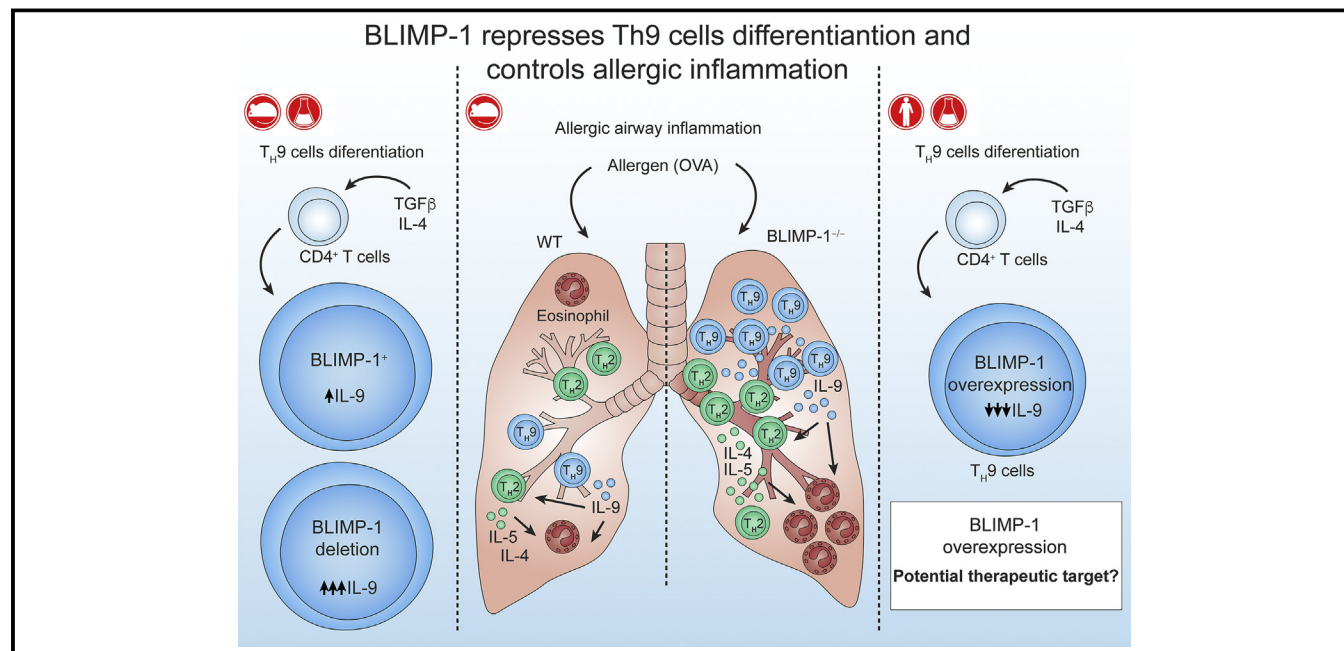


B lymphocyte–induced maturation protein 1 controls T_H9 cell development, IL-9 production, and allergic inflammation



Luciana Benevides, PhD,^a Renata Sesti Costa, PhD,^a Lucas Alves Tavares, MSc,^b Momtchilo Russo, PhD,^c Gislaine A. Martins, PhD,^d Luis Lamberti P. da Silva, PhD,^b L. Karla Arruda, MD, PhD,^e Fernando Q. Cunha, PhD,^f Vanessa Carregaro, PhD,^a and João Santana Silva, PhD^{a,g} *Ribeirão Preto and São Paulo, Brazil, and Los Angeles, Calif*

GRAPHICAL ABSTRACT



Background: The transcriptional repressor B lymphocyte–induced maturation protein 1 (Blimp-1) has a key role in terminal differentiation in various T-cell subtypes. However, whether Blimp-1 regulates T_H9 differentiation and its role in allergic inflammation are unknown.

Objective: We aimed to investigate the role of Blimp-1 in T_H9 differentiation and in the pathogenesis of allergic airway inflammation.

Methods: *In vitro* T_H9 differentiation, flow cytometry, ELISA, and real-time PCR were used to investigate the effects of Blimp-

1 on T_H9 polarization. T cell–specific Blimp-1–deficient mice, a model of allergic airway inflammation, and T-cell adoptive transfer to recombination-activating gene 1 (Rag-1)^{−/−} mice were used to address the role of Blimp-1 in the pathogenesis of allergic inflammation.

Results: We found that Blimp-1 regulates T_H9 differentiation because deleting Blimp-1 increased IL-9 production in CD4⁺ T cells *in vitro*. In addition, we showed that in T cell–specific Blimp-1–deficient mice, deletion of Blimp-1 in T cells worsened airway disease, and this worsening was inhibited by IL-9

From the Departments of ^aBiochemistry and Immunology, ^bCellular and Molecular Biology, and ^cPharmacology, Ribeirão Preto Medical School University of São Paulo, and ^dFiocruz-Bi-Institutional Translational Medicine Platform, Ribeirão Preto; ^ethe Department of Immunology, Institute of Biomedical Sciences, University of São Paulo; ^fthe F. Widjaja Foundation Inflammatory Bowel and Immunobiology Research Institute and Department of Medicine and Biomedical Science, Cedars-Sinai Medical Center (CSMC), Los Angeles; and ^gthe Department of Clinical Medicine, Clinical Hospital of Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto. Supported by the Fundação de Amparo a Pesquisa do Estado de São Paulo–FAPESP (grant 2013/08216-2 [Center for Research in Inflammatory Disease] and grant 2012-08240-8 [scholarship to L.B.]) and Conselho Nacional de Desenvolvimento Científico e Tecnológico–CNPq (grant 445983/2014-0) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior–CAPES (a scholarship to L.B.).

Disclosure of potential conflict of interest: The authors declare that they have no relevant interests to disclose.

Received for publication November 16, 2017; revised May 8, 2018; accepted for publication June 29, 2018.

Available online August 7, 2018.

Corresponding author: João Santana Silva, PhD, Av. Bandeirantes, 3900, 14049-900, Ribeirão Preto, São Paulo, Brazil. E-mail: jsdsilva@fmrp.usp.br.

The CrossMark symbol notifies online readers when updates have been made to the article such as errata or minor corrections

0091-6749/\$36.00

© 2018 American Academy of Allergy, Asthma & Immunology

<https://doi.org/10.1016/j.jaci.2018.06.046>

neutralization. In asthmatic patients CD4⁺ T cells in response to TGF- β plus IL-4 increased IL-9 expression and downregulated Blimp-1 expression compared with expression in healthy control subjects. Blimp-1 overexpression in human T_H9 cells inhibited IL-9 expression.

Conclusion: Blimp-1 is a pivotal negative regulator of T_H9 differentiation and controls allergic inflammation. (J Allergy Clin Immunol 2019;143:1119-30.)

Key words: Allergy, Blimp-1, IL-9, T_H9 differentiation

Adaptive immune responses are orchestrated by different T_H cell subsets, including T_H1, T_H2, T_H17, and regulatory T (Treg) cells, after encountering antigens in different cytokine microenvironments. IL-9-producing T_H9 cells are generated by TGF- β plus IL-4 during cell stimulation.¹⁻³ T_H9 cells have been shown to play a role in allergic airway inflammation,^{4,5} autoimmune diseases,^{6,7} and cancer.^{8,9} Although the transcription factors PU.1,¹⁰ interferon regulatory factor (IRF) 4,¹¹ signal transducer and activator of transcription (STAT) 6,¹² and basic leucine zipper ATF-like transcription factor¹³ are all involved in T_H9 cell differentiation, the transcriptional networks that control T_H9 differentiation are not yet fully understood.

B lymphocyte-induced maturation protein 1 (Blimp-1) is a transcription factor that plays crucial roles in regulating B- and T-lymphocyte function.¹⁴⁻¹⁶ Blimp-1 regulates differentiation of cytotoxic T lymphocytes,^{17,18} T_H1 cells,¹⁹ T_H17 cells,^{20,21} IL-10-expressing Treg cells,²² follicular helper T cells,²³ and conventional T cells.²⁴ In addition to transcription factors, T_H subset differentiation involves various cytokines and costimulatory signals.²⁵ TGF- β mediates differentiation of T_H17 and Treg cells, depending on the presence or absence of IL-6, respectively.²⁶ Expression of Blimp-1 in T cells can be induced by several cytokines, including IL-2, IL-12, and IL-4. Once expressed, Blimp-1 can control the expression of multiple transcription factors, including T-box-containing protein, IRF-4, and B-cell lymphoma 6, which are required for the functions of T_H1, Treg, and follicular helper T cells, respectively.^{19,22,23} Because T_H9 cell differentiation requires IL-4 signaling, we investigated whether Blimp-1 plays a role in T_H9 differentiation and the mechanisms that control the T_H9 cell response in patients with inflammatory conditions.

We found that Blimp-1 negatively regulates T_H9 cell differentiation in mice and human subjects. Blimp-1 deletion in T cells increased the differentiation of naive CD4⁺ T cells into T_H9 cells and disease severity in a murine model of allergic airway inflammation. In conclusion, we propose that Blimp-1 is a suppressive transcription factor that plays a critical role in protection against allergic airway inflammation by modulating T_H9 cells.

METHODS

Mice

C57BL/6 Prdm1^{flox/flox} and C57BL/6 CD4^{Cre} mice were obtained from the Jackson Laboratory (Bar Harbor, Me). Recombination-activating gene 1 (Rag-1)^{-/-}, C57BL/6 Prdm1^{flox/flox}CD4^{Cre+} (T cell-specific Blimp-1-deficient [CKO]), and Prdm1^{+/+}CD4^{Cre} (wild-type [WT]) mice were bred in the animal facility at the University of São Paulo, Brazil, and maintained in a pathogen-free environment. All procedures were performed in accordance with the International Guidelines for the Use of Animals and approved by the local Ethics Committee at the University of São Paulo, Brazil (123/2017).

Abbreviations used

Blimp-1: B lymphocyte-induced maturation protein 1
CKO: T cell-specific Blimp-1-deficient mice
Ct: Cycle threshold
IRF: Interferon regulatory factor
OVA: Ovalbumin
OX86: OX40 agonist
qPCR: Quantitative real-time PCR
Rag-1: Recombination-activating gene 1
STAT: Signal transducer and activator of transcription
Treg: Regulatory T
WT: Wild-type

T-cell isolation and *in vitro* T_H differentiation

Naive CD4⁺ (CD25⁻CD44^{low}) T cells were sorted from spleen and lymph node cell suspensions by using a FACS Aria III (BD Biosciences, San Jose, Calif). CD4⁺ T cells were stimulated with anti-CD3 (2 μ g/mL) and anti-CD28 (1 μ g/mL) for 4 days in RPMI-1640 medium supplemented with 5% FBS (Gibco, Carlsbad, Calif), 100 U/mL penicillin/100 μ g/mL streptomycin, 1 mmol/L sodium pyruvate, nonessential amino acids, L-glutamine, and 50 μ mol/L 2-mercaptoethanol. For T_H1 differentiation, 5 ng/mL IL-12, 10 μ g/mL anti-IL-4, and 25 U/mL IL-2 were used; for T_H2 conditions, 10 ng/mL IL-4, 10 μ g/mL anti-IFN- γ and 25 U/mL IL-2 were used; and for T_H9 conditions, 10 ng/mL IL-4, 3 ng/mL TGF- β , and 10 μ g/mL anti-IFN- γ were used in the presence or absence of OX40 agonist (OX86; 30 μ g/mL) for 4 days. All recombinant cytokines were obtained from R&D Systems (Minneapolis, Minn), and neutralizing antibodies were from Bio X Cell (West Lebanon, NH).

Flow cytometric assay

For intracellular cytokine staining, cells were restimulated with phorbol 12-myristate 13-acetate (50 ng/mL), ionomycin (500 ng/mL; Sigma-Aldrich, St Louis, Mo), and brefeldin A (BioLegend, San Diego, Calif) for 4 hours. Then the cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% saponin. The antibodies used are listed in the [Methods](#) section in this article's Online Repository at www.jacionline.org. Data acquisition and analysis were performed with FACSCanto II (BD Biosciences) and FlowJo (TreeStar, Ashland, Ore) software, respectively.

Quantitative real-time PCR

Total RNA isolation and quantitative real-time PCR (qPCR) for mRNA expression analysis were conducted as detailed in the [Methods](#) section in this article's Online Repository. The primers are listed in [Table E1](#) in this article's Online Repository at www.jacionline.org. Analyses were performed by using the cycle threshold (Ct) method, which allows for quantitative expression analysis using the formula $2^{-\Delta\Delta Ct}$.

Lymphocyte proliferation and death cell assays

Carboxyfluorescein succinimidyl ester-labeled purified CD4⁺CD44^{lo} CD25⁻ cells were stimulated with 2 μ g/mL anti-CD3 and 1 μ g/mL anti-CD28 in T_H9 differentiation conditions for 1, 3, and 5 days. The proliferation index was evaluated by using carboxyfluorescein succinimidyl ester dilution (Sigma), and apoptosis was assessed by staining with Annexin V (BD Biosciences).

Induction of allergic airway disease

Mice were subcutaneously sensitized and boosted with 4 μ g of chicken ovalbumin (OVA)/1.6 mg of aluminum hydroxide in 0.2 mL of PBS on days 0 and 7. Airway inflammation was induced by means of 2 intranasal

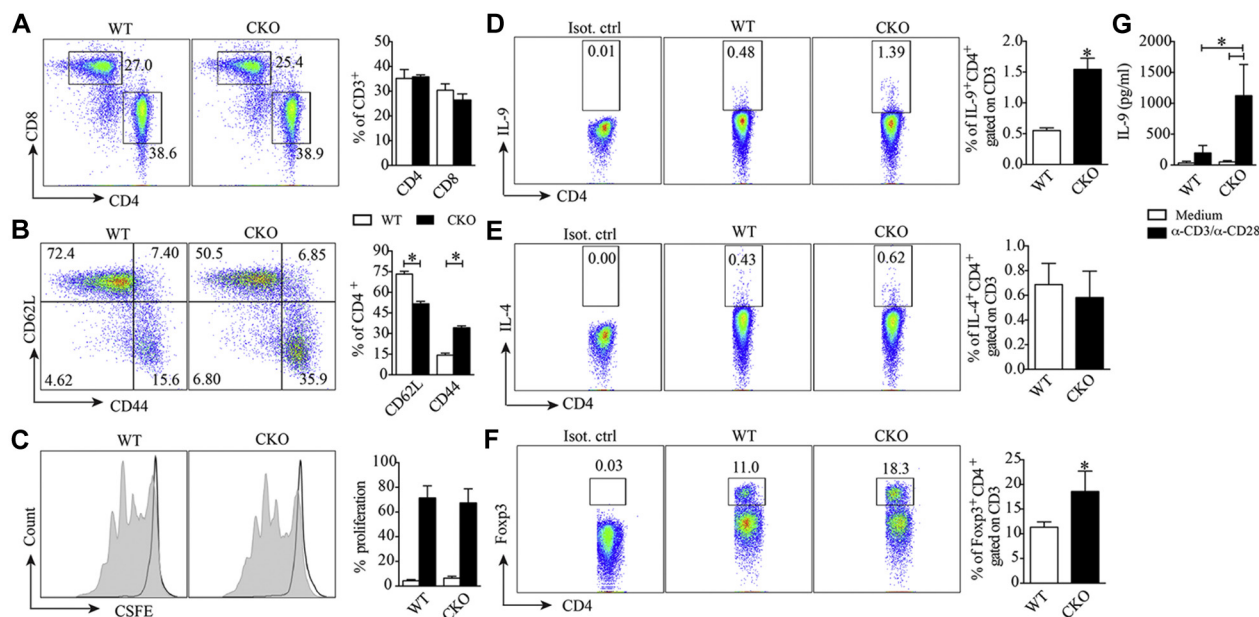


FIG 1. Loss of Blimp-1 in T cells results in accumulation of T_H9. **A** and **B**, Splenocytes from Blimp-1-deficient mice (CKO) and WT mice stained for CD4 and CD8 gated on CD3 (Fig 1, A) and CD44 and CD62L gated on CD4⁺CD3⁺ (Fig 1, B) cells and analyzed by using flow cytometry. Numbers in quadrants indicate percentages of naive (CD4⁺CD62L⁺) and effector/memory (CD4⁺CD44⁺) T cells. **C**, Proliferation of carboxyfluorescein succinimidyl ester (CFSE)-labeled splenocytes from CKO and WT mice activated with anti-CD3 and anti-CD28 for 3 days presented as CFSE dilution. Splenocytes from CKO and WT mice were stimulated *ex vivo* with phorbol 12-myristate 13-acetate plus ionomycin for 4 hours. **D-F**, Percentages of IL-9-expressing (Fig 1, D), IL-4-expressing (Fig 1, E), and forkhead box protein 3 (Foxp3)-expressing (Fig 1, F) CD4⁺ T cells were evaluated by means of flow cytometry and based on the control isotype. **G**, Splenocytes from CKO and WT mice were stimulated *ex vivo* with anti-CD3 and anti-CD28 for 72 hours and analyzed for IL-9 in the supernatant by means of ELISA. Data (means $\pm$ SDs) are representative of 2 experiments. * $P < .05$.

challenges with 10 μ g of OVA on days 14 and 21. For IL-9 and IL-4 neutralization, mice were injected intranasally with anti-IL-9 antibody (10 μ g per dose; R&D Systems), anti-IL-4 antibody (10 μ g per dose; Bio X Cell), or IgG control antibody on days 14 and 21 before challenge. For adoptive transfer experiment, CD4⁺ T cells from WT and CKO mice sensitized as described above were intravenously transferred into Rag-1^{-/-} mice (10 $\times$ 10⁶ cells per mouse). Twenty-four hours later, the recipient mice were challenged intranasally with 2 doses of OVA (10 μ g) at 7-day intervals. Experiments were performed 24 hours after the last intranasal challenge with OVA.

The trachea was cannulated, and the lungs underwent lavage with 1 mL of cold PBS to assay IL-4 and IL-5 by using a sandwich ELISA kit (R&D Systems). Cell numbers in bronchoalveolar lavage fluid were counted with a hemocytometer. Cells were isolated from the lungs, as described in the [Methods](#) section in this article's Online Repository. Lung tissue was embedded in paraffin and stained with hematoxylin and eosin for analysis of inflammatory infiltrate.

Patients

Human PBMCs were obtained from patients with allergic asthma who were seen at the Allergy Clinic at the Clinical Hospital of Ribeirão Preto Medical School, University of São Paulo, and from healthy donors. All subjects signed an informed consent form releasing use of their specimens in the study, which was approved by the Ethics Committee of Ribeirão Preto Medical School Hospital (2261/2011).

Lentivirus production

HEK 293T Peak cells²⁷ were transfected with 0.5 μ g of pLX-TRC317 plasmid (GeneWiz; empty or coding for *PRDM1* cDNA), 0.375 μ g of psPAX2 (catalog no. 12259; Addgene, Cambridge, Mass) and 0.125 μ g of pMD2.G

(catalog no. 12259; Addgene), as described in the [Methods](#) section in this article's Online Repository.

Statistical analysis

Statistical analysis was performed with an unpaired *t* test or ANOVA, followed by Bonferroni multiple comparison tests. The significance of these parameters was calculated by using a log-rank test (GraphPad 5.0 software; GraphPad Software, La Jolla, Calif). All values were considered significantly different at *P* values of less than .05.

RESULTS

Frequency of T_H9 cells increases in the periphery of Blimp-1-deficient mice

To investigate whether Blimp-1 could regulate T_H9 cell function and/or differentiation, we generated T cell-specific Blimp-1-deficient mice (*Prdm1*^{flox/flox}CD4^{cre}-CKO) and evaluated the frequency of IL-9-producing cells and IL-9 production.

First, we analyzed the total frequency of lymphocytes in the periphery using flow cytometry. We found that frequencies of CD4⁺ and CD8⁺ T cells in CKO mice were similar to those in WT mice (Fig 1, A). Moreover, we found a substantially increased frequency of effector/memory (CD44⁺CD62L⁻) T cells (34.13 $\pm$ 1.45 vs 14.26 $\pm$ 1.42), as well as a reduction in numbers of naive (CD62L⁺CD44⁻) T cells (51.60 $\pm$ 1.82 vs 73.23 $\pm$ 2.06), in CKO mice compared with WT mice (Fig 1, B). In addition, T-cell proliferation levels from splenocytes of both CKO and WT mice cultured with anti-CD3 plus

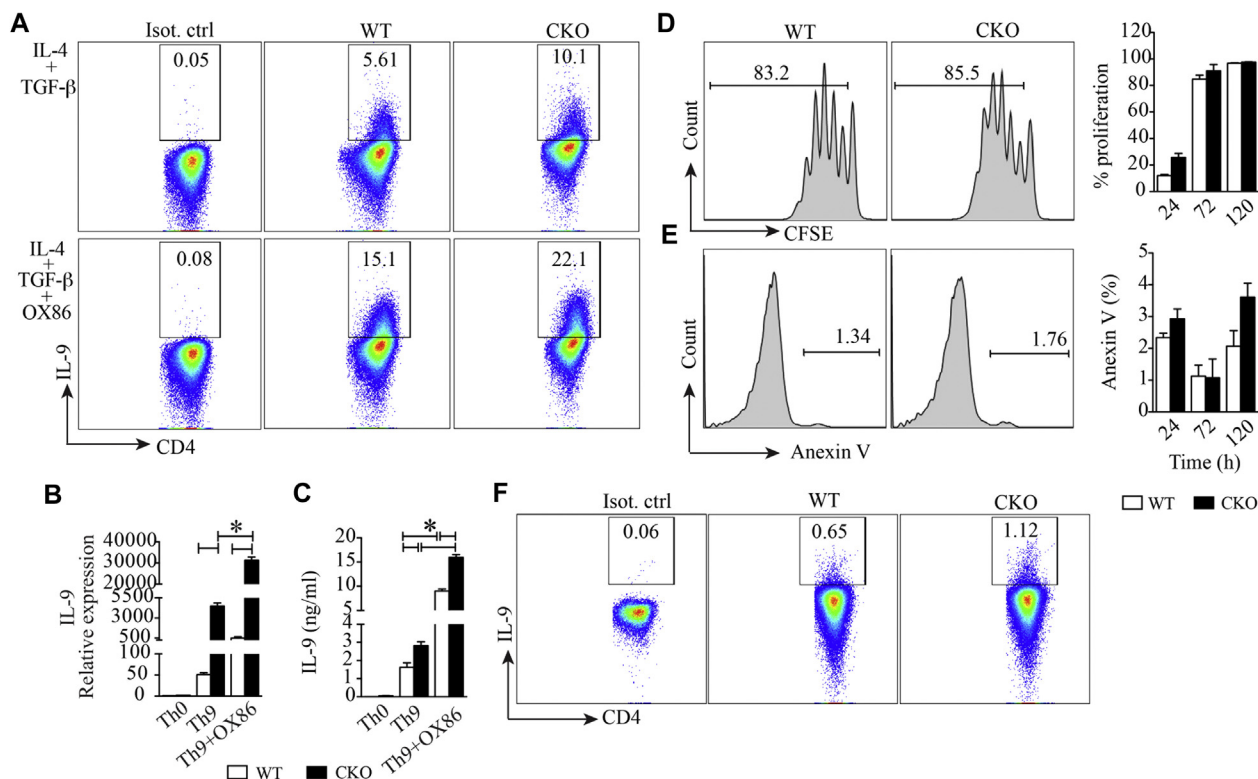


FIG 2. Blimp-1 deficiency enhances T_H9 cell differentiation. **A**, Naive CD4⁺ T cells from WT and CKO mice were differentiated into T_H9 cells with TGF-β and IL-4 plus anti-IFN-γ without (T_H9) or with (T_H9+OX86) OX86. On day 4, differentiated cells were stimulated with phorbol 12-myristate 13-acetate (PMA) plus ionomycin for 4 hours. The frequency of CD4⁺IL-9⁺ cells was determined by means of flow cytometry based on the control isotype. **B**, Expression of IL-9 mRNA in CD4⁺ T cells from WT mice and CKO mice cultured for 4 days under neutral (T_H0) or T_H9-polarizing conditions, as described above. The results are presented relative to those of the control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **C**, IL-9 levels in culture supernatants from T_H0- and T_H9-polarizing conditions were measured by means of ELISA. **D** and **E**, Proliferation (Fig 2, **D**) and survival (Fig 2, **E**) of WT and CKO CD4⁺ T cells activated with anti-CD3 and anti-CD28 in T_H9-polarizing conditions for 1, 3, and 5 days, as assessed by using carboxyfluorescein succinimidyl ester (CFSE) dilution and Annexin V staining. Numbers above bracketed lines indicate percentage cells that proliferated (top) or Annexin V-positive cells (bottom). **F**, Flow cytometric analysis of intracellular IL-9 in (CD4⁺CD44^{high}) effector/memory cells cultured with anti-CD3 and anti-CD28 for 18 hours and stimulated with PMA and ionomycin for 4 hours was determined based on the control isotype. Data (means ± SDs) are representative of 3 (Fig 2, **A-C**) or 2 (Fig 2, **D-F**) experiments. **P* < .05.

anti-CD28 were similar (Fig 1, **C**). On splenocyte stimulation, frequencies of CD4⁺IL-9⁺ cells were 3-fold greater in CKO mice (1.54 ± 0.18) than in WT mice (0.55 ± 0.04 ; Fig 1, **D**). Moreover, levels of IL-9 secreted in the culture supernatant were significantly greater in CKO cells (1123.40 ± 505.00) than in WT mouse cells (196.61 ± 120.80 ; Fig 1, **G**). Moreover, the percentage of CD4⁺IL-4⁺ cells was comparable in cells from WT and CKO mice (Fig 1, **E**). As expected from previously published work,²² we also found that the frequency of Treg (CD4⁺ forkhead box protein 3–positive) cells was greater in the spleens of CKO mice (18.65 ± 4.00) compared with that in WT mice (11.35 ± 4.00 ; Fig 1, **F**). Together, these data suggest that Blimp-1 deletion in T cells favors the T_H9 phenotype *in vivo*.

Blimp-1 is a negative regulator of T_H9 differentiation and IL-9 production

To further elucidate whether Blimp-1 is involved in T_H9 cell differentiation, we cultured naive CD4⁺ T cells from CKO and WT mice under T_H9 conditions for 4 days. The frequency of

IL-9–producing CD4⁺ T cells (Fig 2, **A**) generated from CKO mice was greater than that in WT mice (Fig 2, **A**) and was correlated with enhanced IL-9 mRNA expression (Fig 2, **B**) and levels of IL-9 secreted in the culture supernatants (Fig 2, **C**). The addition of an OX86 antibody, which has been shown previously to potentiate T_H9 differentiation,²⁸ led to increased expression of IL-9, mainly in the absence of Blimp-1 (Fig 2, **B** and **C**). However, Blimp-1 deletion in CD4⁺ T cells did not alter the proliferation (Fig 2, **D**) and survival (Fig 2, **E**) of T_H9 cells during activation with anti-CD3 and anti-CD28. Corroborating these data, sort-purified CD44^{high}CD25[–]CD4⁺ cells from pooled splenocytes of CKO mice exhibited 72% more IL-9⁺ cells compared with control mice (Fig 2, **F**). These data suggest that Blimp-1 represses the T_H9 differentiation program and could be required to control the differentiation of T_H9 cells in tissues.

Blimp-1 expression is not induced in T_H9 cells

We then investigated whether Blimp-1 expression is modulated during T_H9 differentiation. Sorted naive (CD25[–]CD44^{low}) CD4⁺

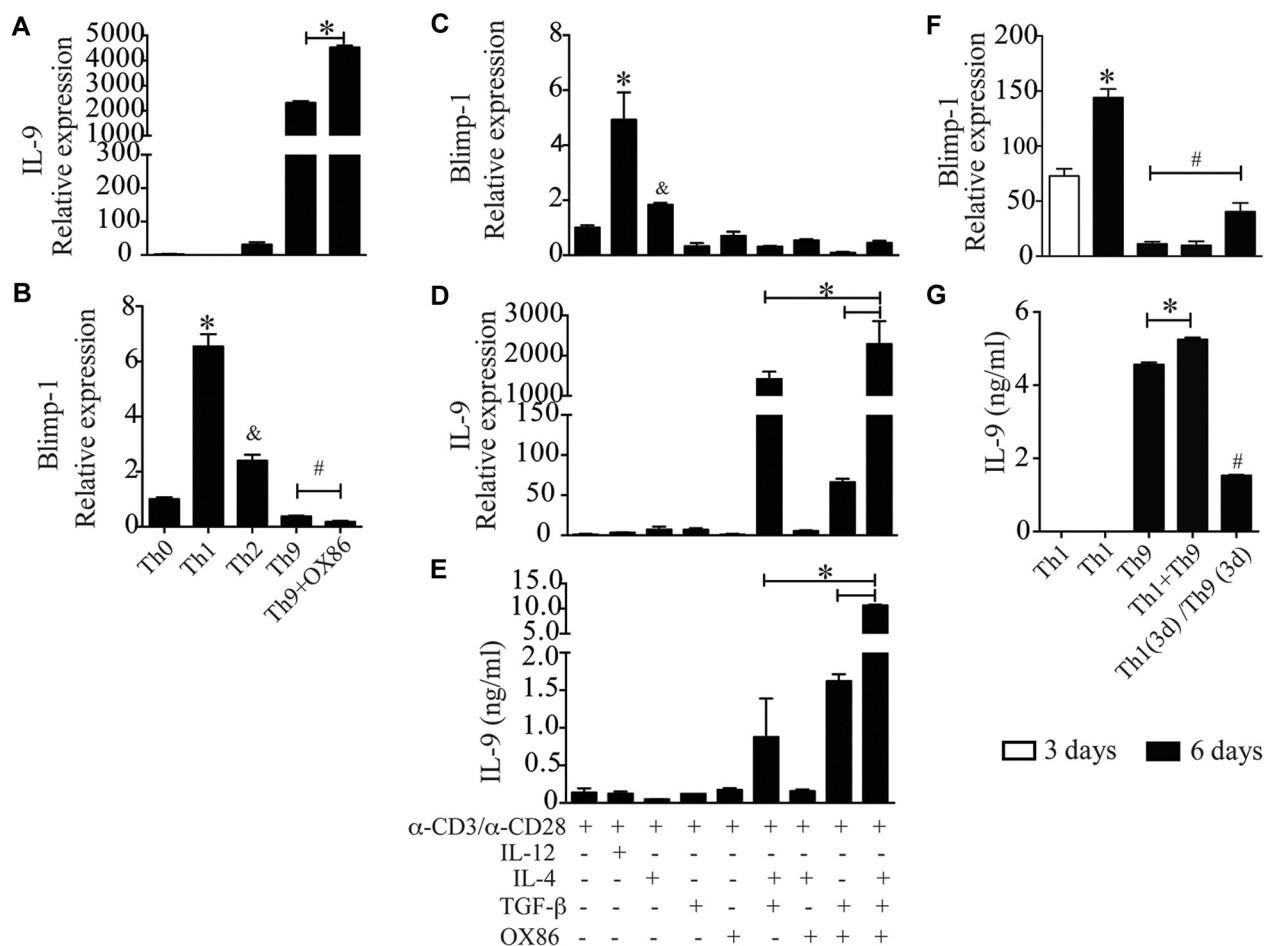


FIG 3. Blimp-1 expression is not induced during T_H9 differentiation. **A** and **B**, Expression of IL-9 and Blimp-1 mRNA in naive $CD4^+$ T cells from WT mice and stimulated with anti-CD3 and anti-CD28 under neutral (medium alone), T_H1 (IL-12 and IL-2 plus anti-IL-4), T_H2 (IL-4 and IL-2 plus IFN- γ), T_H9 (TGF- β and IL-4 plus anti-IFN- γ), and T_H9 plus OX86 (TGF- β , IL-4, OX86, and anti-IFN- γ) conditions. Expression levels of the indicated genes were analyzed by using qPCR. **C-E**, Naive $CD4^+$ T cells from WT mice were cultured in different conditions: stimulation of T-cell receptor alone or in the presence of IL-4, TGF- β , and OX86 or in combination, as shown in Fig 3, E. On day 4, RNA and culture supernatants were collected, expression levels of Blimp-1 (Fig 3, C) and IL-9 (Fig 3, D) mRNA were analyzed by using qPCR, and IL-9 levels (Fig 3, E) were measured by means of ELISA. **F** and **G**, $CD4^+$ T cells were cultured with IL-12 plus IL-2 (T_H1 conditions) for 3 days. In some wells IL-4 plus TGF- β plus OX86 (T_H9 conditions) was added along with IL-2 plus IL-12 (T_H1 plus T_H9 conditions) on the same day or after 3 days (T_H1 -3d/ T_H9) under T_H1 conditions. T_H1 -1 or T_H9 -differentiated cells were used as positive or negative controls. On day 6, RNA and culture supernatants were collected, expression of Blimp-1 mRNA was analyzed by using qPCR (Fig 3, F), and IL-9 levels were measured by means of ELISA (Fig 3, G). Data (means $\pm$ SDs) are representative of 3 experiments. * $P < .05$, & $P < .05$, and # $P < .05$.

T cells from WT mice were stimulated under neutral, T_H1 , T_H2 , and T_H9 conditions, and Blimp-1 mRNA expression was determined by using qPCR. As expected, T_H9 cells showed greater IL-9 mRNA expression than did T_H1 and T_H2 cells (Fig 3, A). As previously shown,^{19,29} stimulation of naive $CD4^+$ T cells with anti-CD3 plus anti-CD28 induced low Blimp-1 expression, whereas cells cultured under T_H1 and T_H2 conditions expressed greater levels of Blimp-1 than did those in neutral conditions. In contrast, cells stimulated under T_H9 conditions exhibited low Blimp-1 expression (Fig 3, B).

We next investigated the factors involved in regulation of Blimp-1 expression in T_H9 cells. WT naive $CD4^+$ T cells cultured with IL-12 or IL-4 showed high Blimp-1 mRNA expression (Fig

3, C). Addition of OX86 and TGF- β alone did not induce Blimp-1 expression, even in the presence of IL-4 (Fig 3, C). As expected, expression of IL-9 mRNA and production of IL-9 were very low in naive $CD4^+$ T cells cultured with IL-4, TGF- β , and OX86 alone (Fig 3, D and E). However, the combinations of IL-4 and TGF- β or OX86 and TGF- β induced significant IL-9 mRNA expression and IL-9 production but did not induce Blimp-1 expression. Moreover, the combination of IL-4, TGF- β , and OX86 potentiated IL-9 mRNA expression and IL-9 secretion into culture supernatants (Fig 3, D and E).

To determine whether Blimp-1 expression was induced or inhibited in T_H9 cells, we cultured $CD4^+$ T cells with IL-2 and IL-12 for 3 days to induce Blimp-1 expression (Fig 3, F) and

added IL-4, TGF- β , and OX86 (T_H9 condition) either at the same time (T_H1 plus T_H9 , IL-12 plus IL-2 plus IL-4 plus TGF- β plus OX86) or after 3 days under T_H1 conditions (T_H1 -3d/ T_H9). Under T_H9 conditions, Blimp-1 expression was significantly reduced after 3 days of T_H1 differentiation (T_H1 -3d/ T_H9 ; 72% inhibition) but was not induced when T_H1 and T_H9 conditions were imposed together (T_H1 plus T_H9 condition; Fig 3, F). Consistently, $CD4^+$ T cells cultured under T_H1 plus T_H9 conditions exhibited IL-9 production as great as that of T_H9 cells, but those cultured under T_H1 -3d/ T_H9 conditions did not (Fig 3, G).

Together, these results indicate that Blimp-1 is not induced during T_H9 differentiation and that inhibition of Blimp-1 by cytokines related to T_H9 differentiation is essential for high IL-9 production.

Absence of Blimp-1 in T cells potentiates allergic airway inflammation

To determine whether the increase in numbers of T_H9 cells caused by Blimp-1 deficiency affects the development of T_H9 -dependent inflammatory diseases, we investigated whether deletion of Blimp-1 in T cells aggravates allergic airway inflammation. We subjected WT and CKO mice to the standard protocol to develop OVA-induced allergic airway inflammation and then evaluated cell influx into the lungs. Compared with WT mice, CKO mice exhibited an intense influx of eosinophils (Siglec-F $^+$ GR-1 $^-$) into the airways (Fig 4, A). Flow cytometric analysis showed that the frequency and total number of leukocytes, more specifically eosinophils (Siglec-F $^+$ GR-1 $^-$), in lung tissues were significantly greater in CKO mice (2.47 ± 0.74) than in WT mice (0.85 ± 0.49 ; Fig 4, B-D). Histologic analysis confirmed that CKO mice had more intense lung parenchymal inflammation, which was characterized by diffuse cell infiltrates, than WT mice (Fig 4, E and F). Consistently, the number of $CD4^+$ IL-9 $^+$ T cells infiltrating the lungs was greater in CKO mice (10.62 ± 4.26) than in WT mice (3.25 ± 0.79 ; Fig 4, G). In addition, together with the enhanced number of eosinophils, numbers of lung $CD4^+$ T cells producing IL-4 and IL-5 were greater in allergic CKO mice (22.61 ± 10.68 and 19.00 ± 6.95 , respectively) than in allergic WT mice (6.08 ± 2.09 and 3.61 ± 1.73 , respectively; Fig 4, H and I). Immunization alone or challenge with OVA did not induce inflammatory infiltrates in the lungs of WT and CKO mice (see Fig E1, A-C, in this article's Online Repository at www.jacionline.org). However, only challenge with OVA induced an increase in IL-9-producing $CD4^+$ cells in the lungs of the CKO mice compared with WT mice (see Fig E1, E and F).

To determine whether Blimp-1 deficiency in $CD4^+$ T cells alone is sufficient to aggravate OVA-induced airway inflammation in a mouse model and whether Blimp-1 has an intrinsic role in controlling T_H9 -mediated inflammation *in vivo*, we sorted $CD4^+$ T cells from CKO and WT mice and adoptively transferred them to Rag-1 $^{-/-}$ mice. Then we evaluated the severity of allergic airway inflammation in the recipient mice after challenge with OVA. Histologic analysis showed increased cell infiltration in the lungs from CKO mice compared with WT mice (Fig 5, A and B). We also detected greater numbers of eosinophils and $CD4^+$ IL-9 $^+$ T cells infiltrating into the lungs of Rag-1 $^{-/-}$ mice that received $CD4^+$ cells from CKO mice than from WT mice (Fig 5, C and D).

Enhancement of $CD4^+$ IL-9 $^+$ T cells was accompanied by an increase in numbers of $CD4^+$ IL-5 $^+$ T cells (Fig 5, F) but not $CD4^+$ IL-4 $^+$ T cells (Fig 5, E).

Therefore Blimp-1 deletion in T cells is sufficient to increase allergic lung inflammation, which is mainly mediated by IL-9, although cytokines of the T_H2 profile, such as IL-5, are also involved in the pathogenesis of allergic airway inflammation. Thus the primary determinant of disease severity in Blimp-1-deficient mice is T-cell intrinsic.

Blimp-1 regulates T_H9 cell pathogenicity in allergic inflammation

We next investigated whether the severity of allergic inflammation in Blimp-1-deficient mice was mediated by IL-9. For this analysis, CKO and WT mice underwent induction of OVA-induced allergic airway inflammation and were treated with anti-IL-9 or anti-IL-4 antibody (10 μ g per mouse) intranasally on days 1 and 2 of the challenge. Twenty-four hours after the last challenge, compared with mice treated with control IgG, CKO mice treated with the anti-IL-9 antibody showed attenuated lung inflammation with decreased peribronchial and perivascular accumulation of leukocytes. In contrast, treatment with anti-IL-4 did not reduce airway inflammation in either experimental group (Fig 6, A). Flow cytometric analysis showed that treatment with the anti-IL-9 antibody reduced numbers of leukocytes and eosinophils in the lungs (Fig 6, B and C). Moreover, IL-9 blockade was associated with a significant reduction in IL-4 and IL-5 expression in pulmonary tissue (Fig 6, D and E) and in bronchoalveolar lavage fluid (Fig 6, F and G) in CKO mice compared with expression in control CKO mice. Taken together, a lack of Blimp-1 in T cells leads to the IL-9-mediated exacerbation of airway inflammation, indicating that Blimp-1 plays a nonredundant role in controlling T_H9 responses *in vivo*.

Blimp-1 controls IL-9 production in human T_H9 cells

Finally, we investigated whether Blimp-1 also acts as a repressor of T_H9 differentiation in human subjects. We isolated $CD4^+$ cells from PBMCs of healthy donors and asthmatic patients and stimulated them with anti-CD3 plus anti-CD28 and T_H9 -polarizing cytokines. Similar to the results obtained with murine cells, TGF- β plus IL-4 induced IL-9 mRNA expression in $CD4^+$ T cells from healthy donors and asthmatic patients (Fig 7, A). However, IL-9 mRNA expression was significantly greater in $CD4^+$ T cells from asthmatic patients than in those from healthy donors (Fig 7, A). Consistent with these findings, Blimp-1 mRNA expression was not induced in human $CD4^+$ T cells in response to TGF- β plus IL-4 (T_H9) compared with that in neutral conditions (T_H0 ; Fig 7, B). These results clearly indicate that in both human subjects and mice, T_H9 cells express low levels of Blimp-1.

To directly test whether Blimp-1 plays a negative role in human T_H9 cell differentiation, we isolated $CD4^+$ T cells from healthy donors and asthmatic patients, stimulated them under T_H9 -polarizing conditions, and transduced them with control (control-LV) or Blimp-1-expressing (Blimp-1-LV) lentivirus, as shown in Fig 7, C. T_H9 cells differentiated *in vitro*, and overexpressing Blimp-1 showed lower IL-9 mRNA expression and IL-9 production than did control-LV-transfected T_H9 cells (Fig 7, D and E). Thus, similar to the observations in murine cells, Blimp-1 repressed IL-9 expression in human $CD4^+$ T cells.

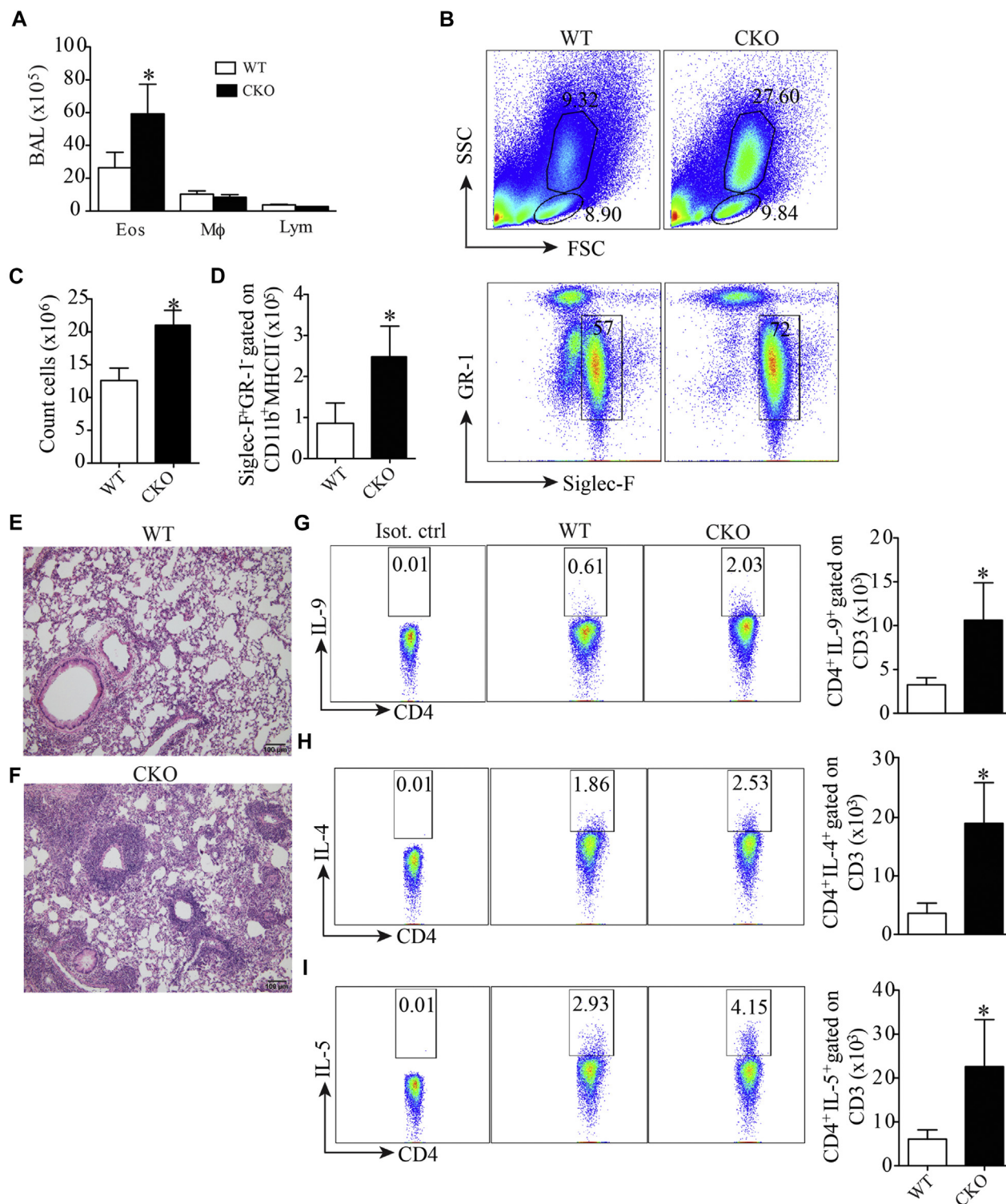


FIG 4. Blimp-1 deficiency in T cells promotes development of allergic inflammation. **A**, Differential counts of cytopsin preparations from bronchoalveolar lavage (BAL) cells of WT and CKO mice 24 hours after the last challenge with intranasal OVA, as outlined in the [Methods](#) section. *Eos*, Eosinophils; *Lym*, lymphocytes; *Mφ*, macrophages. **B-D**, Frequencies and absolute numbers of eosinophils (Siglec-F⁺GR-1⁺) gated on CD11b⁺MHC class II⁺ in lungs of WT and CKO mice were determined by using flow cytometry. *FSC*, Forward scatter; *SSC*, side scatter. **E** and **F**, Representative lung sections of WT (**E**) and CKO (**F**) mice were stained with hematoxylin and eosin for analysis of inflammatory infiltrates. Original magnification ×200. Cells isolated from lungs were stimulated with phorbol 12-myristate 13-acetate and ionomycin for 4 hours for intracellular staining by using flow cytometry. **G-I**, Frequencies and absolute numbers of CD4⁺ cells expressing IL-9 (**G**), IL-4 (**H**), and IL-5 (**I**) gated on CD3 were determined based on the control isotype. Data (means ± SDs) are representative of 3 experiments, with 5 mice per group. **P* < .05.

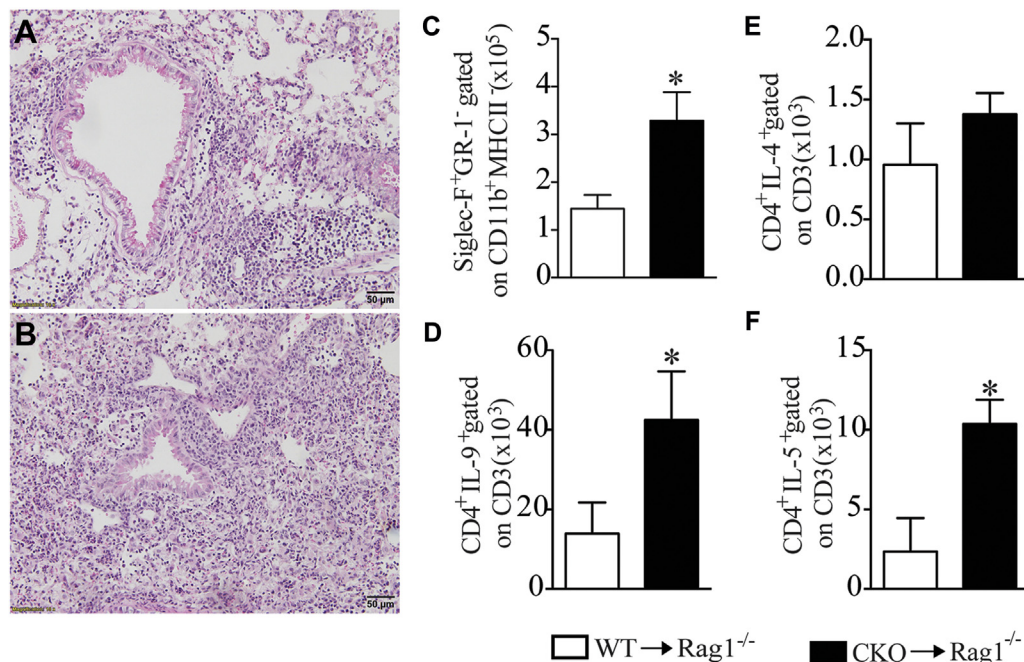


FIG 5. T cell-intrinsic Blimp-1 deficiency in airway inflammation promotes T_H9 cell accumulation. CD4⁺ T cells from WT and CKO mice were adoptively transferred into Rag-1-deficient recipients. Recipients were challenged intranasally with OVA, as described in the [Methods](#) section, before analysis of pulmonary inflammation. **A** and **B**, Representative lung sections of Rag-1^{-/-} recipient mice that received WT (Fig 5, **A**) and CKO (Fig 5, **B**) CD4⁺ cells were stained with hematoxylin and eosin for analysis of inflammatory infiltrates. Original magnification ×200. **C**, Absolute numbers of eosinophils (Siglec-F⁺GR-1⁺) gated on CD11b⁺MHC class II⁺ in the lungs of WT and CKO mice were determined by using flow cytometry. **D-F**, Cells isolated from the lungs were stimulated with phorbol 12-myristate 13-acetate and ionomycin for 4 hours for intracellular staining by flow cytometry. Absolute numbers of IL-9-expressing (Fig 5, **D**), IL-4-expressing (Fig 5, **E**), and IL-5-expressing (Fig 5, **F**) CD4⁺ cells gated on CD3 were determined. Data (means ± SDs) are representative of the 4 mice per group. *P < .05.

DISCUSSION

IL-9-producing T_H cells (ie, T_H9 cells) are a recently described CD4⁺ T-cell subset that plays important roles in airway inflammation. The transcription factors STAT6, STAT5, basic leucine zipper ATF-like transcription factor, PU.1, and IRF-4 have been implicated as positive regulators of T_H9 differentiation.³⁰ However, little is known about the negative regulators of T_H9 programming. Our studies described here support the idea that the transcription factor Blimp-1 functions as a negative regulator of T_H9 differentiation *in vivo* and that its expression in T cells is required to suppress asthma pathogenesis.

Our results showed that Blimp-1 deficiency in T cells promotes increased T_H9 differentiation and accumulation in the periphery. The increase in T_H9 differentiation from Blimp-1-deficient CD4⁺ T cells is unlikely to be secondary to Blimp-1's previously described role in preventing T-cell proliferation and survival^{14,15} because the proliferation and survival of Blimp-1-deficient and sufficient T_H9 cells were comparable. The deletion of Blimp-1 in T cells *in vivo* favored the differentiation/accumulation of T_H9 cells but not T_H2 cells. Moreover, the forced expression of Blimp-1 in human T_H9 cells was sufficient to repress IL-9 expression, further supporting the idea that Blimp-1 is a repressor of the T_H9 differentiation program.

Blimp-1 expression is consistently higher in antigen-experienced T_H1¹⁹ and T_H2³¹ cells than in those in neutral conditions. However, in T_H9 cells, Blimp-1 mRNA expression

remained low as cells differentiated. Impaired expression of Blimp-1 in T_H9 cells is probably due to the presence of TGF-β because TGF-β was previously shown to be a potent repressor of Blimp-1 expression in T_H17 cells,²⁰ and we further observed here that in the presence of TGF-β, Blimp-1 expression was consistently low, even in the presence of an OX86 and IL-4. Furthermore, previous induction of Blimp-1 in CD4⁺ cells, even under T_H9-polarizing conditions, inhibits its expression and IL-9 production. Thus the ideal conditions for high IL-9 expression do not favor Blimp-1 expression.

Mechanisms underlying repression of IL-9 production/T_H9 differentiation by Blimp-1 require further elucidation. Our observation that Blimp-1-deficient CD4⁺ T cells had increased IL-9 mRNA expression suggested that Blimp-1 could regulate IL-9 production at the transcriptional level. This idea is further supported by our observation that forced expression of Blimp-1 in human CD4⁺ T cells resulted in repression of IL-9 mRNA levels.

Our results also support the idea that repression of IL-9 expression by Blimp-1 in T cells is required to prevent severe airway inflammation. Although T_H9 cells have been reported to be important in allergic inflammation, autoimmune diseases, and tumor immunity,³⁰ detection of T_H9 cells is difficult because generation of these cells is transient.

The allergic airway inflammation model is typical and characterized by lung eosinophilia that is dependent on excess activation of T cells, mucus production, and airway

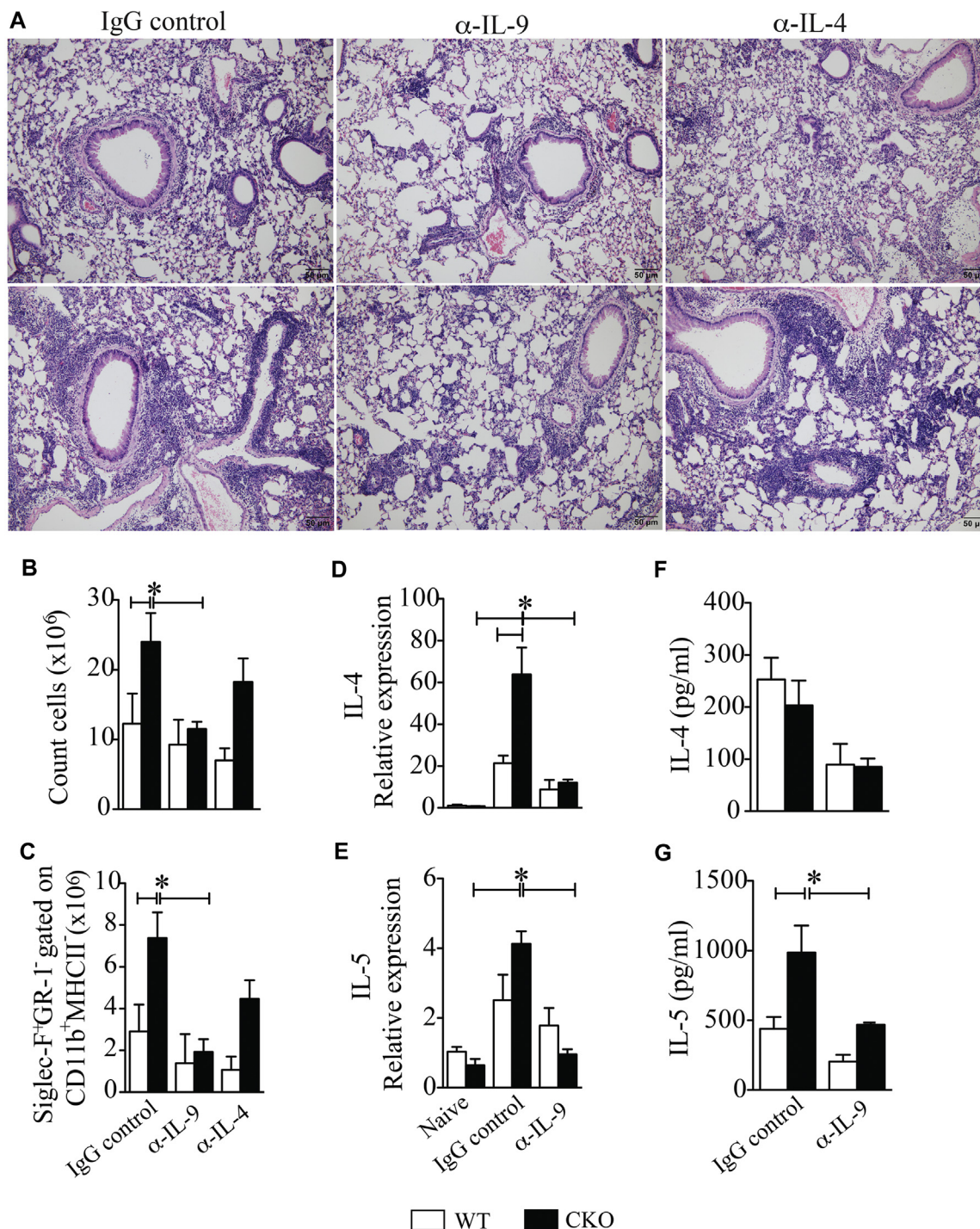


FIG 6. Blimp-1 deletion in CD4⁺ T cells favors the proallergic T_H9 phenotype. WT and CKO mice were treated with IgG control and neutralizing anti-IL-9 or anti-IL-4 antibodies and challenged with OVA, as outlined in the [Methods](#) section. **A**, Representative lung sections of WT (*top panels*) and CKO (*bottom panels*) mice were stained with hematoxylin and eosin for analysis of inflammatory infiltrates. Original magnification $\times 200$. **B** and **C**, Absolute numbers of total leukocytes (Fig 6, **B**) and eosinophils (Fig 6, **C**; Siglec-F⁺GR-1⁺) gated on CD11b⁺MHC class II⁺ and isolated from lung was determined by using flow cytometry. **D** and **E**, Expression of IL-4 (Fig 6, **D**) and IL-5 (Fig 6, **E**) mRNA of lung tissues of WT and CKO mice was performed by using qPCR. **F** and **G**, Concentrations of IL-4 (Fig 6, **F**) and IL-5 (Fig 6, **G**) in bronchoalveolar lavage fluid from WT and CKO mice were determined by using ELISA. Data are representative of 4 mice per group. * $P < .05$.

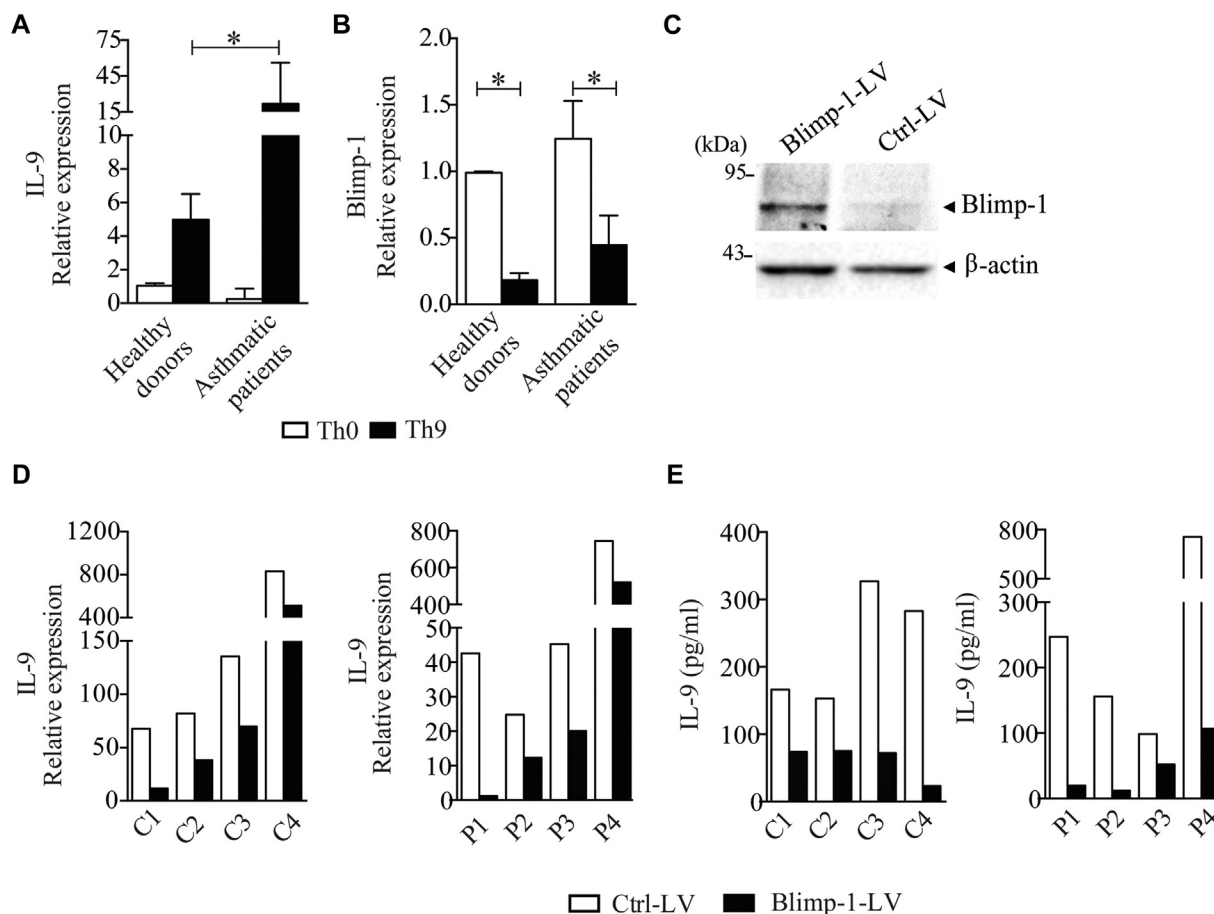


FIG 7. Blimp-1 inhibits differentiation of human TH9 cells. **A**, CD4⁺ T cells isolated from PBMCs of healthy donors and allergic asthmatic patients were differentiated under TH0- and TH9-polarizing conditions. **A** and **B**, After 4 days of culture, RNA was isolated, and IL-9 (Fig 7, **A**) and Blimp-1 (Fig 7, **B**) mRNA levels were determined by using qPCR. Data (means $\pm$ SDs) are representative of 6 patients with allergic asthma and the healthy donor. **C**, Immunoblot analysis of Blimp-1 and β -actin in TH9 cells transduced with control lentivirus (Ctrl-LV) or Blimp-1-expressing lentivirus (Blimp-1-LV); cells were from healthy donors. **D** and **E**, IL-9 mRNA expression (Fig 7, **D**) and IL-9 levels (Fig 7, **E**) in the culture supernatant of TH9 cells transduced with Ctrl-LV or Blimp-1-LV; cells were from healthy donors (C1-C4) or asthmatic patients (P1-P4), and IL-9 mRNA expression and levels were determined by using qPCR and ELISA, respectively. Data are representative of 4 asthmatic patients and 4 healthy donors.

hyperreactivity.³² However, we demonstrated that a lack of Blimp-1 in T cells promotes severe airway inflammation, including intense lung parenchymal inflammation, enhanced eosinophil recruitment, and TH2 and TH9 cell responses. Increases in the number of TH2 and TH9 cells in the lungs can be explained by the need for cooperation of both TH subtypes for development of airway inflammation. Division of labor between these TH subsets in airway inflammation is still unclear. Our observations suggest the importance of TH9 cells in lung tissues for induction of TH2 responses and severity of airway inflammatory disease.

The role of IL-9 in allergic asthma is currently recognized. IL-9 expression is increased in the lungs of asthmatic patients,^{33,34} and transgenic expression of IL-9 results in allergic inflammation.³⁵ Our results showed that blockade of IL-9 inhibited development of the inflammatory profile of TH2 cells and, consequently, eosinophilia. However, blocking IL-4 did not affect the response of TH9 cells and pulmonary inflammation. Interestingly, IL-9

induced during allergic inflammation is the initial trigger for development of an efficient TH2 response. Previous experiments have suggested that IL-9 promotes type 2 cytokine production by innate lymphoid cells in the lungs,³⁶ and IL-13 deficiency inhibits the allergic inflammation initiated by an IL-9 transgene.³⁷

In addition, allergic airway inflammation is characterized by eosinophilia that is dependent on IL-9 production. IL-9 blockade in allergic CKO mice reduces eosinophilia but also impairs IL-5 production. Thus it remains to be determined whether IL-9 has a direct effect on eosinophil counts. Previous reports showed that IL-9 increased the expression of IL-5 receptor on eosinophils and inhibited eosinophil apoptosis, enhancing eosinophil development and promoting eosinophil maturation in synergy with IL-5.^{38,39} Expression of IL-9 in transgenic mice under the control of a lung-specific promoter resulted in severe airway inflammation with eosinophils and lymphocytes, as well as mast cell hyperplasia.⁴⁰ Thus it is difficult to develop an ordered model of TH9 and TH2 cell functions, and each subset likely contributes to

development of allergic inflammation. Our observations further confirm the importance of T_H9 cells in lung tissue for induction of T_H2 responses and the severity of airway inflammatory disease.

Atopic diseases, including atopic dermatitis and asthma, are most commonly associated with T_H2 cytokine responses.³⁰ However, adoptive transfer of T_H9 cells leads to an increase in eosinophil recruitment after OVA challenge and favors allergic airway disease.⁴¹ Recent reports have identified T_H9 cells as major contributors to atopic disease in human subjects.⁴² The adoptive transfer model in Rag-1 knockout mice, which received Blimp-1-deficient CD4⁺ cells, aggravated the pathogenesis of allergic airway inflammation, including increases in numbers of eosinophils and T_H9 cells, suggesting that IL-9 produced by T_H9 cells is essential for the development of disease independent of IL-4, although T_H2 cells are also found during the development of airway inflammation.

In addition, our data showed that T_H9 cells from patients with allergic asthma that were differentiated *in vitro* induce greater IL-9 expression than do those from healthy donors. In addition, similar to the observations in murine cells, Blimp-1 expression was very low in *in vitro*-differentiated human T_H9 cells. Additionally, overexpression of Blimp-1 in CD4⁺ T cells from healthy donors and asthmatic patients inhibited IL-9 production during T_H9 differentiation, suggesting that Blimp-1 could play a role in regulating asthma-associated pathogenesis in human subjects similar to the observations in our murine experimental model.

Overall, the results we describe here uncover a new role for Blimp-1 in repressing T_H9 differentiation and thus controlling allergic airway inflammation. Collectively, these findings might have important implications for the development of new therapeutic approaches to control allergic airway inflammation.

We thank Franciele Pioto, Denise Ferraz, Cristiane Milanezi, Wander Cosme, Wendy Martin Rios, and Luana Sella Motta Maia for technical assistance.

Key messages

- Blimp-1 regulates T_H9 differentiation and IL-9 production.
- Blimp-1-deficient mice show severe IL-9-mediated allergic airway inflammation.
- Blimp-1 overexpression in human T_H9 cells represses IL-9 expression.

REFERENCES

- Schmitt E, Germann T, Goedert S, Hoehn P, Huels C, Koelsch S, et al. IL-9 production of naive CD4⁺ T cells depends on IL-2, is synergistically enhanced by a combination of TGF-beta and IL-4, and is inhibited by IFN-gamma. *J Immunol* 1994;153:3989-96.
- Veldhoen M, Uttenhove C, van Snick J, Helmsby H, Westendorf A, Buer J, et al. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol* 2008;9:1341-6.
- Dardalhon V, Awasthi A, Kwon H, Galileos G, Gao W, Sobel RA, et al. IL-4 inhibits TGF-beta-induced Foxp3(+) T cells and, together with TGF-beta, generates IL-9(+) IL-10(+) Foxp3(-) effector T cells. *Nat Immunol* 2008;9:1347-55.
- Nicolaides NC, Holroyd KJ, Ewart SL, Eleff SM, Kiser MB, Dragwa CR, et al. Interleukin 9: a candidate gene for asthma. *Proc Natl Acad Sci U S A* 1997;94:13175-80.
- Cheng G, Arima M, Honda K, Hirata H, Eda F, Yoshida N, et al. Anti-interleukin-9 antibody treatment inhibits airway inflammation and hyperactivity in mouse asthma model. *Am J Respir Crit Care Med* 2002;166:409-16.
- Jager A, Dardalhon V, Sobel RA, Bettelli E, Kuchroo VK. Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. *J Immunol* 2009;183:7169-77.
- Singh TP, Schon MP, Wallbrecht K, Gruber-Wackernagel A, Wang XJ, Wolf P. Involvement of IL-9 in Th17-associated inflammation and angiogenesis of psoriasis. *PLoS One* 2013;8:e51752.
- Purwar R, Schlappbach C, Xiao S, Kang HS, Elyaman W, Jiang X, et al. Robust tumor immunity to melanoma mediated by interleukin-9-producing T cells. *Nat Med* 2012;18:1248-53.
- Lu Y, Hong S, Li H, Park J, Hong B, Wang L, et al. Th9 cells promote antitumor immune responses *in vivo*. *J Clin Invest* 2012;122:4160-71.
- Chang HC, Sehra S, Goswami R, Yao WG, Yu Q, Stritesky GL, et al. The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nat Immunol* 2010;11:527-34.
- Staudt V, Bothur E, Klein M, Lingnau K, Reuter S, Grebe N, et al. Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity* 2010;33:192-202.
- Goswami R, Jabeen R, Yagi R, Pham D, Zhu JF, Goenka S, et al. STAT6-dependent regulation of Th9 development. *J Immunol* 2012;188:968-75.
- Jabeen R, Goswami R, Awe O, Kulkarni A, Nguyen ET, Attenasio A, et al. Th9 cell development requires a BATF-regulated transcriptional network. *J Clin Invest* 2013;123:4641-53.
- Martins G, Calame K. Regulation and functions of Blimp-1 in T and B lymphocytes. *Annu Rev Immunol* 2008;26:133-69.
- Kallies A, Hawkins ED, Belz GT, Metcalf D, Hommel M, Corcoran LM, et al. Transcriptional repressor Blimp-1 is essential for T cell homeostasis and self-tolerance. *Nat Immunol* 2006;7:466-74.
- Kallies A, Nutt SL. Terminal differentiation of lymphocytes depends on Blimp-1. *Curr Opin Immunol* 2007;19:156-62.
- Kallies A, Xin A, Belz GT, Nutt SL. Blimp-1 transcription factor is required for the differentiation of effector CD8(+) T cells and memory responses. *Immunity* 2009;31:283-95.
- Rutishauser RL, Martins GA, Kalachikov S, Chande A, Parish IA, Meffre E, et al. Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. *Immunity* 2009;31:296-308.
- Cimmino L, Martins GA, Liao J, Magnusdottir E, Grunig G, Perez RK, et al. Blimp-1 attenuates Th1 differentiation by repression of ifng, tbx21, and bcl6 gene expression. *J Immunol* 2008;181:2338-47.
- Salehi S, Bankoti R, Benevides L, Willen J, Couse M, Silva JS, et al. B lymphocyte-induced maturation protein-1 contributes to intestinal mucosa homeostasis by limiting the number of IL-17-producing CD4⁺ T cells. *J Immunol* 2012;189:5682-93.
- Lin MH, Chou FC, Yeh LT, Fu SH, Chiou HY, Lin KI, et al. B lymphocyte-induced maturation protein 1 (BLIMP-1) attenuates autoimmune diabetes in NOD mice by suppressing Th1 and Th17 cells. *Diabetologia* 2013;56:136-46.
- Cretney E, Xin A, Shi W, Minnich M, Masson F, Miasari M, et al. The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. *Nat Immunol* 2011;12:304-11.
- Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 2009;325:1006-10.
- Martins GA, Cimmino L, Shapiro-Shelef M, Szabolcs M, Herron A, Magnusdottir E, et al. Transcriptional repressor Blimp-1 regulates T cell homeostasis and function. *Nat Immunol* 2006;7:457-65.
- Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol* 2010;28:445-89.
- Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 2006;441:235-8.
- Guiraldelli MF, Berenstein EH, Grodzki AC, Siraganian RP, Jamur MC, Oliver C. The low affinity IgG receptor Fc gamma RIIB contributes to the binding of the mast cell specific antibody, mAb BGD6. *Mol Immunol* 2008;45:2411-8.
- Xiao X, Balasubramanian S, Liu W, Chu X, Wang H, Taparowsky EJ, et al. OX40 signaling favors the induction of T(H)9 cells and airway inflammation. *Nat Immunol* 2012;13:981-90.
- Gong D, Malek TR. Cytokine-dependent Blimp-1 expression in activated T cells inhibits IL-2 production. *J Immunol* 2007;178:242-52.
- Kaplan MH, Hufford MM, Olson MR. The development and *in vivo* function of T helper 9 cells. *Nat Rev Immunol* 2015;15:295-307.

31. Wang L, van Panhuys N, Hu-Li J, Kim S, Le Gros G, Min B. Blimp-1 induced by IL-4 plays a critical role in suppressing IL-2 production in activated CD4 T cells. *J Immunol* 2008;181:5249-56.
32. Gonzalo JA, Lloyd CM, Kremer L, Finger E, Martinez AC, Siegelman MH, et al. Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. *J Clin Invest* 1996;98:2332-45.
33. Shimbara A, Christodoulouopoulos P, Soussi-Gounni A, Olivenstein R, Nakamura Y, Levitt RC, et al. IL-9 and its receptor in allergic and nonallergic lung disease: increased expression in asthma. *J Allergy Clin Immunol* 2000;105:108-15.
34. Erpenbeck VJ, Hohlfeld JM, Volkmann B, Hagenberg A, Geldmacher H, Braun A, et al. Segmental allergen challenge in patients with atopic asthma leads to increased IL-9 expression in bronchoalveolar lavage fluid lymphocytes. *J Allergy Clin Immunol* 2003;111:1319-27.
35. Temann UA, Ray P, Flavell RA. Pulmonary overexpression of IL-9 induces Th2 cytokine expression, leading to immune pathology. *J Clin Invest* 2002;109:29-39.
36. Wilhelm C, Hirota K, Stieglitz B, Van Snick J, Tolaini M, Lahl K, et al. An IL-9 fate reporter demonstrates the induction of an innate IL-9 response in lung inflammation. *Nat Immunol* 2011;12:1071-7.
37. Temann UA, Laouar Y, Eynon EE, Homer R, Flavell RA. IL9 leads to airway inflammation by inducing IL13 expression in airway epithelial cells. *Int Immunol* 2007;19:1-10.
38. Gounni AS, Gregory B, Nutku E, Aris F, Latifa K, Minshall E, et al. Interleukin-9 enhances interleukin-5 receptor expression, differentiation, and survival of human eosinophils. *Blood* 2000;96:2163-71.
39. Louahed J, Zhou Y, Maloy WL, Rani PU, Weiss C, Tomer Y, et al. Interleukin 9 promotes influx and local maturation of eosinophils. *Blood* 2001;97:1035-42.
40. Temann UA, Geba GP, Rankin JA, Flavell RA. Expression of interleukin 9 in the lungs of transgenic mice causes airway inflammation, mast cell hyperplasia, and bronchial hyperresponsiveness. *J Exp Med* 1998;188:1307-20.
41. Niedbala W, Besnard AG, Nascimento DC, Donate PB, Sonogo F, Yip E, et al. Nitric oxide enhances Th9 cell differentiation and airway inflammation. *Nat Commun* 2014;5:4575.
42. Brough HA, Cousins DJ, Munteanu A, Wong YF, Sudra A, Makinson K, et al. IL-9 is a key component of memory TH cell peanut-specific responses from children with peanut allergy. *J Allergy Clin Immunol* 2014;134:1329-38.e10.

METHODS

Flow cytometric assay

For T-cell and eosinophil analyses, antibodies against the following mouse proteins were obtained from BioLegend: CD3 (145-2C11), CD4 (RM4-5), IL-9 (RM 9A4), IL-4 (11B11), IL-5 (JES1-39D10), CD11b (M1/70), MHC class II (2G9), GR-1 (RB6-8C5), and Siglec-F (E50-2440) or their respective isotype controls.

qPCR

RNA was extracted with the RNeasy Micro Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. cDNA was synthesized through reverse transcription (Kit High Capacity; Applied Biosystems, Foster City, Calif). Real-time PCR for quantitative mRNA expression analyses was performed on a StepOne Plus Real-Time PCR System (Applied Biosystems) with a goTaq qPCR Master Mix fluorescence quantification system (Promega, Madison, Wis), and the primers are listed in Table E1. Standard PCR conditions were as follows: 50°C for 2 minutes, 95°C for 2 minutes, and 40 cycles of 15 seconds at 95°C, 30 seconds at 58°C, and 30 seconds at 72°C, followed by a standard denaturation curve. Samples were normalized to glyceraldehyde-3-phosphate dehydrogenase, β -actin, or β_2 -microglobulin. Analyses were performed with the Ct method, which allows for quantitative expression analysis using the formula $2^{-\Delta\Delta Ct}$.

Lung cell isolation

Lungs were collected, minced, placed in RPMI-1640 containing 2 mg/mL collagenase IV and 1 mg/mL DNase I (Sigma-Aldrich), and incubated at 37°C for 30 minutes. For single-cell suspensions, the remaining tissue was forced through a 70-mm cell strainer. Cells were pelleted, and erythrocytes were lysed with 1 mL of ACK lysis buffer. The remaining cells were washed with PBS, and viable cells were counted by using trypan blue exclusion. Lung cells were placed in 48-well plates and stimulated for 4 hours with phorbol 12-myristate 13-acetate (50 ng/mL) plus ionomycin (500 ng/mL; Sigma) and brefeldin A (BioLegend) for analysis of intracellular cytokines by using flow cytometry.

Patients and *in vitro* analysis of human T cells

Human PBMCs from allergic asthmatic patients and healthy donors were isolated, and CD4⁺ T cells were purified with a CD4⁺ T Cell Isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4⁺ T cells were cultured for 4 days with plate-bound anti-CD3 (2 μ g/mL) and soluble anti-human CD28 (1 μ g/mL; both from BD Biosciences) alone or in combination with TGF- β (2 ng/mL), IL-4 (10 ng/mL), and anti-IFN- γ (10 μ g/mL). Expression levels of IL-9 and Blimp-1 mRNA were measured by using qPCR.

Lentivirus production

HEK 293T peak cells^{E1} were placed in each well of a 6-well plate and transfected with 0.5 μ g of pLX_TRC317 plasmid (GeneWiz; empty or coding for

PRDM1 cDNA), 0.375 μ g of psPAX2 (catalog no. 12259; Addgene), and 0.125 μ g of pMD2.G (catalog no. 12259; Addgene). After 15 hours, culture medium was removed, and 1 mL of RPMI medium supplemented with 30% FBS (without antibiotics) was added to each well. Fresh viruses were collected from the supernatants 5 days later, combined, and subsequently cleared by means of centrifugation for 10 minutes at 2000g and 4°C and used to infect T cells. For T-cell infection, PMBCs provided by healthy donors and asthmatic patients were purified with a CD4⁺ T-cell isolation kit. Then CD4⁺ T cells were stimulated with anti-CD3 (2 μ g/mL) and anti-CD28 (1 μ g/mL) antibodies for 18 hours. Next, activated T cells (1×10^6) were infected with 1 mL of viral supernatant (7 ng/mL previously quantified by using a RETRO-TEK HIV-1 p24 Antigen ELISA kit, according to the manufacturer's recommendations) in the presence of 8 μ g/mL polybrene (Sigma) and incubated at 37°C for 24 hours. After this period, the viral supernatant was removed, and the cells were cultured under T_H9 cell conditions for 4 days. Levels of IL-9 mRNA and IL-9 were measured by using qPCR and ELISA, respectively.

SDS-PAGE and immunoblot analysis

Cells were lysed for 20 minutes on ice with lysis buffer (50 mmol/L Tris-HCl [pH 7.5], 150 mmol/L NaCl, 10% [vol/vol] glycerol, 5 mmol/L EDTA, and 1% [vol/vol] Triton X-100) supplemented with protease inhibitor (catalog no. P8340; Sigma-Aldrich) and centrifuged for 20 minutes at 16,000g and 4°C. Supernatants were collected, mixed with sample buffer (4% SDS, 160 mmol/L Tris-HCl [pH 6.8], 20% [vol/vol] glycerol, 100 mmol/L dithiothreitol, and 0.1% bromophenol blue), and boiled for 5 minutes. Equal amounts of protein extracts were resolved by using SDS-PAGE and transferred onto a nitrocellulose membrane (GE Healthcare, Fairfield, Conn). The membrane was incubated in blocking solution (PBS-Tween supplemented with 5% nonfat dry milk and 1% BSA) for 1 hour and then incubated with anti-PRDM1/Blimp-1 (dilution 1:1000; Abcam, Cambridge, United Kingdom) or anti- β -actin (Santa Cruz Biotechnology, Dallas, Tex) in PBS-Tween and 1% BSA overnight at 4°C. The membrane was then washed 5 times with PBS-Tween and incubated for 1 hour with horseradish peroxidase-conjugated donkey anti-mouse IgG secondary antibody (dilution 1:10,000; GE Healthcare) in blocking solution. After 5 washes with PBS-Tween, proteins were detected by using enhanced chemiluminescence solutions (solution 1: 1 mol/L Tris-HCl [pH 8.5], 250 mmol/L luminol, and 90 mmol/L p-coumaric acid; solution 2: 30% H₂O₂ and 1 mol/L Tris-HCl [pH 8.5]) and visualized with a Bio-Rad ChemiDoc MP Imaging System (Bio-Rad Laboratories, Hercules, Calif).

REFERENCE

- E1. Guiraldelli MF, Berenstein EH, Grodzki AC, Siraganian RP, Jamur MC, Oliver C. The low affinity IgG receptor Fc gamma RIIB contributes to the binding of the mast cell specific antibody, mAb BGD6. *Mol Immunol* 2008;45:2411-8.

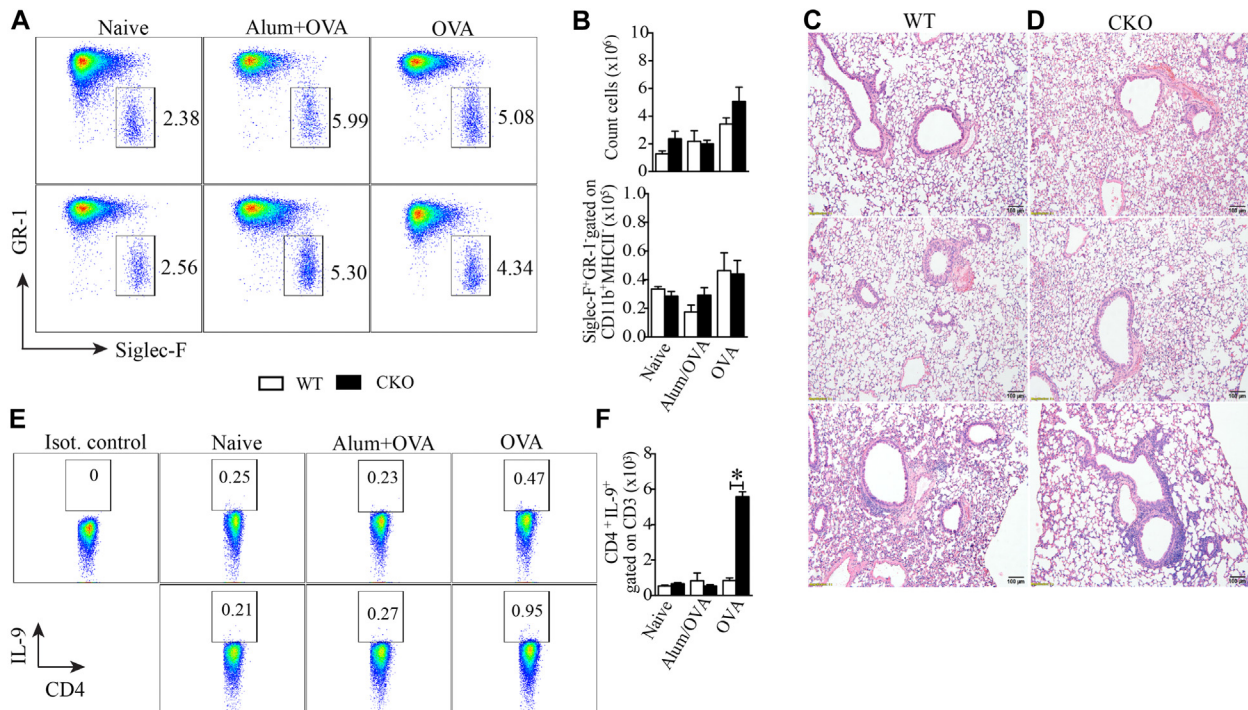


FIG E1. Sensitization and challenge with OVA alone. WT and CKO mice were subcutaneously sensitized with OVA plus alum alone or intranasally challenged with OVA alone and 24 hours after the last challenge. **A** and **B**, Frequencies and absolute numbers of eosinophils (Siglec-F⁺GR-1⁺) gated on CD11b⁺MHC class II⁺ in the lungs were determined by using flow cytometry. **C** and **D**, Representative lung sections of WT (Fig E1, C) and CKO (Fig E1, D) mice were stained with hematoxylin and eosin for analysis of inflammatory infiltrates. *Top panels* show lung cells of naive mice. *Middle panels* show lung cells of mice sensitized with OVA plus alum. *Bottom panels* show lung cells from mice challenged with OVA alone. Original magnification $\times 200$. Cells were isolated from lungs and stimulated with phorbol 12-myristate 13-acetate and ionomycin for 4 hours for intracellular staining by flow cytometry. **E-G**, Frequencies and absolute numbers of CD4⁺ cells expressing IL-9 gated on CD3. Data (means $\pm$ SDs) are representative of 2 experiments, with 3 mice per group. * $P < .05$.

TABLE E1. Primer sequences used in the study

Gene	Forward (5'-3')	Reverse (5'-3')
Mu IL-9	CGTCCCAACGTCACAGGACATTG	TTCTGTGTGGCATTGGTTCA
Mu Blimp-1	GACGGGGGTACTTCTGTTCA	GGCATTCTTGGGAAGTGTGT
Mu IL-4	AAGAGCATCATGCAAATGGA	TTAAAGCATGGTGGCTCAGTAC
Mu IL-5	GAGGTTACAGCAATGCACCATT	TCAGTTGGTAACATGCACAAAG
Mu β -actin	CCCTCGCTGCACAAATACTC	ACGACCCGATGGCCATAG
Mu GAPDH	TGCCAAGTATGATGACATCAAGAAG	TGTTGAAGTCGCAGGAGACAA
Hu IL-9	GTCTCTCACTGAAGCATGGTC	CCTGGACATCAACTTCCTCATC
Hu Blimp-1	CAGAGTTCATTTTCTCAGTGCTC	GAAAGGCTTCACTACCCTTATCC
Hu β_2 -microglobulin	ACCTCCATGATGCTGCTTAC	GGACTGGTCTTTCTATCTCTTGT

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; *Hu*, sequence of human primer; *Mu*, sequence of murine primer.