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The critical role of epithelial-derived Act1 in IL-17- and IL-25-mediated pulmonary inflammation[Ω]

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Abstract

IL-25 initiates, promotes, and augments Th2 immune responses. We here report that Act1, a key component in IL-17-mediated signaling, is an essential signaling molecule for IL-25 signaling. While Act1-deficient mice showed reduced expression of KC (CXCL1) and neutrophil recruitment to the airway compared to wild-type mice in response to IL-17 stimulation, Act1 deficiency abolished IL-25-induced expression of IL-4, IL-5, IL-13, eotaxin-1 (CCL11), and pulmonary eosinophilia. Using a mouse model of allergic pulmonary inflammation, we observed diminished Th2 responses and lung inflammation in Act1-deficient mice compared to wild-type mice. Importantly, Act1 deficiency in epithelial cells reduced the phenotype of allergic pulmonary inflammation due to loss of IL-17-induced neutrophilia and IL-25-induced eosinophilia, respectively. These results demonstrate the essential role of epithelial-derived Act1 in allergic pulmonary inflammation through the distinct impact of the IL-17R-Act1 and IL-25R-Act1 axes. Such findings are crucial for the understanding of pathobiology of atopic diseases including allergic asthma, which identifies Act1 as a potential therapeutic target.

Introduction

Allergic asthma is a chronic inflammatory disorder of the lung with a prevailing CD4+ T-cell infiltrate in the airways, leading to bronchial hyperreactivity, recruitment of neutrophils, eosinophils, mast cells, and lymphocytes, and hyperplasia of smooth muscle, often associated with elevated serum IgE concentrations(1–3). CD4+ Th cells are essential regulators in chronic allergic diseases. Upon activation, Th cells undergo differentiation into functionally distinct effector subsets(4–8). Th1 cells produce IFNγ and regulate cellular immunity, whereas Th2 cells produce IL-4, IL-5, and IL-13 and mediate humoral immunity and allergic responses. It is well known that antigen-induced allergic airway inflammation is mediated, in part, by Th2 cells and their cytokines (IL-4, IL-5 and IL-13). A novel Th cell subset expressing IL-17 has also recently been shown to regulate tissue inflammatory responses, including allergic airway inflammation(9).

IL-17A, produced by Th17 cells, is the prototypic IL-17 family member, exerting its actions either as a homodimer or as a heterodimer with IL-17F (10). IL-17A causes accumulation of

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neutrophils in the bronchoalveolar of rats and mice in vivo. The main function of IL-17A is to coordinate local tissue inflammation via the upregulation of pro-inflammatory and neutrophil-mobilizing cytokines and chemokines [including IL-6, G-CSF, TNFα, IL-1, CXCL1 (KC), CCL2(MCP-1), CXCL2(MIP-2), CCL7(MCP-3), and CCL20(MIP-3A)] as well as matrix metalloproteases (MMPs) to allow activated T cells to penetrate extracellular matrix. IL-17A deficiency leads to diminished antigen-specific T cell mediated immune responses, including allergen induced pulmonary inflammation and airway hyperresponsiveness(11,12). Elevated IL-17 concentrations were found in the lung and blood of allergic asthma patients and linked to severity of asthma.

Homology-based cloning has revealed five additional IL-17 family members, termed IL-17B to IL17F. The most divergent known member of the IL-17 family is IL-17E (IL-25); it is expressed in mouse T lymphocytes of the CD4+ subset with a Th2 profile and human innate effector eosinophils and basophils(13,14). IL-25 has been shown to play a critical role in the initiation and propagation of the Th2 immune response(14–17). Transgenic expression as well as recombinant IL-25 has been shown to induce Th2 immunity, increase Th2 cytokines IL-4, IL-5, IL-13, eosinophilia and IgE(13,18,19). IL25−/− mice demonstrate a delayed expulsion of helminth parasites, indicative of an impairment of Th2 response(20,21). Further, endogenous IL-25 has been shown to be critical in allergen-induced pulmonary inflammation in a mouse asthma model(17). Elevated IL-25 and IL-25R expression were detected in asthmatic lung tissues, linking their roles in allergic pulmonary inflammation(14). While previous studies showed that the cell type responsible for production of Th2 cytokines following IL-25 exposure is of a nonlymphocyte, non-NK, and non-granulocyte lineage, the identity of the IL-25 responsive cell type(s) remains elusive(13).

IL-17A signals through a heteromeric receptor complex, consisting of IL-17R (IL-17RA) and IL-17RC, which are single-pass transmembrane proteins and ubiquitously expressed in various cell types, including epithelial cells, fibroblasts and astrocytes(22,23). IL-25 signals through IL-25R (IL-17RB, also known as IL-17RH1), which is expressed in human lung, kidney, pancrease, liver, brain, and intestine. IL-17A receptor (IL-17RA and IL-17RC) and IL-25R (IL-17RB) belong to a newly defined SEFIR protein family, due to a conserved sequence segment called SEFIR in their cytoplasmic domain(24). We recently found that a novel signaling molecule, Act1 is a key component in IL-17A signaling(25). Act1 contains two TRAF binding sites, a helix-loop-helix domain at the N-terminus, and a SEFIR domain at the C-terminus and therefore Act1 is a member of the SEFIR protein family. Although the Act1 gene (Traf3ip2) was first cloned as an NFκB activator, our later studies showed that Act1, recruited to CD40 and the TNF family member BAFF receptor in B cells through its interaction with TRAF3, negatively regulates B cell survival(26,27). Balb/c Act1-deficient mice also develop Sjogren's disease in association with lupus nephritis due to the hyper B cell function(28). Distinct from these previous findings, we recently reported that upon IL-17 stimulation, Act1 is recruited to IL-17 receptor through the SEFIR domain, followed by the recruitment of TAK1 and TRAF6, mediating NFkB activation. Our data demonstrate the essential function of Act1 for IL-17-dependent signaling and autoimmune inflammatory disease(25).

In this study, we report a novel function of Act1 in mediating IL-25 signaling and IL-25-dependent Th2 pulmonary responses. Using a mouse model of allergic pulmonary inflammation, we observed reduced Th2 responses and lung inflammation in C57BL/6 Act1-deficient mice compared to littermate control wild-type mice. Importantly, Act1 deficiency in epithelial cells reduced IL-17-induced pulmonary neutrophilia and IL-25-mediated eosinophilia in the airway, indicating the essential role of epithelial-derived Act1 in IL-17- and IL-25-mediated pulmonary inflammation.

Material and Methods

Mice, cell culture and biological reagents

Act1-deficient (Traf3ip2−/−) C57BL/6 (B6) mice were generated as described previously(27). Mice were maintained in a temperature-controlled (23°C) facility with a strict 12-h light–dark cycle and were given free access to food and water. The experiments were performed with gender-matched mice aged 6–8 wk. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of The Cleveland Clinic Foundation [fully accredited by Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC)]. (29). HeLa cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (FBS; Hyclone), penicillin G (100 μg/ml) and streptomycin (100 μg/ml). anti-V5 and anti-FLAG (M2) were from Sigma; anti-phospho-JNK, anti-phospho-ERK were from Cell Signaling.

Transfection and Coimmunoprecipitation

HeLa cells were transiently transfected with 5μg of each Flag-tagged mAct1 and V5-tagged mouse IL-25 R using Fugene6 (Roche) according to the manufacturer's instruction. For coimmunoprecipitations, cells were harvested, washed once with cold phosphate buffered saline and lysed in a TritonX-100 containing buffer (0.5% TritonX-100, 20 mM Hepes pH 7.4, 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 10 mM NaF, 2 mM DTT, 1 mM sodium orthovanadate, 2 mM EGTA, 20 μM aprotinin, 1 mM phenylmethylsulfonyl fluoride). Cell extracts were incubated with 1 μg of Ab (anti-Flag (M2 or anti-V5) or normal IgG (negative control) for 2 h, followed by incubation for 2 h with 30μl of protein A-Sepharose beads (prewashed and resuspended in lysis buffer at a 1:1 ratio). After incubations, the beads were washed 4 times with lysis buffer, separated by SDS-PAGE, and analyzed by immunoblotting.

Mouse model of allergic pulmonary inflammation—Mice were immunized with 100ul intraperitoneal injection containing 10ug of ovalbumin (chicken OVA, grade V; Sigma, St. Louis, MO) and 20mg aluminum hydroxide (Sigma, St. Louis, MO). Sham immunization with Intraperitoneal injection of PBS and 20mg aluminum hydroxide was used as a negative control for immunization procedure Intraperitoneal injection of PBS was used as a negative control for immunization procedure. Wild-type and Act1-deficient mice on a C57BL/6 background were immunized by two intraperitoneal injection on day 1 and day 8.

Control mice (K18-Cre$^{+/-}$*Traf3ip2*$^{+/\text{flox}}$) and epithelial-specific Act1-deficient mice (K18-Cre$^{+/-}$*Traf3ip2*$^{-/\text{flox}}$) on BALBc/J background were immunized by one intraperitoneal injection on day 1. Mice were challenged on day 14,15,16, and 17 by exposure to approximately 40 minutes of 1% ovalbumin aerosol diluted in PBS. This was accomplished by placing mice in a 30cmx60cm acrylic box ventilated by NOUVAG Ultrasonic 2000 nebulizer (NOUVAG USA Inc. Lake Hughes, CA). Mice were sacrificed and processed on day 18, 24 hours after last aerosol challenge.

Intranasal instillation of IL-17 and IL-25—Mice were anaesthetized with isoflurane. Carrier free, recombinant murine IL-17 or IL-25 (R&D systems, Minneapolis, Minnesota) resuspended in sterile saline (0.9%) was instilled into nasal opening in 50 μl aliquot per mouse.

BAL and Tissue collection—Mice were sacrificed at the times indicated. 0.7ml of HL-1 media (BioWhittaker, Walkersville, MD) was used to obtain Bronchoalveolar Lavage fluid (BAL) through trachea using a blunt needle and 1ml syringe. Cytospin slide preparations where obtained using shandon CytoSpin III Cytocentrifuge (Shandon/thermo scientific). Differential leukocyte counts where obtained on cytospin slide preparation after diff quik Giemsa stain. Lungs were collected and snap frozen immediately in liquid nitrogen container. Total RNA was obtained by using Trizol (invitrogen) and OMNI TH tissue homogenizer (omni, international). H&E staining was obtained on lung tissue after fixation in 10% neutral buffered formalin and paraffin embedding.

Primary Tracheal epithelial Cell Culture—Mice were sacrificed and tracheas were excised and placed in medium containing 0.15% pronase and incubated overnight at 4°C. Tracheal epithelial cells were harvested from supernatant and plated on collagen coated cell culture plates. Total RNA isolated using Trizol.

Ovalbumin Specific Immunoglobulin ELISA—Ovalbumin (OVA)-specific Antibodies were determined using enzyme-linked immunsorbent assay (ELISA). Briefly, 96-well, flat-bottom protein absorbent polystyrene plates were coated with Ovalbumin(OVA) (Grade V, Sigma Chemical Company) at 10 μg/well. Plates were then blocked with 2% fetal bovine serum (FBS)/PBS. Plates were washed with PBS. Serum samples and standards (OVA specific IgG2a and IgE) were incubated at 4°C overnight. Biotinylated anti-mouse IgG2a or IgE was used followed by streptavidin conjugated horseradish peroxidase (HRP). Plates were developed and using 2, 2′-azino-di(3-ethylbenzthiazoline-6-sulfonate) ABTS containing solution and quantified using Molecular devices plate reader. Data was analyzed using Softmax pro v.5 software.

Statistical analysis

The data are presented as the mean ± SEM with $n = 4$ animalsper condition. The significance of differences between two groups was determined by Student *t* test (two-tailed). Statistical significance was reported if $P < 0.05$ was achieved.

Quantitative real-time PCR—3μg of total RNA was then used for the reverse transcription reaction using Super Script II-reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed in AB 7300 RealTime PCR System and the gene expression was examined by SYBR GREEN PCR Master Mix (Applied Biosystem). PCR amplification was performed in triplicate, and water was used to replace cDNA in each run as a negative control. The reaction protocol included preincubation at 95°C to activate FastStart DNA polymerase for 10 min, amplification of 40 cycles that was set for 15 s at 95°C, and the annealing for 60 s at 60°C. The results were normalized with the housekeeping gene mouse β-actin. The specific primer sequences used in reaction listed as follows:

Gene	**Primer 1**	**Primer 2**
mouse β-actin:	5′-GGTCATCACTATTGGCAACG-3′	5′-ACGGATGTCAACGTCACACT-3′
mouse IL-5	5′-CTCACCGAGCTCTGTTGACAAG-3′	5′-CCAATGCATAGCTGGTGATTTTTAT-3′
mouse IL-4	5′-CTCATGGAGCTGCAGAGACTCTT-3′	5′-CATTCATGGTGCAGCTTATCGA-3′
mouse IL-13	5′-TGACCAACATCTCCAATTGCA-3′	5′-TTGTTATAAAGTGGGCTACTTCGATTT-3′
mouse eotaxin-1	5′-CCCAACTCCCTGCTGCTTTA-3′	5′-AGATCTCTTTGCCCAACCTG-3′
mouse KC (CXCL1)	5′-TAGGGTGAGGACATGTGTGG-3′	5′-AAATGTCCAAGGGAAGCGT-3′
mouse IL-25R	5′-GACGCGAAGGGACAGTTG-3′	5′-CAGCAGCACCAGGAAGAGAG-3′
mouse IL-17	5′-CTCCACCGCAATGAAGAC-3′	5′-CTTTCCCTCCGCATTGAC-3″
mouse Act1	5′-GCTTTGGCAGACTCCTTCAG-3′	5′-GGTAACACGAGGAGGTGAGG-3′
mouse IFNγ	5′-TGATGGCCTGATTGTCTTTCAA-3′	5′-GGATATCTGGAGGAACTGGCAA-3′;
mouse TARC	5′-CAGGAAGTTGGTGAGCTGGT-3′	5′-GGGTCTGCACAGATGAGCTT-3′

mouse IL-4R	5′-AGGCCCCAGTACAGAATGTG-3′	5′-CCAACAAGTCGGAAAACAGG-3′
mouse MCP-1	5′-GCTGGAGCATCCACGTGTT-3′	5′-ATCTTGCTGGTGAATGAGTAGCA-3′

Results

Act1 is recruited to IL-25R through the SEFIR domain

While IL-25 is the most divergent known member of the IL-17 family (also referred as IL-17E), sequence analysis has shown that IL-25R also belongs to the defined SEFIR protein family, due to a conserved sequence segment called SEFIR in its cytoplasmic domain (Fig. 1A). We have previously reported that the adaptor molecule Act1 contains a SEFIR domain at its C-terminal region, through which Act1 is recruited to IL-17R(29). To investigate the possibility that Act1 also functions as an adaptor molecule for IL-25 receptor, we co-expressed flag-tagged Act1 and V5-tagged IL-25R in HeLa cells. Cell lysates from these transfected cells were immunoprecipitated with anti-flag (M2) or anti-V5, followed by western analyses with anti-flag and anti-V5. As shown in Fig. 1B–D, the co-immunoprecipitation experiments indicate that Act1 forms a complex with IL-25R, implicating the possible role of Act1 as an adaptor for IL-25R. IL-25 stimulation enhanced the interaction between Act1 and IL-25R (Fig. 1E). Transfection of the same expression construct of IL-25R into mouse embryonic fibroblasts (MEFs) rendered them responsiveness to IL-25 (induction of G-CSF), indicating that the epitope tag on the IL-25R does not interfere with signaling (data not shown). It is important to note that the interaction between Act1 and IL-25 receptor was abolished when the SEFIR domain was deleted either from Act1 or from IL-25R, indicating the recruitment of Act1 to IL-25 receptor is through the dimerization of the SEFIR domain (Fig. 1F–G).

Act1 is required for IL-25-induced pulmonary eosinophilia and Th2 immune response

Previous studies have shown that IL-25 and IL-25R play an important role in mediating the initiation and propagation of the Th2 immune response, eosinophilia and allergic lung inflammation. Intranasal injection of recombinant IL-25 was able to induce a Th2 response in the airway manifested by an increase in Th2 cytokines, IL-4, IL-5, IL-13 and marked eosinophilia. To examine the role of Act1 in IL-25R signaling in vivo, wild-type and Act1-deficient mice on a C57BL/6 background were treated with recombinant IL-25 through intranasal injection. As shown in Fig. 2A, the eosinophils in the BAL were significantly reduced in Act1-deficient mice compared to that seen in wild-type mice 24 hours after IL-25 stimulation. The expression of IL-25-induced Th2 associated genes was abolished or greatly reduced in Act1-deficient lung tissue, including cytokines IL-4, IL-5, IL-13 and chemokine eotaxin (a potent eosinophil chemokine) (Fig. 2B). Taken together, these results demonstrate a previously unreported requirement of Act1 in mediating IL-25-induced eosinophilia and Th2 immune response. It is important to point out that IL-25-induced pulmonary responses (after 24 hours of intranasal IL-25 delivery) were not reduced in IL-17-deficient mice, suggesting that the observed IL-25 unresponsiveness in Act1-deficient mice was probably not due to the lack of IL-17 signaling.

Act1 is required for IL-17-induced pulmonary neutrophilia

The main function of IL-17A is to coordinate local tissue inflammation via the upregulation of pro-inflammatory and neutrophil-mobilizing cytokines and chemokines. We have recently reported that Act1 is a key component in IL-17A signaling and required for IL-17-mediated inflammatory responses. To examine the impact of Act1 deficiency on IL-17-induced pulmonary inflammation, wild-type and Act1-deficient mice on a C57BL/6 background were treated with recombinant IL-17 through intranasal injection. IL-17-induced neutrophil recruitment was significantly reduced in Act1-deficient mice compared to that in wild-type mice (Fig. 2C and SFig. 1). It is important to note that IL-17 stimulation does not lead to eosinophilia in the wild-type or Act1-deficient mice, which is consistent with the fact that IL-17 is a strong inducer for neutrophilia, but not eosinophilia. Th2 associated genes were not induced by IL-17 intranasal injection (data not shown). Instead, IL-17-induced expression of KC(CXCL1) (a potent in neutrophil chemokine) and cytokine IL-6 was abolished in the lung of Act1-deficient mice compared to that in wild-type mice (Fig. 2D). Taken together, these results indicate that while Act1 plays an important role in IL-25-induced pulmonary eosinophilia, Act1 is required for IL-17-mediated neutrophilia in the airway.

Epithelial-derived Act1 is required for IL-17- and IL-25-mediated pulmonary inflammation

While previous studies have shown that the cell type responsible for production of Th2 cytokines following IL-25 exposure is of a nonlymphocyte, non-NK, and non-granulocyte lineage, the identity of the IL-25 responsive cell type(s) in the airway remains to be elusive(13). On the other hand, IL-17-mediated signaling has been implicated in the up-regulation of cytokines and chemokines in airway epithelial cells. Since Act1 is highly expressed in epithelial cells, including airway epithelial cells(30), we decided to determine the contribution of IL-17 and IL-25 signaling for the function of epithelial-derived Act1 in lung inflammation(31).

We have previously generated epithelial cell specific Act1-deficient mice(25). Act1-deficient (*Traf3ip2*$^{-/-}$) mice were first bred onto K18-Cre transgenic mice (K18-Cre$^{+/+}$) to generate K18-Cre$^{+/-}$*Traf3ip2*$^{+/-}$ mice(32). These mice were further bred onto Act1 floxed mice (*Traf3ip2*$^{\text{flox/flox}}$) to generate the control mice (K18-Cre$^{+/-}$*Traf3ip2*$^{+/\text{flox}}$) and epithelial-specific Act1-deficient mice (K18-Cre$^{+/-}$*Traf3ip2*$^{-/\text{flox}}$). While Act1 expression was greatly reduced in colon epithelial cells from epithelial-specific Act1-deficient mice (K18-Cre$^{+/-}$*Traf3ip2*$^{-/\text{flox}}$)(25), Act1 expression was also abolished in airway epithelial cells from these epithelial-specific Act1-deficient mice (Fig. 3A). Control mice (K18-Cre$^{+/-}$*Traf3ip2*$^{+/\text{flox}}$) and epithelial-specific Act1-deficient mice (K18-Cre$^{+/-}$*Traf3ip2*$^{-/\text{flox}}$) were treated with recombinant IL-25 or IL-17 through intranasal injection. Importantly, IL-25-induced eosinophilia was significantly reduced in epithelial-specific Act1-deficient mice (K18-Cre$^{+/-}$*Traf3ip2*$^{-/\text{flox}}$) (Fig. 3B). Consistent with this, IL-25 induced low levels of Th2 associated gene expression in epithelial-specific Act1-deficient mice (K18-Cre$^{+/-}$*Traf3ip2*$^{-/\text{flox}}$) lung tissue, including cytokines IL-5 and IL-13 and IL-4 and chemokine eotaxin (Fig. 3C). On the other hand, IL-17-induced BAL cellularity and neutrophil recruitment were also significantly diminished in epithelial specific Act1-deficient mice (K18-Cre$^{+/-}$*Traf3ip2*$^{-/\text{flox}}$) compared to that in wild-type mice (Fig. 3D–E). Taken together, these results indicate that epithelial-derived Act1 plays an important role in IL-25-induced eosinophilia and IL-17-mediated neutrophilia during lung inflammation.

Act1 is required for allergen-induced pulmonary inflammation

It is well known that antigen-induced allergic pulmonary inflammation is associated with Th2 immune response. Since Act1 is a key signaling component for both IL-25-mediated Th2 immune response and IL-17-induced inflammatory response, it is important to investigate the role of Act1 in allergic pulmonary inflammation. We examined the impact of Act1 deficiency on allergen-induced pulmonary inflammation using an OVA-induced model of allergic pulmonary inflammation. While Act1-deficient mice on Balb/c background display B cell-mediated autoimmune phenotypes as early as three weeks old(27), Act1-deficient mice in C57BL/6 background have a delayed onset of this phenotype (older than 6 months) and a reduced onset of B cell-mediated autoimmune phenotypes. We therefore used female C57BL/6 Act1-deficient mice (n = 5) or wild-type littermates (n = 5) bred 6 generations for the allergen-induced pulmonary inflammation model. OVA/alum-sensitized wild-type and Act1-deficient mice on a C57BL/6 background were challenged with OVA aerosol or saline as described in methods. Twenty-four hours after the last challenge, the mice were analyzed for Bronchalveolar lavage (BAL) cells and lung inflammation. Lung and airway recruitment of granulocytes, predominantly eosinophils, were reduced in Act1-deficient mice compared to wild-type mice (Fig. 4A–B). The eosinophils, macrophages and lymphocytes in the BAL were also decreased in Act1-deficient mice (Fig. 4C). The low grade of pulmonary inflammation in the Act1-deficient mice correlates with decreased expression of chemokines [including eotaxin (eosinophil recruitment), TARC (thymus-and activation-regulated chemokine/CCL17), and MCP1 (recruitment of macrophages), KC (recruitment of neutrophils)] and Th2 cytokines (IL-5 and IL-13) in the lung tissues (Fig. 3D and SFig. 2). The induction of pulmonary eosinophilia in the mouse model of asthma requires the induction of both cellular as well as humoral immunity. Therefore, we also examined the induction of Ovalbumin specific antibody production in wild-type and Act1-deficient animals after ovalbumin sensitization. Interestingly, wild-type and Act1-deficient mice demonstrated equivalent induction of Ovalbumin specific IgE and IgG2a (Fig. 4E).

Act1 deficiency in epithelial cells reduced allergen-induced pulmonary inflammation

As shown above, epithelial-derived Act1 plays an important role in IL-25-induced eosinophilia and IL-17-mediated neutrophilia during lung inflammation. Thus we examined the impact of epithelial-specific Act1 deficiency in allergen-induced pulmonary inflammation. Importantly, we found that the allergen-induced pulmonary inflammation is reduced in the epithelial specific Act1-deficient mice (K18-Cre$^{+/-}$*Traf3ip2*$^{-/\text{flox}}$). Histologic analysis showed that Act1 deficiency in epithelial cells leads to reduced lung inflammation (Fig. 5A). The total number of BAL cells (including lymphocytes and eosinophils) were reduced in the epithelial-specific Act1-deficient mice (K18-Cre$^{+/-}$*Traf3ip2*$^{-/\text{flox}}$) (Fig. 5B). The reduced pulmonary inflammation in the epithelial-specific Act1-deficient mice correlates with decreased expression of some of Th2-associated genes especially cytokine IL-13 in the lung tissues (Fig. 5C), although epithelial-specific Act1 deficiency had less impact on gene expression than complete Act1 deficiency. We also examined the induction of Ovalbumin specific antibody production in wild-type and epithelial-specific Act1-deficient mice (K18-Cre$^{+/-}$*Traf3ip2*$^{-/\text{flox}}$) after ovalbumin sensitization. Interestingly, wild-type and epithelial-specific Act1-deficient mice (K18-Cre$^{+/-}$*Traf3ip2*$^{-/\text{flox}}$) demonstrated similar levels of Ovalbumin specific IgE and IgG2a (Fig. 5D).

Discussion

Although IL-25 is an important regulator for the induction of Th2 immunity, the detailed molecular mechanism by which IL-25 signals is not yet clear. We here show that Act1, a key component in IL-17-mediated signaling, is also an essential signaling molecule for IL-25 signaling. We previously reported that adaptor molecule Act1 contains a SEFIR domain at its C-terminal region, through which Act1 is recruited to IL-17R (IL-17RA). While IL-25 is the most divergent known member of the IL-17 family (also referred as IL-17E), sequence analysis showed that IL-25R also belongs to the SEFIR protein family. Co-immunoprecipitation experiments indicate that Act1 forms a complex with IL-25 receptor (IL-17RB), confirming the role of Act1 as an adaptor for IL-25 receptor (IL-17RB). It is important to note that recent study by Rickel et al demonstrated an essential role for IL-17RA in IL-25 (IL-17E) signaling through the IL-25 receptor (IL-17RB), suggesting the possibility for heterodimerization of IL-17RA and IL-17RB in mediating IL-25 (IL-17E) signaling**(36)**. While the SEFIR domain of IL-17RA is required for its interaction with Act1(29), we now found that the SEFIR domain of IL-17RB is also required for the recruitment of Act1 to the IL-25 receptor (IL-17RB), suggesting the importance of both IL-17RA and IL-17RB in signaling.

It is important to note that IL-25 signaling is significantly distinct from IL-17 signaling in that it induces different biological responses linked to specific pathologies of various inflammatory diseases. Using IL-17 deficient mice, we have now confirmed that IL-25-induced pulmonary responses are independent of IL-17. Therefore, the discovery of a common adaptor (Act1) downstream of IL-17 and IL-25 receptor provides an opportunity to investigate specific aspects of IL-17- and IL-25-mediated signaling during inflammatory responses. It is important to note that both IL-17 and IL-25 signaling participate in antigen-induced allergic airway inflammation. In particular, the role of IL-25 has been reported to be crucial in the initiation and propagation of antigen induced allergic inflammation. Ballantyne et. al. recently reported that blocking IL-25 signaling (IL-25 neutralizing antibodies) reduced allergic pulmonary inflammatory responses in ova-induced asthma model (19). We demonstrate the requirement for Act1 in the Th2-associated responses in mouse model of allergic pulmonary inflammation. While Act1 plays an important role in IL-25-induced expression of Th2-associated genes (including IL-5, IL-13 and eotaxin-1), and pulmonary eosinophilia, Act1 is required for IL-17-mediated KC expression and neutrophilia in the airway. These results demonstrate the essential role of Act1 in allergic pulmonary inflammation through the differential impact of the IL-17R-Act1 and IL-25R-Act1 axes. Future studies are required to distinguish the relative contribution of IL-17-Act1 versus IL-25-Act1 axis in antigen-induced allergic pulmonary inflammation.

Both IL-17 and IL-25 signaling participate in antigen-induced allergic airway inflammation. One important task is to identify the cell types where Act1 functions to mediate IL-17 and IL-25 signaling, contributing to allergic pulmonary inflammation. Intriguingly, Act1 deficiency in epithelial cells reduced the phenotype of allergic pulmonary inflammation. IL-17-induced BAL cellularity and lung inflammation were significantly reduced in epithelial specific Act1-deficient mice compared to that in wild-type mice. In support of this, IL-17-induced Act1-mediated signaling has also been shown to enhance cytokine expression in human airway epithelial cells **(37)**. These findings are consistent with our previous report that epithelial-specific Act1-deficient mice had reduced colitis due to reduced IL-17-dependent induction of chemokines that recruit neurtrophils to the gut in a murine model of DSS-induced colitis.

Previous studies have clearly shown that IL-25 plays important roles in promoting Th2 cell-mediated inflammation characterized by the infiltration of eosinophils. Recent studies in murine models suggest that IL-25 produced by lung epithelial cells may promote Th2 cell differentiation(17). To determine whether Act1 deficiency has any impact on the activation of Ova-specific T cells, we examined responses of Act1-deficient and wild-type splenocytes 15 days after immunization. Splenocytes from wild-type and Act1-deficient mice showed similar frequencies of T cells secreting IL-5 (data not shown). Therefore, it is clear that the activation of IL-5 producing Th2 cells was not inhibited in Act1-deficient mice, suggesting that the reduced pulmonary inflammation in Act1-deficient cells is not due to lack of Th2 cell differentiation. Previous studies have shown that the cell type responsible for production of Th2 cytokines following IL-25 exposure is of a non-lymphocyte, non-NK, and non-granulocyte lineage(15). Intriguingly, IL-25-induced eosinophilia was significantly reduced in epithelial-specific Act1-deficient mice. These results indicate a definitive role of epithelial-derived Act1 in IL-25-mediated Th2 immune response in vivo.

In summary, we found that Act1 deficiency in epithelial cells abolished IL-17-induced neutrophilia and IL-25-induced eosinophilia, leading to reduced allergic pulmonary inflammation. These results demonstrate the essential role of epithelial-derived Act1 in allergic pulmonary inflammation through the IL-17R-Act1 and IL-25R-Act1 axes. Further mechanistic study of IL-17R-Act1 versus IL-25R-Act1 signaling is crucial for our understanding of pathobiology of atopic diseases including allergic asthma and atopic dermatitis, which will facilitate pursuing Act1 as a potential therapeutic target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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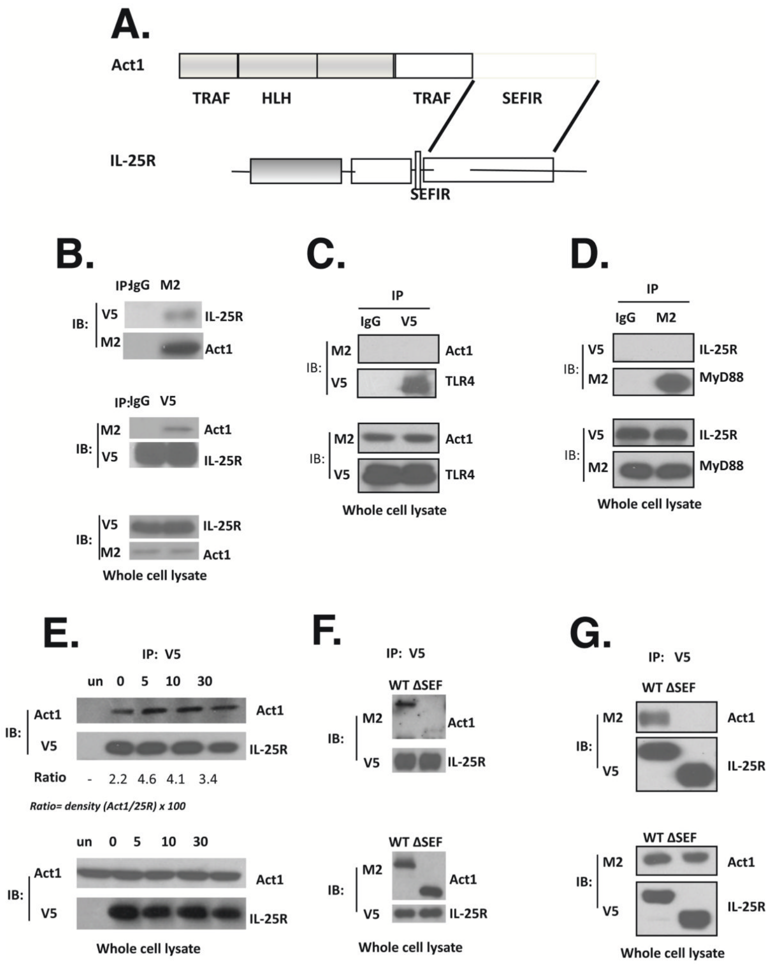

Figure 1. Act1 interacts with IL-25R

(A) Domain structure of Act1 and IL-25 receptor. The C-terminus of Act1 contains a SEFIR domain that is a conserved sequence segment in the cytoplasmic domain of IL-17 receptor family members including IL-25R. **(B-G)** Interaction between Act1 and IL-25R. **(B-D)** Hela cells were transiently transfected with Flag-tagged mAct1 and V5-tagged mouse IL-25R **(B)**; Flag-tagged mAct1 and V5-tagged TLR4 **(C)**; and Flag-tagged MyD88 and V5-tagged IL-25R **(D)**. The lysates of transfected cells were immunoprecipated (IP) with anti-Flag (M2) or anti-V5 antibodies or IgG (control), followed by western analysis (IB) with anti-M2 and anti-V5 antibodies. Western blot analysis (IB) of whole cell lysates for Act1 and IL-25R expression by anti-M2 and anti-V5 antibodies. **(E)** Hela cells were transiently transfected with V5-tagged mouse IL-25R. The cells were treated with IL-25 (50ng/ml) for 0, 5, 10 and 30 min respectively. The lysates of untransfected(un) and transfected cells were immunoprecipated (IP) with anti-V5, followed by western analysis (IB) with anti-Act1 and anti-V5 antibodies. Western blot analysis (IB) of whole cell lysates for Act1 and IL-25R expression by anti-Act1 and anti-V5 antibodies. The intensity of the immunoprecipitated Act1 bands was analyzed by Scion Image 1.62C alias and presented as percentage of the immunoprecipiated IL-25R bands (Ratio). **(F-G)** Hela cells were transiently transfected with Flag-tagged mAct1 or Act1 SEF deletion mutant (ΔSEF) and V5-tagged mouse IL-25R**(F)**; Flag-tagged mAct1 and V5-tagged mouse IL-25R or IL-25R SEF deletion mutant (ΔSEF) **(G).** The lysates of transfected cells were immunoprecipated (IP) with anti-V5, followed by western analysis (IB) with anti-M2 and anti-V5 antibodies. Western blot analysis (IB) of whole cell lysates for mAct1 and IL-25R expression by anti-M2 and anti-V5 antibodies.

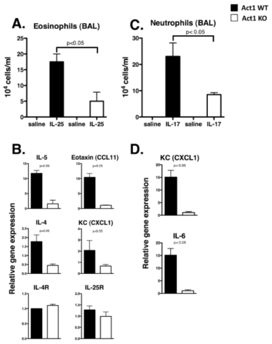

Figure 2. Act1 is required for IL-25 and IL-17 induced responses

Wild-type and Act1-deficient female mice on C57BL/6 background received intranasal administration of saline or recombinant IL-25(5μg/mouse) or IL-17(5μg/mouse) and where sacrificed and analyzed 24hours later. **(A)** Reduced eosinophil accumulation in BAL from Act1-deficient mice compared to wild-type after IL-25 intranasal administration. **(B)** Pulmonary expression (relative to wild-type saline treated mice) of IL-25 associated genes [IL-5, Eotaxin (CCL11), IL-13, and IL-4] in addition to IL-25R, KC (CXCL1), and IL-4R from real-time PCR analysis of total mRNA from lung tissue. **(C)** Reduced neutrophil accumulation in BAL from Act1-deficient mice compared to wild-type after IL-17 intranasal administration. **(D)** Pulmonary expression (relative to wild-type saline treated mice) of IL-17 induced genes [KC(CXCL1), and IL-6 from real-time PCR analysis of total mRNA from lung tissue.

Data represents means (n=4)±S.E.M, p-value indicated. The experiment was repeated three times.

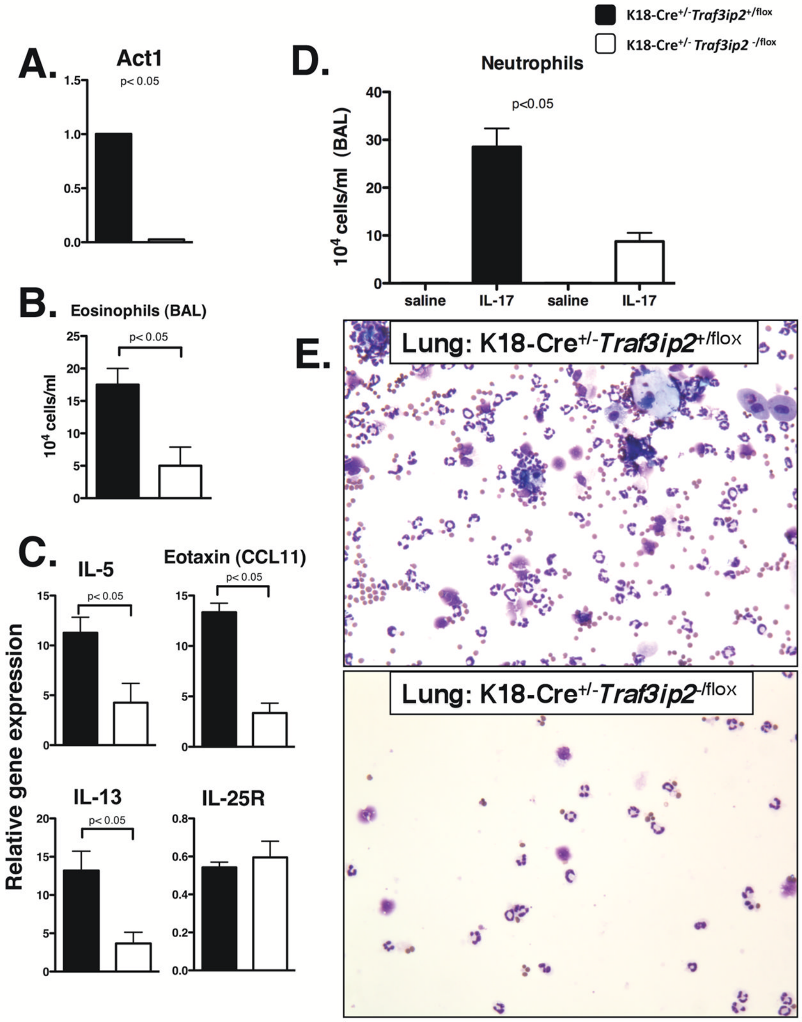

Figure 3. Epithelial specific Act1 deficiency results in reduced IL-25- and IL-17-mediated pulmonary inflammation

(A) Act1 expression from real-time PCR analysis of total mRNA of tracheal epithelial cells isolated from control mice (K18-Cre$^{+/-}$*Traf3ip2*$^{+/\mathrm{flox}}$) and epithelial-specific Act1-deficient mice (K18-Cre$^{+/-}$*Traf3ip2*$^{-/\mathrm{flox}}$). Intranasal administration of recombinant IL-25 (5μg/mouse, **B-C**) or recombinant IL-17 (5μg/mouse, **D-E**) mice were sacrificed and analyzed 24hours later. **(B)** Reduced IL-25-induced eosinophil accumulation in BAL from epithelial-specific Act1-deficient mice compared to wild-type. **(C)** IL-25-induced pulmonary expression (relative to wild-type saline treated mice) of Th2-associated genes [IL-5, Eotaxin (CCL11), IL-4 and IL-13] in addition to IL-4R and IL-25R from real-time PCR analysis of total mRNA from lung tissue. **(D)** Reduced IL-17-induced neutrophil accumulation in BAL from epithelial-specific Act1-deficient mice compared to wild-type. **(E)** BAL cytospin preparations stained with Wright-Giemsa demonstrating reduced IL-17-induced airway inflammatory cell recovery in epithelial-specific Act1 deficient mice compared to wild-type. Data represents means (n=4)±S.E.M, p-value indicated. The experiment was repeated three times.

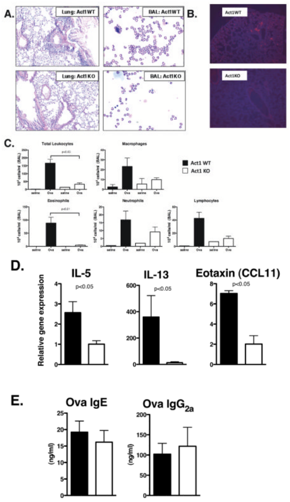

Figure 4. Reduced allergen-mediated pulmonary inflammatory responses in Act1-deficient mice

Wild-type and Act1-deficient female mice on C57BL/6 background where subjected to mouse model of allergen-mediated pulmonary inflammation as described in the methods section. **(A)** H&E staining of lung tissue demonstrating reduced lung inflammation in Act1-deficient mice compared to wild-type. BAL cytospin preparations stained with Wright-Giemsa demonstrating reduced airway inflammatory cell recovery in Act1- deficient mice compared to wild-type. **(B)** Immunoflouresence staining of lung sections using anti-mouse MBP goat antibody (red) indicative of reduced eosinophil Infiltration in Act1 deficient mice compared to wild-type. **(C)** Differential count analysis of BAL leukocytes demonstrating reduced eosinophils, as well as macrophages and lymphocytes in Act1-deficient mice compared to wild-type. **(D)** Reduced pulmonary expression (relative to Ova/Alum immunized wild-type mice following saline aerosol challenge) of Th2 associated genes [IL-4, IL-5, IL-13, Eotaxin (CCL11), IL-4R and] from real-time PCR analysis of total mRNA from lung tissue. **(E)** Equivalent ovalbumin specific IgE and IgG2a induction following Ovalbumin/Alum sensitization (n=3, repeated twice). Data represents means (n=5, unless indicated otherwise)±S.E.M, p-value indicated. The experiment was repeated three times.

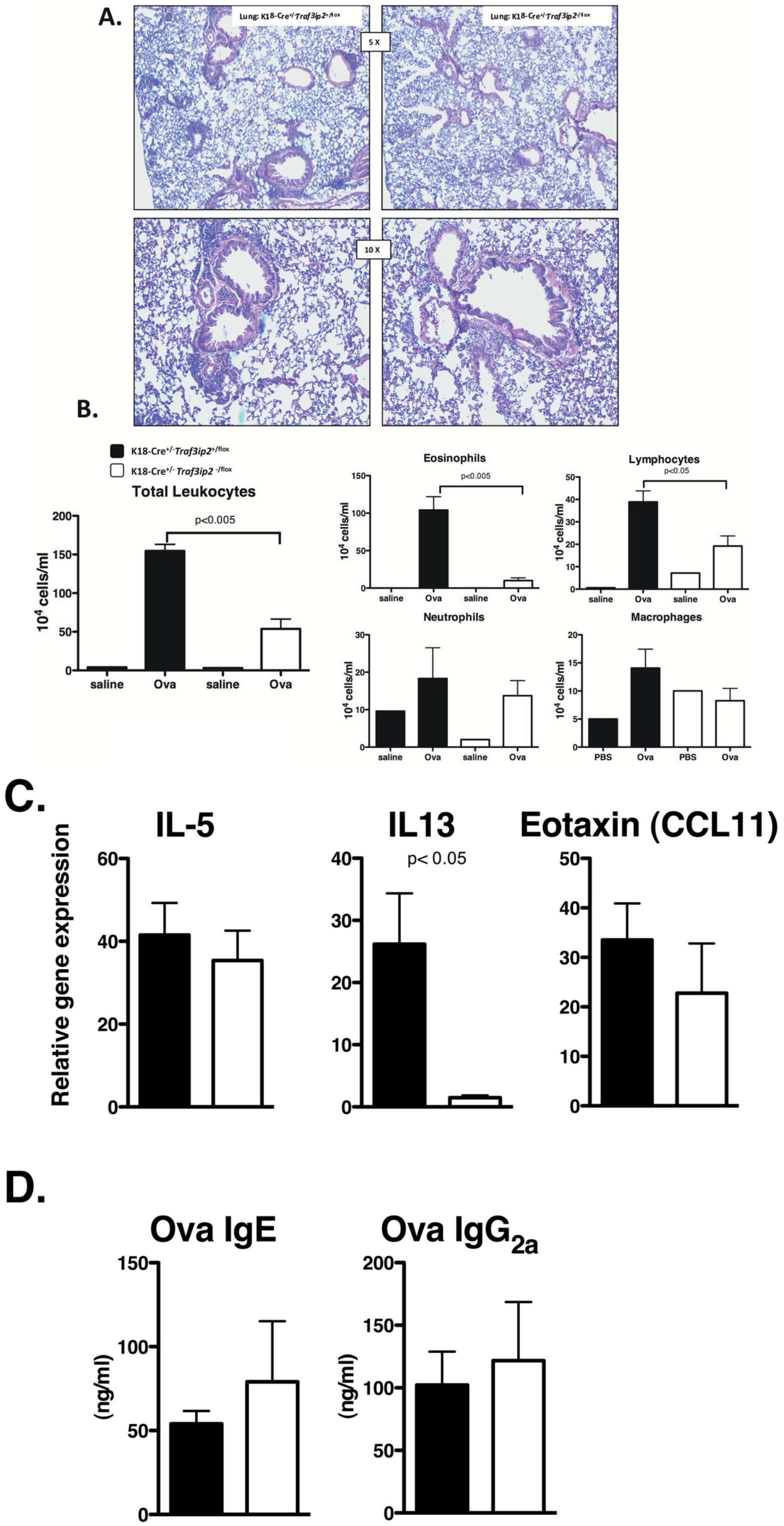

Figure 5. Epithelial specific Act1-deficiency results in reduced allergen-mediated pulmonary inflammatory responses

Control mice (K18-Cre$^{+/-}$*Traf3ip2*$^{+/\text{flox}}$) and epithelial-specific Act1-deficient mice (K18-Cre$^{+/-}$*Traf3ip2*$^{-/\text{flox}}$) where subjected to mouse model of allergen-mediated pulmonary inflammation as described in the methods section. **(A)** H&E staining of lung tissue demonstrating reduced lung inflammation in epithelial-specific Act1-deficient mice (K18-Cre$^{+/-}$*Traf3ip2*$^{-/\text{flox}}$) compared to Control mice (K18-Cre$^{+/-}$*Traf3ip2*$^{+/\text{flox}}$). **(B)** Differential count analysis of BAL leukocytes demonstrating reduced eosinophils, as well as lymphocytes in epithelial-specific Act1-deficient mice (K18-Cre$^{+/-}$*Traf3ip2*$^{-/\text{flox}}$) compared to Control mice (K18-Cre$^{+/-}$*Traf3ip2*$^{+/\text{flox}}$). **(C)** Pulmonary expression (relative to Ova/Alum immunized wild-type mice following saline aerosol challenge) of Th2 associated genes [IL-5, IL-13, Eotaxin (CCL11) expression from real-time PCR analysis of total mRNA from lung tissue. **(D)** Equivalent ovalbumin specific IgE and IgG2a induction following Ovalbumin/Alum sensitization (n=3, repeated twice). Data represents means (n=5, unless indicated otherwise)±S.E.M, p value indicated. The experiment was repeated three times.