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Research Article **Immunology**

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Th9 cell development requires a BATF-regulated transcriptional network

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T helper 9 (Th9) cells are specialized for the production of IL-9, promote allergic inflammation in mice, and are associated with allergic disease in humans. It has not been determined whether Th9 cells express a characteristic transcriptional signature. In this study, we performed microarray analysis to identify genes enriched in Th9 cells compared with other Th subsets. This analysis defined a transcriptional regulatory network required for the expression of a subset of Th9-enriched genes. The activator protein 1 (AP1) family transcription factor BATF (B cell, activating transcription factor–like) was among the genes enriched in Th9 cells and was required for the expression of IL-9 and other Th9-associated genes in both human and mouse T cells. The expression of BATF was increased in Th9 cultures derived from atopic infants compared with Th9 cultures from control infants. T cells deficient in BATF expression had a diminished capacity to promote allergic inflammation compared with wild-type controls. Moreover, mouse Th9 cells ectopically expressing BATF were more efficient at promoting allergic inflammation than control transduced cells. These data indicate that BATF is a central regulator of the Th9 phenotype and contributes to the development of allergic inflammation.

Introduction

Immunity to pathogens and the development of inflammatory diseases rely upon the development of specialized subsets of $CD4^+$ T helper (Th) cells. Th cell subsets differentiate in the presence of a polarizing cytokine environment. Th1 cells develop in the presence of IL-12 and IFN-γ and Th2 cells in the presence of IL-4 (1). The cytokine environment, generally through the phosphorylation of STAT proteins, activates a differentiation program that includes the induction of transcription factors that maintain subset identity and of genes involved in cell migration and cytokine production that are essential for the ability of the Th subset to regulate immune responses. Although there is often thought to be a "master regulator" of each lineage, T-bet for Th1 and GATA3 for Th2, for example, activation of the differentiation program requires the coordinated function of a network of transcription factors.

Th9 cells are the most recent addition to the spectrum of Th cell subsets that differentiate in the presence of a balanced combination of TGFβ and IL-4 (2–4). Th9 cells promote allergic inflammation, antitumor immunity, and may contribute to the regulation of autoinflammatory disease (5, 6). Based on the common requirement for IL-4 in promoting differentiation, Th9 and Th2 cells share a requirement for several transcription factors including STAT6, GATA3, and IRF4 (2–4, 7). PU.1 is an ETS family transcription factor that specifically promotes the development of IL-9–secreting cells, as it represses the Th2 genetic program, making it a switch factor between the two subsets (8–11). Much of the work in Th9 cells has focused on the regulation of *Il9*, and the expression of additional genes that may be restricted to the Th9 cell subset has not been defined.

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BATF (B cell, activating transcription factor–like) is a basic leucine zipper transcription factor that functions by dimerizing with Jun family members and binds to AP-1 DNA sequences (12). BATF is required for the development of Th17 cells, T follicular helper cells, and possibly Th2 cells (13–15). BATF binds to multiple genes within these subsets, as part of a BATF/Jun/IRF4 complex that regulates the Th17 phenotype (16–18). Whether BATF contributes to additional lineages is not yet clear.

In this report, we define a Th9 transcriptional program, including the preferential expression of BATF in the Th9 lineage. We demonstrate that BATF is required for IL-9 production in both human and mouse Th9 cells and that it cooperates with IRF4 in binding to and activating the *Il9* locus. The ability of BATF to activate Th9 genes corresponds to a requirement for BATF in T cells to promote allergic inflammation and an enhanced ability of BATF-expressing cells to promote allergic inflammation. Thus, BATF is a critical component of the transcription factor network inducing the Th9 cell phenotype.

Results

Th9 cells have a distinct transcriptional signature. Th9 cells are specialized for the production of IL-9. Yet, it is not clear that they represent a completely separate cell phenotype. The ability of TGFβ to convert Th2 into Th9 cells suggested that these cell types might be subsets of the same lineage. To begin to define the identity of Th9 cells, we performed a microarray analysis comparing Th9 cells (differentiated with IL-4 and TGFβ) with Th2 cells (differentiated with IL-4 alone) and inducible Treg cells (differentiated with TGFβ alone) (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI69489DS1). Clustering analysis indicated that Th2 and Th9 cells were more similar than Th9 and inducible Treg (iTreg) cells (Figure 1A). Yet, despite deri-

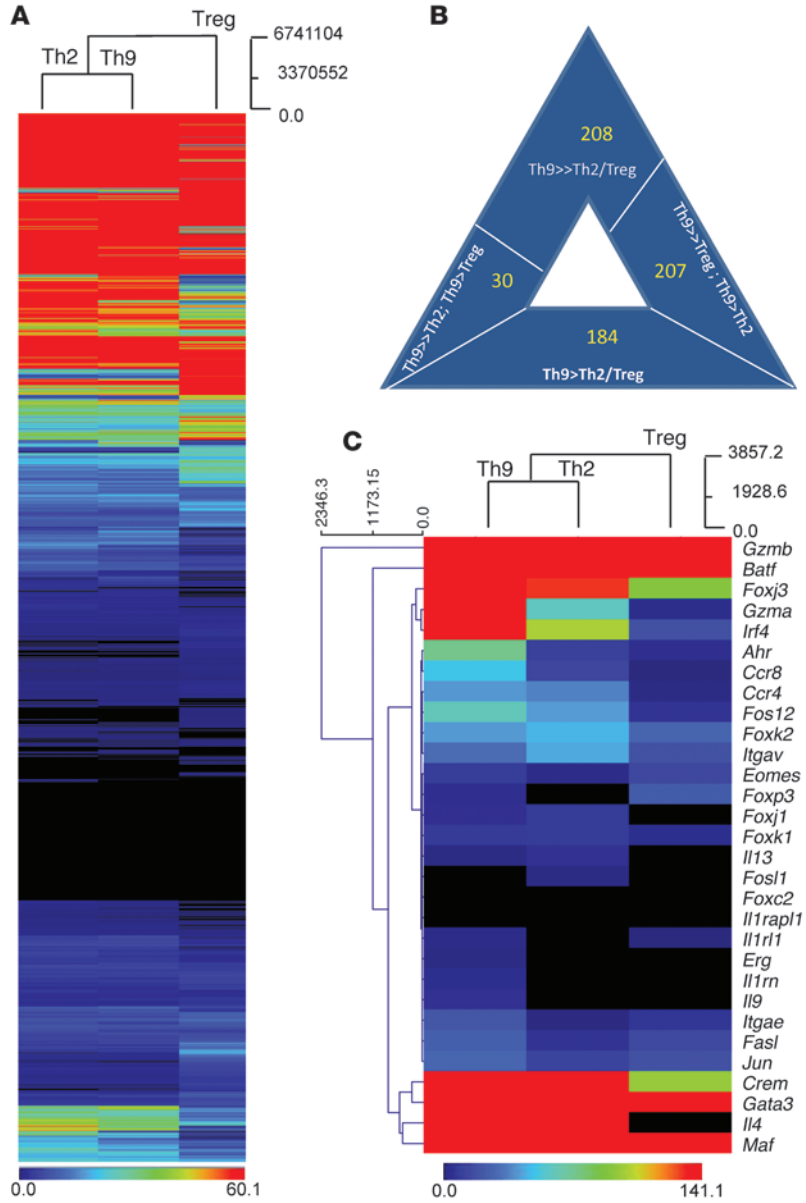

Figure 1
Microarray analysis of the Th9 transcriptional signature. Naive CD4+ T cells were differentiated under Th2, Th9, or iTreg polarizing conditions for 5 days before RNA was isolated for microarray analysis. (**A**) Heatmap comparison of transcript levels in Th2, Th9, and iTreg cells. Hierarchical clustering was performed using a Pearson's correlation with MeV software. (**B**) Graphical representation of 629 genes that were enriched in Th9 cells at least 2-fold compared with Th2 or iTreg cells. Genes were subdivided as enriched in Th9 cells by 5-fold (indicated by ">>") or enriched by 2- to 5-fold (indicated by ">") compared with the other Th subsets. (**C**) Heatmap of selected genes in Th9, Th2, and iTreg cells. Genes were selected based on functions that include transcription factors, cytokines, and surface receptors. Clustering was performed using Manhattan distance analysis.

vation following stimulation with a combination of cytokines that separately promote Th2 or Treg differentiation, Th9 cells have a gene signature that is distinct from either subset.

To further examine the Th9 gene signature, we defined the subset of genes among Th2, Th9, and Treg cells that were at least 2-fold enriched in the Th9 subset versus the other two subsets. We found 629 genes enriched in the Th9 subset (Figure 1B and Supplemental Table 1). Of this subset of genes, 208 showed a 5-fold or greater enrichment compared with Th2 or iTreg cells (Figure 1B and Supplemental Table 1). The shared identity of Th9 cells with Th2 and iTreg cells was also observed in this subset of genes. There were 207 genes that were greater than 5-fold more enriched in Th9 cells than in Tregs, but these were only 2- to 5-fold more enriched than Th2 cells (Figure 1B and Supplemental Table 1). Conversely, there were 30 genes that were expressed in Th9 cells that displayed a 5-fold greater expression than in Th2 cells, but had only a 2- to 5-fold greater expression than was found in iTreg cells (Figure 1B). To compare gene expression at a higher resolution, we generated a heatmap that focused on transcription factors, cytokines, and surface receptors expressed in Th9 cells. Among this subset of genes, we similarly observed Th2 and Th9 cells clustered more closely than either subset to the Treg cells (Figure 1C).

We also compared changes in gene expression between resting Th9 cells and Th9 cells activated with anti-CD3 for 6 hours. Following anti-CD3–mediated activation of Th9 cells, there were 1,006 genes induced more than 5-fold, 252 genes induced more than 10-fold, and 361 genes decreased more than 10-fold (Supplemental Figure 1B). Among the genes that demonstrated the greatest induction were cytokines and chemokines, including *Il2*, *Il3*, *Il21*, *Areg*, *Xcl1*, *Ccl3*, *Ccl4*, *Ccl22*, and *Il9* (Supplemental Figure 1, B and C).

Having established a transcriptome expressed in Th9 cells at greater levels than in Th2 or Treg cells, we chose a panel of genes based on their potential relatedness to Th9 function and confirmed Th9-specific expression in a larger panel of Th subsets. Of the genes examined, *Ccr8*, *Crem*, and *Tnfsf13b* showed the greatest expression in Th9 cells and were among the genes that demonstrated shared expression with Th2 cells (Figure 2A). We included analysis of *Ccr4* in this subset, a chemokine receptor gene that had greatest expression in both Th9 and Th2 cells. *Batf*, *Cxcl3*, *Ahrr*, and *Maf* expression was greatest in Th9 cells, but also had selective expression in Th2 and Th17 cells (Figure 2A). *Fasl* and *Furin* demonstrated shared expression in Th9 and Th1 cells (Figure 2A and data not shown). *Itgae* demonstrated shared expression in Th9 and iTreg cells (Figure 2A). Importantly, several genes including *Il17rb*, *Il1rn*, *Erg*, and *Ahr* showed selective expression in Th9 cells (Figure 2A).

We next tested whether differential expression of these genes conferred differences in protein expression and/or function among the subsets. We used flow cytometry to detect integrin αe (CD103) on a higher percentage of Th9 cells than Treg cells and found CCR4 on a higher percentage of Th9 cells than Th2 cells

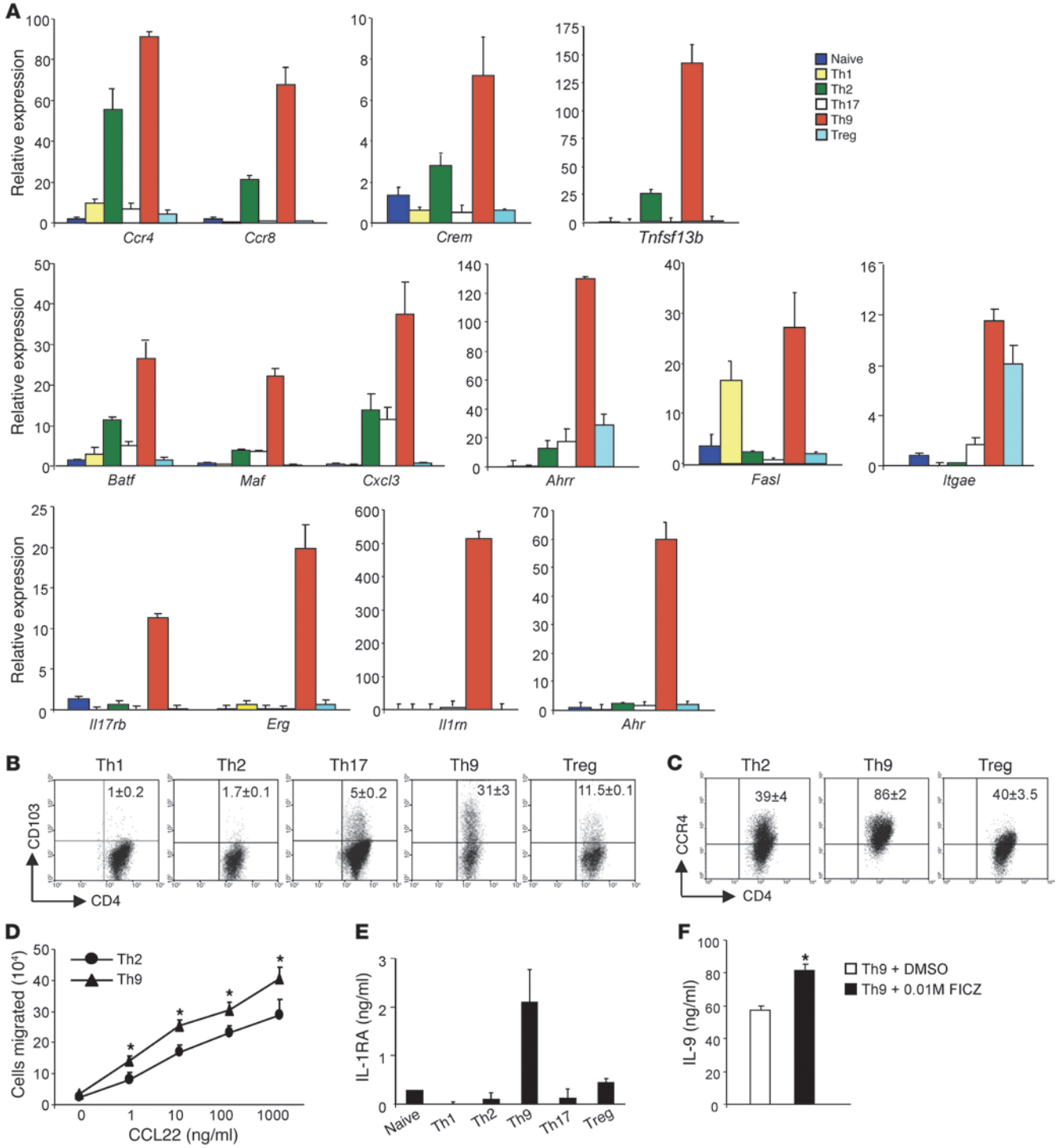

Figure 2

Expression and function of the Th9 gene signature. Naive CD4 T cells were differentiated under polarizing conditions to generate the indicated Th subsets. (**A**) RNA was isolated from Th subsets for expression analysis of the indicated genes using qRT-PCR. Expression is relative to naive CD4+ T cells and normalized to β-2 microglobulin expression. (**B**) Flow cytometric analysis of CD103 expression on the surface of Th subsets. Numbers indicate the average percentage ± SD of positive cells from at least three experiments. (**C**) Flow cytometric analysis of CCR4 expression on the surface of Th subsets. Numbers indicate the average percentage ± SD of positive cells from at least three experiments. (**D**) Chemotaxis assay of Th2 and Th9 cell response to increasing concentrations of CCL22. Migrated cells after 4 hours of incubation were counted in a hemacytometer. Results indicate the average ± SD of triplicate determinations and are representative of three experiments. (**E**) The concentration of IL-1RA was determined in the supernatant 24 hours following anti-CD3 stimulation of the indicated Th subsets. Results are the average ± SD of three experiments. (**F**) Naive CD4+ T cells were cultured under Th9 polarizing conditions in the absence (DMSO, vehicle) or presence of FICZ at the indicated concentration. IL-9 concentration was determined 24 hours after anti-CD3 stimulation. *$P < 0.05$.

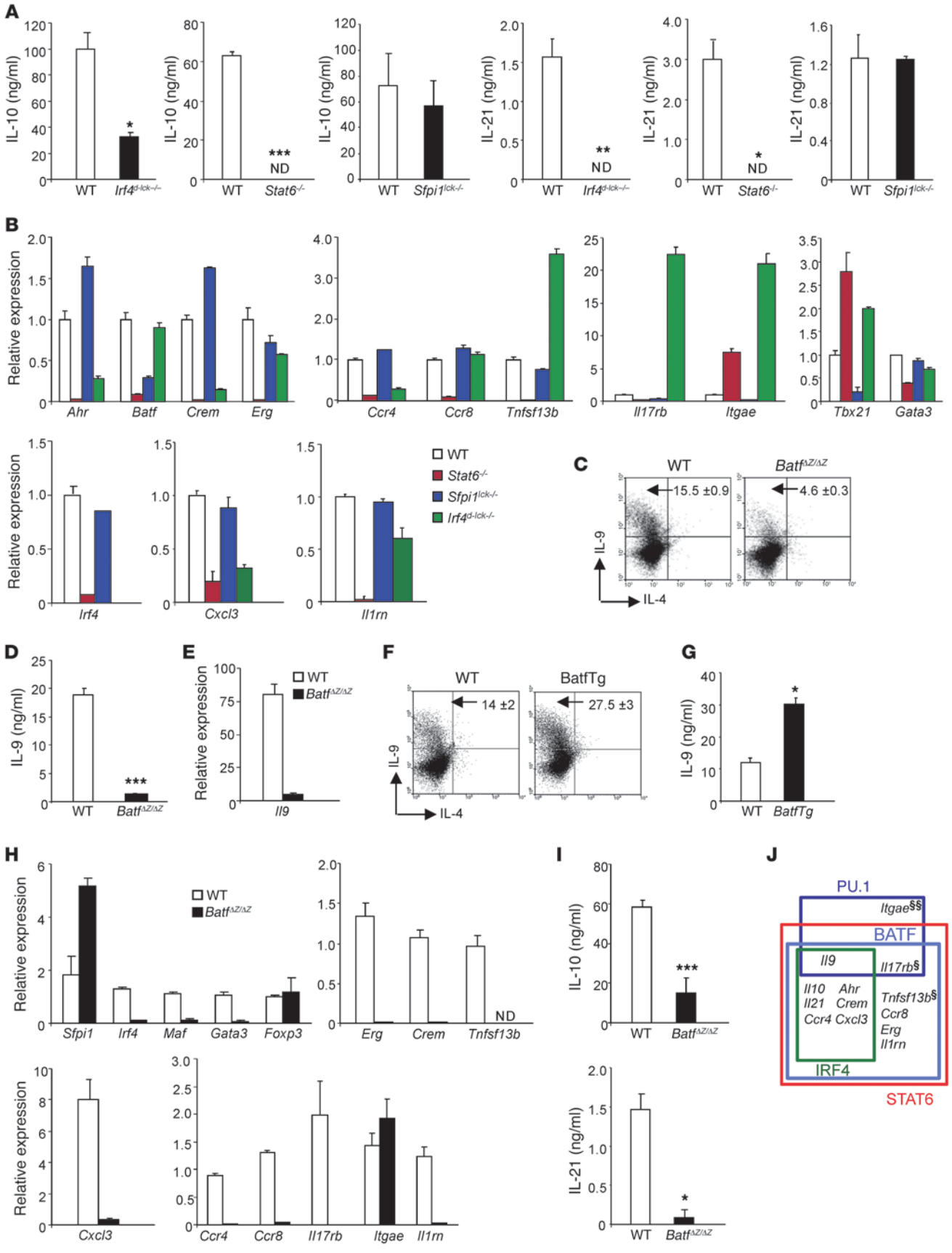
A
IL-10 (ng/ml)
WT
Irf4d-lck-/-
Stat6-/-
Sfpi1lck-/-
ND
IL-21 (ng/ml)
B
Relative expression
Ahr
Batf
Crem
Erg
Ccr4
Ccr8
Tnfsf13b
Il17rb
Itgae
Tbx21
Gata3
Irf4
Cxcl3
Il1rn
WT
Stat6-/-
Sfpi1lck-/-
Irf4d-lck-/-
C
WT
BatfΔZ/ΔZ
15.5 ±0.9
4.6 ±0.3
IL-9
IL-4
D
IL-9 (ng/ml)
BatfΔZ/ΔZ
E
Relative expression
Il9
F
BatfTg
14 ±2
27.5 ±3
G
IL-9 (ng/ml)
BatfTg
H
Sfpi1
Irf4
Maf
Gata3
Foxp3
Erg
Crem
Tnfsf13b
ND
I
IL-10 (ng/ml)
IL-21 (ng/ml)
J
PU.1
Itgae§§
BATF
Il9
Il17rb§
Il10
Ahr
Il21
Crem
Ccr4
Cxcl3
Tnfsf13b§
Ccr8
Erg
Il1rn
IRF4
STAT6

Figure 3
Transcription factor regulation of Th9-associated genes. Naive CD4+ T cells from mice of the indicated genotypes were differentiated under Th9 conditions. (**A**) Supernatants were isolated following anti-CD3 stimulation for 24 hours, and cytokine concentration was determined by ELISA. ND, not detected. (**B**) RNA was isolated from Th9 cells of each genotype for expression analysis of the indicated genes using qRT-PCR. Expression is relative to wild-type Th9 cells. Data are the average ± SD of three to six experiments. (**C**–**E**) Naive CD4+ T cells were isolated from wild-type and BATF-deficient mice and differentiated under Th9 polarizing conditions before analysis of IL-9 production using intracellular cytokine staining (**C**), ELISA following 24-hour stimulation with anti-CD3 (**D**) and qRT-PCR of mRNA following 6 hours of stimulation with anti-CD3 (**E**). (**F** and **G**) Naive CD4+ T cells from wild-type and *Batf* transgenic mice were differentiated under Th9 polarizing conditions before analysis of IL-9 production using intracellular cytokine staining (**F**) and ELISA following 24-hour stimulation with anti-CD3 (**G**). (**H**) RNA was isolated from wild-type and BATF-deficient Th9 cells for expression analysis of the indicated genes using qRT-PCR. (**I**) Supernatants were isolated following anti-CD3 stimulation for 24 hours and tested for cytokine concentration by ELISA. For all data, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$. (**J**) Diagram summarizing transcription factor regulation of genes in Th9 cells. Genes regulated by each transcription factor are within each box color-coded to the transcription factor. §Negatively regulated by IRF4; §§negatively regulated by IRF4 and STAT6.

(Figure 2, B and C). Moreover, in a Transwell experiment, Th9 cells migrated more efficiently toward increasing concentrations of CCL22, a ligand for CCR4 and CCR8, than did Th2 cells (Figure 2D). Supernatants from Th9 cells had the greatest concentrations of IL-1RA, encoded by *Il1rn*, which were detected using ELISA (Figure 2E). The addition of the AHR ligand FICZ to differentiation cultures increased the production of IL-9 from Th9 cells, but not from other Th cell subsets (Figure 2F and data not shown). Thus, Th9 cells have an identifiable transcriptional signature that results in unique functional properties.

BATF is part of a Th9 transcriptional network. IL-9 production in Th9 cells is dependent on transcription factors including PU.1, STAT6, and IRF4 (2–4, 7, 9). IL-10 and IL-21, which are also produced by Th9 cells (2, 19), are expressed independently of PU.1 (9, 11), but rely upon STAT6 and IRF4 (Figure 3A).

Having established an additional panel of genes demonstrating preferential expression in Th9 cells, we wanted to determine whether there were common requirements for Th9-inducing transcription factors in the expression of these genes. Consistent with IRF4 being downstream of STAT6 (3), there was a common requirement for both factors in the expression of *Ahr*, *Crem*, *Ccr4*, and *Cxcl3*, and both factors had negative effects on the expression of *Itgae* and *Tbx21*, the latter a transcription factor that negatively regulates IL-9 production (Figure 3B). The expression of *Batf*, *Gata3*, and *Ccr8* were dependent on STAT6 but not IRF4 (Figure 3B). In contrast, STAT6 was required for *Il1rn*, *Erg*, *Il17rb*, and *Tnfsf13b*, and IRF4 had a more modest or a negative effect on the expression of these genes (Figure 3B). PU.1 was required for the expression of a smaller subset of genes including *Il17rb*, *Itgae*, *Tbx21*, and *Batf*, had modest negative effects on the expression of *Ahr* and *Crem*, and did not have effects on the expression of other Th9 genes (Figure 3B).

The preferential expression of BATF in Th9 cells, coupled with the requirement for IRF4 in Th9 gene expression (ref. 7 and Figure 3B), and recent reports demonstrating IRF4 and BATF function cooperatively in Th17 cell development (16–18) suggested that BATF might contribute to Th9 generation. To test this, we differentiated naive CD4+ T cells from wild-type and BATF-deficient ($Batf^{\Delta Z/\Delta Z}$) mice under Th9 culture conditions. We observed a significant decrease in IL-9 production in BATF-deficient Th9 cultures, as assessed using intracellular cytokine staining and ELISA (Figure 3, C and D). Similarly, we observed decreased *Il9* mRNA in BATF-deficient Th9 cultures (Figure 3E). Importantly, in the absence of BATF, Th9 cells did not differentiate into other Th cell subsets because there were no changes in the production of IFN-γ, IL-4, and IL-17 or in the expression of FOXP3 (data not shown). In parallel experiments, we observed increased IL-9 production from *Batf* transgenic (*Batf* Tg) Th9 cultures compared with wild-type cells (Figure 3, F and G). The expression of all other Th9 genes identified, except for *Itgae*, was dependent on BATF (Figure 3H). In addition, we found that the expression of other transcription factors that promote Th9 development, including *Irf4* and *Gata3*, was also decreased, although BATF had a negative effect on *Sfpi1* expression (Figure 3H). Moreover, BATF was required for IL-10 and IL-21 production by Th9 cells (Figure 3I). Importantly, the addition of IL-21 to wild-type Th9 cultures modestly diminished IL-9 production, and neutralizing IL-21 modestly increased IL-9 production (data not shown), suggesting that the lack of IL-21 in BATF-deficient cultures is not linked to deficient IL-9 production. The role of BATF and the other Th9 transcription factors in regulating this set of genes is summarized in Figure 3J. Thus, BATF is a primary factor in the generation of the Th9 phenotype.

BATF is clearly downstream of STAT3 in Th17 and Th2 cells (20, 21) and, as shown here, is downstream of STAT6 in Th9 cells. Both of these STAT proteins are required for Th2 development, but despite this reliance, there are conflicting data on whether BATF is required for Th2 development (13, 15). To further explore this issue, we differentiated wild-type and BATF-deficient CD4+ T cells under Th2 conditions. We observed a decreased production of Th2 cytokines from BATF-deficient cultures compared with the amount produced by wild-type cells (Supplemental Figure 2, A–C). We further observed diminished expression of GATA3 in BATF-deficient Th2 and Th9 cells compared with control cells (Supplemental Figure 2D). Thus, similar to its requirement in Th9 differentiation, BATF is required for the normal development of murine Th2 cells.

BATF and IRF4 cooperate in activating the Th9 phenotype. Since IRF4 and BATF cooperate in the development of Th17 cells, we tested whether these factors also cooperate in the induction of IL-9. Wild-type, IRF4-deficient, and BATF-deficient naive CD4+ T cells were cultured under Th9 conditions and transduced with retroviruses expressing BATF or IRF4, or with both retroviruses and the corresponding control vectors. Although the transduction of BATF resulted in a 3- to 4-fold increase in the percentage of IL-9–positive cells when transduced into wild-type or BATF-deficient Th9 cultures, it did not induce IL-9 production from transduced Th2 or Th17 cells (Figure 4A). BATF transduction resulted in a 2-fold increase in IL-9 production when transduced into IRF4-deficient cells, suggesting that it had less of an effect in the absence of IRF4 (Figure 4A). IRF4 transduction had more modest effects, with less than a 2-fold increase in IL-9 production in wild-type Th9 cells (Figure 4B). Although IRF4 transduction resulted in a 2-fold increase in IL-9 production by IRF4- and BATF-deficient Th9 cells, the percentage of IL-9–positive cells was still less than the percentage of IL-9–positive control transduced wild-type cells (Figure 4B).

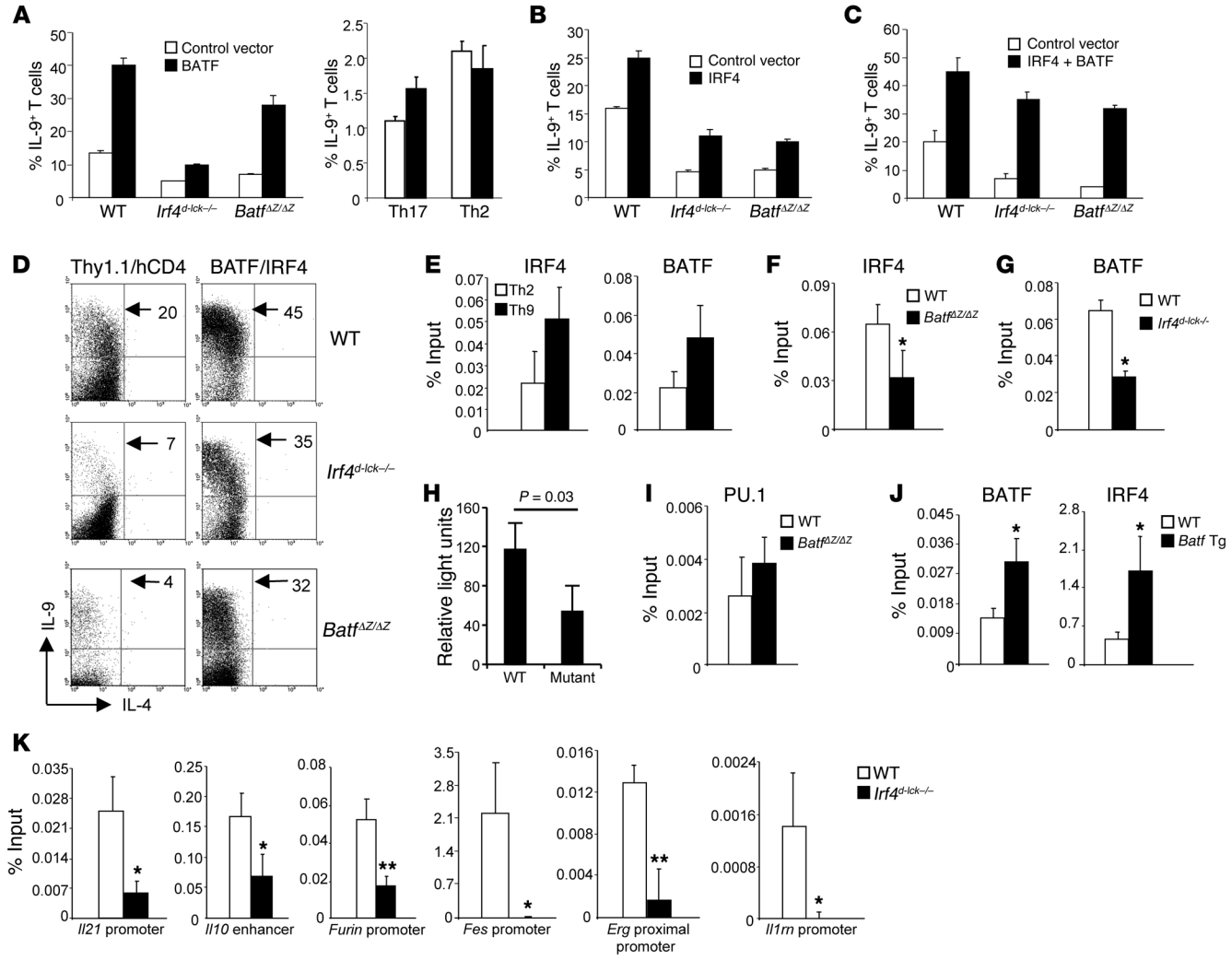

Figure 4

BATF and IRF4 cooperate in the development of Th9 cells. Wild-type, *Irf4*$^{d\text{-}lck-/-}$, or *Batf*$^{\Delta Z/\Delta Z}$ naive CD4$^+$ T cells were differentiated under Th9 polarizing conditions. (**A**–**D**) Differentiating Th9 cells were transduced with control (empty vectors expressing only Thy1.1 or hCD4), BATF-, and/or IRF4-expressing retroviruses. After 5 days of differentiation, cells were stimulated with PMA and ionomycin for 5 hours before intracellular staining for IL-9 and IL-4. Dot plots (**D**) are gated on cells that were double-positive for Thy1.1 and human CD4. Numbers indicate the average percentage ± SD of at least three mice. (**E**) ChIP assays were performed for BATF and IRF4 binding to the *Il9* promoter in Th2 and Th9 cells. (**F**) ChIP assay of IRF4 binding to the *Il9* promoter in wild-type and BATF-deficient Th9 cells. (**G**) ChIP assay of BATF binding to the *Il9* promoter in wild-type and IRF4-deficient Th9 cells. (**H**) Reporter assay of wild-type and mutant *IL9* promoter luciferase reporter vector cotransfected into HEK293 cells with vectors expressing BATF and IRF4. Mean ± SD of four experiments. (**I**) ChIP assay of PU.1 binding to the *Il9* promoter in wild-type and BATF-deficient Th9 cells. (**J**) ChIP assay of BATF and IRF4 binding to the *Il9* promoter in wild-type and *Batf* transgenic Th9 cells. (**K**) ChIP assay of BATF binding to the indicated gene promoters in wild-type and IRF4-deficient Th9 cells. All ChIP data represent the average ± SD of at least three experiments. **P* < 0.05; ***P* < 0.01.

However, cotransduction of BATF and IRF4 resulted in a dramatic increase in IL-9–positive wild-type cells and induced IL-9 production from IRF4- and BATF-deficient Th9 cells in amounts greater than that produced in wild-type control transduced cells (Figure 4, C and D). These results suggest that BATF induces IL-9 more efficiently in the context of IRF4.

To further define the basis for cooperation between BATF and IRF4 in Th9 cells, we performed ChIP assays for both factors at the promoter of *Il9* and other Th9 genes. Both IRF4 and BATF were bound in greater amounts at the *Il9* promoter in Th9 cells than in Th2 cells (Figure 4E). Optimal binding of BATF and IRF4 to the *Il9* promoter required the presence of the other factor, as demonstrated by decreased binding of each factor in the reciprocal gene-deficient cells (Figure 4, F and G). To further support the function of BATF-IRF4 cooperation, mutation of a near match to the consensus for a composite AP-1-IRF4 site at -185 in the *Il9* promoter (17) resulted in decreased *IL9* promoter reporter activity (Figure 4H). As a control, we observed that BATF was not required for PU.1 binding to the *Il9* locus (Figure 4I). Consistent with results from BATF-deficient cells, increased BATF binding in *Batf* transgenic Th9 cultures resulted in increased IRF4 binding (Figure 4J). We expanded these observations to examine other

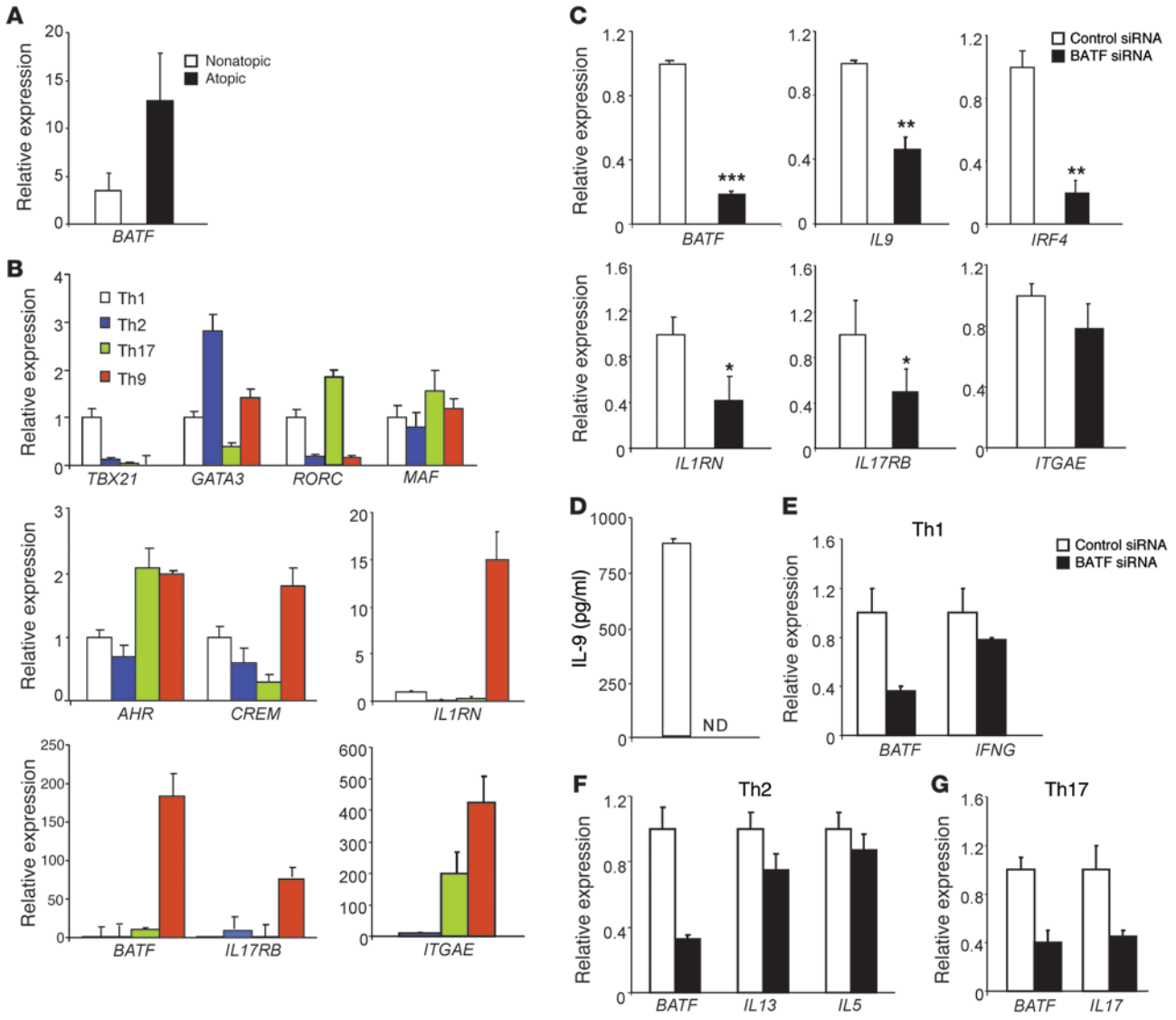

Figure 5

BATF is required for Th9 differentiation in human T cells. (**A**) Naive human CD4+ T cells isolated by nonatopic or atopic infants were differentiated under Th9 polarizing conditions. After 5 days of culture, RNA was isolated and *BATF* mRNA was determined using qRT-PCR (n = 5–8). (**B**) Naive human T cells were differentiated under conditions for each of the indicated subsets. After 2 weeks of differentiation, RNA was isolated to assess the expression of the indicated genes by qRT-PCR. (**C**–**G**) Naive human CD4+ T cells were differentiated under Th9 (**C** and **D**), Th1 (**E**), Th2 (**F**), or Th17 (**G**) conditions and transfected with control or BATF-specific siRNA. After 5 to 6 days of differentiation, cells were stimulated with anti-CD3, and RNA was isolated for the assessment of gene expression by qRT-PCR (**C** and **E**–**G**), or supernatants were assessed for IL-9 production by ELISA (**D**). Results represent the average of values from four donors. *P < 0.05; **P < 0.01; ***P < 0.0001.

BATF target genes in Th9 cells (Figure 3H and data not shown) and observed a broad requirement for IRF4 in the binding of BATF to target genes in Th9 cells (Figure 4K).

BATF is required for human Th9 cells. Since BATF is critical for the Th9 phenotype and Th9 cells promote allergic inflammation, we wanted to test whether BATF is associated with allergic disease. We first examined samples from patients' Th9 cells differentiated from naive CD4+ T cells isolated from atopic or nonatopic infants. We have previously shown that Th9 cells from atopic donors produce more IL-9 and have greater expression of *SPI1* compared with nonatopic donors (22). We observed that *BATF* expression was increased in cultures from atopic donors (Figure 5A).

To determine whether *BATF* demonstrated enriched expression in human Th subsets, we differentiated naive CD4+ T cells from multiple donors under polarizing conditions. Appropriate differentiation was confirmed by the expression of the lineage-associated genes *TBX21*, *GATA3*, and *RORC* (Figure 5B). Although IRF4 was not differentially expressed among the subsets, we observed that *BATF* expression was higher in Th9 cells than in Th1, Th2, or Th17 cultures (Figure 5B). Moreover, human Th9 cultures demonstrated an enriched expression of genes observed in mouse Th9 cultures including *AHR*, *IL17RB*, *IL1RN*, *CREM*, and *ITGAE* (Figure 5B).

The requirement for other transcription factors that promote Th9 development, such as PU.1, is conserved between mouse and

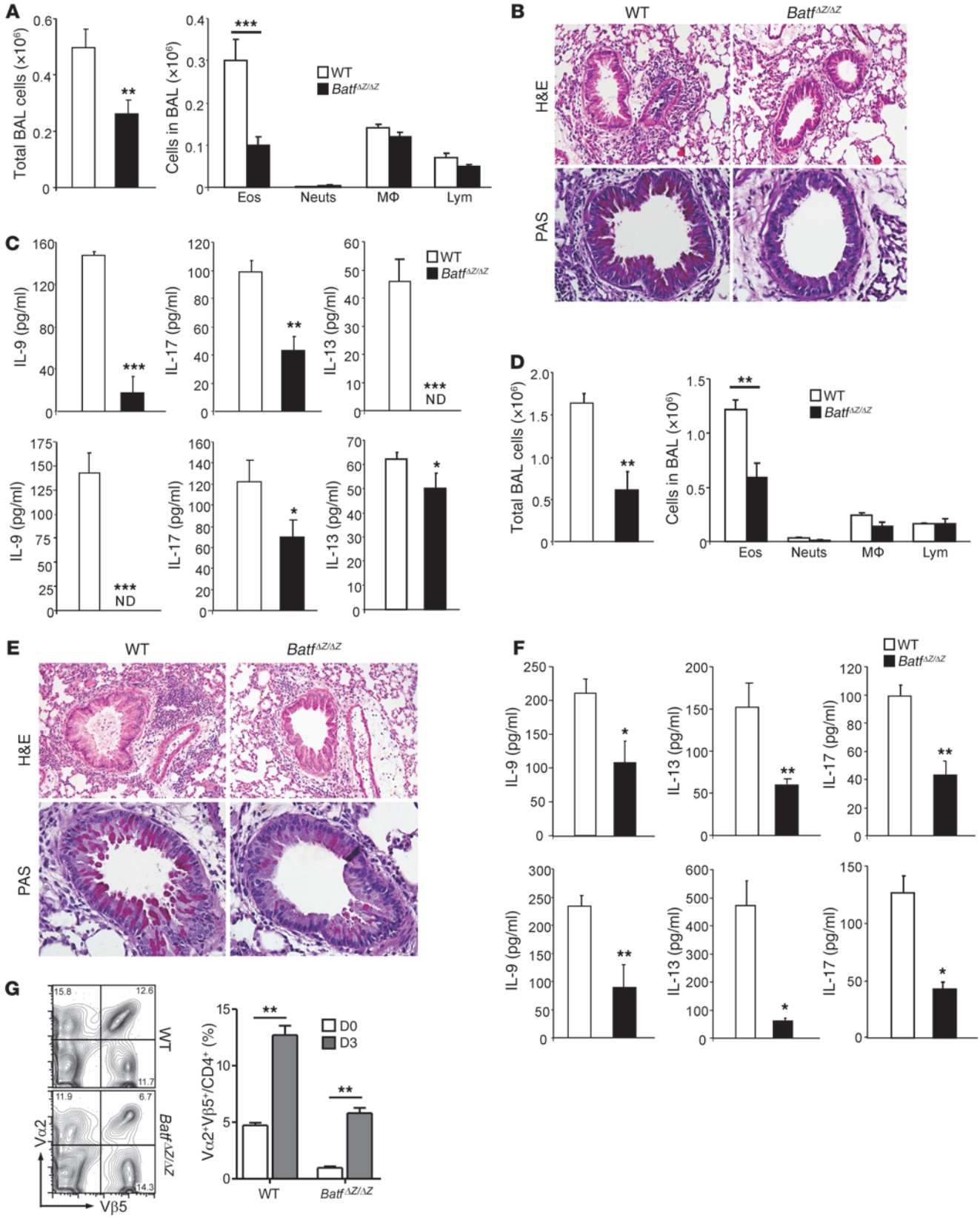
A
Total BAL cells (×10⁶)
Cells in BAL (×10⁶)
WT
Batf^ΔZ/ΔZ
Eos
Neuts
MΦ
Lym
B
WT
Batf^ΔZ/ΔZ
H&E
PAS
C
IL-9 (pg/ml)
IL-17 (pg/ml)
IL-13 (pg/ml)
ND
D
Total BAL cells (×10⁶)
Cells in BAL (×10⁶)
E
WT
Batf^ΔZ/ΔZ
H&E
PAS
F
IL-9 (pg/ml)
IL-13 (pg/ml)
IL-17 (pg/ml)
G
WT
Batf^ΔZ/ΔZ
Vα2
Vβ5
Vα2⁺Vβ5⁺/CD4⁺ (%)
D0
D3

Figure 6
BATF in T cells is required for the development of allergic inflammation. (**A**–**C**) CD4+ T cells from wild-type or BATF-deficient mice were transferred into RAG-deficient recipients. Recipients were challenged i.n. with OVA and TSLP as described in Methods before analysis of pulmonary inflammation. (**A**) Cell numbers from the BAL (average ± SEM of 7 to 9 mice). (**B**) Airway histology from mice. Top panels are stained with H&E. Bottom panels are stained for mucus production with PAS. Original magnification, ×40. (**C**) ELISA analysis of cytokine concentration in the BAL (top) or in supernatants from OVA-stimulated mediastinal lymph node cells (bottom). (**D**–**G**) Wild-type and BATF-deficient OTII cells were polarized in vitro under Th9 conditions and transferred into wild-type recipients before daily i.n. challenges with OVA and TSLP for 5 days and subsequent analysis of pulmonary inflammation. (**D**) Cell numbers from the BAL (average ± SEM of 7 to 8 mice). (**E**) Airway histology from mice treated as indicated. Top panels show cells stained with H&E. Bottom panels show cells stained for mucus production with PAS. Original magnification, ×40. (**F**) ELISA analysis of cytokine concentration in the BAL (top) or in supernatants from OVA-stimulated mediastinal lymph node cells (bottom). (**G**) Quantification of OTII cells from mediastinal lymph node before (D0) and after (D3) OVA stimulation by flow cytometry (contour plots; graph indicates the average ± SEM of all samples). Eos, eosinophils; Neuts, neutrophils; Mϕ, macrophages; Lym, lymphocytes. *$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$.

human cells (9, 23). To directly test the requirement for BATF in human Th9 differentiation, we differentiated naive CD4+ T cells from healthy donors under Th9 polarizing conditions and transfected them with *BATF*-specific or control siRNA. *BATF*-specific siRNA treatment decreased *BATF* mRNA levels compared with the control transfected cells and resulted in decreased expression of *IL9*, diminished production of IL-9, and decreased expression of other Th9-associated genes including *IL1RN* and *IL17RB*, but not *ITGAE*, which was also BATF independent in the mouse system (Figure 5, C and D). As with the murine system, BATF contributes to cytokine production in additional human Th lineages. BATF siRNA resulted in a significant decrease in the expression of *IL17* in Th17 cells, but no significant effects on cytokine expression from Th1 and Th2 cells were detected (Figure 5, E–G). Thus, BATF function in Th9 and Th17 cells is conserved between mouse and human Th cells.

BATF expression in T cells is required for the development of allergic inflammation. Since Th9 cells promote allergic inflammation and BATF promotes the Th9 phenotype, we wanted to determine whether BATF promotes allergic inflammation. However, testing the development of allergic disease in BATF-deficient mice is not informative because in addition to lacking Th2 and Th17 cells, subsets that are required for most of the disease models, they might also have defects in antigen presentation (13, 15, 24). Thus, we employed several approaches to define the requirement for T cell BATF expression in models of allergic inflammation.

Wild-type or BATF-deficient CD4 T cells were transferred into RAG-deficient recipients and challenged i.n. with OVA and TSLP every 48 hours for 2 weeks, a protocol we have previously shown to be IL-9 and Th9 dependent (25). We observed decreased cell numbers, particularly eosinophils, in recipients of BATF-deficient T cells compared with recipients of wild-type T cells (Figure 6A). Histological analysis revealed that recipients of BATF-deficient T cells also had diminished inflammation and airway mucus production compared with wild-type T cell recipients (Figure 6B). Consistent with the decreased inflammation and with the in vitro analysis of the effects of BATF on Th cytokine production, we observed diminished amounts of IL-9, IL-13, and IL-17 in the BAL fluid and in supernatants from OVA-stimulated mediastinal lymph node cells of BATF-deficient T cell recipients compared with those found in wild-type recipients (Figure 6C).

As a parallel approach, we polarized wild-type and BATF-deficient OTII cells under Th9 conditions and transferred them into wild-type recipients. Recipients were challenged with OVA and thymic stromal lymphopoietin (TSLP) daily for 5 days before analysis of lung inflammation. We observed decreased cell numbers, particularly eosinophils, in recipients of BATF-deficient OTII cells compared with recipients of wild type OTII cells (Figure 6D). We found that recipients of BATF-deficient OTII cells also had diminished inflammation and airway mucus production, as assessed by histological analysis, compared with wild-type OTII cell recipients (Figure 6E). Consistent with the decreased inflammation, we observed diminished amounts of IL-9, and IL-13 in the BAL fluid and in supernatants from OVA-stimulated mediastinal lymph node cells of BATF-deficient OTII cell recipients compared with the amounts found in wild-type recipients (Figure 6F). To define the OTII response from these mice, we assessed the percentage of cells positive for the OTII TCR (Vα2/Vβ5) in mediastinal lymph nodes harvested after the final challenge, before and after 3 days of culture with OVA. Although there were decreased percentages of Vα2/Vβ5-positive cells in the lymph nodes from recipients of BATF-deficient cells, commensurate with the decreased inflammation in those mice, BATF-deficient OTII cells had a greater fold increase in these percentages following OVA stimulation than was detected in wild-type cells, confirming the ability of BATF-deficient T cells to respond to antigen in this model (Figure 6G).

Finally, we determined whether IL-9 was necessary for BATF to promote allergic inflammation. CD4+ T cells from D011.10 TCR transgenic mice were transduced with control or BATF-expressing retroviruses and differentiated under Th9 polarizing conditions. Transduced cells were sorted and transferred into recipient BALB/c mice that were subjected to i.n. OVA challenge for 5 days. On days 1, 3, and 5 of challenge, mice received an i.v. injection of 10 μg anti–IL-9 or rat IgG2B control antibodies. Twenty-four hours after the last challenge, mice were analyzed for pulmonary inflammation and cytokine expression. While mice receiving no Th9 cells (PBS group) showed no signs of airway inflammation after OVA challenge, the transfer of control retrovirus–transduced Th9 cells resulted in a significant increase in inflammation and in the number of cells recovered from the BAL, particularly in the macrophage and eosinophil populations (Figure 6, A and B). The transfer of BATF-expressing retrovirus-transduced Th9 cells resulted in greater inflammation and an increase in these cell populations, and coinjection of anti–IL-9 diminished the effect of BATF transduction (Figure 7, A and B).

The transfer of BATF-transduced Th9 cells led to increased mRNA in the recipient lung for genes associated with goblet cell metaplasia (*Gob5*, *Muc5ac*), and this correlated with the amount of PAS staining of airway tissue (Figure 7, B and C). The transfer of BATF-transduced Th9 cells led to a similar increase in mRNA in the recipient lung for *Il9*, but had insignificant effects on *Il13* compared with control transduced cells (Figure 6C). There was a similar increase in IL-9 present in the BAL fluid (Figure 7D). IL-13 and IL-17 concentrations were also increased in the BAL fluid from recipients of Th9 cells transduced with BATF-expressing retrovirus (Figure 7D). Importantly, the treatment of mice receiving BATF-transduced T cells with anti–IL-9 decreased gene

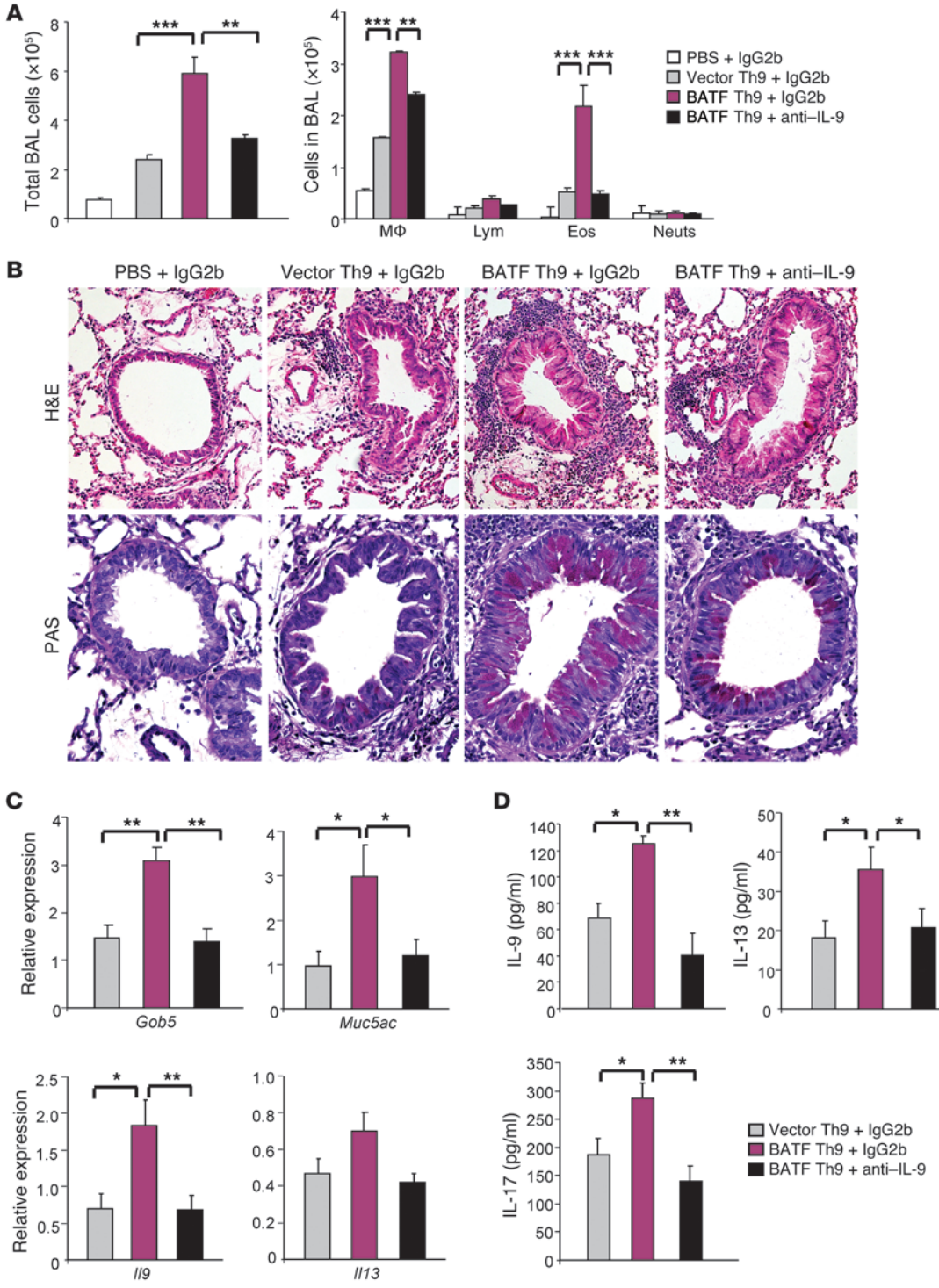

Figure 7

BATF promotes a proallergic Th9 phenotype. DO11.10 TCR transgenic cells were cultured under Th9 polarizing conditions and transduced with control or BATF-expressing bicistronic retrovirus coexpressing Thy1.1 as a marker. After 5 days of culture, Thy1.1$^+$ cells were isolated and transferred into BALB/c recipient mice that underwent i.n. challenges of OVA daily for 5 days. Recipients were treated with control or anti–IL-9 antibodies as indicated. Mice were sacrificed for analysis 24 hours after the last challenge. Data are shown as the average ± SEM of 5 to 8 mice per group and are representative of two experiments. (**A**) Cell numbers in the BAL and differential counts are indicated for each of the treatment groups. (**B**) Airway histology from mice treated as indicated. Top panels show cells stained with H&E. Bottom panels show cells stained for mucus production with PAS. Original magnification, ×40. (**C**) RNA was isolated from the lungs of mice in each of the three treatment groups, and analysis of gene expression was performed using qRT-PCR for the indicated genes. (**D**) Cytokine concentration in the BAL fluid was determined by ELISA. *P < 0.05; **P < 0.01; ***P < 0.0001.

expression, mucus production, and cytokine concentrations to amounts observed in the control transduced cells (Figure 7, B–D), suggesting that BATF-transduced T cells increase pulmonary inflammation through IL-9–dependent mechanisms. Together, these data support a central role for BATF in promoting Th9 development and function.

Discussion

The Th cell subset paradigm has evolved from a model of distinct lineages to one where there is considerable plasticity among the acquired phenotypes (26, 27). Yet, Th cells with polarized cytokine-secreting potential are observed in vivo, and thus regardless of plasticity, it is still important to define how the potential for secretion of a particular cytokine and other functional properties are established. In this report, we have defined a Th9 cell gene signature, and within a subset of genes in that signature, we have described how transcription factors that promote IL-9 production contribute to the regulation of that gene signature. One of the genes in the signature, *Batf*, is required for the production of IL-9 and for the expression of most genes in the Th9 signature. Moreover, BATF in T cells is required for the development of allergic inflammation, and Th9 cells ectopically expressing BATF were more efficient in the induction of allergic inflammation than the control transduced cells. These studies indicate a central role for BATF in Th9 differentiation and in the development of allergic inflammation.

BATF promotes Th9 development in both human and mouse T cells as part of a transcription factor network downstream of the Th9-inducing signals from IL-4 and TGFβ. STAT6 is required for BATF expression, and BATF may be a primary target of STAT6, because additional transcription factors required for Th9 cell differentiation, including *Gata3* and *Irf4*, require both STAT6 and BATF for expression. Each of these factors, along with PU.1, form a network that regulates *Il9* and other Th9-associated genes. STAT6 and BATF are required for all Th9-associated genes, except for the subset more specifically induced by TGFβ signaling, such as *Itgae*. IRF4 also contributes to the expression of a majority of Th9 genes, though some genes were IRF4 independent, and some, such as *Il17rb* and *Tnfsf13b*, were negatively regulated by IRF4. In addition to *Il9*, PU.1 regulated a smaller subset of genes, including *Il17rb* and *Itgae*, but also contributed to *Batf* expression. Together, these data suggest that the Th9 transcriptional signature arises from the balanced activity of multiple transcription factors during differentiation.

It is still not clear whether any of the transcription factors that promote Th9 development could be termed a master regulator. Although that term may be antiquated by studies showing cooperation among many factors in establishing Th cell subset identity (16, 28, 29), the ability of GATA3 and T-bet to induce cytokines in opposing lineages (30, 31) still distinguishes them from other factors. In contrast to lineage-defining factors, there are additional transcription factors that have lineage-specific functions in several Th subsets. For example, BATF and IRF4 are required for Th2 and Th17 subsets as well as for Th9 cell development (13, 15, 32, 33). Although BATF and IRF4 induce IL-9 in Th9 cultures, they do not induce IL-9 when transduced into T cells cultured under other polarizing conditions. Moreover, the cotransduction of IRF4 and BATF into *Stat6*$^{-/-}$ Th9 cultures is not sufficient to induce IL-9, suggesting that additional STAT6-regulated genes are required to establish an IL-9–secreting T cell phenotype. PU.1 is still the closest to a master regulator of the Th9 phenotype, as it is able to convert Th2 cells into Th9 cells by both inducing IL-9 and repressing Th2 cytokines (9, 10). However, PU.1 has a limited ability to induce IL-9 in other polarizing Th cell cultures and, as we have shown in this report, is only required for a subset of the Th9 gene signature. Whether additional transcription factors are critical for establishing Th9 identity is still not known.

BATF and IRF4 have recently been demonstrated by several groups to function as a module in Th17 cells based on genome-wide studies identifying a broad overlap between the binding sites for both factors (16–18). Although a genome-wide analysis for these factors in other Th subsets where they are required has not yet been performed, our focused analysis supports extending this paradigm to Th9 cells. We observed that binding of IRF4 and BATF to target genes was diminished in the reciprocal gene-deficient cells at the *Il9* and other Th9 loci, although PU.1 binding was not affected. Since BATF regulates IRF4 expression, some of the decrease in IRF4 binding observed in BATF-deficient cells could be due to reduced amounts of IRF4 within the cell. However, there was strong cooperation of IRF4 and BATF when they were cotransduced into developing Th9 cells. In contrast to PU.1 and IRF4 cooperation in B cells (34), we demonstrated that PU.1 interferes with IRF4 function in Th2 cells (35), and this is further substantiated in Th9 cells, wherein PU.1 and IRF4 had opposing effects on several Th9 genes. These data suggest that the ability of PU.1 to promote Th9 development is functionally distinct from the activity of the BATF-IRF4 module.

The requirement for BATF in the development of human IL-9–secreting T cells underscores the potential importance of Th9 cells in atopic disease. IL-9–secreting T cells are increased in patients with allergies, and there is increased allergen-specific IL-9 produced by T cells isolated from atopic infants compared with controls (22, 36). More recently, serum IL-9 in infants correlated with concentrations of TSLP, a cytokine that broadly promotes allergic inflammation (25, 37). In human T cell cultures, TSLPR expression was greatest on Th2 and Th9 cells, and TSLP selectively increased IL-13 from Th2 cells and IL-9 from Th9 cells (25). Our data in this report, and previous work from our lab and others, suggest that the transcriptional network promoting the development of IL-9–secreting T cells and requiring STAT6, PU.1, IRF4, and BATF is similar in mouse and human T cells (7, 9, 22, 23). This conservation will be critical in further defining the development of proallergic Th9 cell function.

In addition to BATF, the microarray studies we performed defined a broader set of genes that may be functionally linked to Th9 biology. The expression of receptors for chemokines (CCR4, CCR8) associated with allergic inflammation correlates with the presence of IL-9–secreting T cells at sites of allergic inflammation (6). The production of IL-1RA could be related to reports that Th9 cells suppress Th17-mediated inflammation (38, 39). Th9 cells could be the source of BAFF released following allergen challenge of patients (40). The expression of CD103 by Th9 cells is consistent with a role for these cells at mucosal surfaces. Importantly, we observed that BATF in T cells was required for the development of allergic airway inflammation and that BATF-transduced Th9 cells demonstrated a potent ability to promote allergic inflammation in vivo. Although this could be the result of many factors, IL-9 was critical in this biological function. Yet, the selective depletion of additional BATF target genes might define a role for additional contributions to the development of allergic disease. Further work will define how additional components of the Th9 cell signature contribute to immune responses in vivo.

Methods

Mice. C57BL/6 and BALB/c mice were purchased from Harlan Laboratories. *Stat6*$^{-/-}$ (BALB/c), *Sfpi1*$^{fl/fl}$ Lck-Cre (C57BL/6), and *Batf*$^{\Delta Z/\Delta Z}$ (C57BL/6) and *Batf* transgenic (FVB background crossed to C57BL/6) mice were previously described (9, 13, 41–43), and control littermates were used where appropriate. OTII, *Irf4*$^{fl/fl}$, and DO11.10 TCR transgenic mice were purchased from The Jackson Laboratory, *Batf*$^{\Delta Z/\Delta Z}$ mice were mated to OTII transgenic mice (44), and *Irf4*$^{fl/fl}$ mice were mated to *d-Lck-Cre* transgenic mice.

Patient samples. The population of atopic infants has been previously described in detail (45).

Th cell differentiation. Human and mouse Th cell differentiation was performed as described (9, 22). After 5 to 6 days of culture, differentiated cells were restimulated for 24 hours with plate-bound anti-CD3 (2 μg/ml), and cell-free supernatants collected after centrifugation were stored at –20°C for measuring cytokine production by ELISA. In some experiments, cell pellets were collected after 6 hours of restimulation for RNA extraction and analysis of gene expression by quantitative RT-PCR (qRT-PCR), normalized to β-2 microglobulin expression. For intracellular staining, cells were restimulated with PMA and ionomycin for 5 hours, and monensin was added to the cells for the final 2 hours of stimulation. Cells were fixed with 4% neutral buffered PFA and permeabilized with saponin. Cells were then stained with fluorochrome-conjugated anti-mouse IL-9, IL-4, or IL-17, as indicated in the Figure legends (eBioscience or BioLegend).

ChIP. ChIP assay was performed as previously described (11). Immunoprecipitations were performed with rabbit polyclonal antibodies (rabbit control IgG, IRF4, or BATF) (Santa Cruz Biotechnology Inc.) (46). Quantification of binding DNA was performed with SYBR Green Fast PCR Master Mix (primers for the *Il9* promoter are described in ref. 9) using the ABI 7500 Fast Real-time PCR System (both from Applied Biosystems). To quantify immunoprecipitated DNA, a standard curve was generated from serial dilutions of input DNA. To calculate ChIP results as a percentage of input, the amount of the immunoprecipitated DNA from the IgG control was subtracted from the amount of the immunoprecipitated DNA from the specific antibody ChIP, followed by normalizing against the amount of the input DNA.

Reporter assay. HEK293T cells were transfected with WT or a mutant human IL-9 (hIL9) promoter *Renilla* luciferase reporter construct (SwitchGear Genomics; the mutant lacking the IRF4 binding portion [-188 to -197] of the composite site was generated using the QuikChange II Mutagenesis Kit from Stratagene) and IRF4 and BATF retroviral plasmids. Cells were harvested 24 hours after transfection and assayed for *Renilla* luciferase activity.

Microarrays and analysis. RNA was isolated from Th2, Th9, and Treg cells, each differentiated for 5 days in vitro and performed in triplicate, and submitted to Miltenyi Biotec for microarray analysis using the Agilent Whole Mouse Genome Oligo Microarray 4x44K. Signals in the Microarray Scanner System were processed with Feature Extraction Software (both from Agilent). The generated datasets were normalized using $\log_2$ and quantile transformation. Detection *P* values less than 0.01 were tagged as significant expression and used for further analysis. Hierarchical clustering of total array data was performed using MultiExperiment Viewer (MeV) software, version 4.8, with Pearson's correlation and of the selected gene list with Manhattan distance analysis. We initially performed pairwise comparisons among the Th subsets to generate gene lists based on fold change differences in expression, and these lists were compared to generate a list of genes unique (greater than 2-fold enriched) for the Th9 subset. This was further divided into genes that were 5-fold enriched in Th9 cells versus the other subsets and genes that were 5-fold enriched in one subset but in the range of 2- to 5-fold enriched in the other subset. Raw microarray data were submitted to the Gene Expression Omnibus database (GEO GSE44937).

Retroviral transduction. Bicistronic retroviral vectors encoding mouse IRF4 and human CD4 (35) and BATF subcloned into the bicistronic retroviral vector containing the marker Thy1.1 and Thy1.1 empty vector (provided by S. Goenka (Indiana University, Indianapolis, Indiana, USA) were used to generate virus as described (9). After 2 days of differentiation, Th cells were transduced with retroviral supernatant containing polybrene. On day 5, transduced cells (Thy1.1 or hCD4$^+$ cells) were analyzed by flow cytometry for IL-9 and IL-4 secretion.

Adoptive transfer experiments and IL-9 neutralization. Total CD4 T cells were isolated from WT or BATF-deficient mice, and 10×10^6 cells were adoptively transferred into *Rag*-deficient mice. Mice were challenged i.n. with 100 μg OVA plus 500 ng TSLP every 48 hours for 2 weeks and sacrificed 24 hours after the last challenge for further analysis. Wild-type and BATF-deficient OTII cells were differentiated under Th9 conditions, and $3–4 \times 10^6$ cells were transferred into wild-type recipients. Recipients were then challenged with 100 μg OVA plus 500 ng TSLP daily for 5 days. Mice were sacrificed 24 hours after the last challenge for further analysis. Differentiated and BATF- or vector-transduced OVA-specific DO11.10 Th9 cells ($2–3 \times 10^6$ T cells in 200 μl PBS) were adoptively transferred into wild-type BALB/c recipient mice via tail-vein injection. Twenty-four hours after cell transfer, mice were challenged i.n. with 100 μg OVA for 5 days. To neutralize IL-9 cytokine in vivo, mice were injected via the tail vein with anti–IL-9 (10 μg/dose) or IgG2b control Ab (10 μg/dose) (R&D Systems) on days 1, 3, and 5. Mice were then sacrificed 24 hours after the last challenge for further analysis. All values are the average ± SEM of the number of mice indicated and are representative of two to three experiments.

BAL, tissue fixation, and staining. Mice were euthanized and BAL was performed as described previously (47). Briefly, lungs were washed three times with cold PBS. BAL fluid fractions were centrifuged at 1,400 *g* for 5 minutes at 4°C. Pellets were resuspended and counted. Cytospin preparations were stained with modified Wright-Giemsa stain, and differential cell counts were evaluated by counting at least 200 cells for determination of the relative percentage of each cell type in the BAL. The cytokine levels in BAL fluid were assessed using ELISA. After lavage, lungs were excised from the thoracic cavity, inflated with 4% neutral buffered formaldehyde, and fixed overnight at room temperature. Tissues were embedded in paraffin, sectioned, and stained with H&E or PAS.

Statistics. All statistical analysis was performed using a Student's *t* test, and a *P* value of less than 0.05 was considered significant.

Study approval. The IACUC of Indiana University approved all protocols using mice. All blood draws and human cell experimentation were performed with the approval of the IRB of Indiana University.

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