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B cell activating factor is central to bleomycin- and IL-17-mediated experimental pulmonary fibrosis

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Abbreviations: APRIL: proliferation-inducing ligand, BAFF: B cell activating factor, BALF: bronchoalveolar lavage fluid, BLM: bleomycin, CAB: Chromotrope aniline blue, IPF: idiopathic pulmonary fibrosis, MMP: matrix metallopeptidase, pSS: primary Sjögren's syndrome, SSc: systemic sclerosis, TIMP: tissue inhibitor of metalloproteinase

Highlights

- B-cells contribute to the pathogenesis of fibrosis, but little is known regarding the role of B-cell activating factor (BAFF), a pivotal cytokine for B-cell activation, in fibrosis.

- BAFF levels are elevated in bronchoalveolar lavages of patients with idiopathic pulmonary fibrosis and in bleomycin-induced lung fibrosis.

- Genetic ablation of BAFF or BAFF neutralization by a soluble receptor significantly attenuates pulmonary fibrosis and IL-1β levels in bleomycin-induced lung fibrosis.

- Bleomycin-induced BAFF expression and lung fibrosis are IL-1β- and IL-17A-dependent.

- BAFF is required for rIL-17A-induced lung fibrosis and elevated IL-17A secretion by CD3^{+} T cells from murine fibrotic lungs.

ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a progressive devastating, yet untreatable fibrotic disease of unknown origin. We investigated the contribution of the B-cell activating factor (BAFF), a TNF family member recently implicated in the regulation of pathogenic IL-17-producing cells in autoimmune diseases. The contribution of BAFF was assessed in a murine model of lung fibrosis induced by airway administered bleomycin.We show that murine BAFF levels were strongly increased in the bronchoalveolar space and lungs after bleomycin exposure.We identified Gr1$^+$ neutrophils as an important source of BAFF upon BLM induced lung inflammation and fibrosis. Genetic ablation of BAFF or BAFF neutralization by a soluble receptor significantly attenuated pulmonary fibrosis and IL-1β levels. We further demonstrate that bleomycin-induced BAFF expression and lung fibrosis were IL-1β and IL-17A dependent. BAFF was required for rIL-17A-induced lung fibrosis and augmented IL-17A production by CD3$^+$ T cells from murine fibrotic lungs *ex vivo*.Finally we report elevated levels of BAFF in bronchoalveolar lavages from IPF patients.Our data therefore support a role for BAFF in the establishment of pulmonary fibrosis and a crosstalk between IL-1β, BAFF and IL-17A.

Keywords: BAFF, pulmonary fibrosis, IL-1β, IL-17, systemic sclerosis, autoimmunity, bleomycin

1. Introduction

Fibrosis is a devastating untreatable feature of numerous human diseases, including chronic heart, kidney or liver insufficiency, idiopathic pulmonary fibrosis(IPF)[1]and autoimmune diseases such as systemic sclerosis (SSc)[2]or primary Sjögren's syndrome (pSS)[3]. IPF is a progressive, chronic and irreversible interstitial lung disease of unknown etiology that is usually lethal[1]. A growing body of evidence suggests that the disease process is initiated through alveolar epithelial cell microinjuries that lead to a persistent immuno-inflammatory phase with production of cytokines, chemokines and growth factors responsible for the expansion of fibroblast and myofibroblast populations[4]leading to dysregulated tissue repair[5]and parenchyma destruction[6,7].These fibroblastic foci secrete exaggerated amounts of extracellular matrix components that destroy the lung parenchyma[6,7]. Many forms of the disease are believed to be induced, at least initially by a strong inflammatory response[4]andrecent workshops on research future directions in IPF recommended to promote studies,among six critical areas, on the role of inflammation and immunity in fibrotic diseases[8,9]. To date, no therapy has been clearly shown to prolong IPF patient survival. In systemic sclerosis (SSc)fibrosis[10,11], a skin fibrotic disease often associated with pulmonary fibrosis, levels of the tumor necrosis factor (TNF) family members B cell activating factor (BAFF; also known as TNFSF13B, BLyS or TALL1) correlate with disease progression[12–14].Recently, Xue et al, showed that plasma concentrations of BAFF were significantly greater in IPF than control patients and that increased BAFF levelswere associated with diminished survival[15].Many important advances have been generated using mouse models of IPF. Using the bleomycin (BLM)-induced pulmonary fibrosis model, we previously reported that pulmonary inflammation and fibrosis are mediated by secretion of the pro-inflammatory and pro-fibrotic cytokine IL-1β through Nlrp3 inflammasome

activationand IL-1R1/MyD88 signaling[16–18].We also identified the existence of an early IL-1β-IL-23-IL-17A axis in pulmonary inflammation leading to late fibrosis in this model[19] and IL-17-producing Th17 cells were shown to be a critical mediator of BLM and/or IL-1β-mediated pulmonary fibrosis[7,20,21].

Here we hypothesized that BAFF and/or the proliferation-inducing ligand (APRIL, TNFSF13) couldbe involved in the physiopathology of pulmonary fibrosis.BAFF and APRIL are produced by innate immune cells[22]such as neutrophils[23], macrophages, monocytes, dendritic cells (DCs) or follicular DCs[24], but also T cells[25] , B cells[26]or non-hematopoietic cells such as fibroblast-like synoviocytes from patients with rheumatoid arthritis[27] or salivary gland and conjunctival epithelial cells from patients with Sjögren's syndrome[28,29]. Receptors for BAFF and APRIL are mainly expressed by B cells but also by T cells, monocytes or DCs, indicating that the role of BAFF and APRIL extends beyond that of B cell biology[22]. An excess of BAFF may lead to development of autoimmune disorders in mice and humans[30–32].

Here wereportthe first evidence of a critical role of BAFF in experimental pulmonary fibrosis in mice. Strong overexpression of BAFF was induced in both bronchoalveolar space and lung after bleomycin exposure in this model. Genetic ablation of BAFF or neutralization of BAFF by a soluble receptor significantly attenuated the experimental pulmonary fibrosis.We identified Gr1^{+} neutrophils as an important source of BAFF upon BLM-induced lung inflammation and fibrosis. We show that rIL-17A-induced fibrosis is dependent on BAFF and that BAFF amplifies IL-17-production by T cells from mouse fibrotic lungs. Finally, we report for the first time increased BAFF levels in the bronchoalveolar lavages of IPF patients. Our data therefore support a role for BAFF in the establishment of pulmonary fibrosis and a crosstalk between IL-1β, BAFF and IL-17A.

2. Material and methods

2.1 Mice

BAFF-/-[33], IL-1R1-/- [34], IL-17A-/- and IL-17RA-/- [35] backcrossed 10 times on the wild-type C57BL/6 genetic background were used. Mice were bred in our animal facility (CNRS, Orleans). Six to ten weeks used animals were kept in sterile isolated ventilated cages. All animal experiments complied with the French Government's ethical and animal experiment regulations.

2.2 Treatments

Bleomycin sulfate (5 mg/kg; Bellon Laboratories), rBAFF, rIL1β or rIL-17A (1µg/mice, once at day 0) in saline or saline alone, were given through the airways by nasal instillation in a volume of 40 µl under light ketamine-xylasine anesthesia. The BAFF-R-Ig (provided by Dr Anne Davidson, The Feinstein Institute for Medical Research, Manhasset, NY) or control isotype IgG2a (eBiosciences) were injected at 5mg/kg i.p. at the time of BLM administration (or 3 days later) and every 3 days until the mice were sacrificed.

2.3 Bronchoalveolar Lavage Fluid (BALF), cell count and determination

BALF, cell count and determination were performed as previously described[17].

2.4 Lung homogenization and total lung collagen measurements

Lungs were homogenized as described [19]. Aliquots of lung homogenate (50 μl) were then assayed for lung collagen levels and compared with a standard curve prepared from bovine skin using the Sircol collagen dye binding assay according to the manufacturer's instructions (Biocolor Ltd.).

2.5 Mediator quantification by ELISA

Levels of BAFF, IL-1β, IL-17A, latent TGF-β1, TIMP-1, IL-6 and IL-23 p19 were determined using ELISA assay kits (all from R&D Systems), according to manufacturer's instructions.

2.6 RT-qPCR

Total RNA extracted were reverse transcribed using iScript™ cDNA synthesis kit (BioRad) according to the manufacturer's instructions. Real-time quantitative PCR were performed in a total volume of 20 μl with a SensiMix Plus SYBR® (Quantace) in Rotor-Gene™ 6000 real-time PCR machine (Corbett Life Science®) using the gene-specific primers for Baff, April, Col1a1, Col1a2, Col3a1 and Gapdh. Gapdh was used as a control. Amplification products were detected as an increased fluorescent SYBR Green signal during the amplification cycles. The relative expression of target transcript PCR products was determined using the ΔΔCt method[36].

2.7 Histology

After BAL and lung perfusion, the large lobe was fixed in 4% buffered formaldehyde for standard microscopic analysis. Sections (3 μm) were stained with Chromotrope Aniline Blue as described previously[16]. The severity of the morphological changes (infiltration by

neutrophils and mononuclear cells, destruction and thickening of the alveolar septae and fibrosis) were assessed semi quantitatively using a score of 0–5 by 2 independent observers.

2.8 Reagents and cell culture

For *ex vivo* cell culture, lung cells were isolated through a cell strainer (70 µM), washed, and plated at 5×10^5 cells per well of a 96well plate. Cells were stimulated with 1 µg/ml of antiCD3ε antibody or 5µg/mL of anti-IgM antibody (eBioscience) and 1, 10, 25, 50 or 100 ng/mL of recombinant murine BAFF or 100ng/mL of rIL17A or rIL1β (R&D Systems).

2.9 FACS sorting of lung cells and qPCR analysis

For cell sorting experiments, mice were administrated with 5mg/kg of BLM and sacrificed after 7 days. Lung cells were isolated through a cell strainer (70µM), washed and then labeled with anti-CD115 (clone AFS98), anti-CD3 (clone 17A2), anti-CD19 (clone eBio1D3) and anti-Gr1 (clone RB6-8C5) antibodies (eBiosciences) and sorted with FACS Aria II. Cell purity was checked by flow cytometry analysis for each marker. Total RNA was extracted and reverse-transcribed and Baff mRNA expression was evaluated by qPCR. Expression of Gapdh was used for normalization. Relative expression was normalized to Baff expression in CD19$^+$ cells (cells having the lower Baff expression level).

2.10 Patients and control subjects

The study was approved by the Institutional Review Board of the French learned society for respiratory medicine-Société de Pneumologie de Langue Française-N° 2012-016 (Bichat hospital, Paris) ; written informed consent was obtained from the participants to the study (Biocollection DC 2010-1216, CHU Tours); clinical charts and functional records were collected on a standardized and anonymous collection form. Bronchoalveolar lavage was

performed as previously described[37]. Briefly, BALF of 10 patients with stable idiopathic pulmonary fibrotic (IPF), 5 patients with exacerbated IPF and 15 patients explored for hemopoietic spittle without identified respiratory disease (control) was collected. Patients with symptoms of acute infections or malignancies were excluded. All participants gave their written consent. BALF was collected after lavage with 150ml of physiological serum and wascentrifugated 10min1500t/min at 4°C then aliquoted in the 30 min following the lavage and 2ml of lavage supernatant were kept for collection at -80°C.

2.11 Statistics

Statistical evaluation of differences between the experimental groups was determined by non-parametric Mann-Whitney test using Prism software. *P* values of <0.05 were considered statistically significant.

3. Results

3.1*Bleomycin-treated mice have exacerbated BAFF expression in lungs and BALF*

In order to investigate the contribution of BAFF in idiopathic pulmonary fibrosis (IPF), we used the bleomycin (BLM) model of lung injury and fibrosis in mice[16]. BLM instillation resulted in an increase of BAFF mRNA in the lungs compared with saline-treated littermates, which started after 1 day, peaked at day 7 and returned to basal levels at day 14 (Fig. 1a), while APRIL mRNA levels were not modified (Supplementary Fig. S1). At the protein level, a significant increase of BAFF in lung homogenates started after 1 day and increased with time until day 14 (Fig. 1b). BAFF protein upregulation was even higher in the BALF than in lung homogenates with a 10-fold increase of BAFF compared with saline-treated littermates after 4 days (Fig. 1c). Expression of BAFF protein in BALF was concomitant to the recruitment of neutrophils, which were highest at days 1 and 7, but were still elevated at days

10 and 14 (Fig. 1d), lymphocytes present at days 7, 10 and 14 (Fig. 1e) andmacrophages (Fig. 1f) into the bronchoalveolar space. Therefore, our data indicate that a rapid and marked induction of BAFF after BLM administration precedes lung fibrosis.

3.2 Neutrophils are the major source of BAFF upon BLM-induced injury

In order to identify BAFF-producing cells in the airways upon lung injury, lung cells from BLM-treated mice were collected at day 7, labeled with anti-CD115, anti-CD3, anti-CD19 and anti-Gr1 antibodies and sorted by FACS. CD115, the receptor for macrophage colony stimulating factor (M-CSF)is expressed by monocytes and macrophages whereas CD3, CD19 and Gr1 are markers of T cells, B cells and neutrophils, respectively. BAFF mRNA expression was evaluated by qPCR. Surprisingly, $Gr1^+$ neutrophilsexpress abundantBAFF mRNA in response to BLM-induced injury, and toa lesser extent $CD115^+$monocytes,while $CD3^+$ or $CD19^+$lymphocytes express little BAFF (Fig. 2).Therefore, the cellular sourceof BAFF in lung at day 7 in response to BLM-induced injury is mainly inflammatory neutrophils and monocytes, rather than infiltrating T or B lymphocytes, although strong lymphocyte recruitment is observed.

3.3 Reduced BLM-induced lung fibrosis in BAFF-deficient mice

In order to investigate whether BAFF plays an important role in lung fibrosis, we evaluated the effect of BLM in BAFF-deficient (BAFF-/-) mice. On day 14 after BLM administration, BAFF-/- mice showed a significant reduction of total collagen and IL-1β in the lungs compared to C57BL/6 wild-type (WT) mice (Fig. 3a and b). Tissue inhibitor of matrix metalloproteases-1 (TIMP-1), a marker of evolution towards fibrosis, was significantly lower in BAFF-/- mice compared to BLM-treated WTmice (Fig. 3c). Since TGF-β1 is essential in

the development of pulmonary fibrosis, TGF-β1 levels were analyzed in BALF and lung homogenates. Latent TGF-β1 levels were significantly lower in BALF and lungs of BAFF-/- compared with BLM-treatedWT mice (Fig. 3d and e). Histological analysis of the lungs revealed that BAFF-/- mice presented reduced tissue injury, cellular infiltration, collagen deposition and fibrosis in comparison with BLM-treatedwild-typemice (Fig. 3f and g). Moreover, bodyweight loss was stabilized in BAFF-/- mice after 3 days whereas wild-type mice continued to lose weight until day 14 after BLM exposure(Fig. 3h). Thus, the data strongly suggest thatBAFF contributes to BLM-induced pulmonary IL-1β, TGF-β1 and TIMP-1 production and lung fibrosis.

3.4 Neutralization of BAFF reduces BLM-induced lung fibrosis in mice

With a therapeutic perspective regarding diseases as yet untreatable such as IPFand systemic sclerosis, we next investigated whether BAFF neutralization could prevent lung fibrosis in the BLM model. First, we treatedWT mice with BAFF-R-Ig or isotype control (IgG2a) both at 5mg/kg i.p., every third day, starting at day 0 after BLM challenge. BAFF neutralization with BAFF-R-Ig significantly reduced the BLM-induced overexpression of col1a1, col1a2 and col3a1 mRNA in the lungs (Fig. 4a). Total collagen contents were also significantly decreased in the lungs of BLM mice treated with BAFF-R-Ig, compared to saline-treated littermates, at 7 days (Fig. 4b) and 14 days (Fig. 4c) after BLM administration. Interestingly, BLM exposure induced an increase in pulmonary IL-1β levels that were also significantly reduced in lungs from BAFF-R-Ig treated mice (Fig. 4d). Histological analysis of the lungs revealed that BAFF-R-Ig treated, BLM-exposed mice had reduced cellular infiltration, collagen deposition and fibrosis (Fig. 4e andf), associated with a partial recovery of bodyweight after 3 days (Fig. 4g). Second, in order to evaluate a therapeutic potential of BAFF-R-Ig, we administrated BAFF-R-Ig or control IgG2a, starting only 3 days after BLM exposure. Bothtotal collagen

content and fibrosis scores were significantly decreased in the lungs of mice, when treated with BAFF-R-Ig 3 days after BLM challenge, as compared to mice treated with isotype control, similar to the effect seen when BAFF-R-Ig treatment was started on day 0 (Fig. 4h andi). Therefore, the data obtained with BAFF neutralization demonstrated a significantreduction of lung collagen deposition, IL-1β production, body-weight loss and lung fibrosis upon BLM exposure.

3.5 BAFF expression depends on IL-1β and IL-17A pathways

IL-1β and IL-17A are critical mediators of pulmonary fibrosis through IL-1 receptor 1 (IL-1R1) and IL-17RA signaling, respectively[16,38]. In the BLM model, IL-1β and IL-17A are expressed early and mediate late fibrosis[19,38]. We investigated whether IL-1β and IL-17A may regulate BAFF expression by using IL-1R1-/-, IL-17A-/- and IL-17RA-/- mice. As previously reported, collagen lung deposition and TGF-β1 levels in lungs were significantly reduced in IL-1R1-/-, IL-17A-/- and IL-17RA-/- mice on day 14 post BLM exposure (Fig. 5a-d),confirming the fact that IL-1β and IL-17A are major effectors of pulmonary fibrosis in the BLM model. Interestingly, BAFF levels were significantly reduced in both lung homogenates and BALF of mice deficient for IL-1R1, IL-17A or IL-17RA, indicating that BAFF expression is dependent on early IL-1β and IL-17 release (Fig. 5e and f). Indeed, exogenous administration of rIL-1β or rIL-17A induced the local release of BAFF in the lung (Fig. 5g). Altogether, these data demonstrate that BAFF expression is dependent on IL-1β and IL-17A pathways and suggest a pathogenic involvement of IL-1β, IL-17A and BAFF in BLM-induced lung fibrosis.

3.6 Lung fibrosis triggered by IL-17A is reduced in BAFF-deficient mice

To further investigate the crosstalk between IL-1β, IL-17A and BAFF pathways, we next evaluated the effect of exogenous administration of rIL-1β, rIL-17A or rBAFF, both in wild-type and BAFF-deficient mice. Total collagen in the BALF and lungs was significantly increased by instillation of rIL-17A or rIL-1β (Fig. 6a and b), although less than after BLMexposure. Interestingly, the effect of rIL-17A on collagen induction was abolished in BAFF-deficient mice (Fig. 6a and b), but not that of rIL-1β. Administration ofrIL-1β or rIL-17A enhanced latent TGF-β1 levels in the BALFs (Fig. 6c). The direct administration of exogenous soluble rBAFF had no effect on collagen expression or on latent TGF-β1 levels (Fig. 6a and c) suggesting a role for cell-associated BAFF. These results indicate that, IL-17A-induced pulmonary fibrosis is dependent on BAFF pathway, while IL-1β-induced lung fibrosis is not, and that BAFF acts downstream of IL-17A.

3.7 BAFF amplifies IL-17 production by T lymphocytes from fibrotic lungs

In order to investigate the role of BAFF in the establishment of pulmonary fibrosis after BLM-induced injury, we isolated cells from lung homogenates, 7 days after BLM or NaCl instillation and cultured them *ex vivo*. Pulmonary cells from BLM-treated mice presenteda significant increase in BAFF expression in comparison to NaCl-treated mice (Fig.7a).Addition of rIL-1β or rIL-17Ahad no significant effect on BAFF expression. Stimulation of the isolated cells with anti-CD3 or anti-IgM antibodies,in the presence or absence of rIL-1βor rIL-17A, had no significant effect on BAFF release by pulmonary cells from BLM-treated mice, confirming that T and B lymphocytes recruited to the lungs are not the main source of BAFF (Fig. 7a). *Ex vivo* stimulation of pulmonary cells from BLM-, but not from NaCl-treated mice with anti-CD3 antibodies triggered a strong IL-17 production, confirming that BLM induces the recruitment of IL-17A producing T lymphocytes into the

lungs (Fig. 7b). Interestingly, rBAFFdose-dependently increased IL-17A production by pulmonary, anti-CD3 activatedT lymphocytes (Fig. 7b). These results suggest that IL-17A producing T lymphocytes recruited into the lung are able to respond to local BAFF produced after BLM-induced injury, augmentingIL-17A expression. Similarly,IL-6 release was increased in the presence of rBAFF, indicating that local BAFF may augment the production of IL-6 by infiltrating pulmonary T lymphocytes (Fig. 7c). Moreover, restimulation with anti-CD3 antibodies increased TGF-β1 expression, indicating that T cells produce TGF-β1 in fibrotic mice. However, rBAFF did not potentiate TGF-β1 production by pulmonary T cells from BLM treated mice(Fig. 7d).In contrast, pulmonary cells isolated from mice seven 7 days after BLM instillation did not present detectable IL-1β after restimulation with anti-CD3 or anti-IgM antibodies, in the presence or absence of rBAFF suggesting that T and B lymphocytes recruited in lungs after BLM, do not produce IL-1β (not shown). In summary,our results indicate that BAFF is not produced by T or B lymphocytes from fibrotic lungs, and demonstrate that exogenous BAFF potentiates IL-17A and IL-6 but not TGF-β1 release by T lymphocytes recruited to the lungs.

3.8 Increased BAFF levels in the bronchoalveolar lavage of fibrotic patients

To assess the functional relevance of the key role of BAFF in experimental fibrosis, BAFF and APRIL levels were measured in bronchoalveolar lavage fluid (BALF) of 7 patients with stable idiopathic pulmonary fibrosis (IPF), 5 patients with acute IPF exacerbation, and 7 control patients with no identified respiratory disease. BAFF levels were significantly increased in BALF from patients with stable or exacerbated IPF as compared with controls (Fig. 8a) whereas APRIL was undetectable (data not shown). The pro-inflammatory cytokines IL-1β (Fig. 8b) and IL-6 (Fig. 8c)were only slightly detected and not modified in bronchoalveolar lavage in patients, in contrast to the chemokine CCL2/MCP-1,which was

greatly increased in IPF patients (Fig. 8d) as previously reported[38, 39]. Thus, IPF and IPF exacerbations were associated with increased levels of BAFF, but not APRIL, in the BALF corroborating our results in mice showing that BAFF is a key player in the establishment of experimental pulmonary fibrosis.

4. Discussion

For the first time, our study presents evidence that BAFF is a critical mediator in experimental pulmonary fibrosis. We show that BAFF but not APRIL is strongly induced in bronchoalveolar spaces and lungs in the bleomycin induced pulmonary fibrosis model, in an IL-1β- and IL-17A-dependent manner. BAFF expression was rapidly upregulated during acute inflammation and strongly increased during the development of fibrosis.Importantly, we report that BAFF deficiency or BAFF neutralization using soluble receptors reduces the development of pulmonary fibrosis, thus demonstrating that BAFF plays a critical role in the establishment of experimental lung fibrosis.In particular, BAFF deficiency reduced the production of TGF-β1, an essential pro-fibrotic factor, and collagen deposition.

We identified Gr1^{+} infiltrating neutrophils as an important source of BAFF in mouse fibrotic lungs.Human neutrophils were shown to release biologically active BAFF after *in vitro* incubation with G-CSF[39]. BAFF is produced by tissue infiltrating human neutrophils or bone marrow neutrophils in several diseases[23,40–44]. BAFF secreted by inflammatory neutrophils has been implicated in B-cell, but not in T-cell, responses in humans.

The expression of BAFF by infiltrating cells from BLM-injured lungs was not increased after *in vitro*restimulation with anti-CD3 antibodies or anti-IgM confirming that BAFF is not produced byinfiltrating pulmonary T or B lymphocytes. Since non-hematopoietic cells were shown to produce BAFF during pathogenic conditions, we cannot exclude that epithelial cells or fibroblasts may produce BAFF protein in response to lung injury. Nevertheless, BAFF

expression preceded fibroblast accumulation and lung fibrosissuggesting that fibroblasts are not responsible for the early high BAFF production, although they may contribute to the sustained high BAFF levels on day 14 to 21, whenneutrophils may be less important.

We investigated the relationship between BAFF and IL-1βand showed that IL-1βcould regulate BAFF expression. Moreover, BAFF isalso involved in the regulation of IL-1β production,as demonstrated by the decrease of IL-1βpulmonary levels in bleomycin-challenged mice treated with BAFF-R-Ig or in mice genetically deficient for BAFF. Toour knowledge, it is the first time the interaction between IL-1βand BAFF pathways was reported. Analyzing the link between IL-17A and BAFF, wereport that IL-17A or IL-17RA gene disruption resulted in a marked decrease of BAFF secretion in the bleomycin-induced model of fibrosis.Local administration of rIL-1βor rIL-17A, revealeda crosstalk between IL-1β,IL-17A and BAFF. We show that rIL-17A-induced lung fibrosis is dependent on the presence of BAFF,indicating that BAFF is downstream of rIL-17A. In contrast, BAFF-dependent and BAFF-independent pathways may exist in IL-1β-induced fibrosis.Fibrotic pulmonary cells restimulated with anti-CD3 or anti-IgM antibodies did not produce IL-1β even in presence of rBAFF.Administration of solubleBAFF did not induce the expression of the fibrotic markers collagen and TGF-β1,suggesting that soluble BAFF alone is not pro-fibrotic, and requires other cytokines that could, for example, recruit BAFF-responsive cells in the lungs, or because it is unable to stimulate some of the BAFF receptors, such as TACI, that may preferentially respond to membrane-boundBAFF[45]. Interestingly, we show that addition of BAFF amplifies anti-CD3-induced production ofIL-17A by pulmonary T cellsfrom fibrotic lungs. BAFF-related induction of Th17 cells could be related to the amplification by BAFF of the secretion of IL-6, a pivotal cytokine for Th17 polarization[46], as shown previously by our group[47,48] and others.. Our work shows for the first time that BAFF is a critical mediator in experimental pulmonary fibrosis, through activation of IL-17 producing

cells.BAFF was recently shown to contribute to pathogenic Th17 cell responses and increased IL-17A expression in autoimmune animal models[49–51]. Previous studies have shown a role of B cells in liver fibrosis[52]and BLM-induced pulmonary fibrosis[53].Thus, we propose the schematic diagram illustrating the IL-1β-IL-17A-BAFF crosstalk after BLM-induced lung injurypresented in Fig. 9.

In addition, we showed that BAFF but not APRIL expression was markedly increased in the alveolar space of stable and exacerbated IPFpatients. These results indicate that BAFF expression is associated with the fibrotic state in human disease. BAFF expression wasslightlyenhanced during IPF exacerbation characterized by a reactivation of the inflammatory responses and followed by lung scarring and sometimes by patient's death. Our resultsare in line with therecent study describing a correlation between IPF, circulating B lymphocyte differentiation and elevated BAFF levels in plasma, in which patients with higher BAFF plasma levels presented a reduced survival[15].

5. Conclusions

Our study demonstrates that BAFF is a critical mediator in experimental pulmonary fibrosis and supports a link between IL-1β, BAFF and IL-17A in the establishment of the disease in mice, with BAFF serving as an amplifier of IL-17-production by T lymphocytes from injured fibrotic lungs. The key role of BAFF in the experimental lung fibrotic model and the enhanced BAFF expression in the airways of IPF patients suggest the role of BAFF asa critical player in the establishment of human pulmonary fibrosis.

Acknowledgments

References

[1]   Zeki AA, Schivo M, Chan AL, Hardin KA, Kenyon NJ, Albertson TE, et al. Geoepidemiology of COPD and idiopathic pulmonary fibrosis. J Autoimmun 2010;34:J327–338.

[2]   Hudson M, Fritzler MJ. Diagnostic criteria of systemic sclerosis. J Autoimmun 2014;48-49:38–41.

[3]   Barrera MJ, Bahamondes V, Sepúlveda D, Quest AFG, Castro I, Cortés J, et al. Sjögren's syndrome and the epithelial target: a comprehensive review. J Autoimmun 2013;42:7–18.

[4]   Wynn TA. Integrating mechanisms of pulmonary fibrosis. J Exp Med 2011;208:1339–50.

[5]   Wolters PJ, Collard HR, Jones KD. Pathogenesis of idiopathic pulmonary fibrosis. Annu Rev Pathol 2014;9:157–79.

[6]   King TE, Pardo A, Selman M. Idiopathic pulmonary fibrosis. Lancet 2011;378:1949–61.

[7]   Wilson MS, Wynn TA. Pulmonary fibrosis: pathogenesis, etiology and regulation. Mucosal Immunol 2009;2:103–21.

[8]   Blackwell TS, Tager AM, Borok Z, Moore BB, Schwartz DA, Anstrom KJ, et al. Future directions in idiopathic pulmonary fibrosis research. An NHLBI workshop report. Am J Respir Crit Care Med 2014;189:214–22.

[9]   Crystal RG, Bitterman PB, Mossman B, Schwarz MI, Sheppard D, Almasy L, et al. Future research directions in idiopathic pulmonary fibrosis: summary of a National Heart, Lung, and Blood Institute working group. Am J Respir Crit Care Med 2002;166:236–46.

[10]   Bhattacharyya S, Wei J, Tourtellotte WG, Hinchcliff M, Gottardi CG, Varga J. Fibrosis in systemic sclerosis: common and unique pathobiology. Fibrogenesis Tissue Repair 2012;5:S18.

[11]   Luo Y, Wang Y, Wang Q, Xiao R, Lu Q. Systemic sclerosis: genetics and epigenetics. J Autoimmun 2013;41:161–7.

[12]   Lahiri A, Pochard P, Le Pottier L, Tobón GJ, Bendaoud B, Youinou P, et al. The complexity of the BAFF TNF-family members: implications for autoimmunity. J Autoimmun 2012;39:189–98.

[13]   Nezos A, Papageorgiou A, Fragoulis G, Ioakeimidis D, Koutsilieris M, Tzioufas AG, et al. B-cell activating factor genetic variants in lymphomagenesis associated with primary Sjogren's syndrome. J Autoimmun 2014;51:89–98.

[14]   Matsushita T, Hasegawa M, Yanaba K, Kodera M, Takehara K, Sato S. Elevated serum BAFF levels in patients with systemic sclerosis: enhanced BAFF signaling in systemic sclerosis B lymphocytes. Arthritis Rheum 2006;54:192–201.

[15]   Xue J, Kass DJ, Bon J, Vuga L, Tan J, Csizmadia E, et al. Plasma B lymphocyte stimulator and B cell differentiation in idiopathic pulmonary fibrosis patients. J Immunol Baltim Md 1950 2013;191:2089–95.

[16]   Gasse P, Mary C, Guenon I, Noulin N, Charron S, Schnyder-Candrian S, et al. IL-1R1/MyD88 signaling and the inflammasome are essential in pulmonary inflammation and fibrosis in mice. J Clin Invest 2007;117:3786–99.

[17]   Gasse P, Riteau N, Charron S, Girre S, Fick L, Pétrilli V, et al. Uric acid is a danger signal activating NALP3 inflammasome in lung injury inflammation and fibrosis. Am J Respir Crit Care Med 2009;179:903–13.

[18]   Riteau N, Gasse P, Fauconnier L, Gombault A, Couegnat M, Fick L, et al. Extracellular ATP is a danger signal activating P2X7 receptor in lung inflammation and fibrosis. Am J Respir Crit Care Med 2010;182:774–83.

[19]   Gasse P, Riteau N, Vacher R, Michel M-L, Fautrel A, di Padova F, et al. IL-1 and IL-23 mediate early IL-17A production in pulmonary inflammation leading to late fibrosis. Plos One 2011;6:e23185.

[20]   Mi S, Li Z, Yang H-Z, Liu H, Wang J-P, Ma Y-G, et al. Blocking IL-17A promotes the resolution of pulmonary inflammation and fibrosis via TGF-beta1-dependent and -independent mechanisms. J Immunol Baltim Md 1950 2011;187:3003–14.

[21]   Sonnenberg GF, Nair MG, Kirn TJ, Zaph C, Fouser LA, Artis D. Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A. J Exp Med 2010;207:1293–305.

[22]   Mackay F, Schneider P. Cracking the BAFF code. Nat Rev Immunol 2009;9:491–502.

[23]   Scapini P, Carletto A, Nardelli B, Calzetti F, Roschke V, Merigo F, et al. Proinflammatory mediators elicit secretion of the intracellular B-lymphocyte stimulator pool (BLyS) that is stored in activated neutrophils: implications for inflammatory diseases. Blood 2005;105:830–7.

[24]   Mackay F, Schneider P, Rennert P, Browning J. BAFF AND APRIL: a tutorial on B cell survival. Annu Rev Immunol 2003;21:231–64.

[25]   Mackay F, Leung H. The role of the BAFF/APRIL system on T cell function. Semin Immunol 2006;18:284–9.

[26]   Chu VT, Enghard P, Riemekasten G, Berek C. In vitro and in vivo activation induces BAFF and APRIL expression in B cells. J Immunol Baltim Md 1950 2007;179:5947–57.

[27]   Alsaleh G, Messer L, Semaan N, Boulanger N, Gottenberg J-E, Sibilia J, et al. BAFF synthesis by rheumatoid synoviocytes is positively controlled by alpha5beta1 integrin stimulation and is negatively regulated by tumor necrosis factor alpha and Toll-like receptor ligands. Arthritis Rheum 2007;56:3202–14.

[28]   Gottenberg J-E, Cagnard N, Lucchesi C, Letourneur F, Mistou S, Lazure T, et al. Activation of IFN pathways and plasmacytoid dendritic cell recruitment in target organs

of primary Sjögren's syndrome. Proc Natl Acad Sci U S A 2006;103:2770–5.

[29]   Ittah M, Miceli-Richard C, Gottenberg J-E, Sellam J, Lepajolec C, Mariette X. B-cell-activating factor expressions in salivary epithelial cells after dsRNA virus infection depends on RNA-activated protein kinase activation. Eur J Immunol 2009;39:1271–9.

[30]   Fletcher CA, Groom JR, Woehl B, Leung H, Mackay C, Mackay F. Development of autoimmune nephritis in genetically asplenic and splenectomized BAFF transgenic mice. J Autoimmun 2011;36:125–34.

[31]   Mackay F, Sierro F, Grey ST, Gordon TP. The BAFF/APRIL system: an important player in systemic rheumatic diseases. Curr Dir Autoimmun 2005;8:243–65.

[32]   Mackay F, Woodcock SA, Lawton P, Ambrose C, Baetscher M, Schneider P, et al. Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. J Exp Med 1999;190:1697–710.

[33]   Schiemann B, Gommerman JL, Vora K, Cachero TG, Shulga-Morskaya S, Dobles M, et al. An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. Science 2001;293:2111–4.

[34]   Glaccum MB, Stocking KL, Charrier K, Smith JL, Willis CR, Maliszewski C, et al. Phenotypic and functional characterization of mice that lack the type I receptor for IL-1. J Immunol Baltim Md 1950 1997;159:3364–71.

[35]   Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, et al. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. J Exp Med 2001;194:519–27.

[36]   Meijerink J, Mandigers C, van de Locht L, Tönnissen E, Goodsaid F, Raemaekers J. A novel method to compensate for different amplification efficiencies between patient DNA samples in quantitative real-time PCR. J Mol Diagn Jmd 2001;3:55–61.

[37]   Crestani B, Dehoux M, Hayem G, Leçon V, Hochedez F, Marchal J, et al. Differential role of neutrophils and alveolar macrophages in hepatocyte growth factor production in pulmonary fibrosis. Lab Investig J Tech Methods Pathol 2002;82:1015–22.

[38]   Wilson MS, Madala SK, Ramalingam TR, Gochuico BR, Rosas IO, Cheever AW, et al. Bleomycin and IL-1beta-mediated pulmonary fibrosis is IL-17A dependent. J Exp Med 2010;207:535–52.

[39]   Scapini P, Nardelli B, Nadali G, Calzetti F, Pizzolo G, Montecucco C, et al. G-CSF-stimulated neutrophils are a prominent source of functional BLyS. J Exp Med 2003;197:297–302.

[40]   Holden NJ, Williams JM, Morgan MD, Challa A, Gordon J, Pepper RJ, et al. ANCA-stimulated neutrophils release BLyS and promote B cell survival: a clinically relevant cellular process. Ann Rheum Dis 2011;70:2229–33.

[41]   Jablonska E, Slodczyk B, Wawrusiewicz-Kurylonek N, Garley M, Dziemianczyk D, Kretowski A, et al. Overexpression of B cell-activating factor (BAFF) in neutrophils of oral cavity cancer patients - preliminary study. Neoplasma 2011;58:211–6.

[42]   Krumbholz M, Specks U, Wick M, Kalled SL, Jenne D, Meinl E. BAFF is elevated in serum of patients with Wegener's granulomatosis. J Autoimmun 2005;25:298–302.

[43]   Palanichamy A, Bauer JW, Yalavarthi S, Meednu N, Barnard J, Owen T, et al. Neutrophil-mediated IFN activation in the bone marrow alters B cell development in human and murine systemic lupus erythematosus. J Immunol Baltim Md 1950 2014;192:906–18.

[44]   Sawicka-Powierza J, Jablonska E, Kloczko J, Piszcz J, Garley M, Ratajczk-Wrona W. Evaluation of TNF superfamily molecules release by neutrophils and B leukemic cells of patients with chronic B - cell lymphocytic leukemia. Neoplasma 2011;58:45–50.

[45]   Bossen C, Cachero TG, Tardivel A, Ingold K, Willen L, Dobles M, et al. TACI, unlike BAFF-R, is solely activated by oligomeric BAFF and APRIL to support survival of activated B cells and plasmablasts. Blood 2008;111:1004–12.

[46]   Dagur PK, Biancotto A, Wei L, Sen HN, Yao M, Strober W, et al. MCAM-expressing CD4(+) T cells in peripheral blood secrete IL-17A and are significantly elevated in inflammatory autoimmune diseases. J Autoimmun 2011;37:319–27.

[47]   François A, Chatelus E, Wachsmann D, Sibilia J, Bahram S, Alsaleh G, et al. B lymphocytes and B-cell activating factor promote collagen and profibrotic markers expression by dermal fibroblasts in systemic sclerosis. Arthritis Res Ther 2013;15:R168.

[48]   Yoshimoto K, Tanaka M, Kojima M, Setoyama Y, Kameda H, Suzuki K, et al. Regulatory mechanisms for the production of BAFF and IL-6 are impaired in monocytes of patients of primary Sjögren's syndrome. Arthritis Res Ther 2011;13:R170.

[49]   Doreau A, Belot A, Bastid J, Riche B, Trescol-Biemont M-C, Ranchin B, et al. Interleukin 17 acts in synergy with B cell-activating factor to influence B cell biology and the pathophysiology of systemic lupus erythematosus. Nat Immunol 2009;10:778–85.

[50]   Lai Kwan Lam Q, King Hung Ko O, Zheng B-J, Lu L. Local BAFF gene silencing suppresses Th17-cell generation and ameliorates autoimmune arthritis. Proc Natl Acad Sci U S A 2008;105:14993–8.

[51]   Zhou X, Xia Z, Lan Q, Wang J, Su W, Han Y-P, et al. BAFF promotes Th17 cells and aggravates experimental autoimmune encephalomyelitis. Plos One 2011;6:e23629.

[52]   Novobrantseva TI, Majeau GR, Amatucci A, Kogan S, Brenner I, Casola S, et al. Attenuated liver fibrosis in the absence of B cells. J Clin Invest 2005;115:3072–82.

[53]   Komura K, Yanaba K, Horikawa M, Ogawa F, Fujimoto M, Tedder TF, et al. CD19 regulates the development of bleomycin-induced pulmonary fibrosis in a mouse model. Arthritis Rheum 2008;58:3574–84.

Figures Legends

Fig. 1 BAFF production after bleomycin (BLM)-induced lung injury in mice. Mice were challenged with BLM (5mg/kg) or saline solution instillation and sacrificed after 1, 4, 7, 10, 14 or 21 days. BAFF mRNA expression was measured in lungs by qRT-PCR (A). Expression of GAPDH was used for normalization. Relative expression was normalized to BAFF expression in the lungs of NaCl-treated mice. BAFF levels were measured in lungs homogenates (B) and BALF (C) by ELISA. Neutrophils (D) lymphocytes (E) and macrophages (F) numbers were evaluated in BALF. One of two independent experiments is shown and data are expressed as mean values $\pm$ SEM (n=5 mice per group; *p<0.05, **p<0.01 using a Mann-Whitney test).

Fig. 2 Strong overexpression of BAFF in neutrophils in BLM-induced Lung Fibrosis.
Lung cells purified from mice 7 days after BLM (5mg/kg) instillation were labeled with anti-CD115, anti-CD3, anti-CD19 and anti-Gr1 antibodies and individual cell populations sorted by FACS. BAFF mRNA expression was evaluated by qPCR on each sorted cell population. Expression of GAPDH was used for normalization. Relative expression was normalized to BAFF expression in CD19$^+$ cells. One of two independent experiments is shown and data are expressed as mean values $\pm$ SEM."

Fig. 3 BLM-induced lung fibrosis is reduced in BAFF deficient mice. B6 and BAFF-/- mice were sacrificed 14 days after BLM challenge (5mg/kg). Lung collagen (A), lung IL-1β (B), lung TIMP-1 (C), BALF latent TGF-β1 (D) and lung latent TGF-β1 (E) levels were evaluated

by the Sircol assay and ELISA. Fibrosis score (F) was evaluated from histology sections (G). (Chromotrope aniline blue staining. Magnification x50 and x200). Body weight loss was evaluated at different time points after BLM instillation (H). One of two independent experiments is shown and data are expressed as mean values ± SEM (n=4 mice per group; *p<0.05, **p<0.01 using a Mann-Whitney test).

Fig. 4 Neutralization of BAFF prevents BLM-induced lung fibrosis in mice. B6 mice challenged with BLM (5mg/kg) were treated with BAFF-R-Ig (5mg/kg i.p., every 3 days) and sacrificed after 7 or 14 days. *Col1a1*, *Col1a2* and *Col3a1* mRNA levels in lungs were measured 7 days after BLM challenge by qRT-PCR (A). Lung collagen was evaluated 7 (B) and 14 (C) days after BLM-challenge by the Sircol assay. IL-1β was measured by ELISA in lung homogenates (D). Fibrosis score (F) was evaluated from histology sections (E). (Chromotrope aniline blue staining. Magnification x50 and x200). Body weight loss was evaluated at different time points after BLM instillation (G). Mice were treated with isotype control (IgG2a) or BAFF-R-Ig (both at 5mg/kg i.p., every 3 days) starting at day 0 or day 3 after BLM challenge. Lung collagen (H) and fibrosis score (I) was evaluated 14 days after BLM-challenge. One of two independent experiments is shown and data are expressed as mean values ± SEM (n=4 mice per group; *p<0.05, **p<0.01 using a Mann-Whitney test).

Fig. 5 BAFF expression depends on IL-1β and IL-17A.B6, IL-1R1-/-, IL-17A-/- and IL-17RA-/- mice were sacrificed 14 days after BLM challenge (5mg/kg). Lung collagen (A) and lung latent TGF-β1 (D) levels were evaluated by the Sircol assay and ELISA. Fibrosis score (C) was evaluated from histology sections (B). (Chromotrope aniline blue staining. Magnification x50 and x200).BAFF levels were measured in BALF (E) and lung homogenates (F) by ELISA. B6 mice were sacrificed 14 days after BLM (5mg/kg), rIL1β or rIL-17A (1µg/mice) instillations and lung BAFF levels were evaluated by ELISA (G). One of

two independent experiments is shown and data are expressed as mean values ± SEM (n=4 mice per group; *p<0.05, **p<0.01 using a Mann-Whitney test).

Fig. 6 IL-17-induced lung fibrosis is reduced in BAFF-deficient mice. B6 and BAFF-/- mice were sacrificed 14 days after BLM (5mg/kg), rBAFF, rIL1β or rIL-17A (1µg/mice) instillations. BALF (A) and lung (B) collagen and BALF latent TGF-β1 (C) levels were evaluated by the Sircol assay and ELISA. Data are expressed as mean values ± SEM (n=4 mice per group; *p<0.05, **p<0.01 and ***p<0.001 using a Mann-Whitney test).

Fig. 7 BAFF amplifies IL-17 production by T lymphocytes from fibrotic lungs.Lung cells were isolated from BLM or NaCl treated mice 7 days after instillation and restimulated in vitro with anti-IgM antibodies (5µg/mL) or with anti-CD3 antibodies (1µg/ml) in the presence or absence of murine rIL-1β or rIL-17A (100ng/mL each) and BAFF content was evaluated in the supernatant at 72h of stimulation (A). Lung cells were restimulated *in vitro* with anti-CD3 antibodies (1µg/ml) in the presence or absence of different concentrations of murine recombinant BAFF (rBAFF) and IL-17A (B), IL-6 (C) and TGF-β1 (D) production was evaluated at 72h of stimulation. One of two independent experiments is shown and data are expressed as mean values ± SEM (n=4 mice per group; *p<0.05, **p<0.01 using a Mann-Whitney test).

Fig. 8 Increased BAFF levels in BALF from IPF patients. BALF of 7 patients with stable idiopathic pulmonary fibrosis (IPF), 5 patients with acute IPF exacerbation and 7 control patients with no identified respiratory disease were assayed for BAFF (A), IL-1β (B), IL-6 (C) or CCL2/MCP-1 (D) by ELISA. (One of two independent experiments is shown; *p<0.05, **p<0.01 using a Mann-Whitney test).
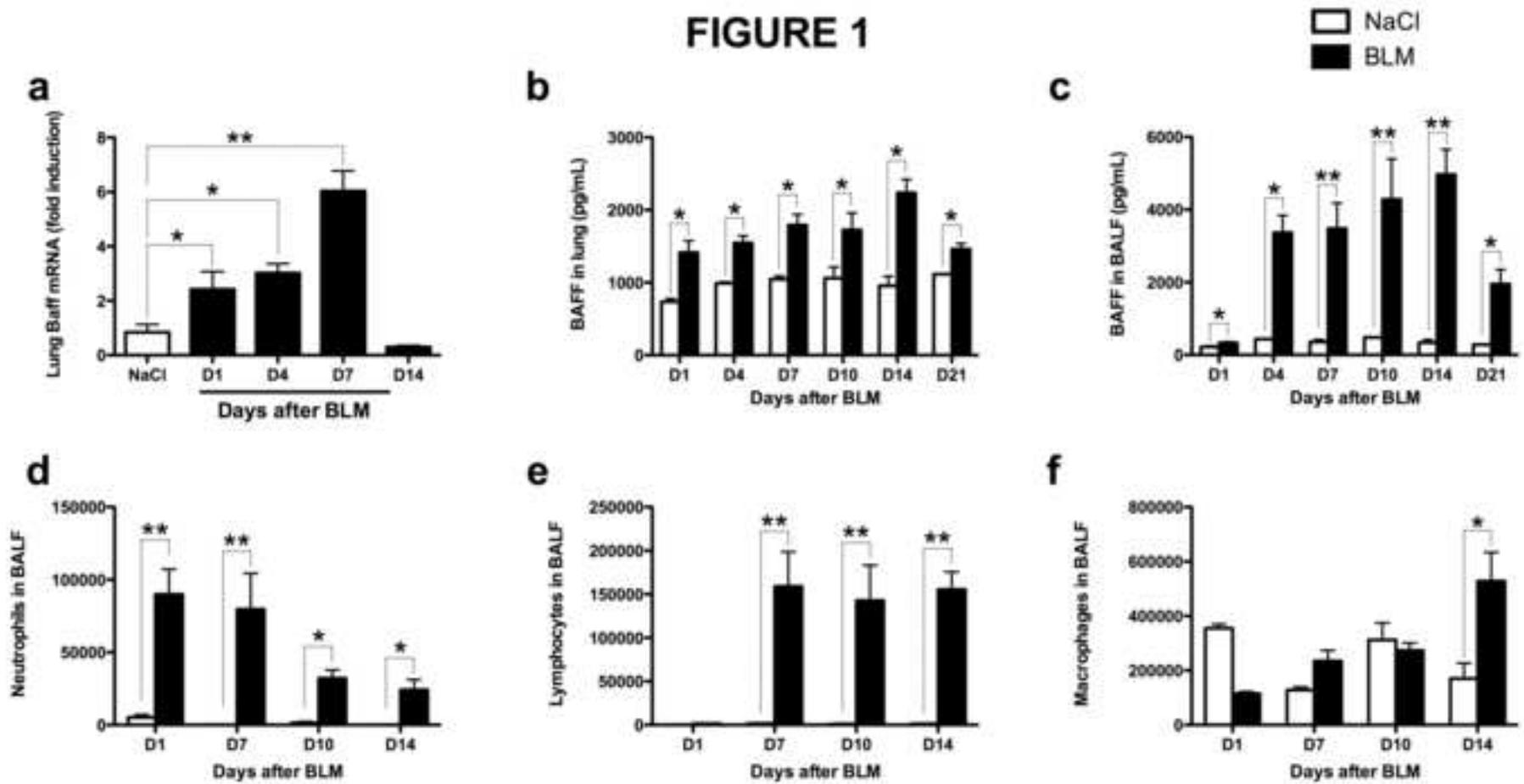
Fig. 9 Schematic diagram illustrating the IL-1β-IL-17A-BAFF crosstalk after BLM-induced lung injury. Upon bleomycin-induced damage, lung epithelial cells release danger signals

[17,18], triggering maturation and secretion of IL-1β by alveolar macrophages, monocytes and/or dendritic cells[14]; IL-1βand a first wave of IL-17A produced by innate γδT lymphocytes[19] at the early phase of the fibrotic process, trigger BAFF expression, essentially by inflammatory neutrophils present early in lung tissues and bronchoalveolar space; increased local concentrations of BAFF in turn activate the Th17 lymphocytes recruited locally to secrete a second wave of IL-17. In addition, BAFF also increases IL-1β production by an amplification loop. BAFF is also capable to activate B lymphocytes, promoting their survival and differentiation leading to autoantibody and proinflammatory cytokine secretion, which might contributeto the pathogenesis of lung fibrosis induced by BLM exposure deserves further investigations. BAFF also activates other lung infiltrating cells to produce TGF-β1, an essential pro-fibrotic factor which promotes transformation of epithelial cells and resident fibroblasts into myofibroblasts leading to excessive remodeling and fibrosis.
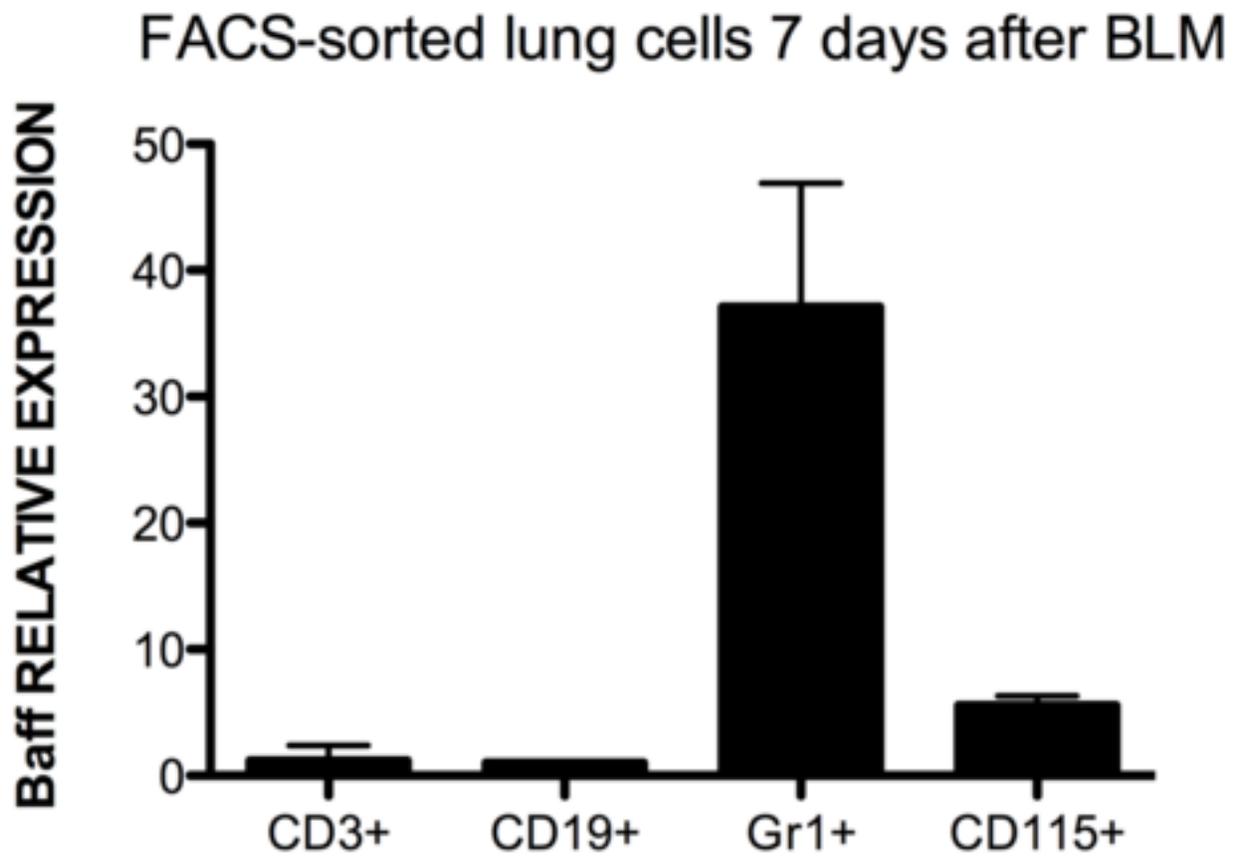
Supplementary Fig. S1 APRIL mRNA expression in lung was unchanged after BLM challenge. Mice were challenged with BLM (5mg/kg) or saline solution instillation and sacrificed after 1, 4, 7 or 14 days. APRIL mRNA expression was measured in lungs by qRT-PCR. Expression of GAPDH was used for normalization. Relative expression was normalized to APRIL expression in the lungs of NaCl-treated mice. One of two independent experiments is shown and data are expressed as mean values ± SEM (n=4 mice per group).

FIGURE 1

FIGURE 2

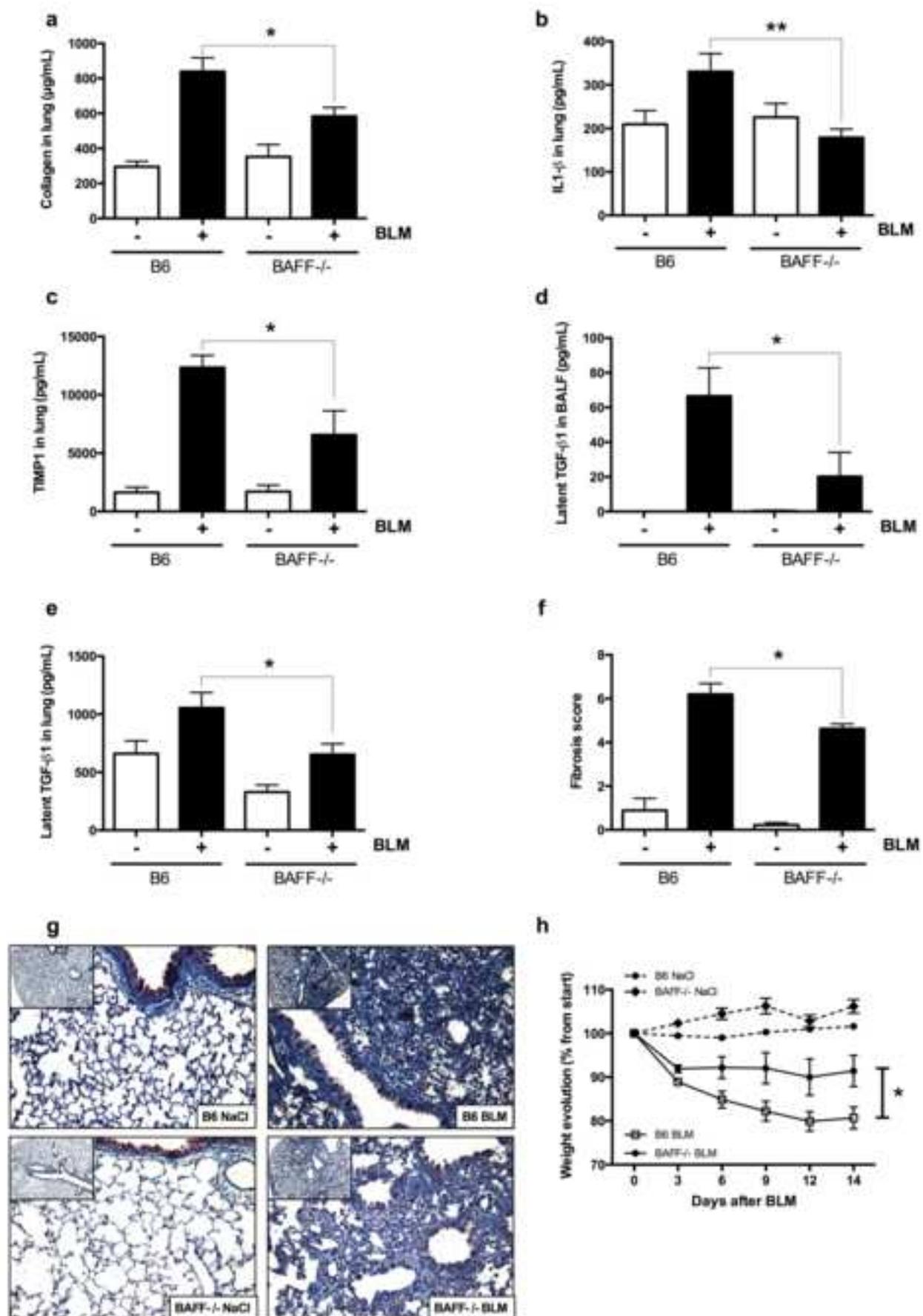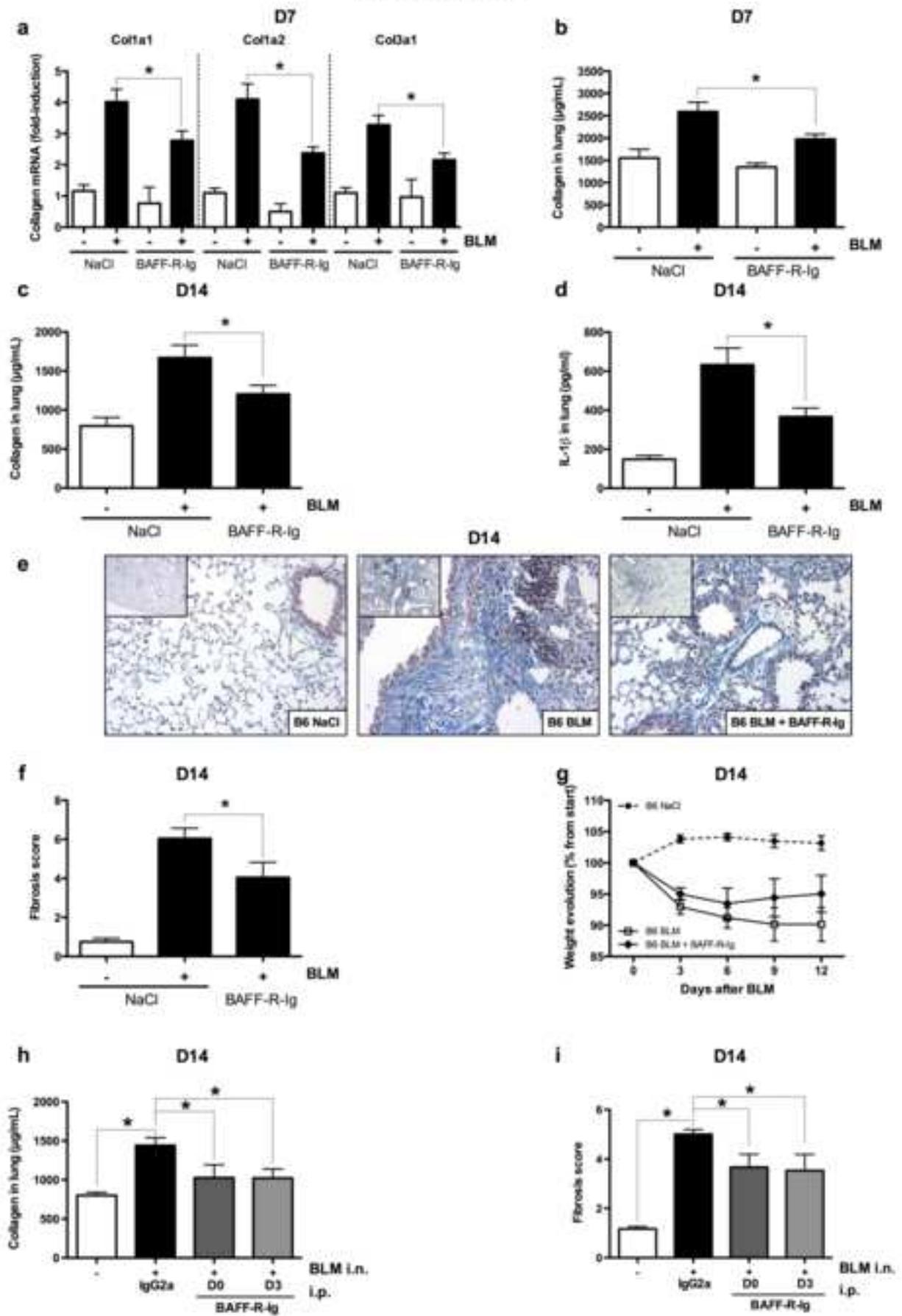FACS-sorted lung cells 7 days after BLM
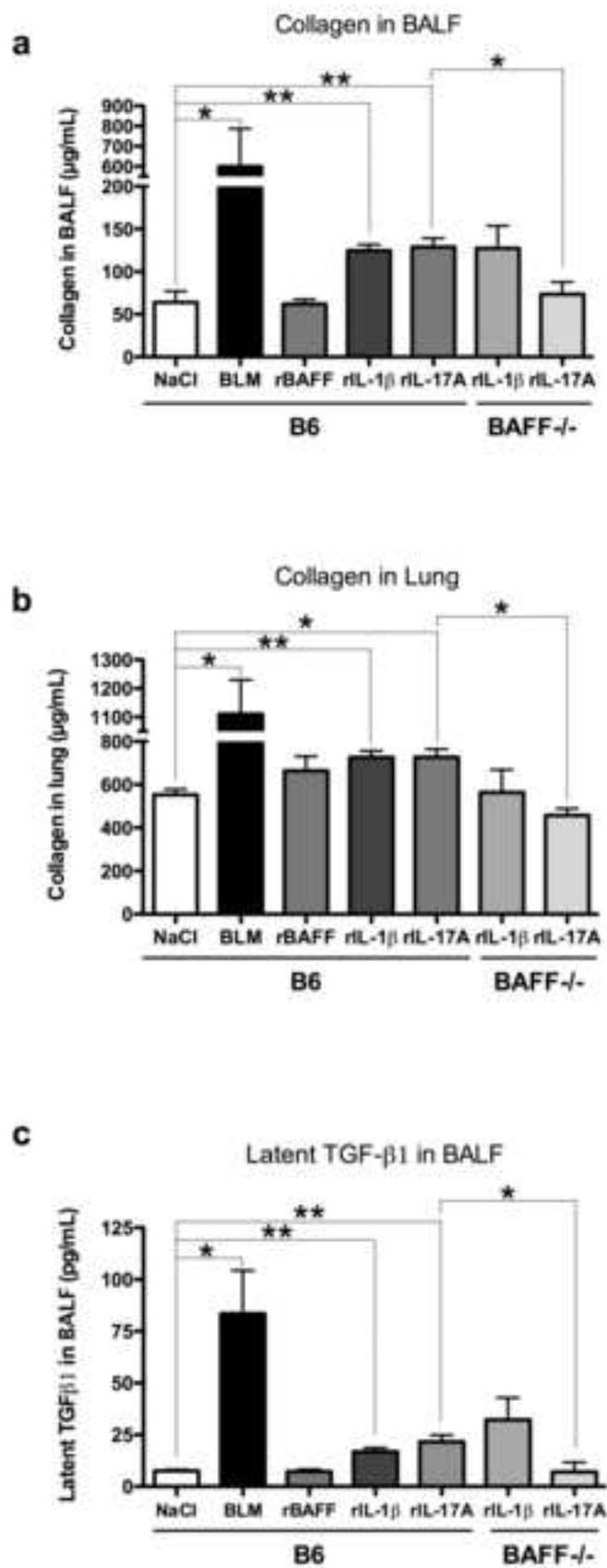
Figure 3
Click here to download high resolution image

FIGURE 3

FIGURE 4

FIGURE 5

FIGURE 6

a Collagen in BALF

b Collagen in Lung

c Latent TGF-β1 in BALF
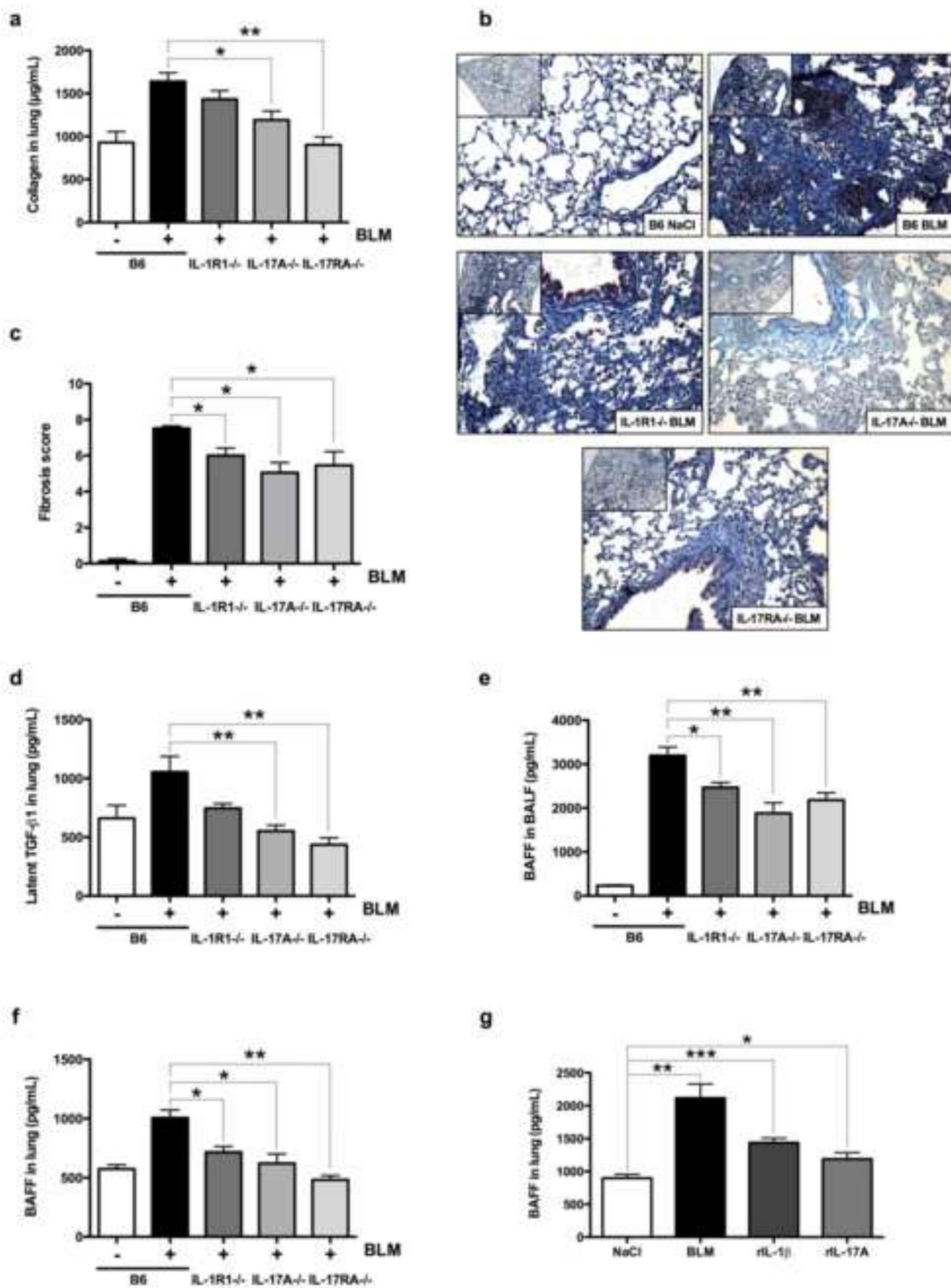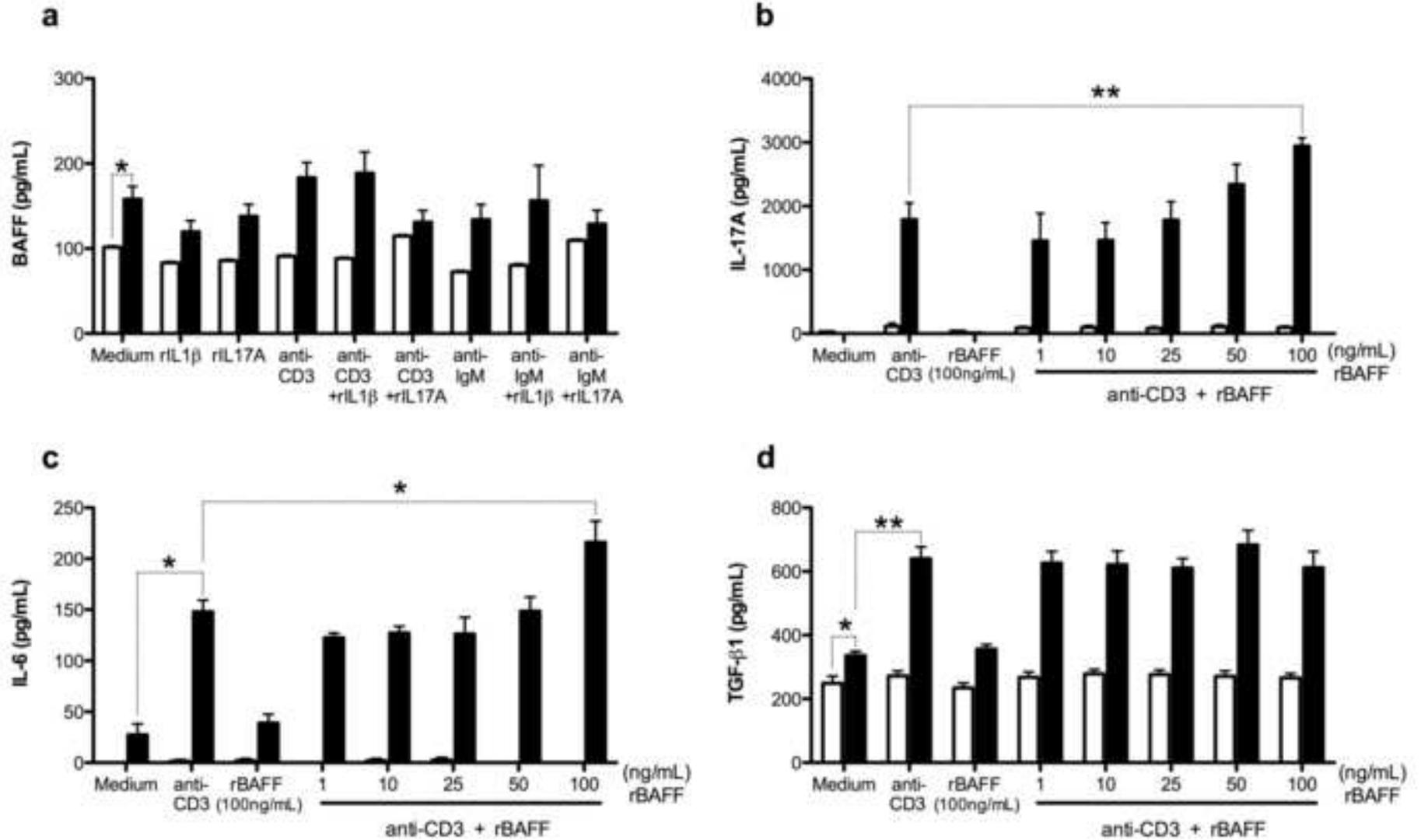
FIGURE 7

FIGURE 8

Figure 9

FIGURE 9