Original article

T_H17 cells mediate pulmonary collateral priming

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Background: Our laboratory has shown that inhalational sensitization to new antigens is facilitated through an ongoing T_H2-polarized inflammation of the lung, a phenomenon we call "collateral priming."

Objective: We were interested to analyze whether a T_H1-polarized pulmonary inflammation also facilitates priming toward new antigens and which cytokine or cytokines are involved.

Methods: T_H1-polarized T cells were generated *in vitro* and transferred into congenic mice. Mice were challenged initially with cognate antigen and an unrelated antigen; consecutively, they received cognate antigen or the secondary antigen. Airway inflammation, antigen-specific IgG2a levels, and airway hyperresponsiveness were assessed to determine the inflammatory phenotype, with antibody blocking studies used to determine cytokine requirements for T_H1 collateral priming.

Results: Our experiments revealed that ongoing inflammation of the lung induced by the transfer of T_H1-polarized cells also facilitates priming toward new antigens, which results in lymphocytic inflammation of the lung. Interestingly, blocking studies identified IL-17A as a major contributor to this pathology. Accordingly, we could demonstrate for the first time that T_H17-polarized cells alone can facilitate priming toward new antigens, inducing lymphocytic airway inflammation and strong airway hyperresponsiveness. Flow cytometric analysis revealed priming of endogenous T cells for IL-17A secretion with a distinct memory/effector phenotype compared to T_H1 cells, thus presenting an exciting model to further elucidate differentiation of T_H17 cells.

Conclusions: We show that airway inflammation mediated by T_H17 cells facilitates sensitization to new antigens and confers increased airway responsiveness in a murine model of polysensitization, suggesting a mechanism involving IL-17A behind the increased risk for allergic sensitization in polysensitized subjects. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: *Asthma, IL-17A, T_H cell, polysensitization*

A wealth of animal data supports the pivotal role of T_H2-polarized T cells in the initiation and propagation of allergic airway disease.[1] Yet some murine studies suggest a less unequivocal situation with regard to the role of T_H1 polarization in allergic airway disease. Some murine data show a clear-cut effect of T_H1-polarized immune responses in inhibiting T_H2-polarized airway disease,[2,3] and other studies show a significant exacerbation by T_H1 cells.[4,5]

The expansion of the T_H1/T_H2 dichotomy to include a new distinct cell type, T_H17 cells,[6] has added more complexity to this question. IL-17A has been shown, in different chronic inflammatory diseases, to underlie effects formerly ascribed to T_H1 cytokines.[7] In the context of (allergic) airway inflammation, IL-17A has been described to promote different aspects of the disease[8-12] but might also play a protective role.[13]

Studies in human subjects have generally been less clear cut with regard to the T_H1/T_H2 polarity of allergic airway disease. Many studies have found that both T_H1 and T_H2 cytokine levels are increased in the blood and airways of asthmatic patients,[14-16] with T_H1 cytokine levels correlating with disease severity, and similar data have been obtained for IL-17A.[17] Additionally, human studies, particularly in childhood, point to a role of T_H1-polarized viral infections in the promotion of allergic airway disease.[18-20] These data, some of which have been translated into murine models,[21] clearly dispute the hygiene hypothesis.[22] These controversies show that the immunologic effects of a concomitant airway inflammation on subsequent immune responses to unrelated antigens, such as aeroallergens, remain controversial or unknown. We therefore initially sought to address the question of how a T_H1-polarized airway inflammation influences subsequent sensitizations toward neoantigens.

We have recently described a model showing that ongoing T_H2-polarized airway inflammation facilitates priming toward secondary unrelated allergens.[23] We therefore sought to delineate whether similar mechanisms also promote T_H1-polarized airway responses. We show here that ongoing airway inflammation induced by the transfer of T_H1-polarized T cells also facilitates pulmonary priming toward a secondary unrelated antigen. Surprisingly, T_H1-induced collateral priming was not dependent on IFN-γ but does depend on IL-17A. Analysis of the transferred T_H1 cells revealed a small IL-17A–producing population of antigen-specific $CD4^+$ cells, demonstrating that some T_H17 cells

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Abbreviations used
AHR: Airway hyperresponsiveness
APC: Antigen-presenting cell
BAL: Bronchoalveolar lavage
KLH: Keyhole limpet hemocyanin
LN: Lymph node
mLN: Mediastinal lymph node
OVA: Ovalbumin
PMA: Phorbol 12-myristate 13-acetate

can escape the *in vitro* T_H1-polarizing conditions. We could further demonstrate that T_H17-polarized cells by themselves facilitate priming, revealing a novel role for this cell type in the induction of secondary airway inflammation and airway hyperresponsiveness (AHR).

METHODS

Mice

BALB/cJ (wild-type) mice, T-cell receptor–transgenic OT-II mice (C57BL/6-Tg [TcrαTcrβ]425Cbn/J on a C57/Bl6 background), and IFN-γ receptor–deficient mice (B6.129S7-Ifnγr1tm1Agt/, C57/Bl6 background) were purchased from the Jackson Laboratory (Bar Harbor, Me). T-cell receptor–transgenic DO11.10 mice (C.Cg-Tg[DO11.10]10Dlo/J) backcrossed onto an $\alpha\beta^{-/-}$ background were bred in our facility. Six- to 10-week-old female mice were used in all experiments. All experimental methods described in this article were performed as approved by the respective institutional animal care and use committees.

Generation of polarized T_H cells, adoptive transfer, and *in vitro* restimulation

$CD4^+$ T and syngeneic T-depleted splenocytes were prepared as described previously.[23] $CD4^+$ T cells and antigen-presenting cells (APCs) were cultured with 5 μg/mL OVA peptide 323–339, 25 U/mL recombinant murine IL-2 (Roche, Mannheim, Germany), 5 ng/mL recombinant murine IL-12 (Strathmann Biotec GmbH, Hamburg, Germany), and anti–IL-4 (11B11) to generate T_H1 cells. For generation of T_H17 cells, $CD4^+$ T cells and APCs were cultured with 5 μg/mL pOVA323–339, 20 ng/mL recombinant murine IL-23 (eBioscience, San Diego, Calif), 2 ng/mL recombinant human TGF-β (Peprotech, Rocky Hill, NJ), 40 ng/mL recombinant murine IL-6 (Miltenyi Biotec, Bergisch Gladbach, Germany), anti–IL-4 (11B11), and anti–IFN-γ (XMG1.2). Cells were split 1:2 on day 3 and harvested on day 7.

T_H2, T_H1, or T_H17 cells (5×10^6) were injected intravenously into BALB/cJ mice. Purity before injection ranged from 92% to 98% $CD4^+KJ1\text{-}26^+$ cells, and an additional aliquot of the cells was retained for *in vitro* restimulation and analysis by means of ELISA.

Collateral priming protocol

For primary challenge, 24 hours after the transfer of T_H cells, mice were exposed to either 5 μg of ovalbumin (OVA; grade V; Sigma-Aldrich, St Louis, Mo) and 5 μg of BSA (fraction V; Invitrogen, Carlsbad, Calif) or keyhole limpet hemocyanin (KLH; Sigma-Aldrich) intranasally on days 0 and 1. Secondary challenge was performed with either 5 μg of OVA or BSA or KLH on days 18 and 19. Mice were killed on day 22 (Fig 1, *A*). For induction of memory, rechallenge was applied on days 74 and 75, with doses as used for secondary challenges.

Analysis of lung function

Mice were anesthetized and intubated orotracheally for lung function measurements, as described previously.[24] AHR was assessed based on increases in lung resistance during provocation with increasing doses of aerosolized methacholine defined by means of a feedback-dose control system.[25]

Antibody treatment

For blocking studies against IFN-γ (clone XMG1.2) or IL-17A (clone 50104.11; R&D Systems, Minneapolis, Minn), mice were treated with 100 μg of anti–IFN-γ intraperitoneally on days −1, 0, 1, 2, and 3 or with 50 μg of anti–IL-17A intraperitoneally and intranasally on days 0, 1, 2, and 3 of the collateral priming protocol (Fig 1, *A*) 1 hour before primary antigen application.

Analysis of bronchoalveolar lavage fluid

Bronchoalveolar lavage (BAL) inflammatory cells were obtained by means of lavage of the airway lumen with PBS, prepared, stained, and differentiated microscopically, as previously described.[26]

Determination of serum antibody concentration

Antigen-specific (KLH, BSA, and OVA) antibodies in sera were determined by means of ELISA, as described previously.[26]

Lymph node and lung cell preparation and restimulation

Mediastinal lymph nodes (mLNs) and lungs were harvested on days 4 or 22 of the collateral priming protocol (Fig 1, *A*) and pooled from each group at the time of death. Single-cell suspensions were obtained as described previously.[26] Cells harvested on day 22 were stimulated immediately, whereas cells harvested on day 4 were stimulated after a predetermined 4-day resting period.

Restimulation was performed with 1 μg/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 0.75 μg/mL ionomycin (Sigma-Aldrich) for 4 hours (Figs 3, *A* and *B*, and 5, *A* and *B*, and see Figs E5, *A*, and E6, *A*, in this article's Online Repository at www.jacionline.org), with 5 μg of OVA peptide and freshly isolated APCs (Fig 2, *A*), 200 μg/mL OVA, BSA, or KLH for 48 hours (Figs 2, *B*, and 3, *C*) or with GM-CSF differentiated bone marrow–derived dendritic cells from wild-type mice and 200 μg/mL OVA, KLH, or medium for 48 hours (Fig E6, *B*). For intracellular cytokine staining, GolgiPlug was added to PMA and ionomycin stimulation, according to the manufacturer's instructions (BD Biosciences, Mississauga, Ontario, Canada), for 4 hours (Figs 3, *A*, and 5, *A*).

Measurement of cytokine production

The cytokines IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IFN-γ, and TNF-α were measured by using Multiplex-based bead technology (Millipore, Temecula, Calif; Fig 2, *A* and *B*) or ELISA (R&D Systems; Figs 3, *B* and *C*, and 5, *B*). As per manufacturer's response, the R&D Systems ELISA used for determination for IL-17 is specific for IL-17A.

Flow cytometric analysis

All staining procedures were performed on ice. Cell surfaces were blocked with anti-FcR (24G2) antibody. The presence of OVA-transgenic T cells was determined with an anti-clonotypic mAb (KJ1-26) antibody, simultaneously identifying $CD4^+$ cells (clone RM4-5, eBioscience), as well as $CD44^+$ (clone IM7, eBioscience) and $CD62L^+$ (clone MEL-14, eBioscience) cells, after fixation and permeabilization and staining for IL-4–producing (clone 11B11), IL-17A–producing (clone TC11-1810.1), and IFN-γ–producing (clone XMG1.2) cells (all from BD PharMingen, San Jose, Calif). Cells were analyzed on a LSRII (Becton Dickinson) flow cytometer in association with FlowJo (Treestar, Inc, Ashland, Ore) software.

Determination of statistical significance

Statistical significance was determined by using the Mann-Whitney *U* test, unless otherwise stated. A *P* value of less than .05 was considered significant.

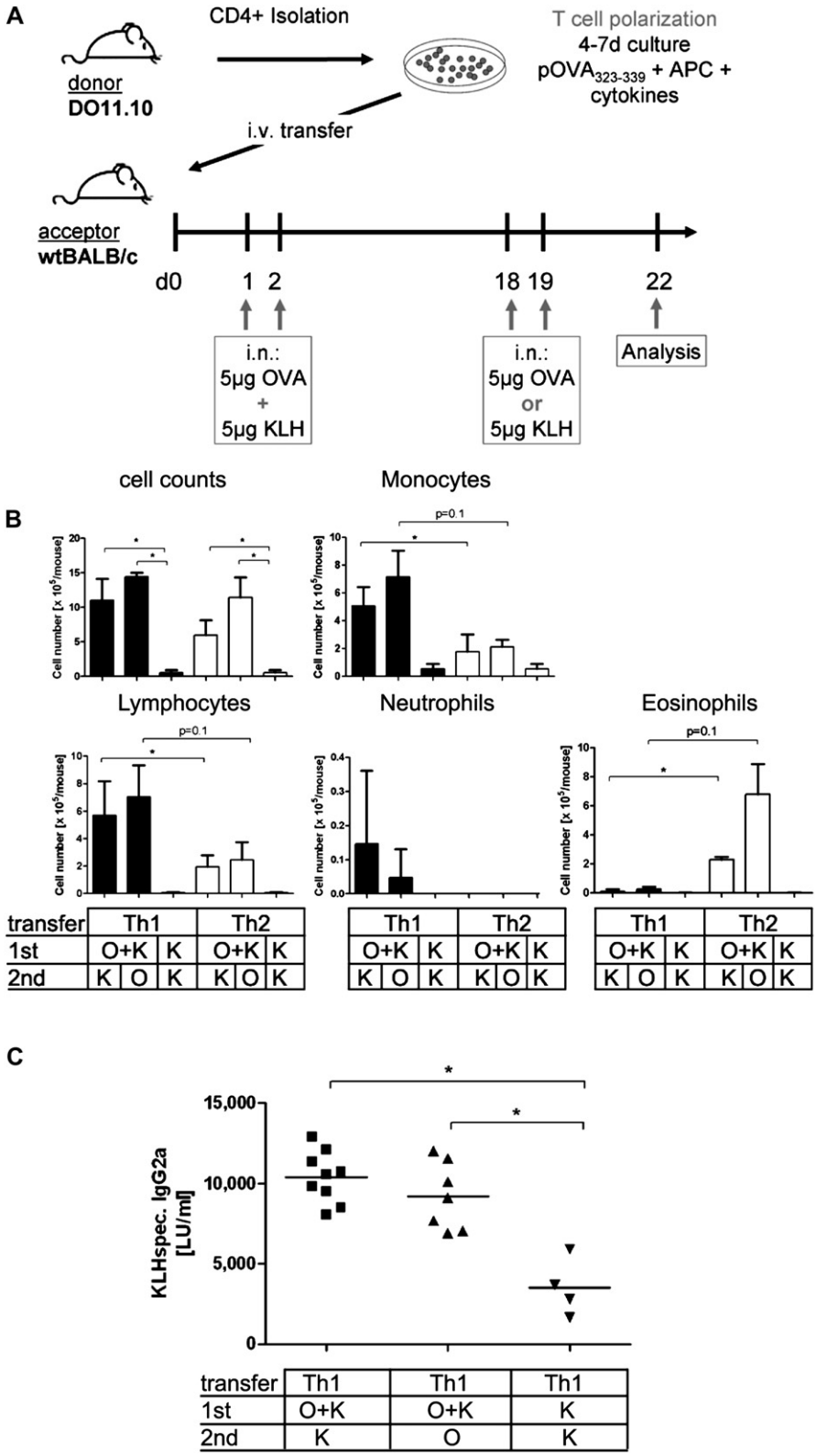

FIG 1. Protocol and phenotype of T_H1 collateral priming. **A**, Collateral priming protocol. *i.n.*, Intranasal; *i.v.*, intravenous. **B**, BAL pattern of T_H1 versus T_H2 (transfer) collateral priming, with OVA and KLH *(O+K)* applied during the first and OVA *(O)* or KLH *(K)* applied during the second challenge phase. **C**, KLH-specific serum IgG2a levels in T_H1 collateral priming (representative experiments; n = 3-5 animals per group). Experiments were performed 3 to 7 times. $*P < .05$.

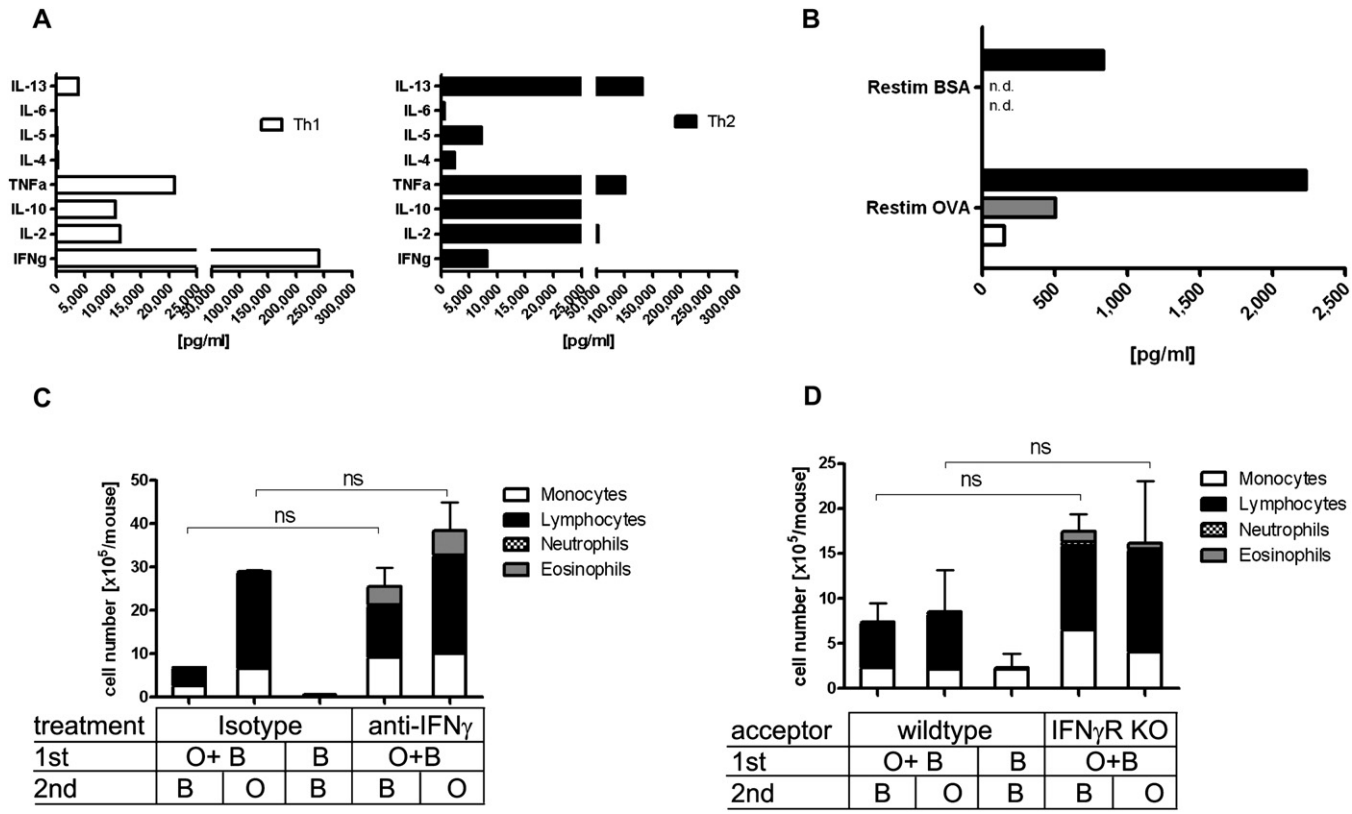

FIG 2. Cytokine profiles of T_H1 collateral priming and independence from IFN-γ. **A**, Cytokine profiles of *in vitro* polarized T_H1 *(white)* and T_H2 *(black)* cells (duplicates). The experiment was performed 17 times. **B**, LN cultures obtained at day 4 of T_H1 collateral priming (*black*, IFN-γ; *gray*, IL-5; *white*, IL-13; mean values of duplicates, experiment performed 5 times). *n.d.*, Not detectable. **C** and **D**, BAL cells from mice undergoing the T_H1 collateral priming protocol receiving IFN-γ–neutralizing antibody or isotype (treatment; Fig 2, *C*) or mice being deficient for IFN-γ receptor versus wild-type mice (acceptor; Fig 2, *D*). *B*, BSA; *1st*, first challenge; *ns*, not significant; *O*, OVA; *2nd*, second challenge. Experiments were performed 3 to 4 times.

Unless indicated otherwise, 5 mice were used for each condition studied in an individual experiment. Each treatment condition was repeated at least 3 times.

RESULTS

Phenotype of T_H1 collateral priming

To test our hypothesis that collateral priming is a phenomenon that pertains not only to T_H2-polarized airway inflammation, we adapted our model for T_H2 collateral priming[23] to a T_H1-polarized response (Fig 1, *A*). This model allowed Eisenbarth et al[23] to determine that an ongoing T_H2-polarized airway inflammation facilitates sensitization toward a secondary unrelated antigen.

The collateral priming model consists of the transfer of polarized transgenic DO11.10 T cells and 2 airway challenges. A first airway challenge is performed with the cognate antigen OVA together with a secondary unrelated antigen (BSA or KLH). After the initial antigen-driven inflammation has resolved, we challenged a second time with either cognate antigen or the secondary unrelated antigen (Fig 1, *A*). Comparing the transfer of T_H2-polarized T_H cells with the transfer of T_H1-polarized T_H cells, we observed that a secondary challenge ("second: K") with an unrelated antigen, KLH, introduced during the first challenge with the cognate antigen OVA ("first: O+K") facilitates priming toward this antigen, regardless of the polarization (transfer T_H1 vs T_H2) of the transferred T cells (Fig 1, *B*). The dose of the secondary antigen alone is not sufficient to induce significant airway inflammation ("first: K, second: K").

Obviously the composition of the BAL inflammatory influx differs when transferring T_H1 versus T_H2 cells. T_H2 collateral priming induces a typical T_H2-polarized airway inflammation, while T_H1 collateral priming induces an inflammatory influx that is dominated by lymphocytes and cells of monocytic morphology (Fig 1, *B*). Transfer of naive transgenic T cells followed by 2 consecutive OVA challenges or transfer of T_H1-polarized transgenic T cells and primary challenge with PBS followed by challenge with OVA during secondary challenge did not result in significant airway inflammation (see Fig E1, *B*, in this article's Online Repository at www.jacionline.org), demonstrating that activation of the transferred, polarized T-cell population and the ensuing inflammation are necessary for collateral priming to occur.

The increase in levels of KLH-specific IgG2a antibodies in peripheral blood (Fig 1, *C*) confirmed the induction of a T_H1-polarized, systemic KLH-specific antigen response. Finally, rechallenge 8 weeks after initial priming demonstrated that collateral priming induces sustained memory formation (see Fig E1, *A*).

Independence of T_H1 collateral priming from IFN-γ

We carefully examined the cytokine profile of our transferred T_H1 cells before transfer and the cytokine profile of mLN cells

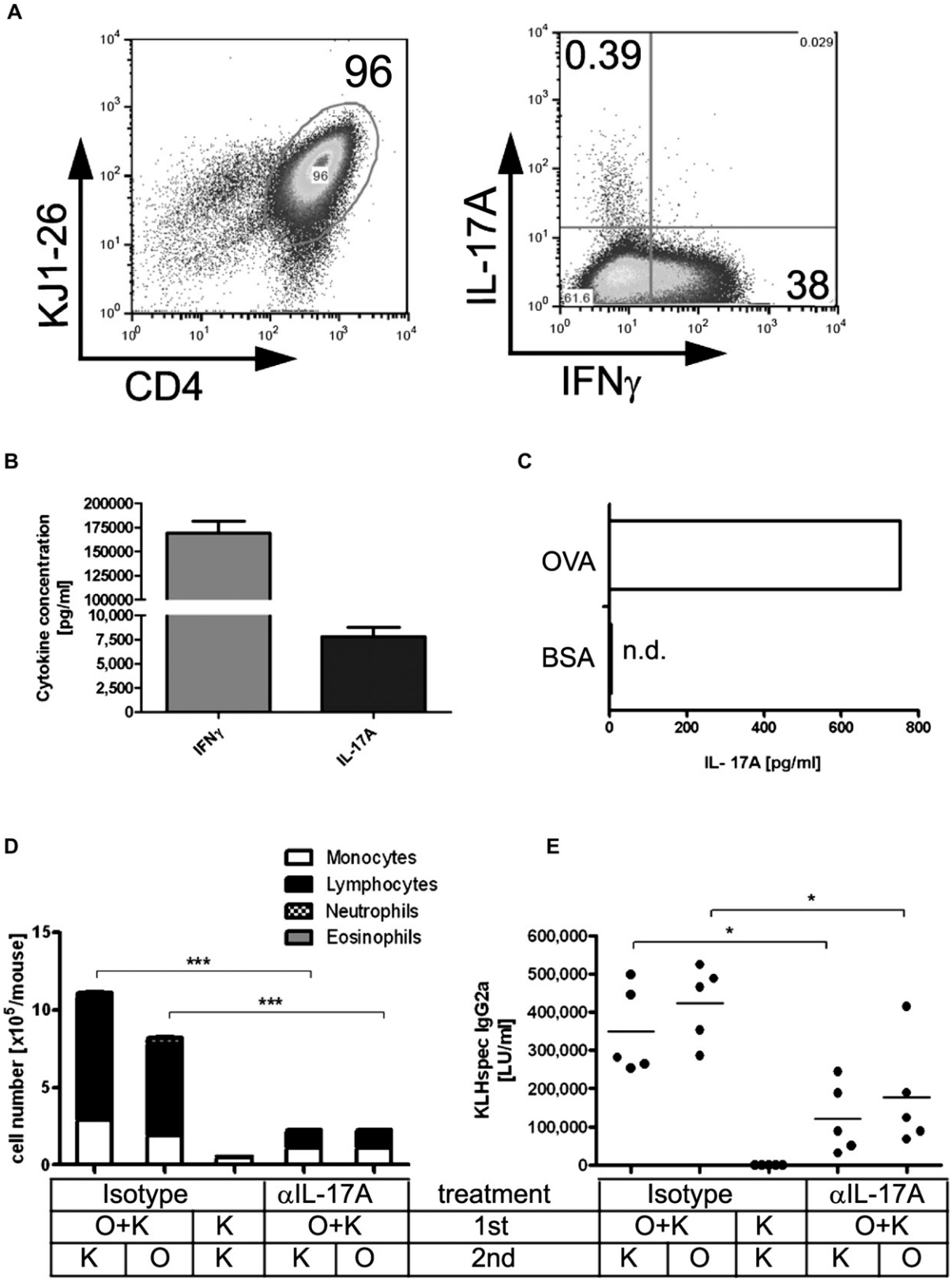

FIG 3. Role of IL-17A in T_H1 collateral priming. **A** and **B**, Intracellular cytokine analysis (Fig 3, *A*) and supernatant ELISA levels (Fig 3, *B*) of IL-17A and IFN-γ production from *in vitro* polarized T_H1 cells (*gray*, IFN-γ; *black*, IL-17A). **C**, Multiplex analysis of IL-17A production as in Fig 2, *B*. *n.d.*, Not detectable (duplicates). **D** and **E**, BAL cells and KLH-specific serum IgG2a levels from mice undergoing the T_H1 collateral priming protocol receiving IL-17A–neutralizing antibody (treatment). *1st*, First challenge; *K*, KLH; *O*, OVA; *2nd*, second challenge. n = 5 animals per group. $^*P < .05$. $^{***}P < .001$. Experiments were performed 3 to 5 times.

upon restimulation after transfer to identify possible mediators responsible for the T_H1 collateral priming phenomenon. As expected, *in vitro* restimulation of T_H1 cells with OVA peptide and APCs elicited a typical T_H1 profile: large amounts of IFN-γ; some IL-2, IL-10, and TNF-α; and negligible amounts of IL-4, IL-5, IL-6, and IL-13 compared with cells polarized toward a T_H2 phenotype (Fig 2, *A*). The *ex vivo* cytokine profile of mLN cells upon *in vitro* restimulation after an initial intranasal challenge also revealed a typical T_H1 profile (Fig 2, *B*).

In spite of a predominance of IFN-γ, blocking studies with an anti–IFN-γ antibody showed that T_H1 collateral priming is independent from the presence of IFN-γ. Administration of anti–IFN-γ antibody during the first antigen challenge did not diminish T_H1 collateral priming (Fig 2, *C*). Similar results were obtained in an OT-II transfer system comparing wild-type with IFN-γ receptor–deficient mice as acceptor mice in the collateral priming protocol (Fig 2, *D*).

IL-17A is a potent mediator of T_H1 transfer–mediated collateral priming

Consecutively, we extended our search for possible mediators of T_H1 collateral priming. We included IL-17A in our analysis, considering that in some studies T_H17 cells have replaced T_H1 cells as principal culprits in certain diseases.[7,27]

In vitro analyses of T_H1-polarized transgenic cells by means of intracellular cytokine staining revealed that among the transgenic T-cell population, we could identify a distinct IL-17A–producing population (Fig 3, *A*). Although IL-17A–producing T cells represented only a small population compared with IFN-γ–producing cells, considerable amounts of IL-17A were secreted during restimulation, as determined by means of ELISA (Fig 3, *B*). Additionally, OVA-specific *in vitro* restimulation of mLN cells after the first antigen challenge revealed significant amounts of IL-17A in the culture supernatants (Fig 3, *C*). Thus we identified a small population of IL-17A–producing antigen-specific $CD4^+$ cells in our T_H1-polarized cultures. These results encouraged us to test whether IL-17A might be a decisive mediator for T_H1 collateral priming.

Administering an anti-IL-17A antibody intraperitoneally and intranasally on days 0 to 3 during the first antigen challenge, we observed a significant reduction in T_H1 collateral priming, based on a significant reduction in BAL cell counts (Fig 3, *D*), as well as a significant reduction in KLH-specific IgG2a levels in peripheral blood (Fig 3, *E*).

IL-17A–secreting cells confer collateral priming *in vivo*

Because our results revealed IL-17A and not IFN-γ as an important mediator in collateral priming, we sought to investigate whether the effects observed by T_H1 cell transfer would be similar when transferring IL-17A–producing T_H17 cells. To this end, we performed the collateral priming protocol (Fig 1, *A*) with *in vitro* T_H17-polarized DO11.10 cells. Analysis of the BAL fluid after the second airway challenge showed that priming toward the unrelated antigen KLH was facilitated by T_H17 cell transfer. T_H17-polarized collateral priming led to an influx of monocytes and numerous lymphocytes into the airways (Fig 4, *A* and *B*), resembling the BAL differential observed in T_H1 collateral priming. Systemic sensitization toward the secondary antigen was confirmed by the induction of KLH-specific IgG2a antibodies (Fig 4, *C*). Finally, T_H17 collateral priming led to significant AHR compared to that seen in control mice (Fig 4, *D*, and see Fig E2 in this article's Online Repository at www.jacionline.org).

T_H17 collateral priming initiates priming of endogenous T_H17 cells

Similar to observations by Eisenbarth et al,[23] KJ1-26$^+$CD4$^+$ T cells could not be detected in the lungs after the second challenge (see Fig E3 in this article's Online Repository at www.jacionline.org), suggesting that priming of endogenous T cells and subsequent T_H17 polarization of these cells occurred. Detailed flow cytometric analysis of the cells recruited to the lung after T_H17 collateral priming revealed a substantial increase in $CD4^+$ cells, whereas the number of $CD8^+$ cells did not change in comparison with the control group (see Fig E4 in this article's Online Repository at www.jacionline.org). Intracellular cytokine staining of the $CD4^+$ cells after PMA/ionomycin restimulation revealed induction of endogenous T_H17 and T_H1 cells in the lung, as shown by a significant production of IL-17A, as well as IFN-γ (Fig 5, *A*, and see Fig E5 in this article's Online Repository at www.jacionline.org). Restimulation of lymph node (LN) cells, however, led to significantly lower secretion of either cytokine (Fig 5, *A*, and see Fig E5). ELISA measurements of IL-17A versus IFN-γ secretion in the supernatants of lung and LN cells (Fig 5, *B*) confirmed the intracellular cytokine data in that they also revealed significant secretion of IL-17A and IFN-γ in restimulated lung cells, with secretion of these cytokines in restimulated LN cells being less than the detections limits of the ELISAs. Direct comparison of IL-17A and IFN-γ secretion by lung and LN cells that were restimulated either nonspecifically with PMA/ionomycin or specifically with antigens (OVA/KLH in the presence of bone marrow–derived dendritic cells) revealed a similar pattern and confirmed priming toward KLH (see Fig E6 in this article's Online Repository at www.jacionline.org) because KLH-specific IL-17A, as well as IFN-γ, production could be demonstrated (see Fig E6, *B*, in this article's Online Repository at www.jacionline.org).

When examining the phenotype of pulmonary $CD4^+$ cells producing IL-17A or IFN-γ more closely, we observed another interesting difference. Similar to published data in a model of bacterial infection,[28] the IL-17A–producing cells in the lung are $CD44^+$ and $CD62L^+$, phenotypically resembling central memory T cells, whereas their IFN-γ^+ counterparts show much higher percentages of $CD44^+/CD62L^{inter\text{-}low}$ cells, thus resembling effector memory cells (Fig 5, *C*, and see Fig E5). Moreover, IL-17A–producing lung cells express CD62L in higher amounts compared with the IFN-γ–producing population, regardless of central memory or the effector memory phenotype (Fig 5, *D*).

DISCUSSION

Our current study extends previous findings demonstrating that an ongoing T_H2-polarized pulmonary inflammation facilitates priming toward unrelated antigens.[23,26,29,30] We identify IL-17A as the key cytokine to facilitate priming toward new antigens during concomitant T_H1-polarized airway inflammation and demonstrate that T_H17 cells by themselves can facilitate priming of endogenous T cells for IL-17A production. IL-17A seems to be

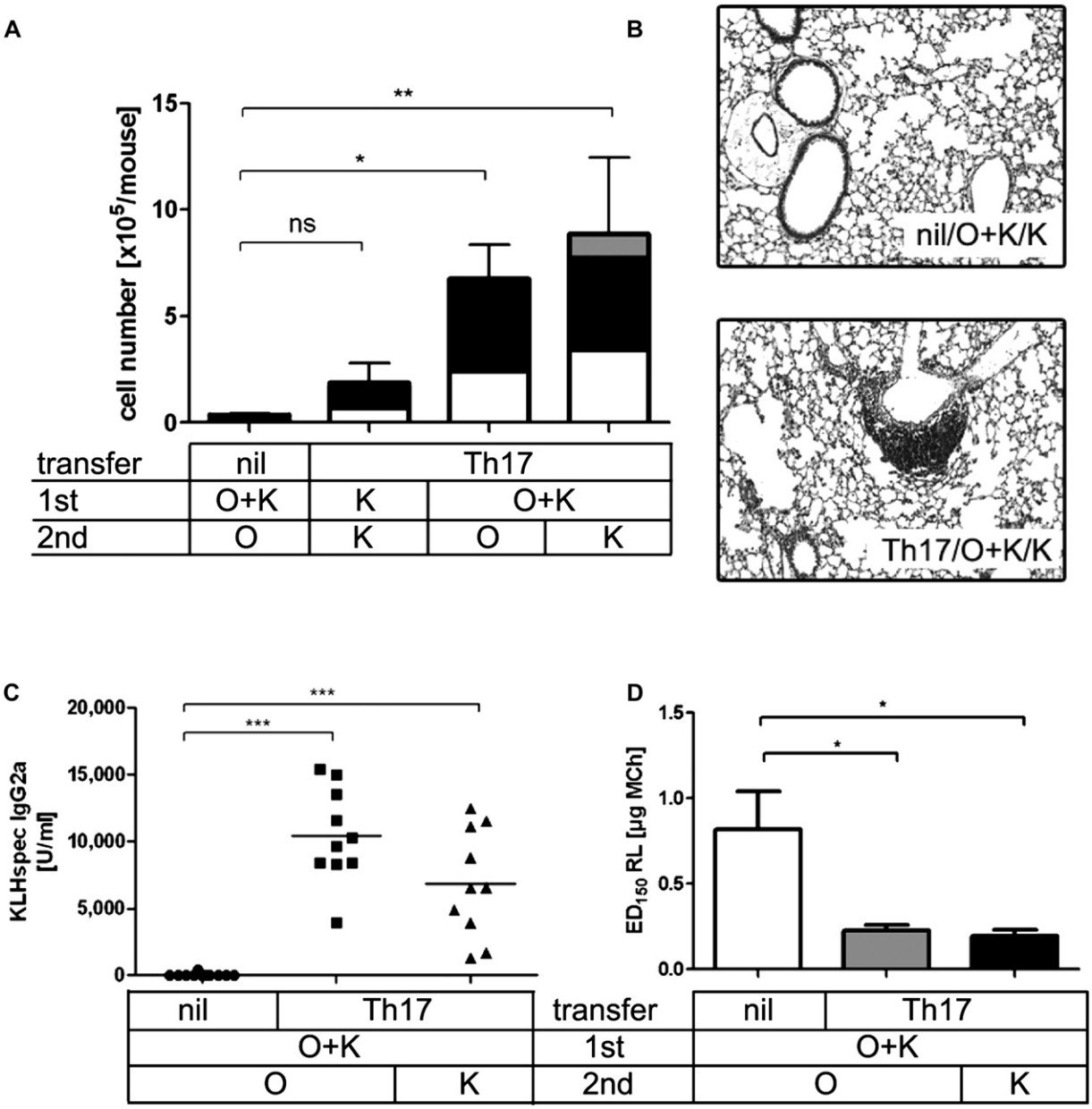

FIG 4. T_H17 cells mediated collateral priming. BAL differential **(A)**, lung hematoxylin and eosin staining **(B)**, KLH-specific serum IgG2a levels **(C)**, and invasive lung function measurements **(D)** of mice subjected to T_H17 collateral priming. Control mice received either no cells or only KLH *(K)* in the first *(1st)* and second *(2nd)* challenge phases. Fig 4, *B*: magnification ×100x. Fig 4, *D*: *Bars* represent the effective inhalational dose *(ED_{150})* of methacholine in micrograms eliciting a 150% increase in lung resistance *(R_L)* compared with baseline. Fig 4, *D*: n = 9-10 (**P* < .05, unpaired Student *t* test). Fig 4, *A* and *C*: n = 3-10 (**P* < .05, ***P* < .01, and ****P* < .001). *ns*, Not significant; *O*, OVA. Experiments were performed 8 times.

a major contributor to both T_H1 and T_H17 collateral priming: neutralization of IL-17A in T_H1 (Fig 3, *D*) and T_H17 (see Fig E7 in this article's Online Repository at www.jacionline.org) collateral priming significantly reduces BAL inflammatory cell influx, suggesting that IL-17A is an important contributor to the collateral priming process under both circumstances. However, neutralization of IL-17A during cell transfer and the first challenge phase did not affect T_H2 collateral priming (see Fig E7).

Human studies show that exacerbation and chronicity of asthma are linked to T_H1-polarized pulmonary inflammation, which often occurs because of viral infections,[18-20,31] thus underlining an important role not only for T_H2- but also T_H1-polarized lymphocytes in asthmatic patients. Our results revealed that collateral priming can be induced through transfer of T_H1-polarized cells, possibly modeling the increased risk of allergic sensitization after viral infections seen in children.[18-20] However, in spite of the importance of IFN-γ as the key T_H1 cytokine, T_H1 collateral priming depends on IL-17A. Since the paradigm of a T_H1/T_H2 dichotomy in inflammatory disorders has been revised to include T_H17 cells, a wealth of data has been generated linking these cells to deleterious effects that were previously thought to be T_H1-driven diseases.[7,27,32,33] Additionally, a critical role of

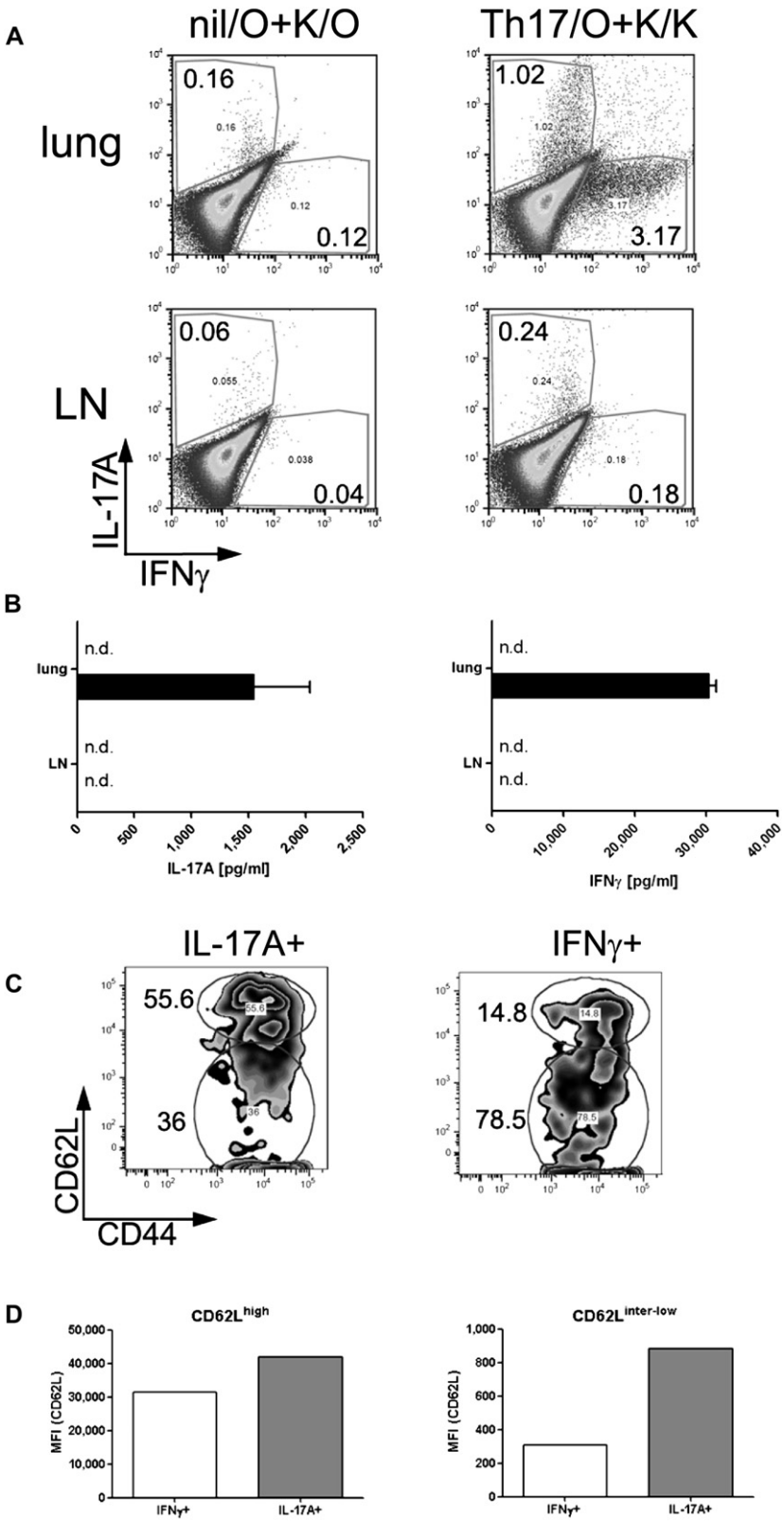

FIG 5. Phenotype of endogenous T_H17 cells. **A** and **B**, Intracellular cytokine staining (Fig 5, *A*) and ELISA supernatant analysis (Fig 5, *B*) for IL-17A and IFN-γ secretion from $CD4^+$ cells of lung/LN cultures from control mice *(nil/O+K/O)* and mice subjected to collateral priming *(Th17/O+K/K)*. **C** and **D**, CD44 and CD62L surface expression (Fig 5, *C*) and CD62L mean fluorescence intensity (*MFI*; Fig 5, *D*) of $CD62L^{high}$ and $CD62L^{inter\text{-}low}$ populations of $CD4^+$ lung cells producing IFN-γ or IL-17A obtained from lungs of mice subjected to T_H17 collateral priming. Experiments were performed 3 to 6 times. *n.d.*, Not detected. Fig 5, *A* and *C*: For gating strategy, see Fig E5 in this article's Online Repository.

T_H17 cells in host defense, including viral infections, has been demonstrated.[34,35] In patients with allergic airway disease, IL-17A levels correlate with disease severity and the influx of neutrophils.[36-38] Rodent models of allergic airway inflammation revealed a role for IL-17A in the recruitment of neutrophils, as well as eosinophils,[10,39] and complement factor C3a as an important regulator of the IL-23/T_H17 axis in patients with severe asthma.[40] Our studies confirm the crucial role of T_H17 in pulmonary priming for airway inflammation and reactivity and thus underline the need to address these cells in more detail when searching for new interventional strategies in patients with allergic airway disease.

The development of an IL-17A–producing subpopulation of $CD4^+$ cells under T_H1-polarizing conditions was surprising because IFN-γ is described to counterbalance T_H17 polarization.[41] However, in light of *in vivo* circumstances in which developing T_H17 cells might encounter IFN-γ and other cytokines favoring or opposing T_H17 cell development, the presence of a minor IL-17A–producing population that is not susceptible to IFN-γ suppression is not inconceivable. In fact, our own *in vivo* data on cytokine secretion by lung and LN cells after the secondary challenge in T_H17 collateral priming (Fig 5, *A* and *B*) showed secretion of IL-17A and large amounts of IFN-γ by distinct populations of lung cells, suggesting that T_H17 cells can escape the suppressive effects of IFN-γ during their development. Indeed, studies have suggested that under certain circumstances, the presence of IL-17A might even be important for recruitment of T_H1 cells in patients with bacterial infections.[42]

Because IL-17A, in contrast to IL-4, which was identified as a crucial cytokine for T_H2 collateral priming, is not directly acting as a T-cell differentiation factor, the mechanism behind IL-17A–mediated collateral priming might be more indirect. Various cell types, including bronchial epithelial cells and fibroblasts, are described to secret chemokines and cytokines (eg, T_H17 differentiation factor IL-6) and upregulate leukocyte adhesion molecules, such as intercellular adhesion molecule 1, in response to IL-17A stimulation.[43] Together with the presence of other inflammatory cells during the first challenge phase, such as macrophages,[44] which under inflammatory conditions have been described to be sufficient for T_H17 polarization of naive T cells, this might provide a milieu sufficient for T_H17 differentiation.

Contrary to some studies that point toward a pivotal role of IL-17A in neutrophilia,[10,34,45] we found a lymphocytic influx into the BAL fluid on day 22 after T_H17 collateral priming with few neutrophils. This discrepancy might depend on different protocols for the induction of airway inflammation, in particular with regard to the amount of antigen used. Higher amounts of antigen invariably increase the amount of antigen-contaminating LPS, which dose dependently induces neutrophilia.[46] Additionally, analysis of BAL cells at different time points after challenge revealed that neutrophils appear in the lung 24 hours after the first challenge phase (day 4), as well as 24 hours after the second challenge phase (day 20), but decrease in number with time, as seen at 72 hours after the second challenge (day 22), suggesting that early neutrophilia might be missed in our protocol (see Fig E8 in this article's Online Repository at www.jacionline.org). Recent clinical data also suggest a role for IL-17A (and IL-17F) in patients with chronic obstructive pulmonary disease and asthma but show no correlation with neutrophilic airway inflammation.[47]

Several studies have shown a seminal role for the IFN-γ/IL-12 axis in the induction of AHR,[48,49] whereas other studies demonstrate contribution of the T_H17/IL-23 axis and neutrophil recruitment in conferring AHR.[39,45] However, a recent study describes conversion of T_H17 into IFN-γ producers *in vivo* as a prerequisite for AHR,[50] which constitutes a combination of both axes and is conceivable in the light of potential T_H17 cell plasticity.[51] Additional T_H plasticity has been observed in patients with allergic airway disease, in whom a subset of IL-17A–secreting T_H2 cells has been detected at increased levels in asthmatic patients and proinflammatory cytokine stimulation was shown to induce IL-17A secretion from classical T_H2 cells.[52] Our studies addressed the role of IL-17A versus IFN-γ during the collateral priming process, where we did observe a role for IL-17A but not for IFN-γ (Fig 3, *D* and *E*). However, at the present time, we can neither distinguish between a singular contribution of IL-17A versus IFN-γ toward the induction of AHR nor exclude a contribution of IL-17A–producing T_H2 cells to collateral priming.

Our findings concerning the role of IL-17A in pulmonary priming might be particularly critical with regard to steroid-resistant asthma, which has been described to be mediated by T_H17 cells.[12] Extrapolating from our data, patients with this form of asthma would be at a particular risk for new sensitizations.

Evolutionarily, collateral priming might have evolved to ensure that during an ongoing immune response bystander cells with different antigen specificity could be more easily recruited to become polarized effector cells. At least partially, we draw on this effect when boosting for vaccination to enhance a response against a given pathogen. However, in the context of allergic responses, this beneficial effect can have deleterious consequences because any lung inflammation, regardless of its origin (allergic, viral, and environmental) or polarization, will increase the risk of *de novo* sensitization toward unrelated harmless antigens. Ultimately, this process leads to polysensitization, a subtype of allergic disease with a much worse clinical course that is immensely more difficult to treat[53-55] and thus needs to become a focus of future research directions.

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Clinical implications: Our findings bring mechanistic knowledge to the phenomena observed when boostering for vaccination and the phenomenon of polysensitization and primary sensitization in asthma, particularly in the context of concomitant viral infections.

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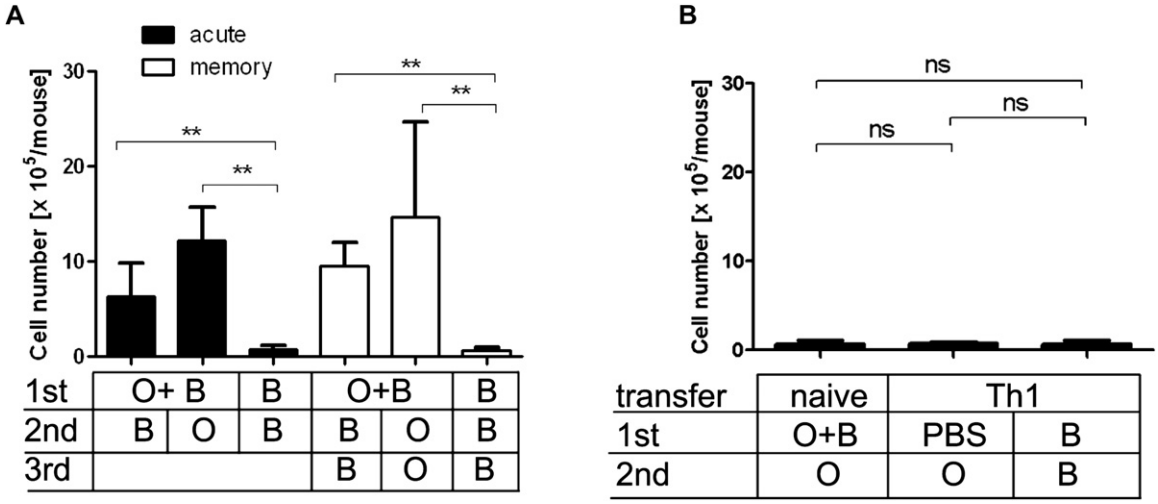

FIG E1. Memory response and control groups for T_H1 collateral priming. **A**, BAL cell numbers of mice that underwent T_H1 collateral priming. Mice received T_H1-polarized cells and were consecutively challenged with OVA and BSA *(O+B)* or BSA alone *(B)* during the first challenge phase and analyzed after the second challenge phase with either BSA *(B)* or OVA (*O*) or after a third challenge phase 8 weeks later. **B**, Comparison of BAL cell numbers from different control mice that were subjected to the collateral priming protocol. *naive*, Transfer of naive (not polarized) transgenic T cells; *ns*, not significant; *PBS*, mice received buffer in the first challenge phase (n = 5 animals per group). Experiments were performed 2 to 3 times. $^{**}P > .01$.

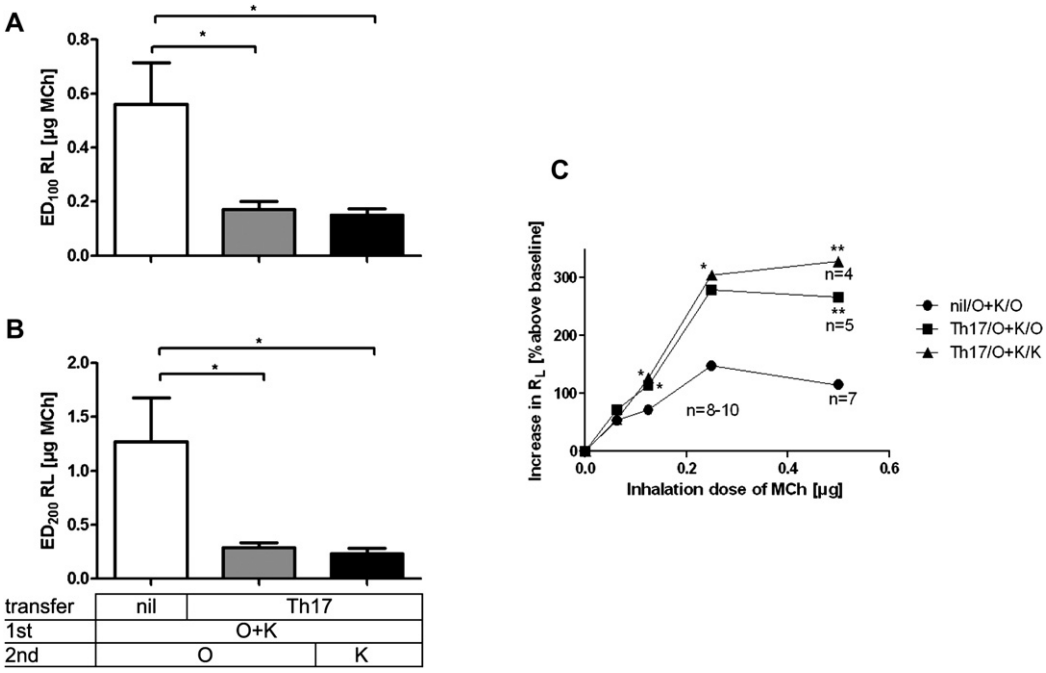

FIG E2. Invasive lung function after T_H17 collateral pulmonary priming. ED_{100} **(A)**, ED_{200} **(B)**, and dose-response curve **(C)** values of mice undergoing T_H17 collateral pulmonary priming. *1st*, first challenge with OVA and KLH *(O+K)*; *2nd*, second challenge with OVA *(O)* or KLH *(K)*; *transfer*, T_H17 cells or buffer. Fig E2, *A* and *B*: *Bars* represent the effective inhalational dose (ED_{100} and ED_{200}) of methacholine *(MCh)* in micrograms calculated from the individual dose-response curves eliciting a 100% or 200% increase in lung resistance *(R_L)* compared with baseline. Fig E2, *C*: Dose-response curve (methacholine vs lung resistance) of the same mice. Because of the experimental protocol (animals are not provoked further with methacholine once they reach maximal bronchoconstriction, as evidenced by no further increase of bronchoconstriction with higher methacholine doses), the animal number decreases with increasing methacholine dosages. Fig E2, *A* and *B*: n = 8-10. Fig E8, *C*: n as depicted in graph. $*P < .05$ and $**P < .01$, unpaired *t* test for Th17/O+K/O or Th17/O+K/K versus negative control (nil/O+K/K). The experiment was performed once.

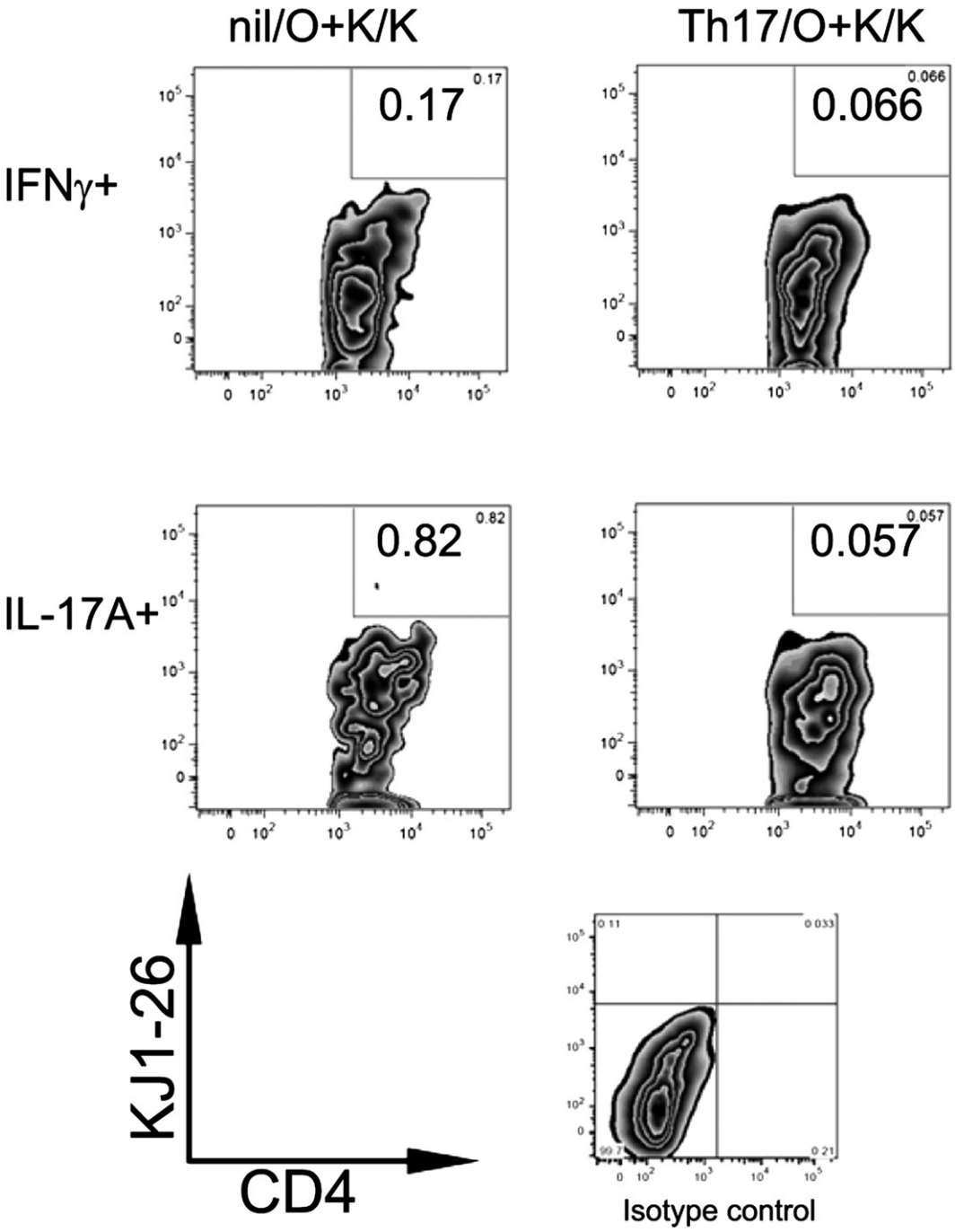

FIG E3. Absence of transferred transgenic T cells after collateral priming protocol. Expression of CD4 and transgenic OVA-specific T-cell receptor (KJ1-26^{+}) by IL-17A– and IFN-γ–producing lung cells after unspecific restimulation from control mice (ie, receiving no cells *[nil/O+K/O]*) and mice subjected to collateral priming *(Th17/O+K/K)*. Analysis was performed on day 22 of the collateral priming protocol. Lung cells were pooled from each group at the time of death (n = 5 animals per group). The experiment was performed once. For gating strategy, see Fig E5.

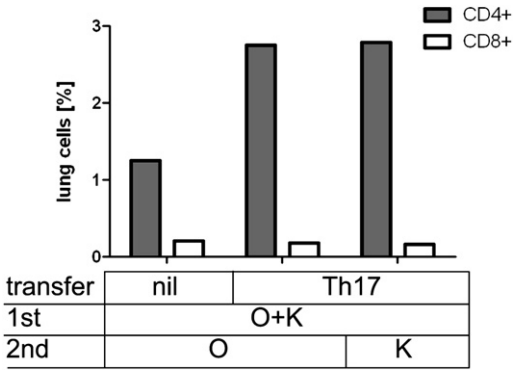

FIG E4. Increase of $CD4^+$ cell proportion in lungs after T_H17 collateral priming. FACS analysis of $CD4^+$ *(gray)* and $CD8^+$ *(white)* populations among total lung cells from animals that received T_H17 cells compared with control mice *(nil)*. All groups were challenged with OVA and KLH *(O+K)* during the first challenge phase and either OVA *(O)* or BSA *(B)* alone during the second challenge phase. Lung cells were pooled from each group at the time of death (n = 3 animals per group). The experiment was performed once.

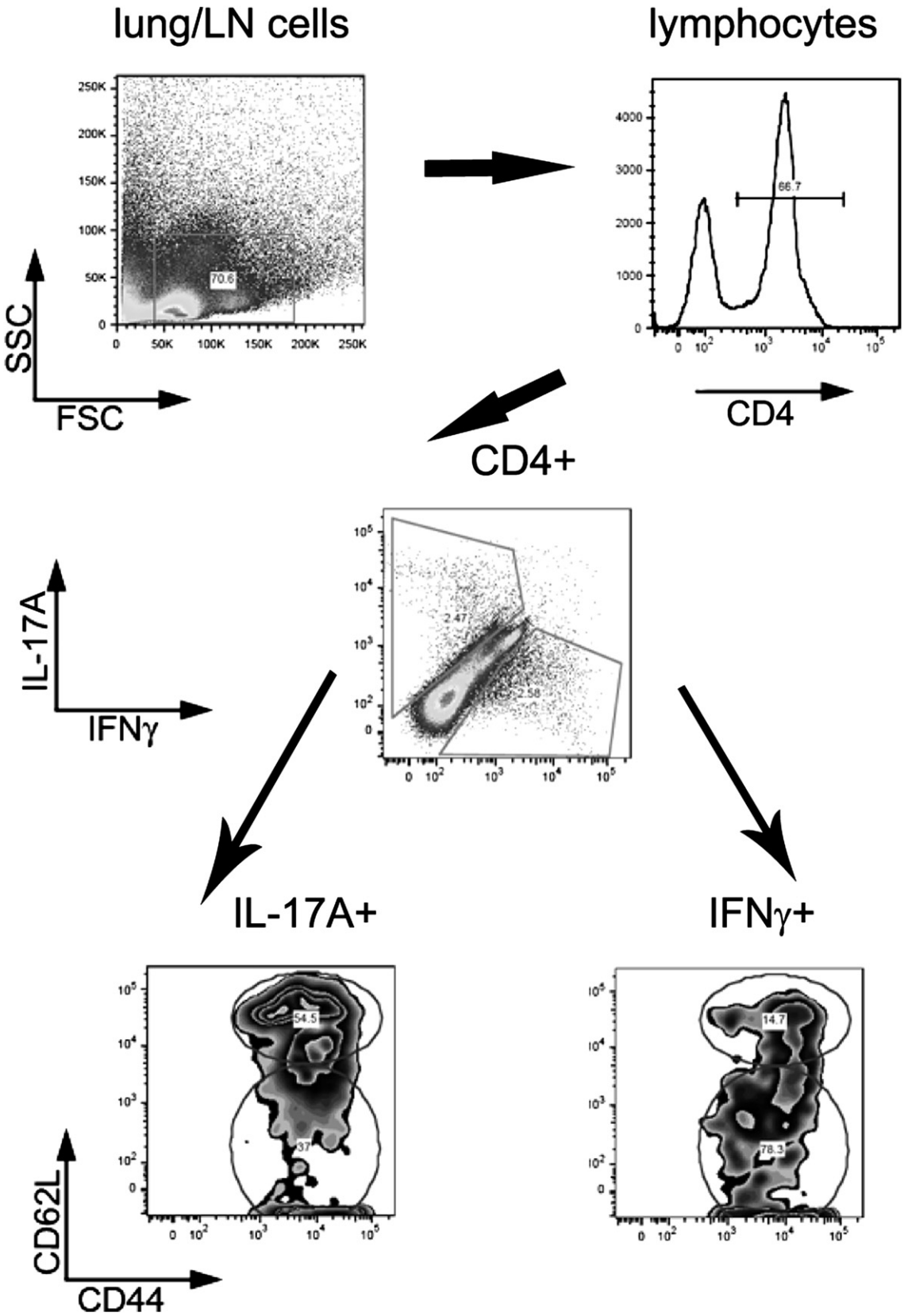

FIG E5. Gating strategy for intracellular cytokine staining of lung and LN cells. Lymphocytes from lungs or LNs were gated regarding their forward-scatter *(FSC)* and side-scatter *(SSC)* properties. $CD4^+$ lymphocytes were analyzed regarding their intracellular IL-17A and IFN-γ expressions. The resulting IL-17A and IFN-γ single-positive populations were further analyzed with regard to CD62L and CD44 surface expression. Experiments were performed 3 times.

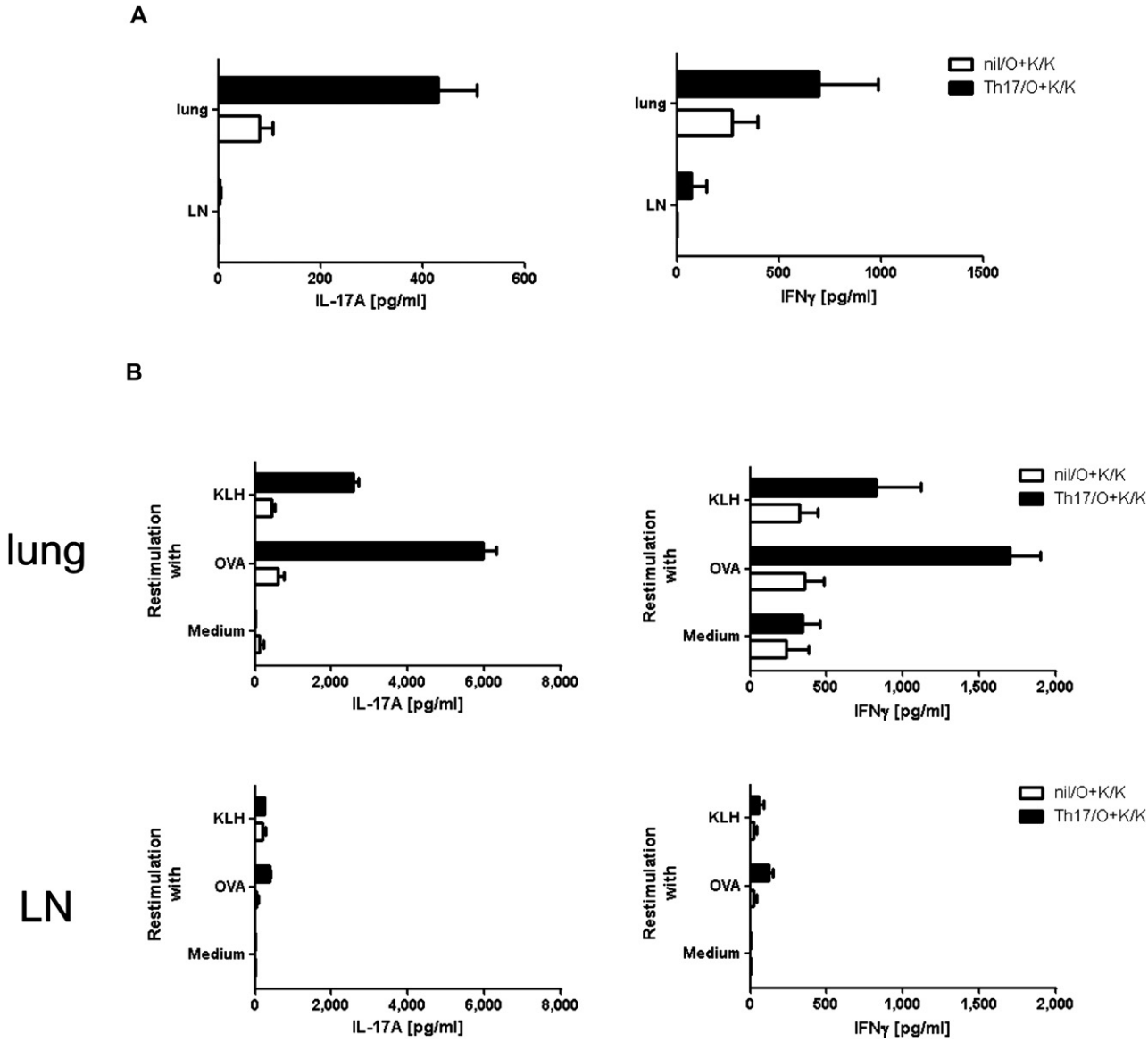

FIG E6. Comparison of cytokine release from lung and LN cells after specific and unspecific restimulation. ELISA measurements of IL-17A and IFN-γ production by LN and lung cell cultures from control mice (*white*, *nil/O+K/O*) and mice subjected to T_H17 collateral priming (*black*, *Th17/O+K/K*) at day 22. Cells were restimulated at 4 hours with PMA/ionomycin (**A**, triplicates) or 48 hours with KLH or OVA, respectively, in the presence of bone marrow–derived dendritic cells, with medium serving as a negative control (**B**, duplicates). Lung and LN cells were pooled from each group at the time of death (n = 5 animals per group). The experiment was performed once.

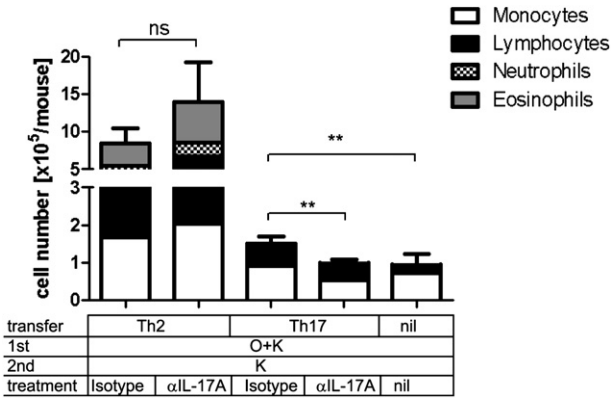

FIG E7. Independence of T_H2 collateral priming from IL-17. BAL differential pattern of collateral priming induced by T_H17 versus T_H2 cell transfer subjected to anti–IL-17A antibody (αIL-17A) or matching isotype control treatment during cell transfer and the first challenge phase. OVA and KLH *(O+K)* were applied during the first challenge phase, and KLH *(K)* was applied during the second challenge phase. Control mice did not receive cells or antibody (*nil*; n = 4-10 animals per group). The experiment was performed once. $^{**}P > .01$. *ns*, Not significant.

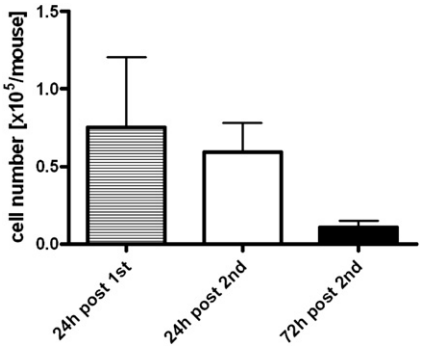

FIG E8. Neutrophil influx during the T_H17 collateral priming protocol. Comparison of neutrophil numbers in BAL fluid from mice that underwent the T_H17 collateral priming protocol 24 hours after the first *(striped)*, 24 hours after the second *(white)*, and 72 hours after the second *(black)* challenge phase (n = 3 animals per group). The experiment was performed once.