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Identification of Functional Roles for Both IL-17RB and IL-17RA in Mediating IL-25-Induced Activities

Erika A. Rickel,* Lori A. Siegel,* Bo-Rin Park Yoon,* James B. Rottman, † David G. Kugler,* David A. Swart,* Penny M. Anders,* Joel E. Tocker,* Michael R. Comeau,* and Alison L. Budelsky1*

IL-25 (IL-17E) is a unique IL-17 family ligand that promotes Th2-skewed inflammatory responses. Intranasal administration of IL-25 into naïve mice induces pulmonary inflammation similar to that seen in patients with allergic asthma, including increases in bronchoalveolar lavage fluid eosinophils, bronchoalveolar lavage fluid IL-5 and IL-13 concentrations, goblet cell hyperplasia, and increased airway hyperresponsiveness. IL-25 has been reported to bind and signal through IL-17RB (IL-17BR, IL-17Rh1). It has been demonstrated recently that IL-17A signals through a heteromeric receptor composed of IL-17RA and IL-17RC. We sought to determine whether other IL-17 family ligands also utilize heteromeric receptor complexes. The required receptor subunits for IL-25 biological activities were investigated in vitro and in vivo using a combination of knockout (KO) mice and antagonistic Abs. Unlike wild-type mice, cultured splenocytes from either IL-17RB KO or IL-17RA KO mice did not produce IL-5 or IL-13 in response to IL-25 stimulation, and both IL-17RB KO and IL-17RA KO mice did not respond to intranasal administration of IL-25. Furthermore, treatment with antagonistic mAbs to either IL-17RB or IL-17RA completely blocked IL-25-induced pulmonary inflammation and airway hyperresponsiveness in naïve BALB/c mice, similar to the effects of an antagonistic Ab to IL-25. Finally, a blocking Ab to human IL-17RA prevented IL-25 activity in a primary human cell-based assay. These data demonstrate for the first time that IL-25-mediated activities require both IL-17RB and IL-17RA and provide another example of an IL-17 family ligand that utilizes a heteromeric receptor complex.


Materials and Methods

Mice

C57BL/6 and BALB/c wild-type (WT) mice were obtained from Charles River Laboratories and Taconic Farms. The generation of IL-17RA−/− (IL-17RA KO) mice has been described previously (16), and the IL-17RA KO mouse breeding colony is maintained at Taconic Farms. IL-17RB−/− (IL-17RB KO) mice were generated as follows. A gene targeting vector containing IL-17RA and IL-17RC (15). In light of this information, we investigated the receptor subunits required for IL-25 activities using a combination of KO mice and antagonistic Abs. The roles for both IL-17RB and IL-17RA in mediating IL-25-induced events were first investigated using IL-17RB KO and IL-17RA KO mice. KO mouse experiments were validated by studies using antagonistic Abs to these receptors. Finally, the mouse data were followed up by examining the role for IL-17RA in IL-25-mediated activity in a human primary cell-based assay. Taken together, these data provide compelling evidence that IL-25 biological activities require both IL-17RB and IL-17RA, identifying IL-25 as another IL-17 family cytokine that utilizes IL-17RA in a heteromeric receptor complex.

The biological effects of IL-25 are reported to be mediated through IL-17RB (also known as IL-17BR or IL-17Rh1), a member of the IL-17R family of cytokine receptors (1, 2). IL-25 binds with high affinity to and activates signaling pathways through IL-17RB (11–14). It has, however, recently been reported that IL-17A and IL-17F signal through a heteromeric receptor complex containing IL-17RA and IL-17RC (15). In light of this information, we investigated the receptor subunits required for IL-25 activities using a combination of KO mice and antagonistic Abs. The roles for both IL-17RB and IL-17RA in mediating IL-25-induced events were first investigated using IL-17RB KO and IL-17RA KO mice.

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2 Abbreviations used in this paper: KO, knockout; WT, wild type; BALF, bronchoalveolar lavage fluid; BAL, bronchoalveolar lavage; AHR, airway hyperresponsiveness; MCh, methacholine; PAS, periodic acid-Schiff; TSLP, thymic stromal lymphopoietin; i.n. = intranasal; PNiH, enhanced pause; TRAF6, TNFR-associated factor 6.

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designed to remove a region of exon 3 of the murine IL-17RB gene encoding a H335-R73. 129-derived embryonic stem cells were electroporated with the targeting vector and selected in the presence of G418 and ganciclovir as previously described (17). Embryonic stem cell clones carrying the targeted insertion in IL-17RB were identified by a combination of PCR and genomic Southern blot analyses and were injected into Swiss Black blastocysts. PCR genotyping was performed using primers 3291-82 (5′-CCAAGTCCCAGAGTTAGCT-3′) and 3291-83 (5′-CCCATGGTCCCTCCCTTATGTC-3′) that produce a 336-bp product for the WT allele and a 280-bp product for the KO allele. Male chimeras were crossed to Swiss Black females to generate mice heterozygous for the IL-17RB mutation (IL-17RB+/−). IL-17RB−/− mice were subsequently intercrossed to generate IL-17RB−/− mice (IL-17RB KO). IL-17RB KO mice were used at a C57BL/6 background using Marker-Assisted Accelerated Backcrossing (MAX-BAX) technology at Charles River Laboratories. Mice were bred to be 95–95% C57BL/6 were used to block Ag-specific responses in mice expressing the WT allele and to block IL-17RB−/− mice were used at 8–12 wk of age and were age and sex matched within experiments.

**Generation of mAbs to mouse IL-17B, mouse IL-25, mouse IL-17RA, and mouse IL-17A**

A mAb to mouse IL-17B was generated in IL-17RB KO mice using standard hybridoma techniques. Briefly, IL-17RB KO mice were immunized with recombinant mouse IL-17RB.hIg (R&D Systems). Spleens and inguinal lymph node cells from mice treated with the respective Ag-positive mAb to mouse IL-17RB were fused with equal numbers of NS-1 mouse myeloma cells. Ag-positive hybridomas were identified by ELISA and the resulting hybridomas were cloned by limiting dilution. IgG was purified from cell culture supernatants from subcloned hybridoma lines and tested for their ability to block IL-17B-induced IL-5 production in a mouse splenocyte assay. An Ab to mouse IL-17B was chosen for in vivo use that completely blocked IL-17B-induced IL-5 production in a mouse splenocyte assay (IC50 ~8 nM).

A mAb to mouse IL-25 was generated using standard hybridoma techniques in Lewis rats. Briefly, Lewis rats were immunized with mouse IL-25 (R&D Systems). Spleen and inguinal lymph node cells from rats treated with a positive Ab were fused with NS-1 mouse myeloma cells, and the resulting Ag-positive hybridomas were cloned by limiting dilution. IgG was purified from cell culture supernatants from subcloned hybridoma lines and tested for their ability to block IL-25-induced IL-5 production in a mouse splenocyte assay. An Ab to mouse IL-25 was chosen for in vivo use that completely blocked IL-25-induced IL-5 production in a mouse splenocyte assay (IC50 ~37 nM).

A mAb to mouse IL-17A was generated using standard hybridoma techniques in Lewis rats. Briefly, rats were immunized with recombinant mouse IL-17RA.Fc (R&D Systems), spleens and inguinal lymph node cells were fused with NS-1 mouse myeloma cells, and the resulting Ag-positive hybridomas were cloned by limiting dilution. IgG was purified from cell culture supernatants from subcloned hybridoma lines and tested for their ability to block IL-17A-induced IL-6 production from cultured NIH-3T3 cells (18). An Ab to mouse IL-17A was chosen for in vivo use that completely blocked IL-17A-induced IL-6 production in the 3T3 cell assay (IC50 ~500 nM). This Ab does not bind mouse IL-17B.hIg or mouse IL-17B.hIg by ELISA.

A mAb to mouse IL-17A was developed as described previously (18).

**Splenocyte cultures**

Spleens were removed aseptically from C57BL/6 or BALB/c mice and treated with 0.4 mg/ml collagenase D (Roche) and 0.1% DNase I (Roche) in RPMI 1640 (Life Technologies) to generate single-cell suspensions. Splenocytes were cultured for 72 h at 37°C in a 5% CO2 humidified incubator at 2.0 × 107 cells/ml in complete DMEM (Life Technologies) alone or with the addition of 1 µg/ml Con A (Sigma-Aldrich), mouse IL-25 (Amgen), mouse IL-17A (R&D Systems), or mouse IL-17F (R&D Systems) at the indicated final concentrations. Inhibition experiments were done in the presence of 100 µg/ml anti-mouse IL-17RB mAb, anti-mouse IL-17RA mAb, or a control mAb. Abs were preincubated with splenocytes for 45 min before addition of mouse IL-25. The cell supernatants were examined for IL-5 and IL-13 concentrations by ELISA (R&D Systems).

**Intranasal cytokine challenge protocol**

Mice were intranasally dosed with 0.5 µg of murine IL-25 (Amgen), 0.5 µg of murine IL-13 (Biosystems), 0.5 µg of murine IL-17F (R&D Systems), or an equal volume (50 µl) of vehicle. Cytokines were diluted in PBS (Life Technologies) containing 10 µg/ml mouse serum albumin (Sigma-Aldrich) or in PBS alone. IL-25, IL-13, and IL-17F intranasal challenge was performed once daily for 5 consecutive days. All mice were analyzed 24 h after the final intranasal challenge.

**Ab treatment protocol**

BALB/c mice were injected i.p. once daily for 4 consecutive days with 250 µg of anti-IL-25 mAb (Amgen), 250 µg of anti-IL-17B mAb (Amgen), 200 µg of anti-IL-17RA-mAb (Amgen), 200 µg of anti-IL-17A mAb (Amgen), or appropriate protein control 3 h before receiving the intranasal challenge.

**Bronchovascular lavage fluid cellularity and cytokine concentrations**

Bronchovascular lavage (BAL) was performed by intubating mice anesthetized with a 300-µl i.p. injection of 2.5% Avertin (2,2,2-trimethoxy-ethanol; Sigma-Aldrich) and flushing the lungs with 2 × 600-µl volumes of ice-cold Dulbecco’s PBS (Life Technologies). BAL fluid cells were pelleted by centrifugation at 0.1 × g in an Eppendorf centrifuge model 5415D for 10 min, resuspended in PBS plus 5% FBS (HyClone), and analyzed using an Advia 120 hematology machine (Bayer). A correlation between the BAL cellularity counts provided by the AutoBAL software and by manual differentials was confirmed using a linear regression analysis. The cleared BAL fluid was analyzed by ELISA for IL-5, IL-13, IL-17A, or IL-17F levels according to the manufacturer’s directions (R&D Systems). The limit of detection for each ELISA used in this study is as follows: IL-5, 31 pg/ml; IL-13, 61 pg/ml; IL-17A, 15 pg/ml; and IL-17F, 23 pg/ml. Samples with cytokine concentrations below the limit of detection were reported as the limit of detection for the ELISA.

**Airway hyperresponsiveness measurements**

Airway hyperresponsiveness (AHR) to acetyl-l-methylcholine (MCh; Sigma-Aldrich) challenge was measured noninvasively in conscious, unrestrained mice using a whole-body plethysmograph (Buxco Electronics) as described previously (19). Briefly, mice were placed into individual plethysmograph chambers and exposed to aerosolized vehicle (distilled water) followed by increasing concentrations of aerosolized MCh (3–50 mg/ml). Enhanced pause (PENH) was recorded and expressed as the percent change relative to baseline in response to MCh challenge.

AHR to MCh challenge was also measured in anesthetized and mechanically ventilated mice. Mice were sedated with xylazine hydrochloride (20 mg/kg i.p.) and anesthetized with sodium pentobarbital (100 mg/kg i.p.). The trachea was cannulated with a metal needle and the mouse was connected to a small animal ventilator (flexVent, SCIREQ; Scientific Respiratory Equipment). Each mouse was ventilated twice to total lung capacity and passive expiration with a rate of 150 breaths/minute and amplitude of 10 ml/kg mouse weight. A positive end expiratory pressure of 3.0 cm of H2O was established by the connection of the mouse to a water column. After the mouse was ventilated for 1 min, the lungs were expanded twice to total lung capacity (amplitude pressure of 30 cm of H2O). An aerosol of either saline or increasing concentrations of MCh was delivered to the lung for 15 s followed by 15 s of ventilation. Following aerosol and ventilation, a 2.5-Hz volume-driven oscillation was applied to the airway opening. Each of the 10- to 2.5-Hz volume-driven oscillations had 0.20-ml amplitude and lasted 1.25 s. Before the next dose of MCh, lungs were expanded twice to total lung capacity. Pressure and volume measurements over time in the respiratory system were recorded by the small animal ventilator, and respiratory system resistance (R) was calculated by fitting the data to the single compartment model of the respiratory system where PR = RV + EV + P0 (P0 = tracheal pressure, V = volume/time, E = elastance= pressure/volume, V = volume, P0 = baseline pressure).

**Lung histology**

Mouse lungs were perfused with 1 ml of 10% neutral-buffered formalin solution (Sigma-Aldrich) instilled directly into the trachea and then immersed in neutral-buffered formalin solution for 18 h. Lungs were then processed, embedded in paraffin, sectioned at a thickness of 6 µm, and stained with H&E or periodic acid-Schiff (PAS). Lung inflammation was assessed by a pathologist blinded to the treatment groups. The following scoring system was used for assessing goblet cell hyperplasia in PAS-stained sections: 0 = normal; 1 = minimal, goblet cell hyperplasia in large
bronchioles; 2 = mild, goblet cell hyperplasia in large and medium bronchioles; 3 = moderate, goblet cell hyperplasia in large, medium, and some small bronchioles; and 4 = marked, goblet cell hyperplasia in all airways.

Quantitative real-time PCR analysis of whole lung tissue

Total RNA was isolated from individual frozen lungs using a Qiagen RNeasy Maxi kit following the manufacturer’s instructions. cDNA was prepared from lung total RNA using a High Capacity cDNA Archive Kit (Applied Biosystems) following the manufacturer’s instructions. The following Assay on Demand TaqMan primers were purchased (PE Applied Biosystems): mouse CCL11 (Mm00441224_m1), CCL12 (Mm00441225_m1), IL-5 (Mm00439646_m1), IL-9 (Mm00434305_m1), IL-10 (Mm00439616_m1), IL-13 (Mm00432404_m1), IL-17A (Mm00439619_m1), IL-17RA (Mm00434214_m1), IL-17RB (Mm00444706_m1), and IL-17RC (Mm01184649_m1). TaqMan analysis was performed on the Applied Biosystems Prism 7900HT Fast RT-PCR System (PE Applied Biosciences). Each data point represents expression results from individual mouse lungs.

The relative expression of each gene to GAPDH (PE Applied Biosystems) gene expression in each treatment group was determined by SDS 2.2.3 (PE Applied Biosciences).

**Human PBMC IL-25 bioassay**

PBMC were isolated from heparinized human whole blood obtained from normal donors and set up in culture at 5 × 10^6 cells/ml for 24 h with 100 ng/ml recombinant human thymic stromal lymphopoietin (TSLP; Amgen) in X-VIVO 15 (Lonza) plus 5% human AB serum (Lonza). PBMC were added to the PBMC culture at the time of IL-2 plus IL-25 restimulation.

After 3 days of culture, supernatants were harvested and assessed for IL-5 and IL-17RB were preincubated with PBMC for 30 min at room temperature before restimulation with IL-2 plus IL-25. The Ab to human IL-17A was added to the PBMC culture at the time of IL-2 plus IL-25 restimulation. After 3 days of culture, supernatants were harvested and assessed for IL-5 production by ELISA (R&D Systems). The goat anti-human IL-17A Ab (R&D Systems; catalog no. AF177) and mouse anti-human IL-17A mAb clone 41809 (R&D Systems; catalog no. MAB317), or 10 µg/ml mouse anti-human IL-17RA mAb clone 133621 (R&D Systems; catalog no. MAB1771), 10 µg/ml mouse human IL-17RA.Fc (Amgen) (20), 10 µg/ml mouse anti-human IL-17A mAb clone 41809 (R&D Systems; catalog no. MAB317), or 10 µg/ml of an appropriate protein control. Soluble human IL-17RB.Fc and soluble human IL-17RA.Fc were preincubated with IL-2 plus IL-25 for 30 min before addition to the PBMC cultures. Abs to human IL-17A and IL-17RB were preincubated with PBMC for 30 min at room temperature before restimulation with IL-2 plus IL-25. The Ab to human IL-17A was added to the PBMC culture at the time of IL-2 plus IL-25 restimulation.

As previously reported (22), intranasal IL-25 increased total BALF leukocytes, in-duced BALF cellularity, BALF IL-5 and IL-13 concentrations, or lung proinflammatory gene mRNA levels in response to intranasal administration of IL-25.

**Results**

**Splenocytes from IL-17RB KO and IL-17RA KO mice do not produce IL-5 or IL-13 in response to IL-25 stimulation**

To begin investigating the relationship between IL-25 and IL-17RB, we generated IL-17RB KO mice on a C57BL/6 background. IL-17RB KO mice develop normally and produce litters of normal size with equivalent numbers of males and females. There was no obvious phenotype when IL-17RB KO mice were examined using routine anatomic and clinical pathology tests. A role for IL-17RB in mediating IL-25-induced responses was first analyzed in splenocytes from IL-17RB KO mice. Cultured splenocytes from IL-17RB KO mice did not produce IL-5 or IL-13 in response to IL-25 stimulation, unlike WT splenocytes (Fig. 1A). IL-17RB KO splenocytes did produce significantly increased concentrations of IL-5 and IL-13 in response to Con A stimulation compared with stimulation with medium alone; however, IL-17RB KO splenocyte responses was next analyzed in splenocytes from IL-17RA KO mice. Cultured splenocytes from IL-17RA KO mice also did not produce IL-5 or IL-13 in response to IL-25 stimulation (Fig. 1B). Similar to IL-17RB KO splenocytes, IL-17RA KO splenocytes produced significantly increased concentrations of both IL-5 and IL-13 in response to Con A stimulation compared with stimulation with medium alone (Fig. 1B). These data suggest for the first time that IL-17RA is required for mediating IL-25-induced activities.

Interestingly, IL-17RA KO splenocytes produced significantly more IL-5 and IL-13 compared with WT splenocytes in response to Con A stimulation (Fig. 1B). It is unclear at this time why Con A stimulation elicited a lower response from IL-17RB KO splenocytes compared with WT splenocytes, but elicited a higher response from IL-17RA KO splenocytes compared with WT splenocytes; however, one possibility is that a cell type important for normal immune responses is absent in these KO mice spleens. To further investigate the roles of IL-17RB and IL-17RA in WT splenocytes, antagonistic Abs to IL-17RB or IL-17RA were tested in splenocyte experiments using cells from WT BALB/c mice. IL-25 induced more IL-5 production from BALB/c splenocytes compared with WT splenocytes, as might be expected because Th2 responses are easier to induce in BALB/c mice compared with C57BL/6 mice (21). Anti-mouse IL-17RB mAb and anti-mouse IL-17RA mAb treatment significantly blocked IL-25-induced IL-5 production from WT BALB/c splenocytes (Fig. 1C). The combination of splenocyte data from IL-17RB KO mice, IL-17RA KO mice, and the antagonistic Ab experiments provide the first in vitro evidence that IL-25 activities may require both IL-17RB and IL-17RA.

IL-17RA has been reported previously to be required for activity of both IL-17A and IL-17F (15). We therefore next examined the potential role of IL-17A and IL-17F in mediating IL-25-induced activities in this BALB/c splenocyte assay. IL-17A and IL-17F concentrations were not detectable by ELISA in the cell culture supernatants from IL-25-stimulated splenocytes (data not shown), suggesting that IL-25 does not induce IL-17A or IL-17F production. In addition, IL-17A and IL-17F stimulation of mouse splenocytes did not induce IL-5 or IL-13 production even at concentrations up to 100 ng/ml (data not shown). Together these data suggest that IL-25-induced IL-5 and IL-13 production from splenocytes is not dependent on IL-17A or IL-17F, providing support for a direct role for IL-17RA in mediating IL-25 responses.

IL-17RB KO and IL-17RA KO mice do not show increased BALF cellularity, BALF IL-5 and IL-13 concentrations, or lung proinflammatory gene mRNA levels in response to intranasal administration of IL-25.

The in vivo roles for IL-17RB and IL-17RA in mediating IL-25-induced activities were next examined using an intranasal cytokine challenge protocol in IL-17RB KO mice and IL-17RA KO mice. As previously reported (22), intranasal IL-25 increased total BALF leukocyte numbers in WT mice, including increases in BALF eosinophils, neutrophils, and, to a lesser extent, lymphocytes and macrophages (Fig. 2A). Intranasal IL-25, however, did not increase BALF leukocyte numbers in IL-17RB KO mice (Fig. 2A). Intra-nasal IL-13 administration increased total BALF leukocytes, including BALF eosinophils, neutrophils, and lymphocytes to a similar degree in both IL-17RB KO mice and WT mice (Fig. 2B), providing evidence that these mice can recruit leukocytes to the
The induction of proinflammatory genes in the lungs in response to intranasal IL-25 challenge was next examined in WT and IL-17RB KO mice. Transcript levels of proinflammatory cytokines and chemokines previously identified as being up-regulated by IL-25 or other IL-17 receptor and ligand family members were chosen for evaluation (4, 7, 23). Intranasal IL-25 administration significantly increased lung expression of CCL2 and CCL11 mRNAs in WT mice, but did not increase expression of either of those genes in IL-17RB KO mice (data not shown). There was a trend toward an increase in IL-17A mRNA in IL-25-treated WT mice, but not in IL-25-treated IL-17RB KO mice (Fig. 2F). Expression of IL-17RA and IL-17RC mRNAs was equivalent in WT and IL-17RB KO mice lungs (Fig. 2G), providing evidence that disruption of expression of IL-17RB by homologous recombination did not affect expression of these other IL-17R family members. Together with the BALF data, the lung mRNA analyses show that IL-17RB KO mice do not respond to IL-25 stimulation, providing evidence that IL-25 activities require IL-17RB.

The effects of intranasal IL-25 administration in WT and IL-17RA KO mice were next examined. Intranasal IL-25 administration did not increase total BALF leukocyte numbers in IL-17RA KO mice, but did in WT mice (Fig. 3A). Intranasal IL-13 administration, however, increased total BALF leukocytes, including BALF eosinophils, neutrophils, and lymphocytes to an equal extent in both IL-17RA KO and WT mice (Fig. 3B), demonstrating that these mice could recruit BALF leukocytes in response to a different cytokine stimulus. Intranasal IL-25 administration also did not increase BALF IL-5 and IL-13 concentrations in IL-17RA KO mice, but did in WT mice (Fig. 3C). Finally, intranasal IL-25 administration did not significantly increase the lung mRNA levels.
FIGURE 2. Intranasal IL-25 administration did not increase BALF cellularity, BALF IL-5 and IL-13 concentrations or expression of proinflammatory genes in IL-17RB KO mice. IL-17RB KO or WT mice (n = 5 per group) were i.n. dosed with vehicle, 0.5 µg of IL-25, or 0.5 µg of IL-13 for 4 days, and BALF and lungs were analyzed 24 h after the final dose. A and B, Differential counts were performed on the BALF. The mean total number of BALF cells ± SE is shown for each group. C, BALF IL-5 and IL-13 concentrations were measured by ELISA from individual mice. The mean concentrations ± SE are plotted. RNA was isolated from individual lungs (n = 4 per group), and expression levels of CCL2 (D), CCL11 (E), IL-17A (F), IL-17RA (G), and IL-17RC (G) were evaluated by TaqMan. Gene expression levels were normalized to GAPDH expression. The data are representative of two independent experiments. Statistical analyses of WT and KO mice were performed using a t-test.
Intranasal IL-25 administration did not increase BALF cellularity, BALF IL-5 and IL-13 concentrations, or expression of proinflammatory genes in IL-17RA KO mice. IL-17RA KO or WT mice (n = 5 per group) were i.n. dosed with vehicle, 0.5 μg of IL-25, or 0.5 μg of IL-13 for 4 days, and BALF and lungs were analyzed 24 h after the final dose. A and B, Differential counts were performed on the BALF. The mean total number of BALF cells ± SE is shown for each group. C, BALF IL-5 and IL-13 concentrations were measured by ELISA from individual mice. The mean concentrations ± SE are plotted. RNA was isolated from individual lungs (n = 4 per group), and expression levels of CCL2 (D), CCL11 (E), IL-17A (F), IL-17RB (G), and IL-17RC (G) were evaluated by TaqMan. Gene expression levels were normalized to GAPDH expression. Data are representative of two independent experiments. Statistical analyses of WT and KO mice were performed using a t test.
of CCL2, CCL11, or IL-17A in IL-17RA KO mice, but did in WT mice (Fig. 3, D–F). In addition, expression of IL-5, IL-13, IL-9, and IL-10 mRNAs were not up-regulated in IL-17RA KO mice treated with intranasal IL-25, but were in WT mice (data not shown). Lung expression levels of IL-17RB and IL-17RC mRNAs in IL-17RA KO mice were similar to those seen in WT mice, providing evidence that disruption of IL-17RA did not affect expression of these other IL-17R family members (Fig. 3G). These data show that IL-17RA KO mice do not respond to IL-25 stimulation, providing the first in vivo evidence that IL-25 activities require IL-17RA.

**IL-17RB KO and IL-17RA KO mouse lungs do not display IL-25-induced histological signs of inflammation**

Intranasal administration of IL-25 caused a significant increase in BALF cellularity in WT mice but not in IL-17RB KO or IL-17RA KO mice. To address the possibility that leukocytes in the lungs of IL-17RB KO or IL-17RA KO mice were unable to extravasate into the airways, the lungs of intranasally dosed WT, IL-17RB KO, and IL-17RA KO mice were examined histologically for signs of inflammation. Intranasal administration of vehicle alone had no inflammatory effect on the lung pathology of any of the mice examined (Fig. 4, A–F). Intranasal IL-25 administration in WT mice induced a prominent inflammatory response in the region around the vessel and in the vessel wall of most of the pulmonary blood vessels, including pulmonary arteries, arterioles, veins, and venules, but not capillaries (Fig. 4, G and H). There was a robust accumulation of eosinophils beneath the endothelium lining these vessels and prominent endothelial hyperplasia (Fig. 4H). In addition to eosinophils, the inflammatory cell infiltrate also consisted of neutrophils, lymphocytes, and monocytes/macrophages (Fig. 4, G and H). The inflammation extended beyond the blood vessels to adjacent bronchioles, and the inflammatory cells traversed the walls to reach the lumen of the airways (Fig. 4G). In addition to the
FIGURE 5. Intranasal IL-25-induced lung inflammation was blocked by anti-IL-25 mAb, anti-IL-17RB mAb, and anti-IL-17RA mAb treatment. BALB/c mice (n = 5 per group) were treated with either control Ab or blocking Abs to IL-25, IL-17RB, IL-17RA, or IL-17A 3 h before each i.n. dose of IL-25. Mice were i.n. dosed with 0.5 μg of IL-25 for 4 days. BALF and lungs were analyzed 24 h after the final IL-25 dose. A, Total number of BALF leukocytes. B, PENH (% above baseline). C, Total number of BALF leukocytes. D, BALF eosinophils. E, Total number of BALF neutrophils. F, IL-5 and IL-13 levels in BALF. G, [PENH, % above baseline]. H, R (cm·H₂O·s/mL).
inflammatory cell infiltrate observed in H&E-stained sections, PAS staining revealed robust goblet cell hyperplasia in the lungs of WT mice intranasally dosed with IL-25 (Fig. 4, M and N). In contrast, intranasal IL-25 administration did not induce any of these histological changes in either IL-17RB KO mice (Fig. 4, I, J, and M) or IL-17RA KO mice (Fig. 4, K, L, and N). The combined BALF, lung mRNA, and lung histology analyses from IL-17RB KO and IL-17RA KO mice administered intranasal IL-25 provide the first in vivo evidence that both IL-17RB and IL-17RA are required for IL-25 responsiveness.

**Anti-mouse IL-17RB mAb treatment or anti-mouse IL-17RA mAb treatment blocks IL-25-induced lung inflammation in BALB/c mice**

To further investigate the essential role of IL-17RB in mediating IL-25 activities in vivo, we compared the effects of an antagonistic Ab to mouse IL-17RB with the effects of an antagonistic Ab to mouse IL-25 on IL-25-induced inflammation in the lung. Naïve BALB/c mice were treated with either anti-IL-17RB mAb or anti-IL-25 mAb before intranasal challenge with IL-25 in the 4-day challenge model. Both anti-IL-17RB mAb and anti-IL-25 mAb treatment inhibited IL-25-induced activities in the lung, including increased BALF leukocytes (Fig. 5A), BALF IL-5 and IL-13 concentrations (data not shown), and AHR (Fig. 5B).

The role of IL-17RA in mediating IL-25 activities in vivo was next examined using an antagonistic Ab to mouse IL-17RA. Naïve BALB/c mice were treated with anti-IL-17RA mAb before intranasal challenge with IL-25 in the 4-day challenge model. Anti-IL-17RA mAb treatment blocked all measured IL-25-induced responses in the lung, including BALF cellularity (Fig. 5, C–E), BALF IL-5 and IL-13 concentrations (Fig. 5F), and AHR (Fig. 5, G and H).

Although we were unable to detect IL-17A protein in the BALF of IL-25-treated WT BALB/c mice (data not shown), the up-regulation of IL-17A lung transcript by IL-25 (Figs. 2F and 3F) suggested the possibility that IL-17A might be playing a role in IL-25-mediated activities. To more closely examine the role of IL-17A in IL-25-induced biological activities, the effects of an antagonistic Ab to mouse IL-17A were also examined in mice intranasally dosed with IL-25. Anti-IL-17A mAb treatment inhibited IL-25-induced BALF neutrophilia (Fig. 5E), but had no effect on IL-25-induced BALF eosinophils (Fig. 5D), lymphocytes (data not shown), and macrophages (data not shown), BALF IL-5 and IL-13 concentrations (Fig. 5F), or AHR (Fig. 5G). These data provide evidence that IL-17A may be playing a role in IL-25-induced BALF neutrophilia in this mouse model, but that all other IL-25-induced activities are independent of IL-17A activity.

The abilities of the anti-IL-17RB mAb and of the anti-IL-17RA mAb to block IL-25-induced activities were further investigated through histological analysis of the lungs from these mice. Intranasal IL-25 administration into BALB/c mice induced pulmonary inflammation (Fig. 6B) and goblet cell hyperplasia (Fig. 6G) similar to that described in WT C57BL/6 mice (Fig. 4, G, H, M, and N). Pulmonary inflammation induced by intranasal IL-25 administration was at the level of background in mice treated with either leukocytes in mice i.n. dosed with IL-25 and treated with control Ab, anti-IL-25 mAb, or anti-IL-17RB mAb. Each dot represents BALF cellularity from one mouse. **B**, AHR in mice treated with control Ab, anti-IL-17RB mAb, or anti-IL-25 mAb. The mean percent change in PENH relative to baseline is reported for each treatment group ± SE. Total BALF leukocytes (**C**), eosinophils (**D**), and neutrophils (**E**) from mice treated with control Ab, anti-IL-17RA mAb, or anti-IL-17A mAb. Each dot represents BALF cellularity from one mouse. **F**, BALF IL-5 and IL-13 concentrations from mice treated with control Ab, anti-IL-17RA mAb, or anti-IL-17A mAb. The bars represent the mean concentration ± SE. **G**, AHR in mice treated with control Ab, anti-IL-17RA mAb, or anti-IL-17A mAb. The mean percent change in PENH relative to baseline is reported for each treatment group ± SE. **H**, AHR was also measured in mechanically ventilated mice treated with vehicle and control Ab (n = 5), IL-25 and control Ab (n = 7), or IL-25 and anti-IL-17RA mAb (n = 8). Mean airway resistance (R) is shown for each treatment group ± SE. Data shown are representative of two independent experiments for A and B, six independent experiments for C–F, and two independent experiments for G and H. Statistical analyses of control and Ab-treated mice were performed using a one-way ANOVA (A and C–F) or a repeated measure ANOVA (H).

**FIGURE 6.** Abs to IL-17RB, IL-17RA, or IL-25 block IL-25-induced histological signs of pulmonary inflammation. BALB/c mice were i.n. dosed with vehicle (A) or 0.5 μg of IL-25 (B–F) for 4 days, and lungs were harvested for histological analysis 24 h after the final dose. Mice (n = 5 per group) were treated with control Ab (B) or blocking Abs to IL-17RB (C), IL-25 (D), IL-17RA (E), or IL-17A (F) 3 h before each i.n. dose. Formalin-fixed lung tissue sections were stained with H&E (A–F) or PAS (G) for analysis. **A–F,** Original magnification, ×200. **G,** Goblet cell hyperplasia was assessed in PAS-stained lung sections as described in Materials and Methods. Statistical analyses were performed using a nonparametric one-way ANOVA.
the anti-IL-17B mAb (Fig. 6C), anti-IL-25 mAb (Fig. 6D), or anti-IL-17RA mAb (Fig. 6E), similar to that seen in mice intranasally dosed with vehicle (Fig. 6A). Moreover, IL-25-induced goblet cell hyperplasia was dramatically reduced in mice treated with anti-IL-17B mAb, anti-IL-25 mAb, or anti-IL-17RA mAb (Fig. 6G). Anti-IL-17A mAb treatment had no effect on IL-25-induced pulmonary lesions (Fig. 6F) or goblet cell hyperplasia (Fig. 6G), providing further evidence that although IL-17A may be involved in IL-25-induced BALF neutrophilia, all other IL-25-induced activities measured in this study are independent of IL-17A activity.

An Ab to IL-17RA can block IL-5 production from IL-25-stimulated human PBMC cultures

The data generated in mice provide substantial evidence of a role for IL-17RA in IL-25-induced activities, leading us to next investigate this relationship in human cells. We first developed a human IL-25 cell-based bioassay and then analyzed the effects of anti-human IL-17RA Abs in that assay. TSLP-stimulated dendritic cells have been shown to induce expression of IL-17RB mRNA in memory Th2 T cells and to expand these IL-25-responsive cells (24, 25). We tested whether or not TSLP stimulation could create an IL-25-responsive environment in a culture of PBMC. In fact, TSLP-stimulated PBMC produced IL-5 in response to IL-25 stimulation, and our subsequent experiments showed that IL-17RB and IL-17RA are required for IL-25 activities. In the present study, we have provided evidence that IL-25-induced activities require both IL-17RB and IL-17RA. IL-17RB has been shown previously to bind IL-25 and to induce activation of multiple signaling molecules in response to IL-25 stimulation (11, 12, 14). Our experiments using IL-17RB KO mice and an antagonistic Ab to IL-17RB provide additional functional data supporting a role for IL-17RB in mediating IL-25-induced activities. A surprising finding was that IL-17RA KO mice also did not respond to IL-25 stimulation, and our subsequent experiments showed that human IL-17RA Ab can block IL-17A activities, unlike the polyclonal Ab to IL-17RA which is known to block IL-17A activities.

Given the polyclonal anti-IL-17RA Ab we tested is reported to provide further evidence that IL-17A does not play a role in the IL-25-induced IL-5 production in this PBMC assay, the effects of two IL-17A antagonists on IL-25-induced IL-5 production were tested. Blockade of endogenous IL-17A using either soluble IL-17RA.Fc or a neutralizing mAb to IL-17A did not inhibit IL-5 production in response to IL-25 stimulation alone (data not shown) or in response to IL-17A plus IL-2 (Fig. 7B). In addition, IL-17A plus IL-25 plus IL-2 produced similar amounts of IL-5 as IL-25 plus IL-2 (data not shown). These data provide evidence that the TSLP-stimulated PBMC do not respond to IL-17A.

We have not been able to detect endogenous IL-17A protein in the PBMC culture medium (data not shown); however, to more closely examine the role of endogenous IL-17A in this PBMC assay, the effects of two IL-17A antagonists on IL-25-induced IL-5 production were tested. Blockade of endogenous IL-17A using either soluble IL-17RA.Fc or a neutralizing mAb to IL-17A did not inhibit IL-5 production in response to IL-25 plus IL-2 (Fig. 7B). The lack of inhibition seen with IL-17A antagonists provide further evidence that IL-17A does not play a role in the IL-25 plus IL-2-induced IL-5 production in this PBMC assay. Taken together, these data demonstrate that IL-17RA plays a functional role in the human IL-25R complex in vitro that is independent of IL-17A, providing an encouraging complement to our data generated in mice.

**Discussion**

In the present study, we have provided evidence that IL-25-induced activities require both IL-17RB and IL-17RA. IL-17RB has been shown previously to bind IL-25 and to induce activation of multiple signaling molecules in response to IL-25 stimulation (11, 12, 14). Our experiments using IL-17RB KO mice and an antagonistic Ab to IL-17RB provide additional functional data supporting a role for IL-17RB in mediating IL-25-induced activities. It is currently unknown whether or not this mouse monoclonal anti-IL-17RB mAb can block IL-17A activities, unlike the polyclonal Ab to IL-17RA which is known to block IL-17A activities.

FIGURE 7. Goat anti-human IL-17RA Ab treatment but not mouse anti-human IL-17A mAb treatment can block IL-25-induced IL-5 production in an in vitro human PBMC assay. A, Human PBMC were cultured in TSLP for 24 h, harvested, and restimulated with IL-2 and IL-25 for an additional 72 h in the presence or absence of soluble IL-17RB.Fc protein, IL-17RA Abs, or appropriate control proteins. The production of IL-5 was determined in the culture supernatants by ELISA and is reported as the mean ± SD. Data shown are from one PBMC donor and are representative of three experiments performed using three different donors. B, Human PBMC were cultured in TSLP for 24 h, harvested, and restimulated with IL-2, IL-2 and IL-25, or IL-2 and IL-17A for an additional 72 h. Inhibition experiments were done in the presence or absence of soluble IL-17RB.Fc, soluble IL-17RA.Fc protein, a blocking IL-17A mAb, or an appropriate control protein. The production of IL-5 was determined in the culture supernatants by ELISA and is reported as the mean ± SD. Data shown are from one PBMC donor and are representative of two experiments performed using two different donors.
shown that IL-25 but not IL-17A induced IL-5 production from published data). Finally, in our human PBMC assay, we have shown that intranasal administration of IL-17A increased BALF influx (22). In addition to increasing BALF neutrophils, we have viral expression of IL-17A in mice induced neutrophil influx into the lung while adenoviral expression of IL-25 induced eosinophil influx (22); therefore, it is not surprising that this component of our mouse experiment could be attributed to IL-17A. The fact that all of the other IL-25-induced activities were independent of IL-17A activity, both in our mouse and human experiments, supports our hypothesis that IL-25 acts directly through a receptor complex comprised of at least IL-17RB and IL-17RA. We did not expect IL-17A to mediate all of the downstream effects of IL-25 that were measured (Figs. 5 and 6). In our human PBMC assay, a mouse anti-human IL-17A mAb did not inhibit IL-25-induced IL-5 production (Fig. 7B). IL-17A mediates recruitment of neutrophils (22); therefore, it is not surprising that this component of our mouse experiment could be attributed to IL-17A. The fact that all of the other IL-25-induced activities were independent of IL-17A activity, both in our mouse and human experiments, supports our hypothesis that IL-25 acts directly through a receptor complex comprised of at least IL-17RB and IL-17RA. We did not expect IL-17A to mediate all of the downstream effects of IL-25 that were measured because it has been shown that IL-17A and IL-25 mediate different activities in vivo and in vitro. For example, adenoviral expression of IL-17A in mice induced neutrophil influx into the lung while adenoviral expression of IL-25 induced eosinophil influx (22). In addition to increasing BALF neutrophils, we have shown that intranasal administration of IL-17A increased BALF KC concentrations 1 h after dosing, while IL-25 did not (our unpublished data). Finally, in our human PBMC assay, we have shown that IL-25 but not IL-17A induced IL-5 production from TSLP-stimulated PBMC (Fig. 7B and data not shown).

Another IL-17 family ligand reported to require IL-17RA for activity is IL-17F (15, 27). Overexpression of IL-17F in the lung has been reported to increase numbers of neutrophils in BALF (24) and lung tissue (22). In contrast, intranasal administration of IL-17F to naive BALB/c mice has been reported to not induce BALF neutrophils (28). To investigate the potential role for IL-17F in mediating IL-25-induced activities, especially BALF neutrophilia, we compared the effects of IL-17F and IL-25 in vitro and in vivo. IL-17F did not induce IL-5 and IL-13 production from cultured BALB/c splenocytes (data not shown) and intranasal IL-17F did not increase BALF leukocytes or BALF IL-5 and IL-13 concentrations (data not shown). These data suggest that IL-17F and IL-25 do not have similar activities in vitro or in vivo. Furthermore, IL-25 did not induce detectable concentrations of IL-17F in stimulated splenocyte cultures or in BALF from intranasally dosed mice (data not shown). Together, these data provide initial evidence that IL-17F is not involved in IL-25-mediated activities. However, future experiments using antagonistic Abs to IL-17F will provide further clarity as to the potential role IL-17F may have in mediating IL-25-induced activities.

The signaling pathways activated by IL-17A and IL-25 include many of the same proteins. For example, stimulation of different cell types with either IL-17A or IL-25 activates TNFR-associated factor 6 (TRAF6), ERK, JNK, and p38, but the mechanisms by which these signaling pathways are activated have been reported to be different (12–14). For example, IL-17A does not contain a TRAF6-binding motif but it has been reported to recruit TRAF6 by directly associating with the adaptor protein Act1 which does contain a TRAF6-binding motif (29). IL-17A and Act1 interact through homotypic interaction of their SEFIR (similar expression to fibroblast growth factor genes and IL-17R) domains, which share similar residues with two of the three conserved motifs present in TIR (Toll/IL-1R) domains (30). Signaling through IL-17RB, in contrast, has been reported to be through direct interaction of the receptor with TRAF6 (12). Unlike IL-17A, the cytosolic region of IL-17RB contains a TRAF6-binding motif but does not contain a SEFIR domain (12, 30). Interestingly, IL-17RC, which has also been reported to interact with IL-17RA, also does not contain a SEFIR domain (15, 30). It is possible that the SEFIR domain of IL-17RA may be important for initiating intracellular signaling pathways, either in response to IL-17A when complexed with IL-17RC or in response to IL-25 when complexed with IL-17RB. Elucidation of the precise functional role of each of these receptors in activating signaling pathways in response to ligand stimulation will require continued investigation.

Collectively, these data demonstrate for the first time that IL-25-mediated activities require the presence of both IL-17RB and IL-17RA, identifying IL-25 as another IL-17 ligand family cytokine that utilizes a receptor comprised of at least two distinct subunits. Additional studies are required to determine how IL-17RA interacts with IL-17RB and in what stoichiometry for mediating IL-25-induced activities. IL-17RA has now been identified to be involved in the receptor complex for IL-17A, IL-17F, and IL-25, and it will be interesting to compare how IL-17RA interacts with other IL-17R family members in these different receptor complexes.

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Disclosures


References

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