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T Cell-Derived Act1 Is Necessary for IL-25–Mediated Th2 Responses and Allergic Airway Inflammation

Shadi Swaidani,^{*,†,‡} Katarzyna Bulek,^{*} Zizhen Kang,^{*} Muhammet Fatih Gulen,^{*} Caini Liu,^{*} Weiguo Yin,^{*} Amina Abbadi,^{*} Mark Aronica,[†] and Xiaoxia Li^{*}

The cellular and molecular mechanisms driven by IL-25 and its cognate receptor IL-17RB necessary for the promotion of Th2-mediating pathogenic pulmonary inflammation remains to be defined. We have previously reported the critical role of the U-box-type E3 ubiquitin ligase Act1 (1) for the downstream signaling of the IL-17 cytokine family including the Th2-promoting cytokine IL-25 (IL-17E) (2). In this study, we report that IL-25–driven but not conventional IL-4–driven Th2 polarization and cytokine production is impaired in Act1-deficient T cells. Also, Act1 deficiency in the T cell compartment results in the abrogation of eosinophilic airway infiltration as well as airway hyperresponsiveness in mouse models of Ag-induced airway inflammation. The in vivo generation of Ag-specific Th2 cytokine-producing cells is defective in the absence of Act1 expression in T cells after OVA/aluminum hydroxide immunization. Notably, the production of OVA-specific IgG₁ but not IgG_{2a} or IgE is also impaired. At the molecular level, we report that IL-25–mediated induction of Th2 master regulator GATA-3 and the transcription factor GFI-1 is attenuated in Act1-deficient T cells. Taken together, our findings indicate that Act1 expression in T cells is required for cellular and humoral Th2-mediated allergic responses and the development of airway hyperresponsiveness, in part, through Act1's function in IL-25–induced development of Th2 T cells. *The Journal of Immunology*, 2011, 187: 000–000.

Chronic pulmonary inflammation in allergic asthma is associated with airway hyperresponsiveness (AHR) and is pathologically marked by an infiltration of CD4⁺ Th2 cells, NKT cells, neutrophils, eosinophils, and mast cells and is typically associated with an elevation of serum IgE (3, 4). The sensitization and progression toward allergic asthma involves the reactivity of epithelial and innate immune cells to allergens and the subsequent induction of adaptive immune responses where T cells producing Th2 cytokines (IL-4, IL-5, IL-9, and IL-13) mediate allergen-induced pulmonary eosinophilic inflammation (5, 6). IL-25 (also known as IL-17E) is a disulfide-linked homodimeric glycoprotein expressed and secreted by a variety of cells including airway epithelial cells, eosinophils, basophils, mast cells, and macrophages as well as CD4⁺ cells (7–9). IL-25 has been shown to initiate Th2 immunity by inducing the expression of Th2 cytokines (IL-4, IL-5, IL-9, IL-13), tissue eosinophilia, serum IgG1 and IgE, and AHR (10–13). Furthermore, endogenous IL-25 has been shown to be critical for allergen-induced pulmonary inflammation and AHR in experimental asthma models (7, 14).

We recently reported that Act1 is an essential adapter molecule for IL-25 signaling (2, 15). Both Act1 and IL-25 receptor (IL-17RB) contain a similar expression to FGF genes and IL-17R

(SEFIR) domain and belong to the STIR (SEFIR+TIR) superfamily of proteins that also include TIR and the adapter molecule MyD88 (16). Our biochemical studies have demonstrated that Act1 and IL-25 receptor do indeed interact, noting that this interaction was amplified by ligand treatment and required SEFIR–SEFIR domain on both IL-25 receptor and Act1 (2). Because the role of IL-25 in Th2 immunity has been well established, it is important to elucidate the mechanistic actions of the IL-25–induced Act1-mediated pathway in Th2 responses and allergic pulmonary inflammation. Allergic pulmonary inflammation requires delicate interplay between multiple cell types, including dendritic cells, lymphocytes, and the airway epithelial cells. Cell type-specific deletion of Act1 provides important tools to investigate how different cell types coordinately participate in the initiation and effector stages of allergic pulmonary inflammation. Act1 deficiency in epithelial cells reduced IL-25–induced eosinophilia and the phenotype of allergic pulmonary inflammation. However, the development of AHR and the generation of OVA-specific IgG1 and IgE remained intact in epithelial-specific Act1-deficient mice after OVA aerosol challenge following OVA/aluminum hydroxide (alum) sensitization. This was not completely surprising given that in addition to epithelial cells, other cell types including T cells have also been reported to respond to IL-25.

It has been shown that IL-25 responsiveness requires the expression of the IL-25 receptor (IL-17RB or IL-17Rh1) in addition to the IL-17RA receptor (17). IL-25 receptor (IL-17RB) expression has been reported in epithelial cells, CD4⁺ T cells, invariant NKT cells, and eosinophils (7, 8). In the T cell compartment, IL-25 receptor is expressed on naive T cells; this expression is upregulated and maintained after Th2 polarization and commitment (7). In addition, in Th2-prone mouse strains like BALB/cJ, IL-25R⁺ invariant NKT cells are necessary and sufficient for the induction of AHR after IL-25 treatment (11, 13). More recently, IL-17RB was also reported on IL-9–producing Th9 cells where IL-25 was reported to induce IL-9 expression (18). Thus, despite our improved understanding of the role of IL-25–mediated

^{*}Department of Immunology, Cleveland Clinic, Cleveland, OH 44195; [†]Department of Pathobiology, Cleveland Clinic Foundation, Cleveland, OH 44195; and [‡]Department of Molecular Medicine, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland, OH 44195

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Address correspondence and reprint requests to Dr. Xiaoxia Li and Dr. Mark Aronica, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195. E-mail addresses: lix@ccf.org and aronicm@ccf.org

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; MBP, major basic protein; SEFIR, similar expression to FGF genes and IL-17R.

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responses, the molecular mechanism for how IL-25 promotes T cell-mediated Th2 immunity remains elusive and poorly defined.

In this study, we examined the requirement of Act1 in the T cell compartment for the development of T cell-mediated Th2 responses. Act1 is required for robust IL-25-dependent, but not IL-4-dependent, *ex vivo* generation of Th2 cytokine-producing T cells. Furthermore, Act1 deficiency in T cells resulted in an abrogation of eosinophilic airway infiltration as well as AHR in response to methacholine challenge after OVA aerosol challenge following OVA/alum sensitization. Act1-deficient T cells exhibited defective IL-25-driven Th2 differentiation and cytokine production marked by attenuated induction of the key Th2 transcription factors GATA-3 and GFI-1. Taken together, our findings suggest that Act1 expression in T cells is required for T cell-mediated Th2 allergic responses through, in part, Act1's critical impact on the IL-25R–Act1 axis.

Materials and Methods

Mice

Mice were maintained at the Cleveland Clinic Lerner Research Biological Resource Unit in a temperature-controlled (23°C) facility with an automatic 12-h light–dark cycle and were given free access to food and water. The experiments were performed with gender-matched mice aged 8–12 wk. All animal protocols were approved by the Institutional Animal Care and Use Committee of The Cleveland Clinic Foundation (fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International).

T cell differentiation

Naive CD4⁺ T cells from spleens and peripheral lymph nodes were enriched by negative selection using EasySep CD4⁺ T cell reagents and magnets (StemCell) followed by positive selection for CD62L and Easy-step Biotin Selection Kit (StemCell). We maintained 1×10^6 cells/ml in complete medium: RPMI 1640 (Invitrogen Life Technologies) culture medium supplemented with 10% heat-inactivated FBS, sodium bicarbonate, 2 mM L-glutamine, penicillin/streptomycin, and 5×10^{-4} M 2-mercaptoethanol. All Abs and cytokines (except IL-12 and IL-25) were purchased from BD Pharmingen (San Diego, CA). T cell polarization was conducted as previously described (19). Cells were stimulated with Dynabeads mouse T-Activator CD3/CD28 (Invitrogen) or plate-bound anti-CD3/CD28 (3 g/ml each). For Th1 conditions (polarization), anti-IL-4 (11B11, 10 µg/ml) plus IL-12 (10 ng/ml) were used; for Th2 conditions (polarization), anti-IFN-γ (XMG1.2, 10 µg/ml), anti-IL-12 (C17.8, 10 µg/ml), plus IL-4 (1000 U/ml); for Th25 polarization, rIL-25 (R&D Systems; concentrations as indicated), anti-IL-12 (C17.8, 10 µg/ml), and anti-IFN-γ (XMG1.2, 10 µg/ml); and for anti-Th1 conditions, anti-IFN-γ (XMG1.2, 10 µg/ml) and anti-IL-12 (C17.8, 10 µg/ml). For intracellular staining, cells were stimulated with PMA and ionomycin in the presence of GolgiStop for 4 h, after which IL-4- and IFN-γ-producing cells were analyzed with intracellular staining.

ELISA

IL-4, IL-5, and IL-13 production was measured using DuoSet (R&D Systems, Minneapolis, MN), following the manufacturer's instructions.

Intranasal instillation of rIL-25

Mice were exposed to inhaled anesthesia with isoflurane. Recombinant murine IL-25, carrier-free (R&D Systems) and resuspended in sterile saline (0.9%), was instilled into a nasal opening in a 50-µl aliquot per mouse (1 µg/mouse).

Mouse model of allergic pulmonary inflammation

Mice (8–12 wk) were immunized with a 100 µl i.p. injection containing 10 µg OVA (chicken OVA, grade V; Sigma, St. Louis, MO) and 20 mg alum (Sigma). Sham immunization with i.p. injection of PBS and 20 mg alum was used as a negative control for the immunization procedure. Control mice (Lck-Cre^{+/+}-*Traf3ip2*^{fllox}) and T cell-specific Act1-deficient mice (Lck-Cre^{+/+}-*Traf3ip2*^{fllox}) on C57BL/6 background were immunized by two i.p. injections on day 1 and day 8. Mice were sacrificed and processed on day 14 for Ig ELISAs and ELISPOT analysis. Mice were then challenged on days 14, 15, 16, and 17 by exposure to ~40 min of 1% OVA

aerosol diluted in PBS or PBS alone. This was accomplished by placing mice in a 30 cm × 60 cm acrylic box ventilated by a NOUVAG Ultrasonic 2000 nebulizer (NOUVAG USA, Lake Hughes, CA). Mice were sacrificed, and further analysis was performed on day 18, 24 h after the last aerosol challenge. Baseline responses were measured in control treatment and constituted nebulized PBS aerosol challenge of immunized mice.

AHR and lung mechanics

AHR and lung mechanics were measured in mice in response to increasing doses of inhaled methacholine as described (20). Mice were anesthetized with i.p. pentobarbital sodium (60 mg/kg). After the intubation and the placement of a 19-gauge cannula through a tracheotomy incision, the cannula was affixed with a silk suture ligation. Mice were connected to a computer-controlled piston ventilator run at a rate of 150 breaths/min, a tidal volume of 0.2 ml, and a positive end-expiratory pressure of 2–3 cm H₂O (Flexivent; Scireq, Montreal, Canada). Mice were then administered a muscle relaxant (pancuronium bromide, 800 µg/kg), and the lungs were expanded twice to total lung capacity at an amplitude pressure of 30 cm H₂O. Each mouse was aerosolized with methacholine in saline at doses of 0 mg/ml, 3.12 mg/ml, 6.25 mg/ml, and 12.5 mg/ml, delivered over 10 s through an in-line nebulizer. FlexiVent 5.2 (Flexivent; Scireq) software was used to obtain resistance measurements through multiple linear regression fitting of measured pressure and volume from each mouse to the model of linear motion of the lung. Coefficient of determination <0.90 was excluded. Lung resistance (R_L) was obtained through forced oscillation technique, and airway resistance (R_n) was obtained by a constant phase model.

ELISPOT assays

Single-cell suspensions were prepared from spleens of immunized mice and cultured using HL-1 medium (BioWhittaker, Walkersville, MD) supplemented with 1 mM L-glutamine and penicillin/streptomycin (Invitrogen/Life Technologies). Purification was done by negative selection using EasySep mouse CD4⁺ T cell reagents and magnets (StemCell). Cells (100×10^3) were cultured with 200×10^3 mitomycin C-treated stimulator cells (panned splenocytes from naive mice) and incubated with 10 µg/ml OVA (chicken OVA, grade V) for 4 h at 37°C and 5% CO₂, followed by three washes with HL-1 medium in 96-well ImmunoSpot M200 plates (Cellular Technology). The plates were previously coated with capture Abs for IL-5 or IL-13 (eBioscience) (4 µg/ml Ab in PBS overnight at 4°C), blocked with PBS plus 1% BSA (Sigma-Aldrich), and washed three times with PBS plus 0.5% Tween 20. After 48-h incubation at 37°C and 5% CO₂, the plates were washed three times with PBS plus 0.5% Tween 20, and biotinylated detection Abs for IL-5 or IL-13 (eBioscience) in PBS plus 1% BSA were added to the wells for 2 h, followed by washing and incubation for 2 h with streptavidin-alkaline phosphatase (R&D Systems). Spots were developed with 5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine salt and nitro blue tetrazolium chloride in organic solvent (R&D). The plates were analyzed with an ImmunoSpot Series 2 Analyzer (Cellular Technology, Shaker Heights, OH).

Bio-Plex cytokine assay

Cell culture supernatant or bronchoalveolar lavage (BAL) fluid was collected and centrifuged at 13,000 rpm for 10 min at 4°C and stored at –80°C until further analysis. A custom Bio-Plex (Bio-Rad) kit was used. The Bio-Plex assay was performed following the manufacturer's guidelines. The Bio-Plex Plate was analyzed using Bio-Plex manager software version 4.1 and a Bio-Plex system reader.

BAL fluid and tissue collection

Mice were sacrificed at the times indicated. HL-1 medium, 0.7 ml (Bio-Whittaker), was used to obtain BAL fluid through the trachea using a blunt needle and 1-ml syringe. Cytospin slide preparations were obtained using a Shandon CytoSpin III Cyto centrifuge (Shandon/Thermo Scientific). Differential leukocyte counts were obtained on cytospin slide preparation after Diff-Quik Giemsa stain. Lungs were collected and snap frozen immediately in a liquid nitrogen container. Total RNA was obtained by using TRIzol (Invitrogen) and Omni TH tissue homogenizer (Omni, International). H&E staining was performed on lung tissue after fixation in 10% neutral buffered formalin and paraffin embedding.

OVA-specific Ig ELISA

OVA-specific Abs were determined using ELISA. Briefly, 96-well, flat-bottom, protein-absorbent polystyrene plates were coated with OVA (grade V; Sigma) at 10 µg/well. Plates were then blocked with 2% FBS/

PBS and washed with PBS. Serum samples and standards (OVA-specific IgG₁ and IgE) were incubated at 4°C overnight. Biotinylated anti-mouse IgG₁ or IgE was used followed by streptavidin-conjugated HRP. Plates were developed, stained with 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonate), and quantified using a Molecular Devices plate reader. Data were analyzed using Softmax Pro v.5 software.

Immunohistochemistry

Fresh-frozen sections were fixed in cold acetone/methanol 50%/50% for 10 min. Sections were then blocked with 3% BSA/PBS for all Abs used. After 3% BSA/PBS blocking, slides were incubated with goat polyclonal Ab against eosinophil major basic protein (1:50 dilution; Santa Cruz). After washing, secondary reagents were added and incubated for 1 h at room temperature in the dark. Sections were examined with a Leica fluorescence microscope, and images were captured with a Retiga 2000R camera and QCapture pro6 software and quantitated by Image-Pro plus 7.0 software.

Statistical analysis

The data are presented as the mean $\pm$ SEM; *n* is indicated in the figure legends for representative experiments. 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. Data analysis was performed and figures generated using Prism version 5.0a (GraphPad Software).

Results

Generation of T cell-specific deletion of *Act1*

Because it has been shown that IL-25 promotes Th2 cell differentiation and memory T cell function, it is important to examine the role of T cell-derived Act1 in IL-25-dependent allergic pulmonary inflammation *in vivo*. Thus, we have generated T cell-specific Act1-deficient mice by breeding Act1-deficient (*Traf3ip2*^{-/-}) mice onto Lck-Cre transgenic mice (Lck-Cre^{+/+}) to generate Lck-Cre^{+/+}-*Traf3ip2*^{+/-} mice. These mice were further bred onto Act1 floxed mice (*Traf3ip2*^{flox/flox}) (21) to generate the control mice (Lck-Cre^{+/+}-*Traf3ip2*^{+/-flox}) and T cell-specific Act1-deficient mice (Lck-Cre^{+/+}-*Traf3ip2*^{-flox}). To verify the efficiency and specificity of Act1 distribution in the T cell compartment, Act1 mRNA levels were examined in freshly sorted double-positive thymocytes (Fig. 1A), splenic T cells (CD3⁺; Fig. 1C), and B cells (CD19⁺, B220⁺; Fig. 1B) by real-time PCR. Act1 mRNA was diminished in the thymic as well as splenic T cells obtained from T cell-specific Act1-deficient mice (Lck-Cre^{+/+}-*Traf3ip2*^{-flox}) compared with control mice (Lck-Cre^{+/+}-*Traf3ip2*^{+/-flox}), whereas Act1 expression in B cells remained the same in these mice. In addition, protein expression from purified splenic CD4 T cells was assessed by Western blotting using a commercially available Ab specific for Act1 (Fig. 1D).

Act1 in T cells is necessary for IL-25-induced Th2 polarization and cytokine expression

We tested the role of Act1 in Th1, Th2 cell polarization *in vitro*. CD62L^{hi} naive CD4⁺ T cells from T cell-specific Act1-sufficient or -deficient mice were polarized to Th2 and Th25 cells in the presence of IL-4 or IL-25, respectively (Fig. 2A). Intracellular cytokine staining showed that Act1 deficiency in T cells had no substantial impact on IL-4 production from IL-4-mediated Th2 polarization, whereas the IL-25 induction of IL-4-producing cells was decreased by more than 50% in Act1-deficient cells (Fig. 2A). Importantly, IFN- γ was 5-fold higher in Act1-deficient Th25 cells compared with that in Act1-sufficient cells undergoing Th25 differentiation conditions. These results suggest that in addition to the promotion of Th2 cytokine production during Th25 differentiation, IL-25-induced Act1-mediated signaling is also important for the suppression of Th1 cytokines (i.e., IFN- γ). Importantly, the decreased IL-4 expression we observed in T cell-specific Act1-deficiency during Th25 cultures was independent of a feedback

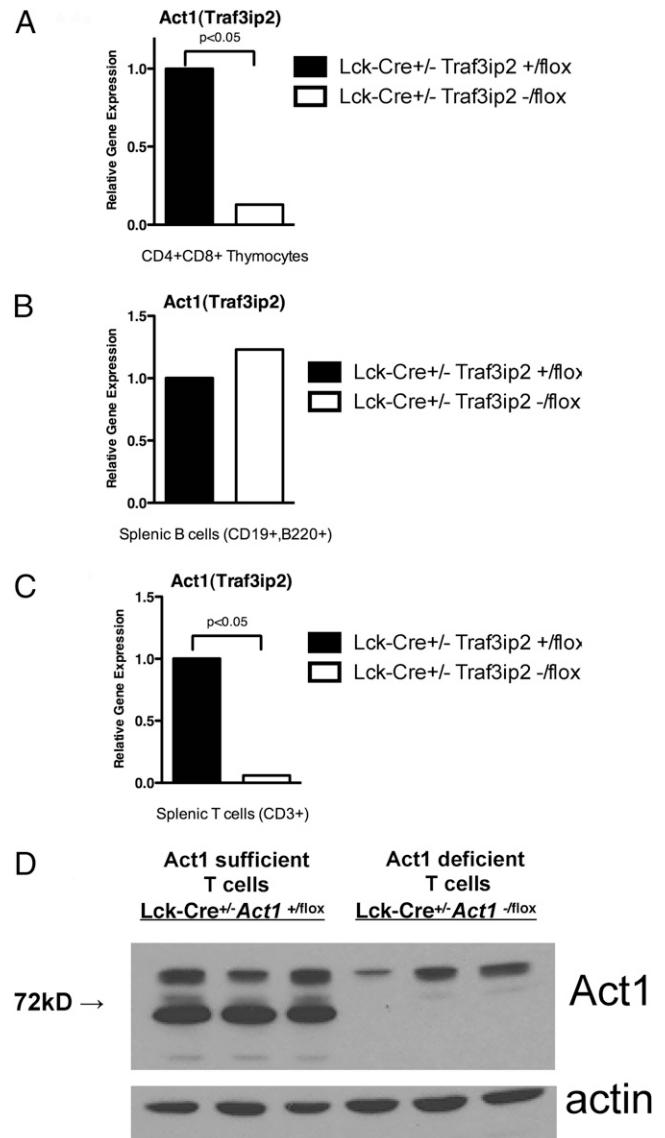
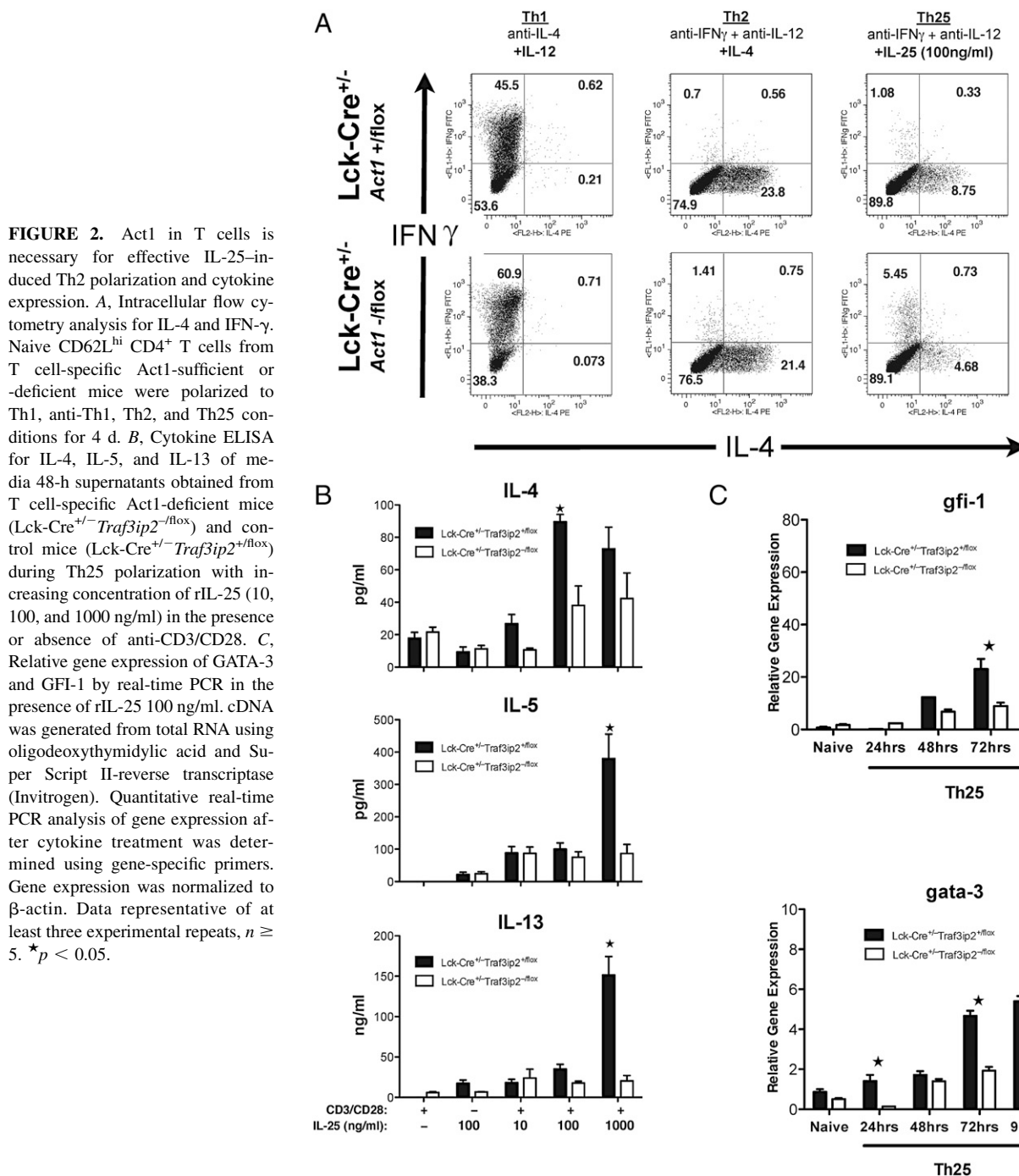


FIGURE 1. T cell-specific deletion of *Traf3ip2/Act1*. A–C, Act1 expression from real-time PCR analysis of total mRNA obtained from purified double-positive thymocytes (A), splenic B cells (B), and splenic T cells (C) isolated from control mice (Lck-Cre^{+/+}-*Traf3ip2*^{+/-flox}) and T cell-specific Act1-deficient mice (Lck-Cre^{+/+}-*Traf3ip2*^{-flox}) demonstrating specific abolishment of *Traf3ip2/Act1* expression in the T cell compartment. D, Immunoblot analysis for the expression of Act1 in T cells from control mice (Lck-Cre^{+/+}-*Traf3ip2*^{+/-flox}) and T cell-specific Act1-deficient mice (Lck-Cre^{+/+}-*Traf3ip2*^{-flox}). Data representative of at least three experimental repeats.

effect of increased IFN- γ (Supplemental Fig. 1). Notably, increased IFN- γ was observed only under Th1, Th2, and Th25 polarization conditions and not under Th0, ThN, or Th17 polarization conditions (Supplemental Fig. 2).

We next examined the early effect of rIL-25 on Th2 cytokine production during the *ex vivo* Th25 differentiation. This was accomplished by the measurement of IL-4, IL-5, and IL-13 in supernatant of Act1-sufficient or -deficient CD62L^{hi} naive CD4⁺ T cells cultured in the presence of anti-CD3/CD28, anti-IFN- γ , and anti-IL-12 with increasing concentrations of IL-25 (10, 100, and 1000 ng/ml). Whereas IL-25 was able to induce the production of IL-4, IL-5, and IL-13 in a dose-dependent manner in Act1-sufficient T cells, reduced production of IL-4, IL-5, and IL-13 was observed in Act1-deficient T cells (Fig. 2B). Given the



clear defect in the IL-25-induced generation of Th2 cytokine-producing T cells in the absence of Act1 expression, we next examined the expression of critical regulators and transcription factors known to facilitate the Th2 differentiation. GATA-3 has been demonstrated to be necessary and sufficient for Th2 differentiation and cytokine production (22–24). The induction of GATA-3 expression during the Th25 culture conditions was impaired in Act1-deficient naive T cells (Fig. 2C). Furthermore, the expression of GFI-1, which has also been demonstrated to be critical for the expansion of Th2 cells (25) and suppression of other types of T cell differentiation (26), was diminished in the absence of Act1 (Fig. 2C). Thus, Act1 expression in naive T cells is crucial for the effective IL-25-driven promotion of Th2 differentiation and cytokine production through the induction of key Th2 transcription factors GATA-3 and GFI-1.

Act1 in T cells is required for the development of IL-25-induced pulmonary eosinophilia

IL-25 has been demonstrated to induce and promote eosinophilic inflammation by driving the expression of prototypical Th2 cytokines IL-4, IL-5, and IL-13 (10, 12). This effect is dependent on IL-25 cognate receptors IL-17RA and IL-17RB (17). The blockade or gene deletion of either of these molecules leads to an attenuation of the IL-25 responses. We have recently reported that Act1 is an essential adapter molecule for IL-25 signaling and induces biological responses where Act1 interacts with IL-25 receptors through a SEFIR domain-dependent manner. To examine the role of T cell-derived Act1 in IL-25 receptor signaling in vivo, wild-type and T cell-specific Act1-deficient mice were treated with rIL-25 through the intranasal route. T cell-specific Act1 deficiency abolished IL-25-induced eosinophilia and the

induction of Th2 proteins IL-5 and IL-13 (Fig. 3A, 3B). Notably, the reduced eosinophilic infiltration observed in T cell-specific Act1-deficient mice after intranasal IL-25 administration was accompanied by a significant increase in neutrophilic lung accumulation. Thus, T cell-specific Act1-deficient mice are unable to induce a Th2-biased eosinophilic pulmonary response in response to IL-25 treatment or in response to house dust mite challenge (Supplemental Fig. 3).

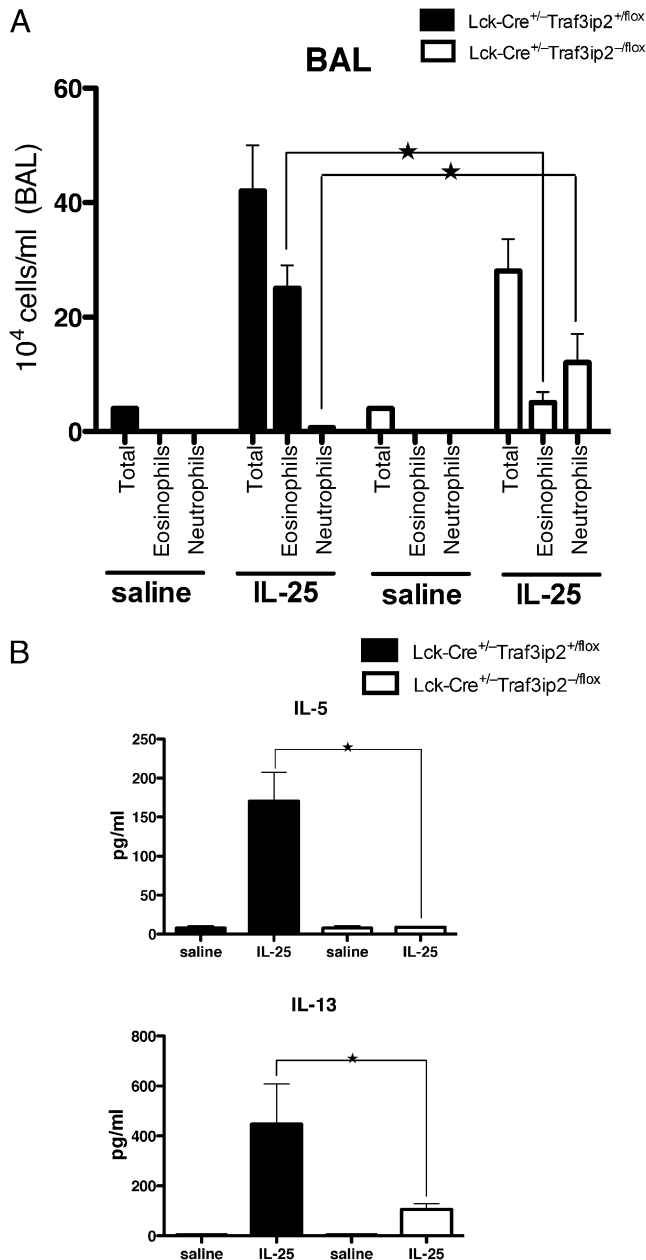


FIGURE 3. Act1 in T cells is required for the development of IL-25-induced pulmonary eosinophilia. Control mice (Lck-Cre^{+/+}Traf3ip2^{+/flox}) and T-specific Act1-deficient mice (Lck-Cre^{+/+}Traf3ip2^{-flox}) received intranasal administration of saline or rIL-25 (5 μ g/mouse), and mice were sacrificed and analyzed 4 d later. **A**, Reduced eosinophil accumulation in BAL fluid from T cell-specific Act1-deficient mice (Lck-Cre^{+/+}Traf3ip2^{-flox}) compared with that in BAL fluid from control littermates (Lck-Cre^{+/+}Traf3ip2^{+/flox}) after IL-25 intranasal administration. **B**, IL-5 and IL-13 ELISA was performed on BAL fluid obtained after intranasal administration of saline or rIL-25. Data represent means ($n = 8$) $\pm$ SEM. * $p < 0.05$.

Act1 in T cells is required for the development of Ag-induced airway inflammation, eosinophilia, and AHR

To examine the in vivo role of Act1 in the T cell compartment during the development of Ag-induced airway inflammation, we used the OVA model of airway inflammation. The OVA model of airway inflammation has been used extensively to examine the in vivo development of eosinophilic inflammation and the associated increase in AHR in which T cell-mediated Th2 immunity is heavily implicated (27, 28). A protocol optimized for the induction of pulmonary eosinophilia and AHR in the C57BL/6 strain was used (2, 29) (Fig. 4A). Control mice (Lck-Cre^{+/+}Traf3ip2^{+/flox}) and T cell-specific Act1-deficient mice (Lck-Cre^{+/+}Traf3ip2^{-flox}) were immunized with chicken OVA in conjunction with the adjuvant alum. Enumeration of leukocyte infiltration of the BAL fluid revealed a significant reduction in the total leukocytes infiltration (121.6 ± 22.3 versus 44.60 ± 9.2 , $p < 0.05$) including eosinophils (79.2 ± 15.7 versus 34.4 ± 5.2 , $p < 0.05$), neutrophils (13.7 ± 4 versus 4.5 ± 1), and macrophages (20.6 ± 5 versus 5.820 ± 1.3 , $p < 0.05$) in T cell-specific Act1-deficient mice (Lck-Cre^{+/+}Traf3ip2^{-flox}) compared with that of their control littermates (Lck-Cre^{+/+}Traf3ip2^{+/flox}) (Fig. 4C, 4D). Gross histological analysis of lung tissue after H&E staining revealed a marked abrogation of perivascular as well as peribronchial leukocytic infiltration in T cell-specific Act1-deficient mice (Fig. 4B). Notably, periodic acid-Schiff staining revealed an equivalent amount of goblet cell hyperplasia.

To investigate the infiltration and accumulation of eosinophils, which are important mediators of pulmonary pathologies associated with allergic pulmonary responses, we performed immunofluorescence staining on lung tissue for eosinophil granule major basic protein (MBP), a constituent of the crystalline core of the eosinophil granule and a marker for eosinophils. MBP staining was markedly increased in T cell-specific Act1-sufficient mice after OVA aerosol challenge following OVA/alum sensitization; nevertheless, T cell-specific Act1-deficient mice undergoing similar treatment demonstrated minimal MBP staining (Fig. 5A, 5B). The marked decrease of MBP-positive eosinophils is consistent with the BAL fluid enumeration as well as the gross histological analysis (Fig. 4B). Notably, IL-5 protein levels obtained from multiplex analysis of recovered BAL fluid revealed no apparent reduction of the levels of this essential regulator for the terminal differentiation of eosinophil progenitors (Fig. 5B). Similarly, levels of IL-12p40, RANTES, and KC were comparable among experimental groups. In contrast, IL-13, GM-CSF, eotaxin-1, and IL-9 were robustly induced in immunized T cell-specific Act1-sufficient mice after OVA aerosol challenge yet were below the detection threshold in BAL fluid of immunized T cell-specific Act1-deficient mice (Fig. 5C). Altogether, these results demonstrate an attenuation of eosinophilic pulmonary infiltration after Ag sensitization and aerosol challenge in T cell-specific Act1-deficient mice and implicate associated mediators including IL-13, GM-CSF, eotaxin-1, and IL-9.

Next, we investigated the pathophysiological development of AHR typically associated with Ag-induced pulmonary eosinophilia in a mouse model of allergic asthma. In our previous study, we observed that the development of AHR in epithelial-specific Act1-deficient mice remained intact despite the marked attenuation of pulmonary inflammation, suggesting that Act1 in the epithelial compartment was not necessary for the development of AHR despite the reduced epithelial responsiveness to IL-25 in these mice. Because the role of Act1 in T cells as it relates to the development of AHR during a murine model of asthma is not known, we therefore analyzed the changes in AHR in response to increasing doses of

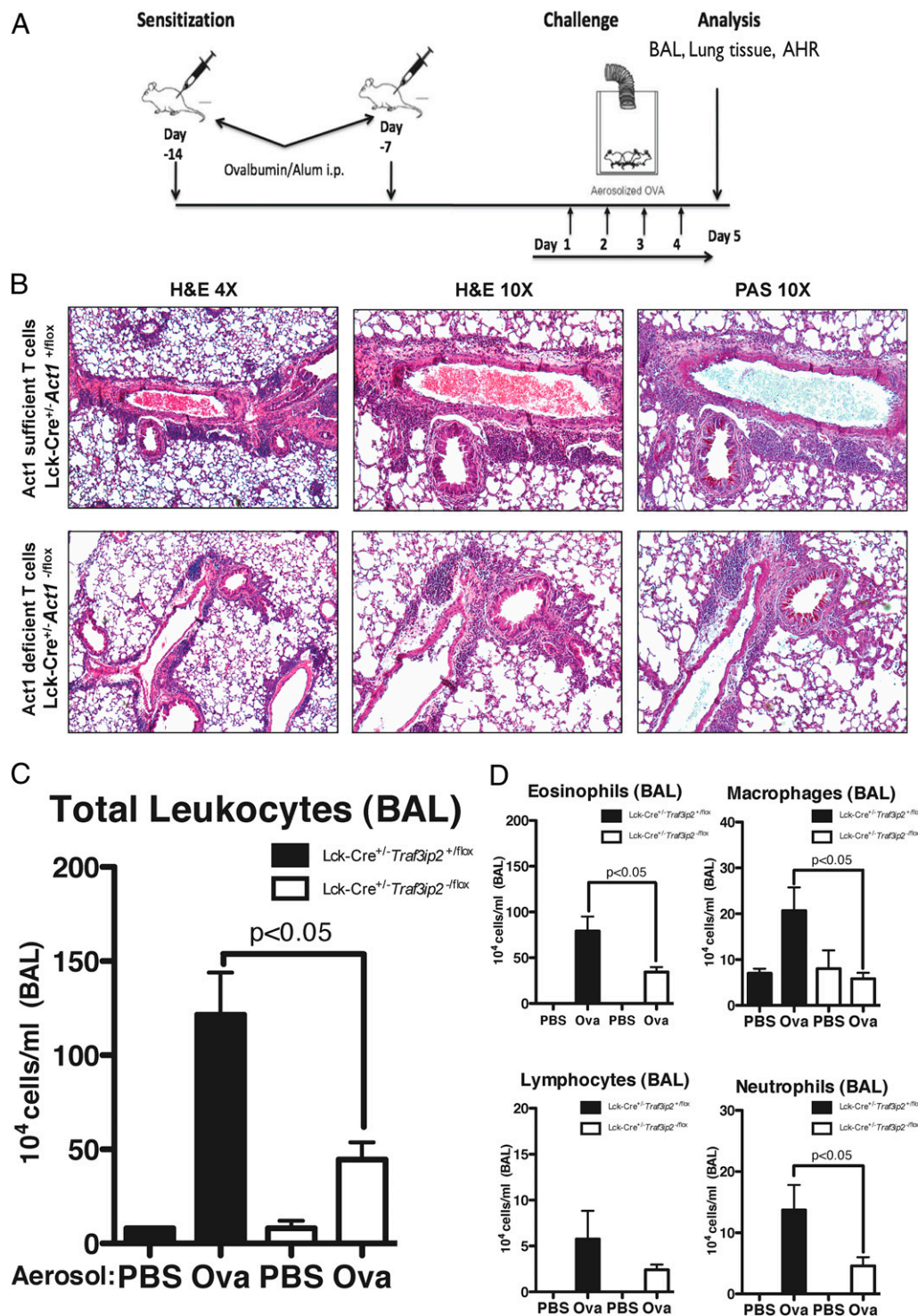


FIGURE 4. T cell-specific deletion of Traf3ip2/Act1 leads to an attenuation of pulmonary inflammation in a mouse model of allergic pulmonary eosinophilia. Control mice (Lck-Cre^{+/+}-Traf3ip2^{+/lox}) and T cell-specific Act1-deficient mice (Lck-Cre^{+/+}-Traf3ip2^{-/lox}) were subjected to a mouse model of allergen-mediated pulmonary inflammation as described in *Materials and Methods*. **A**, Schematic representation of the OVA/alum mouse asthma model. **B**, H&E and periodic acid-Schiff (PAS) staining of lung sections. **C** and **D**, Differential count analysis of BAL cytospin preparations stained with Wright-Giemsa. Data represent means ($n \geq 5$) $\pm$ SEM; four independent experiments. * $p < 0.05$.

methacholine 24 h after the last aerosol challenge. AHR assessment in a murine model of acute allergic pulmonary inflammation is performed by assessing lung mechanics in response to methacholine after the induction of allergic pulmonary inflammation (30). The parameters we measured include lung resistance (R_L), which is a dynamic resistance measure obtained through forced oscillation technique by assessing the level of constriction in total

lung tissue, which reflects narrowing of the conducting airways as well as alterations in the peripheral lung tissue (constriction of distal airways and changes in the intrinsic mechanical properties of the parenchyma). Also, we measured airway resistance (R_n), which is a Newtonian resistance parameter that is obtained by use of a constant phase model and represents the resistance of the central airways only. OVA aerosol-challenged mice were compared with

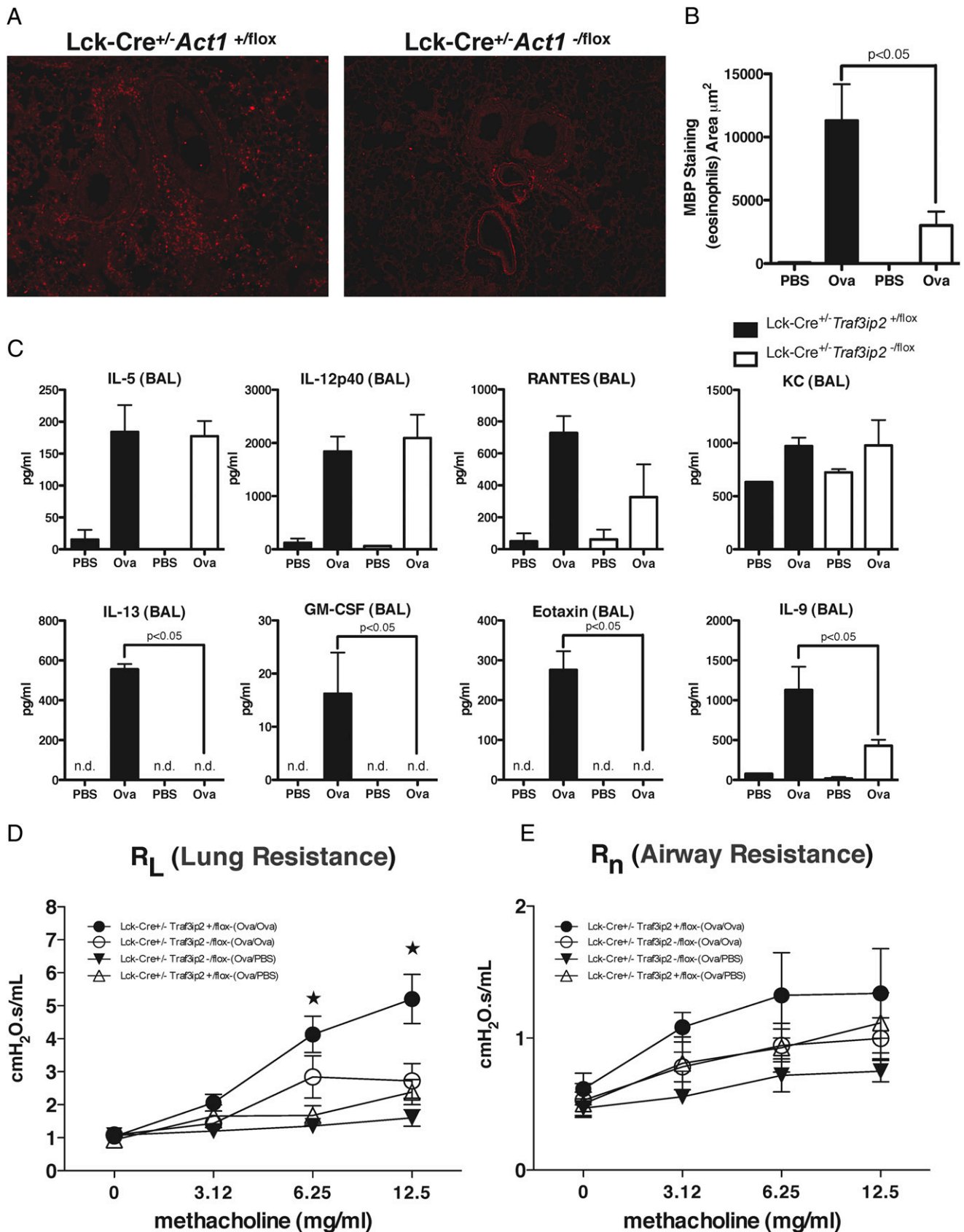


FIGURE 5. T cell-specific deletion of Traf3ip2/Act1 leads to an attenuation of pulmonary eosinophilic inflammation and AHR. **A** and **B**, Immunofluorescence staining of frozen lung sections using anti-mouse MBP goat Ab (red) in T cell-specific Act1-deficient mice (Lck-Cre^{+/+}Traf3ip2^{-flox}) compared with that in control mice (Lck-Cre^{+/+}Traf3ip2^{+/flox}) (original magnification $\times 10$). **C**, Multiplex protein analysis of BAL fluid from mice after the induction of mouse asthma model. **D** and **E**, AHR measurement using FlexiVent (Scireq). Lung resistance (**D**) and airway resistance (**E**) in response to increasing doses of aerosol methacholine delivery after the OVA or PBS challenge of OVA/alum-sensitized T cell-specific Act1-deficient mice (Lck-Cre^{+/+}Traf3ip2^{-flox}) compared with that in control mice (Lck-Cre^{+/+}Traf3ip2^{+/flox}). Data represent means $\pm$ SEM, $n = 7$ for all groups. Representative experiment of four independent experiments. $\star p < 0.05$.

PBS-challenged mice after OVA/alum immunization of T cell-specific Act1-sufficient or -deficient mice. A dramatic decrease of total lung resistance (R_L) was observed in the T cell-specific Act1-deficient mice (Fig 5D, 5E). In addition, although airway resistance (R_n) exhibited a reduced trend of induction, these changes were statistically significant.

Act1 in T cells is required for the development of cellular and humoral Th2 responses

The abrogation of eosinophilic airway infiltration as well as AHR in T cell-specific Act1-deficient mice after the induction of a murine OVA model of airway inflammation raised the question whether Act1 expression in T cells is necessary for the induction of Th2 responses after the OVA challenge in sensitized mice. To test this, we first analyzed the development of IL-5- and IL-13-

producing CD4⁺ T cells in both T cell-specific Act1-sufficient or -deficient mice after OVA/alum sensitization. A significant decrease in Ag-induced production of the Th2 cytokines IL-5 and IL-13 was observed in T cell-specific Act1-deficient mice compared with that in T cell-specific Act1-sufficient mice, indicating defective Th2 responses in T cell-specific Act1-deficient mice (Fig. 6A).

IL-25-induced Th2 immunity is associated with increased concentrations of serum IgG1 and IgE. We analyzed OVA-specific Igs from both T cell-specific Act1-sufficient or -deficient mice after OVA/alum sensitization or after sham immunization with alum alone. Act1 deficiency in the T cell compartment did not result in any apparent abnormality in the levels of total Ig concentration (Fig. 6B). Furthermore, there was no significant difference in the generation of OVA-specific IgG2a and IgE after OVA/alum

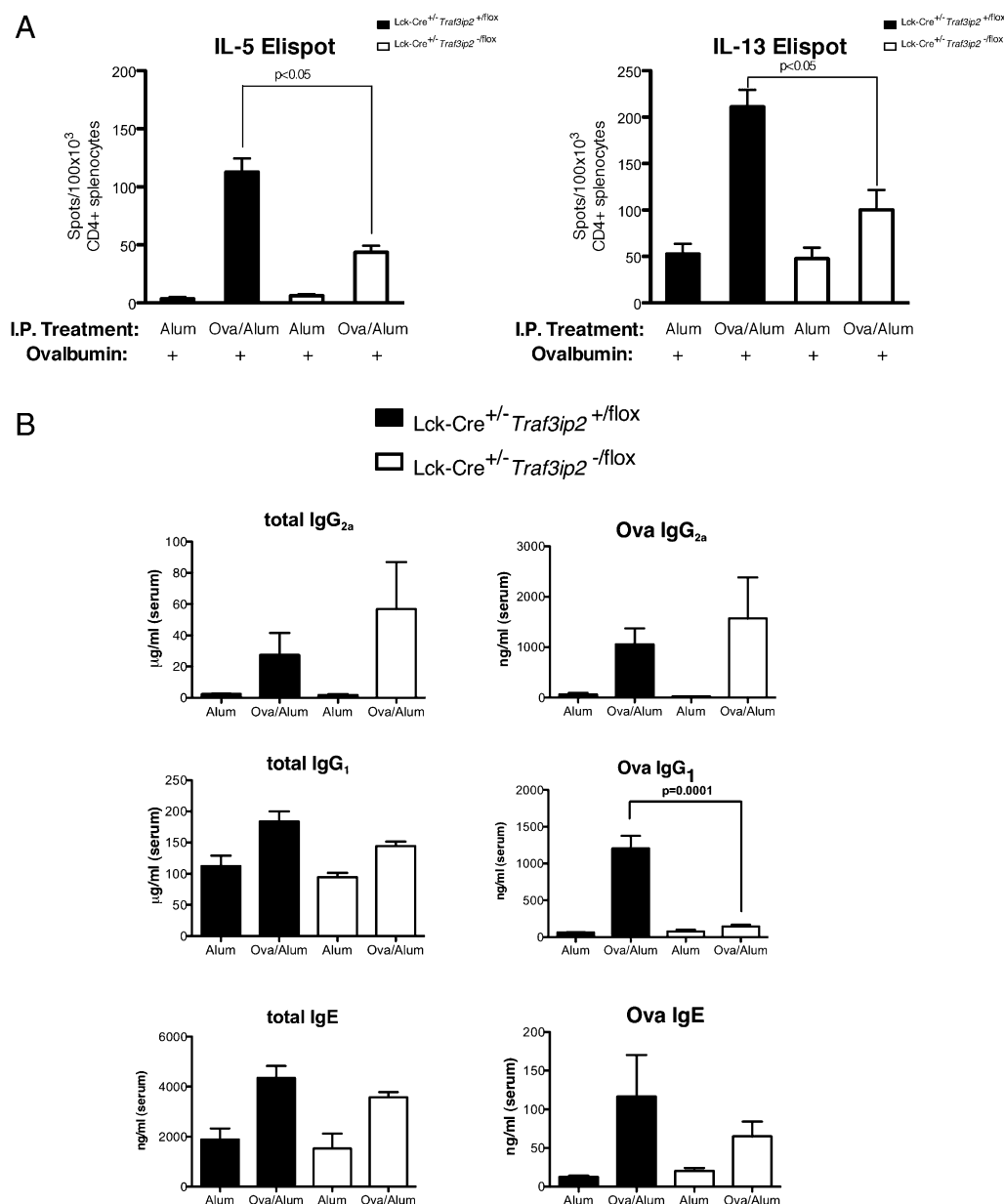


FIGURE 6. Act1 in T cells is required for the development of cellular and humoral Th2 responses. A, IL-5 and IL-13 ELISPOT assays of CD4⁺ enriched splenocytes obtained from OVA/alum-sensitized or alum sham-sensitized T cell-specific Act1-deficient mice (Lck-Cre^{+/-} Traf3ip2^{-/-flox}) and control mice (Lck-Cre^{+/-} Traf3ip2^{+/-flox}); 100 × 10³ CD4⁺ enriched splenocytes. B, Total and OVA-specific Abs after OVA/alum sensitization in T cell-specific Act1-deficient mice (Lck-Cre^{+/-} Traf3ip2^{-/-flox}) and control mice (Lck-Cre^{+/-} Traf3ip2^{+/-flox}). Data represent means ± SEM for (A) n ≥ 5 and (B) n ≥ 5. Three independent experiments. The p values < 0.05 are indicated on the figure.

immunization (Fig. 6B). In contrast, the generation of OVA-specific IgG1 was completely abolished in T cell-specific Act1-deficient mice after immunization ($1,205 \pm 169.9$ ng/ml versus 142.8 ± 24.33 ng/ml, $p = 0.0001$) (Fig. 6B). Thus, Act1 in the T cell compartment leads to a particular defect in the generation of Ag-specific IgG1 isotype.

Discussion

IL-25 (IL-17E) has been shown to drive Th2 immunity including pathogenic responses associated with allergic pulmonary inflammation (7–9). However, it is unclear how IL-25 drives these allergic/atopic responses through the interplay between the immune and parenchymal compartments, particularly during the initiation, propagation, and effector phases of the allergic immune response (5). Recent studies have demonstrated that lung epithelial cells can produce IL-25 in response to allergic triggers (31). Further, IL-25 can elicit recently described novel populations of innate helper type 2 (32–34). Nonetheless, the cellular network involving IL-25 responsiveness warrants additional investigation.

Using cell type-specific deletion of the U-box-type E3 ubiquitin ligase Act1 (CIKS/Traf3ip2), previously reported to be a critical adaptor molecule for the IL-17 cytokine family including IL-25/IL-17E (1, 2), we demonstrated that epithelial-derived Act1 was necessary for robust IL-25-induced as well as allergen-mediated eosinophilic pulmonary inflammation. Nevertheless, epithelial-specific Act1-dependent signaling was dispensable for the development of AHR and the generation of allergic humoral responses associated with the allergic sequelae.

In this study, we used cell-specific deletion of Act1 to identify the role of Act1 in the T cell compartment, while IL-25 receptor (IL-17RB) has been reported to mediate T cell-mediated responses relevant to allergic responses (11, 13, 18, 35). We show that Act1-deficient T cells have a limited capacity to differentiate into Th2 subtype in response to IL-25 stimulation, consistent with their attenuated induction of the key Th2-associated transcription factors GATA-3 and GFI-1. Notably, conventional IL-4-driven Th2 differentiation was intact in Act1-deficient T cells. Further, our *in vivo* studies show that the defect in Th2 differentiation was also relevant to exogenous IL-25 as well as Ag-dependent Th2 responses, particularly the development of Ag-induced eosinophilic pulmonary inflammation, AHR, and allergen-induced eosinophilia. Thus, Act1 in the T cells plays a nonredundant and necessary role in the induction of Th2 immunity and allergic immune responses.

Therefore, by the targeted disruption of Act1 (CIKS/Traf3ip2) expression in a different tissue compartment, we have better defined the role of Act1 in the biological and pathological responses induced by IL-25 (IL-17E). Robust induction of IL-25-mediated responses required the expression of Act1 in both epithelial and T cells. Yet, the Act1 requirement in the epithelium is distinct from its requirement in the T cell compartment. Act1 deficiency in the T cell compartment, but not in the epithelial compartment, resulted in an abrogation in allergic humoral responses and AHR. Based on these findings, we conclude that IL-25-induced Act1-mediated signaling is essential for Th2 cell responses and allergic pulmonary inflammation through the differential and synergistic impact on the epithelial and T cell compartments and consequent propagation of allergic immune responses.

Disclosures

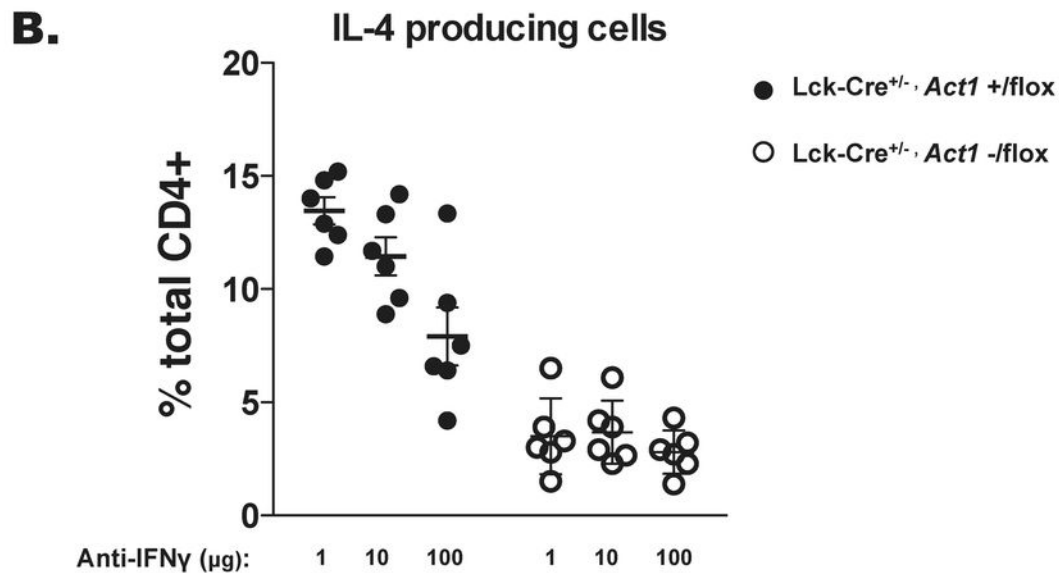
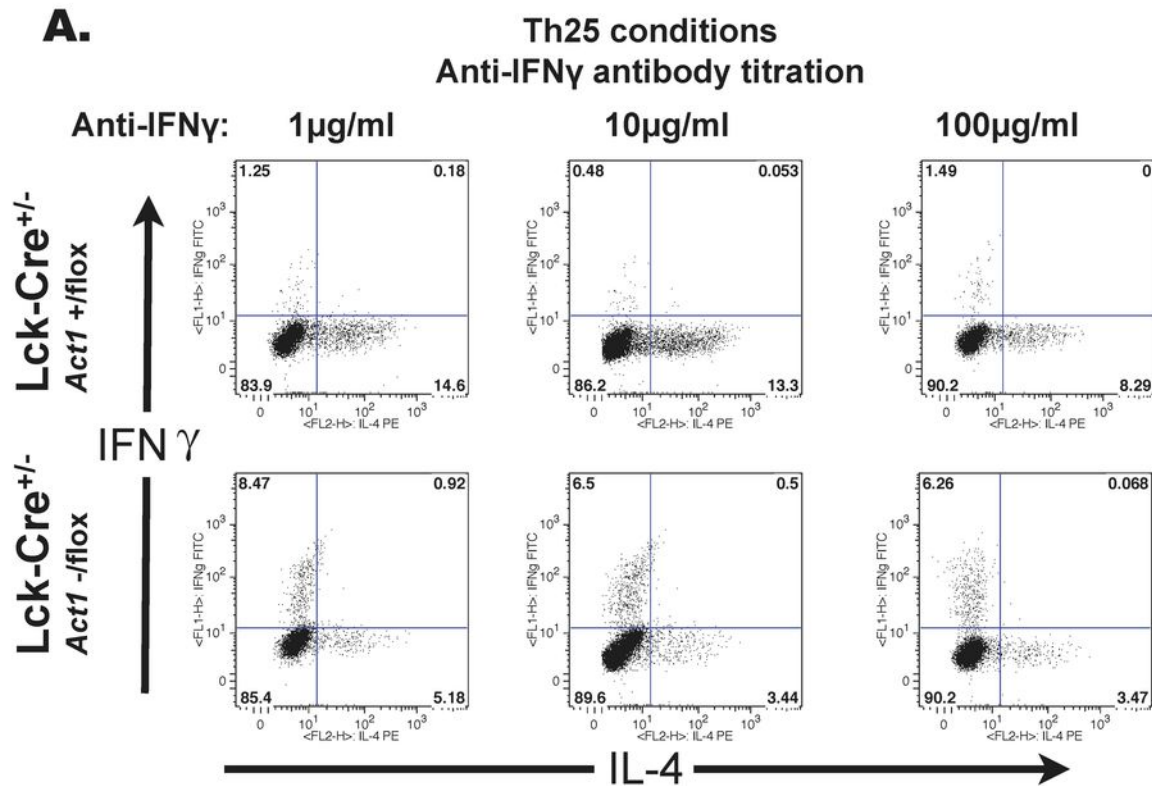
The authors have no financial conflicts of interest.

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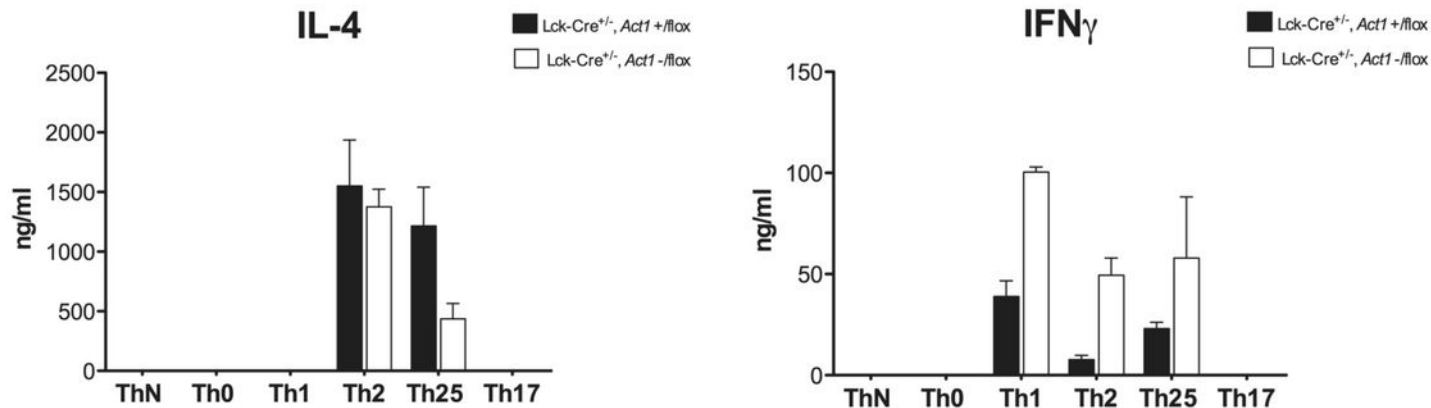
Supplementary Figure 1: (A.), (B.)



Supplementary figure 1: Decreased IL-4 expression is independent of increased IFN γ in T cell-specific Act1-deficiency . (A.) Intracellular flow cytometry analysis for IL-4 and IFN γ . Naive CD62L^{hi} CD4⁺ T cells from T cell-specific Act1 sufficient or deficient mice were polarized to Th25 conditions for 4 days in the presence of anti-IFN γ antibody at 1 μ g/ml, 10 μ g/ml, and 100 μ g/ml. **(B)** Percentage of IL-4 producing T cells from T cell-specific Act1 sufficient or deficient mice polarized in vitro under Th25 conditions for 4 days in the presence of anti-IFN γ antibody at 1 μ g/ml, 10 μ g/ml, and 100 μ g/ml. Data represents means ($n \geq 5$) $\pm$ S.E.M.

Supplementary Figure 2:

IL-4 and IFN γ production during *in vitro* T cell polarization

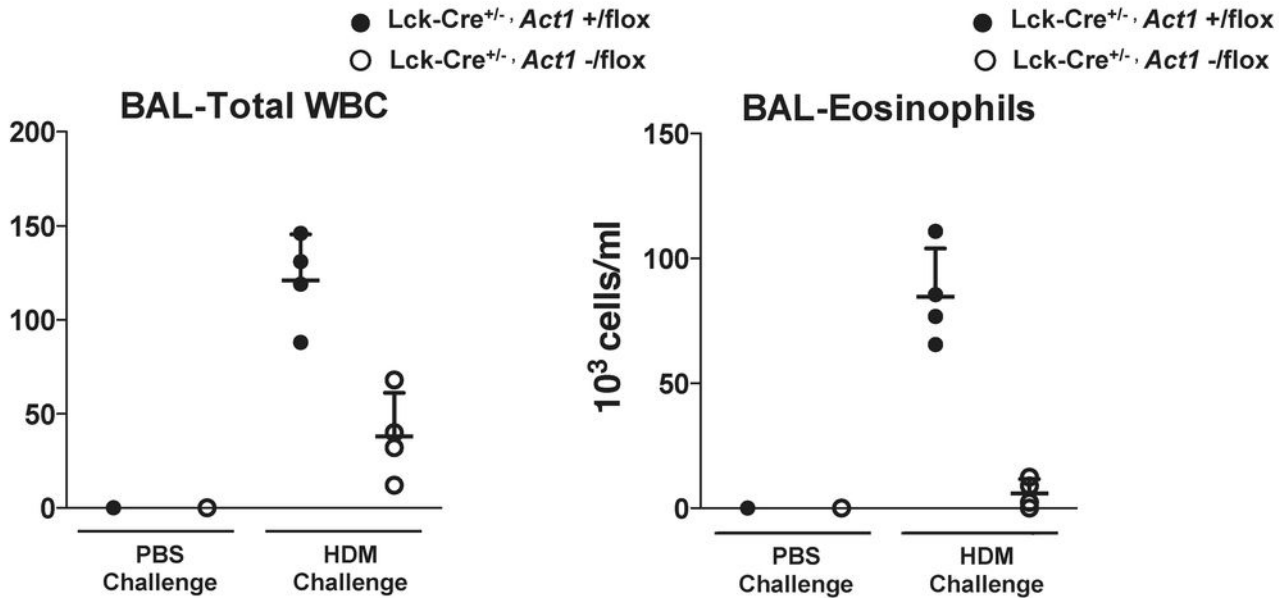


Supplementary figure 2: Cytokine Th1 vs. Th2 cytokine production, ELISA for IL-4 and IFN γ of media supernatant from T cells cultures (from T cell-specific Act1-deficient mice (Lck-Cre^{+/+}-Traf3ip2^{-/lox}) and Control mice (Lck-Cre^{+/+}-Traf3ip2^{+/lox})) that underwent ThN, Th0, Th1, Th2, Th25, and Th17 polarization conditions for five days, followed by two days resting and re-stimulation. ThN (no antibodies or recombinant cytokines added), Th0 (10 μ g/ml anti-IFN γ , 10 μ g/ml anti-IL-12/23, and 10 μ g/ml anti-IL-4), Th1 (10 ng/ml IL-12 and 10 μ g/ml anti-IL-4), Th2 (1000 U/ml IL-4, 10 μ g/ml anti-IFN γ , and 10 μ g/ml anti-IL-12/23), Th17 (1 ng/ml TGF β , 20 ng/ml IL-6, , 10 ng/ml IL-23, 10 μ g/ml anti-IFN γ , and 10 μ g/ml anti-IL-4). Data represents means ($n \geq 5$) $\pm$ S.E.M.

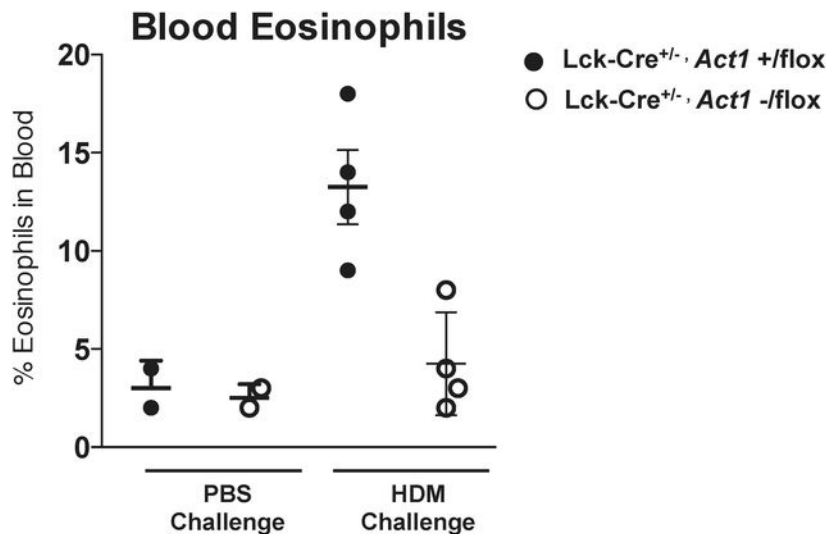
Supplementary Figure 3: (A.), (B.)

A.

House dust mite Challenge



B.



Supplementary figure 3: T cell specific deletion of Traf3ip2/Act1 leads to an attenuation of pulmonary and systemic eosinophilia in response to house dust mite challenge. (A) Differential count analysis of BAL cytopsin preparations stained with Wright-Giemsa from control mice (Lck-Cre^{+/+}-Traf3ip2^{+/lox}) and T cell-specific Act1-deficient mice (Lck-Cre^{+/+}-Traf3ip2^{-/lox}) following exposure to house dust mite challenge as previously described (36). (B) Blood eosinophils were assessed using a blood analyzer (Advia 120, Bayer). Data represents means (n≥4)±S.E.M. Four independent experiment.