Transcription repressor Bach2 is required for pulmonary surfactant homeostasis and alveolar macrophage function

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Pulmonary alveolar proteinosis (PAP) results from a dysfunction of alveolar macrophages (AMs), chiefly due to disruptions in the signaling of granulocyte macrophage colony-stimulating factor (GM-CSF). We found that mice deficient for the B lymphoid transcription repressor BTB and CNC homology 2 (Bach2) developed PAP–like accumulation of surfactant proteins in the lungs. Bach2 was expressed in AMs, and Bach2-deficient AMs showed alterations in lipid handling in comparison with wild-type (WT) cells. Although Bach2-deficient AMs showed a normal expression of the genes involved in the GM–CSF signaling, they showed an altered expression of the genes involved in chemotaxis, lipid metabolism, and alternative M2 macrophage activation with increased expression of Ym1 and arginase-1, and the M2 regulator Irf4. Peritoneal Bach2-deficient macrophages showed increased Ym1 expression when stimulated with interleukin-4. More eosinophils were present in the lung and peritoneal cavity of Bach2-deficient mice compared with WT mice. The PAP–like lesions in Bach2-deficient mice were relieved by WT bone marrow transplantation even after their development, confirming the hematopoietic origin of the lesions. These results indicate that Bach2 is required for the functional maturation of AMs and pulmonary homeostasis, independently of the GM–CSF signaling.

The normal function of the lung, including gas exchange, requires the production of pulmonary surfactant. Pulmonary alveolar proteinosis (PAP) is a rare fatal syndrome accompanied by an abnormal accumulation of pulmonary surfactant in the alveolar spaces of the lungs (Trapnell et al., 2003; Borie et al., 2011). Pulmonary surfactant is composed mainly of phospholipids, neutral lipids (mainly of cholesterol), and four kinds of protein, known as surfactant protein (SP)–A (encoded by Sftpa1), SP-B (Sftpb), SP-C (Sftpc), and SP-D (Sftpd). Surfactant makes up the air–liquid interface of the alveolar wall to prevent alveolar collapse. It also contributes to pulmonary host defense by opsonization of microbial pathogens. Surfactant homeostasis is regulated by two types of cells. Surfactant is produced and secreted by the alveolar type 2 epithelial cells, and is taken up by them and reused. In addition, excess surfactant is phagocytized by alveolar macrophages (AMs), the resident mononuclear phagocytes in the respiratory tract, and is catabolized (Trapnell et al., 2003).

Clinically, PAP is classified into three types: acquired, congenital, and secondary. Several observations have suggested that dysfunctions of AMs are causative to PAP. Acquired PAP is a result of neutralizing autoantibodies against...
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In response to Th2 cytokines IL-4 and IL-13, they become polarized to show the alternative M2 phenotype, which is important for debris scavenging, tissue remodeling, and wound healing after inflammation (Gordon, 2003; Mosser and Edwards, 2008; Martinez, 2011). Although the functional diversity of macrophages has been appreciated for a long while, the mechanisms that regulate the acquisition of particular functional states in terms of gene expression are just emerging. Transcription factors Irf4 and Stat6 promote M2 polarization (Satoh et al., 2010; Lawrence and Natoli, 2011; Nelson et al., 2011), and Spi-C is required for the differentiation of red pulp macrophages of the spleen specialized in iron homeostasis (Kohyama et al., 2009).

GM-CSF (Kitamura et al., 1999; Inoue et al., 2008), indicating that GM-CSF signaling is essential for AM maturation and function. Congenital PAP is associated with mutations in genes encoding the GM-CSF receptor βc chain (Csf2rb; Dirksen et al., 1997), SP-B (Nogee et al., 1993), or SP-C (Nogee et al., 2001). Secondary PAP, which is the most common form of PAP, accompanies other diseases such as hematological cancers (Buechner and Ansari, 1969; Ruben and Talamo, 1986; Cordonnier et al., 1994; Keller et al., 1995; Ladeb et al., 1996; Trapnell et al., 2003). Although secondary PAP may also involve a dysfunction of AMs, its etiology is unclear. As a result, patients with secondary PAP are difficult to cure, thereby necessitating further elucidation of the functions and regulation of AMs.

Macrophages play diverse functions such as innate immunity, inflammation, tissue repair, and iron homeostasis depending on their location and the stimuli they receive (Mosser and Edwards, 2008). Macrophages show functionally polarized phenotypes in a signal- and context-dependent manner. In response to cytokines such as interferon-γ and bacterial components like LPS, macrophages become polarized to show the classical activated M1 phenotype with increased microbicidal activity mediating the initial inflammatory response (Gordon, 2003; Mosser and Edwards, 2008; Martinez, 2011). In response to Th2 cytokines IL-4 and IL-13, they become polarized to show the alternative M2 phenotype, which is important for debris scavenging, tissue remodeling, and wound healing after inflammation (Gordon, 2003; Mosser and Edwards, 2008; Martinez, 2011). Although the functional diversity of macrophages has been appreciated for a long while, the mechanisms that regulate the acquisition of particular functional states in terms of gene expression are just emerging. Transcription factors Irf4 and Stat6 promote M2 polarization (Satoh et al., 2010; Lawrence and Natoli, 2011; Nelson et al., 2011), and Spi-C is required for the differentiation of red pulp macrophages of the spleen specialized in iron homeostasis (Kohyama et al., 2009).

BTB and CNC homology 2 (Bach2) has been regarded as a B cell–specific transcription factor which associates with the Maf recognition elements (MARs) of DNA (Oyake et al., 1996). Its expression is high from pro–B cells to mature B cells, whereas it is low in terminally differentiated plasma cells (Muto et al., 1998). Genetic ablation of Bach2 results in severe reduction in DNA class switch recombination (CSR) and somatic hypermutation, with a concomitant increase in IgM production (Muto et al., 2004). Bach2 represses the

Figure 1. PAP-like disease in Bach2-deficient mice. (a) The body weight curves of WT and Bach2-deficient male and female mice (n = 10/group). The data from the WT mice are shown as the mean with the SD, whereas the data from Bach2-deficient mice are shown individually because Bach2-deficient mice showed individual variability in body weight and longevity. (b) The survival curves of WT and Bach2-deficient mice. WT male (M) and female (F) mice were combined. The curves for Bach2-deficient male (light blue, n = 10) and female (pink, n = 10) mice are shown separately. (c) The macroscopic appearance of the lungs of WT and Bach2-deficient mice at indicated time points. (d) HE staining of paraffin-embedded lungs from the WT and Bach2-deficient mice. Bars: 500 µm; (insets) 50 µm.
be filled with pink materials and inflammatory cells. Proliferation of the alveolar epithelial cells was also apparent (Fig. 1 d). These changes further deteriorated at 16 wk of age (Fig. 1, c and d). The bronchoalveolar lavage (BAL) fluid of Bach2-deficient mice showed a milky appearance, thus suggesting the accumulation of pulmonary surfactant (unpublished data). A cytological examination of the BAL cells showed that the majority of the cells were AMs in the WT mice. In contrast, we observed an increased number of AMs which looked foamy in appearance, and more eosinophils and neutrophils were also recruited in the BAL fluid of Bach2-deficient mice (Fig. 2, a and b). The presence of eosinophils and neutrophils were confirmed by flow cytometry of BAL cells using F4/80 and Gr-1 antibodies (unpublished data). A similar infiltration of Blimp-1, the key regulator of plasma cell differentiation, and thereby inhibits plasma cell differentiation (Ochiai et al., 2006; Muto et al., 2010). The Bach2-mediated repression of the Blimp–1 gene is essential for CSR and the expression of its executing molecule AID (Muto et al., 2010). Although Bach2 is also expressed in hematopoietic progenitors (unpublished data), its expression and function in macrophages have not yet been reported. We herein report our discovery that Bach2-deficient mice develop PAP-like pulmonary histopathology. Our data were obtained by analyzing AMs and using BM transplantation, and suggest that Bach2 has an important role in tuning the function of AMs with regard to surfactant homeostasis in the lungs. We further show that Bach2 represses the M2 polarization of alveolar and peritoneal macrophages, extending its function to innate immunity.

RESULTS
Bach2-deficient mice develop PAP-like lesions with progressive dyspnea
Bach2-deficient mice were born at the Mendelian ratio and thrived almost as much as WT mice for the first 3 mo. However, they gradually started to show slow movement with progressive dyspnea and body weight loss irrespective of sex (Fig. 1 a), and started to die as early as 18 wk of age (Fig. 1 b). Because Bach2-deficient mice became moribund with dyspnea, we examined the alterations in their lungs over time. Although the sizes of the lungs in 8-wk-old Bach2-deficient mice were similar to those in WT mice (Fig. 1 c), the alveolar spaces of Bach2-deficient mice were infiltrated with large AMs and granulocytes (Fig. 1 d). At 12 wk of age, the lungs of the Bach2-deficient mice became obviously larger than those of WT mice (Fig. 1 c). Their alveolar spaces began to

![Figure 2. PAP-like disease in Bach2-deficient mice.](image)

(a) May–Giemsa staining of BAL cells from WT and Bach2-deficient mice. Bars, 100 µm. An enlarged panel is at the bottom to show eosinophils (bar, 10 µm). (b) Cell concentrations of AMs in BAL fluids from WT and Bach2-deficient mice. The mean values from five mice for each group are shown. Error bars represent SD. P-values were obtained by two-tailed Student’s t-tests. *, P < 0.05. (c) Crystal polygons (arrows) in the BAL fluid from Bach2-deficient mice. Bar, 20 µm.

![Figure 3. The accumulation of SPs in Bach2-deficient lungs.](image)

(a) The mRNA levels of SP-A, -B, -C, and -D in the lungs were determined by quantitative RT-PCR. mRNA of β-Actin was used as a normalization control. The mean values from five mice for each group are shown. P-values were obtained by two-tailed Student’s t-tests. **, P < 0.01. (b) SPs were compared by Western blot analysis of the BAL fluids from WT and Bach2-deficient mice (n = 5). (c and d) An immunohistochemical analysis of WT (c) and Bach2-deficient (d) lungs using antibodies against SP-A, -B, -C, -D, or control IgG. The results of PAS staining are also shown. Bars, 50 µm.
of granulocytes in the lungs is also found in secondary PAP patients (Ando et al., 2002). We also observed crystal polyn-
gon in the BAL fluid (Fig. 2 c) which looked like the Ym1 crystal observed in SHIP-deficient mice (Rauh et al., 2005).

We assessed the expression of SPs in the lungs. The mRNA expression levels of SP-A and SP-D were increased in Bach2-
deficient mice (Fig. 3 a). An immunoblotting analysis of BAL fluids revealed that the amounts of SP-A, SP-C, and SP-D were increased in Bach2-deficient mice (Fig. 3 b). In an immuno-
histochemical analysis of WT mice, SPs were detected in the alveolar type 2 epithelial cells and alveolar walls (Fig. 3 c). In
contrast, the accumulated materials in the alveolar spaces of Bach2-deficient mice were positive for all SPs (Fig. 3 d). More-
over, the deposits in the alveolar spaces were periodic acid-
Schiff (PAS)–positive (Fig. 3 d), indicating that the accumulated pink materials were pulmonary surfactant (Trapnell et al.,
2003). Collectively, these findings showed that Bach2-deficient mice developed PAP-like disease.

**Altered lipid handling of Bach2-deficient AMs**

Because pulmonary surfactant is secreted by alveolar type 2 epithelial cells and is phagocytized by AMs, we investigated the expression of Bach2 in these cells. The expression of Bach2 was hardly detected in the isolated alveolar type 2 epithelial cells by the RT-PCR (Fig. 4 a) or immunohistochemical analyses of the lungs using anti-Bach2 antibodies (not depicted). In contrast, we detected Bach2 in WT but not Bach2-deficient AMs by immunostaining (Fig. 4 b).

A substantial part of pulmonary surfactant is lipid, including phospholipids and cholesterol, which are taken up by AMs
(Trapnell et al., 2003). The foamy AMs associated with PAP are due to their ingestion of a large amount of lipids (Iyonaga et al., 1999). We detected a prominent accumulation of lipids in Bach2-deficient AMs by Oil-Red O staining (Fig. 4 c). We also found that Bach2-deficient AMs showed an enhancement in cholesterol uptake in vitro compared with WT control cells (Fig. 4 d). Peroxisome proliferator-activated receptor γ (Pparg) and liver X receptor α (Lxra) play pivotal roles in the regulation of cholesterol metabolism in macrophages. These transcrip-
tion factors are activated by oxysterols, oxidized derivatives of cholesterol, and transactivate ATP-binding cassette trans-
porters A1 (Abca1) and G1 (Abcg1), which excrete excess cho-
lesterol from macrophages (Chawla et al., 2001; Bonfield et al., 2003; Ricote et al., 2004; Bates et al., 2005; Baker et al., 2010). Although the mRNA levels of Pparg1 and Lxra were increased in Bach2-deficient AMs compared with WT cells, the mRNA levels of Abca1 and Abcg1 were decreased (Fig. 4 e). These results suggest that the enhanced uptake of cholesterol and the changes in gene expression related to cholesterol ef-
flux in Bach2-deficient AMs were connected with the onset of the PAP-like disease.

**Phospholipid compositions of AMs were compared using liquid chromatography–mass spectrometry (LC–MS/MS) analysis.** We focused on phosphatidylcholines (PCs), including palmitoyl-palmityl PC (PC16:0/16:1, characterized by the presence of one saturated and one unsaturated fatty acid), PC16:0/18:2, PC16:0/18:1, and other related PC species which are the major surfactant lipids (Postle et al., 2006). The representative mass chromatograms by precursor ion scanning mode are shown in Fig. 5 a. As shown in Fig. 5 b, many of
foamy AMs found in PAP. We therefore looked for an alteration in the GM-CSF pathway in Bach2-deficient mice. The mRNA levels of GM-CSF in the lungs and the concentrations of GM-CSF in the BAL fluids were not decreased in the Bach2-deficient mice (unpublished data). Furthermore, the mRNA levels of GM-CSFRα and GM-CSFRβc and PU.1 in sorted AMs were not decreased by Bach2 deficiency (unpublished data).

Immunostaining of BAL cells verified that PU.1 was expressed in these PC species were reduced in Bach2-deficient AMs compared with WT AMs. We identified two oxidized forms of PCs which were increased in Bach2-deficient AMs (Fig. 5c). These altered levels of PCs and oxidized PCs point to a defective handling of the surfactant lipids by Bach2-deficient AMs.

The GM-CSF–PU.1 pathway is not impaired in Bach2-deficient mice

GM-CSF stimulation is essential for the functional maturation of AMs. GM-CSF binds to the GM-CSF receptor to induce PU.1 expression, which then drives the terminal differentiation of AMs (Shibata et al., 2001; Trapnell et al., 2009). A segment of PAP patients (Dirksen et al., 1997; Kitamura et al., 1999) and GM-CSF-deficient mice (Dranoff et al., 1994) show a disruption of the GM-CSF signaling in AMs, thus resulting in impaired phagocytosis (Harris, 1979; Paine et al., 2001; Yoshida et al., 2001) and cholesterol efflux (Thomassen et al., 2007; Blanchard et al., 2012), which contribute to the foamy AMs found in PAP. We therefore looked for an alteration in the GM-CSF pathway in Bach2-deficient mice. The mRNA levels of GM-CSF in the lungs and the concentrations of GM-CSF in the BAL fluids were not decreased in the Bach2-deficient mice (unpublished data). Furthermore, the mRNA levels of GM-CSFRα and GM-CSFRβc and PU.1 in sorted AMs were not decreased by Bach2 deficiency (unpublished data). Immunostaining of BAL cells verified that PU.1 was expressed...
Altered expression of genes for immune cell trafficking in macrophages

To reveal the genes downstream of Bach2 in AMs, we compared the gene expression profiles of AMs using a microarray analysis. We focused on genes whose expression changed >10-fold in the absence of Bach2 (Fig. 6a). Consistent with the increased infiltration of AMs and granulocytes in Bach2-deficient mice (Fig. 2), gene ontology (GO) analysis of these genes revealed that GO categories related to inflammatory response and immune cell trafficking tended to be enriched in Bach2-deficient AMs (Table S2). These categories included genes such as Ccl2, Ccl7, Ccl8, Csf1, S100a9, Adam8, and MafB (Fig. 6a). The altered lipid handling of Bach2-deficient macrophages was also supported by the enrichment of GO terms related to lipid binding (e.g., Dgts). The altered expression of genes for immune cell trafficking in macrophages was also supported by the enrichment of GO terms related to lipid binding (e.g., Dgts). The higher expression of Ccl2, which encodes monocyte migration cytokine (MCP-1) in Bach2-deficient AMs than control AMs, was confirmed using an RT-PCR analysis (Fig. 6b). Ccl24 encoding an eosinophil migration cytokine (eotaxin-2) and Cxcl1 encoding a neutrophil migration chemokine (KC) also

In humans, PAP frequently involves anti–GM-CSF autoantibodies (Kitamura et al., 1999). Therefore, we investigated whether anti–GM-CSF antibodies were involved in the pathogenesis of PAP in the Bach2-deficient mice because they tend to produce autoantibodies (unpublished data). Blimp-1 (Prdm1) is the master regulator of plasma cells, and Blimp-1–deficient mice cannot secrete any antibodies (Shapiro-Shelef et al., 2003). We crossed Bach2–deficient mice and B cell–specific Blimp-1–deficient mice (Shapiro-Shelef et al., 2003) and obtained Bach2-deficient mice which could not secrete any antibodies. Although the Blimp-1–deficient mice did not develop PAP, the Bach2 and Blimp-1 double-deficient mice developed PAP as severely as did the Bach2-deficient mice (unpublished data). These results showed that autoantibodies were not involved in the pathogenesis of PAP in Bach2-deficient mice. Collectively, these data suggested that the dysfunction of Bach2-deficient AMs was independent of the GM-CSF-PU.1 pathway.

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tended to be up-regulated in Bach2-deficient AMs in the array analysis (unpublished data) and RT-PCR analysis (Fig. 6 b). Increased levels of Ccl2/MCP-1 and Cxcl1/KC were confirmed by a cytokine antibody array analysis of the BAL fluid (unpublished data). These results suggest that the enhanced recruitment of AMs and granulocytes in the lung of Bach2-deficient mice was due, at least in part, to the changes in the expression of genes related to immune cell trafficking in AMs. By focusing on genes whose expression was reduced more than fourfold, Bach2 deficiency was found to affect MHC class II components (Table S3). Interestingly, expression of MHC class I components such as H2-M2 were increased (Fig. 6 a and not depicted). These observations together suggest that the lung pathology we observed may involve deregulation of the immune system at multiple points.

Aberrant activation of M2-related genes and Irf4 in Bach2-deficient AMs

The expression of M1- and M2-related genes was also altered. Among the M1-related genes, although Nos2 encoding iNOS was not affected, a fraction of the genes showed either decreased (Cxcl10 and Ifn5) or increased (Il-6 and Il-1b) expression (Fig. 7, a and b). Higher levels of Il-6, Il-12, and soluble TNFR1 (sTNFR1) were also present in the BAL fluids from Bach2-deficient mice (unpublished data). Many of the M2-related genes were up-regulated in the Bach2-deficient AMs, including Arg1 encoding arginase and Ym1 encoding chitinase-3–like 3 (Fig. 7, c and d). The expression of two other M2-related genes, Retnla and Mrc1, also tended to be higher. IL-4 receptor α mRNA was also higher in Bach2-deficient AMs than WT cells, raising the possibility of increased IL-4 signaling in Bach2-deficient AMs (Fig. 7 e). These results suggested that Bach2-deficient AMs showed aberrant activation of M1 and M2 phenotypes.

Irf4 is a critical regulator of M2 polarization (Satoh et al., 2010). We found that the expression of Irf4 was elevated in Bach2-deficient AMs (Fig. 7 f). Bach2 represses the expression of Blimp-1, which in turn activates Irf4 in B lymphoid cells (Sciannimas et al., 2006; Igarashi et al., 2007; Muto et al., 2010). However, the expression of Blimp-1 was not elevated in Bach2-deficient AMs (Fig. 7 f). These results suggest that Bach2 represses Irf4 in a Blimp-1–independent manner in AMs and that the overexpression of Irf4 might contribute to the activation of the M2 phenotype in Bach2-deficient AMs.

Based on the above results, we surmised that the altered gene expression in Bach2-deficient AMs would affect the production of surfactants in the alveolar cells. To address this possibility, we focused our analysis on Cxcl11, Il-12 p40/Il-12b, Il-6, Ccl2/MCP-1, and sTNF-R1, which showed higher expression in Bach2-deficient macrophages than in control WT cells (Figs. 6 and 7) and/or was present at higher levels in BALs of Bach2-deficient mice than those of WT mice (not depicted). We used human NCI-H441 cell line which exhibits the characteristics of alveolar type II epithelial cells. As shown in Fig. 7 g, Il-6 enhanced SP-A expression, whereas other factors did not show such an effect. Therefore, the altered expression of Il-6 in Bach2-deficient AMs may be responsible, at least in part, for the enhanced production of SP-A (Fig. 3 a).

Aberrant activation of M2 phenotype in Bach2-deficient peritoneal macrophages

To investigate whether the effect of Bach2 deficiency upon the macrophage phenotype is restricted to AMs or not, we compared peritoneal macrophages in WT control and Bach2-deficient mice. Bach2-deficient mice tended to contain higher numbers of peritoneal cells and resident macrophages (Fig. 8, a and b). As was the case in the lung, more F4/80low CD11b− SiglecF+ eosinophils were present in the peritoneal cavity of Bach2-deficient mice than WT mice (Fig. 8, a and b), suggesting that Bach2 regulated the recruitment of eosinophils. Isolated peritoneal macrophages were stimulated with GM-CSF in vitro in the presence of the M1 inducer LPS or the M2
Because one of the critical downstream effectors of IL-4 in macrophages is STAT6 (Takeda et al., 1996; Kang et al., 2008; Lawrence and Natoli, 2011), we compared phosphorylation of STAT6 in BM-derived macrophages (BMDMs) isolated from Bach2-deficient and control WT mice. We found an enhancement in STAT6 phosphorylation in response to IL-4 in Bach2-deficient BMDM as compared with WT cells (Fig. 9 e). Therefore, the enhanced response to IL-4 may contribute the altered phenotypes of Bach2-deficient macrophages. The higher expression of IL-4 receptor mRNA (Fig. 7 e) may be causative to this enhanced STAT6 phosphorylation.

The expression of Bach2 in hematopoietic cells is essential for pulmonary homeostasis

To confirm the involvement of altered hematopoietic cells in the development of PAP-like disease in the Bach2-deficient mice, we performed BM transplantation using Bach2-deficient mice (CD45.2+) and WT mice (CD45.1+CD45.2+). First, to examine whether WT BM could prevent the onset of PAP in Bach2-deficient mice, we transplanted WT and Bach2-deficient BM into 8-wk-old Bach2-deficient mice (Fig. 10 a), which we had shown to be at preclinical stage (i.e., less accumulation of surfactant in the alveolar spaces; Fig. 2 a). At 2 mo after transplantation, a flow cytometric analysis of the peripheral blood cells showed efficient replacement by WT cells in Bach2-deficient mice transplanted with WT BM (Fig. 10 b). The Bach2-deficient mice transplanted with WT BM looked free from dyspnea. Pink materials did not accumulate in the alveolar spaces, and inflammatory cells were not recruited in these mice (Fig. 10 c). In contrast, the Bach2-deficient mice transplanted with Bach2-deficient BM showed progressive dyspnea and developed PAP as severely as nontreated Bach2-deficient mice (Fig. 10, b and c). Therefore, the PAP-like disease with the infiltration of inflammatory cells was caused by Bach2 deficiency in hematopoietic cells.

Next, to examine whether BM transplantation could ameliorate the PAP-like disease of Bach2-deficient mice, we transplanted WT BM into Bach2-deficient mice >16 wk of age (Fig. 10 d), which we expected to have already accumulated large amounts of surfactant in the alveolar spaces (Fig. 1). This expectation was confirmed by the CT imaging of these mice before transplantation (Fig. 10 f). Indeed, these Bach2-deficient mice had already shown progressive dyspnea. At 2 mo after transplantation, the flow cytometric analysis of peripheral blood cells showed that a part of the BM had been replaced by WT cells (Fig. 10 e). This was because we applied only 500 rads upon the transplantation due to their disease condition (Fig. 10, figure legend). The mice started to show calm and normal breathing after the WT BM transplantation. Before BM transplantation, we observed the diffuse distribution of reticular shadows and partial consolidation shadows in the CT images of the lungs of these mice (Fig. 10 f). After transplantation, these findings dramatically improved, and the diffuse reticular shadows became lighter and the consolidation shadows almost disappeared (Fig. 10 f). Although foamy AMs and granulocytes still existed in the alveolar...
spaces, the surfactant accumulation was greatly reduced (Fig. 10 g). Though we did not measure the function of lungs or saturation level of oxygen in these mice, we judged that they were undoubtedly improved based on the changes in the pathology and CT images. These results showed that the presence of Bach2 in the BM cells prevented the onset of PAP-like disease in Bach2-deficient mice and that the BM transplantation was effective for ameliorating PAP even after its onset in this model.

DISCUSSION

Tissue macrophages are not a homogenous population, and it is generally thought that they represent a spectrum of activated phenotypes rather than a discrete stable subpopulation (Mosser and Edwards, 2008). AMs maintain the surfactant homeostasis and play a key regulatory role in pro- and anti-inflammatory responses by acquiring distinct functional phenotypes. However, the factors that regulate the differentiation and function of AMs and the surfactant homeostasis are largely unknown (Lawrence and Natoli, 2011). In this study, we showed that Bach2 was essential for the maintenance of surfactant homeostasis and that this new function of Bach2 involved the regulation of AMs.

Bach2-deficient mice developed the PAP-like accumulation of SPs in the alveolar spaces (Fig. 1). Our results suggest that multiple defects contributed to this phenotype. First, we found that AMs in Bach2-deficient mice showed more accumulation of lipids, elevated levels of cholesterol uptake, and faulty handling of PCs compared with WT AMs (Fig. 4, c–e; and Fig. 5). Second, we also observed a reduction in the expression of genes involved in cholesterol metabolism (Fig. 4 e). Lipid homeostasis is regulated by the Ppar–Abc transporter pathway (Chawla et al., 2001; Ricote et al., 2004), a dysfunction of which results in the formation of foamy AMs (Bates et al., 2005; Thomassen et al., 2007; Baker et al., 2010). Expression of Ppary and Abcg1 are decreased in the foamy AMs of PAP patients with anti–GM-CSF antibodies and in GM-CSF–deficient mice (Thomassen et al., 2007). Because the mRNA levels of Abca1 and Abg1 were both decreased in AMs of Bach2-deficient mice (Fig. 4 e), these changes are expected to result in an accumulation of cholesterol, contributing to the generation of foamy AMs observed in Bach2-deficient mice.
Although Bach2 is involved in macrophage regulation, it is not clear whether this reflects cell-intrinsic function or not. The altered responses of isolated peritoneal macrophages support the role of Bach2 in macrophages. In addition, we have recently found that the phosphatidylinositol 3-kinase pathway, which is known to promote M2 polarization (Martinez, 2011), inhibits the expression of Bach2 in the peritoneal macrophages (unpublished data). In contrast, we did not observe an elevation in the levels of IL-4 or IL-13 in the lung (unpublished data). These observations support cell-autonomous function of Bach2 in macrophages. Consistent with this interpretation, the expression level of Ifi4 was elevated in Bach2-deficient AMs (Fig. 7 f), which is known to promote the M2 phenotype (Cao et al., 2002; Satoh et al., 2010). Thus, we suggest that Bach2 is one of the critical regulators of M1-M2 polarization, limiting the manifestation of a subprogram of M2 phenotype. Because some of the M1-related genes such as Il6 were expressed at higher levels without Bach2, Bach2 deficiency did not lead to a simple, general suppression of M1 and activation of M2 genes. It may regulate subprograms for both M1 and M2 phenotypes. However, further studies are required, including cell-specific deletion of Bach2, to conclude this interpretation. Identification of direct target genes of Bach2 in macrophages will also be critical.

It remains possible that the altered macrophage phenotype in Bach2-deficient mice reflects alterations in both macrophages and microenvironment. This is likely because Bach2 is an important regulator of B cells (Igarashi et al., 2007) and T cells (Roychoudhuri et al., 2013; Tsukumo et al., 2013). It has been reported recently that B1 B cells drive M2 polarization of macrophages in mice (Wong et al., 2010). A recent report suggests a connection between eosinophils and M2-like macrophages (Satoh et al., 2013). Therefore, it is possible that Bach2-deficient B cells, T cells, and eosinophils may further contribute to the M2 polarization and the PAP-like disease in the Bach2-deficient mice. To further clarify this point, a conditional knockout of Bach2 in hematopoietic cells will be important. Based on the observations and discussion above, we propose that Bach2-deficient mice provide a valuable mouse model for secondary PAP associated with hematologic disorders. Although an effective treatment for secondary PAP associated with hematologic disorders has not been established, there have been reports that secondary PAP was improved by BM transplantation (Cordonnier et al., 1994; Ladeb et al., 1996; Ishii et al., 2011). In this study, even a partial replacement of Bach2-deficient BM with WT BM could rescue the accumulation of surfactant in the alveolar spaces (Fig. 10, c and g), showing that BM transplantation is effective for certain types of PAP. The present results also suggest that one should look into the feasibility of identifying therapeutic targets of PAP and other macrophage-related diseases from the downstream target genes or upstream regulators of Bach2.

**MATERIALS AND METHODS**

**Mice.** Bach2-deficient mice were described previously (Muto et al., 2004). B cell-specific Blimp–1-deficient mice (Shapiro-Shale et al., 2003) were provided by K. Calame (Columbia University, New York, NY). *Bach2*<sup>+/−</sup> *Blimp-1*<sup>−/−</sup>
mice were generated by breeding these mice. CD45.1 congenic mice were purchased from Sankyo Lab Service Corporation, Inc. All experiments involving mice were approved by the Institutional Animal Care and Use Committee of the Tohoku University Environmental & Safety Committee.

Body weight and survival rate curves. WT and Bach2-deficient mice were housed under specific pathogen-free conditions. The body weight of the mice was measured every week from 4 until 50 wk of age. Averaged body weight curves were generated for WT mice. Because Bach2-deficient mice showed individual variability in body weight and longevity, the data from individual mice were presented separately. The body weight curves and the survival rate curves were generated using Excel (Microsoft) and Delta Graph software (Nihon Poladigital, K.K.) programs, respectively.

Histology and immunohistochemistry. Mice were euthanized using diethyl ether and the lungs were removed, fixed with 4% paraformaldehyde, and embedded in paraffin. Lung sections were stained with hematoxylin and eosin (HE) or PAS stain. Deparaffinized lung sections were incubated with antibodies against SP-A (1:500; Millipore), SP-B (1:500; Millipore), SP-C (1:100; Santa Cruz Biotechnology, Inc.), or SP-D (1:500; Millipore) and visualized by a secondary antibody conjugated with horseradish peroxidase.

BAL fluids and cells. Mice were euthanized using diethyl ether and the trachea was exposed and cannulated. The lungs were flushed two times with 0.8-ml aliquots of PBS. The aliquots were combined and centrifuged at 300 g for 5 min to separate the supernatants and BAL cells. The supernatants were stored at −80°C. BAL cells were used for experiments.

Flow cytometry. The collected BAL cells were suspended with staining buffer (PBS with 3% fetal bovine serum) and stained with fluorochrome-conjugated antibodies specific for F4/80 (eBioscience) and Gr-1 (BD). The cells were sorted with a FACSAria II (BD). The data were analyzed using FlowJo software (Tree Star).

The collected peritoneal cells were similarly suspended with staining buffer (PBS with 3% fetal bovine serum) and stained with fluorescent-conjugated antibodies specific for F4/80 (eBioscience) and Gr-1 (BD). The cells were sorted with a FACSAria II (BD). The data were analyzed using FlowJo software.

Immunofluorescence staining. BAL cells were placed on Matsunami Adhesive silane (MAB)–coated slides and stained with biotin-conjugated antibodies specific for NICD11c (BD) and anti-Bach2 antisem (F69-1), or an anti-PU.1 antibody (Santa Cruz Biotechnology, Inc.). Cells were then incubated with streptavidin–conjugated FITC and Alexa Fluor 555–conjugated anti–rabbit IgG antibody. Samples were examined with a confocal laser-scanning microscope (FV-100; Olympus). Photoshop (Adobe) was used for the presentation of images.

Immunoblotting, ELISA, and cytokine antibody array. The BAL fluid was mixed with SDS and heat denatured. Samples were loaded onto SDS–polyacrylamide gels at 15 µl/well. The immunoblotting analysis was performed with antibodies against SP-A, SP-B, SP-C, or SP-D. The GM-CSF ELISA of BAL fluid was performed using a Mouse GM-CSF ELISA kit (Thermo Fisher Scientific). The cytokine antibody array using the BAL fluid was performed with a RayBio Mouse Cytokine Antibody Array 2 (RayBiotech).

RT-PCR and microarray analysis. The RNA from the lungs and alveolar type 2 epithelial cells was prepared by using the TRizol reagent (Invitrogen). RNA from the AMs was prepared using an RNeasy Plus Micro kit (Qiagen). Complementary DNA was synthesized using an Omniscript RT kit (Qiagen) for RNA from the lungs and a SuperScript First III kit (Invitrogen) for the RNA of AMs. The methods of quantitative PCR and microarray analysis were described previously (Dohi et al., 2008). The analysis of genes was performed using Gene Spring software (Agilent Technologies) and Ingenuity Pathways Analysis software (IPA 9.0; Ingenuity Systems). The primers used are listed in Table S1.

Quantification of internalized NBD-cholesterol. AMs were incubated with RPML-1640 (2 × 10⁵/100 µl in glass-bottom dishes) and allowed to adhere for 1 h. The cells were added with 1 µM NBD–cholesterol. The dishes were then washed gently with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. After washing with PBS, fluorescence intensity of the cells with internalized fluorescent cholesterol conjugates was observed upon excitation at 488 nm at a constant intensity in all experiments.

LC-MS/MS analysis. LC-MS/MS was consisted with a NANSOPACE SI-2 HPLC system (Shiseido) and a TSQ Quantum Ultra (Thermo Fisher Scientific) triple quadrupole mass spectrometer equipped with a heated ESI source. Samples were analyzed by precursor ion scanning mode in a range of m/z 20 to 1,000 in positive ionization mode via collision induced dissociation (CID). After fragmentation of CID gas with 31 eV of collision energy, PC molecules produced a fragment with m/z 184 corresponding to the protonated phosphocholine head group according to a previous study (Brügger et al., 1997). The optimum value of the MS parameters for spray voltage, sheath gas pressure, auxiliary gas pressure, vaporizer temperature, capillary temperature, and collision pressure were set at 3.0 kV, 35 psi, 20 psi, 350°C, 270°C, and 0.7 mTorr, respectively. Liquid chromatographic separation was performed using a CapCell Pak Silica SG120 (150 mm × 2 mm i.d., 5-µm particle size; Shiseido) column with a hydrophilic interaction chromatography elution. The mobile phases were 5 mmol/liter ammonium formate (A) and acetonitrile (B). The gradient program was as follows: initial elution with 100% B followed by a linear gradient to 82.5% B from 0 to 15 min, and immediately decreased 30% B at 17 min. Then, we returned to the initial conditions for 2 min until the end of the run. The total run time was 20 min. The mobile phase flow rate was 400 µl/min. Column effluent was introduced into the MS between 3.0 and 22.5 min after injection.

Sample preparation for LC-MS/MS was done according to a previous study (Saiga et al., 2012). An automated cell counter was used to calculate cell numbers and 25,000 cells were used for the sample preparation. 100 µl methanol was added into the sample of AMs. Because cell debris may be counted, we may have overestimated the numbers of the cells. The resulting mixture was homogenized for 5 min in an ultrasonic bath. After centrifugation at 14,600 g for 10 min, the supernatant was transferred into a new 2.0-ml siliconized plastic tube. Then, an internal standard (IS; PC14:0/14:0, 1 µmol/liter, 50 µl) was added, and the mixture was homogenized for 5 min in an ultrasonic bath and centrifuged at 16,400 g for 10 min. Then, the supernatant was transferred into a new siliconized plastic tube and passed through a filter (pore size: 0.2 µm; YMCA). Subsequently, 5 µl of the filtered solution was injected into the LC-MS/MS system. The values of composition were calculated by the ratio of IS, and comparisons between WT and KO groups were made using Student’s independent t test. The representative spikes of PCs in lung macrophages were extracted according to a previous study (Postle et al., 2006) and assigned with the Lipid Search (Mitsui Knowledge Industry) software using additional independent high-resolution data by a Q Exactive (Thermo Fisher Scientific).

Isolation of alveolar type 2 epithelial cells. Alveolar type 2 epithelial cells were isolated as described previously (Corti et al., 1996) with some modifications. Anti-CD32 antibody was omitted for the purification.

Peritoneal macrophages and BMDMs. Peritoneal macrophages and BMDMs were isolated from WT or Bach2-deficient mice, as previously described (Fleetwood et al., 2007; Satoh et al., 2010), and were cultured in RPMI-1640 medium containing 10% (vol/vol) fetal bovine serum and 10 ng/ml GM-CSF; 10 µg/ml LPS (InvivoGen) and 20 ng/ml IL-4 (Sigma-Aldrich) were added as M1 and M2 macrophage inducer, respectively.

Intracellular staining. IL-4–stimulated cells were washed in staining buffer, PBS containing BSA (0.5%), and sodium azide (0.05%) and blocked with
http://dx.doi.org/10.1194/JLR-M081653

http://dx.doi.org/10.1152/ajplung.00234.2005

http://dx.doi.org/10.1194/jlr.M021485

http://dx.doi.org/10.1165/rcmb.2003-0148OC

http://dx.doi.org/10.1183/09059180.0001311

http://dx.doi.org/10.1073/pnas.94.6.2339

http://dx.doi.org/10.1016/s0367.4274


http://dx.doi.org/10.1016/S1097-2765(01)00164-2

http://dx.doi.org/10.1164/ajrccm.149.3.8118651

http://dx.doi.org/10.1165/ajrcmb.14.4.8609933

http://dx.doi.org/10.1172/JCI119758

http://dx.doi.org/10.1038/nmb1516

http://dx.doi.org/10.1126/science.8171324


http://dx.doi.org/10.1038/nri978

http://dx.doi.org/10.1016/j.bbamem.2007.10.027

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Table S1. Sequences of the primers used for RT-PCR

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Table S2. GO analysis of genes with >10-fold up-regulation (Continued)

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**Table S3.** GO analysis of genes with more than fourfold down-regulation

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