Th2 Cytokines Augment IL-31/IL-31RA Interactions via STAT6-dependent IL-31RA Expression

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Background: IL-31RA is a novel Type I cytokine receptor that pairs with oncostatin M receptor to mediate IL-31 signaling. Binding of IL-31 to its receptor results in the phosphorylation and activation of STAT6, MAPK, and JNK signaling pathways. IL-31 plays a pathogenic role in tissue inflammation, particularly in allergic diseases. Recent studies demonstrate IL-31RA expression and signaling in non-hematopoietic cells, but this receptor is poorly studied in immune cells. Macrophages are key immune-effector cells that play a critical role in Th2-cytokine-mediated allergic diseases. Here, we demonstrate that Th2 cytokines IL-4 and IL-13 are capable of up-regulating IL-31RA expression on both peritoneal and bone marrow-derived macrophages from mice. Our data also demonstrate that IL-4Rα-driven IL-31RA expression is STAT6 dependent in macrophages. Notably, the inflammation-associated genes Fizz1 and serum amyloid A (SAA) are significantly up-regulated in M2 macrophages stimulated with IL-31, but not in IL-4 receptor-deficient macrophages. Furthermore, the absence of Type II IL-4 receptor signaling is sufficient to attenuate the expression of IL-31RA in vivo during allergic asthma induced by soluble egg antigen, which may suggest a role for IL-31 signaling in Th2 cytokine-driven inflammation and allergic responses. Our study reveals an important counter-regulatory role between Th2 cytokine and IL-31 signaling involved in allergic diseases.

Interleukin 31 receptor α (IL-31RA) is a novel Type I cytokine receptor that pairs with oncostatin M receptor to mediate IL-31 signaling. Binding of IL-31 to its receptor results in the phosphorylation and activation of STAT6, MAPK, and JNK signaling pathways. IL-31 plays a pathogenic role in tissue inflammation, particularly in allergic diseases. Recent studies demonstrate IL-31RA expression and signaling in non-hematopoietic cells, but this receptor is poorly studied in immune cells. Macrophages are key immune-effector cells that play a critical role in Th2-cytokine-mediated allergic diseases. Here, we demonstrate that Th2 cytokines IL-4 and IL-13 are capable of up-regulating IL-31RA expression on both peritoneal and bone marrow-derived macrophages from mice. Our data also demonstrate that IL-4Rα-driven IL-31RA expression is STAT6 dependent in macrophages. Notably, the inflammation-associated genes Fizz1 and serum amyloid A (SAA) are significantly up-regulated in M2 macrophages stimulated with IL-31, but not in IL-4 receptor-deficient macrophages. Furthermore, the absence of Type II IL-4 receptor signaling is sufficient to attenuate the expression of IL-31RA in vivo during allergic asthma induced by soluble egg antigen, which may suggest a role for IL-31 signaling in Th2 cytokine-driven inflammation and allergic responses. Our study reveals an important counter-regulatory role between Th2 cytokine and IL-31 signaling involved in allergic diseases.

IL-31 is a cytokine with a predicted arrangement consisting of a four-helix bundle structure and based on homology, it is a member of the IL-6 family of cytokines (1). IL-31 signals through a heterodimeric receptor complex of interleukin 31 receptor α (IL-31RA)3 and oncostatin M receptor β (OSMRβ) subunits. IL-31 is the only known ligand for IL-31RA, which belongs to the large family of glycoprotein 130 (gp130) receptors (2). In the mouse, IL-31RA transcripts exist in two different isoforms: IL-31RAv1 and IL-31RAv2 (1). IL-31RAv1 is homologous to human IL-31RAv4 and is expressed as a membrane-bound form, whereas IL-31RAv2 is a soluble form consisting of a cytokine-binding domain and fibronectin type III domains (1, 2). Both the membrane-bound and soluble forms of the receptor bind to IL-31 with comparable affinity and initiate intracellular signal transduction resulting in secretion of several proinflammatory cytokines and chemokines, which play a critical role in Th2 cytokine-mediated inflammatory diseases (3, 4). Although the expression and distribution of IL-31RA in different cells or tissues have not been well studied, IL-31RA is constitutively expressed by non-hematopoietic cells, such as lung epithelial cells, and also exhibits limited expression on activated monocytes and eosinophils (1, 5, 6). Recently, it has been suggested that IL-31 is selectively produced by activated CD4+T cells, skewed toward a Th2-type cytokine profile (5). Human eosinophils and epidermal keratinocytes also have been shown to express IL-31RA and produce pro-inflammatory cytokines in response to IL-31 (6).

The transcripts and protein levels of IL-31 and the number of Th2 cells that secrete IL-31 are elevated in various skin diseases including atopic dermatitis, atopic contact dermatitis, and purigo nodularis (7–9). IL-31 is thought to play an important role in the induction of pruritus, which is a characteristic feature of these skin diseases (10). Notably, IL-31 expression has been shown to correlate with the expression of Th2 cytokines IL-4 and IL-13 in human skin diseases (11). In support of this, overexpression of IL-31 in transgenic mice results in a severe pruritus, alopecia, and skin lesions (1). Moreover, neutralization of IL-31 has been shown to ameliorate scratching behavior.

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3 The abbreviations used are: IL-31RA, interleukin 31 receptor α; OSMRβ, oncostatin M receptor β; BM, bone marrow; SEA, soluble egg antigen; BMDM, bone marrow-derived macrophages; OSM, oncostatin M; STAT6, signal transducer and activator of transcription 6; SAA, serum amyloid A.
in a mouse model of atopic dermatitis (12). Recent studies suggest that IL-31 signaling is required to limit Th2 cytokine-driven inflammation in the lung and that a complete genetic loss of IL-31RA might result in increased activity and signaling by oncostatin M (OSM) via OSMRβ (13). These reports strongly support a need to investigate the molecular mechanisms that regulate IL-31/IL-31RA interactions involved in inflammation and tissue remodeling.

Macrophages are key regulators of the inflammatory response in tissues and exhibit diversity in phenotypes and plasticity based on tissue environment and cytokine stimuli (14). The Th1 cytokine IFN-γ polarizes macrophages toward classically activated macrophages (M1 phenotype) with an induced expression of iNOS. In contrast, Th2 cytokines IL-4 and IL-13 skew macrophages toward alternatively activated macrophages (M2 phenotype) with expression of Ym1, Arg1, and Fizz1 (15, 16). These macrophage phenotypes are responsible for effective immune responses in tissues to target antigens or pathogens. However, downstream mediators of different macrophage phenotypes involved in allergic diseases are largely unknown (17). A recent study showed that IL-31 stimulation of lung epithelial cells along with Th2 cytokines IL-4 and IL-13 induces the expression of epidermal growth factors, vascular endothelial growth factors, and chemokines, all of which are known to contribute to bronchial inflammation (18). However, mechanisms that explain the cross-talk between the Th2 cytokine and IL-31 pathways during inflammatory and allergic responses are poorly defined. In the current study, we explored the immunoregulatory role of Th2 cytokine in altering IL-31/IL-31RA interactions and signaling involved in inflammation and tissue remodeling. Our findings demonstrate that IL-4 and IL-13 increase the expression of IL-31RA and alter the IL-31 signaling involved in allergic responses.

Materials and Methods

Mice—C57BL/6 (Jackson Laboratories, Bar Harbor, ME), Balb/C (Jackson Laboratories), and IL-4Ra knock-out (KO) (C57BL/6 background, inbred) mice at 8–14 weeks of age were used for all of the experiments. IL-13Ra1−/− and IL-13Ra2−/− mice (both on a C57BL/6 background >10 generations) were obtained from the NIAID-Taconic contract. Mice were housed in either the Cincinnati Children’s Hospital Medical Center animal facility or at the National Institutes of Health in facilities approved by the American Association for the Accreditation of Laboratory Animal Care. All mice were maintained under aseptic conditions and received sterile food and water. Experiments were performed following IACUC regulations.

Macrophage Isolations and Treatments—A 4% thioglycollate medium (BD Bioscience, Sparks, MD) was prepared in a sterile saline solution and 3 ml was injected intraperitoneally into each mouse. On day 5 after injection, mice were sacrificed and peritoneal macrophages were harvested with 5 ml of phosphate-buffered saline (PBS) washing containing 2% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA). Cells were washed twice with RPMI 1640 medium (Gibco™, Life Technologies™, ThermoFisher Scientific, Waltham, MA) containing 5% FBS and 1% each of penicillin and streptomycin (Gibco) and cultured overnight in 12-well plates. Non-adherent cells were removed and the medium was replaced with fresh complete RPMI 1640 medium.

Bone marrow (BM)-derived macrophages were generated from mouse BM cells isolated from the tibia and femur by flushing the bones with sterile PBS. Red blood cells (RBC) were lysed with RBC lysis buffer (Gibco). Cells were re-suspended and plated with 15 ng/ml of M-CSF (PeproTech, Rocky Hill, NJ) for 7 days with a one-time medium change on day 3 (19). Macrophages with M1 and M2 phenotype were generated by stimulating cells for 48 h with IFN-γ and IL-13 and/or IL-4 (R&D Systems, Minneapolis, MN), respectively. To study IL-31 signaling, macrophages were stimulated with recombinant IL-31 at 200 ng/ml for 12 h and measured for IL-31-dependent RNA transcripts.

Animal Model of SEA-induced Allergic Asthma—Allergic asthma in animals was induced according to a previously published method (20). In brief, mice were sensitized twice (days 0 and 14) by intraperitoneal injection of 10 μg of soluble egg antigen (SEA) extracted from Schistosoma mansoni parasitic eggs. On days 28 and 31 mice were anesthetized with a mixture of xylazine and ketamine and given an intratracheal airway challenge with 10 μg of SEA. Mice were sacrificed 24 h after the final airway challenge (day 32), and lungs were collected in RNAlater solution (Applied Biosystems™, Life Technologies™, ThermoFisher Scientific) and stored at −80 °C until use.

RNA Isolation, cDNA Synthesis, and Quantitative PCR—RNA was isolated using the RNeasy kit (Qiagen Sciences, Valencia, CA) as described previously (21). A total of 1 μg of RNA was used for cDNA synthesis, and gene expression was measured using the StepOnePlus™ sequence detection system (Applied Biosystems). Relative gene expression was quantified using SYBR® Green PCR Master Mix or Taqman® assay (Applied Biosystems), and gene expression was normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) or 18S RNA. The data were analyzed with StepOnePlus™ software 2.1 (Applied Biosystems) as described by the manufacturer. The mouse probes and primers used in this study are shown in Tables 1 and 2.

Chromatin Immunoprecipitation (ChIP)—ChIP was performed using the ChIP assay kit (Millipore, Temecula, CA). Briefly, 1 × 10⁷ cells were grown on 100-mm dishes and stim-
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TABLE 3
The sequences of the primer sets used for ChIP Seq

| Location of STAT6 relative to ATG | Sequence of primers | Annealing temperatures | PCR product sizes |
|----------------------------------|---------------------|------------------------|------------------|
| Site 1 (−456)                    | F-ATPATTGGAAGGCGCTCTTG | 62 °C                  | 216 bp          |
| Site 2 (−5694)                   | R-CCCACTCAAATGAACACTC | 58 °C                  | 200 bp          |
| Site 3 (−8991)                   | F-CCAGATATTTTCCTTCCTTCC | 58 °C                  | 225 bp          |
| Sites 4 and 5 (−9300 and −9334) | R-AGGTGCTCATTCCCTTCA | 58 °C                  | 212 bp          |

Flow Cytometry—Thioglycollate-induced peritoneal macrophages were generated from C57BL/6 and IL-4Rα mice and plated overnight at 37 °C and 5% CO2 in 12-well tissue culture plates (BD Biosciences). Non-adherent cells were removed, and the medium was replaced with fresh RPMI 1640 medium containing 5% FBS with penicillin and streptomycin with or without rIL-13 for 24 or 48 h. Cells were dissociated with TripLE Express buffer (Gibco) and stained using a LIVE/DEAD® Fixable Aqua Dead-Cell Stain Kit (Molecular Probes™, Life Technologies™, ThermoFisher Scientific). Stained cells were incubated in Fc receptor-blocking medium for 10 min on ice followed by rat anti-mouse IL-31RA antibody for 30 min on ice. Excess antibody was removed by washing the cells with FACS buffer (0.5% BSA in PBS), followed by the addition of a secondary antibody, anti-rat Alexa Fluor® 594 (Molecular Probes), and then cells were washed twice with FACS buffer. Cells were acquired using a BD™ LSR II flow cytometer (BD Biosciences), and only live cell-gated data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Immunofluorescence—Peritoneal macrophages were plated overnight on polylysine-coated coverslips (Sigma). Non-adherent cells were removed and plated with fresh RPMI 1640 medium containing 5% FBS and 1% each of penicillin and streptomycin. Cells were stimulated with IL-4 (10 ng/ml) and IL-13 (20 ng/ml) and incubated at 37 °C and 5% CO2 for either 12 or 24 h. Cells were fixed with 4% paraformaldehyde for 20 min and washed three times with PBS. Fixed cells were permeabilized with 0.01% of Triton® X-100 (Sigma) for 5 min and washed with PBS. Cells were then treated with anti-mouse IL-31RA antibody (Bristol-Myers Squibb, Seattle, WA) and a co-localization membrane lipid-raft marker, cholera toxin subunit B conjugated to Alexa Fluor® 594 antibody (Molecular Probes) for 1 h, followed by three washes with PBS. Cells were stained with Alexa Fluor® 594 or Alexa Fluor® 488-conjugated secondary antibody (Molecular Probes) for 30 min, nuclei were stained with DAPI for 5 min, and mounted onto microscope slides with mounting medium. Staining was visualized using a Nikon A1Rsi inverted confocal microscope (Nikon Instruments Inc., Melville, NY), and data were analyzed with Imaris software (Bitplane AG, Zurich, Switzerland).

Statistical Analysis—The data were analyzed using GraphPad Prism (version 6; GraphPad, La Jolla, CA). An unpaired Student’s t test was used for comparing between two groups. One-way analysis of variance with Tukey’s multiple comparison test was used for comparison of different experimental groups. p values less than 0.05 were considered statistically significant.
IL-4 and IL-13 Up-regulate IL-31RA Expression in Macrophages—To investigate the role of Th2 cytokines in regulating the expression of IL-31RA and OSMR, we isolated thioglycollate-induced peritoneal macrophages from C57BL/6 mice stimulated with IL-4 and IL-13. Both of the Th2 cytokines were capable of up-regulating IL-31RA transcripts in a dose-dependent manner, compared with media-treated macrophages (Fig. 1, A and B). However, IL-4-stimulated macrophages had significantly higher expression of IL-31RA transcripts compared with IL-13-stimulated macrophages. We also studied the time kinetics in IL-4-stimulated peritoneal macrophages. IL-31RA expression was significantly up-regulated by IL-4 in time-dependent manner (Fig. 1C). Notably, no significant difference was observed in OSMRβ transcripts when stimulated with either IL-4 or IL-13 (Fig. 1D). These data suggest that Th2 cytokines are involved in IL-31RA expression, but not OSMRβ expression. To investigate whether the expression of IL-31RA was restricted to only thioglycollate-induced macrophages or also occurred in other macrophages, we studied the effect of IL-4 and IL-13 cytokines on IL-31RA expression using bone marrow-derived macrophages (BMDM). Bone marrow cells were isolated from wild-type (WT) mice and differentiated into macrophages with M-CSF according to standard protocols (19). On day 6, BMDM were stimulated with either IL-4 or IL-13. As observed for peritoneal macrophages, Th2 cytokines IL-4 and IL-13 significantly increased IL-31RA expression in BMDM (Fig. 1E).

Macrophages express both Type I and Type II Th2 cytokine receptors involved in signaling for IL-4 and IL-13 (15). To determine the role of different Th2 cytokine receptors in IL-31RA expression, macrophages were treated with IL-4 or IL-13 in the presence or absence of neutralizing antibodies to IL-4Rα or IL-2RγC that can selectively block signaling receptors for IL-4 and IL-13 (21). IL-4, but not IL-13 signals via the Type I IL-4 receptor, which is a heterodimeric complex com-

**Results**

IL-4 and IL-13 Up-regulate IL-31RA Expression in Macrophages—To investigate the role of Th2 cytokines in regulating the expression of IL-31RA and OSMRβ, we isolated thioglycollate-induced peritoneal macrophages from C57BL/6 mice stimulated with IL-4 and IL-13. Both of the Th2 cytokines were capable of up-regulating IL-31RA transcripts in a dose-dependent manner, compared with media-treated macrophages (Fig. 1, A and B). However, IL-4-stimulated macrophages had significantly higher expression of IL-31RA transcripts compared with IL-13-stimulated macrophages. We also studied the time kinetics in IL-4-stimulated peritoneal macrophages. IL-31RA expression was significantly up-regulated by IL-4 in time-dependent manner (Fig. 1C). Notably, no significant difference was observed in OSMRβ transcripts when stimulated with either IL-4 or IL-13 (Fig. 1D). These data suggest that Th2 cytokines are involved in IL-31RA expression, but not OSMRβ expression. To investigate whether the expression of IL-31RA was restricted to only thioglycollate-induced macrophages or also occurred in other macrophages, we studied the effect of IL-4 and IL-13 cytokines on IL-31RA expression using bone marrow-derived macrophages (BMDM). Bone marrow cells were isolated from wild-type (WT) mice and differentiated into macrophages with M-CSF according to standard protocols (19). On day 6, BMDM were stimulated with either IL-4 or IL-13. As observed for peritoneal macrophages, Th2 cytokines IL-4 and IL-13 significantly increased IL-31RA expression in BMDM (Fig. 1E).

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**FIGURE 1.** Th2 cytokines IL-4 and IL-13 signal through IL-4Rα and enhance IL-31RA expression. A, IL-31RA expression was measured by quantitative RT-PCR in peritoneal macrophages of C57BL/6 wild-type mice stimulated with the indicated concentrations of IL-4 for 24 h. B, IL-31RA expression was measured by quantitative RT-PCR in peritoneal macrophages of C57BL/6 wild-type mice stimulated with the indicated concentrations of IL-13 for 24 h. C, IL-31RA expression was measured by quantitative RT-PCR in peritoneal macrophages of wild-type mice treated with IL-4 (10 ng/ml) for the indicated time points. D, OSMRβ expression was measured by quantitative RT-PCR in peritoneal macrophages of C57BL/6 wild-type mice stimulated with IL-4 (20 ng/ml) and IL-13 (20 ng/ml) for 24 h. E, IL-31RA expression was measured by quantitative RT-PCR in bone marrow-derived macrophages of C57BL/6 wild-type mice stimulated with IL-4 (20 ng/ml) and IL-13 (20 ng/ml) for 24 h. F, peritoneal macrophages from wild-type mice were cultured with IL-4 (20 ng/ml) and IL-13 (20 ng/ml) along with anti-4Rα and anti-2RγC for 24 h, and IL-31RA expression was analyzed by quantitative RT-PCR. G, wild-type and IL-13Rα1-/ mice were cultured with IL-4 (20 ng/ml) and IL-13 (20 ng/ml), and mRNA level of IL-31RA expression was measured. Data are representative of three independent experiments and are expressed as mean ± S.E. *, p < 0.05; ***, p < 0.001; ****, p < 0.0001.
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prising IL-4Rα and IL-2RγC. Blockade of Type I IL-4 receptor with anti-IL-2RγC attenuated IL-4-driven IL-31RA expression (Fig. 1F). Both IL-4 and IL-13 bind and signal through Type II IL-4 receptor, which is a heterodimeric receptor complex formed by IL-4Rα and IL-13Rα1. Notably, neutralizing antibodies to IL-4Rα reduced both IL-4- and IL-13-induced expression of IL-31RA (Fig. 1F). These data suggest that IL-4Rα signaling is indispensable for IL-4/IL-13-induced IL-31RA expression. To further identify IL-13Rα1 receptor signaling in IL-4/IL-13-induced IL-31RA expression, we measured IL-31RA transcripts in peritoneal macrophages of IL-13Rα1 KO mice treated with IL-4 and IL-13. Consistent with our other findings, the absence of IL-13Rα1 attenuated IL-13-driven IL-31RA expression (Fig. 1G). Conversely, the absence of IL-13Rα1 augmented the expression of IL-31RA in IL-13Rα1-deficient macrophages compared with WT cells treated with IL-4. These findings suggest that the presence of IL-13Rα1 receptor (Type II IL-4 receptor) antagonizes Type I IL-4 receptor-driven IL-31RA expression in macrophages (Fig. 1G).

IL-4Rα-driven IL-31RA Expression is STAT6-dependent—STAT6 is a major transcription factor involved in the expression of IL-4/IL-13-driven genes (22, 23). To confirm whether IL-4Rα-mediated STAT6 signaling is necessary for IL-31RA expression, peritoneal macrophages of WT and IL-4Rα or STAT6 KO mice were treated with IL-13. Notably, WT macrophages had a severalfold increase in IL-31RA expression; however, no significant changes were observed in the IL-13-induced IL-31RA expression in either IL-4Rα KO or STAT6 KO macrophages compared with media-treated controls (Fig. 2, A and B). We also investigated whether IL-31 regulates its own receptor in an autocrine fashion by measuring IL-31RA expression in IL-13- or IL-31/IL-31-stimulated macrophages, and found that IL-13 and IL-31 in combination had no further effect on IL-31RA expression compared with IL-13 alone (supplemental Fig. S1). These results suggest that IL-4Rα-mediated STAT6 signaling is crucial for IL-31RA expression.

To determine the location of Th2 cytokine-induced STAT6 in the mouse IL-31RA gene, we performed a computational analysis of the sequence, which revealed the presence of several STAT6 binding sites in the promoter region of the gene (Fig. 2C). To further validate the binding capacity of the STAT6 motifs identified in the mouse IL-31RA promoter, peritoneal macrophages from WT mice were stimulated with IL-4 (10 ng/ml) for 2 h, and ChIP assays were performed. ChiP analysis using STAT6-specific antibodies revealed that the transcription factor bound more efficiently to Site 2 (−5669) and Site 3 (−8991) as compared with Site 1 (−456) of the IL-31RA promoter (Fig. 2D), suggesting that Sites 2 and 3 are more relevant for IL-4-mediated IL-31RA expression. In contrast, no signal was observed with primers designed for Sites 4 and 5 (−9300 and −9334) of the IL-31RA promoter, suggesting that these sites may play a limited role in IL-4-mediated IL-31RA expression. Together, these data confirm that IL-4-induced signaling for IL-31RA expression was mediated by binding of the STAT6 transcription factor to Sites 2 and 3 of the IL-31RA promoter.

Surface Expression of IL-31RA on Macrophages—IL-31RA is expressed as two different variants (IL-31RAv1 and IL-31RAv2) and IL-31RAv2 is a soluble form of IL-31RA that can decoy the signaling of IL-31 (2). To assess whether the membrane-bound form of IL-31RA protein (IL-31RAv1) was being expressed in our experiments, we developed a flow cytometry method to detect the membrane-bound form of IL-31RA using a mouse anti-IL-31RA antibody. Peritoneal macrophages isolated from WT and IL-4Rα KO mice were stimulated with IL-13 for 12 and 24 h and stained for IL-31RA. Significantly increased expression of the membrane-bound form of IL-31RA was observed in IL-13-induced WT macrophages; however, no difference was observed in IL-4Rα KO macrophages (Fig. 2, A and B). These results are consistent with the level of IL-31RA transcripts measured using RT-PCR. Furthermore, macrophages stained for IL-31RA also were used to obtain immunofluorescence images by fluorescence microscopy. IL-13-stimulated macrophages had increased expression of IL-31RA protein at 12 and 24 h compared with unstimulated macrophages (Fig. 3C), suggesting that Th2-cytokines up-regulate the membrane form of IL-31RA, which was absent in IL-4Rα KO macrophages. Notably, a similar expression of IL-31RA along with co-localization with a membrane lipid-raft marker, cholera toxin, was observed
in IL-4-stimulated peritoneal macrophages (Fig. 3D). To further determine IL-31RA expression on macrophages with M1 or M2 phenotypes, we stimulated macrophages with IL-13 and IFN-γ individually and also in combination. Not surprisingly, macrophages transformed into M1 or M2 phenotypes, expressing iNOS or Arg1/Ym1, respectively (supplemental Fig. S2). IFN-γ stimulation that induced an M1 phenotype had no effect on IL-31RA expression; however, the presence of IFN-γ attenuated IL-13-driven IL-31RA expression on both peritoneal macrophages and BMDM (Fig. 4, A and B).

**Th2 Cytokines Augment IL-31RA-driven Signaling**—To study the effects of Th2-cytokine-induced IL-31RA expression on IL-31 signaling, we pre-treated peritoneal macrophages from WT and IL-4Rα KO mice with both IL-4 and IL-13 for 48 h and stimulated cells with media or IL-31 alone for 12 h. Macrophages treated with IL-31 expressed more inflammatory-re-
Altered IL-31RA Expression in Vivo during SEA-induced Allergic Disease—The SEA of S. mansoni has been shown to induce strong Th2 responses. Previously, we have demonstrated that SEA acts as a potent allergen and induces Th2 cytokine-driven allergic inflammation and airway hyper-responsiveness in a mouse model of allergic asthma (20, 24). To identify the effect of Type II Th2 cytokine receptors on IL-31RA expression in vivo during allergic lung disease, we studied IL-31RA expression in WT and IL-13RA1−/− mice exposed to SEA. As shown in Fig. 6A, mice were sensitized and challenged twice with either SEA or PBS as a control. As described previously, SEA-induced allergic disease requires no adjuvant and is sufficient to induce inflammation and hyper-responsiveness in airways (20, 24). PBS- or SEA-challenged lungs were collected at 24 h after the final challenge. Mice exposed to SEA showed a significant increase in the expression of IL-31RA compared with mice exposed to PBS (Fig. 6B). Notably, the increase in IL-31RA transcripts during SEA-induced allergic disease was attenuated in mice deficient for Type II IL-4 receptors. Conversely, an increase in IL-31RA expression was observed in IL-13RA1−/− mice during SEA-induced allergic disease (Fig. 6B). OSM is another member of the IL-6 group of cytokines that is also known to play an important role in Th2 cytokine-mediated diseases (13). OSM signals through a heterodimeric receptor, consisting of OSMRβ and gp130. To determine whether IL-13RA1 signaling alters other members of the IL-6 group of cytokines and their receptor subunits, we quantified OSM, OSMRβ, and gp130 expression during SEA-induced allergic disease in IL-13RA1−/− and IL-13RA2−/− mice. No significant differences were observed in OSM, OSMRβ, or gp130 expression between the different groups of mice (Fig. 6C–E), suggesting that IL-13RA1 signaling was involved in altering IL-31/IL-31RA interactions, but had no effect on OSM and OSMRβ expression in the lungs during SEA-induced allergic disease in mice.

Discussion

IL-31RA is a gp130-like Type I cytokine receptor that pairs with OSMRβ and forms a signaling receptor for IL-31 (1). Binding of IL-31RA to the IL-31 ligand activates downstream signaling and induces the expression of proinflammatory cytokines and chemokines (2). Therefore, the study of the IL-31/IL-31RA interaction may provide clinical implications for treatment of IL-31-associated diseases. In the present study, we investigated the role of the Th2 cytokines IL-4 and IL-13 in regulating IL-31RA expression and downstream signaling in mouse macrophages. Macrophages stimulated with IL-4 and IL-13 had significantly increased IL-31RA expression, suggesting that Th2 cytokine signaling plays an important role in IL-31RA expression. IL-4 and IL-13 are the key inducers in development of M2 macrophages (15) and signal through binding to a common IL-4Rα chain. IL-2RγC and IL-13Rα1 are the other active constituents to form Type I (IL-4Rα/IL-2RγC) and Type II (IL-4Rα/IL-13Rα1) IL-4 receptors, respectively (25). These receptor complexes are phosphorylated upon ligation with IL-4 and IL-13 and mediate distinct signaling pathways. Studies of mice have shown that IL-4Rα signaling plays a crucial role in the development of M2 macrophages and is associated with Type II immune responses (26). To determine the role of IL-4Rα in Th2 cytokine-mediated IL-31RA expression, we blocked the signaling pathway mediated by Type I IL-4 receptor (IL-4Rα/IL-2Rγ) and measured IL-31RA transcripts in the Th2 cytokine-induced macrophages. IL-31RA expression was attenuated in the presence of antibodies against anti-IL-4Rα, which may suggest that IL-4Rα plays a critical role in Th2 cytokine-mediated IL-31RA expression. However, macrophages deficient for IL-
13Rα1−/− displayed heightened IL-31RA expression with IL-4 stimulation, whereas no differences were observed with IL-13. The increased expression of IL-31RA in the absence of the IL-13Rα1 receptor may suggest that Type II IL-4 receptors compete for IL-4 and function as a negative regulator of Type I IL-4 receptor-driven signaling (27). However, mice deficient for IL-13Rα1 showed no significant increase in IL-31RA expression compared with wild-type mice during SEA-induced allergic asthma. One possible explanation for no increase in IL-31RA transcripts in IL-13Rα1−/− mice could be in vivo dependence of IL-13 to induce the pathophysiological features of asthma (21, 25, 27, 28). In support, the absence of IL-13Rα2, a decoy receptor of IL-13 has resulted in a further increase in IL-31RA expression compared with wild-type mice during SEA-induced allergic asthma. Furthermore, macrophages deficient for either IL-4Rα or STAT6 showed no increases in IL-31RA expression. In summary, our findings demonstrate that Th2 cytokine-induced IL-31RA expression is regulated by IL-4Rα and STAT6 signaling in murine macrophages.

Previous studies have demonstrated that the IL-31RA protein is expressed as multiple isoforms that are either membrane bound or secreted as a soluble decoy receptor (1, 2). Our flow cytometry and immunofluorescence data demonstrated that IL-4Rα-mediated signaling increased the expression of the membrane-bound form of IL-31RA. Future studies are needed to determine whether any soluble form of IL-31RA protein also is expressed and secreted by macrophages. Previous in vitro studies have shown that the Th1 cytokine IFN-γ up-regulates the expression of IL-31RA in monocytes/macrophages and keratinocytes (1, 29). Therefore, we attempted to determine the effect of IFN-γ alone or in combination with the Th2 cytokine IL-13 on IL-31RA expression. In contrast to observations made using human peripheral blood mononuclear cells (16), we found that mouse macrophages stimulated with IFN-γ did not have altered IL-31RA expression, but did have inhibited IL-13-induced IL-31RA expression. In addition, previous studies also suggest opposing effects of IFN-γ on IL-4/IL-13 signaling and their target gene expression in human primary airway epithelial cells and also airway smooth muscle cells via inhibition of STAT6 activation (30, 31). However, future studies are needed to identify mechanisms that might be involved in IFN-γ inhibition of Th2 cytokine-driven IL-31RA gene expression. Macrophages with an M2 phenotype selectively express Fizz1 and are involved in recruitment of immune cells and tissue remodeling.
during Th2 cytokine-driven allergic diseases (32). Our new findings demonstrate that enhanced IL-31/IL-31RA interactions also contribute to Fizz1 expression and possibly to elevated inflammatory responses. Furthermore, we observed a significant increase in the expression of SAA in macrophages stimulated with IL-31, but not in macrophages deficient for Th2 cytokine signaling. Together, our findings suggest that IL-4Rα signaling is critical for IL-31-driven Fizz1 and SAA expression involved in inflammatory and allergic responses.

Lung allergic responses induce Th2 cells to secrete IL-4 and IL-13, which increases bronchial smooth muscle contractility, mucus production by goblet cells in airway epithelium, and production of excess collagen by fibroblasts (33–35). The absence of IL-13Rα1 or an excess of soluble IL-13Rα2 has been shown to attenuate these pathologic responses during an allergic asthma (27, 34). To substantiate our findings that Th2 cytokines alter IL-31RA expression, we studied a murine model of SEA-induced allergic disease, as well as an in vivo model for Th2-driven inflammation and remodeling of the lung (20). SEA-challenged IL-13Rα1−/− mice exhibited a significant decrease in expression of IL-31RA compared with WT mice. In contrast, significantly increased expression of IL-31RA was observed in SEA-challenged IL-13Rα2−/− mice. Previous studies have shown that IL-13Rα2 functions as a decoy receptor of IL-13 and inhibits Type II IL-4 receptor complex-dependent STAT6 signaling (36). Therefore, lack of the IL-13Rα2 gene may have augmented IL-13-driven IL-31RA expression during SEA-induced allergic disease. Several previous studies (1, 37, 38) have shown that human IL-31RA is expressed on monocytes/macrophages, keratinocytes, epithelial cells, and dendritic cells. In addition, increased IL-31 and IL-31RA levels have been found in Th2-related allergic diseases, such as atopic dermatitis (39), which is characterized by the polarization of antigen-presenting cells and activation of a Th2 response (40). In a mouse model of atopic dermatitis, increased IL-31 transcripts were associated with scratching behavior (41), whereas a recent study showed that anti-IL-31 neutralizing antibody reduces scratching behavior and dermatitis in mice (12). Based on an accumulating understanding of IL-31 signaling and atopic dermatitis, our findings suggest that signaling induced by Th2 cytokines is critical in regulating IL-31/IL-31RA interactions involved in inflammatory and remodeling responses. Thus, our finding of Th2-dependent IL-31RA expression may provide new insights into the regulation of IL-31 and subsequent immunopathogenesis associated with elevated Th2 responses.

In conclusion, the current study demonstrates that Th2 cytokines are the key inducers for IL-31RA expression and therefore play a critical role in Th2-mediated IL-31/IL-31RA interactions (Fig. 7). We also showed that both Type I and Type II IL-4 receptor mediated STAT6 signaling is crucial for IL-31RA expression. Moreover, our in vivo SEA-induced allergic disease model reveals that IL-13Rα2, a decoy receptor for IL-13, acts as a potent negative regulator of IL-31RA expression. Although future studies warrant delineation of the role of Th2-mediated IL-31/IL-31RA signaling in the manifestation of Th2 cytokine-related inflammatory disease, our current data highlight the counter-regulatory roles between Th2 cytokine signaling and IL-31/IL-31RA interactions involved in inflammatory and remodeling responses.
IL-31/IL-31RA interactions in allergic and inflammatory diseases.

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References
1. Dillon, S. R., Sprecher, C., Hammond, A., Bilsborough, J., Rosenfeld-Franklin, M., Presnell, S. R., Haugen, H. S., Maurer, M., Harder, B., Johnston, J., Bort, S., Mudri, S., Kuijper, J. L., Bukowski, T., Shea, P., Dong, D. L., Dasovich, M., Grant, F. J., Lockwood, L., Levin, S. D., LeCiel, C., Waggi, K., Day, H., Topouzis, S., Kramer, J., Kuestner, R., Chen, Z., Foster, D., Parrish-Novak, J., and Gross, J. A. (2004) Interleukin 31, a cytokine produced by activated T cells, induces dermatomic in mice. Nat. Immunol. 5, 752–760
2. Zhang, Q., Putheti, P., Zhou, Q., Liu, Q., and Gao, W. (2008) Structures and biological functions of IL-31 and IL-31 receptors. Cytokine Growth Factor Rev. 19, 347–356
3. Chaotopadhay, S., Tracy, E., Liang, P., Robledo, O., Rose-John, S., and Baumann, H. (2007) Interleukin-31 and oncostatin-M mediate distinct signaling reactions and response patterns in lung epithelial cells. J. Biol. Chem. 282, 3014–3026
4. Cornelissen, C., Lüscher-Firzlaff, J., Baron, J. M., and Lüscher, B. (2012) Signaling by IL-31 and functional consequences. Eur. J. Cell Biol. 91, 552–566
5. Maier, E., Werner, D., Duschl, A., Bohle, B., and Horejs-Hoeck, J. (2014) Human Th2 but not Th9 cells release IL-31 in a STAT6/NF-kB-dependent way. J. Immunol. 193, 645–654
6. Cheung, P. F., Wong, C. K., Hu, S., Chen, D. P., and Lam, C. W. (2009) Anti-interleukin-31/IL-31RA interactions in allergic and inflammatory dermatitis. J. Invest. Dermatol. 122, 437–447
7. Takeda, K., Tanaka, T., Shi, W., Matsumoto, M., Minami, M., Kashiwamura, S., Nakanishi, K., Yoshida, N., Kishimoto, T., and Akira, S. (1996) Essential role of Stat6 in IL-4 signalling. Nature 380, 627–630
8. Kaplan, M. H., Schindler, U., Smiley, S. T., and Grusby, M. J. (1996) Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. Immunity 4, 313–319
9. Wilson, M. S., Pesce, J. T., Ramalingam, T. R., Thompson, R. W., Cheever, A. W., and Wynn, T. A. (2008) Suppression of murine allergic airway disease by IL-2-antii-IL-2 monoclonal antibody-induced regulatory T cells. J. Immunol. 181, 6942–6954
10. Thompson, R. W., Pesce, J. T., Ramalingam, T. R., Wilson, M. S., Minniccozzi, S., Cheever, A. W., Thompson, R. W., Miettink-Kane, M. M., and Wynn, T. A. (2010) Matrix metalloproteinase 12-deficiency augments extracellular matrix degrading metalloproteinases and attenuates IL-13-dependent fibrosis. J. Immunol. 184, 3955–3963
11. Leung, D. Y., Maurer, M., Howell, M., Boguniewicz, M., Boguniewicz, M., Yeo, L., Storey, H., LeCiel, C., Harder, B., and Gross, J. A. (2006) IL-31 is associated with cutaneous lymphocyte antigen-positive skin homing T cells in patients with atopic dermatitis. J. Allergy Clin. Immunol. 117, 418–425
12. Neis, M. M., Peters, B., Dreuw, A., Wenzel, J., Bieber, T., Mauch, C., Krieg, T., Stanzel, S., Heinrich, P. C., Merk, H. F., Bosio, A., Baron, J. M., and Hermans, H. M. (2006) Enhanced expression levels of IL-31 correlate with IL-4 and IL-13 in atopic and allergic contact dermatitis. J. Allergy Clin. Immunol. 118, 930–937
13. Sonkoly, E., Muller, A., Lauerma, A. I., Pivarcis, A., Soto, H., Kemeny, L., Alenius, H., Dieu-Nosjean, M. C., Meller, S., Rieker, J., Steinhoff, M., Hoffmann, T. K., Ruzicka, T., Zlotnik, A., and Homey, B. (2006) IL-31: a new link between T cells and pruritus in atopic skin inflammation. J. Allergy Clin. Immunol. 117, 411–417
14. Gonzales, A. J., Humphrey, W. R., Messamore, J. E., Fleck, T. J., Fici, G. J., Shelly, J. A., Teel, J. F., Bammert, G. F., Dunham, S. A., Fuller, T. E., and McCafl, R. B. (2013) Interleukin-31: its role in canine atopic dermatitis. Vet. Dermatol. 24, 48–53
15. Heller, N. M., Matsukura, S., Georas, S. N., Boothby, M. R., Rothman, P. B., Stellato, C., and Schleimer, R. P. (2004) Interferon-γ inhibits STAT6 signal transduction and gene expression in human airway epithelial cells. Am. J. Respir. Cell Mol. Biol. 31, 573–582
16. Pesce, J. T., Ramalingam, T. R., Wilson, M. S., Miettink-Kane, M. M., Thompson, R. W., Cheever, A. W., Urban, J. F., Jr., and Wynn, T. A. (2009) Rtnla (relmβ) suppresses helminth-induced Th2-type immunity. PLoS Pathog. 5, e1000393
17. Kuperman, D. A., Huang, X., Nguyen, L., Holscher, C., Brombacher, F., and Erle, D. J. (2005) IL-4 receptor signaling in Clara cells is required for...
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allergen-induced mucus production. *J. Immunol.* **175**, 3746–3752

34. Ohta, Y., Hayashi, M., Kanemaru, T., Abe, K., Ito, Y., and Oiko, M. (2008) Dual modulation of airway smooth muscle contraction by Th2 cytokines via matrix metalloproteinase-1 production. *J. Immunol.* **180**, 4191–4199

35. Perkins, C., Yanase, N., Smulian, G., Gildea, L., Orekov, T., Potter, C., Brombacher, F., Aronow, B., Wills-Karp, M., and Finkelman, F. D. (2011) Selective stimulation of IL-4 receptor on smooth muscle induces airway hyperresponsiveness in mice. *J. Exp. Med.* **208**, 853–867

36. Kawakami, K., Taguchi, J., Murata, T., and Puri, R. K. (2001) The interleukin-13 receptor α2 chain: an essential component for binding and internalization but not for interleukin-13-induced signal transduction through the STAT6 pathway. *Blood* **97**, 2673–2679

37. Kasraie, S., Niebuhr, M., and Werfel, T. (2010) Interleukin (IL)-31 induces pro-inflammatory cytokines in human monocytes and macrophages following stimulation with staphylococcal exotoxins. *Allergy* **65**, 712–721

38. Horejs-Hoeck, J., Schwarz, H., Lamprecht, S., Maier, E., Hainzl, S., Schmittner, M., Posselt, G., Stoecklunger, A., Hawranek, T., and Duschl, A. (2012) Dendritic cells activated by IFN-γ/STAT1 express IL-31 receptor and release proinflammatory mediators upon IL-31 treatment. *J. Immunol.* **188**, 5319–5326

39. Nobbe, S., Dzianycz, P., Mühleisen, B., Bilsborough, J., Dillon, S. R., French, L. E., and Hofbauer, G. F. (2012) IL-31 expression by inflammatory cells is preferentially elevated in atopic dermatitis. *Acta Derm. Venereol.* **92**, 24–28

40. Brandt, E. B., and Sivaprasad, U. (2011) Th2 cytokines and atopic dermatitis. *J. Clin. Cell. Immunol.* **2**, 110

41. Takaoka, A., Arai, I., Sugimoto, M., Honma, Y., Futaki, N., Nakamura, A., and Nakaie, S. (2006) Involvement of IL-31 on scratching behavior in NC/Nga mice with atopic-like dermatitis. *Exp. Dermatol.* **15**, 161–167