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IL-31 Receptor (IL-31RA) Knockout Mice Exhibit Elevated Responsiveness to Oncostatin M

Janine Bilsborough,[1] Sherri Mudri,[1] Eric Chadwick,[2] Brandon Harder,[3] and Stacey R. Dillon

IL-31 signals through the heterodimeric receptor IL-31RA and oncostatin M receptor (OSMR), and has been linked with the development of atopic dermatitis, a Th2 cytokine-associated disease in humans. However, recent studies of IL-31RA knockout (KO) mice have suggested that IL-31 signaling may be required to negatively regulate Th2 type responses rather than exacerbate them. Because those studies were performed on genetically modified mice, we examined whether neutralizing IL-31 with a specific mAb would give similar results to IL-31RA KO mice in two Th2 cytokine-associated immune models. We report no difference in lymphocyte Th2-type cytokine production after Ag immunization between IL-31RA KO mice, mice treated with the IL-31 mAb, or control animals. Second, we tested whether the absence of the IL-31RA subunit in IL-31RA KO mice may allow for increased pairing of the OSMR subunit with another cytokine receptor, gp130, resulting in overrepresentation of the heterodimeric receptor for OSM and increased responsiveness to OSM protein. We found that intranasal OSM challenge of IL-31RA KO mice resulted in increased IL-6 and vascular endothelial growth factor production in the lung compared with wild-type littermate control animals. Moreover, PBS-challenged IL-31RA KO mice already had increased levels of vascular endothelial growth factor, which were further increased by OSM challenge. These data imply that IL-31RA–deficient mice produce increased levels of OSM-inducible cytokines during airway sensitization and challenge, which may be the driving force behind the apparent exacerbation of Th2-type inflammatory responses previously observed in these mice. ***The Journal of Immunology*****, 2010, 185: 6023–6030.**

Interleukin-31 is produced by activated $CD4^+$ T cells, particularly those skewed toward a Th2-type cytokine profile (1). Overexpression of IL-31 in transgenic mice results in a pruritic skin condition that is similar to human atopic dermatitis (AD) (1). Moreover, analysis of IL-31 levels in human dermatitis samples has shown increased expression of IL-31 in AD, prurigo nodularis, and allergic contact dermatitis compared with healthy control populations (2–5). IL-31 expression has also been shown to correlate with the expression of the Th2 cytokines IL-4 and IL-13 in human skin disease samples, implicating IL-31 in the pathogenesis of allergic skin diseases (3).

More recently, IL-31 protein has been associated with allergic asthma, because IL-31 levels were found to be increased in serum of patients with allergic asthma compared with normal control subjects (6). Asthma and AD are frequently associated, with AD often preceding the development of asthma or allergic rhinitis, a concept often referred to as the "atopic march" (reviewed in Ref. 7), where epicutaneous sensitization to Ag is thought to induce a systemic allergic response that results in airway involvement. Notably, IL-31R, a heterodimeric complex composed of a gp130-like receptor (IL-31RA) and oncostatin M receptor (OSMR), is expressed on bronchial epithelial cell lines. Preliminary studies in a murine model of asthma suggest the receptor for IL-31 is upregulated after Ag sensitization and challenge in the lung (1). Stimulation of lung epithelial cell lines with IL-31 induces STAT phosphorylation and activation of the MAPK pathways p38, JNK, ERK, and Akt (8, 9). Moreover, IL-31 stimulation of lung epithelium alone or in conjunction with the Th2 cytokines IL-4 and IL-13 induces the production of factors known to contribute to bronchial inflammation, tissue damage, and lung remodeling, including epidermal growth factor, vascular endothelial growth factor (VEGF), and MCP-1 (9).

In contrast with the theory that IL-31 is actively involved in the promotion of Th2-type diseases, others have suggested that IL-31 negatively regulates the development of the Th2-type response. This theory was proposed after analysis of the development of Th2-dependent granuloma formation in IL-31RA-deficient mice using a model of *Schistosoma mansonii* infection (10) and more recently extended to intestinal helminth infection (11). In both studies, infected IL-31RA knockout (KO) mice developed more severe inflammation and increased production of Th2 cytokines by Ag-specific activated T cells compared with infected wild-type (WT) mice. Moreover, macrophages from IL-31RA KO mice were reported to enhance Th2 cytokine expression and enable proliferation of Th2-type cells during Ag stimulation (10). However, because these studies were performed in IL-31RA KO animals, and not with direct neutralization of IL-31 with an Ab, it was possible these observations were due to the physical absence of the IL-31RA subunit and not a result of the absence of signaling through the IL-31RA.

IL-31RA pairs with OSMR to form the functional heterodimer for IL-31, and the absence of the IL-31RA receptor chain may

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Abbreviations used in this paper: AD, atopic dermatitis; BALF, bronchoalveolar lavage fluid; ES, embryonic stem; i.n., intranasally; KC, keratinocyte chemoattractant; KO, knockout; OSM, oncostatin M; OSMR, OSM receptor; TSLP, thymic stromal lymphopoietin; VEGF, vascular endothelial growth factor; WT, wild-type.

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therefore allow for increased availability of the constitutively expressed OSMR subunit. This could result in an artificially increased level of expression of an alternative cytokine receptor containing OSMR as a contributing subunit. The receptor for OSM is a heterodimeric receptor, consisting of OSMR and gp130. OSM is known to contribute to pulmonary inflammation (12, 13) and may play a significant role in the induction of inflammation in mice where IL-31RA is absent and the functional receptor for OSM is overrepresented.

To test whether the reported development of an exacerbated Th2-type response in IL-31RA KO mice (10, 11) was due to the absence of negative signaling of IL-31 or an exacerbated response to OSM, we performed two different Ag-driven Th2-skewed models in the presence of the an anti–IL-31 neutralizing Ab and checked for the level of Th2-type responses, and we investigated the effect of OSM delivery on IL-31RA KO mice.

Materials and Methods

Animals

Eight- to 10-wk-old BALB/c or C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were acclimatized for 2 wk before performing experiments. IL-31RA KO mice were generated at Lexicon Genetics (The Woodlands, TX) as previously described (1). In brief, *IL31RA* genomic clones were identified in a genomic library from mouse strain 129S5 (129SvEvBrd). A replacement-type targeting vector was made to delete 3.4 kb of IL-31RA including exons 4–6, encoding the cytokine-binding domain 2 of IL-31RA. This deleted region was replaced with an IRESbGal-poly(A)-MC1neo-poly(A) selectable marker cassette. The linearized targeting vector was electroporated in 129S5-derived Lex-1 embryonic stem (ES) cells, and subjected to positive and negative selection with G418 and fialuridine. The targeted ES clones were identified by Southern blotting using a 215-bp intron 1 probe, amplified by PCR primers ZG15.28 (5′-TTGGAATGTGTAGTGGATGGG-3′) and ZG15.29 (5′-ACTGAAGCCTCACGCAGTAGC-3′). Chimeras were generated by injection of targeted ES cells in C57BL/6-Tyrc-Brd blastocysts and transplantation into pseudopregnant females. Progeny were genotyped by Southern blotting using the intron 1 probe on tail genomic DNA digested with SacI to identify a 10.3-kb WT and 11.5-kb targeted band. The IL-31RA KO mice were subsequently identified by PCR genotyping strategy. Three PCR primers, #43416 (5′-GATACCACTAATGTTTCATGG-3′), #43419 (5′-CAGGTCTGCAACCTCACAGG-3′), and #43420 (5′-CGCAAGCCCGGTGCCTGA-3′) were used in a multiplex PCR reaction to detect the WT allele and mutant allele. The WT allele yields a DNA fragment 290 bp in length, whereas the mutated allele generates a DNA fragment of 552 bp in length (data not shown). Analysis of IL-31RA mRNA expression was performed using RT-PCR to confirm deletion of IL-31RA in RNA from skin, lung, and colons of homozygous IL31RA KO mice versus their heterozygous and WT littermates. Animals were backcrossed to the C57BL/6 background for these studies. All animal procedures were approved by the ZymoGenetics Institutional Animal and Care and Use Committee.

Ab reagents

The rat anti-mouse IL-31 IgG1 Ab was generated as follows. Three-mo-old female Sprague–Dawley rats (Charles River Laboratories) were immunized with recombinant Glu-Glu tagged mouse IL-31 produced in BHK cells. Single-cell suspensions prepared from the spleen and lymph nodes from the rat showing the greatest IL-31 inhibitory titer were fused to the Sp2/0 mouse myeloma cell line (14). The fusion was screened for hybridomas secreting Abs that bound to IL-31 by ELISA and inhibited IL-31 in an in vitro bioassay. The rat anti-mouse IgG1 mAb used for the in vivo studies was designated as clone 271.26.6.6.1 and was shown to specifically inhibit mouse IL-31 in an in vitro bioassay using BaF3 cells transfected with the murine IL-31R complex (IL-31RA and OSMR), and a luciferase reporter. The IC_{50} of the Ab was ~3 ng/ml or 18.5 pM (Fig. 1*A*). A rat IgG1 mAb directed against an irrelevant human protein was produced at ZymoGenetics (Seattle, WA) and used as the Ig control mAb (ZGEN, clone 262.17.1.3.2.2). This IgG1 rat anti-human mAb does not cross-react with mouse protein.

In vivo neutralization of IL-31–induced pruritus and alopecia

BALB/c mice were given 10 μg/day purified recombinant murine IL-31 protein for 7 d, delivered s.c. via a miniosmotic pump (Alzet Osmotic Pump; Durect Corporation, Cupertino, CA). One day before pump implantation and then again on day 4, mice were treated with either 10 mg/kg (Fig. 1*B*, *left graph*) or 20 mg/kg (Fig. 1*B*, *right graph*) of the neutralizing rat anti-mouse IL-31 mAb, a control mAb, or were left untreated. Mice were monitored daily and assigned a score for pruritus (scratching behavior) and hair loss according to the following scale: 0, no scratching or hair loss; 0.5, scratching with no evidence of hair loss; 1, scratching with patches of thinning hair; 2, scratching with small patches of hair loss; 3, increased scratching with moderate hair loss; 4, extreme scratching with severe hair loss. Severity of scratching was based on intensity of scratching behavior during a 5-min observation period.

Immune response model

Mice were immunized s.c. in both flanks with total of 100 μg OVA (32467; Calbiochem, EMD, San Diego, CA) in 1:1 mix with Imject alum (77161; Pierce, Thermo Scientific, Rockford, IL). Twenty-four hours before s.c. immunization, mice received 200-μg injections of test Abs i.p. Mice were euthanized 10 d postimmunization, and the draining inguinal lymph nodes were collected and processed for Ag-specific ex vivo cytokine assays.

Airway hyperresponsiveness model

Mice were sensitized with Ag on days 0 and 7 by i.p. injection with 10 μg OVA in a 1:1 mix with Imject alum. Mice were then challenged with Ag on days 14 and 15 with 20 μg OVA delivered intranasally (i.n.). Groups of mice were treated with test Abs twice weekly with 200 μg/mouse i.p. injections starting 1 wk before the first OVA sensitization and throughout the course of the model for a total of seven injections. Mice were euthanized 48 h after the second i.n. challenge, and bronchoalveolar lavage fluid (BALF) was collected for infiltrating cell and cytokine analysis.

Ex vivo cytokine production by draining lymph nodes

Draining lymph nodes were harvested and processed into single-cell suspensions. Cell cultures were maintained in RPMI 1640 (Life Technologies, Carlsbad, CA) supplemented with L-glutamine, sodium pyruvate, 50 μM β-mercaptoethanol, 10% FBS, and penicillin, streptomycin, and neomycin. Cells were plated at 200,000 cells/well in 96-well round bottom sterile tissue culture plates (Falcon) and activated with titrations of OVA (50, 10, 2, 0 μg/ml). Culture supernatants were collected at 72 and 96 h and stored at −20°C for cytokine detection assays.

Analysis of BALF cell infiltrate

Lungs were lavaged with 3 × 0.5-ml aliquot PBS containing 0.5% FBS. The lavage fluids were centrifuged, BALF cells isolated, and the supernatant frozen for later analysis. RBCs were lysed using ACK lysis buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$, 0.0001 M EDTA, pH 7.3; ZymoGenetics), bronchoalveolar lavage cell pellets were resuspended at 2×10^6 cells/ml, and 100 μl was used for total and differential cell counts. Total BALF leukocyte counts were determined for each mouse via light microscopy using trypan blue exclusion. Differential cell counts were determined by H&E staining (Diff-Quik; Merz & Dade, Dubingen, Switzerland) of air-dried and fixed cytospin slides. Cell differentials were determined by Phoenix Central Laboratory (Everett, WA) by examining 100 cells per cytospin.

Oncostatin M challenge

Groups of mice received 5 μg OSM (495-MO-025; R&D Systems, Minneapolis, MN) in 50 μl PBS i.n. on day 0. Twenty-four hours later, BALF was collected and analyzed for cytokine concentration by Luminex (Luminex, Austin, TX).

Detection of cytokines

Cytokine levels in BALF and cell culture supernatants were measured using custom Mouse Cytokine detection kits from Millipore (Milliplex assays; Billerica, MA) and Invitrogen (Carlsbad, CA) as per the manufacturer's instructions. Data were read using the Luminex100 plate reader (Luminex). Quantification of cytokines was performed by regression analysis from a standard curve generated from cytokine standards included in the kit. Thymic stromal lymphopoietin (TSLP) was detected using an ELISA kit from R&D Systems, IL-23 was detected using an ELISA kit from eBioscience (San Diego, CA), and tissue inhibitor of metalloproteinases-1 was detected using an ELISA kit from R&D Systems (MTM100).

Statistical analysis

Statistical analysis was performed with GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, CA). A two-way ANOVA with

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Bonferroni's posttest was performed when analyzing multiple groups and different concentrations of mAb. A one-way ANOVA with Bonferroni's posttest was performed when analyzing multiple groups at a single time point. The unpaired Student t test was used to determine statistical significance when comparing data between two groups. The p values <0.05 were considered statistically significant.

Results

Anti–IL-31 neutralizing Ab

We identified a neutralizing mAb specific for mouse IL-31 after immunization of rats with recombinant murine IL-31 protein. The rat anti-mouse IL-31 mAb was shown to specifically inhibit activity of mouse IL-31 in an in vitro bioassay using BaF3 cells transfected with the murine IL-31R complex (IL-31RA and OSMR), and a luciferase reporter (previously described in Ref. 1) (Fig. 1*A*). The IC_{50} for the mAb in this assay was estimated to be ~3 ng/ml (Fig. 1*A*). This Ab has also been used in vivo to ameliorate the scratching behavior of NC/Nga mice that spontaneously develop AD (15). We further confirmed the ability of this Ab to neutralize IL-31 activity in vivo using a biological readout of hair loss in IL-31–treated animals. Subcutaneous delivery of IL-31 by osmotic pump in mice induces progressive alopecia, generally beginning 3 d after pump implantation (1). The anti-mouse IL-31 mAb was delivered i.p. on day 1 of IL-31 delivery, and the animals were subsequently observed for evidence of alopecia and scored based on severity of symptoms. Compared with both the vehicle control and the control mAb-treated groups, the anti–IL-31 mAb significantly inhibited the degree of alopecia observed in IL-31–treated animals (Fig. 1*B*).

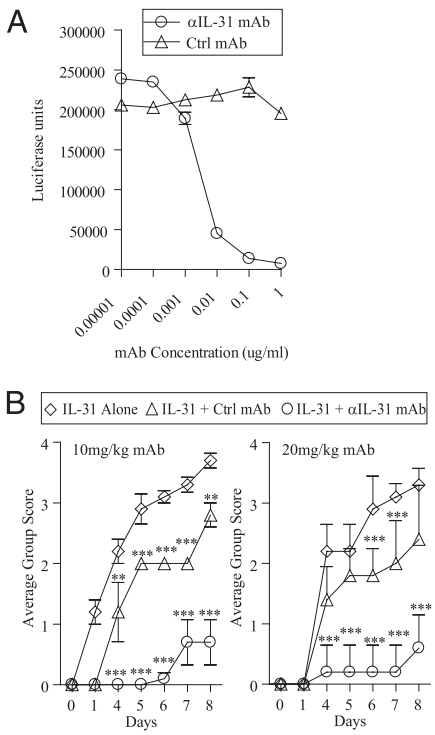

FIGURE 1. Inhibition of IL-31 activity in vitro and in vivo. *A*, Inhibition of IL-31–induced luciferase activity by an anti-mouse IL-31 mAb (circles) compared with an irrelevant control Ab (triangles) on IL-31R transfected BaF3 cells. *B*, In vivo neutralization of IL-31–induced alopecia by 10 (*left graph*) or 20 mg/kg (*right graph*) of the anti-IL31 mAb (circles) compared with either a matched dose of control mAb (triangles) or a vehicle (no mAb) control (diamonds). $n = 5$ mice per group. Data are representative of three independent experiments. $***p < 0.001$; $**p < 0.01$, two-way ANOVA with Bonferroni's multiple-comparison posttest.

In vivo neutralization of IL-31 does not affect Th2-type cytokine production after Ag immunization

Alum adjuvants are known to drive Ag-specific immune responses toward a Th2-type cytokine profile (16), especially when delivered to BALB/c mice that have a strong bias toward the development of Th2-type immune response after immunization. To determine whether neutralization of IL-31 exacerbates Th2-driven responses to Ag in vivo, we delivered the anti-mouse IL-31 neutralizing mAb before immunization with OVA + alum, as described in *Materials and Methods*. One week later, cytokine secretion from cells within the draining lymph nodes were examined. Analysis of supernatants showed that although Th2 cytokines were produced after Ag-specific stimulation of T cells, neutralization of IL-31 in vivo had no effect on the level of Th2-type cytokines produced at either 72 (Fig. 2*A*) or 96 h (data not shown). Moreover, when IL-31RA KO mice were tested in the same immunization model, there was no increase in Th2-type cytokine production compared with WT mice (Fig. 2*B*).

IL-31 neutralization during Ag-induced airway inflammation does not increase Th2 cytokine production

Although blocking IL-31 in a systemic Ag-specific immune response model did not appear to skew mice toward an exacerbated Th2-type response, it was possible that previous observations of Th2 skewing in IL-31RA KO mice were particular to the lung environment (10). We therefore tested the anti–IL-31 neutralizing mAb in a mouse model of airway hyperresponsiveness. In this model, $CD4^+$ cells, and the Th2 cytokines IL-4 and IL-5, are necessary for the development of eosinophilic lung inflammation (17–19). The anti–IL-31 neutralizing mAb was delivered twice weekly throughout the OVA sensitization and challenge protocol, starting 1 d before the first Ag sensitization. Analysis of BALF showed no difference in the total number of cells (Fig. 3*A*, *left graph*), or in the percentage of cell subsets within the BAL including neutrophils, lymphocytes, macrophages, or eosinophils, between treatment groups (Fig. 3*A*, *right graph*). Moreover, although cytokine levels were significantly increased in OVA-sensitized and -challenged mice compared with naive animals, delivery of the anti–IL-31 mAb did not significantly alter the level of Th2-type cytokines in the BAL compared with either an irrelevant mAb or a vehicle control, although there was a slight trend toward decreasing Th2 cytokine levels after anti–IL-31 mAb treatment (Fig. 3*B*). We have previously shown that IL-31 is produced by in vitro derived Th1 and Th2 cells, with greater levels of IL-31 mRNA present in Th2 cells. Moreover, the receptors for IL-31 are increased in the lung in the OVA-induced airway hyperresponsiveness model (1). Our current data suggest that although IL-31 may be associated with cells that produce Th2-type cytokines and the receptor is upregulated in Th2-type disease models, IL-31 does not appear to be a key inducer of Th2-type cytokines. This is consistent with the fact that IL-31 transgenic mice do not have increased levels of Th2 cytokines in the serum.

Further analysis of BAL cytokines showed no difference in eotaxin, IL-9, MIP-2, or RANTES levels (data not shown). IFN-γ, IL-10, IL-12p40, IL-12p70, IL-17, IL-23, and TSLP were investigated but not detected (data not shown). In addition, there was no effect of IL-31 neutralization on the production of cytokines from lung draining lymph nodes at 48 or 72 h after Ag restimulation in vitro (data not shown and Fig. 3*C*, respectively).

IL-31RA KO mice, but not mice treated with the anti–IL-31 neutralizing mAb, demonstrate an exacerbated inflammatory response in the lung

To better reconcile the findings between our studies and those showing increased susceptibility of IL-31RA KO mice to pul-

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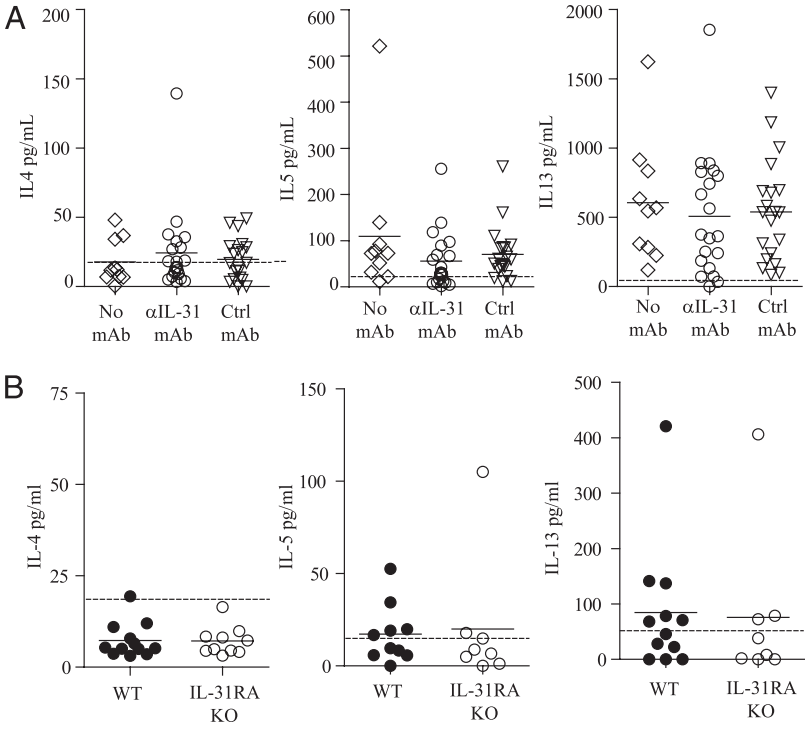

FIGURE 2. Production of Th2- type cytokines by in vitro activated Ag-specific T cells from (*A*) mice pretreated either with vehicle control (diamonds, n = 10), anti–IL-31 mAb (circles, n = 20), or an irrelevant control mAb (triangles, n = 20), or (*B*) WT littermate controls (open circles, n = 12) and IL-31RA KO mice (closed circles, n = 10). Supernatants were collected at 72 h. Dotted lines indicate the lower level of detection for each assay. Data are representative of three independent experiments.

monary inflammation in a *S. mansonii* model (10), we compared the effect of neutralizing anti–IL-31 mAb treatment to IL-31RA KO responses in our murine model of airway hyperresponsiveness. Because the IL-31RA KO mice were on a C57BL/6 background, we repeated the anti–IL-31 neutralizing mAb studies in C57BL/6 mice. Analysis of BAL cell infiltrates showed that, similar to the studies in BALB/c mice, inhibition of IL-31 with a neutralizing mAb in C57BL/6 mice did not significantly alter the number of infiltrating cells in the BALF (Fig. 4*A*). Also similar to the BALB/c studies, anti–IL-31 mAb treatment of C57BL/6 mice did not alter the levels of IL-4, IL-5, or IL-13 found in the BAL, although, as expected, the levels of production of these cytokines was significantly lower in C57BL/6 mice compared with BALB/c animals (data not shown).

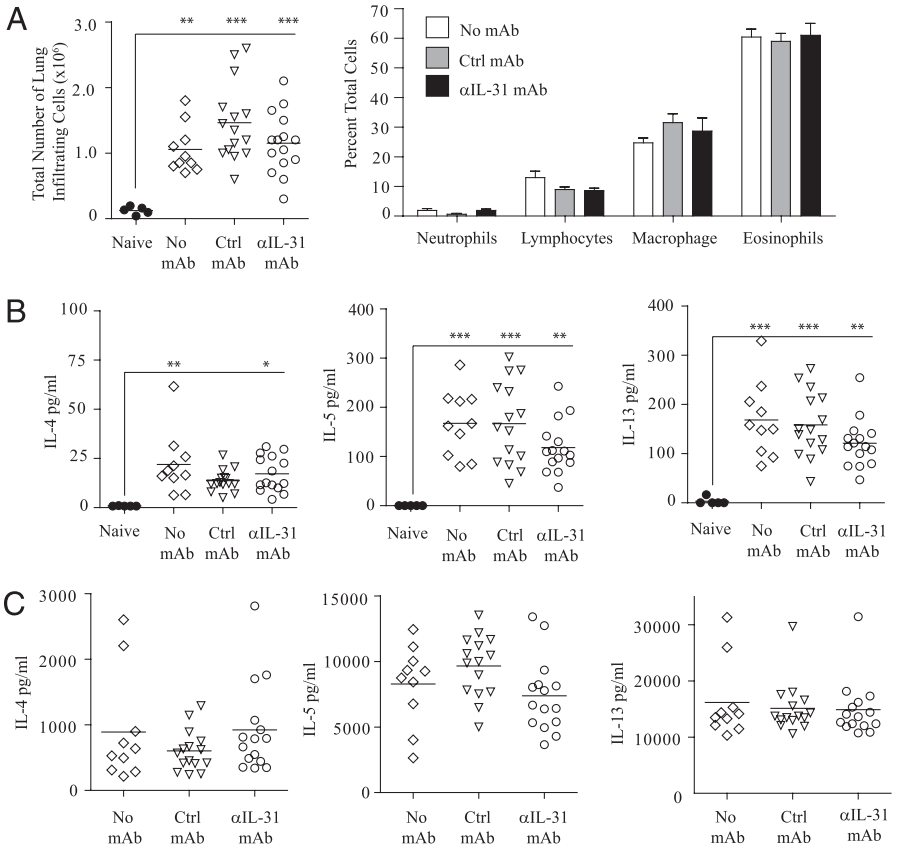

FIGURE 3. Lung cell infiltrates and cytokine production after airway Ag sensitization and challenge of anti–IL-31–treated mice. *A*, Total cell infiltrates in BALF from naive mice (closed circles, n = 4) or mice sensitized and challenged with OVA but pretreated with vehicle only (no mAb; diamonds, n = 10), an irrelevant control mAb (triangles, n = 15), or with anti–IL-31 mAb (open circles, n = 15) (*left graph*). BAL cell differentials from OVA-sensitized and challenged mice pretreated with vehicle only (no mAb; white bars, n = 10), an irrelevant control mAb (gray bars, n = 15), or an anti–IL-31 mAb (black bars, n = 15) (*right graph*). *B*, Cytokine levels in BALF and (*C*) cytokine levels in supernatants of lung draining lymph node cells at 72 h (similar data were observed for 48 h supernatants: data not shown). Data are representative of two independent experiments. $*p < 0.01$; $**p < 0.001$; $***p < 0.0001$, one-way ANOVA with Bonferroni's multiple-comparison posttest.

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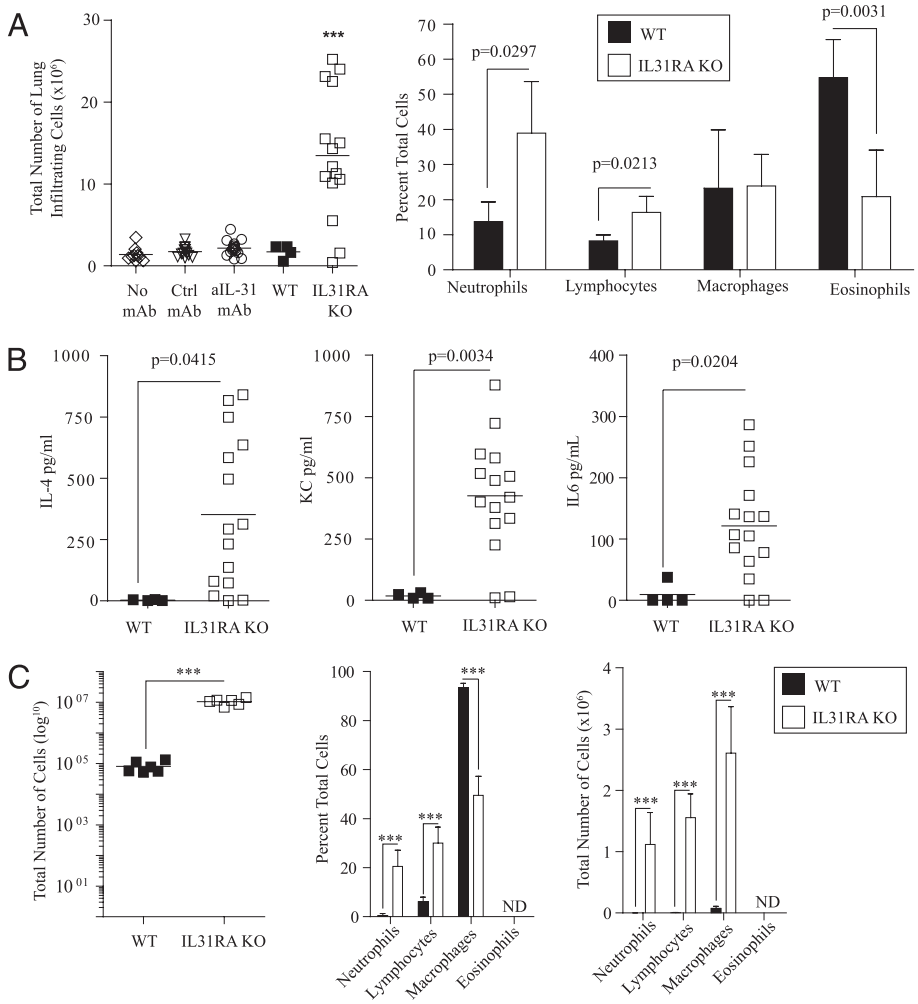

FIGURE 4. Lung cell infiltrates and cytokine production after airway Ag sensitization and challenge of IL-31RA KO mice. *A*, Total cell infiltrates in BALF from mice sensitized and challenged with OVA but pretreated with vehicle only (no mAb; diamonds, $n = 10$), an irrelevant control mAb (triangles, $n = 14$), or with anti–IL-31 mAb (circles, $n = 15$) compared with WT littermate controls (closed squares, $n = 4$) and IL-31RA KO mice (open squares, $n = 15$). $***p < 0.0001$ using one-way ANOVA (*left graph*). BAL cell differentials from OVA sensitized and challenged WT littermate control mice (black bars, $n = 4$) and IL-31RA KO mice (white bars, $n = 15$). The p values were calculated via unpaired t test (*right graph*). *B*, Cytokine levels in BALF of IL-31RA KO mice ($n = 15$) and WT control littermates ($n = 4$). The p values were calculated using unpaired t test. Data are representative of two independent experiments. *C*, Total cell numbers in BALF from nonsensitized, naive IL-31RA KO mice ($n = 6$) and WT control littermates ($n = 6$) (*left graph*). Percent cell types in BAL from nonsensitized, naive IL-31RA KO mice and WT control littermates (*middle graph*). Total number of cell types from nonsensitized, naive IL-31RA KO mice and WT control littermates (*right graph*). Data are representative of three independent experiments. $***p < 0.0001$ using unpaired t test. ND, not detected.

In striking contrast, however, the number of BAL cells infiltrating the lung of IL-31RA KO mice after Ag challenge was significantly increased compared with those in the lungs of WT littermate control animals (Fig. 4*A*, *left graph*). In contrast with the increased levels of eosinophils in WT mice, a finding typical for this model, the increased cells in the BALF of IL-31RA KO mice largely consisted of neutrophils and lymphocytes (Fig. 4*A*, *right graph*). In addition, IL-4, keratinocyte chemoattractant (KC), and IL-6 were significantly increased in the BALF of IL-31RA KO mice compared with WT control animals (Fig. 4*B*). Eotaxin, IL-17, RANTES, and VEGF also showed trends toward increases in IL-31RA KO mice, but the concentrations of these cytokines in the BALF were close to the limit of detection of the assay (data not shown). IL-5, IL-9, IL-13, and MIP-2 were detected but not significantly changed between groups, and IFN-γ IL-10, IL-12p70, IL-23, and TSLP were investigated but not detected (data not shown).

When BALF was collected from IL-31RA KO mice before OVA Ag sensitization or challenge, we found a striking increase in the number of cells in the lung compared with WT mice (Fig. 4*C*, *left graph*). Differential analysis of infiltrating cell populations in the lung of WT and IL-31RA KO mice showed that IL-31RA KO mice had significantly increased percentages of neutrophil and lymphocyte populations compared with WT mice (Fig. 4*C*, *middle graph*). Because of the increase in total cell numbers present in the lung of IL-31RA KO mice, this translated to increased total numbers of neutrophils, lymphocytes, and macrophages compared with WT mice (Fig. 4*C*, *right graph*). These data demonstrate that naive IL-31RA KO mice already show abnormal levels of infiltrating cell numbers in their lungs before any Ag sensitization.

IL-31RA KO mice exhibit increased responsiveness to oncostatin M intranasal delivery and constitutively express VEGF

The increased number of cells in the lung of naive IL-31RA KO mice suggested an alteration in the lung microenvironment in the absence of IL-31RA. Because both IL-31RA and gp130 can colocalize with OSMR to form the receptor for either IL-31 or OSM, respectively, we hypothesized that the absence of IL-31RA may allow for a relative overrepresentation of the OSM heterodimeric receptor, gp130/OSMR. The increased availability of this receptor would result in increased reactivity to OSM, which could lead to inflammation and tissue remodeling (12, 13). Moreover, an increased responsiveness to OSM in the lung of IL-31RA KO mice may explain both the increased level of lung infiltrating cells in unchallenged mice and the increased susceptibility of IL-31RA KO mice to pulmonary inflammation after Ag challenge.

We tested the hypothesis that IL-31RA KO mice had increased functional OSM receptor by examining production of cytokines and chemokines in the lung BALF of IL-31RA KO or WT littermate controls after i.n. delivery of recombinant OSM. Data summarized in Fig. 5 show that IL-31RA KO mice are more responsive to OSM compared with WT littermate control mice as evidenced by a significant increase in the levels of IL-6 and VEGF in the BALF after i.n. treatment with OSM. In contrast, IL-4 and

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KC are two cytokines that we have found consistently upregulated in IL-31RA KO mice compared with WT animals, but these proteins were not directly induced by i.n. delivery of OSM at the time point tested. However, VEGF is known to be involved in enhanced Th2 sensitization, cytokine production, and lung remodeling (20), suggesting a secondary effect of OSM signaling through VEGF production might be involved in the observed increase in IL-4 and KC in sensitized IL-31RA KO mice. Other cytokines that were tested but not found to be differentially expressed included fibroblast growth factor, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-10, IL-12, IL-13, IL-17, IP-10, MCP-1, MIG, MIP-1α, and TNF-α (data not shown).

Of note, when the levels of IL-6 and VEGF were compared between OSM- or PBS-challenged IL-31RA KO mice, IL-6 levels were found to be specifically induced by OSM treatment, whereas VEGF levels were already significantly increased in IL-31RA KO mice and were found to increase further after OSM treatment (Fig. 5, *left* and *right graphs*, respectively). These data suggest that the absence of IL-31RA expression in mice not only results in increased responsiveness to OSM but results in increased basal levels of VEGF in the lung of naive animals, which may promote their susceptibility to Th2 sensitization (21).

To further test the hypothesis that increased OSM responsiveness was driving enhanced Th2 responses in IL-31RA KO mice, we attempted to neutralize OSM during the airway hyperresponsiveness model using a commercially available OSM neutralizing Ab. To determine whether anti-OSM treatment was effective, we analyzed BALF levels of IL-6, VEGF, and TIMP-1, three factors known to be induced by OSM stimulation (22). No significant decline in the levels of any of these cytokines was found in the BALF of sensitized and anti-OSM–treated mice, suggesting that OSM neutralization was not effective (Fig. 6*A*). The anti-OSM Ab used in this study has been reported to have limited neutralizing efficacy in vivo (23) (H. Arnett, personal communication), and although analysis of OSM in the BALF showed a trend in decreasing OSM levels, no significant difference in concentration between groups treated or not with the anti-OSM Ab was observed (with the caveat that it was not known whether the OSM assay used can detect both bound and neutralized OSM, as well as unbound OSM; Fig. 6*B*). Our results did indicate that although airway-sensitized IL-31RA KO and WT mice had similar levels of OSM in the BALF, the level of IL-6, VEGF, and TIMP-1 in the BALF of IL-31RA KO mice was 10- to 100-fold greater than in WT mice (Fig. 6). Taken together with our results from the OSM challenge of IL-31RA KO mice (Fig. 5), these data demonstrate that IL-31RA KO mice exhibit an increased response

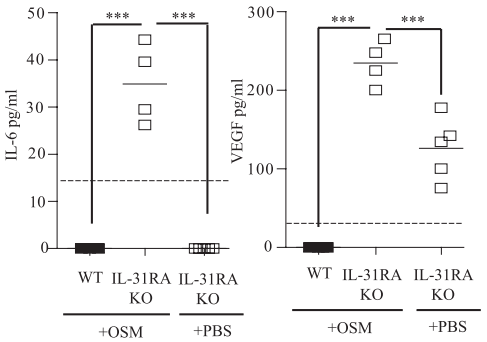

FIGURE 5. Levels of IL-6 (*left graph*) and VEGF (*right graph*) in BALF from WT littermate control mice challenged i.n. with OSM ($n = 4$), IL-31RA KO mice challenged i.n. with OSM ($n = 4$), and IL-31RA KO mice challenged i.n. with PBS ($n = 5$). Data are representative of two independent experiments. $***p < 0.0001$, one-way ANOVA.

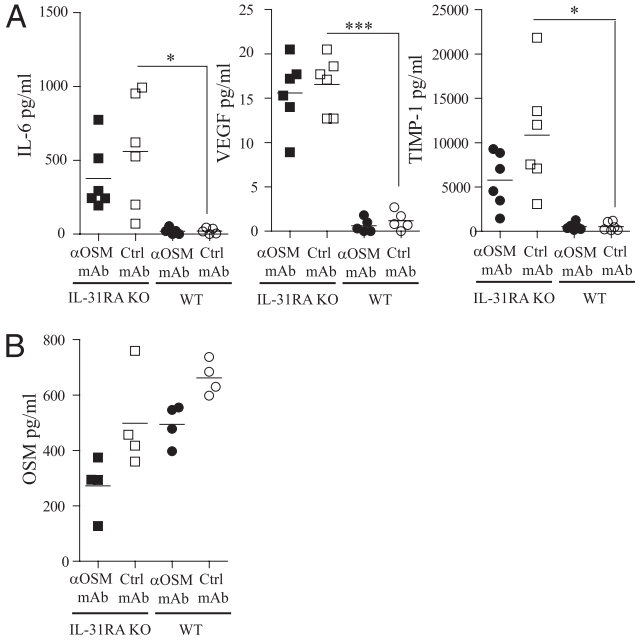

FIGURE 6. Levels of cytokines in BALF of Ag-sensitized mice after treatment with anti-OSM mAb. *A*, IL-6, VEGF, and TIMP-1 levels in BALF from IL-31RA KO mice (squares; $n = 6$) and WT littermate controls (circles; $n = 6$) treated with an anti-OSM Ab (closed symbols) or isotype control (open symbols). $*p < 0.05$; $***p < 0.0001$, unpaired t test. *B*, Levels of OSM in BALF from IL-31RA KO mice (open squares; $n = 4$) and WT littermate control mice (closed squares; $n = 4$) treated with an anti-OSM Ab or isotype control.

to OSM and produce exacerbated levels of OSM-inducible cytokines such as IL-6, VEGF, and TIMP-1 during airway sensitization and challenge.

Discussion

Recent studies investigating parasitic infections of IL-31RA KO mice have suggested that IL-31 signaling is required to negatively regulate Th2-mediated inflammation (10, 11). These results are somewhat contrary to studies that suggest IL-31 plays a role in the development and exacerbation of the Th2-associated disease AD. The latter hypothesis was formed following a number of published studies from various groups. Investigation of IL-31 transgenic mice showed that overexpression of IL-31 resulted in the development of AD-like lesions, and that IL-31 expression was highly associated with Th2-skewed T cells (1). In a nontransgenic murine model of spontaneous AD development, increased levels of IL-31 mRNA were associated with scratching behavior (24, 25), and subsequent inhibition of scratching was achieved using a neutralizing anti–IL-31 mAb (15). Finally, increased levels of IL-31 mRNA have been associated with samples from human AD patients (2, 5), and these IL-31 levels were further shown to correlate with increases in IL-4 and IL-13 (3).

Because the discrepancy surrounding IL-31 biology may be a result of using genetically modified animals to investigate IL-31 function, we tested whether neutralization of IL-31 in vivo using an mAb would result in similar findings to those of IL-31RA KO mice. The anti–IL-31 mAb used in our studies has been shown to neutralize biological activity of IL-31 in vitro and in vivo (15), and in this study the same mAb was used to inhibit IL-31 activity in two Th2 cytokine-associated immune response models. In this paper, we report no alteration or exacerbation of the Th2-cytokine profile produced by Ag-specific T cells in the presence of IL-31 neutralization in either the Ag-driven systemic immunization model or in an Ag-specific airway sensitization model.

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We hypothesized that the disparity between our results, using a neutralizing anti–IL-31 mAb, and the studies using IL-31RA KO mice might be explained by the nature of the family of receptors to which the IL-31 heterodimeric receptor belongs. It is well established that IL-31 signals through the heterodimeric receptor composed of IL-31RA and OSMR (1). The OSMR subunit is also shared by the type II heterodimer receptor for OSM, which comprises OSMR and gp130. The OSMR/gp130 receptor complex is expressed on numerous cell types including epithelial cells, fibroblasts, endothelial cell, hepatocytes, chondrocytes, and cells within the neuronal system (reviewed in Refs. 21, 26). OSM is functionally pleiotropic and has been associated with hematopoiesis, inflammation, and cell survival (reviewed in Ref. 27). Given the interchangeability of the IL-31RA, OSMR, and gp130 receptor subunits, we hypothesized that genetic deletion of IL-31RA could leave the OSMR subunit free to pair with gp130, resulting in overabundance of the functional receptor for OSM and an increased responsiveness to OSM protein.

Strikingly, naive IL-31RA KO mice had abnormally high levels of lung cell infiltrates, which largely consisted of neutrophils, lymphocytes, and macrophages, with few to no eosinophils. In contrast, published studies testing delivery of recombinant OSM in mice have shown lung cell infiltrates to contain a large eosinophil component, although this finding is mouse strain dependent (12, 13). The primary difference between these reports and our current study is that we are using IL-31RA KO mice, which lack expression of IL-31RA throughout development. The prolonged absence of IL-31RA, which we hypothesize leads to enhanced OSM signaling, may alter the profile of cellular infiltrates over time. Alternatively, lack of IL-31RA expression by certain cell populations may alter downstream cytokine and chemokine profiles, which could result in the observed differences compared with the previously published studies.

To directly test the hypothesis that IL-31RA KO mice exhibit increased responsiveness to OSM, we challenged IL-31RA KO mice with recombinant OSM and analyzed the subsequent response compared with WT mice. Intranasal challenge with OSM resulted in statistically significant increases in the OSM-associated cytokines IL-6 and VEGF in the IL-31RA KO mice compared with WT animals. OSM is known to induce VEGF production from airway smooth muscle cells (28), and VEGF is known to induce remodeling and enhance Th2-mediated sensitization in the lungs (20, 29). Our studies also demonstrated that although OSM protein is produced in BALF of both Ag-sensitized IL-31RA KO and WT mice at comparable levels, the concentration of IL-6, VEGF, and TIMP-1, three cytokines known to be induced by OSM, was 10- to 100-fold greater in the BALF of IL-31RA KO mice compared with control animals. These data support our hypothesis that IL-31RA KO mice are more responsive to OSM, and this heightened sensitivity would likely be exacerbated during Ag sensitization in the lungs, where OSM is present. Thus, the enhanced Th2 responses observed in IL-31RA KO after *S. mansonii* infection (10) may be a result of increased levels of factors such as VEGF, induced through OSM signaling, rather than absence of negative regulation by IL-31.

In an effort to definitively demonstrate that an increased responsiveness to OSM was the contributing factor to the enhanced Th2-type responses by IL-31RA KO mice, we attempted to neutralize OSM during our airway sensitization and challenge model with a commercially available anti-OSM Ab. However, in agreement with others (22) (H. Arnett, personal communication), we found this Ab to have limited neutralizing activity in vivo, as evidenced by the lack of significant decreases in OSM, IL-6, VEGF, or TIMP-1 in the BALF after Ab treatment. Thus, because of the unavailability of a suitable reagent, we were unable to definitively demonstrate that enhanced OSM signaling is directly responsible for the exacerbated Th2 responses demonstrated in these mice.

In summary, our focus was to determine whether neutralization of IL-31 in vivo with a specific neutralizing mAb would result in skewing of an Ag-specific immune response. Our data demonstrate that in vivo neutralization of IL-31 does not exacerbate the production of Th2 cytokines in two Th2 cytokine-associated immune response models. Moreover, we found that IL-31RA KO mice exhibit an increased response to OSM, producing VEGF, among other cytokines, which is known to increase susceptibility to Th2 sensitization (28). Our data, therefore, offer an alternative explanation to recent suggestions that IL-31 negatively regulates Th2-type responses and instead indicate that the susceptibility of IL-31RA KO mice to exacerbated Th2-type diseases is an indirect result of IL-31RA deletion that causes an increased responsiveness to OSM.

Disclosures

S.R.D. is a current employee and stockholder of ZymoGenetics, Inc. J.B., S.M., E.C., and B.H. are ZymoGenetics, Inc. stockholders and former employees. ZymoGenetics, Inc. has filed various patent applications related to IL-31.

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