

The activating protein 1 transcription factor basic leucine zipper transcription factor, ATF-like (BATF), regulates lymphocyte- and mast cell-driven immune responses in the setting of allergic asthma

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Background: Mice without the basic leucine zipper transcription factor, ATF-like (BATF) gene (*Batf*^{−/−}) lack T_H17 and follicular helper T cells, which demonstrates that *Batf* is a transcription factor important for T- and B-cell differentiation. **Objective:** In this study we examined whether BATF expression would influence allergic asthma.

Methods: In a cohort of preschool control children and children with asthma, we analyzed *BATF* mRNA expression using real-time PCR in PBMCs. In a murine model of allergic asthma, we analyzed differences in this allergic disease between wild-type, *Batf* transgenic, and *Batf*^{−/−} mice.

Results: In the absence of corticosteroid treatment, children with recurrent asthma have a significant increase in *BATF* mRNA expression in their PBMCs. *Batf*^{−/−} mice display a significant reduction in the pathophysiologic responses seen in asthmatic wild-type littermates. Moreover, we discovered a decrease in IL-3 production and IL-3-dependent mast cell development in *Batf*^{−/−} mice. By contrast, IFN-γ was induced in lung CD4⁺ and CD8⁺ T cells. Intranasal delivery of anti-IFN-γ antibodies induced airway hyperresponsiveness and inflammation in wild-type but not in *Batf*^{−/−} mice. Transgenic overexpression of *Batf* under the control of the CD2 promoter/enhancer augmented lung inflammation and IgE levels in the setting of experimental asthma.

Conclusion: *BATF* is increased in non-steroid-treated asthmatic children. Targeting *BATF* expression resulted in amelioration of the pathophysiologic responses seen in children with allergic asthma, and *BATF* has emerged as a novel target for antiasthma interventions. (J Allergy Clin Immunol 2013;■■■■:■■■■-■■■■.)

Key words: *BATF*, asthma, T_H2, T_H17, mast cells, T cells

Allergic asthma is often associated with activated T_H2- and T_H17-type cytokines in the airways.^{1,2} Here we investigated the role of basic leucine zipper transcription factor, ATF-like (BATF), an activating protein 1 transcription factor important for T_H2, T_H17, and B-cell differentiation in patients with allergic asthma.^{3–7} To this aim, we analyzed *BATF* mRNA expression in 2 cohorts of children with and without asthma and studied transgenic *Batf* and *Batf*^{−/−} mice in experimental models of asthma.

METHODS

Human studies: Detection of *BATF* mRNA in PBMCs

We analyzed 2 cohorts of children with and without asthma at the age of 4 to 6 years. The clinical outcomes of the recruited children in this study are reported in Table E1 in this article's Online Repository at www.jacionline.org. This study was approved by the Ethics Committee of the Friedrich-Alexander University Erlangen-Nürnberg, Germany (Re-No 4435). Informed consent was obtained from the parents of all participants of this study.

Mice

Batf^{−/−} and wild-type mice on a 129 SvEv or BALB/c genetic background and of mixed sex and CD2-*Batf* transgenic mice on a BALB/c genetic background were used in this study at the age of 6 to 8 weeks. Additionally, DO11.10 transgenic T-cell receptor transgenic and DO11.10-CD2-*Batf* double-transgenic mice were used. The mice were maintained under specific pathogen-free conditions, and all experiments were undertaken with approved licenses (23-177-07/G09-1-008 and 23177-07/051-1V2 from ethical review board Rheinland-Pfalz and 54-2532.1-2/10 from the government of Mittelfranken, Bavaria).

Intranasal αIFN-γ antibody treatment in ovalbumin-sensitized mice

Mice were treated intranasally with 100 μg of αIFN-γ antibodies (XMG 1.2 hybridoma cell line) per mouse or a polyclonal IgG control antibody (Sigma Aldrich, Steinheim, Germany, and Dianova, Hamburg, Germany) at the same concentration. The treatment was performed 30 minutes before ovalbumin (OVA) challenge at days 18 and 20, respectively.

Airway hyperresponsiveness

To measure airway reactivity, we used invasive whole-body plethysmography 24 hours after the last OVA treatment, as previously described in a

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Abbreviations used

AHR:	Airway hyperresponsiveness
ATF:	Activating transcription factor
BAL:	Bronchoalveolar lavage
BALF:	Bronchoalveolar lavage fluid
BATF:	Basic leucine zipper transcription factor, ATF-like
BMMC:	Bone marrow-derived mast cell
Foxp3:	Forkhead box protein 3
IRF4:	Interferon regulatory factor 4
MCh:	Methacholine
OVA:	Ovalbumin
P _{enh} :	Enhanced pause
pSTAT5:	Phospho-signal transducer and activator of transcription 5
RORγt:	Retinoic acid-related orphan receptor γt
SCF:	Stem cell factor
STAT:	Signal transducer and activator of transcription
T-bet:	T-box transcription factor
Treg:	Regulatory T

Buxco Electronics system.⁸ Data are expressed as mean values of “airway resistance [RL]/baseline RL” ± SEMs. Additionally, a FlexiVent FX1 device (SCIREQ Scientific Respiratory Equipment, Montreal, Quebec, Canada) was used for measuring invasive whole-body plethysmography (see the [Methods](#) section in this article’s Online Repository at www.jacionline.org).

Collection and analysis of bronchoalveolar lavage fluid

Twenty-four hours after the last OVA challenge, bronchoalveolar lavage (BAL) of the right lung was performed, as previously described, and processed as further described in the [Methods](#) section in this article’s Online Repository.⁹

Histology

Lungs were removed, fixed in 10% formalin-PBS solution, dehydrated, and embedded in paraffin (see the [Methods](#) section in this article’s Online Repository).

Isolation and culture of lung cells

Lungs were removed from *Batf*^{−/−} or wild-type mice, and total cells were isolated, as previously described.¹⁰ CD4⁺ T cells were positively sorted by using magnetic bead isolation (MACS; Miltenyi Biotec, Bergisch-Gladbach, Germany), according to the manufacturer’s protocol. The cells were plated at a density of 1 million per milliliter in RPMI 1640 medium and supplemented with 10% FCS, 1% L-glutamine, and 1% penicillin and streptomycin, respectively, for 24 hours at 37°C and 5% CO₂. For cell stimulation, plate-bound anti-CD3 (2 μg/mL) and anti-CD28 (2 μg/mL) antibodies were used. OVA rechallenge was performed with 500 μg OVA/mL medium.¹¹

Statistical analysis

Differences were evaluated for significance by using the Student 2-tailed *t* test for independent events (Excel, version 2003; Microsoft, Redmond, Wash) or by using 1-way ANOVA, as indicated in the figure legends. Data are shown as means ± SEMs.

RESULTS

Increased *BATF* mRNA in PBMCs isolated from asthmatic children not treated with glucocorticoids

At the time of recruitment, we found a statistically significant increase in *BATF* mRNA expression in PBMCs isolated from asthmatic children who were not treated with glucocorticoids and came later to the Children’s Hospital for a symptomatic visit,

compared with glucocorticoid-treated asthmatic children without a symptomatic visit and control children ([Fig 1, A](#)).

Batf^{−/−} mice are protected against experimental allergic asthma

In the presence of immunologic sensitization and challenge with OVA ([Fig E1, A](#)), *Batf*^{−/−} mice showed significantly suppressed responses to methacholine (MCh) compared with wild-type mice, as measured by using both the noninvasive enhanced pause (P_{enh}) response method (see [Fig E1, B](#), in this article’s Online Repository at www.jacionline.org) and 2 invasive plethysmographic systems ([Fig 1, B and C](#), invasive Buxco and SCIREQ, respectively). These findings suggest that BATF is an important regulator of airway hyperresponsiveness (AHR) in the setting of experimental asthma.

In *Batf*^{−/−} mice numbers of total BALF cells ([Fig 1, D](#)), airway neutrophils ([Fig 1, E](#)), and eosinophils ([Fig 1, F](#)), but not monocytes ([Fig 1, G](#)), were found to be markedly downregulated compared with those seen in wild-type mice after allergen sensitization and challenge. Total cell lung analysis did not show a decrease in CD11c⁺ cell compartments (see [Fig E1, C and D](#)). Total CD3⁺ T-cell numbers were found to be increased in *Batf*^{−/−} mice (see [Fig E1, E](#)). These data are consistent with no changes in GM-CSF and TNF-α levels in the bronchoalveolar lavage fluid (BALF) of *Batf*^{−/−} mice (see [Fig E1, F and G](#)). Airway inflammation was also found to be significantly downregulated in the absence of BATF in this model of allergic asthma ([Fig 1, H](#)). In addition, we found decreased airway mucus production, a pathoclinical parameter associated with IL-17A production,¹² in the absence of BATF compared with that seen in wild-type control animals ([Fig 1, I](#)).

CD2-*Batf* transgenic mice have increased lung inflammation and serum IgE levels in the setting of allergic asthma

To analyze the effect of BATF on antigen-specific T-cell activation in a model of allergic asthma, we exposed DO11.10 T-cell receptor transgenic mice and DO11.10-CD2-*Batf* double-transgenic mice, which overexpress BATF in CD4⁺, CD8⁺, and natural killer cells, to OVA intranasally. We found that OVA-specific activation of T cells that overexpress BATF results in a significant increase in lung inflammation compared with that seen in DO11.10 transgenic mice, which did not overexpress BATF ([Fig 2, A](#)). BATF also induced IL-10 levels in the lungs of transgenic mice ([Fig 2, B](#)) and reduced IFN-γ levels ([Fig 2, C](#)).

The DO11.10 model of asthma without OVA systemic priming is characterized by increased AHR without an increase in total serum IgE levels.¹³ Consistent with a role for BATF in B cells, we found increased IgE production in the sera of DO11.10-CD2-*Batf* transgenic mice in the absence of systemic sensitization ([Fig 2, D](#)). The effect of BATF on IgE levels was even more evident in the classical model of allergic asthma (OVA/OVA) using the CD2-*Batf* transgenic mice on a BALB/c genetic background ([Fig 2, E](#)). In conclusion, BATF induces inflammation and IgE through an antigen-specific effect on T and B cells, respectively.

Batf^{−/−} mice have a defect in OVA-specific IgE levels, IL-3 secretion, and mast cell development

We found a profound decrease in IgE and IgG_{2a} levels in the sera of *Batf*^{−/−} mice compared with their wild-type littermates

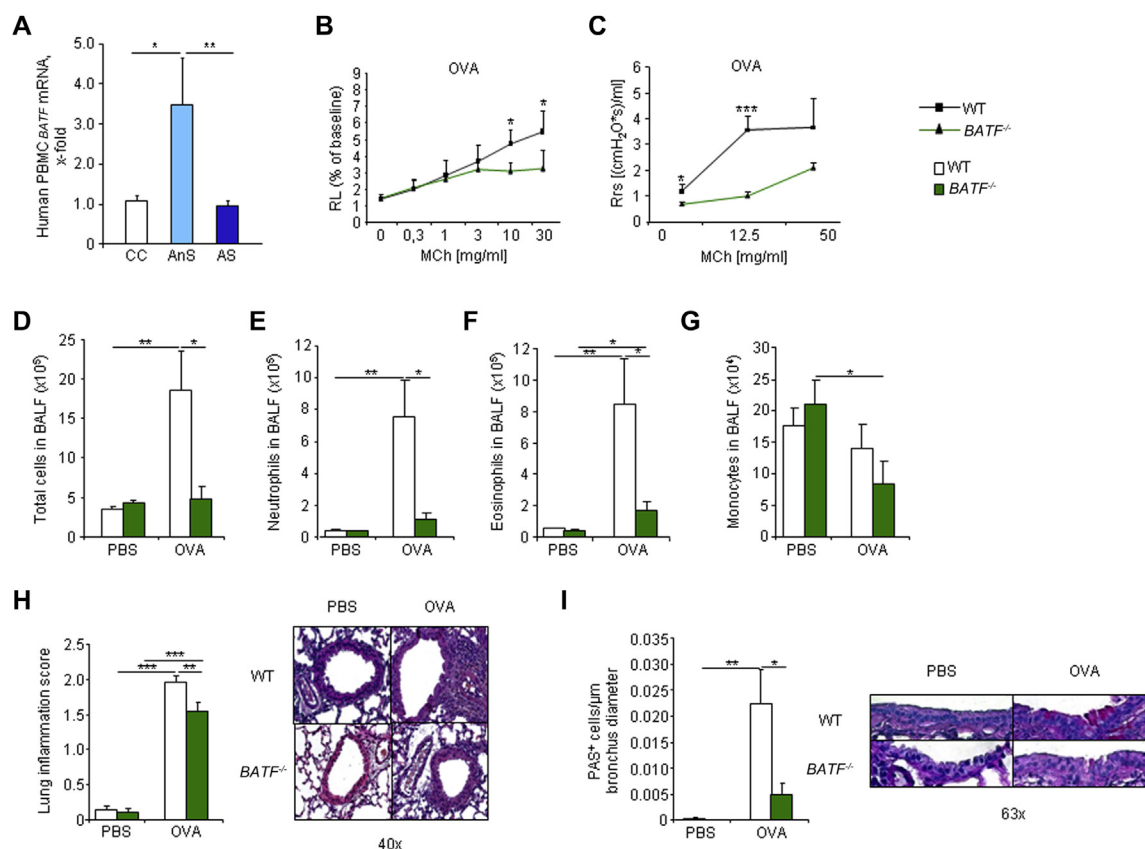


FIG 1. Increased *BATF* mRNA expression in the setting of allergic asthma. **A**, *BATF* mRNA expression in PBMCs of children with asthma not treated with steroids (*AnS*), children with asthma treated with steroids during reactivation (*AS*), and control children (*CC*; *CC*, *n* = 9; *AnS*, *n* = 4; and *AS*, *n* = 6). **B** and **C**, AHR (Buxco [Fig 1, *B*] and SCIREQ [Fig 1, *C*], respectively; *n* = 3-7 per group). *RL*, Airway resistance; *Rrs*, respiratory system resistance. **D-G**, BAL differential cell counts (*n* = 3-9 per group). **H** and **I**, Hematoxylin and eosin and periodic acid-Schiff (*PAS*) staining of lung sections (*n* = 3-9 per group). **P* < .05, ***P* < .01, and ****P* < .001, ANOVA (Fig 1, *B* and *C*) or Student 2-tailed *t* test (Fig 1, *A* and *D-I*). *WT*, Wild-type.

(Fig 2, *F* and *G*), which is consistent with the lack of immunoglobulin class-switching described in these mice.⁵ Next, we looked at mast cells.¹⁴ Mucosal mast cells develop from progenitor cells in the bone marrow under the influence of IL-3. By contrast, stem cell factor (SCF), the c-kit ligand produced by structural cells, supports connective-type mast cells in the lung but not bone marrow-derived mast cells (BMMCs).¹⁵ Here we observed that asthmatic *Batf*^{-/-} mice have a significant reduction in the number of lung c-kit⁺FcεRIα⁺CD123⁺ mast cells compared with wild-type mice (Fig 2, *H*). However, histamine levels in the BALF of these mice are comparable (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org). When we analyzed the secretion of IL-3 in cell-culture supernatants of total lung cells and lung CD4⁺ T cells from OVA-sensitized and OVA-challenged mice, we detected a strong decrease in IL-3 production in the absence of *BATF* (Fig 2, *I* and *J*). These results suggested defective mast cell development because of the lack of IL-3 production in *BATF* deficiency. We next confirmed a defect in BMMCs in *Batf*^{-/-} mice (Fig 2, *K*, left panel). These mast cells were well granulated (Fig 2, *K*, right panel) and contained similar amounts of histamine as those isolated from wild-type littermates (Fig 2, *L*). We then performed deprivation of growth factors for 72 hours in these mast cells and found that they were IL-3 dependent, because they underwent apoptosis after IL-3 deprivation (Fig 2, *M*, and see Fig E2, *B*). In

summary, we demonstrate the functional pathogenetic importance of *BATF* for IL-3-dependent mast cell development and IgE responses in a murine model of allergic asthma.

Batf^{-/-} asthmatic mice have a decrease in T_H17- and T_H2-related cytokine levels in the airways

Next, we addressed the presence of T_H17 cells in the airways in the setting of experimental asthma because *BATF* plays an important role in inducing IL-17A production.⁶ We could not detect IL-17A levels in supernatants from lung CD4⁺ T cells of *Batf*^{-/-} mice, regardless of PBS or OVA treatment. In contrast, there was an increase of IL-17A production in wild-type mice after allergen challenge (Fig 3, *A*). Because *Batf*^{-/-} T cells have been described not to induce factors required for T_H17 differentiation, such as the cytokine IL-21 and the transcription factor retinoic acid-related orphan receptor γt (RORγt; encoded by *Rorc*),⁶ we next analyzed the expression of these factors in experimental asthma. It was found that levels of both IL-21 and *Rorc*γt were significantly induced in wild-type mice treated with OVA compared with those seen in untreated control mice. In contrast, no induction was noted in *Batf*^{-/-} mice (Fig 3, *B* and *C*). It has been described recently that the T_H17 lineage is composed of at least 2 types of cells, one of which secretes IL-17A and another with more anti-inflammatory properties producing IL-17A in conjunction with

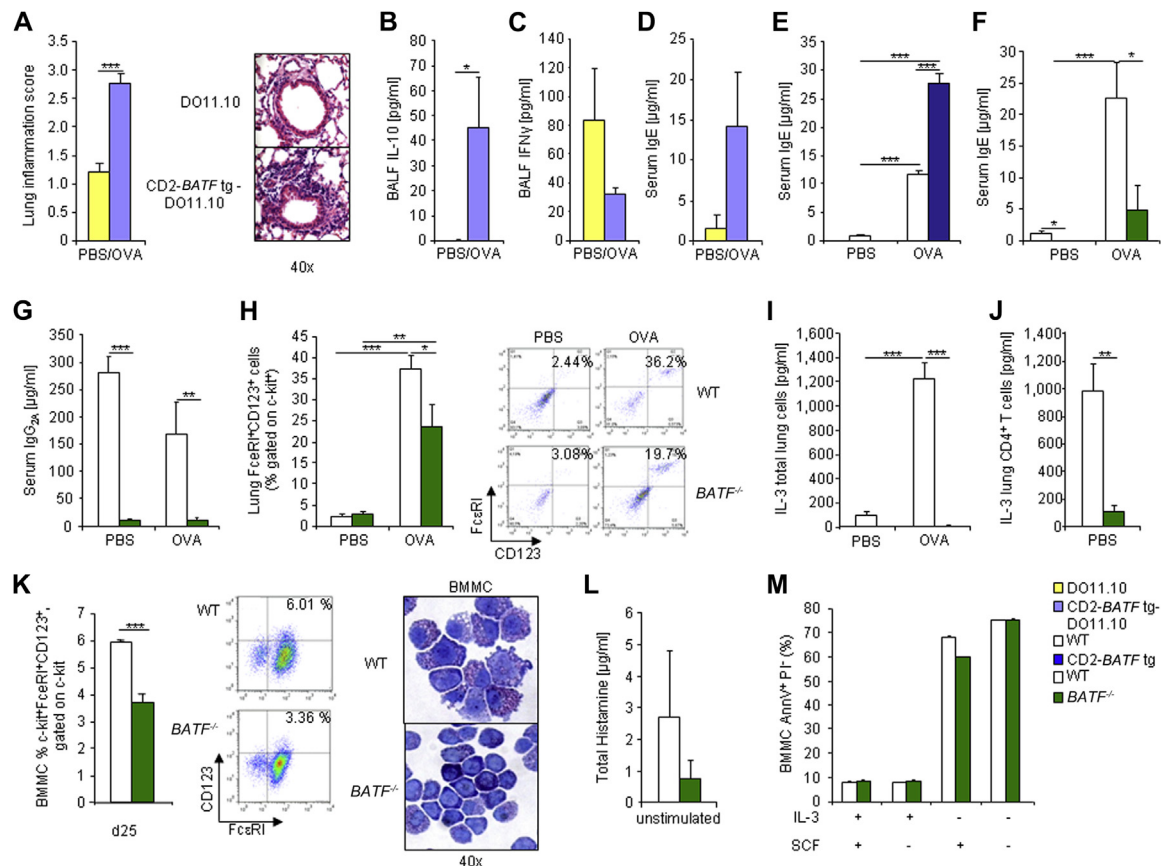


FIG 2. *Batf*^{-/-} mice have a defect in OVA-specific IgE and IL-3 levels and mast cell development. **A-E**, Lung inflammation and BALF cytokine or serum immunoglobulin levels ($n = 3$ per group). **F** and **G**, Serum IgE and IgG_{2a} levels ($n = 3-6$ per group). **H**, Lung mast cells ($n = 4$ per group). **I** and **J**, ELISA for IL-3 ($n = 3-4$ per group). **K**, Flow cytometry (left) and cytospin (right) of d25 BMMCs ($n = 3$ per group). **L**, BMMC histamine ($n = 5$ per group). **M**, BMMC apoptosis (Annexin V⁺ propidium iodide-negative cells). * $P < .05$, ** $P < .01$, and *** $P < .001$, Student 2-tailed t test. WT, Wild-type.

the immunosuppressive cytokine IL-10.¹⁶ Here we found a profound defect in IL-10 levels in mice with experimental asthma in the absence of BATF (Fig 3, D). Taken together, these findings suggest that BATF is a strong inducer of IL-10 production in T_H17 cells. These data are in agreement with a recent finding on the IL-10 promoter, in which BATF and c-Jun N-terminal kinase interact with interferon regulatory factor 4 (IRF4) under T_H17-skewing conditions.^{17,18}

IRF4 is also a strong inducer of T_H9 cells, and T_H9 cells have been demonstrated to play a pathogenetic role in asthma.¹⁹ These cells are driven by TGF- β and IL-4 levels. We then examined whether the defect in IL-10 would also be associated with reduced IL-9 levels because T_H9 cells produce both IL-10 and IL-9.^{19,20} IL-9 was found to be significantly downregulated in the BALF of *Batf*^{-/-} mice compared with that seen in wild-type mice in the murine OVA model of asthma (Fig 3, E). Thus BATF synergizes with IRF4 to induce IL-10 and IL-9 after allergen challenge in the setting of experimental asthma. This suggests that IRF4 without BATF cannot fully support IL-10 and IL-9 production in the lungs in mice with allergic asthma.

Consistent with a pathogenetic role of T_H2 cytokines in asthmatic patients, we found that lung CD4⁺ T cells isolated from *Batf*^{-/-} mice secreted lower levels of IL-4 and statistically significantly decreased amounts of IL-5 and IL-13 after

allergen challenge, whereas wild-type cells produced large amounts of these cytokines (Fig 3, F). Consistently, *Gata3* mRNA expression was significantly reduced in the absence of BATF in isolated lung CD4⁺ T cells compared with that seen in wild-type control specimens (Fig 3, G). Similar results were obtained for IL-4, IL-5, IL-13, and IL-10 (see Fig E3, A-D, respectively, in this article's Online Repository at www.jacionline.org) when total lung cells from untreated or OVA-sensitized and OVA-challenged mice were restimulated in cell culture with OVA.

Decreased IL-2 levels and numbers of CD4⁺CD25⁺ effector and CD4⁺CD25⁺ forkhead box protein 3-positive regulatory T cells in lungs of *Batf*^{-/-} mice

IL-2 has been shown to be a key factor for proliferation and expansion of effector but also regulatory T (Treg) cells.²¹ Signal transducer and activator of transcription 5 (STAT5) is activated through phosphorylation downstream of IL-2.²² We then analyzed phospho-STAT5 (pSTAT5) in the lungs of wild-type and *Batf*^{-/-} mice (Fig 3, H) by means of Western blot analysis and detected a decrease in pSTAT5 levels in the lungs of *Batf*^{-/-} asthmatic mice compared with those seen in their wild-type littermates. IL-2 levels were found to be reduced in the supernatants of

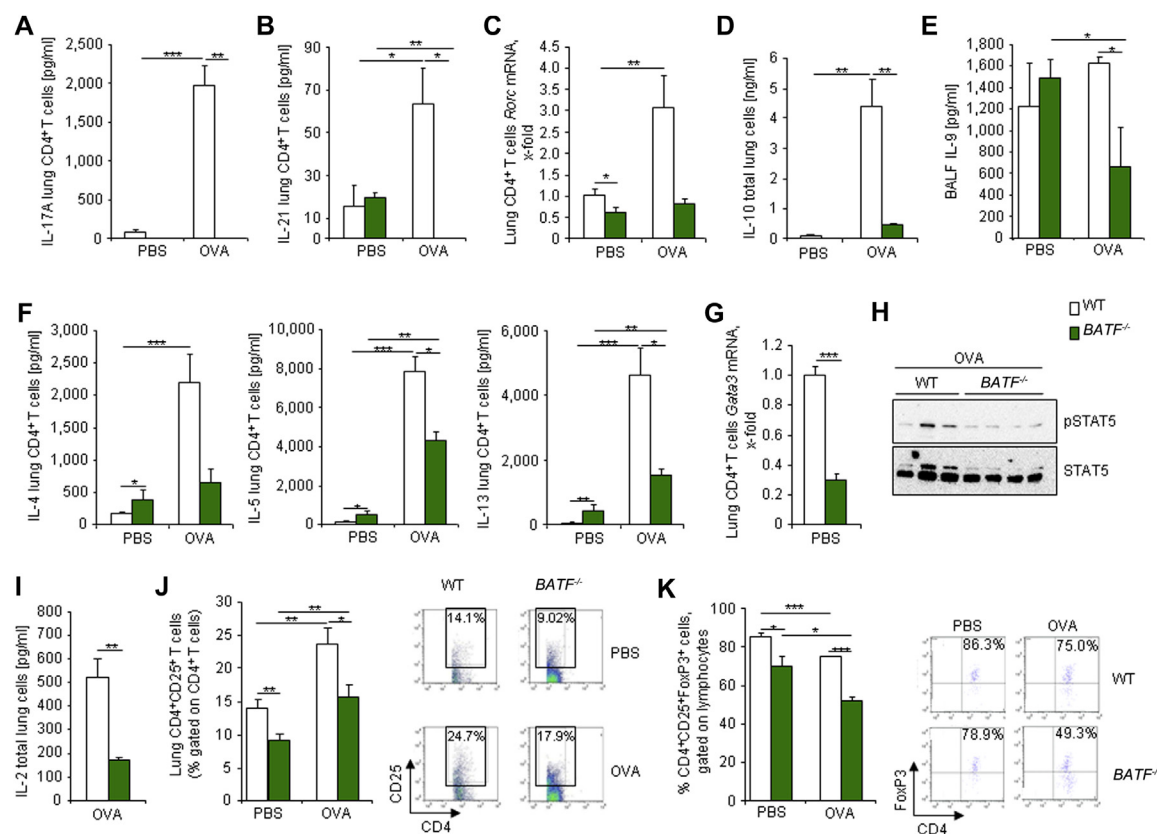


FIG 3. BATF controls T_H17 and T_H2 cells in the setting of asthma. **A** and **B**, Supernatants were analyzed for IL-17A (Fig 3, **A**) and IL-21 (Fig 3, **B**) production. **C**, *Rorc* mRNA expression. **D**, Lung IL-10 levels ($n = 3-6$ per group). **E**, BALF IL-9 levels ($n = 5$). **F**, Lung CD4⁺ cytokine secretion. **G**, *Gata3* mRNA expression ($n = 3-6$ per group). **H**, Lung tissue pSTAT5 and STAT5 levels in asthmatic mice ($n = 3-4$ per group). **I**, Lung IL-2 levels ($n = 3-4$ per group). **J**, Lung activated T cells ($n = 8-10$ per group). **K**, Lung Treg cells ($n = 4$ per group). * $P < .05$, ** $P < .01$, and *** $P < .001$, Student 2-tailed t test. WT, Wild-type.

total lung cells from OVA-treated *Batf*^{-/-} mice (Fig 3, **I**), which is consistent with a reduction in CD4⁺CD25⁺ T effector and CD4⁺CD25⁺ forkhead box protein 3 (Foxp3)⁺ Treg cell numbers in the lungs of these mice (Fig 3, **J** and **K**, and see Fig E3, **E** and **F**) compared with wild-type asthmatic mice. This finding is consistent with a role of pSTAT5 in effector and Treg cells.²³

Taken together, these data suggest that BATF deficiency impairs the functional plasticity of activated lung type 2 and type 17 CD4⁺ T helper cells in a murine model of asthma.

Increased IFN- γ production in CD3⁺, CD4⁺, and CD8⁺ T cells in the airways of *Batf*^{-/-} mice

In subsequent studies we then looked at IFN- γ production because IFN- γ has been previously reported to inhibit the development of peripheral Treg cells.^{24,25} In our model of allergic asthma, we observed a significant increase in IFN- γ secretion in the BALF of *Batf*^{-/-} mice compared with that seen in control animals (Fig 4, **A**) in experimental allergic asthma induced by OVA sensitization and challenge. On further investigation, we found increased numbers of IFN- γ -producing T cells in the CD3⁺, CD4⁺, and CD8⁺ T-cell compartments in the lungs of naive and asthmatic *Batf*^{-/-} mice (Fig 4, **B-D**), whereas there was no significant difference in other cell types, such as dendritic cells or monocytes (see Fig E4, **A-C**, in this article's Online

Repository at www.jacionline.org). These findings were confirmed also when the total cell numbers of gated cells were taken into consideration (see Fig E4, **D-I**), suggesting that IFN- γ production by lung T cells is a key target of BATF in experimental asthma.

Local treatment with anti-IFN- γ antibodies induced Treg cells and reduced mast cell numbers and AHR in *Batf*^{-/-} mice

On the basis of the above findings, we explored in subsequent studies the concept that the increase in IFN- γ levels in *Batf*^{-/-} mice would be important in the asthma protection seen in these mice. Accordingly, we thus inhibited airway IFN- γ function with a neutralizing antibody during allergen challenge (see Fig E5, **A**, in this article's Online Repository at www.jacionline.org). We found that this treatment did not alter serum IgE levels (Fig 5, **A**) and airway inflammation, which were significantly reduced compared with that observed in the lungs of anti-IFN- γ antibody-treated wild-type littermates (Fig 5, **B**). Mast cell numbers were further inhibited by anti-IFN- γ antibody treatment (Fig 5, **C**). These data were confirmed when the total cell number was taken into consideration and mast cells were gated on total granulocytes or on c-kit⁺ cells (see Fig E5, **F**).

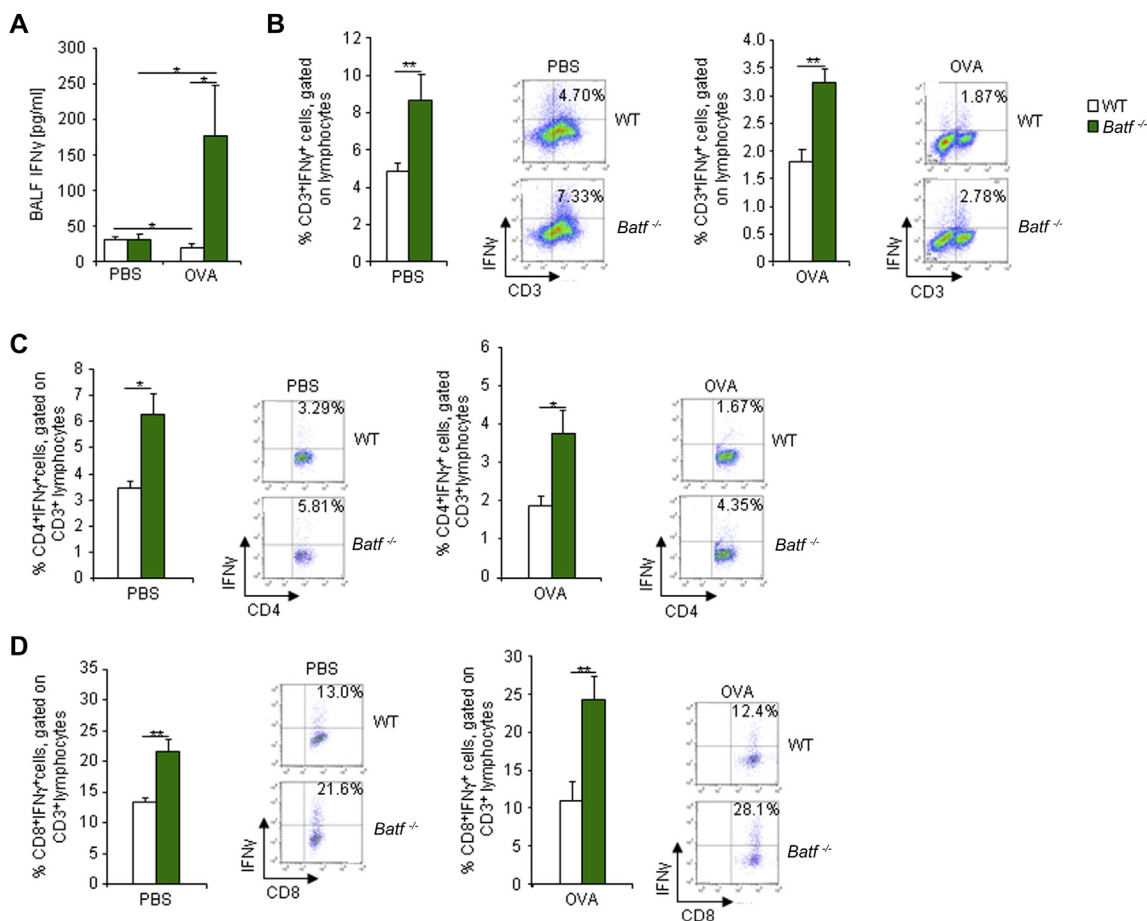


FIG 4. Increased IFN- γ production in CD4⁺ and CD8⁺ T cells in the airways of *Batf*^{-/-} mice. **A**, BALF IFN- γ levels. **B-D**, Cells were stained for IFN- γ and T-cell surface markers ($n = 3-4$ per group). * $P < .05$ and ** $P < .01$, Student 2-tailed t test. WT, Wild-type.

Consistent with a regulatory role of IFN- γ in asthma, anti-IFN- γ antibody treatment resulted in a significant increase in AHR in wild-type littermates. Targeted deletion of BATF abolished the effect of anti-IFN- γ treatment on AHR (Fig 5, D). IFN- γ is known to counteract IL-17A and IL-10, 2 cytokines controlled by BATF. Indeed, blockade of IFN- γ in the airways resulted in an increase in IL-17A levels and a significant upregulation of IL-10 levels in wild-type but not *Batf*^{-/-} mice (see Fig E6, A and B, respectively, in this article's Online Repository at www.jacionline.org). Consistent with the inhibition of IL-9 and IL-13 in the *Batf* knockout mice and with a pathogenetic role of these 2 cytokines in allergic asthma, we found that anti-IFN- γ antibody treatment did not significantly change these cytokines in total cells stimulated with anti-CD3/anti-CD28 antibodies (see Fig E6, C and D, respectively) in the lungs of treated *Batf*^{-/-} mice.

The number of Treg cells was increased by anti-IFN- γ antibody treatment in *Batf*^{-/-} asthmatic mice (Fig 5, E). These data indicated an inhibitory function of IFN- γ on the number of adaptive Treg cells in the absence of BATF. These data were confirmed when the total numbers of gated cells was taken into consideration (see Fig E6, E).

These data suggest that BATF controls Treg and mast cells in the setting of experimental asthma through its effects on IFN- γ production in lung T cells.

DISCUSSION

Here we report a crucial role for BATF in allergic asthma. In fact, in 2 cohorts of preschool children, we observed an increase in *BATF* levels in a subgroup of children with recurrent asthma that was not treated with steroids compared with those seen in steroid-treated asthmatic children. Several mechanisms might contribute to the effects of corticosteroids on *BATF* expression.^{26,27} *Batf* mRNA transcription is induced by IL-6 signaling, particularly in the absence of T-box transcription factor (T-bet).^{4,28} In addition, in asthmatic children the expression of *TBET* negatively correlates with *IL6* levels.²⁸ Thus it is possible that steroids in asthma treatment target *IL6* mRNA stability and that the impaired IL-6 signaling decreases *BATF* gene expression. Furthermore, activating protein 1 can stimulate the expression of its own component, c-Jun, and the c-Jun gene can be repressed through binding of the steroid-receptor complex.^{29,30} Therefore *BATF* mRNA expression might be regulated in a similar way. Because the exact effects of steroids on intracellular pathways are not thoroughly resolved, *BATF* mRNA expression might also be influenced by other factors that require separate investigations.

By comparing *Batf*^{-/-} mice with wild-type control animals, we showed for the first time that BATF deficiency protected these mice from experimental asthma. Consistently, BATF deficiency

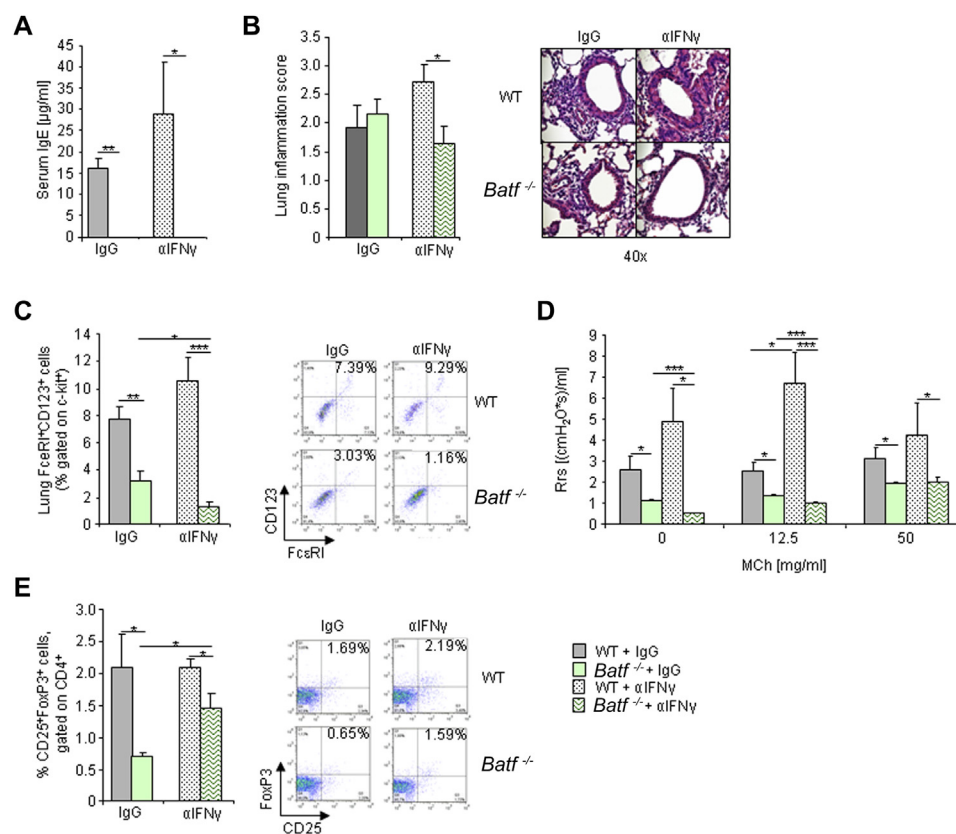


FIG 5. Local treatment with anti-IFN- γ antibody in *Batf*^{-/-} mice and wild-type (WT) littermates. **A**, Serum IgE. **B**, Hematoxylin and eosin–stained lung sections (n = 4 per group). **C–E**, Lung mast cells (Fig 5, **C**), invasive AHR (Fig 5, **D**), and Lung Treg cells (Fig 5, **E**; n = 3 per group). **P* < .05, ***P* < .01, and ****P* < .001, ANOVA (Fig 5, **D**) or Student 2-tailed *t* test (Fig 5, **A–C** and **E**).

suppressed the pathophysiologic asthmatic responses, such as AHR, airway eosinophilia, and mucus production, as well as serum IgE levels and lung mucosal mast cell and BMMC development, compared with those seen in control mice. The phenotype of the asthmatic *Batf*^{-/-} mice was found to be the opposite of that observed in mice with T-bet deficiency because T-bet-deficient mice have increased IgE levels, AHR, and lung inflammation and induction of T_H2 and T_H17 cytokines.^{25,28}

As anticipated, T_H2 and T_H17 cytokine levels were found to be decreased in the absence of BATF in this model of allergic asthma.^{6,17} Our study also demonstrates, for the first time, that BATF is essential for the development of IgE antibodies, which is consistent with a defect in the immunoglobulin class-switching already described for these mice in detail for other immunoglobulin subclasses.⁵ This was demonstrated not only in *Batf*^{-/-} mice but also in DO11.10-CD2-*Batf* transgenic mice, in which lung inflammation and serum IgE were induced in an antigen-specific manner.

Mast cells are also important in the development of allergic asthma.¹⁴ They can be of connective or mucosal subtypes and require growth factors, such as SCF or IL-3.^{9,31} Because the *Batf*^{-/-} mice had a defect in IgE production and less IL-3 secretion and IL-3 receptor expression, we reasoned that these lung mast cells could not react to allergen challenge.

IL-2 is an important cytokine for T-cell proliferation and the expansion of both effector and Treg cells.^{22,32} Here we showed that IL-2 secretion from total lung cells after OVA treatment is

markedly reduced and that this suppression was associated with reduced numbers of activated CD4⁺CD25⁺ T cells in *Batf*^{-/-} mice. We further demonstrated that the defect in IL-2 results in reduced pSTAT5 levels in the lungs of *Batf*^{-/-} mice. This supports the notion that Treg cells are downregulated in *Batf*^{-/-} mice because STAT5-deficient mice do not have Treg cells.³³

Because both T_H2 and T_H17 responses were found to be decreased in a murine model of asthma in mice with BATF deficiency, we took a closer look at the T_H1 response, which is supposed to be beneficial in asthma.²⁵ Our first observation was that higher levels of IFN- γ were present in the BALF of OVA-sensitized and OVA-challenged *Batf*^{-/-} mice. Moreover, in the lungs of OVA-treated animals, we found a higher numbers of IFN- γ ⁺ T cells in the CD3⁺, CD4⁺, and CD8⁺ T-cell compartments in the absence of BATF. Brand et al³⁴ described an increase in DNA methylation on the *Ifng* promoter after OVA treatment of mice. Therefore it would be interesting to analyze the methylation pattern on the *Ifng* promoter in *Batf*^{-/-} mice in the setting of allergic asthma. Cytokine responses were also confirmed to be antigen specific because IFN- γ was found to be downregulated and IL-10 to be upregulated in the lungs after transgenic overexpression of BATF on a DO11.10 background. The increase in IL-10 levels is probably unrelated to the Treg cells in this model.

Treg cells play a protective role in asthma.^{35–37} We found that Foxp3⁺ Treg cell numbers were significantly decreased in both our treated and untreated groups defective in BATF. This is in accordance with a finding by Caretto et al,²⁴ in which a

STAT1-dependent inhibition of peripheral Treg cell development by IFN- γ was observed. To further investigate the role of IFN- γ in the absence of BATF, we applied an anti-IFN- γ antibody intranasally during the challenge phase in this murine model of allergic asthma. We found that the allergic trait of *Batf*^{-/-} mice was not changed after anti-IFN- γ antibody treatment, indicating a direct control of BATF on the T_H2 and T_H17 genes, as previously demonstrated by others.^{3,6} The IgE defect also indicates an intrinsic effect of BATF on B cells.⁵ Interestingly, for the first time, we have found a direct effect of BATF on the *Il3* gene that was not reversed by anti-IFN- γ antibody treatment, which requires further investigation. Consistently, the mast cell defect was not reversed by anti-IFN- γ antibody treatment. Furthermore, we noted that IFN- γ blocks the development of CD4⁺CD25⁺Foxp3⁺ T cells in the lungs of *Batf*^{-/-} asthmatic mice. These adaptive Treg cells were upregulated after anti-IFN- γ antibody treatment. This is a relevant finding for the understanding of Treg cells in the setting of asthma and requires a separate investigation. Another important parameter that we found to be influenced by IFN- γ in the presence of BATF was AHR. This pathogenetic effect mediated by BATF in a reduced IFN- γ milieu is probably the result of the decrease in Treg cell numbers and increase in mast cell numbers.

In conclusion, in this report we show that BATF deficiency is beneficial in the setting of experimental asthma. The potential clinical relevance of these findings was underlined by a study in children showing that patients with allergic asthma had augmented *BATF* levels in PBMCs. Thus targeting BATF might be a new avenue for medical intervention in patients with allergic diseases.

We thank A. Geiger and D. Engelbrecht for animal husbandry and M. Schubert, M. Mathews, A. Mattil, S. Trump, H. A. Lehr, S. Koch, and B. E. Koch for their assistance. We also thank Professor Edgar Schmitt, Institute of Immunology, University of Mainz, for providing us the anti-IFN- γ antibodies for the *in vivo* studies.

Clinical implications: Current antiasthma steroid therapies in children might target BATF. Targeting BATF might ameliorate allergic asthma through upregulation of IFN- γ and downregulation of T_H2 and T_H17 cells and IL-3 cytokine production in the lung.

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METHODS

Human studies: Isolation of PBMCs from children with and without asthma and real-time PCR for *BATF*

In this study we analyzed 2 cohorts of children with and without asthma at the age of 4 to 6 years (Study Predicta, Registration no. Deutsches Register Klinischer Studien [German Clinical Trials Register] DRKS00004914). The children with asthma were instructed to come to the study center at the children's hospital within the next 2 days if they had a respiratory tract infection/cold or exacerbation of asthma-related symptoms or decreased FEV₁ of greater than 15% or peak expiratory flow of greater than 30% (symptomatic visit). The clinical outcomes of the recruited children in this study are reported in Table E1. At the time of recruitment (day 0), PBMCs were isolated from heparinized blood with Ficoll, and total RNA from the PBMCs was extracted by using RNeasy RNeasy Pure (Qiagen, Hilden, Germany), according to the manufacturer's protocol. RNA (1 µg) was then reverse transcribed with the first-strand cDNA synthesis kit for RT-PCR (MBI Fermentas, St Leon-Rot, Germany). The resulting template cDNA was amplified by using quantitative real-time PCR with SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Munich, Germany). Quantitative PCR was performed with a cycle of 2 minutes at 98°C; 50 cycles of 5 seconds at 95°C, 10 seconds at 60°C, followed by 5 seconds at 65°C; and 5 seconds at 95°C in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). The primers and sequences used for human samples were as follows: *hBATF* (forward: 5'- AGC GAA GAC CTG GAG AAA CA-3', reverse: 5'- TTC AGC ACC GAC GTG AAG TA-3'). *BATF* gene expression was normalized to the expression of the housekeeping gene *hHPRT* (forward: 5'-TGA CAC TGG CAA AAC AAT GCA-3', reverse: 5'- GGT CCT TTT CAC CAG CAA GCT-3').

OVA sensitization and challenge

Batf^{-/-}, wild-type BALB/c, or 129 SvEv mice received intraperitoneal injections of 500 µg/mL OVA (Calbiochem, Gibbstown, NJ) complexed with 10% alum (Sigma-Aldrich, Steinheim, Germany) on days 0 and 7, as previously described. On days 18, 19, and 20, the animals received 50 µg of OVA in PBS administered intranasally (2 mg of OVA/mL PBS), as previously described (OVA/OVA model).^{E1} In the PBS/OVA model DO11.10 T-cell receptor transgenic mice were injected intranasally with OVA on 3 consecutive days. All control animals were left untreated (PBS). Mice were killed 24 hours after the last intranasal application to isolate total lung cells, as previously described (see Sauer et al^{E2} until step 25).

AHR

Invasive plethysmography with a FlexiVent FX1 device (SCIREQ Scientific Respiratory Equipment) using the FlexiWare7.2.2 software was used in pentobarbital-anesthetized, spontaneously breathing mice. A cannula was inserted into the trachea, and the animal was attached to the device. During mechanical ventilation, first PBS and then increasing doses of aerosolized MCh (Sigma-Aldrich) were applied to the lung of the mouse. Lung resistance (respiratory system resistance) was measured 12 times for each dose. Data are expressed as mean values of respiratory system resistance ± SEM.

Noninvasive whole-body plethysmography was also performed at least 2 hours after the last challenge with OVA by using a Buxco Electronics apparatus (Buxco Research Systems, Wilmington, NC). Here mice were challenged with increasing doses of nebulized MCh in an exposure chamber, and Penh responses were measured. Data are expressed as mean values of enhanced pause ± SEMs.

Collection and analysis of BALF

After centrifugation, BALF supernatants were frozen (-20°C) and subsequently analyzed by using ELISA. The cell pellet was resuspended in PBS, counted, and subsequently used for cytospin analysis after staining with May-Grünwald-Giemsa solution (Carl Roth, Karlsruhe, Germany).

Histology

Five-micrometer-thick lung sections from paraffin blocks were stained with hematoxylin and eosin for quantification of inflammation. The scoring of

inflammation was done blindly by a pathologist (R.J.R.), as previously described.^{E3} For quantification of mucus production, lung sections were stained with periodic acid-Schiff, and the number of periodic acid-Schiff-positive cells per micrometer of bronchus was determined, as previously described.^{E4} If BAL was performed before lung tissue collection on the right lobe, only left lobes were used for histology.

Mast cell differentiation from BMMCs

To mature mast cells from bone marrow using IL-3 and SCF, we isolated bone marrow from femurs and tibias of 8- to 12-week-old mice. Cells were grown in RPMI 1640 medium supplemented with 10% FBS, 100 µg each of penicillin and streptomycin per milliliter, 2 mmol/L L-glutamine, 50 µmol/L β-mercaptoethanol, 10 mmol/L HEPES, and 10 ng/mL recombinant murine IL-3 and SCF (PeproTech, Hamburg, Germany) for 4 weeks at 37°C in 5% CO₂. After 4 weeks of cell culture, more than 80% of the cells were c-kit and FcεRI positive in flow cytometry, as assessed by using anti-c-kit and anti-FcεRI antibody staining, respectively. BMMC morphology was assessed with cytospin and staining with May-Grünwald-Giemsa. An apoptosis assay was performed after 72 hours of growth factor deprivation, as described in the BD Pharmingen Annexin V-FITC Apoptosis Detection Kit I (containing propidium iodide staining solution). Histamine release was measured after culturing differentiated mast cells in Tyrode buffer (pH 7.4) for 10 minutes at 37°C. After a washing step, the supernatant was collected, and the cell pellet was resuspended in Tyrode buffer. The supernatant and cell pellet were incubated for 10 minutes at 95°C and stored at -80°C. Total histamine levels were then determined by using a Histamine ELISA^{FastTrack} Kit (Labor Diagnostika Nord GmbH & Co KG, Nordhorn, Germany).

Flow cytometric analysis

Freshly isolated cells were used unstimulated, whereas for intracellular cytokine staining, cells were incubated with phorbol 12-myristate 13-acetate (50 ng/mL), ionomycin (500 ng/mL), and a protein transport inhibitor (BD GolgiStop) for 4 hours at 37°C. Surface markers were stained with an antibody cocktail for 30 minutes at 4°C, washed in PBS, and then analyzed on a FACSCalibur (BD Pharmingen, Heidelberg, Germany). For intracellular staining, cells were incubated with Fixation/Permeabilisation solution (eBioscience, Frankfurt, Germany) for 35 minutes at 4°C and then washed and stained with an antibody in Permeabilisation buffer (eBioscience) for 30 minutes at 4°C. After washing in PBS, the cells were analyzed, as mentioned above. The following fluorescein isothiocyanate-, phycoerythrin-, PerCP-, or allophycocyanin-conjugated mAbs were used for flow cytometry: anti-CD4, anti-CD11c (BD Bioscience), anti-CD3, anti-CD8, anti-CD25, anti-CD45R, anti-CD117 (= c-kit), anti-CD123, anti-F4/80, anti-FcεRI, anti-IFN-γ, anti-MHC class II, anti-NK1.1 (eBioscience), and anti-FoxP3 (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) antibodies. The samples were analyzed with CellQuest Pro (BD Bioscience) and FlowJo software (TreeStar, Ashland, Ore).

ELISA

Serum or supernatants from cultured total lung cells or lung CD4⁺ T cells, as well as BALF, were subsequently analyzed by using ELISA. Mouse IL-2, IL-3, IL-4, IL-5, IL-10, IFN-γ, IgG_{2a}, and TNF-α were detected by using OptEIA sandwich ELISA kits from BD Bioscience. Mouse IL-13, IL-17A, IL-21, and GM-SCF were detected by using a DuoSet sandwich ELISA kit from R&D Systems (Wiesbaden, Germany). Mouse IL-9 was detected by using the Mouse IL-9 Ready-SET-Go! ELISA Set from eBioscience.

Murine RNA isolation and quantitative real-time PCR

Murine total lung or lung CD4⁺ T cells were lysed and total mRNA was extracted by using RNeasy RNeasy Pure, according to the manufacturer's protocol (Qiagen). RNA was reverse transcribed with the first-strand cDNA synthesis kit for RT-PCR (MBI Fermentas). The cDNA was amplified by means of quantitative real-time PCR with SsoFast EvaGreen Supermix (Bio-Rad Laboratories). Quantitative PCR was performed with a cycle of 2 minutes at

98°C; 50 cycles of 5 seconds at 95°C, 10 seconds at 60°C, followed by 5 seconds at 65°C; and 5 seconds at 95°C in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). The primers and sequences used were as follows: *mGata3* (forward: 5'- GTC ATC CCT GAG CCA CAT CT-3', reverse: 5'- TAG AAG GGG TCG GAG GAA CT-3'), *mRorc* (forward: 5'- GTG TGC TGT CCT GGG CTA CC-3', reverse: 5'-AGC CCT TGC ACC CCT CAC AG-3'). The mRNA of the genes of interest was normalized with the housekeeping gene *Hprt* (forward: 5'-GCC CCA AAA TGG TTA AGG TT-3', reverse: 5'-TTG CGC TCA TCT TAG GCT TT-3').

Protein isolation and Western blotting

Lung tissues were homogenized with Minilys (PeqLab) and SpeedMill+ (Analytik Jena, Jena, Germany) in lysis buffer containing 1% NP-40, 20 mmol/L TRIS HCl (pH 8), 137 mmol/L NaCl, 5% glycine, 2 mmol/L EDTA, and protein inhibitors cocktail (cOmplete, Mini; Roche Diagnostics, Mannheim, Germany). Proteins were further isolated by means of centrifugation, and their concentrations were determined with Bradford Assay (Protein Assay Dye Reagent Concentrate, Bio-Rad Laboratories). After denaturation (at 95°C for 5 minutes) in reducing loading buffer (Roti Load 1; Carl Roth, Karlsruhe, Germany), 30 µg of protein was loaded on a 10% polyacrylamide gel (PAGEr EX Gels; Lonza, Basel, Switzerland) and separated in a Biometra EcoMini EBC Device. The proteins were blotted on a Protran Nitrocellulose membrane (0.45 mm; Whatman, Kent, United Kingdom) in a Fastblot B33 chamber (Biometra, Göttingen, Germany) for 45 minutes. Membranes were then washed in 1× PBS plus Tween 0.05%, and unspecific binding sites were blocked (5% milk in 1× PBS + 0.05% Tween 20) for 1 hour at room temperature. Primary antibodies (1:1000) were incubated overnight at 4°C on a shaking device, according to the manufacturer's instructions. Horseradish peroxidase-conjugated secondary antibodies (1:2000) were incubated for 1 hour at room temperature on a shaking device. Specific bands were detected with WesternBright Quantum HRP substrate (Advansta, Menlo Park, Calif) and a FluorChem FC2 device (Biozym, Hessisch Oldendorf, Germany). Primary antibodies were pStat5 (Tyr694; C11C5) rabbit mAb (Cell Signaling

Technology, Danvers, Mass) and Stat5 (3H7) rabbit mAb (Cell Signaling Technology). The secondary antibody was ECL rabbit IgG, horseradish peroxidase-linked whole antibody (from donkey; GE Healthcare Life Sciences, Freiburg, Germany).

RESULTS

Local treatment with anti-IFN-γ antibodies reduced eosinophilia in *Batf*^{-/-} mice compared with that seen in anti-IFN-γ antibody-treated wild-type mice.

BALF eosinophilia was found to be reduced in the lungs of *Batf*^{-/-} mice treated with anti-IFN-γ antibody (Fig E5, B). Moreover, we discovered an effect of the isotype control on lung neutrophilia in *Batf*^{-/-} mice, which was suppressed by anti-IFN-γ antibody treatment (Fig E5, B). IL-4, IL-5, and IL-3 production was not affected by anti-IFN-γ antibody treatment (Fig E5, C-E, respectively) in *Batf*^{-/-} mice.

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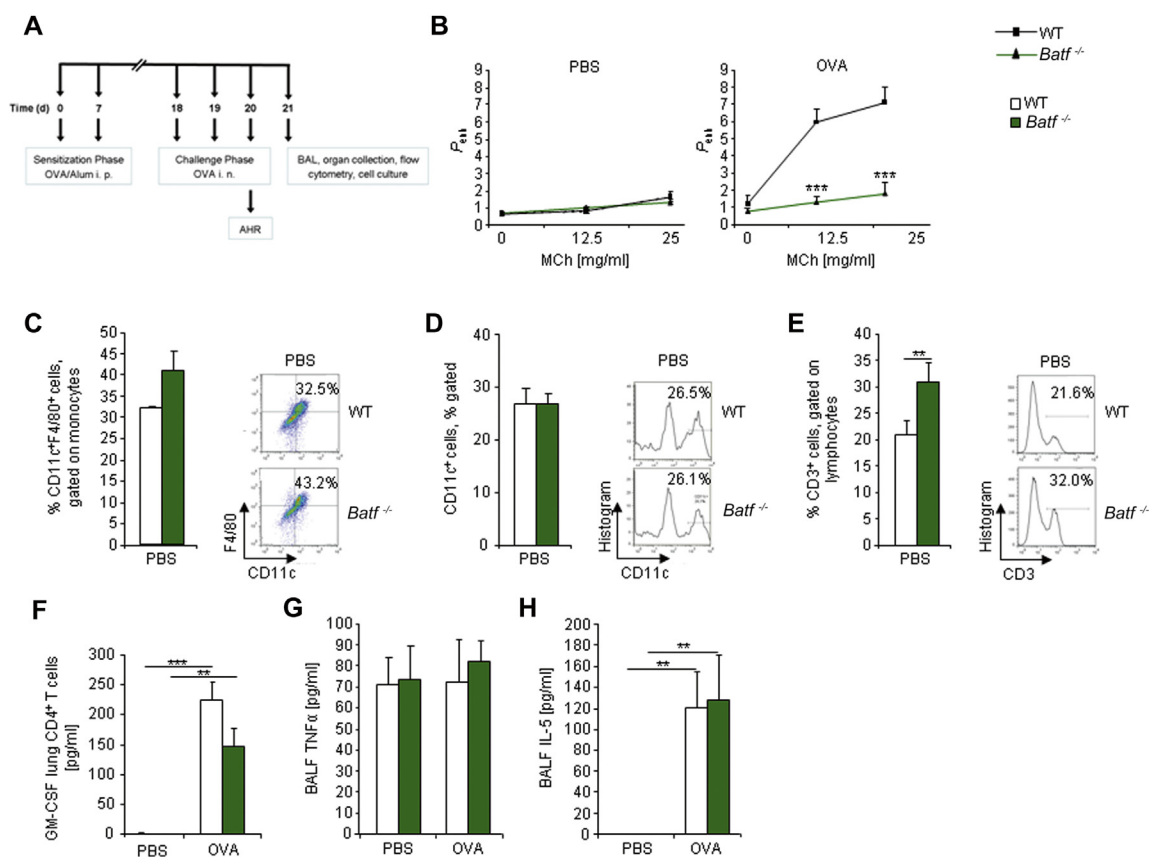


FIG E1. Measurement of noninvasive AHR and characterization of lung cell populations and cytokine levels in BALF. **A**, Schematic representation of the experimental asthma protocol used in this study with 2 intra-peritoneal injections of OVA/alum on days 0 and 7, followed by intranasal challenge with OVA on days 18, 19, and 20. **B**, AHR was measured by using noninvasive plethysmography in naive mice and after OVA treatment ($n = 5-7$ per group). **C-E**, Total lung cells from naive wild-type and *Batf*^{-/-} mice were analyzed by means of flow cytometry for surface markers of macrophages (Fig E1, C), dendritic cells (Fig E1, D), and CD3⁺ T cells (Fig E1, E). **F-H**, GM-CSF (Fig E1, F), TNF- α (Fig E1, G), and IL-5 (Fig E1, H) secretion from untreated and OVA-sensitized and OVA-challenged mice was measured with an ELISA ($n = 3-6$ per group). Data are presented as mean values $\pm$ SEMs. ** $P \leq .01$ and *** $P \leq .001$, Student 2-tailed t test. WT, Wild-type.

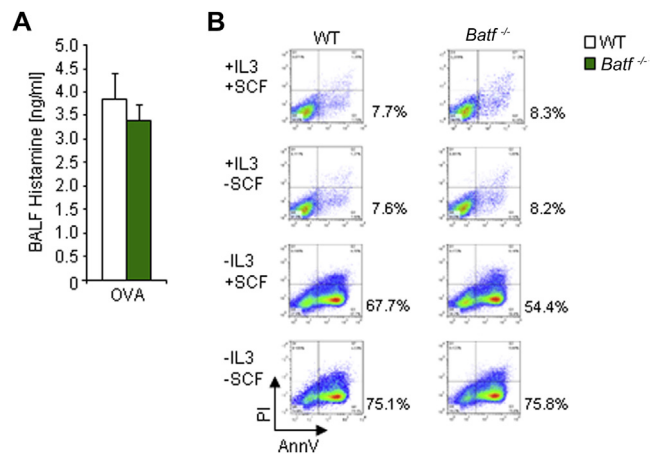


FIG E2. BALF histamine levels and IL-3 dependency of BMMCs. **A**, Total histamine levels were determined with ELISA in BALF of OVA-treated wild-type and *Batf*^{-/-} mice. **B**, Dot plots from an apoptosis assay after cytokine deprivation of BMMCs ($n =$ at least 3 readouts per group). Data are mean values $\pm$ SEMs. WT, Wild-type.

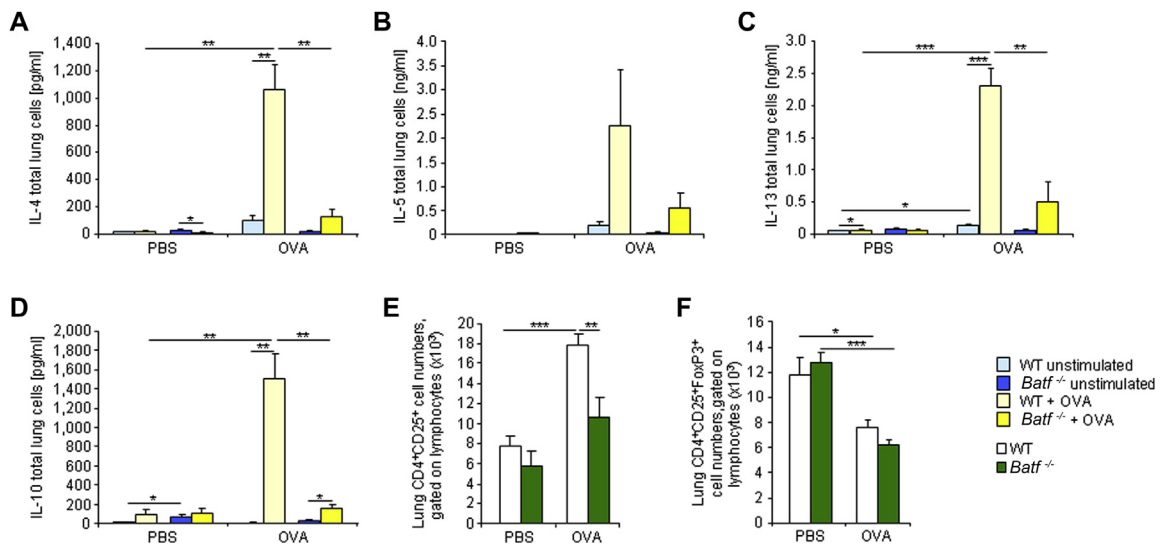


FIG E3. Cytokine levels of OVA-restimulated total lung cells and decreased numbers of activated T cells and Treg cells in lungs of *Batf*^{-/-} mice. **A-D**, Total lung cells from control or OVA-treated mice were restimulated with OVA *in vitro*, and levels of cytokines (IL-4, IL-5, IL-13, and IL-10) were measured in the supernatants (n = 3 per group). **E** and **F**, CD4⁺CD25⁺ activated T-cell numbers (Fig E3, **E**) and CD4⁺CD25⁺FoxP3⁺ Treg cell numbers (Fig E3, **F**) were determined by using cytometry, and the number of gated lymphocytes was analyzed (n = 4-10 per group). Data are presented as mean values ± SEMs. **P* ≤ .05, ***P* ≤ .01, and ****P* ≤ .001, Student 2-tailed *t* test.

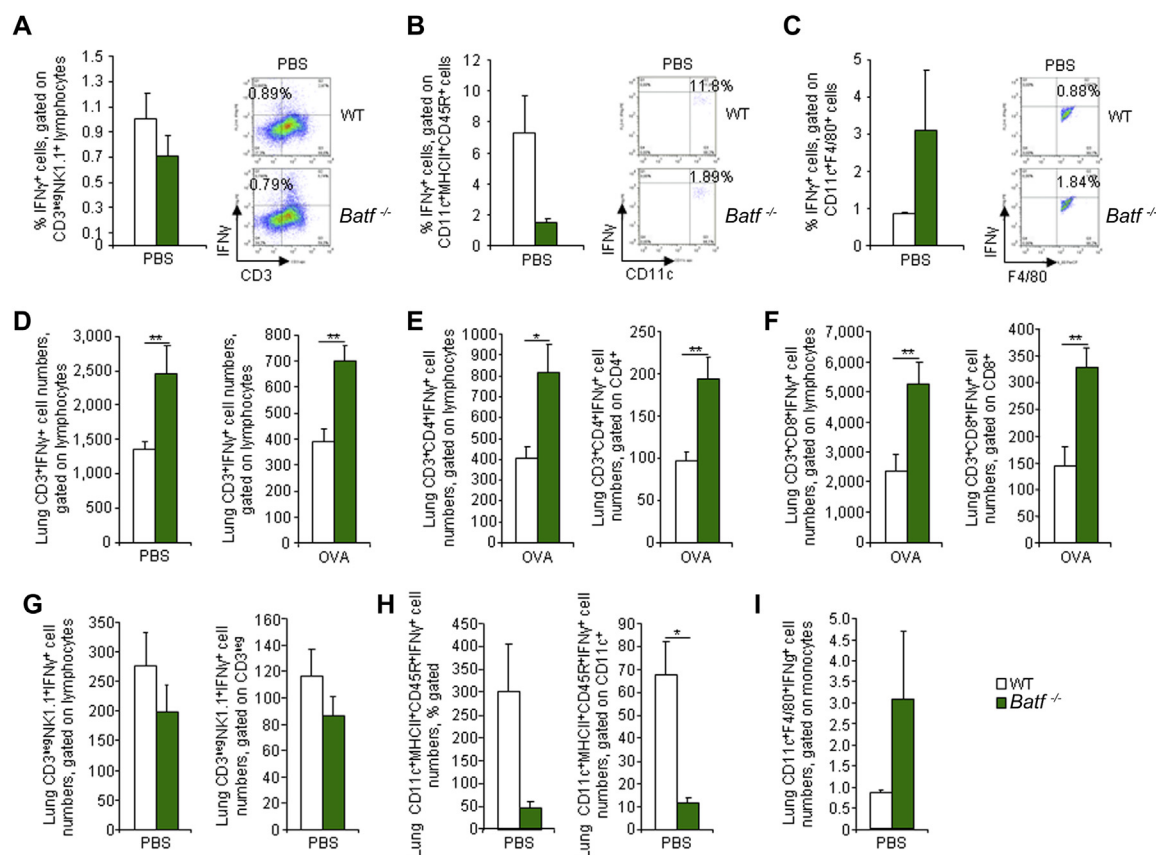


FIG E4. Determination of IFN- γ $^{+}$ lung cells in untreated and OVA-treated mice by using flow cytometry. **A-C**, Percentage of IFN- γ $^{+}$ cells in the natural killer cell, dendritic cell, and macrophage compartments in naive wild-type and *Batf* $^{-/-}$ mice. **D-I**, IFN- γ $^{+}$ cell numbers calculated with the number of gated cells ($n = 3-6$ per group). Data are mean values $\pm$ SEMs. * $P \leq .05$ and ** $P \leq .01$, Student 2-tailed t test. WT, Wild-type.

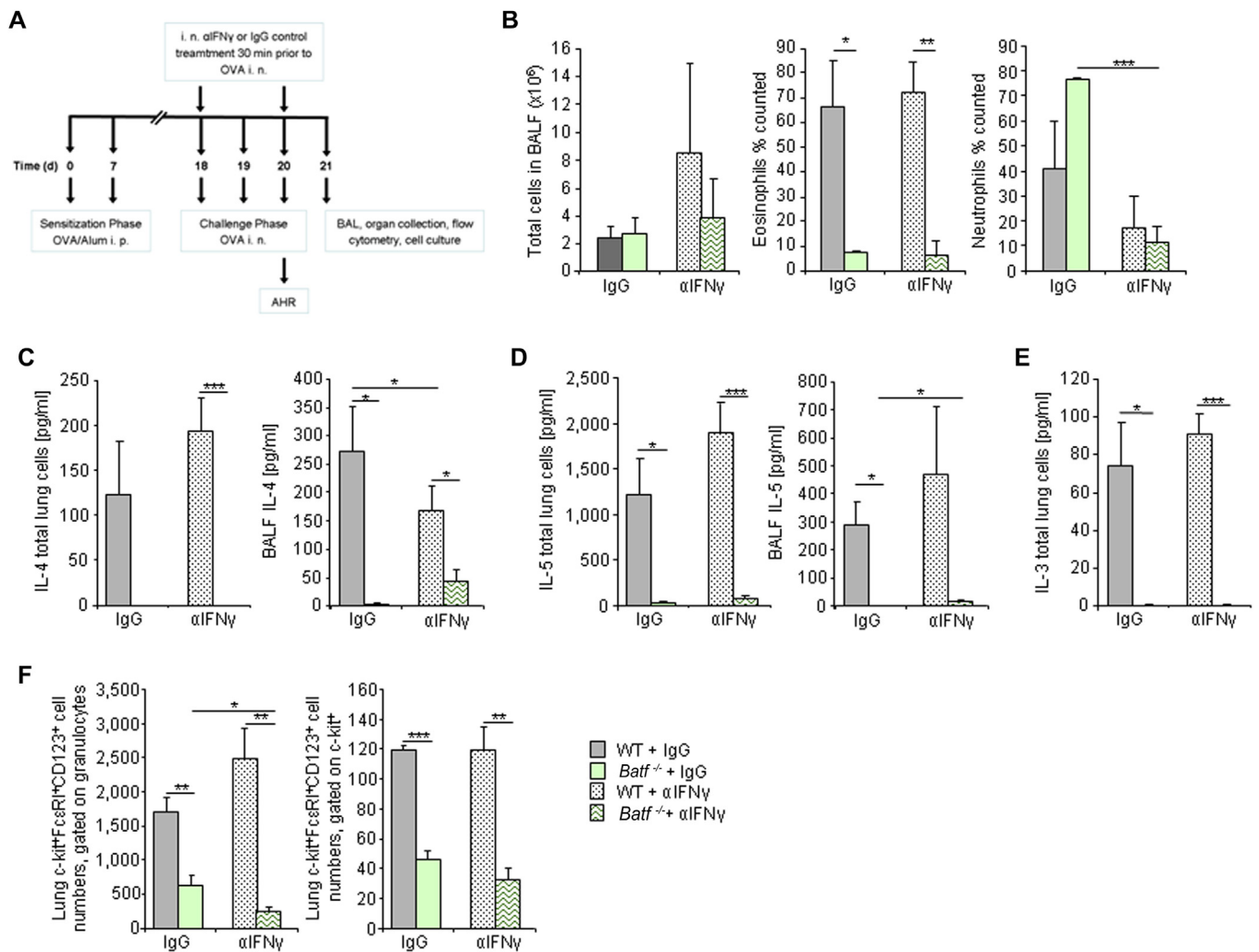


FIG E5. Effects of anti-IFN- γ antibody treatment on cytokine secretion and mast cell numbers. **A**, Schematic representation of the asthma protocol used in this study. On days 18 and 20, mice received 100 μ g of either control IgG or anti-IFN- γ antibodies per day. **B**, BALF cells were stained with May-Grünwald-Giemsa and analyzed by using light microscopy. **C** and **D**, Analysis of IL-4 and IL-5 levels from total lung cells and in BALF of IgG or anti-IFN- γ -treated wild-type or *Batf*^{-/-} mice in a murine model of experimental asthma. **E**, IL-3 secretion from total lung cells. **F**, Lung mast cells were measured with flow cytometry, and cell numbers of gated cells were determined ($n = 4$ -5 per group). Data are mean values $\pm$ SEMs. * $P \leq .05$, ** $P \leq .01$, and *** $P \leq .001$, Student 2-tailed t test. WT, Wild-type.

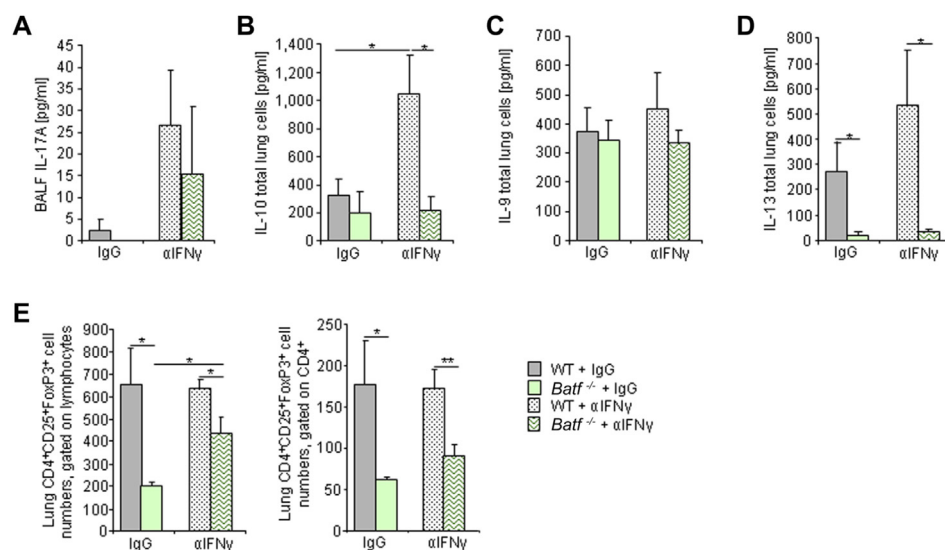


FIG E6. Cytokine levels and Treg cell numbers after anti-IFN- γ antibody treatment. **A-D**, IL-17A, IL-10, IL-9, and IL-13 levels were measured by means of ELISA in BALF (Fig E6, A) or total lung cell supernatants (Fig E6, B-D) from mice treated with anti-IFN- γ antibodies or control IgG in a murine model of asthma. **E**, Lung Treg cell numbers were determined with flow cytometry, and the number of gated cells was taken into account ($n = 4-5$ per group) Data are mean values $\pm$ SEMs. * $P \leq .05$ and ** $P \leq .01$, Student 2-tailed t test.

TABLE E1. Clinical outcome and treatment of children with asthma and control children participating in the Predicta Study

Group/patient	Age (y)	Sex	Treatment	Symptomatic visit
No asthma				
C1	6	Female	X	X
C2	5	Female	X	X
C3	4	Male	X	X
C4	6	Male	X	X
C5	4	Female	X	X
C6	6	Male	X	X
C7	4	Male	X	X
C8	5	Female	X	X
C9	5	Female	X	X
Mean	5.00			
SEM	0.29			
Asthma				
A1	5	Female	Steroid	X
A2	6	Male	Steroid + nonsteroid	X
A3	5	Male	Steroid + nonsteroid	X
A4	5	Female	Steroid + nonsteroid	X
A5	6	Female	Nonsteroid	✓
A6	4	Male	Steroid + nonsteroid	X
A7	5	Male	X	✓
A8	4	Male	Nonsteroid	✓
A9	5	Male	X	✓
A10	4	Male	Steroid + nonsteroid	X
Mean	4.90			
SEM	0.23			

X, No treatment and no symptomatic visit, respectively; ✓, symptomatic visit.