Role of IL-4 receptor α–positive CD4$^+$ T cells in chronic airway hyperresponsiveness

Frank Kirstein, PhD, Natalie E. Nieuwenhuizen, PhD,* Jaisubash Jayakumar, PhD, William G. C. Horsnell, PhD, and Frank Brombacher, PhD *Cape Town, South Africa, and Berlin, Germany*

Background: T_H2 cells and their cytokines are associated with allergic asthma in human subjects and with mouse models of allergic airway disease. IL-4 signaling through the IL-4 receptor α (IL-4Rα) chain on CD4$^+$ T cells leads to T_H2 cell differentiation *in vitro*, implying that IL-4Rα–responsive CD4$^+$ T cells are critical for the induction of allergic asthma. However, mechanisms regulating acute and chronic allergen-specific T_H2 responses *in vivo* remain incompletely understood.

Objective: This study defines the requirements for IL-4Rα–responsive CD4$^+$ T cells and the IL-4Rα ligands IL-4 and IL-13 in the development of allergen-specific T_H2 responses during the onset and chronic phase of experimental allergic airway disease.

Methods: Development of acute and chronic ovalbumin (OVA)–induced allergic asthma was assessed weekly in CD4$^+$ T cell–specific IL-4Rα–deficient BALB/c mice (Lck^{cre}IL-4Rα$^{-/lox}$) and respective control mice in the presence or absence of IL-4 or IL-13.

Results: During acute allergic airway disease, IL-4 deficiency did not prevent the onset of T_H2 immune responses and OVA-induced airway hyperresponsiveness or goblet cell hyperplasia, irrespective of the presence or absence of IL-4Rα–responsive CD4$^+$ T cells. In contrast, deficiency of IL-13 prevented allergic asthma, irrespective of the presence or absence of IL-4Rα–responsive CD4$^+$ T cells. Importantly, chronic allergic inflammation and airway hyperresponsiveness were dependent on IL-4Rα–responsive CD4$^+$ T cells. Deficiency in IL-4Rα–responsive CD4$^+$ T cells resulted in increased numbers of IL-17–producing T cells and, consequently, increased airway neutrophilia.

Conclusion: IL-4–responsive T helper cells are dispensable for acute OVA-induced airway disease but crucial in maintaining chronic asthmatic pathology. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: *T_H2 cell, acute allergic airway disease, chronic asthma, cytokine receptors, IL-4, IL-13, gene-deficient mice*

Allergic asthma is a chronic inflammatory disease of the airways characterized by an inappropriate immune response to harmless environmental antigens. T_H2 cells regulate adaptive immune responses to allergens, and their presence correlates with disease symptoms in human subjects and mice.[1] IL-4 plays a crucial role in the *in vitro* and *in vivo* differentiation of T_H2 cells, suggesting major contributions for IL-4 and its receptor, IL-4 receptor α (IL-4Rα), in regulating allergic T_H2 responses.[2] However, the initiation and maintenance of *in vivo* T_H2 differentiation is incompletely understood. The IL-4/IL-4Rα pathway is not essential for T_H2 polarization in certain *in vivo* settings, and important roles for the cytokines IL-25 and IL-33 and type 2 innate lymphoid cells (ILC2s) have been suggested.[2] Several studies described IL-4–independent T_H2 differentiation in response to allergens or helminth infection.[3,4] Other studies suggested that IL-4 signaling is required for the expansion and maintenance of T_H2 responses after *Nippostrongylus brasiliensis* infection or allergen treatment but not for the initial T_H2 differentiation.[5,6]

Although we and others have described IL-4Rα–independent airway hyperresponsiveness (AHR),[7,8] mouse models of allergic asthma generally highlight the importance of IL-4, IL-5, IL-13, and the IL-4Rα/signal transducer and activator of transcription 6 signaling pathway for the development of allergic pathology, including airway eosinophilia, goblet cell hyperplasia, and AHR. Mice deficient in IL-4Rα showed abrogated T_H2 cell differentiation and were protected from ovalbumin (OVA)–induced allergic airway disease.[9-11] IL-4Rα is ubiquitously expressed, and we and others have successfully used mice with cell type–specific disruptions of the *Il4ra* gene to identify IL-4Rα–dependent disease symptoms mediated by airway epithelial cells,[12] smooth muscle cells,[11,13] and macrophages.[10] The *in vivo* requirements for IL-4Rα signaling on CD4$^+$ T cells in the initiation of allergen-specific T_H2 responses remain incompletely understood. Furthermore, it has been shown that CD4$^+$ cells and IL-4 are required for chronic lung inflammation and AHR but might not be necessary for chronic pathogenesis, including AHR, after acute inflammatory responses have resolved.[14-17]

In the present study we used previously described Lck^{cre}IL-4Rα$^{-/lox}$ mice with a CD4$^+$ T cell–specific IL-4Rα disruption[18] to define whether IL-4Rα–responsive CD4$^+$ T cells and the IL-4Rα ligands IL-4 and IL-13 are required for the initial *in vivo* differentiation of an allergen-specific T_H2 response and the subsequent onset of OVA-induced allergic airway disease.

From the International Centre for Genetic Engineering and Biotechnology (ICGEB) and Division of Immunology, Institute of Infectious Disease and Molecular Medicine, Faculty of Health Science, University of Cape Town.

*Natalie E. Nieuwenhuizen, PhD, is currently affiliated with the Department of Immunology, Max Planck Institut für Infektionsbiologie, Berlin.

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Corresponding author: Frank Brombacher, PhD, Program Coordinator, ICGEB, Level 1 Werner and Beit South, Faculty of Health Science, University of Cape Town Campus, Anzio Road, Observatory, 7925 Cape Town, South Africa. E-mail: frank.brombacher@icgeb.org. Or: frank.brombacher@uct.ac.za.

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Abbreviations used
AHR: Airway hyperresponsiveness
BAL: Bronchoalveolar lavage
FITC: Fluorescein isothiocyanate
ICOS: Inducible costimulator
ILC2: Type 2 innate lymphoid cell
IL-4Rα: IL-4 receptor α
OVA: Ovalbumin
PE: Phycoerythrin

Furthermore, we investigated whether $CD4^+$ T cells require IL-4Rα signals to sustain a T_H2-type phenotype and to promote disease pathology during chronic allergic airway inflammation. We found that acute allergic airway disease and T_H2 differentiation are independent of IL-4 signaling and that IL-13 drives airway pathology. Loss of IL-4Rα signaling on $CD4^+$ T cells resulted in increased IL-17 production and airway neutrophilia. Importantly, IL-4–responsive $CD4^+$ T cells promoted chronic asthmatic disease. In conclusion, IL-4–responsive T helper cells are dispensable for acute OVA-induced airway disease but required to maintain chronic asthmatic pathology.

METHODS

Mice

Six- to 8-week-old female mice were used in the experiments. Generation of Lck^{cre}IL-4Rα$^{-/lox}$ (C.Cg-Il4ra^{tm1Fbb}/Il4ra$^{tm2Fbb\text{-}Tg[Lck\text{-}Cre]/J}$) mice and IL-4R$^{-/lox}$ littermates on a BALB/c background was described previously.[18] Lck^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/lox}$ littermate control mice were intercrossed with IL-4–deficient[19] or IL-13–deficient[20] BALB/c mice for 9 generations. Animal procedures were approved by the University of Cape Town Animal Ethics Committee.

Models of allergic airway disease

Acute OVA-induced allergic airway disease. Mice were sensitized intraperitoneally and challenged intranasally with OVA (Sigma-Aldrich, Aston Manor, South Africa), as previously described.[10] In some experiments mice received 0.2 mg/mouse anti–IL-17A antibody[21] (clone MM17F3, kind gift of C. Uyttenhove) or isotype-matched control antibody by means of intraperitoneal injection on days 20 and 23.

Chronic OVA-induced allergic airway disease. Mice were sensitized subcutaneously with 20 μg of OVA adsorbed to 0.65% alum on days 0, 7, 14, and 21. On days 27 and 29 and then twice weekly for 4 weeks, mice were challenged intranasally with 20 μg of OVA in 50 μL of PBS after achievement of anesthesia.[10]

AHR

Respiratory resistance and compliance of the whole respiratory system and Newtonian resistance of the central airways were determined by means of forced oscillation measurements with a flexiVent system (SCIREQ, Montreal, Quebec, Canada) by using the single-compartment ("snapshot") perturbation or constant phase model, as previously described.[11]

Evaluation of goblet cell hyperplasia

Periodic acid–Schiff reagent–positive goblet cells and total epithelial cell nuclei in 5 to 8 bronchioles per lung section containing 80 to 220 epithelial cells were counted, and the ratio was converted to a percentage.[13]

Measurement of serum antibody levels

Total IgE and allergen-specific IgG antibody levels were measured by means of ELISA, as described previously.[11]

Analysis of airway and lung cell populations

Single-cell suspensions were prepared from lungs of individual mice and stained for intracellular cytokines, as previously described.[10] Bronchoalveolar lavage (BAL)[11] and lung cell composition of individual mice was determined by means of cell-surface staining with Siglec-F–phycoerythrin (PE), CD11c-allophycocyanin, and GR-1–fluorescein isothiocyanate (FITC) for eosinophils, neutrophils, and alveolar macrophages; CD3-PE, CD4–peridinin-chlorophyll-protein complex, and T1/ST2-FITC for T helper cells; and lineage–peridinin-chlorophyll-protein complex–Cy5.5, inducible costimulator (ICOS)–allophycocyanin, and T1/ST2-FITC for ILC2s. Flow cytometry was performed on a FACSCalibur flow cytometer (BD Biosciences, Erembodegem, Belgium), and data were analyzed with FlowJo version 10 software (TreeStar, Ashland, Ore). All antibodies were purchased from BD Biosciences or eBioscience (San Diego, Calif), except T1/ST2 (MD Bioproducts, St Paul, Minn). In some experiments BAL cells were stained with the Rapi-Diff Stain Set (Clinical Diagnostics CC, Johannesburg, South Africa) and analyzed based on differential cell counts, as described previously.[11]

Analysis of lung cytokine responses

Cytokine concentrations in lung tissue homogenate (1 mg/mL protein concentration) were determined by means of ELISA, according to the manufacturer's protocols (IL-4, IL-5, and IFN-γ: BD Biosciences; IL-13, IL-17, IL-25, and IL-33: R&D Systems, Minneapolis, Minn).

Statistical analysis

P values were calculated with GraphPad Prism 4 software (GraphPad Software, San Diego, Calif) by using the nonparametric Mann-Whitney test or Kruskal-Wallis test with the Dunn posttest. Respiratory resistance and compliance data were analyzed with repeated-measures ANOVA with the Bonferroni posttest. *P* values of .05 or less were considered significant.

RESULTS

Development of OVA-induced allergic airway disease in Lck^{cre}IL-4Rα$^{-/lox}$ mice

The role of IL-4Rα–responsive $CD4^+$ T cells in acute OVA-induced allergic airway disease was investigated by using $CD4^+$ T cell–specific IL-4Rα–deficient mice (Lck^{cre}IL-4Rα$^{-/lox}$) and IL-4Rα–responsive IL-4Rα$^{-/lox}$ littermate control mice (Fig 1, *A*). IL-4Rα was efficiently disrupted on the cell surfaces of $CD3^+CD4^+$ T cells but present on $CD3^+CD8^+$ T cells from lungs of OVA-treated Lck^{cre}IL-4Rα$^{-/lox}$ mice, confirming previous results (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org).[18] Development of AHR, airway eosinophilia, and goblet cell hyperplasia are the main characteristics of allergic airway disease and were absent in saline-treated Lck^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/lox}$ littermate control animals (see Fig E1, *B-D*). In OVA-treated mice respiratory resistance and compliance of the whole respiratory system and Newtonian resistance of the central airways were measured in response to increasing doses of inhaled methacholine as indicators of AHR. Importantly, AHR was not affected by the loss of IL-4Rα expression on $CD4^+$ T cells. OVA treatment induced increased respiratory and Newtonian resistance and a decrease in respiratory compliance in $CD4^+$ T cell–specific IL-4Rα–deficient mice (Lck^{cre}IL-4Rα$^{-/lox}$) and IL-4Rα–expressing IL-4Rα$^{-/lox}$ littermate control mice, with no significant differences between these groups (Fig 1, *B*). Furthermore, efficient goblet cell hyperplasia was observed after OVA treatment in Lck^{cre}IL-4Rα$^{-/lox}$ mice and littermate control

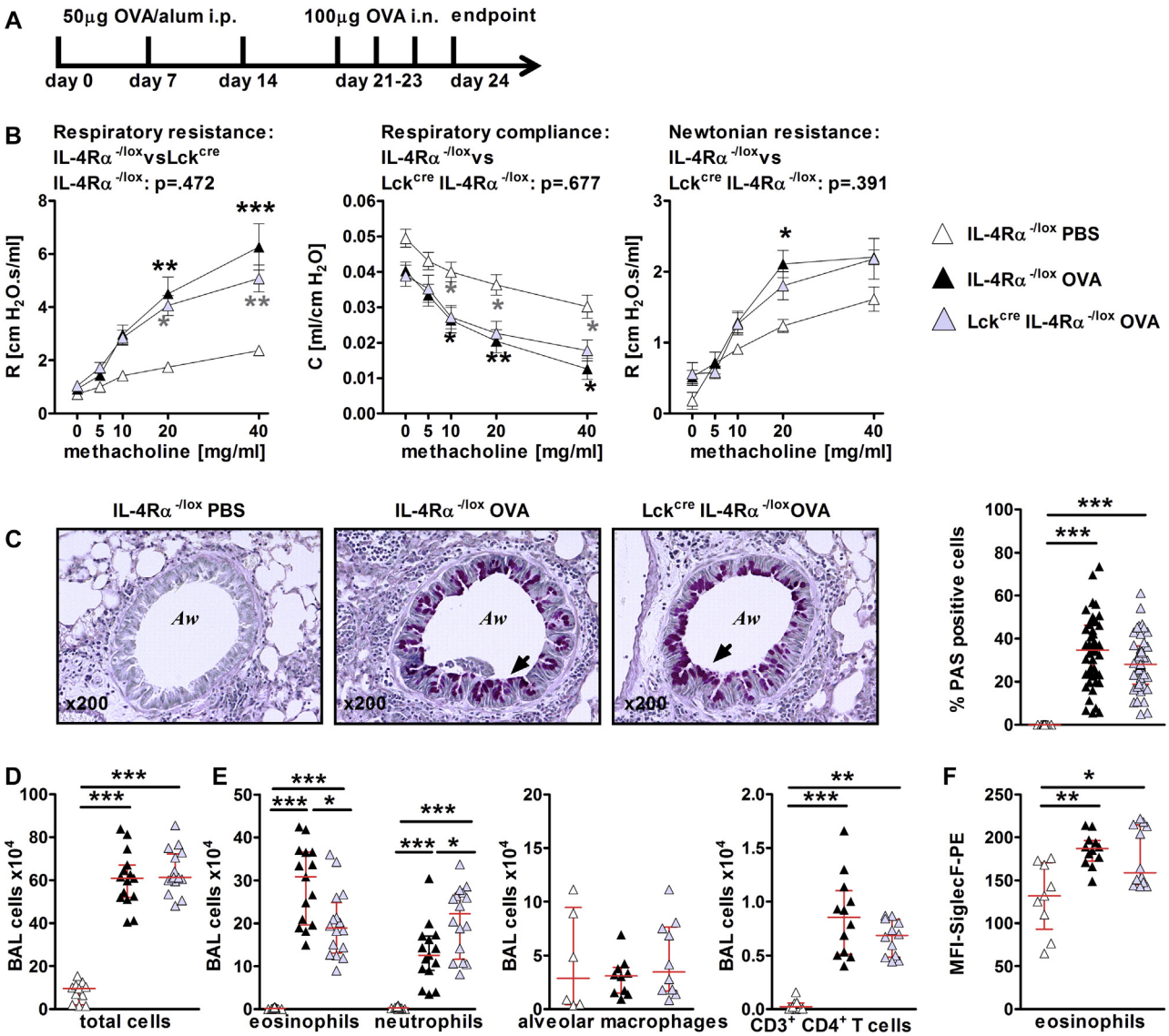

FIG 1. A, Mice received intraperitoneal *(i.p.)* injections of OVA/alum and intranasal *(i.n.)* OVA challenges. **B,** Respiratory resistance, compliance (n = 11, 12, and 14), and Newtonian resistance (n = 5, 8, and 10) in response to methacholine. *P* values are for comparison of dose-response curves by means of 2-way repeated-measures ANOVA (means ± SEMs). **C,** Goblet cell hyperplasia as indicated by *arrows* in representative periodic acid–Schiff (PAS) staining and percentage of PAS-positive bronchial epithelial cells. *Aw*, Airways. **D,** Total cell numbers in BAL fluid (n = 11, 15, and 16). **E,** Detection of eosinophils, neutrophils (n = 11, 15, and 16), alveolar macrophages, and T helper cells in BAL fluid (n = 9, 12, and 12) by means of flow cytometry. **F,** Mean fluorescence intensity *(MFI)* of Siglec-F–PE staining on BAL eosinophils. Data were pooled from 2 to 3 independent experiments. *Lines* represent medians ± interquartile ranges. $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$, Kruskal-Wallis nonparametric test.

mice but not in PBS-treated control animals, with no significant differences between the OVA-treated groups (Fig 1, *C*).

A model of subcutaneous sensitization was then used to investigate whether the route of sensitization affected our results.[10] Subcutaneous sensitization led to comparable pathology with intraperitoneal sensitization in Lck^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/lox}$ littermate control mice with regard to AHR and goblet cell hyperplasia, confirming that these parameters were not dependent on the route of sensitization (see Fig E2 in this article's Online Repository at www.jacionline.org). Allergic airway inflammation is characterized by infiltration of the lungs by eosinophils. OVA treatment led to significant cell infiltration in the BAL, and the total number of cells was not affected by the loss of CD4$^+$ T-cell IL-4Rα (Fig 1, *D*). OVA-treated CD4$^+$ T cell–specific IL-4Rα–deficient mice (Lck^{cre}IL-4Rα$^{-/lox}$) and IL-4Rα–expressing IL-4Rα$^{-/lox}$ littermate control mice showed increased numbers of eosinophils, neutrophils, and CD3$^+$CD4$^+$ T helper cells but not alveolar macrophages in the BAL fluid (Fig 1, *E*) and lung tissue (see Fig E3, *A*, in this article's Online Repository at www.jacionline.org). However, the number of eosinophils was reduced in Lck^{cre}IL-4Rα$^{-/lox}$ mice compared with littermate control

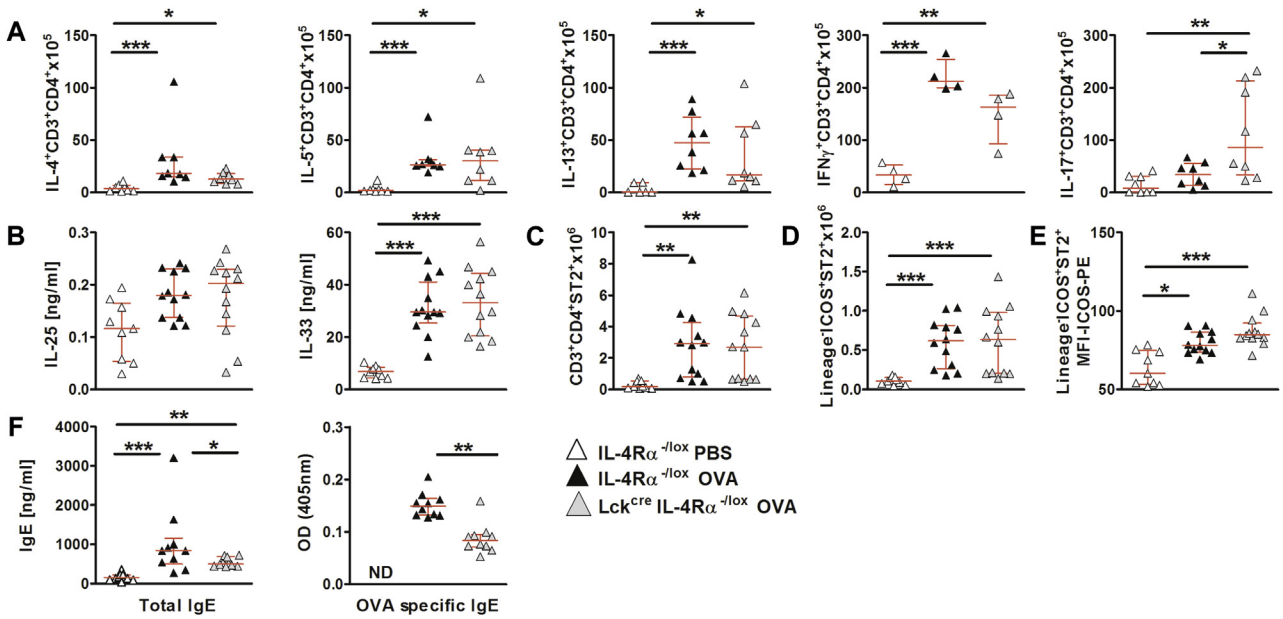

FIG 2. A, Cytokine-producing CD4$^+$ T cells from lung tissue were measured by means of fluorescence-activated cell sorting (n = 8). **B,** IL-25 and IL-33 levels were measured in lung tissue homogenate by using ELISA (n = 9, 12, and 12). **C** and **D,** IL-33 receptor (ST2) expressing CD3$^+$CD4$^+$ T cells (Fig 2, *C*) and lineage$^-$ICOS$^+$ST2$^+$ ILC2 populations (Fig 2, *D*) were detected with fluorescence-activated cell sorting (n = 9, 12, and 12). **E,** Mean fluorescence intensity *(MFI)* of ICOS-PE staining on lung ILC2s. Data were pooled from 2 independent experiments. **F,** Total IgE and OVA-specific IgE levels in blood serum were measured by means of ELISA. Data were pooled from 3 independent experiments. (n = 10). *Lines* represent medians ± interquartile ranges. *$P < .05$, **$P < .01$, and ***$P < .001$, Kruskal-Wallis nonparametric test.

animals, whereas the number of neutrophils was significantly higher in the BAL fluid (Fig 1, *E*). Although eosinophil numbers were increased compared with neutrophil numbers in IL-4Rα$^{-/lox}$ littermate control mice, eosinophil and neutrophil numbers were similar in Lck^{cre}IL-4Rα$^{-/lox}$ mice. BAL and lung tissue eosinophils in OVA-treated Lck^{cre}IL-4Rα$^{-/lox}$ mice and littermate control animals showed similar upregulation of Siglec-F surface expression, indicating an activated phenotype in both strains (Fig 1, *F*, and see Fig E3, B).[22,23] Together, these results demonstrate that IL-4Rα expression on CD4$^+$ T cells is not necessary for the development of major symptoms of allergic airway disease in mouse models of systemic OVA/alum sensitization.

Lck^{cre}IL-4Rα$^{-/lox}$ mice maintain T$_H$2-type cytokine responses to OVA

Because the development of allergic airway disease is regulated by T$_H$2-associated cytokines, we examined the effect of a lack of IL-4Rα responsiveness by lung CD4$^+$ T cells on their cytokine production. OVA treatment increased numbers of CD4$^+$ T cells producing the T$_H$2 cytokines IL-4, IL-5, and IL-13 in OVA-treated Lck^{cre}IL-4Rα$^{-/lox}$ mice and littermate control mice, with no significant differences between these groups (Fig 2, *A*). These results suggest that IL-4Rα signaling on CD4$^+$ T cells is not required for the *in vivo* differentiation of IL-4–, IL-5–, and IL-13–producing T$_H$2 cells. Treatment with OVA also promoted a strong IFN-γ response from CD4$^+$ T cells in both Lck^{cre}IL-4Rα$^{-/lox}$ mice and littermate control mice. IL-17–producing CD4$^+$ T cells were significantly upregulated in Lck^{cre}IL-4Rα$^{-/lox}$ mice compared with IL-4Rα$^{-/lox}$ mice, indicating that IL-4Rα signaling on CD4$^+$ T cells inhibits the development of T$_H$17 responses (Fig 2, *A*). Analysis of lung tissue cytokines and cytokine secretion of *ex vivo*–stimulated lung cells by means of ELISA confirmed a T$_H$2-type cytokine profile in Lck^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/lox}$ mice, whereas IL-17 concentrations were increased only in Lck^{cre}IL-4Rα$^{-/lox}$ mice (see Fig E4 in this article's Online Repository at www.jacionline.org). The cytokines IL-25 and IL-33 have been implicated in the development of type 2 immune responses in allergic diseases.[2] We found no significant increase in IL-25 concentrations in lung tissue homogenates after OVA treatment. However, IL-33 concentrations were upregulated in IL-4Rα$^{-/lox}$ and Lck^{cre}IL-4Rα$^{-/lox}$ mice after OVA treatment (Fig 2, *B*). IL-33 signals through the T1/ST2 receptor, which is primarily expressed on T$_H$2 cells but not on other T-cell subtypes.[24] In agreement with the T$_H$2 cytokine profile in T cells, we found similar numbers of T1/ST2-expressing CD3$^+$CD4$^+$ T cells in the lungs of IL-4Rα$^{-/lox}$ and Lck^{cre}IL-4Rα$^{-/lox}$ mice (Fig 2, *C*). In addition to its role in T$_H$2 cell functions, IL-33 has been implicated in the differentiation of ILC2s, which are able to promote type 2 immune responses and have been linked to allergic airway inflammation.[25] Allergen challenge increased numbers of a lineage-negative ICOS$^+$T1/ST2$^+$ ILC2 population in the lungs of IL-4Rα$^{-/lox}$ and Lck^{cre}IL-4Rα$^{-/lox}$ mice (Fig 2, *D*), which had an activated phenotype, as indicated by increased ICOS surface expression (Fig 2, *E*).[26] In conclusion, the main features of the adaptive and innate type 2 immune response were maintained in the absence of IL-4Rα on T cells, whereas T$_H$17 responses were increased.

Reduced IgE responses to OVA in Lck^{cre}IL-4Rα$^{-/lox}$ mice

T$_H$2-type immune responses are associated with antibody isotype switching to IgE and IgG$_1$, whereas IFN-γ promotes

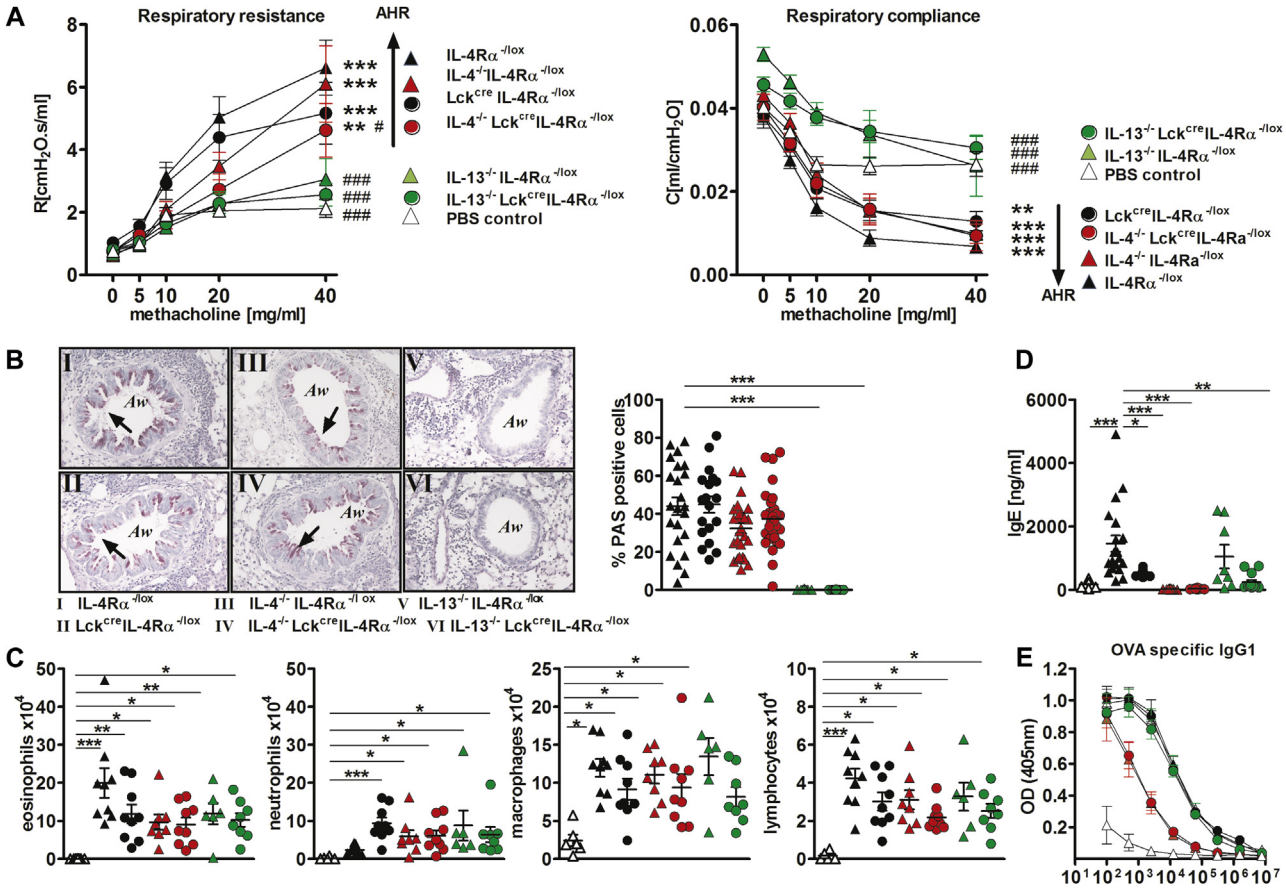

FIG 3. A, Respiratory resistance and compliance in response to methacholine (n = 8-10). **B,** Goblet cell hyperplasia as indicated by *arrows* in representative periodic acid–Schiff (PAS) staining and percentages of PAS-positive bronchial epithelial cells. *Aw*, Airways. **C,** Enumeration of eosinophils, neutrophils, lymphocytes, and macrophages in BAL fluid by differential cell counts (n = 8). **D** and **E,** Levels of total IgE (Fig 3, *D*) and OVA-specific IgG_1 (Fig 3, *E*) in blood serum were measured by means of ELISA (n = 8). Data were pooled from 2 independent experiments. *Lines* represent means ± SEMs. $*P < .05$, $**P < .01$, and $***P < .001$ versus PBS control. $\#P < .05$ and $\#\#\#P < .001$ versus IL-4Rα$^{-/lox}$. Fig 3, *A*, Two-way repeated-measures ANOVA at 40 mg/mL methacholine. Fig 3, *C* and *D*, Kruskal-Wallis nonparametric test.

IgG_{2a} and IgG_{2b} responses. In Lck^{cre}IL-4Rα$^{-/lox}$ mice total and OVA-specific IgE serum concentrations were significantly reduced compared with those in littermate control mice (Fig 2, *F*). IgG responses were similar in CD4$^+$ T cell–specific IL-4Rα–deficient mice (Lck^{cre}IL-4Rα$^{-/lox}$) and littermate control mice, with high OVA-specific IgG_1 and low IgG_{2a} and IgG_{2b} titers (see Fig E5 in this article's Online Repository at www.jacionline.org).

IL-13, but not IL-4, is required for development of OVA-mediated allergic airway disease

To clarify whether disease development in CD4$^+$ T cell–specific IL-4Rα–deficient mice (Lck^{cre}IL-4Rα$^{-/lox}$) requires IL-4 or IL-13, we examined allergic airway responses in these mice in the presence (Lck^{cre}IL-4Rα$^{-/lox}$) or absence of IL-4 (IL-4$^{-/-}$Lck^{cre}IL-4Rα$^{-/lox}$) or IL-13 (IL-13$^{-/-}$Lck^{cre}IL-4Rα$^{-/lox}$). IL-4 was not needed for OVA-induced AHR (Fig 3, *A*) because IL-4–deficient mice in the presence or absence of IL-4Rα–expressing CD4$^+$ T cells (IL-4$^{-/-}$IL-4Rα$^{-/lox}$ mice or IL-4$^{-/-}$Lck^{cre}IL-4Rα$^{-/lox}$) showed similar AHR responses compared with IL-4–competent littermate control animals (IL-4Rα$^{-/lox}$ mice). In stark contrast, IL-13 was crucial for OVA-induced AHR (Fig 3, *A*) because IL-13–deficient mice in the presence (IL-13$^{-/-}$IL-4Rα$^{-/lox}$) or absence (IL-13$^{-/-}$Lck^{cre}IL-4Rα$^{-/lox}$) of IL-4Rα–expressing CD4$^+$ T cells did not have AHR compared with IL-13–competent littermate controls (IL-4Rα$^{-/lox}$). The absence of IL-4 and IL-4Rα–responsive CD4$^+$ T cells did not significantly change the percentage of goblet cells in the airway epithelium, whereas goblet cell hyperplasia was impaired in the absence of IL-13 (Fig 3, *B*). IL-4 and IL-13 were required for the development of allergic airway inflammation because IL-4 or IL-13 deficiency in Lck^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα–expressing littermate control mice reduced eosinophilia and increased neutrophilia in the BALF (Fig 3, *C*). Lymphocyte and macrophage numbers were increased in all treatment groups compared with PBS-treated control mice, with no significant differences between the groups (Fig 3, *D*). As expected from IL-4 being responsible for isotype class-switching to IgE, serum concentrations of IgE were drastically reduced in OVA-challenged IL-4–deficient mouse strains (IL-4$^{-/-}$IL-4Rα$^{-/lox}$ and IL-4$^{-/-}$Lck^{cre}IL-4Rα$^{-/lox}$ mice) compared with those in IL-4Rα$^{-/lox}$ control mice (Fig 3, *E*). Because murine B cells are unresponsive to IL-13, mice deficient in IL-13

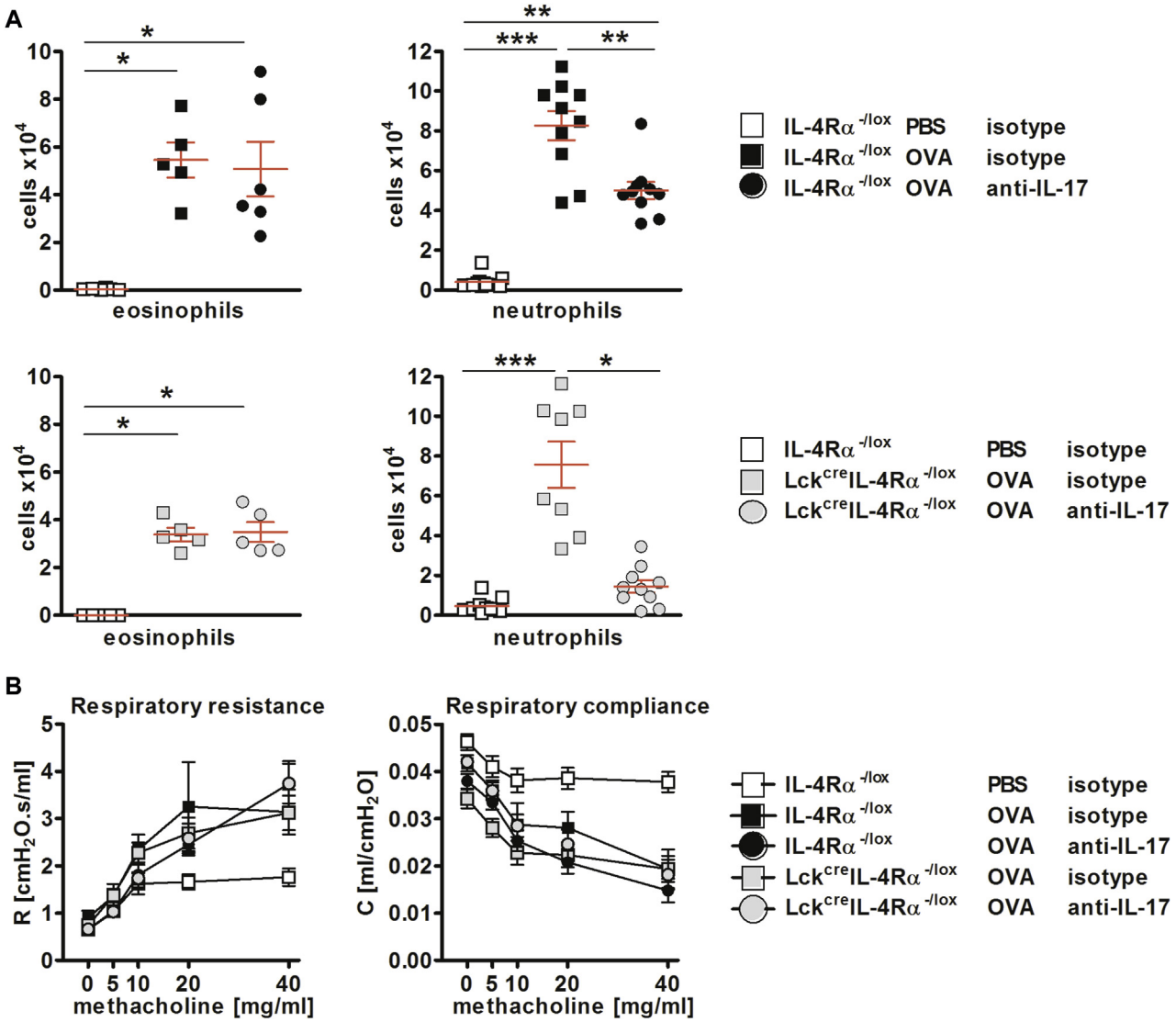

FIG 4. A, Numbers of eosinophils and neutrophils in BAL fluid. **B,** Respiratory resistance and compliance in response to methacholine. Data were pooled from 2 independent experiments. *Lines* represent means ± SEMs. $*P < .05$, $**P < .01$, and $***P < .001$, Kruskal-Wallis nonparametric test.

(IL-13$^{-/-}$IL4Rα$^{-/lox}$) responded similarly to OVA challenge compared with IL-4Rα$^{-/lox}$ control mice. However, in mice with IL-13 deficiency and IL-4Rα–unresponsive CD4^{+} T cells (IL-13$^{-/-}$Lck^{cre}IL-4Rα$^{-/lox}$ mice), IgE concentrations were strikingly reduced. This was expected because CD4^{+} T cell–4Rα expression is required for a normal IgE response (Fig 2, *F*).[27] OVA-specific IgG$_1$ concentrations were similar in IL-4Rα$^{-/lox}$, Lck^{cre}IL-4Rα$^{-/lox}$, IL-13$^{-/-}$IL-4Rα$^{-/lox}$, and IL-13$^{-/-}$Lck^{cre}IL-4Rα$^{-/lox}$ mice but reduced in mice with an IL-4–deficient genetic background.

In summary, the data presented here show that acute OVA-mediated allergic airway disease and a T$_H$2-polarized immune response can develop independently of IL-4 and IL-4Rα expression on CD4^{+} T cells. Furthermore, these results show that IL-13, but not IL-4, is mainly responsible for an allergic phenotype.

IL-17 induces neutrophilic airway inflammation in Lck^{cre}IL-4Rα$^{-/lox}$ mice

OVA exposure induced a significant increase in neutrophil counts in BAL fluid (Fig 1, *E*) and in IL-17^{+}CD4^{+} T cells and IL-17 in the lungs (Fig 2, *A*, and see Fig E4) of Lck^{cre}IL-4Rα$^{-/lox}$ mice but not littermate control mice. IL-17 can orchestrate neutrophil infiltration in mouse models of allergic asthma and has been associated with the development of severe asthma and AHR.[28,29] Anti–IL-17 antibodies injected during intranasal OVA challenge significantly reduced neutrophil numbers in Lck^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/lox}$ littermate control mice. Eosinophil numbers were not affected by anti–IL-17 antibody treatment (Fig 4, *A*). Furthermore, pulmonary resistance and compliance in Lck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice were not significantly altered by anti–IL-17 treatment (Fig 4, *B*). Therefore increased production of IL-17 after disrupted

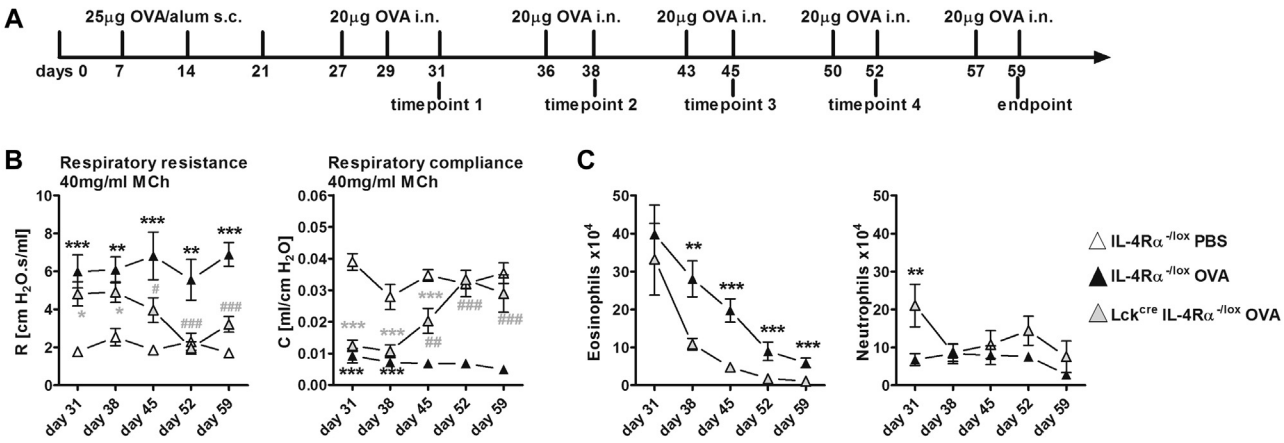

FIG 5. A, Mice received subcutaneous *(s.c.)* injections of OVA/alum followed by intranasal OVA challenges for 5 weeks to induce chronic allergic airway disease. **B,** Respiratory resistance and compliance in response to maximum (40 mg/mL) methacholine challenge. **C,** Eosinophils and neutrophils in BAL fluid were analyzed by using fluorescence-activated cell sorting. Two individual experiments were performed for days 31, 38, 45, 52, and 59. Pooled data are represented as means ± SEMs (n = 10-12). $*P < .05$, $**P < .01$, and $***P < .001$ versus PBS control; $\#P < .05$, $\#\#P < .01$, and $\#\#\#P < .001$, IL-4Rα$^{-/lox}$ OVA versus Lck^{cre}IL-4Rα$^{-/lox}$ OVA, Mann-Whitney nonparametric test.

IL-4Rα expression on CD4$^+$ T cells was responsible for airway neutrophilia but did not contribute to the development of AHR.

IL-4Rα–responsive CD4$^+$ T cells are required to sustain chronic AHR and eosinophilic lung inflammation

Having defined the roles of IL-4–, IL-13–, and IL-4Rα–responsive CD4$^+$ T cells in the onset of OVA-mediated allergic asthma, we investigated the role of IL-4Rα–responsive CD4$^+$ T cells in maintaining chronic allergic airway disease. Mice received systemic OVA sensitization followed by intranasal challenges over 5 consecutive weeks (Fig 5, *A*).[10] Baseline values for respiratory resistance and compliance measured before methacholine challenge were not affected in Lck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice after 5 weeks of chronic airway exposure to allergens, indicating that chronic OVA exposure did not affect general lung function (see Fig E6, *A*, in this article's Online Repository at www.jacionline.org). AHR in response to methacholine did not significantly change during the 5-week course of allergen challenge in OVA-treated IL-4Rα$^{-/lox}$ mice (Fig 5, *B*, and see Fig E7 in this article's Online Repository at www.jacionline.org). However, AHR was significantly reduced in Lck^{cre}IL-4Rα$^{-/lox}$ mice from day 45 onward and decreased to levels similar to those in PBS-treated control groups by day 52 (Fig 5, *B*, and see Fig E7). Chronic airway inflammation was assessed by measuring numbers of BAL cells. BAL fluid eosinophil numbers continuously decreased over the 5-week period of allergen exposure in Lck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice (Fig 5, *C*). However, in CD4$^+$ T cell–specific IL-4Rα–deficient Lck^{cre}IL-4Rα$^{-/lox}$ mice, airway eosinophilia was significantly reduced at every time point compared with that in littermate control mice and was resolved by day 52 (Fig 5, *C*). Surface expression of the activation marker Siglec-F on eosinophils was not significantly different in Lck^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice until day 59, when eosinophils were hard to detect in Lck^{cre}IL-4Rα$^{-/lox}$ mice (see Fig E6, *B*). Airway neutrophilia was significantly increased in Lck^{cre}IL-4Rα$^{-/lox}$ mice compared with that in IL-4Rα$^{-/lox}$ mice after the first week of allergen challenge, and similar numbers were observed afterward in Lck^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/lox}$ littermate control mice (Fig 5, *C*). Numbers of alveolar macrophages did not differ between Lck^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/lox}$ littermate control mice or change significantly over the course of allergen challenge (see Fig E6, C). Together, these results demonstrate that IL-4Rα expression on T cells is critical for maintaining the hallmarks of allergic airway disease, such as AHR and airway inflammation, in response to chronic allergen exposure.

Type 2–specific cytokine responses are maintained after impairment of IL-4Rα responsiveness in CD4$^+$ T cells during chronic allergic asthma

OVA treatment induced T_H2 cytokine production by CD4$^+$ T cells from lungs independently of IL-4Rα responsiveness during chronic allergen challenge (Fig 6, *A*). Numbers of CD4$^+$ T cells producing the T_H2 cytokines IL-4, IL-5, and IL-13 were not significantly different in CD4$^+$ T cell–specific IL-4Rα–deficient Lck^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα–expressing IL-4Rα$^{-/lox}$ littermate control mice throughout the course of OVA challenges. Numbers of IFN-γ– or IL-10–producing CD4$^+$ T cells were similar in control IL-4Rα$^{-/lox}$ mice and Lck^{cre}IL-4Rα$^{-/lox}$ mice. Of note, the numbers of IL-17–producing CD4$^+$ T cells were higher in Lck^{cre}IL-4Rα$^{-/lox}$ mice throughout the course of allergen challenge (Fig 6, *A*). Total IgE and OVA-specific IgE serum concentrations were increased in IL-4Rα$^{-/lox}$ compared with Lck^{cre}IL-4Rα$^{-/lox}$ mice and peaked on day 45 (Fig 6, *B*). OVA-specific IgG_1 antibodies were detected at the highest concentrations on day 45, with no significant differences between IL-4Rα$^{-/lox}$ and Lck^{cre}IL-4Rα$^{-/lox}$ mice (see Fig E8 in this article's Online Repository at www.jacionline.org). Levels of the type 1–associated antibodies IgG_{2a} and IgG_{2b} were increased in Lck^{cre}IL-4Rα$^{-/lox}$ mice compared with those in littermate control mice (see Fig E8). In conclusion, impairment of IL-4Rα responsiveness by CD4$^+$ T cells during chronic disease leads to reduced IgE responses and enhanced T_H17 cytokine responses but does not impair T_H2 cytokine (IL-4, IL-5, and IL-13) production.

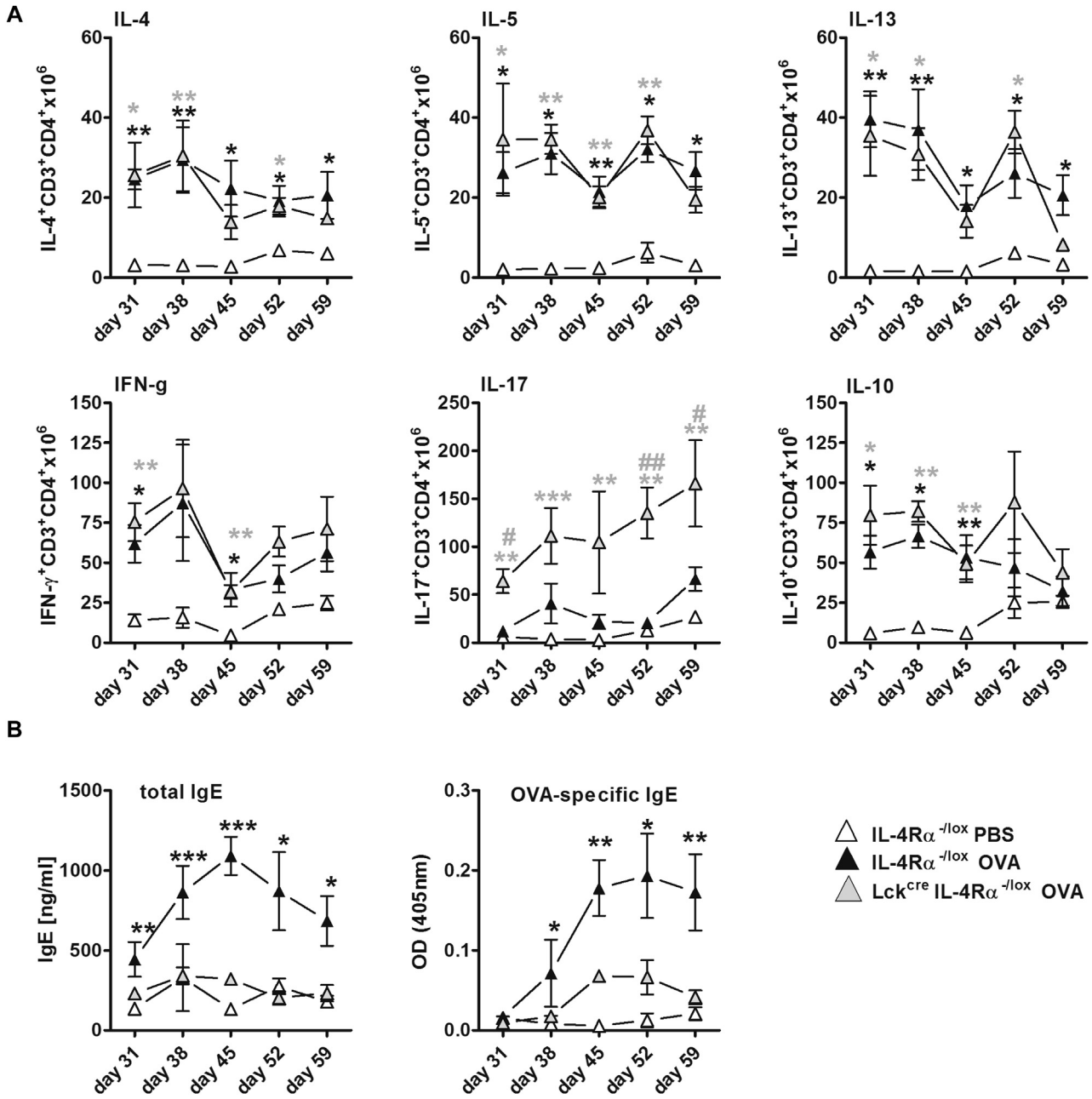

FIG 6. A, Cytokine-producing CD4$^+$ T cells from lung tissue were measured by using fluorescence-activated cell sorting. **B,** Total IgE and OVA-specific IgE levels in blood serum were measured by using ELISA. Specific antibody response is represented as OD values at 1:3 serum dilution. Data were pooled from 2 individual experiments on days 31, 38, 45, 52, and 59 and represented as means ± SEMs. IL-4Rα$^{-/lox}$ PBS, n = 5 to 8; IL-4Rα$^{-/lox}$ OVA and Lck^{cre}IL-4Rα$^{-/lox}$ OVA, n = 7 to 8. *P < .05, **P < .01, and ***P < .001 versus PBS control. #P < .05 and ##P < .01, IL-4Rα$^{-/lox}$ OVA versus Lck^{cre}IL-4Rα$^{-/lox}$ OVA, Kruskal-Wallis nonparametric test.

DISCUSSION

Allergic asthma is considered to be driven by allergen-specific T_H2 immune responses, and CD4$^+$ T cells are an essential source of T_H2 cytokines. According to the paradigm for the development of T_H2 immunity, the IL-4/IL-4Rα signaling pathway on CD4$^+$ T cells is of key importance for T_H2 cell differentiation and subsequently for allergic asthma. However, mechanisms of *in vivo* T_H2 differentiation and the role of IL-4Rα in mouse models are not fully understood, and we and others have demonstrated that T_H2 responses can develop in the absence of the canonical IL-4/IL-4Rα–mediated pathway of T_H2 differentiation.[4,5,30-32]

Our previous studies with CD4$^+$ T cell–specific IL-4Rα–deficient Lck^{cre}IL-4Rα$^{-/lox}$ mice in infectious disease models indicated critical contributions of IL-4Rα$^+$ T cells to T_H2-mediated lung pathology.[33-35] However, our previous studies on mouse models of allergic dermatitis or anaphylaxis indicated that T_H2-type immune responses to allergen exposure can develop in the absence of IL-4 and IL-4Rα–expressing CD4$^+$ T cells.[36,37] The present study demonstrates that IL-4–responsive

T_H2 cells are crucial for chronic but not acute OVA-induced airway disease pathology and that T_H2-type immunity to OVA develops in the absence of IL-4 signaling. Furthermore, the loss of IL-4Rα signaling on $CD4^+$ T cells resulted in increased IL-17 production. IL-17 can orchestrate neutrophil infiltration in mouse models of allergic asthma, whereas IL-5 recruits eosinophils. Accordingly, we found similar numbers of neutrophils and eosinophils in the presence of IL-5 and IL-17 in Lck^{cre}IL-4Rα$^{-/lox}$ mice.

It is now appreciated that other cytokines, such as IL-25 and IL-33, can induce *in vivo* differentiation of T_H2 cells.[2] We found IL-33 and numbers of $CD4^+$ T cells expressing the IL-33 receptor T1/ST2 to be upregulated on airway exposure to OVA, irrespective of the presence of IL-4Rα–responsive $CD4^+$ T cells. IL-33 can induce production of IL-5 and IL-13 in $CD4^+$ T cells independently of IL-4 *in vitro*,[38] and IL-33–primed dendritic cells induce the development of T_H2 cells, which are crucial for allergic airway inflammation.[39] Furthermore, IL-25 and IL-33 have been associated with expansion of ILC2s in allergic responses. A recent study found that these cells drove the initiation of allergic airway inflammation in response to the protease allergen papain and mediated T_H2 responses independently of IL-4.[40] We found similar numbers of activated ILC2s in mice lacking IL-4–responsive T cells and in littermate control mice, which might explain the IL-4–independent development of T_H2 responses. However, a recent study in a similar model of OVA-induced allergic asthma showed that ILC2s were dispensable in the development of T_H2 and, subsequently, the development of allergic airway inflammation responses.[41] Together with the results from our study, this indicates that systemic sensitization with OVA induces a strong T_H2-driven allergic response and ILC2 activation in the absence of the canonical IL-4–driven pathway of T_H2 differentiation.

$CD4^+$ T cells are required for initiation of allergic airway inflammation[42] and development of chronic asthmatic pathology in mouse models.[43] Therefore we wanted to investigate whether IL-4Rα expression by $CD4^+$ T cells contributes to chronic airway inflammation and disease pathology. Strikingly, disruption of IL-4Rα expression on $CD4^+$ T cells was sufficient to abrogate persistent AHR after 2 weeks of chronic OVA exposure. Our results and previous studies[44,45] showed that chronic AHR did not correlate with the degree of eosinophilic airway inflammation or activation status of lung eosinophils. IL-4Rα$^{-/lox}$ mice sustained AHR at a high magnitude despite a decrease in pulmonary eosinophil numbers. We found that IL-4 signaling on $CD4^+$ T cells contributed to the maintenance of chronic airway eosinophilia because in Lck^{cre}IL-4Rα$^{-/lox}$ mice eosinophilia was completely resolved after 5 weeks of OVA exposure. Despite this striking reduction in airway eosinophilia and AHR, Lck^{cre}IL-4Rα$^{-/lox}$ mice still presented with IL-5 and IL-13 cytokine-producing T cells in the lung. This would suggest that IL-4Rα expression on $CD4^+$ T cells contributes to chronic eosinophilia and AHR through factors other than chronic T_H2-type cytokine production, including IL-5 and IL-13. Chronic AHR can be sustained independently of IL-13 and might not correlate with the presence of T_H2 cytokine–producing T helper cells.[15,46] We did not find significant differences in $CD4^+$ T cells producing either IFN-γ or IL-10, cytokines associated with T_H1 or regulatory T-cell responses, respectively. However, IL-17–producing T-cell counts were constantly increased in $CD4^+$ T cell–specific IL-4Rα–deficient (Lck^{cre}IL-4Rα$^{-/lox}$) mice. IL-17 has been found to contribute to the development of allergic asthma but to downregulate established allergic airway inflammation and reduce pulmonary eosinophil recruitment and AHR in mouse models of OVA-induced asthma.[47] This might explain the reduced numbers of eosinophils in the presence of IL-5–producing $CD4^+$ T cells in Lck^{cre}IL-4Rα$^{-/lox}$ mice in which lung IL-17 levels were significantly increased.

In our study IL-17 neutralization in the acute phase of allergic airway disease did not significantly alter AHR development but reduced numbers of BAL neutrophils significantly. In the chronic phase AHR and airway eosinophilia was resolved faster in Lck^{cre}IL-4Rα$^{-/lox}$ mice in the presence of high numbers of IL-17–producing $CD4^+$ T cells. Therefore our study does not support an important role for IL-17 in the development of acute AHR, but we cannot rule out a protective rule of IL-17 during the chronic phase of the disease. On the other hand, T_H17 cells are upregulated in the lungs of asthmatic patients and linked to severe clinical phenotypes, such as neutrophilic steroid-resistant asthma, and are often considered to be detrimental in allergic airway disease.[29]

We found a significant reduction in serum concentrations of total and OVA-specific IgE in Lck^{cre}IL-4Rα$^{-/lox}$ mice in the acute phase and during chronic allergen challenge. An acute allergic airway response can develop independently of IgE in mice, but mast cells and FcεRI contribute to the development of AHR in chronic asthma through antibody-mediated mechanisms.[48-50] Therefore it is possible that the reduced AHR in Lck^{cre}IL-4Rα$^{-/lox}$ mice is due to reduced IgE antibody titers affecting mast cell–mediated responses.

This study extends our understanding of the regulation of acute and chronic allergic airway responses and T_H2-type immune responses. We provide substantial evidence for differential requirements of IL-4Rα–responsive $CD4^+$ T cells during the acute and chronic phases of allergic airway disease. IL-4Rα expression on $CD4^+$ T cells plays a major role in the pathogenesis of chronic asthma and the regulation of allergen-specific T_H17 responses, whereas acute asthmatic pathology and T_H2 responses developed in the absence of IL-4Rα–responsive $CD4^+$ T cells and IL-4. The molecular mechanisms behind IL-4–independent T_H2 differentiation in response to OVA and AHR during chronic allergic airway responses remain to be determined.

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Key messages

- **OVA-mediated acute allergic airway disease is independent of IL-4–promoted T_H2 differentiation but dependent on IL-13 functions.**
- **Loss of IL-4Rα signaling on $CD4^+$ T cells results in increased IL-17 production and airway neutrophilia.**
- **OVA-mediated chronic asthmatic disease is promoted by IL-4–responsive $CD4^+$ T cells.**

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METHODS

Analysis of *in vitro* cytokine production

For analysis of *ex vivo* cytokine production, single-cell suspensions from individual lungs of OVA-treated and control mice were incubated for 120 hours at 4×10^6 cells/mL in 200 μL of IMDM (Gibco, Loughborough, United Kingdom)/10% FCS (Delta, Johannesburg, South Africa) in 96-well plates. Cells were stimulated with OVA (100 μg/mL), and supernatants were collected after 120 hours. Concentrations of IL-5, IFN-γ (BD Biosciences), and IL-17 (R&D Systems) were measured by using sandwich ELISA assays, according to the manufacturer's protocol.

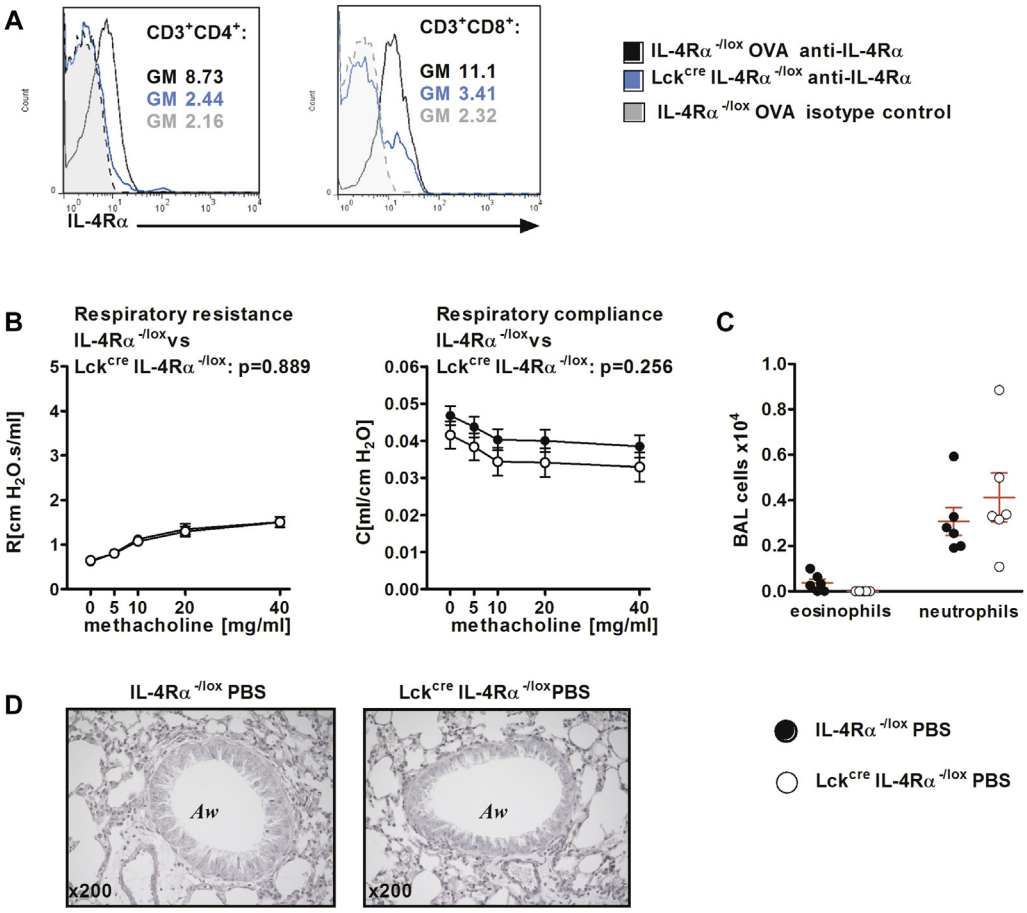

FIG E1. A, Expression of IL-4Rα on CD4$^+$ or CD8$^+$ T cells from lungs of OVA-treated IL-4Rα$^{-/lox}$ and Lck^{cre}IL-4Rα$^{-/lox}$ mice. *GM*, Geometric mean. **B,** Lck^{cre}IL-4Rα$^{-/lox}$ mice and littermate control mice received intraperitoneal *(i.p.)* injections of PBS/alum and intranasal PBS challenges. Respiratory resistance and compliance in response to methacholine (n = 6). **C,** Eosinophils and neutrophils in BAL fluid were analyzed by using fluorescence-activated cell sorting (n = 6). **D,** Representative periodic acid–Schiff staining of lung sections. *Aw*, Airways. *Bars* represent means ± SEMs. Kruskal-Wallis nonparametric test.

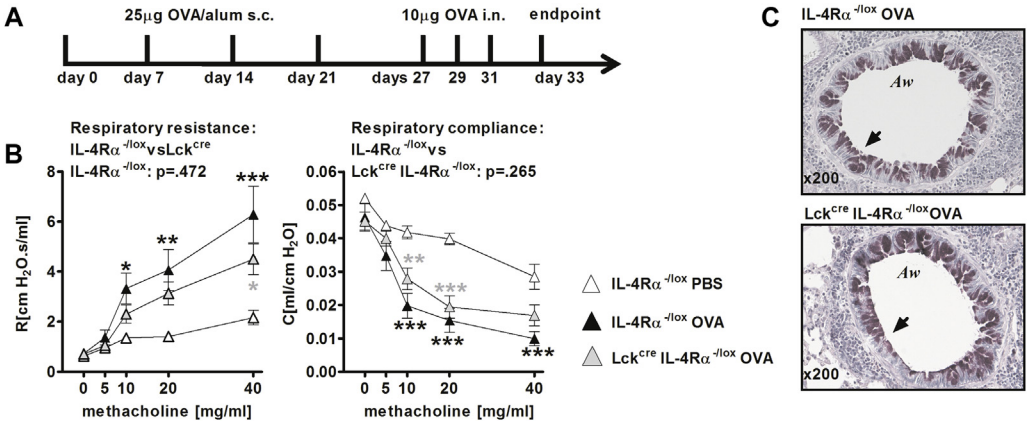

FIG E2. A, Mice received subcutaneous *(s.c.)* injections of OVA/alum and intranasal OVA challenges to induce allergic airway disease. **B,** Airway resistance and compliance in response to methacholine. **C,** Airway mucus secretion and goblet cell hyperplasia as indicated by *arrows* in representative periodic acid–Schiff staining. *Aw,* Airways. Data were pooled from 2 independent experiments. *Bars* represent means ± SEMs. $*P < .05$, $**P < .01$, and $***P < .001$, Kruskal-Wallis nonparametric test.

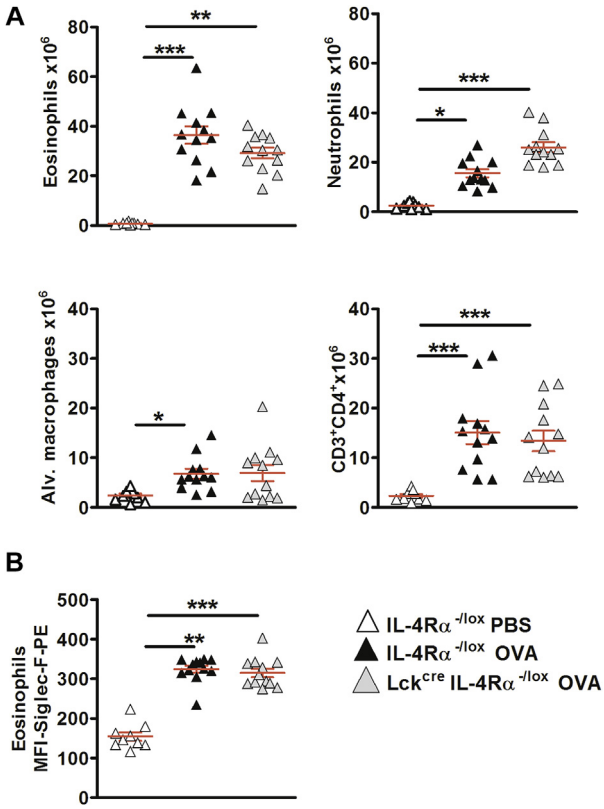

FIG E3. A, Detection of eosinophils, neutrophils, alveolar macrophages, and T helper cells in digested lung tissue (n = 9, 12, and 12) by using flow cytometry. **B,** Mean fluorescence intensity *(MFI)* of Siglec-F–PE staining on eosinophils (n = 9, 12, and 12). Data were pooled from 2 independent experiments. *Bars* represent means ± SEMs. **P* < .05, ***P* < .01, and ****P* < .001, Kruskal-Wallis nonparametric test.

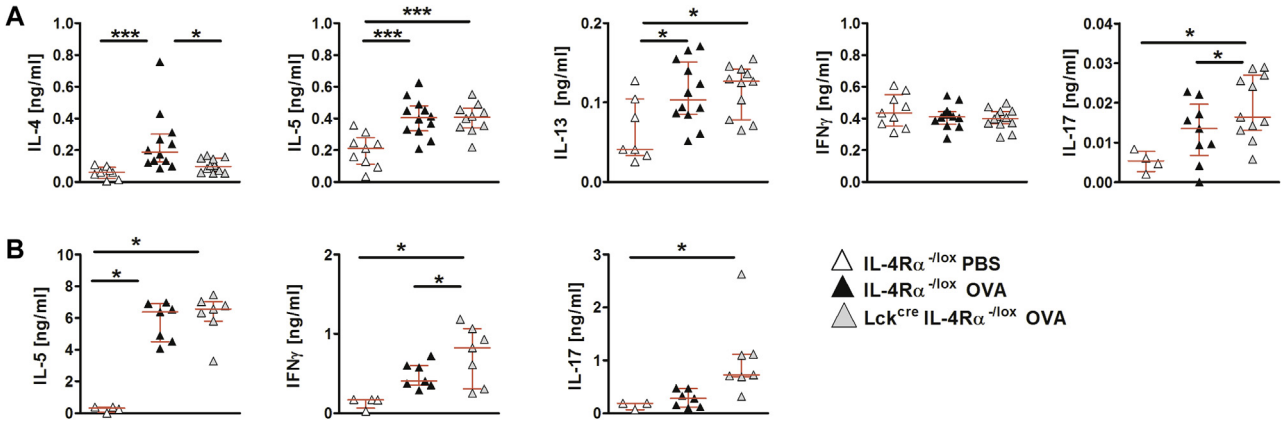

FIG E4. A, Levels of T_H2-, T_H1-, and T_H17-associated cytokines were measured in lung tissue homogenate by using ELISA (n = 9, 12, and 12). **B,** IL-5, IFN-γ, and IL-17 levels were measured in cell-culture supernatants of *ex vivo* lung cells stimulated with OVA (n = 4, 7, and 7). Data are from one of 2 representative experiments. *Lines* represent medians ± interquartile ranges. *$P < .05$, Kruskal-Wallis nonparametric test.

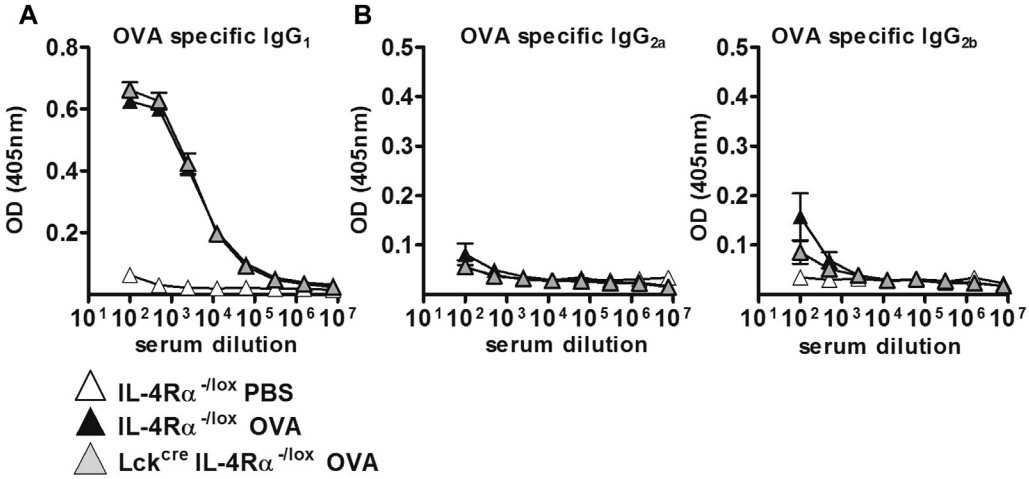

FIG E5. OVA-specific IgG levels in blood serum were measured by using ELISA. Data were pooled from 3 independent experiments. **A,** n = 10. **B,** n = 9, 14, and 12. *Bars* represent means ± SEMs. Kruskal-Wallis nonparametric test.

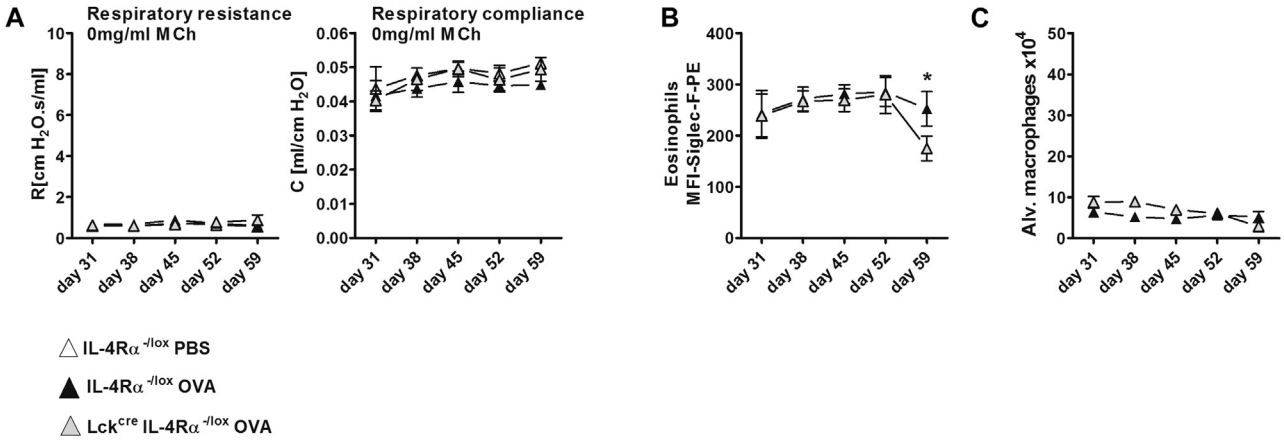

FIG E6. A, Baseline respiratory resistance and compliance before methacholine challenge. **B,** Mean fluorescence intensity *(MFI)* of Siglec-F–PE staining on BAL eosinophils. **C,** Alveolar macrophages in BAL fluid were analyzed by using fluorescence-activated cell sorting. Two individual experiments were performed for days 31, 38, 45, 52, and 59. Pooled data were represented as means ± SEMs (n = 10-12). $*P < .05$, Mann-Whitney nonparametric test.

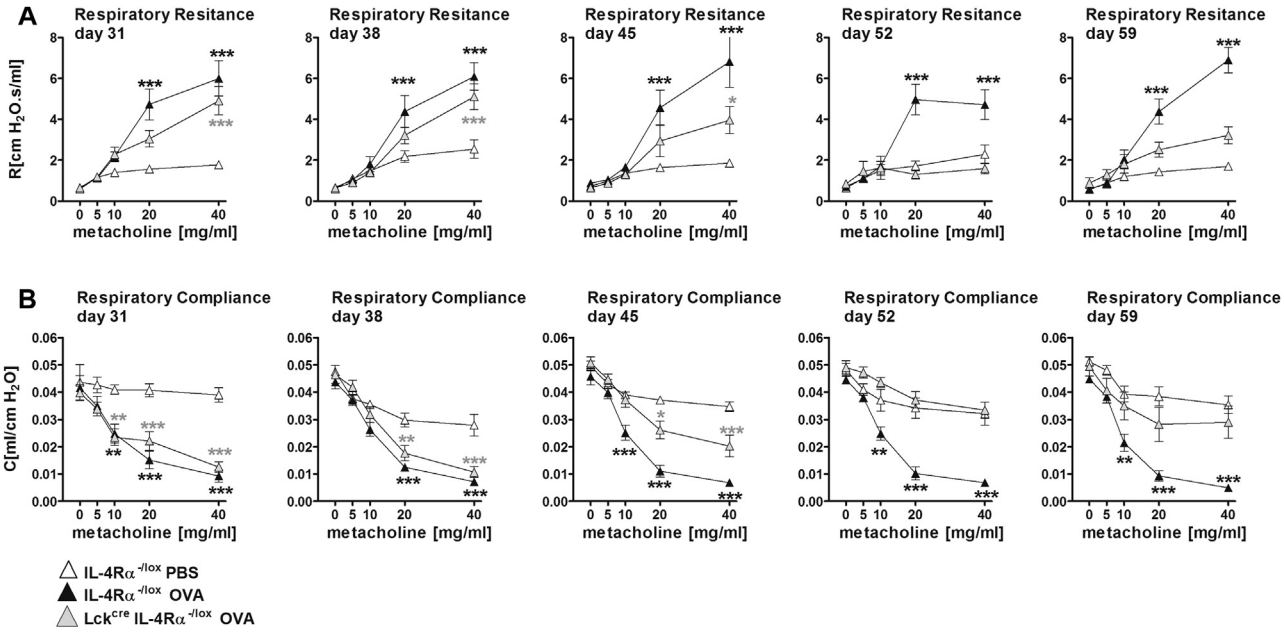

FIG E7. A, Dose-response curves for respiratory resistance in response to methacholine at individual time points during chronic OVA challenge. **B,** Dose-response curves for respiratory compliance in response to methacholine at individual time points during chronic OVA challenge. Pooled data were from 2 experiments for each time point. Data are shown as means ± SEMs (n = 8-12). $^{**}P < .01$ and $^{***}P < .001$ versus PBS control, repeated-measures ANOVA.

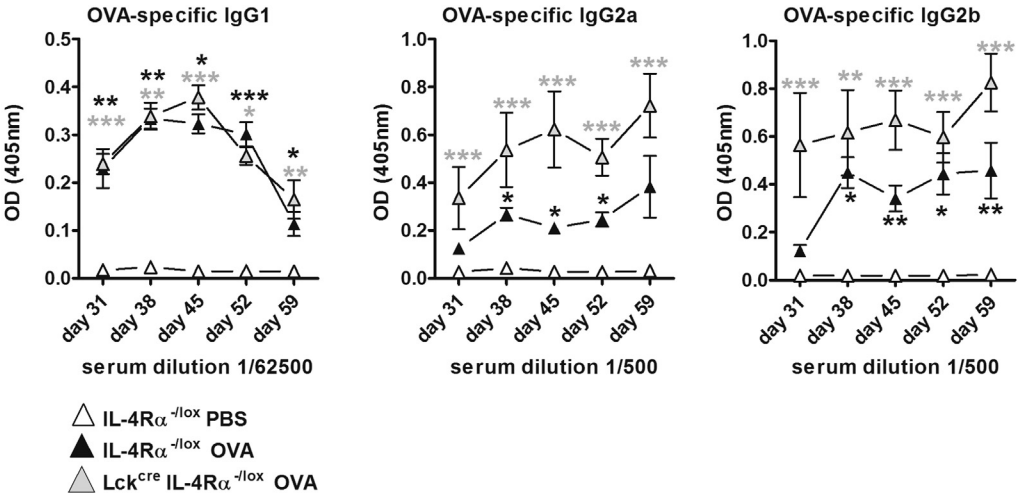

FIG E8. IgG isotypes in blood serum were measured by using ELISA at indicated serum dilutions. Pooled data from 2 individual experiments on days 31, 38, 45, 52, and 59 were represented as means ± SEMs. IL-4R$\alpha^{-/lox}$ PBS, n = 5 to 8; IL-4R$\alpha^{-/lox}$ OVA and Lck^{cre}IL-4R$\alpha^{-/lox}$ OVA, n = 7 to 8. *P < .05, **P < .01, and ***P < .001 versus PBS control, Kruskal-Wallis nonparametric test.