prepared and analyzed by EMSA for DNA-binding activity using a radiolabeled oligonucleotide N4-type GAS probe (SBE1) that preferentially binds activated STAT6 (upper panel) and for STAT1 activity using an N3-type GAS probe (GRR) that has a high affinity for STAT1 (lower panel). **b** Wild-type macrophages were pretreated or not with the anti-IL-4R α mAb, M1, for 30 min at 37°C. IL-4 or IL-13 (20 ng/ml) was then added, and the cultures were incubated for an additional 30 min. Nuclear protein extracts were then prepared and analyzed by EMSA for STAT6-binding activity using the SBE1 probe.

4R α gene KO (*Il4ra*^{-/-}) mice [22]. Bone marrow-derived macrophages from wild-type or *Il4ra*^{-/-} mice were treated with recombinant murine IL-4, IL-13 or IFN- γ (20 ng/ml) for 30 min at 37°C. Nuclear protein extracts were then prepared and analyzed by electrophoretic mobility shift assays (EMSA) for DNA-binding activity using an N4-type GAS probe (SBE1) that preferentially binds activated STAT6 [26]. As shown in figure 1a, both IL-4 and

Fig. 2. Activation of STAT6 by IL-4 is diminished but not abrogated in γ_c -deficient macrophages. **a** Bone marrow-derived macrophages from control (wild-type) and γ_c KO (*Il2rg*^{-/-}) mice (C57BL/6J background) were treated with log₁₀ concentrations of IL-4 ranging from 1,000 to 0.1 ng/ml for 30 min at 37°C. An additional set of bone marrow-derived macrophages from γ_c KO mice were treated with IL-4 in the presence of the neutralizing anti-IL-4R α 1 mAb, M1 (lanes 13–18). Nuclear protein extracts were then prepared and the levels of activated STAT6 were measured by EMSA using a radiolabeled SBE1 probe. **b** Another set of macrophages derived from the same mice were incubated with IL-13 (1,000–10 ng/ml) for 30 min at 37°C (lower panel). Nuclear protein extracts were then prepared and analyzed for STAT6 activity by EMSA using the SBE1 probe.

IL-13 induced activation of STAT6 in wild-type macrophages; however, neither IL-4 nor IL-13 induced STAT6 activity in IL-4R α chain-deficient macrophages. IL-4 induced higher levels of STAT6 DNA-binding activity than IL-13 even though the cells were treated with equivalent concentrations of each cytokine. The inability of IL-4 and IL-13 to induce activation of STAT6 in IL-4R α -null macrophages did not reflect a global inability of these cells to respond to cytokines because treatment with IFN- γ induced equivalent levels of STAT1 activation in wild-type and *Il4ra*^{-/-} macrophages.

To confirm the essential role of the IL-4R α chain for productive signaling by IL-4 and IL-13, we treated normal (wild-type) bone marrow-derived macrophages with IL-4 or IL-13 in the presence or absence of a blocking monoclonal antibody (M1) to the murine IL-4R α chain [30]. As shown in figure 1b, the M1 mAb completely blocked activation of STAT6 by both IL-4 and IL-13. These results confirmed that the IL-4R α chain is an essential component of the receptor complexes for both IL-4 and IL-13 in macrophages.

Deletion of the Common Gamma Chain Diminishes the Ability of IL-4 but Not IL-13 to Activate STAT6 in Macrophages

Most cell types express either the common gamma chain (γ_c) or the IL-13R α 1 chain, but not both. However, monocytes and macrophages are unique because they co-express γ_c and IL-13R α 1 [19, 20, 31]. To determine if

macrophages require γ_c to mediate responsiveness to IL-4 and/or IL-13, we prepared bone marrow-derived macrophages from control (wild-type) and γ_c -deficient (*Il2rg*^{-/-}) mice [23, 32] and examined their responsiveness to IL-4 and IL-13. As shown in figure 2a, IL-4 induced strong activation of STAT6 in wild-type macrophages (lanes 1–6); however, the induction of STAT6 activity by IL-4 was much weaker but not abrogated in γ_c -deficient macrophages (lanes 7–12). Treatment of γ_c KO macrophages with IL-4 in the presence of the inhibitory anti-IL-4R α mAb (M1) blocked the residual activation of STAT6 by IL-4 in these cells (lanes 13–18). Higher concentrations of IL-4 were required to activate STAT6 in γ_c ^{-/-} macrophages compared to wild-type macrophages. Furthermore, IL-4 appeared to function more like IL-13 in γ_c ^{-/-} macrophages because the IL-4-induced dose response curve in *Il2rg*^{-/-} macrophages was very similar to the IL-13-induced dose response in wild-type macrophages. Unlike the response to IL-4, the absence of γ_c expression did not decrease the ability of IL-13 to activate STAT6 in macrophages (fig. 2b). These findings indicate that IL-4 responsiveness is markedly diminished but not abrogated in γ_c -deficient macrophages, whereas responsiveness to IL-13 is unaffected. The residual STAT6 activity induced by IL-4 in *Il2rg*^{-/-} macrophages results from default signaling through type II IL-4 receptor complexes because these cells still express the IL-13R α 1 chain in the absence of γ_c .

Fig. 3. The IL-13R α 1 chain is required for activation of STAT6 by IL-13 but not IL-4 in macrophages. **a** Macrophages derived from wild-type or *Il13ra1*^{-/-} mice (C57BL/6J background) were treated with IL-4 (1,000–0.1 ng/ml) or IL-13 (1,000–10 ng/ml) for 30 min at 37°C. Nuclear protein extracts were then prepared, and STAT6 activity was measured by EMSA using a radiolabeled SBE1 probe. **b** A companion set of whole cell lysates was also prepared from wild-type and *Il13ra1*^{-/-} macrophages, and the levels of tyrosine-phosphorylated STAT6 were measured by Western blotting with rabbit anti-phospho-STAT6 (Tyr⁶⁴¹) antibody. IP = Immunoprecipitation.

The IL-13R α 1 Chain Is Required for Activation of STAT6 by IL-13 but Not IL-4 in Macrophages

Both IL-4 and IL-13 can signal through type II IL-4 receptor complexes in nonhematologic cell types such as fibroblasts and endothelial cells [33, 34]; however, it is not clear if the IL-13R α 1 chain is required for signaling by both IL-4 and IL-13 in macrophages. To define the importance of the IL-13R α 1 chain in IL-4/IL-13 signaling by macrophages, we prepared bone marrow-derived macrophages from control wild-type and *Il13ra1*^{-/-} mice [24, 35], and measured their responsiveness to IL-4 and IL-13. As shown in figure 3a, IL-4 induced equivalent levels of STAT6 activity in wild-type and IL-13R α 1-deficient mac-

rophages. However, although IL-13 induced activation of STAT6 in wild-type macrophages, the ability of IL-13 to activate STAT6 was abrogated in *Il13ra1*^{-/-} macrophages. Similar results were obtained when we measured the levels of tyrosine-phosphorylated STAT6 by Western blotting (fig. 3b). These findings demonstrate that the IL-13R α 1 chain is essential for IL-13 signaling but not for IL-4 signaling in macrophages.

To determine if IL-4 and IL-13 induce similar responses in peritoneal macrophages, we obtained peritoneal macrophages from wild-type and *Il13ra1* KO mice and examined their responsiveness to IL-4 and IL-13. Similar to the findings with bone marrow-derived macrophages,

Fig. 4. The IL-13Rα1 chain is required for induction of STAT6-responsive genes by IL-13 but not IL-4 in macrophages. Bone marrow-derived macrophages from wild-type or *Il13ra1*^{-/-} mice were incubated with medium alone (control), IL-4 (10 ng/ml), IL-13 (100 ng/ml) or IFN-γ (10 ng/ml) for 6 h at 37°C. At the end of this incubation period, RNA extracts were prepared and analyzed by

quantitative RT-PCR to measure expression levels of several IL-4-responsive genes, including *Arg1* (arginase-1; **a**), *Retnla* (Fizz1, Relm-α; **b**), *Ccl11* (eotaxin; **c**), *Clec7a* (dectin-1, β-glucan receptor; **d**), *Nfil3* (E4BP4; **e**) and *Socs1* (SOCS-1; **f**). The gene expression levels were determined relative to the housekeeping gene *Gapdh* and represent the mean of triplicate determinations.

both IL-4 and IL-13 induced activation of STAT6 in wild-type peritoneal macrophages, whereas only IL-4 induced activation of STAT6 in peritoneal macrophages derived from *Il13ra1* KO mice (online suppl. fig. 1; see www.karger.com/doi/10.1159/000376579 for all online suppl. material).

The IL-13Rα1 Chain Is Required for Induction of the AMA Pathway by IL-13 but Not IL-4

Both IL-4 and IL-13 can induce the AMA pathway [4–7]. The phenotype of alternatively activated macrophages is defined in part by expression of a characteristic set of STAT6-responsive genes that includes *Arg1* (arginase-1), *Retnla* (Fizz1), *Ccl11* (eotaxin) and *Clec7a* (dectin-1, β-glucan receptor), among others. To determine if the IL-13Rα1 chain is required for induction of IL-4/IL-13-responsive genes, we treated control wild-type macrophages and IL-13Rα1-null macrophages with IL-4, IL-13 or IFN-γ for 6 h, and then harvested total RNA from these cells to measure the expression levels of several genes that are characteristically expressed by alternatively activated macrophages. As shown in figure 4, both IL-4 and IL-13

induced expression of *Arg1* and *Retnla* in wild-type macrophages; however, only IL-4 induced expression of *Arg1* and *Retnla* in IL-13Rα1-null macrophages. IFN-γ did not induce expression of *Arg1* or *Retnla* in either wild-type or *Il13ra1*^{-/-} macrophages. The inability of IL-13 to upregulate gene expression in IL-13Rα1-deficient macrophages extended to several other genes, including *Ccl11*, *Clec7a* and the transcriptional regulatory genes *Nfil3* (E4BP4) and *Socs1*. Like the *Arg1* and *Retnla* genes, expression of these genes was upregulated by both IL-4 and IL-13 in wild-type macrophages, but only IL-4 upregulated expression of these genes in *Il13ra1*^{-/-} macrophages.

Induction of the AMA pathway by IL-4 or IL-13 is also characterized in part by membrane fusion and the formation of multinucleated giant cells [9, 36, 37]. To determine if the IL-13Rα1 chain is required for membrane fusion and the formation of multinucleated giant cells by IL-4 and/or IL-13, we incubated bone marrow-derived macrophages from control wild-type and *Il13ra1*^{-/-} mice with IL-4 or IL-13 (100 ng/ml) for 48 h, and then measured the percentage of fused cells. As shown in figure 5a, both IL-4 and IL-13 induced membrane fusion and giant cell for-

Fig. 5. Absence of the IL-13Rα1 chain abrogates the ability of IL-13 but not IL-4 to induce macrophage fusion and giant cell formation. Bone marrow-derived macrophages from wild-type and *Il13ra1* KO mice were cultured in the presence or absence of IL-4 or IL-13 (100 ng/ml) for 48 h. The cells were then stained with Hemacolor

solution to facilitate the detection of giant cells (a), and the percentage of fused cells was measured microscopically (b). The results shown represent the mean $\pm$ SD of quadruplicate determinations for each treatment group. ND = Not detectable.

mation by the control wild-type macrophages. However, only IL-4 induced giant cell formation by IL-13Rα1-null macrophages. The percentage of fused cells was quantified microscopically, and the results shown in figure 5b represent the mean $\pm$ SD of quadruplicate determinations for each treatment group. The cell fusion results in figure 5b correlated well with the observed differences in giant cell formation shown in figure 5a. Together, these findings demonstrate that deletion of the IL-13Rα1 chain abrogates the ability of IL-13 but not IL-4 to induce macrophage fusion and giant cell formation.

The IL-13Rα1 Chain Is Required for IL-13 to Inhibit IFN-γ-Induced Signaling and Gene Expression in Macrophages

Previous studies have shown that IL-4 and IL-13 can inhibit classical macrophage activation by IFN-γ [38, 39]. To determine if the IL-13Rα1 chain is required for the ability of IL-4 and/or IL-13 to inhibit signal transduction and gene expression induced by IFN-γ, we pretreated cultures of wild-type or IL-13Rα1-null macrophages overnight (18 h) with IL-4 or IL-13, and then added IFN-γ (10 ng/ml) to the cultures. After incubation for an additional 30 min, nuclear protein extracts were prepared, and the

levels of STAT1 activity were assayed by EMSA using the N3 GAS probe (GRR). As shown in figure 6a, both IL-4 and IL-13 markedly inhibited activation of STAT1 in wild-type macrophages; however, only IL-4 inhibited activation of STAT1 in *Il13ra1*^{-/-} macrophages.

Next, we used a matched set of bone marrow-derived macrophages derived from wild-type and *Il13ra1*^{-/-} mice to determine if the inability of IL-13 to inhibit IFN-γ signaling in IL-13Rα1-null macrophages correlated with an inability to inhibit expression of STAT1-inducible genes. Macrophages derived from wild-type and *Il13ra1* KO mice were pretreated overnight with IL-4 or IL-13, and then IFN-γ (10 ng/ml) was added as indicated. The cultures were incubated for an additional 4 h, and then total RNA extracts were prepared and analyzed by qPCR to measure the levels of *Cxcl9* (Mig) and *Cxcl10* (IP-10) gene expression. As shown in figure 6b, pretreatment with IL-4 or IL-13 markedly suppressed the levels of *Cxcl9* (Mig) and *Cxcl10* (IP-10) gene expression induced by IFN-γ in wild-type macrophages. In contrast, although IL-4 markedly inhibited *Cxcl9* and *Cxcl10* gene expression in IL-13Rα1-null macrophages, IL-13 did not. In separate but related experiments, we also found that IL-4 but not IL-13 markedly inhibited LPS-induced production of IL-12 by

Fig. 6. The IL-13R α 1 chain is required for IL-13 but not IL-4 to inhibit induction of signaling and gene expression by IFN- γ in macrophages. **a** Bone marrow-derived macrophages from control (wild-type) or IL-13R α 1 KO mice were incubated overnight (18 h) with IL-4 (10 ng/ml) or IL-13 (100 ng/ml), and then IFN- γ (10 ng/ml) was added as indicated. After incubation for an additional 30 min, nuclear protein extracts were prepared, and the levels of STAT1 activity were measured by EMSA using a radiolabeled GAS (GRR) probe. **b** A matched set of macrophages derived from wild-type and *Il13ra1*^{-/-} mice were pretreated overnight with IL-4 (10 ng/ml) or IL-13 (100 ng/ml), and then IFN- γ (10 ng/ml) was added as indicated. The cells were incubated for an additional 4 h, and then total RNA extracts were prepared. The levels of *Cxcl9* (Mig) and *Cxcl10* (IP-10) gene expression were measured by qRT-PCR.

Il13ra1 KO macrophages, whereas both IL-4 and IL-13 inhibited IL-12 production by wild-type macrophages (online suppl. fig. 2).

The IL-13R α 1 Chain Is Essential for Signaling by both IL-4 and IL-13 in Fibroblasts

As shown in figure 3, the ability of IL-4 to activate STAT6 was essentially unchanged in *Il13ra1*^{-/-} macrophages because, despite the absence of IL-13R α 1, these cells still express γ_c , and IL-4 can signal effectively via type I IL-4 receptor complexes. In contrast to monocytes and macrophages which coexpress γ_c and IL-13R α 1, nonhematologic cell types, such as fibroblasts and endothelial cells, express IL-13R α 1 but not γ_c [33, 34]. We compared the ability of IL-4 and IL-13 to activate STAT6 in macrophages and fibroblasts derived from wild-type mice and *Il13ra1*^{-/-} mice. The fibroblasts used in these experiments were generated from the lungs of wild-type and *Il13ra1*^{-/-} mice as described previously [24]. As shown in figure 7a, both IL-4 and IL-13 induced activation of STAT6 in wild-type macrophages, but only IL-4 was able to activate STAT6 in *Il13ra1*^{-/-} macrophages. In contrast, although both IL-4 and IL-13 induced activation of STAT6 in wild-type fibroblasts, neither IL-4 nor IL-13 induced activation of STAT6 in *Il13ra1*^{-/-} fibroblasts. Similar results were obtained when we measured the levels of tyrosine-phos-

phorylated STAT6 by immunoblotting with anti-pY-STAT6 Ab (fig. 7b). Both IL-4 and IL-13 induced phosphorylation of STAT6 in wild-type macrophages, but only IL-4 induced phosphorylation of STAT6 in *Il13ra1* KO macrophages. However, unlike in macrophages, neither IL-4 nor IL-13 induced phosphorylation of STAT6 in fibroblasts. These findings demonstrate that the IL-13R α 1 chain is required for signaling by IL-4 in fibroblasts but not in macrophages. However, in the case of IL-13, the IL-13R α 1 chain is essential for signaling by both macrophages and fibroblasts.

Discussion

Our findings demonstrate that expression of the IL-13R α 1 chain is necessary for induction of the AMA pathway by IL-13 but not IL-4. Macrophages express two distinct receptor complexes by which IL-4 can signal to induce the AMA pathway. The first is mediated by signaling through type I IL-4 receptor complexes that consist of the IL-4R α chain and γ_c . The second is activated by signaling through type II IL-4 receptor complexes which are heterodimers composed of the IL-4R α and IL-13R α 1 chains. In γ_c -deficient macrophages, IL-4 can still signal via type II IL-4 receptor complexes because these cells maintain ex-

Fig. 7. The IL-13R α 1 chain is required for activation of STAT6 by IL-4 in fibroblasts but not in macrophages. **a** Macrophages derived from wild-type or *Il13ra1*^{-/-} mice were treated with IL-4, IL-13 or IFN- γ (20 ng/ml) for 30 min at 37°C. Nuclear protein extracts were then prepared and assayed for STAT6 activity by EMSA using a radio-labeled SBE1 probe. Lung fibroblast cultures were also prepared from wild-type and *Il13ra1*^{-/-} mice, and treated with IL-4, IL-13 or IFN- γ in a similar manner. **b** A companion set of whole cell lysates was prepared from wild-type and *Il13ra1*^{-/-} macrophages and fibroblasts, and the levels of tyrosine-phosphorylated STAT6 were measured by Western blotting with rabbit anti-phospho-Tyr⁶⁴¹ STAT6 antibody. IP = Immunoprecipitation; WB = Western blot.

pression of IL-13R α 1. In other words, the binary complex formed by the initial binding of IL-4 to the IL-4R α chain can still recruit the IL-13R α 1 chain if γ_c is not present on the cell membrane. In contrast to IL-4, IL-13 can only signal via type II IL-4 receptor complexes to induce activation of STAT6 and downstream STAT6-responsive gene expression. Interestingly, we observed that the magnitude of STAT6 activation and gene expression induced by IL-4 in wild-type macrophages is generally greater than that induced by IL-13. These results are consistent with related findings by others [19, 20]. However, in γ_c -deficient macrophages, the magnitude of STAT6 activity induced by IL-13 was comparable to that induced by IL-4. This is likely due to the fact that, in the absence of γ_c , both IL-4 and IL-13 can only signal via type II IL-4 receptor complexes.

IL-4 receptor structural studies have shown that IL-4 and IL-13 catalyze differential assembly of IL-4 receptor complexes [40, 41]. IL-4 binds initially to the IL-4R α chain to generate a binary complex which then recruits either γ_c or the IL-13R α 1 chain to form ternary type I or type II IL-4 receptor complexes, respectively. In contrast, the primary ligand-binding chain for IL-13 is IL-13R α 1, not IL-4R α . Consequently, IL-13 binds initially to the IL-13R α 1 chain to generate IL-13/IL-13R α 1 binary complexes which then recruit the IL-4R α chain to complete assembly of ternary type II IL-4 receptor complexes. Therefore, IL-4 and IL-13 induce rapid assembly of type II IL-4 receptor complexes by catalyzing physical association of the same receptor chains (i.e. IL-4R α and IL-13R α 1), but the order of assembly is reversed. Although

the receptor assembly sequences are distinct for IL-4 and IL-13, the intracellular signal transduction pathway and repertoire of genes induced by these two cytokines are largely the same.

We found that IL-13 failed to upregulate expression of several STAT6-inducible genes in IL-13R α 1-deficient macrophages. These genes included *Arg1* (arginase-1), *Retnla* (Fizz1), *Ccl11* (eotaxin-1), *Clec7a* (dectin-1), *Nfil3* (E4BP4) and *Socs1*. Although the ability of IL-4 and IL-13 to induce expression of the *Arg1*, *Retnla*, *Ccl11* and *Clec7a* genes in macrophages is well established, the ability of IL-4 and IL-13 to induce expression of *Nfil3* and *Socs1* has received less attention. We showed previously that IL-4 and IL-13 induce *Socs1* gene expression in both mouse and human macrophages in a STAT6-dependent manner [28]. We also showed that *Socs1* serves an autoregulatory role in macrophages by feedback inhibiting the expression of STAT6-responsive genes such as *Arg1* and *Retnla*. *Socs1* also strongly inhibits classical macrophage activation by IFN- γ [42, 43] and this may explain, at least in part, how IL-4 and IL-13 suppress the induction of STAT1-responsive genes in macrophages [38, 39].

In addition to the inability of IL-13 to induce expression of STAT6-responsive genes in IL-13R α 1-deficient macrophages, IL-13 also failed to induce membrane fusion and giant cell formation in these cells. The ability of IL-4 and IL-13 to induce membrane fusion and giant cell formation by mononuclear phagocytes was first reported more than 2 decades ago [36, 37]. Although the molecular basis of giant cell formation is not yet fully defined, several studies have shown that this process is STAT6 dependent and requires homotypic adhesion [44, 45]. The ability of IL-4 and IL-13 to induce membrane fusion and multinucleated giant cell formation is consistent with the association of giant cell formation with disease states such as asthma and certain helminth infections that are characterized by chronic activation of Th2 and type 2 innate lymphoid cells (ILC2) [46].

The differential responsiveness of macrophages to IL-4 versus IL-13 cannot be attributed to differences in the binding affinities of IL-4 and IL-13 for γ_c versus the IL-13R α 1 chain. LaPorte et al. [40] showed that the K_d values of IL-4/IL-4R α binary complexes for γ_c versus the IL-13R α 1 chain are low but quite similar. In contrast, IL-13 binds initially to the IL-13R α 1 chain to form IL-13R α 1/IL-13 binary complexes which then heterodimerize with the IL-4R α chain to generate type II IL-4 receptor complexes. The overall binding affinity of fully assembled IL-13R α 1/IL-13/IL-4R α ternary complexes is much greater

($K_d \approx 30$ pM) than the affinities of the individual receptor chains.

Macrophages coexpress γ_c and IL-13R α 1, and can respond to both IL-4 and IL-13. However, we found that IL-4 preferentially uses γ_c , not IL-13R α 1, to mediate signal transduction in macrophages, whereas IL-13 uses the IL-13R α 1 chain, not γ_c , to mediate signaling. If γ_c expression is diminished or absent in macrophages, IL-4 can default signal through type II IL-4 receptor complexes because the cells still express the IL-13R α 1 chain. Our studies demonstrate that the IL-13R α 1 chain is dispensable in the case of the ability of IL-4 to induce the AMA pathway. However, the IL-13R α 1 chain is essential for IL-13 to activate the AMA pathway. IL-4 can activate the AMA pathway by signaling through either type I or type II IL-4 receptor complexes, whereas IL-13 can only signal via type II IL-4 receptor complexes.

Unlike macrophages which coexpress γ_c and IL-13R α 1, nonhematologic cell types such as fibroblasts and endothelial cells do not express γ_c [33, 34]. Therefore, most nonhematologic cell types express only type II IL-4 receptor complexes, and the magnitude of IL-4- and IL-13-induced responses is comparable in these cells. We compared the ability of IL-4 and IL-13 to activate STAT6 in macrophages versus pulmonary fibroblasts derived from wild-type and *Il13ra1* KO mice. We found that the IL-13R α 1 chain is essential for activation of STAT6 by IL-13, but is not required for IL-4 signaling in macrophages. In contrast, IL-13R α 1 was essential for signaling by both IL-4 and IL-13 in fibroblasts. This is because, unlike macrophages which coexpress γ_c and IL-13R α 1, fibroblasts express IL-13R α 1 but do not express γ_c .

It is generally agreed that the production of IL-4 by activated Th2 and ILC2 cells is tightly regulated and largely restricted to the immediate site(s) of immune reactivity. In contrast, IL-13 is often quantitatively more abundant than IL-4 in diseases such as asthma and parasitic infections that are characterized by the presence of activated ILC2 and/or Th2 cells. IL-13 is a primary regulator of airway hyperreactivity and mucus production in allergic lung inflammation, and it has been shown to play a more dominant role than IL-4 as an effector cytokine of Th2-mediated pathogenesis in several animal models [24, 35]. Alternatively activated macrophages are key participants in Th2-mediated inflammatory processes, and development of therapeutic agents that block the AMA pathway may provide an effective means to inhibit these processes.

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