Inflammatory responses induce an identity crisis of alveolar macrophages, leading to pulmonary alveolar proteinosis

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Pulmonary alveolar proteinosis (PAP) is a severe respiratory disease characterized by dyspnea caused by accumulation of surfactant protein. Dysfunction of alveolar macrophages (AMs), which regulate the homeostasis of surfactant protein, leads to the development of PAP; for example, in mice lacking BTB and CNC homology 2 (Bach2). However, how Bach2 helps prevent PAP is unknown, and the cell-specific effects of Bach2 are undefined. Using mice lacking Bach2 in specific cell types, we found that the PAP phenotype of Bach2-deficient mice is due to Bach2 deficiency in more than two types of immune cells. Depletion of hyperactivated T cells in Bach2-deficient mice restored normal function of AMs and ameliorated PAP. We also found that, in Bach2-deficient mice, hyperactivated T cells induced gene expression patterns that are specific to other tissue-resident AMs. Moreover, hyperactivated T cells in Bach2-deficient mice, in which Bach2 then bound to regulatory regions of inflammation-associated genes in myeloid cells. Of note, in AMs, Bach2 restricted aberrant responses to excessive T cell-induced inflammation, whereas, in T cells, Bach2 puts a brake on T cell activation. Moreover, Bach2 stimulated the expression of multiple histone genes in AMs, suggesting a role of Bach2 in proper histone expression. We conclude that Bach2 is critical for the maintenance of AM identity and self-renewal in inflammatory environments. Treatments targeting T cells may offer new therapeutic strategies for managing secondary PAP.

Pulmonary alveolar proteinosis (PAP) is a rare pulmonary disease accompanied by an accumulation of pulmonary surfactant in alveolar spaces (1). Alveolar macrophages (AMs) have critical functions in maintaining homeostasis in the lungs by phagocytosing and catabolizing excess surfactant, consisting of 90% phospholipids and neural lipids and 10% surfactant protein (SP) (1). PAP is clinically classified into four types based on the cause: autoimmune, hereditary, congenital, and secondary. Patients with autoimmune PAP have neutralizing autoantibodies against GM-CSF (2). Hereditary PAP is due to mutations in genes such as GM-CSF receptor (3). Normal signaling of GM-CSF and the resulting expression of Pparg are indispensable for the development of mature AMs (4). Congenital PAP is due to mutation in genes encoding SP-B, SP-C, ABCA3, and NKX2.1 (5–7). Secondary PAP often accompanies hematopoietic diseases such as leukemia, infection, or Behçet’s disease. However, its mechanism is still unclear.

Tissue-resident macrophages are now recognized to possess tissue-specific functions, to originate from monocytes in the fetal liver or yolk sac, and to be maintained after birth by self-

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This article contains supplemental Fig. S1.

The microarray, RNA sequencing, and ChIP sequencing data have been deposited at the Gene Expression Omnibus database under accession codes GSE79139 and GSE79558.

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renewal (8, 9). Transcription factors play critical roles in the development and acquisition of the specific functions of tissue-resident macrophages (10, 11). The expression of such transcription factors and, thus, the identities of tissue-resident macrophages depend on the tissue environment, which collaborates with PU.1 to establish tissue macrophage–specific enhancers (12).

BTB and CNC homology 2 (Bach2) is a transcription repressor and regulates the immune system in multiple cell lineages, including B cells (13, 14), T cells (15–18), and common lymphoid progenitors (19). We previously observed dysfunction of AMs and development of PAP in Bach2-deficient mice (20).

Because these mice did not show abnormal expression of the genes involved in GM-CSF signaling (20), Bach2-deficient mice may be an interesting model of secondary PAP. However, how Bach2 functions to prevent PAP has been unclear (20). It has also been unclear whether a lack of Bach2 only in AMs or T cells would lead to the development of PAP. Therefore, we aimed to identify cell lineages where Bach2 functions to prevent PAP and then to clarify target genes of Bach2 in those cells. With this approach, we tried to understand the precise mechanism whereby deficiency of Bach2 impairs the function of AMs.

Results

Conditional ablation of Bach2 in single cell lineages did not result in PAP

First, to clarify which cells are primarily responsible for the altered function of AMs and the etiology of PAP observed in Bach2-deficient mice, we analyzed a series of genetically modified mice. We first generated Bach2/Rag2 double-deficient mice (21). At 13 weeks of age, there was no sign of PAP in their lungs, and the surface expression pattern of AMs was also normal in Bach2/Rag2 double-deficient mice (F4/80int-hi, CD11bint-hi, SiglecFhi, CD11chi) (Fig. 1, A and B). In contrast, AMs in control Bach2−/−/Rag2−/− mice showed an abnormal pattern of surface marker expression (F4/80int, CD11bhi, SiglecFint, CD11chih) (Fig. 1, A and B). In contrast, AMs in control Bach2−/−/Rag2−/− mice showed an abnormal pattern of surface marker expression (F4/80int, CD11bhi, SiglecFint, CD11chih), showing that T, B, and/or natural killer T (NKT) cells were essential for the onset of PAP in Bach2-deficient mice.

We next deleted Bach2 specifically in B or T cells. We found that Bach2−/−/mb1cre+ mice lacking Bach2 in B cells (22, 23) did not develop PAP when they were 26 weeks of age (Fig. 1C). Blimp-1 (Pdm-1) is the master regulator of plasma cell differentiation, and B cell–specific Blimp-1−/− mice do not secrete any antibodies (24). Although B cell–specific Blimp-1−/− mice did not develop PAP, Bach2/Blimp-1−/− mice still developed PAP (Fig. 1D), showing that autoantibodies were not involved in the pathogenesis of PAP. Therefore, deficiency of Bach2 in B cells did not have any impact on AMs. Bach2−/−/CD4cre/+ mice lacking Bach2 specifically in the T cell lineage (18) showed no pathological features of PAP (Fig. 1E). However, there was an increased number of cells, including eosinophils and lymphoid cells, in the bronchoalveolar lavage (BAL) prepared from Bach2−/−/CD4cre/+ mice at 26 weeks of age compared with control (Bach2+/−/CD4+/+) mice (total cells, 6.7 × 10⁷/mouse versus 1.8 × 10⁷/mouse; eosinophils, 1.3 × 10⁷/mouse versus 8.4 × 10⁶/mouse; lymphocytes, 8.0 × 10⁵/mouse versus 6.4 × 10⁴/mouse). Nonetheless, the AMs of Bach2−/−/CD4cre/+ mice showed a normal expression pattern of surface markers (F4/80int-hi, CD11bint-hi, SiglecFhi, CD11chih) (Fig. 1F). The number of AMs tended to be smaller in Bach2−/−/CD4cre/+ mice than in control mice (1.6 × 10⁷/mouse versus 7.4 × 10⁶/mouse). The expression of genes involved in lipid metabolism such as Aceb1, Rara, and Lsr, whose expression was decreased in Bach2-deficient mice (see below), was not markedly altered in AMs of Bach2−/−/CD4cre/+ mice (Fig. 1G).

Interestingly, the expression of Bach2 was up-regulated in the AMs of Bach2−/−/CD4cre/+ mice (Fig. 1G), indicating that an altered signal from Bach2-deficient T cells promoted the expression of Bach2 in AMs. We next generated Bach2−/−/LysMcre/+ mice (25), lacking Bach2 in the myeloid lineage. These mice showed no pathological finding of PAP in the lungs (Fig. 1H). The pattern of surface markers was normal in the AMs of Bach2−/−/LysMcre/+ mice, and no accumulation of lymphoid cells or eosinophils was observed (Fig. 1I). These results indicate that deficiency of Bach2 in any of the single cell lineages examined was not sufficient to cause PAP.

Depletion of T cells restored functional AMs in Bach2-deficient mice

If the Bach2-deficient AMs were altered in response to the inflammatory environment, then they may be normalized by changing the environment. To examine the plasticity of the abnormal AMs, we administered anti-CD4 and anti-CD8 antibodies to deplete CD4+ T cells and CD8+ T cells, respectively, in 8-week-old Bach2-deficient mice that already had abnormal AMs and PAP. Two weeks after administration of anti-CD4 antibody, we observed an increase in the numbers of normal AMs (F4/80int-hi, CD11bint-hi, SiglecFhi, and CD11chih) in the BAL, although there still remained abnormal AMs (F4/80int, CD11bhi, SiglecFin, and CD11chih) (Fig. 2, A and B). We also found a decrease in the numbers of eosinophils in these mice. Surprisingly, the accumulation of SPs was almost completely resolved in these mice (Fig. 2C). In contrast, we did not observe such curative responses with the anti-CD8 antibody (Fig. 2, A–C). These results indicated that Bach2-deficient CD4+ T cells were required but not sufficient for the induction of abnormal AMs and the onset of PAP.

Loss of the tissue-specific identity in Bach2-deficient AMs

Next, to reveal the gene expression profile of Bach2-deficient AMs, we performed a microarray analysis using normal AMs from WT mice and abnormal AMs from Bach2-deficient mice at 9 weeks of age (supplemental Fig. S1). We used biological triplicates to achieve a more rigorous analysis, including a gene set enrichment analysis (GSEA), which was not possible in our previous analysis (20). Using a threshold of more than 2-fold change (p < 0.05), 3334 probes were up-regulated and 3720 down-regulated in Bach2-deficient AMs compared with WT AMs (Fig. 3A). The numbers of probes with altered expression were much less than reported previously (20), confirming a more strict analysis than the previous one. Tissue-specific macrophages show gene expression patterns specific to the tissues where they locate (26). A GSEA revealed that the AMs of
Figure 1. Effect of Bach2-deficient T cells on AMs. A, microscopic appearance of the lungs of Bach2/Rag2-DKO mice. Shown are H&E and PAS staining of paraffin-embedded lungs. Scale bars = 100 μm and 200 μm (insets). B, flow cytometry of cells in BAL from Rag2<sup>−/−</sup>, Bach2<sup>−/−</sup> Rag2<sup>−/−</sup>, or Bach2/Rag2-DKO mice at 13 weeks of age. Green square, normal AM; blue square, eosinophil; red square, abnormal AM. C, microscopic appearance of the lungs of Bach2<sup>−/−</sup> × mb<sub>1cre</sub> and Bach2<sup>−/−</sup> × mb<sub>1cre</sub> mice at 26 weeks of age. D, microscopic appearance of the lungs of Blimp-1<sup>−/−</sup> and Bach2<sup>−/−</sup> × Blimp-1<sup>−/−</sup> mice at 16 weeks of age. Scale bars = 100 μm and 200 μm (insets). E, microscopic appearance of the lungs of Bach2<sup>−/−</sup> × CD4<sup>−/−</sup> and Bach2<sup>−/−</sup> × CD4<sup>cre</sup> mice at 26 weeks of age. F, flow cytometry of cells in the BAL from Bach2<sup>−/−</sup> × CD4<sup>−/−</sup> and Bach2<sup>−/−</sup> × CD4<sup>cre</sup> mice at 26 weeks of age. Green squares, normal AM; blue squares, eosinophil. G, mean expression of Abcg1, Rara, Lsr, and Bach2 in AMs from Bach2<sup>−/−</sup> × LysM<sup>−/−</sup> and Bach2<sup>−/−</sup> × LysM<sup>cre</sup> mice at 16 weeks of age. H, microscopic appearance of the lungs of Bach2<sup>−/−</sup> × LysM<sup>−/−</sup> and Bach2<sup>−/−</sup> × LysM<sup>cre</sup> mice at 16 weeks of age. Shown are H&E and PAS staining of paraffin-embedded lungs. Scale bars = 100 μm and 200 μm (insets). I, flow cytometry analysis of cells in the BAL from Bach2<sup>−/−</sup> × LysM<sup>−/−</sup> and Bach2<sup>−/−</sup> × LysM<sup>cre</sup> mice at 16 weeks of age. Green squares, normal AM.
Bach2-deficient mice highly expressed genes whose expression is specifically low in AMs (Fig. 3B) (26). We confirmed alterations in the expression of transcription factors included in this category, such as Rcsd1, Prkcb, Nsmaf, and B4galt6, using quantitative RT-PCR (Fig. 3B). In contrast, the expression of genes specifically high in AMs among tissue macrophages (26), including those involved in the metabolism of surfactants such as "lipid catabolic process" and "lipid transporter activity" was lower in Bach2-deficient AMs than in WT cells (Fig. 3C). These results suggested that the AMs of Bach2-deficient mice lost the identity and function of AMs. Furthermore, genes specific to other tissue macrophages (red pulp macrophages, peritoneal macrophages, and microglia) (26) were strongly expressed in the AMs of Bach2-deficient mice compared with WT mice (Fig. 3, D–F). We confirmed the elevated expression of transcription factors important for tissue-specific macrophages (Fig. 3, D–F; right panels). Because deficiency of MafB/c-Maf is reported to enable self-renewal of macrophages (27), their up-regulation may affect the self-renewal process of AMs in Bach2-deficient mice. Consistent with this prediction, we found that Bach2-deficient AMs showed decreased DNA synthesis and an arrested cell cycle (Fig. 3G). Furthermore, we found that many of the genes that are induced in DC under inflammatory conditions were expressed at higher levels in Bach2-deficient AMs compared with WT AMs (Fig. 3H). These results suggest that AMs in Bach2-deficient mice acquired the characteristics of other tissue macrophages and DC, losing their identity and function as AMs.

Bach2 functions in both T cells and AMs

When we analyzed Bach2<sup>F/F</sup>×CD<sup>4cre/+</sup> mice at an earlier time point (16 weeks of age) than in the above experiments, we observed abnormal AMs (F4/80<sup>int</sup>, CD11b<sup>hi</sup>, SiglecF<sup>int</sup> and CD11c<sup>hi</sup>) as well as apparently normal AMs (F4/80<sup>int-hi</sup>, CD11b<sup>int-hi</sup>, SiglecF<sup>hi</sup>, CD11c<sup>hi</sup>) (Fig. 4A). Despite the presence of abnormal AMs, they did not show any pathological features of PAP (data not shown). Taking advantage of the presence of both normal and abnormal AMs in these mice, we compared their gene expression. We sorted normal AMs from control Bach2<sup>F/+</sup>×CD<sup>4cre/+</sup> mice, normal and abnormal AMs from Bach2<sup>F/F</sup>×CD<sup>4cre/+</sup> mice, normal AMs from WT mice, and abnormal AMs from Bach2-deficient mice, and performed RNA sequencing.

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**Figure 2.** CD<sup>4+</sup> T cells contribute to the development of PAP in Bach2-deficient mice. A, flow cytometry of cells in BAL from Bach2<sup>−/−</sup> mice at 10 weeks of age that were injected with control IgG, anti-CD4 (clone Gk1.5), or anti-CD8 (clone Ly2) antibodies at 8 weeks of age. Green squares, normal AM; blue squares, eosinophil; red squares, abnormal AM. B, mean numbers of normal AMs, abnormal AMs, and eosinophils in the BAL from the mice analyzed in A (control, n = 4; Gk1.5, n = 3; Ly2, n = 4). C, microscopic appearance of the lungs of the mice analyzed in A. Scale bars = 500 μm and 200 μm (insets).
In the clustering analysis (Fig. 4B), abnormal AMs from Bach2f/f × CD4cre/mice and Bach2-deficient mice showed gene expression profiles that were distinct from those of normal AMs in WT mice and control Bach2+/+ × CD4+/+ mice, and they were clustered away from the two normal AMs. These observations suggest that the higher expression
of Bach2 in normal AMs from Bach2\(^{+/+}\) \(\times\) CD4\(^{cre/+}\) mice may be important for maintenance of the normal gene expression profile. Consistent with this idea, the expression of Bach2 was indeed elevated in the normal AMs compared with abnormal AMs from Bach2\(^{+/+}\) \(\times\) CD4\(^{cre/+}\) mice (Fig. 4C).

We selected genes whose expression was more than 2-fold different between normal and abnormal AMs from Bach2\(^{+/+}\) \(\times\) CD4\(^{cre/+}\) mice and performed a pathway analysis and a GO analysis to interpret the differences of these AMs (Fig. 4, D and E). The genes up-regulated in abnormal AMs were enriched in KEGG pathways such as “chemokine signaling pathway” and “Jak-STAT signaling pathway.” They were also enriched in GO categories such as “regulation of transcription” and “chromatin organization.” These results suggested that abnormal macrophages with lower expression of Bach2 were transcriptionally activated in response to the inflammatory environment caused by T cells lacking Bach2. Taken together, these results clearly indicate that T cells lacking Bach2 altered the character of AMs, but this alteration was eventually suppressed in AMs when Bach2 was induced.

Figure 4. RNA sequence of AMs from Bach2\(^{+/+}\) \(\times\) CD4\(^{cre/+}\) mice at 16 weeks of age. A, flow cytometry analysis of cells in the BAL from Bach2\(^{+/+}\) \(\times\) CD4\(^{cre/+}\) and Bach2\(^{+/+}\) \(\times\) CD4\(^{cre/+}\) mice at 16 weeks of age. Green squares, normal AM; blue square, eosinophil; red squares, abnormal AM. B, tree diagram of gene expression profile of AMs detected in A. WT, Bach2\(^{+/+}\) mice; KO, Bach2\(^{−/−}\) mice; cKO, Bach2\(^{+/+}\) \(\times\) CD4\(^{cre/+}\) mice; control, Bach2\(^{+/+}\) \(\times\) CD4\(^{cre/+}\) mice. C, Read Per Kilobase of exon per Million mapped reads (RPKM) of Bach2 in the RNA sequence using AMs detected in A. D and E, KEGG pathway (D) and GO analysis (E) of genes showing >2-fold up-regulation only in abnormal AMs observed in Bach2\(^{+/+}\) \(\times\) CD4\(^{cre/+}\) mice, focusing on biological process.

Figure 3. Gene expression profile of AMs and Bach2-deficient mice. A, heatmap of gene expression profiles in AMs from WT and Bach2\(^{−/−}\) mice. Microarray analysis was performed using three mice per genotype. B–F, GSEA of genes specific to each tissue macrophage and mRNA expression of the transcription factors specific to each tissue macrophage (26) in AMs from WT and Bach2\(^{−/−}\) mice; specifically low in alveolar macrophages (B), specific to alveolar macrophages (C), specific to red pulp macrophages (D), specific to peritoneal macrophages (E), and specific to microglia (F). G, cell cycle analysis using FxCycle violet stain and incorporation of EdU in AMs from WT or Bach2\(^{−/−}\) mice. H, GSEA of dendritic cell maturation is enriched in genes showing >2-fold up-regulation in Bach2\(^{−/−}\) AMs compared with WT AMs. Mice were 9 weeks of age. ND, not detected. Data are from 3 mice/genotype. The p value was as compared with the WT (unpaired two-tailed Student’s t test). All genes are ranked in order of their differential expression (left, WT > Bach2\(^{−/−}\); right, WT < Bach2\(^{−/−}\)). Black bars show genes included in gene lists in B–F and H.
Activation of AMs of Bach2-deficient mice by exposure to inflammatory cytokines

Collected cells from the BAL of Bach2-deficient mice highly expressed multiple genes encoding cytokines, including IFN-γ (Fig. 5), suggesting an inflammatory environment in the lung. A GSEA of the up-regulated genes in Bach2-deficient AMs revealed enrichment of genes in the JAK-STAT cascade (Fig. 6A). IFN-γ, upstream of this pathway, was highly expressed in both CD4+ and CD8+ T cells in Bach2-deficient mice (Fig. 6B). Phosphorylation of Stat1 was up-regulated in the BAL from Bach2-deficient mice compared with that from WT mice (Fig. 6, C–E). The expression and phosphorylation of Stat3, which also mediates IFNγ signaling, also tended to increase (Fig. 6, F–H). Importantly, the expression of Bach2 in WT AMs was up-regulated by IFNγ in vitro (Fig. 6I). These results suggested that IFNγ released from T cells affected the AMs.

Bach2 directly regulates identity- and cytokine-related genes in AMs

To identify genes that are critical to keep the functions of AMs, we focused on genes whose expression was elevated in abnormal AMs from Bach2-deficient mice and in abnormal AMs from Bach2-deficient mice at 16 weeks of age. In the intersection of these sets were 721 genes, including Stat4, Gata3, and Ahr (Fig. 7A). Stat4 is up-regulated along with DC maturation (28). Gata3 induces the expression of Il-13 and Il-5 in DCs (29), leading to migration and activation of eosinophils in the airway. Therefore, the DC-like gene expression profile of abnormal AMs and the increased number of eosinophils in the lungs of Bach2-deficient mice and Bach2-deficient mice may involve aberrant expression of Stat4 and Gata3. Ahr is an important regulator of osteoclasts (30) and may alter the specificity of AMs in Bach2-deficient mice. The expression of the Cdkn family (e.g. Cdkn1a) was up-regulated, which likely resulted in cell cycle arrest of AMs from Bach2-deficient mice.

A ChIP-seq analysis of AMs would be ideal to reveal direct target genes of Bach2 in AMs but was technically difficult because of a limited number of cells. Therefore, we used a dataset of our ChIP-seq analysis using myeloblastic leukemia M1 cells, which represent a myeloid lineage and express Bach2 as a proxy (31). The ChIP-seq data were obtained without inducing macrophage differentiation. Within the 2642 genes with nearby Bach2 binding, the expression of 120 genes was up-regulated, and 127 genes were down-regulated in AMs of Bach2-deficient mice and Bach2-deficient mice (Fig. 7, A and B). Binding of Bach2 to inflammation-related genes, including Spic, Irf8, and Il6, was verified using ChIP quantitative PCR (Fig. 7, C and D). These results suggest that Bach2 regulates the AM specificities.
and inflammatory response by binding to the genes related to both a part of the cytokine and signaling systems, including interleukin family, SOCS family, and STAT family and transcription factor genes of the myeloid system.

Because Runx family members are essential regulators of differentiation and inflammatory responses of hematopoietic cells (32), and Runx3 was up-regulated in AMs of both Bach2-deficient and Bach2\textsuperscript{\textalpha\textbeta\textgamma} mice, we generated a bone marrow chimera of Runx3-deficient hematopoietic cells. AMs, peritoneal macrophages, red pulp macrophages, and bone marrow macrophages from Runx3-deficient cells did not show any disorder (Fig. 8). Thus, Runx3 appears to be dispensable for the functions of tissue-specific macrophages, including AMs.


**Alveolar macrophage identity crisis in Bach2-deficient mice**

![Diagram](image)

**Figure 7. ChIP-seq analysis of Bach2 and expression profile of genes with Bach2 binding.** A, Venn diagram of target genes of Bach2, genes showing >2-fold up-regulation in Bach2\(^{-/-}\) AMs compared with WT AMs, and genes showing >2-fold up-regulation or detected only in abnormal AMs compared with normal AMs in Bach2\(^{-/-}\) \(\times\) CD4\(^{cre/+}\) mice. Target genes of Bach2 were detected in ChIP-seq analysis using M1 cells (31). B, Venn diagram of target genes of Bach2, genes showing >2-fold down-regulation in Bach2\(^{-/-}\) AMs compared with WT AMs, and genes showing >2-fold down-regulation or detected only in normal AMs compared with abnormal AMs in Bach2\(^{-/-}\) \(\times\) CD4\(^{cre/+}\) mice. Target genes of Bach2 were detected in ChIP-seq analysis using M1 cells (31). C, Bach2 binding region of genes (Spic, Irf8, and Il6). D, chromatin immunoprecipitation analysis of the binding of Bach2 to the regions of Spic, Irf8, and Il6 in M1 cells. ChIP-seq and the ChIP above were performed using myeloblastic leukemia M1 cells without differentiation to macrophages. The experiment was performed with three lots.* \(p < 0.05\) compared with normal rabbit serum (NRS) (unpaired two-tailed Student’s t test).

**Bach2 is required for the expression of histone genes in AMs**

Considering the fact that T cell–specific knockout of Bach2 did not lead to PAP, we next focused on genes whose expressions were altered only in Bach2-deficient AMs. There were 168 up- AMs from Bach2-deficient mice with binding of Bach2 whose expression was not altered in abnormal AMs from Bach2\(^{-/-}\) \(\times\) CD4\(^{cre/+}\) mice (Fig. 7, A and B). GO analysis revealed that the up-regulated target genes were enriched in GO categories related to proliferation and osteoclasts (Fig. 9A). On the other hand, the down-regulated target genes were enriched in GO categories related to nucleosomes and chromatin (Fig. 9B). Interestingly, many of the histone genes were included in these categories, and their expression was decreased in AMs from Bach2-deficient mice (Fig. 9C). Bach2 was found to bind to clusters of histone genes at multiple sites (Fig. 9D). Therefore, Bach2 may play a role in histone gene expression. The cell cycle arrest observed in AMs from Bach2-deficient mice (Fig. 3G) may be partly due to the reduced expression of histone genes and may have led to the functional defects of AMs.

We also found that genes showing high expression in myeloid lineages, such as Rhou, Cacna1d, Scn3a, Ceacam19, and Slc36a2, were up-regulated in AMs from Bach2-deficient mice, and Bach2 bound to these genes in ChIP-seq using myeloid cells (data not shown), suggesting that Bach2 represses genes related to innate immunity to prevent aberrant inflammation in the lungs. Together with the induction of Bach2 expression in AMs of Bach2\(^{-/-}\) \(\times\) CD4\(^{cre/+}\) mice at 26 weeks of age (Fig. 1G) and the induced expression of Bach2 in normal AMs compared with abnormal AMs from Bach2\(^{-/-}\) \(\times\) CD4\(^{cre/+}\) mice at 16 weeks of age (Fig. 4C), these results suggest that Bach2 in AMs may prevent the development of PAP in the presence of activated T cells by binding to inflammation-related genes and histone genes.

**Discussion**

In this study, we revealed a novel mechanism of PAP in Bach2-deficient mice that we believe is relevant to secondary PAP. Deficiency of Bach2 specifically in either myeloid or T cell lineages alone did not disrupt homeostasis in the lung. Only when Bach2 is reduced in several cell lineages (most likely in AMs and CD4\(^{+}\) T cells) is the homeostasis of the lung ruined, resulting in development of PAP. Deficiency of Bach2 has been reported to cause dysfunction of regulatory T cells (15) and up-regulation of inflammatory cytokines in CD4\(^{+}\) and CD8\(^{+}\) T cells (16). Of note, we found that, although Bach2 bound to Ifng in T cells to suppress excessive production of cytokines (17), Bach2 was up-regulated in AMs by IFN\(\gamma\) and directly repressed genes related to inflammation and specific functions of other tissue macrophages (e.g. Il6 and Spic, respectively). A few reports have found that transcription factors can regulate the
inflammatory response beyond particular single-cell lineages. For example, NF-κB is essential for the development and function of acquired immunity, including both T and B cells and macrophages (33). Our observations here, together with recent reports on T cells (15–17), suggest that Bach2 orchestrates the responses of the immune system at multiple steps in several types of cells. To confirm the importance of Bach2 in both AMs and T cells, we transplanted Bach2-deficient T cells into Bach2/Rag2 double-deficient mice. However, these mice showed normal patterns of surface markers of AMs and did not develop PAP. More numbers of transplanted T cells or a longer period may be required for the development of PAP.

Tissue-resident macrophages show plasticity toward other tissue-resident macrophages, depending on their environment (11, 12, 34, 35). The AMs of Bach2-deficient mice not only lost their function of surfactant metabolism but also gained a mixture of gene expression profiles of other tissue macrophages and may be termed schizophrenic macrophages. Therefore, Bach2 appears to demarcate the gene expression identity of AMs from those of other macrophages. These results further suggest that inflammation is a trigger for tissue macrophages to lose their specific functions when the activity of Bach2 is reduced. It has recently been reported that hyperactivation of Th1 cells causes PAP. In this model, the expression of Bach2 is increased in AMs (36). This observation is consistent with a role of Bach2 in restricting responses to inflammation. In addition, up-regulation of Ifi8, Runx3, and Gata3, which are important for the differentiation and function of the DC lineage (29, 37),

Figure 8. Tissue macrophages derived from Runx3-deficient progenitor cells. A–D, flow cytometry of cells in the BAL (A), cells in the peritoneal cavity (B), splenic cells (C), and bone marrow cells (D) from mice transplanted with WT or Runx3+/−/− fetal liver cells at 21 weeks of age. E and F, ratio of CD45.2 macrophages/nucleated cells (E) and number of CD45.2 macrophages per body (F) observed in mice transplanted with WT or Runx3+/−/− fetal liver cells at 21 weeks of age. Data are from 5 mice/genotype. The p values are as compared with the WT (unpaired two-tailed Student’s t test).

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may contribute to the DC-like phenotype and dysfunction of AMs in Bach2-deficient mice.

Although Bach2 has been reported as a transcription repressor in multiple cell lineages, our findings suggest that Bach2 functions as a transcription activator as well. Specifically, our results suggest for the first time that Bach2 may activate expression of a series of histone genes in myeloid lineages, leading to promotion of cell proliferation. Many transcription factors function both as an activator and a repressor, depending on their interactions with co-factors. We have recently reported that Bach2 interacts with many chromatin-related co-factors, some of which are implicated in transcription activation (38). It is a future task to reveal the precise mechanism of Bach2 to activate gene expression. Direct target genes of Bach2 in AMs remain to be elucidated because we used the ChIP-seq data of M1 cells in this study. It will be important to validate the regulation of putative target genes, including histone genes, by Bach2 in AMs.

Figure 9. GO analysis of target genes up- or down-regulated in AMs of Bach2-deficient mice compared with WT mice and not affected in abnormal AMs in Bach2f/f/CD4cre mice. A, GO analysis of 168 genes shown in Fig. 7A. B, GO analysis of 188 genes shown in Fig. 7B. C, volcano plots for Bach2 AMs compared with the WT AM, by using GeneSpring software. Green, fold change > 1 (log2) in absolute value; red, "Bach2 binding" annotated genes included in the 168 and 120 genes shown in Fig. 7A or in the 188 and 127 genes shown in Fig. 7B; black, histone genes included in the 168 genes shown in Fig. 7A or in the 188 genes shown in Fig. 7B; gray, histone genes included in the 127 genes shown in Fig. 7B. D, ChIP-seq binding profiles for Bach2 in M1 cells and histone H3 lysine 4 monomethylation (H3K4me1), histone H3 lysine 4 dimethylation (H3K4me2), and histone H3 lysine 4 trimethylation (H3K4me3) in bone marrow macrophages (GSE60103). All gene names and the loci of genes in Fig. 7, A and B (arrowheads) are presented under each gene locus. Boldface text and black arrowheads, histone genes included in the 188 genes shown in Fig. 7B; green text and green arrowheads, histone genes included in the 374 genes shown in Fig. 7B; red text and red arrowheads, histone genes included in the 168 genes shown in Fig. 7A; underlined text and orange arrowheads, histone genes included in the 461 genes shown in Fig. 7A.
Depletion of Bach2-deficient CD4+ T cells normalized the accumulation of SPs in alveolar spaces observed in Bach2-deficient mice. Although the PAP phenotype was dependent on the reduced functions of Bach2 in AMs and T cells, removal of altered T cells was sufficient to ameliorate the disease. Our findings may help to develop a new treatment strategy for secondary PAP associated with inflammatory diseases. Bach2 may also be involved in diseases such as diabetes mellitus and systemic lupus erythematosus, which is supported by the presence of multiple reports associating these diseases with BACH2 polymorphisms (39, 40). Determining the roles of Bach2 in these diseases may help us understand the pathology behind them.

**Experimental procedures**

**Mice**

Bach2-deficient mice (13) were generated as described previously (19). To generate Bach2/Rag2-deficient mice, we crossed Rag2 heterozygous mice (21) with Bach2 heterozygous mice. B cell–specific Blimp-1–deficient mice (24) were provided by K. Calame (Columbia University). Bach2−/− Blimp-1−/− mice were generated by breeding Bach2-deficient mice and B cell–specific Blimp-1–deficient mice. To generate Bach2f/+LysMcre mice, we crossed Bach2f/+ mice (22) with LysMcre heterozygous mice (25). These mice were bred at the animal facility of Tohoku University. Mice were housed under specific pathogen-free conditions. All experiments involving mice were performed in accordance with the Animal Care and Use Committee of the Tohoku University Environmental and Safety Committee.

Bach2f/+; mb1cre (22, 23) mice were from the Center for Integrative Medical Sciences, RIKEN. Bach2f/+; CD4cre mice (18) were kindly provided by the Department of Immunology/Infection and Host Defenses at Ehime University. Runx3-deficient mice (32) were provided by the Department of Experimental Therapeutics, Institute for Advancement of Clinical and Translational Science, Kyoto University Hospital. All mice were of the C57BL/6j background.

**Single-cell suspension of organs and collection of BAL fluid and peritoneal cells**

For the analysis of lung, spleen, and bone marrow, organs were isolated from the body and grinded with slide glasses. To collect BAL, the trachea was exposed for cannulation. The lungs were washed three times with 1 ml of 3% FBS/PBS, and this procedure was repeated twice. Peritoneal cells were collected as described previously (41). The fluid was centrifuged at 300 × g for 5 min, and supernatants were removed. Red blood cells were lysed for 5 min at room temperature in 3 ml of osmotic lysis buffer.

**Lung histology**

The trachea and lungs were exposed and cannulated, inflated with fixative (10% v/v formaldehyde), and ligated with sutures while retracting the cannula to seal the lung under pressure. Lung sections were stained with H&E and PAS (periodic acid-Schiff).

**Flow cytometry analysis**

For analysis of BAL cells, cells were stained with anti-F4/80 (BMS, eBiosciences), anti-CD11b (M1/70, Biolegend), anti-SingleF (E50-2440, Pharmingen), anti-CD11c (N418, Biolegend), anti-CD4 (GK1.5, Pharmingen), and anti-CD8 (53–6.7, Pharmingen) antibodies.

For analysis of tissue macrophages (bone marrow macrophages, red pulp macrophages, and peritoneal macrophages), cells were stained with anti-CD45.1 (A70, Biolegend), anti-CD45.2 (104, Biolegend), anti-F4/80 (BMS, eBioscience), anti-CD11b (M1/70, Pharmingen), anti-Gr-1 (1A8, BD Biosciences), anti-CD115 (AFS98, eBioscience), anti-CD3e (145–2C11, Tonbo), and anti-B220 (RA3-6B2, Tonbo) antibodies. The cells were sorted with a FACSaria II (BD Biosciences) and analyzed by FlowJo software (Tree Star).

**EdU pulsing**

Mice were given an intraperitoneal injection of 1 mg/200 ml EdU (Click-IT Plus EdU Alexa Fluor 488 flow cytometry assay kit; Life Technologies) and maintained for 2 h. For analysis of EdU incorporation, AMs were stained, fixed, and permeabilized, and 1 ml of ExCycle violet stain (Invitrogen) was added per 1 ml of cell suspension. Analysis was performed by flow cytometry.

**Microarray analysis**

Total RNA was amplified with an Amino Allyl MessageAmplification kit (Ambion) and labeled with indocarbocyanine with the CyDye post-labeling reactive dye pack (Amersham Biosciences). A Sureprint G3 Mouse GE microarray slide was used according to the instructions of the manufacturer (Agilent). The data were collected on an Agilent scanner. The GeneSpring software package (Agilent) was used for analysis of genes. GSEA and the Database for Annotation, Visualization and Integrated Discovery (DAVID v6.7) were used to assess pathway enrichment.

**Quantitative profiling with reverse transcription**

RNA for quantitative PCR analysis was isolated with a RNeasy micro kit (Qiagen) and converted into cDNA with random primers and Superscript III reverse transcriptase (Invitrogen). Light Cycler FastStrand DNA Master SYBR Green I (Roche) reagents and a Light Cycler nano system (Roche) were used for PCR.

For the PCR analysis using cells in BAL from Bach2f/+; CD4cre/+ mice, total RNA was isolated using TRIzol reagent, and cDNA was synthesized using the Superscript VILO cDNA synthesis kit (Life Technologies). Quantitative RT-PCR was performed as described, using the Step One Plus real-time PCR system (Life Technologies).

**Primers**

Primers used were as follows: β-actin, forward 5′-CGTTGACATCCGTAAAGCCCTC-3′ and reverse 5′-AGCCACCGATCCACACAGA-3′; Rcsd1, forward 5′-ACCACCCAGTAA-GCCAACAAG-3′ and reverse 5′-GCCGGATGGTGATTTCC-TCCTC-3′; Prkcb, forward 5′-GTGTCAAGTCTGCTGCTT-3′ and reverse 5′-GTAGGACTGGAGTACGTGTGG-3′; Nsnaf, forward 5′-CTGAGCCAAGATCTGATAGGG-3′; and reverse 5′-CCAGCAGTACAGCAGA-3′.
RNA sequencing

Total RNA was extracted with the RNeasy micro kit (Qiagen). cDNA was prepared from 100 pg of total RNA per sample using the SMARTer Ultra Low Input RNA Kit for Sequencing v3 (Clontech Laboratories) following the standard protocol with slight modifications (18 PCR cycles). The cDNA was fragmented using a Covaris S2 focused ultrasonicator, and RNA sequencing libraries were prepared from the fragmented cDNA with the Ovation Ultralow DR Multiplex System V2 (Nugen). The DNA libraries were clonally amplified on a flow cell and sequenced on HiSeq2500 (HiSeq control software v2.2.38, Illumina) with a 51-mer paired-end sequence. Image analysis and base calling were performed using Real-Time Analysis software (v1.18.61, Illumina). To quantify gene expression, Bowtie 1.1.2 (42) and eXpress 1.5.1 (43) were used, and edgeR (44) analysis was performed to identify differentially expressed genes. DAVID v6.7 was used to assess pathway enrichment.

Intracellular staining

Cells included in the BAL were fixed by incubation for 10 min at 25 °C with BD Cytofix buffer (BD Biosciences) and washed with stain buffer (BD Biosciences). They were permeabilized in BD Phosflow Perm Buffer III (BD Biosciences) and then stained for 30 min with Alexa Fluor 647–labeled antibody to STAT1 phosphorylated at Tyr-701 (BD Phosflow), Alexa Fluor 647–labeled antibody to STAT3 phosphorylated at Tyr-705 (BD Phosflow), and phosphatidylethanolamine-labeled antibody to STAT6 phosphorylated at Tyr-641 (BD Phosflow). After being washed with stain buffer, cells were analyzed by flow cytometry on a FACSaria II.

Chromatin immunoprecipitation

M1 cells were expanded without differentiation to macrophages, and the collected cells were fixed for 10 min at 37 °C with 1% formaldehyde in culture medium, followed by quenching for 1 min at 37 °C with 125 mM glycine. The cells were then washed twice with ice-cold PBS and once with ice-cold PBS including protease inhibitor (Roche). They were lysed in 1 ml of shearing buffer (50 mM Tris-HCl (pH 7.6), 0.2% Triton X-100, and 1 mM PMSF (Sigma)). The cross-linked chromatin was sheared with a Bioruptor sonicator (CosmoBio).

Chromatin was diluted in dilution buffer (0.01% SDS and 1 mM EDTA), incubated for 10 min on ice, and centrifuged to collect the supernatant. The supernatant was incubated with Dynabeads protein A (Life Technologies) or Dynabeads protein G (Life Technologies) conjugated with antibodies at 4 °C for 8 h. Bach2-containing chromatin complexes bound to magnetic beads were isolated by magnetic sorting and were washed with the following buffers: with low-salt buffer (0.1% SDS, 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.1% DOC, and 1% Triton X) twice, with high-salt buffer (0.1% SDS, 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.1% DOC, 1% Triton X, and 0.3 M NaCl) twice, with LiCl buffer (0.5% Nonidet P-40, 0.21 M LiCl, and 0.5% sodium DOC) twice, and with Tris-EDTA buffer with 0.2% Triton X-100 once. The Bach2–chromatin complexes were released from formaldehyde fixation by incubation at 65 °C for 4 h in the presence of protease inhibitor (Invitrogen). Genomic DNA was isolated with the DNA Clean & Concentrator Kit (Zymo Research) following the standard protocol. Relative immunopre-
cipitation efficiency was calculated by division of the value of the immunoprecipitated sample by that of the input sample. Chromat-in immunoprecipitation was performed using the following anti-sera: anti-Bach2 antibody (N2) (15), rabbit IgG isotype control (Invitrogen), and normal rabbit serum (Jackson ImmunoResearch Laboratories).

ChIP-seq

ChIP-seq libraries were prepared from 1–3 ng of ChIP DNA with the Ovation Ultralow DR Multiplex System (Nugen). The DNA libraries were clonally amplified on a flow cell and sequenced on HiSeq2500 (HiSeq control software v2.2.38, Illumina) with a 51-mer paired-end sequence. Image analysis and base calling were performed using Real-Time Analysis software (v1.18.61, Illumina). The first five bases were trimmed because of low read quality. Reads were mapped with BWA 0.7.12 (45), and peak call was performed with macs2 2.1.0.20150420 (46). We utilized the public dataset GSE60103 (47).

T cell depletion

Anti-mouse CD4 mAb (clone GK1.5) and anti-mouse CD8 mAb (clone Ly2) were purified from hybridomas in nude mice. For anti-CD4 treatment, 8-week-old mice received 100 μg of the anti-mouse CD4 mAb. For anti-CD8 treatment, the mice received 200 μg of the anti-mouse CD8 mAb. We administered 100 μg of rat IgG to control mice. Intraperitoneal injection of the antibodies was performed every 3 days. Mice were analyzed at 10 weeks of age.

Stimulation of AMs with IFNγ

BAL cells were collected from WT mice (almost all cells were AMs) and plated in RPMI 1640 medium including GM-CSF (10 ng/ml) with or without IFNγ (100 ng/ml) for 24 h.

Statistical analysis

The data are presented as means ± S.D. All statistical analyses were done using two-tailed Student’s t test.

Study approval

All experiments involving mice were approved by Tohoku University.

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References

Alveolar macrophage identity crisis in Bach2-deficient mice


