ORIGINAL ARTICLE

B Cell–Activating Factor

An Orchestrator of Lymphoid Follicles in Severe Chronic Obstructive Pulmonary Disease

Francesca Polverino[1,2,3], Borja G. Cosio[4,5], Jaime Pons[4,6], Maria Laucho-Contreras[1], Paula Tejera[7], Amanda Iglesias[4], Angel Rios[4], Andreas Jahn[4], Jaume Sauleda[4,5], Miguel Divo[1,2], Victor Pinto-Plata[1,2], Lynette Sholl[8], Ivan O. Rosas[1,2], Alvar Agusti[5,9], Bartolome R. Celli[1,2], and Caroline A. Owen[1,2]

[1]Division of Pulmonary and Critical Care Medicine and [8]Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts; [2]Lovelace Respiratory Research Institute, Albuquerque, New Mexico; [3]University of Parma, Parma, Italy; [4]Research Unit, FISIB, IdISPa, and [6]Immunology Department, Hospital Son Espases, Mallorca, Spain; [5]Department of Respiratory Medicine, Hospital Son Espases, Ciber de Enfermedades Respiratorias (CIBERES), Mallorca, Spain; [7]Harvard School of Public Health, Boston, Massachusetts; and [9]Thorax Institute, Hospital Clinic, IDIBAPS, University of Barcelona, and CIBERES, FISIB, Mallorca, Spain

Abstract

Rationale: Patients with chronic obstructive pulmonary disease (COPD) have increased pulmonary lymphoid follicle (LF) counts. B cell–activating factor of tumor necrosis factor family (BAFF) regulates B cells in health, but its role in COPD pathogenesis is unclear.

Objectives: To determine whether BAFF expression in pulmonary LFs correlates with COPD severity, LF size or number, and/or readouts of B-cell function in LFs.

Methods: We correlated BAFF immunostaining in LFs in lung explants or biopsies from nonsmoking control subjects (NSC), smokers without COPD (SC), and patients with COPD with the number and size of LFs, and LF B-cell apoptosis, activation, and proliferation. We analyzed serum BAFF levels and BAFF expression in B cells in blood and bronchoalveolar lavage samples from the same subject groups. We assessed whether: (*1*) cigarette smoke extract (CSE) increases B-cell BAFF expression and (*2*) recombinant BAFF (rBAFF) rescues B cells from CSE-induced apoptosis by inhibiting activation of nuclear factor-κB (NF-κB).

Measurements and Main Results: Patients with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage IV COPD had increased numbers and larger pulmonary LFs than patients with GOLD stages I–II COPD and SC. We identified two main types of pulmonary LFs: (*1*) type A, the predominant type in GOLD stages I–II COPD and SC, characterized by abundant apoptotic but few BAFF-positive cells (mostly B cells); and (*2*) type B, the main type in GOLD stage IV COPD, characterized by abundant BAFF-positive cells but few apoptotic cells (mostly B cells). BAFF levels were also higher in blood and bronchoalveolar lavage B cells in patients with COPD versus NSC and SC. Surprisingly, rBAFF blocked CSE-induced B-cell apoptosis by inhibiting CSE-induced NF-κB activation.

Conclusions: Our data support the hypothesis that B-cell BAFF expression creates a self-perpetuating loop contributing to COPD progression by promoting pulmonary B-cell survival and LF expansion.

Keywords: cigarette smoke; chronic obstructive pulmonary disease; B cell–activating factor of tumor necrosis factor family; autoimmunity; lymphoid follicles

(Received in original form January 19, 2015; accepted in final form June 6, 2015)

Supported by National Institutes of Health grants P01 HL105339, P01 HL114501, AI111475-01, and R21 HL111835; BWH-LRRI Research Consortium grants; The Flight Attendants Medical Research Institute (CIA#046123); a research grant from the Spanish Society of Respiratory Medicine; and a short-term fellowship from the joint European Respiratory Society and the Italian Multidisciplinary Association for the Study of Respiratory Diseases.

Author Contributions: F.P., J.P., B.G.C., B.R.C., and C.A.O. conceived the project and designed the experiments. F.P., J.P., A.I., A.J., M.L.-C., P.T., J.S., M.D., V.P.-P., L.S., I.O.R., A.A., B.G.C., B.R.C., and C.A.O. conducted experiments and contributed to data analysis and interpretation. All authors contributed to the writing and editing of the manuscript.

Correspondence and requests for reprints should be addressed to Caroline A. Owen, M.D., Ph.D., Division of Pulmonary and Critical Care Medicine, Brigham and Women's Hospital, Room 855B, Harvard Institutes of Medicine Building, 77 Avenue Louis Pasteur, Boston, MA 02115. E-mail: cowen@rics.bwh.harvard.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 192, Iss 6, pp 695–705, Sep 15, 2015

Originally Published in Press as DOI: 10.1164/rccm.201501-0107OC on June 13, 2015
Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the Subject: An abnormal adaptive immune response is believed to play a pathogenic role in chronic obstructive pulmonary disease (COPD). B cell–activating factor of tumor necrosis factor family (BAFF) is a key regulator of B-cell homeostasis and self-tolerance. BAFF has been implicated in many autoimmune conditions, but BAFF's role in COPD pathogenesis and progression is not known.

What This Study Adds to the Field: This study shows that the expression of BAFF is up-regulated in B cells in pulmonary lymphoid follicles (LFs) and in B cells in blood and bronchoalveolar lavage samples in patients with COPD. The expression of BAFF in B cells in pulmonary LFs correlates directly with the size of the LFs and with COPD severity and indirectly with B-cell apoptosis in LFs in COPD and smoker lungs. We identified two distinct types of LFs based on B-cell BAFF expression and B-cell apoptosis in LFs in COPD and smoker lungs. Also, BAFF rescues B cells from cigarette smoke extract (CSE)-induced apoptosis *in vitro* by inhibiting CSE-induced activation of the transcription factor nuclear factor-κB. These observations support a role for BAFF in promoting the survival of B cells in pulmonary LFs, the expansion of LFs, and disease progression in COPD.

Chronic obstructive pulmonary disease (COPD) is characterized by enhanced pulmonary inflammatory responses to inhaled particles and gases present in cigarette smoke (CS). However, only a percentage of smokers develop COPD, suggesting that as-yet unidentified pathways are crucial for the development of COPD (1, 2).

The adaptive immune response contributes to COPD pathogenesis. COPD lungs have increased numbers of T cells, B cells, and dendritic cells (DCs) but reduced regulatory T-cell counts or activity (3–6). B cells are present in lymphoid follicles (LFs) in the small airways and lung parenchyma (7), especially in severe human COPD and animal models of COPD (6, 8). LFs in patients with COPD result from lymphoid neogenesis (9) and belong to inducible bronchus-associated lymphoid tissue (iBALT). The iBALT in COPD lungs consists of B cells in germinal centers and peripherally located $CD8^+$ and $CD4^+$ T cells (7). LFs may enhance immune responses to pulmonary pathogens. However, LF B cells may produce autoantibodies that may perpetuate CS-initiated pulmonary inflammation and injury (6, 7, 9, 10). Consistent with this notion, FEV_1 measurements in patients with COPD correlate indirectly with the percentage of airways displaying LFs and pulmonary B-cell counts (7). However, it is not clear how LFs develop and expand in COPD lungs.

One potential candidate in the genesis of LFs in COPD lungs is B cell–activating factor (BAFF) or tumor necrosis factor (TNF)-ligand superfamily member-13B. BAFF is produced by monocytes, macrophages, DCs, polymorphonuclear neutrophils, and T cells. BAFF increases B-cell survival, thereby promoting B-cell maturation and adaptive immune responses. BAFF binds to three receptors that are constitutively expressed on B cells (11–14). One of these receptors, BAFF receptor (BAFF-R), is highly expressed on B cells, and BAFF binds to BAFF-R with the highest affinity (15–17). Mature B-cell survival is mediated by BAFF–BAFF-R and B-cell antigen receptor (BCR) signaling (18). BAFF stimulation rescues self-reactive B cells from peripheral deletion, allowing them to migrate into splenic follicular and marginal zones during B-cell development (15, 19). In health, B cells do not produce BAFF but require signals from antigen-activated T-helper cells to proliferate. These signals include CD40 ligand expressed on antigen-activated T cells, which binds to CD40 expressed on B cells (20).

Overexpression of BAFF is associated with autoimmune diseases in humans and mice (21–26). In autoimmune diseases two events often occur: (*1*) BAFF is produced by B cells themselves, thereby stimulating B cells in an autocrine/paracrine fashion;

Table 1. Demographic and Clinical Characteristics of the Lung Tissue Cohort Studied

	NSC*	SC	COPD†	*P* Value‡
No. of subjects	5†	5	19	
Men, %	80	60	42	0.3
Age, yr	48 ± 4	46 ± 7	56 ± 2	0.08
Smoking history, pack-years	0	23 ± 5	28 ± 7	0.006§
Current smokers, %	0	80	16	<0.001§
FEV_1% predicted	Not available	Not available	46 ± 7‖	Not done

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; NSC = nonsmoker control subjects; SC = smoker control subjects.
Data presented as mean ± SEM unless otherwise noted.
*Lung samples from NSC and SC were unused lung transplant donor lungs that were rejected for lung transplantation for reasons other than pulmonary disease. Current smokers were identified as the subjects who were active smokers at the time of the study or stopped smoking less than 1 year from the beginning of the study. These samples were obtained from the Pulmonary and Critical Care Medicine Human Biorepository or the Department of Pathology at Brigham and Women's Hospital, Boston. Pulmonary function tests were not available on the NSC and SC subjects. However, histologic examination of the lung samples by a senior pathologist at Brigham and Women's Hospital revealed no evidence of chronic lung disease, lung cancer, or respiratory tract infection.
†Sections of lung were obtained from bronchial or lung biopsies or explanted lungs from patients with COPD and were provided by the NHLBI-sponsored Lung Tissue Research Consortium (www.ltrcpublic.com). None of the subjects studied had evidence of respiratory tract infection at the time of lung tissue sampling. Three out of 14 patients had a secondary diagnosis of lung cancer.
‡Statistical analyses included one-way analysis of variance tests for continuous variables (age, FEV_1% predicted, and pack-year smoking histories) followed by pairwise comparisons using Student's *t* tests or Mann-Whitney *U* tests. The Chi-square test was used to analyze categorical variables. $P < 0.05$ was considered statistically significant.
§There were statistically significant differences in the pack-year smoking histories of the patients with COPD and the SC versus the NSC group ($P = 0.003$ for both comparisons). However, the pack-year smoking histories of the patients with COPD and the SC were not significantly different. The current smoking status was different between patients with COPD and SC ($P = 0.025$).
‖All patients with COPD had a post-bronchodilator FEV_1/FVC ratio < 0.7.

and (*2*) self-reactive B cells are generated that proliferate without antigen-activated T-helper cells (11, 27, 28). Although BAFF expression is increased in alveolar macrophages and LFs in COPD lungs (29), it is not known whether B-cell production of BAFF occurs in COPD and contributes to self-perpetuating pulmonary inflammation and disease progression.

Our goal was to test the hypothesis that BAFF stimulates adaptive immune responses occurring in patients with advanced (Global Initiative for Chronic Obstructive Lung Disease [GOLD] stages III–IV) COPD. We quantified LF number and size and the number of BAFF-positive B cells in LFs in different COPD stages versus control lungs. We correlated the number of BAFF-positive B cells in LFs with FEV_1; readouts of B-cell apoptosis, activation, or proliferation; and B-cell expression of IgG. We compared BAFF expression in B cells in blood and bronchoalveolar lavage (BAL) leukocytes and sera from patients with COPD and control subjects. We also tested our hypotheses that: (*1*) low concentrations of cigarette smoke extract (CSE) increase BAFF expression by B cells *in vitro*; (*2*) high concentrations of CSE induce B-cell apoptosis, which is attenuated by treating cells with rBAFF; and (*3*) rBAFF activates the nuclear factor-κB (NF-κB) pathway in B cells, as BAFF signaling activates NF-κB in B cells to increase B-cell survival in other model systems (30, 31). Some of the results of these studies have been previously reported in the form of an abstract (32).

Methods

See online supplement for additional methods.

Subjects

This study was approved by institutional ethics committees in Boston and Mallorca. All 146 study subjects signed informed consent forms. Study subjects included active or ex-smokers with COPD (GOLD stage I–IV), healthy smoker control subjects (SC), and healthy nonsmokers (NSC). See Table 1 and Tables E1–E3 in the online supplement for demographic and clinical details. Animal studies were approved by the Harvard Medical School Institutional Animal Care and Use Committee.

Immunostaining of Lung Sections for CD20, BAFF, BAFF-R, and Markers of B-Cell Activation, Proliferation, and Apoptosis

Formalin-fixed sequential lung sections from patients with GOLD stages I–II and IV COPD, SC, and NSC (5–10 subjects/group; Table 1) were triple immunostained for: (*1*) CD20 (to identify B cells), BAFF, and BAFF-R; (*2*) CD20, CD8, or CD4 (to identify T-cell subsets), or CD21 to identify follicular dendritic cells (FDCs); (*3*) CD20, CD83 (activation marker), and Ki67 (proliferation marker); and (*4*) CD20, active caspase-3 (apoptosis marker), and human IgG. Other lung sections were immunostained with nonimmune control antibodies.

BAFF Expression in CSE-treated B Cells

Blood B cells were isolated from six NSCs and incubated with 0 to 5% CSE. At intervals, B cells were fixed, permeabilized, and immunostained with Alexa-546 for CD20 and Alexa-488 for BAFF. BAFF immunostaining in B cells was quantified in cytocentrifuge preparations using MetaMorph software.

B-Cell Apoptosis

Blood B cells from three NSC were incubated at 37°C without or with 10% CSE (to induce B-cell apoptosis) either with or without 100 nM recombinant human BAFF. At intervals, B cells were fixed, permeabilized, and stained with: (*1*) Alexa-488 for active caspase-3 and Alexa-546 for CD20, (*2*) propidium iodide (PI), or (*3*) a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit. Nonimmune antibodies or negative controls were also tested.

Murine splenic B cells isolated from C57BL/6 wild-type mice were incubated with or without 2% CSE (to induce B-cell apoptosis) and with or without 100 nM recombinant murine BAFF (rmBAFF). Cells were stained for intracellular active caspase-3 or with PI or a TUNEL kit, and staining was quantified.

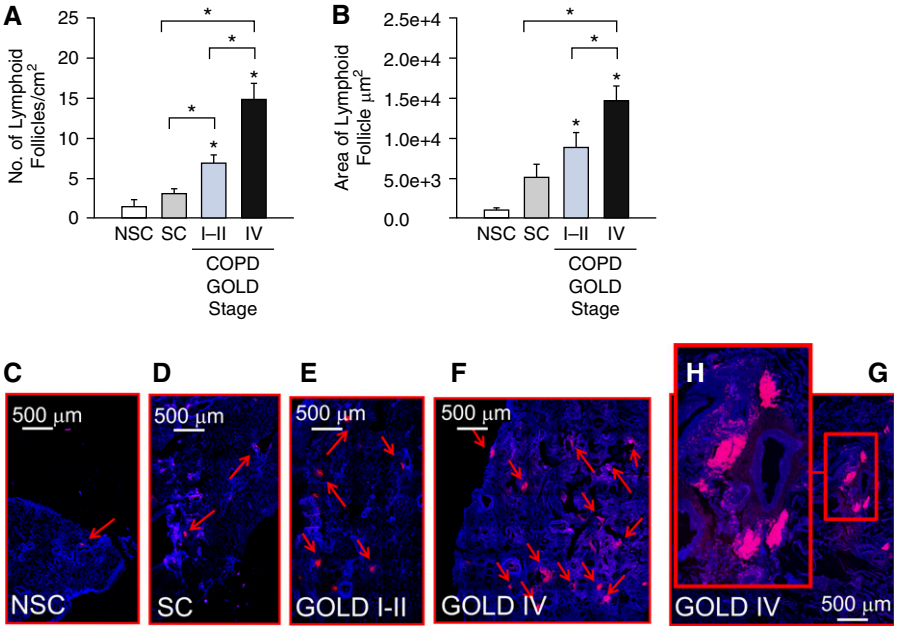

Figure 1. The number and the size of pulmonary lymphoid follicles (LFs) were increased in patients with chronic obstructive pulmonary disease (COPD), especially in the very severe stages of the disease. Quantitation of the number (*A*) and area (*B*) of LFs in the lungs of nonsmoking control subjects (NSC), smokers without COPD (SC), patients with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages I–II COPD, and patients with GOLD stage IV COPD (n = 5/group). The Student's *t* test was used to analyze the data. In *A* and *B*, data are mean + SEM; $^*P < 0.05$. (*C–H*) Representative images of LFs in the lungs of NSC (*C*), SC (*D*), patients with GOLD stages I–II COPD (*E*), and patients with GOLD stage IV COPD (*F–H*), in which B cells are identified by staining with a *red* fluorophore for CD20. In *C–G*, the magnification is ×100. (*H*) *Inset* of an image of typical pulmonary LFs in the lung of the patient with GOLD stage IV COPD shown in *G*.

B-Cell NF-κB Activation

Murine splenic B cells were incubated for 12 hours with or without 2% CSE and with or without 100 nM rmBAFF. B-cell NF-κB activation was assessed using electrophoretic mobility shift assays on nuclear protein extracts. NF-κB p65 subunit localization was assessed by immunostaining permeabilized B cells. Lung sections from patients with GOLD stage I–IV COPD and SC (5–7 subjects/group) were triple immunostained for CD20, BAFF, and the NF-κB p65 subunit to assess cellular localization of p65 in LF B cells.

BAFF and BAFF-R Expression in Blood and BAL Lymphocytes

Phlebotomy and BAL were performed on NSC, SC, and patients with GOLD stage I–IV COPD (*see* Table E2 in the online supplement). Peripheral blood mononuclear cells were isolated using density gradient centrifugation (33). Peripheral blood mononuclear cells and/or BAL leukocytes were double immunostained for markers of B cells (CD79b) or T cells (CD2) and BAFF-R or BAFF. The mean fluorescence intensity and percentage of BAFF- or BAFF-R–positive cells were quantified using flow cytometry. BAFF serum levels (Table E3) were measured using an ELISA.

Statistics

We used one-way analysis of variance tests for continuous variables and *Z* tests or Chi-square tests for categorical variables. For pairwise comparisons, parametric and nonparametric data were analyzed using two-sided Student's *t* tests and Mann-Whitney *U* tests, respectively. Correlation coefficients were calculated using the Pearson or the Spearman rank method or the Dubin-Watson statistical correlation test for nonlinear data. *P* less than 0.05 was considered statistically significant. Analyses were performed using SigmaStat (Systat Software, San Jose, CA).

Results

LFs Were Increased in Number and Size in COPD Lungs

NSC, SC, and patients with COPD did not differ in their age or sex ratios (Table 1; Tables E1–E3). LFs were defined as aggregates containing more than 40 mononuclear cells exhibiting the typical topographical arrangement of cells: (*1*) centrally located CD20-positive B cells along with a few FDCs, and (*2*) $CD4^+$ and $CD8^+$ T cells located in the periphery (Figure E1A). Patients with COPD had more LFs per unit lung area (Figure 1A) than SC and NSC, especially in the parenchyma and also around airways and vessels (Figures E1B–E1D). GOLD stage IV COPD lungs had more numerous and larger pulmonary LFs than GOLD stages I–II COPD lungs, which, in turn, had more numerous and larger LFs than SC and NSC lungs (Figures 1A–1H). On hematoxylin and eosin–stained lung sections, an average of 16% of LFs analyzed in the COPD lungs studied had a recognizable germinal center (GC). Among the nine LFs analyzed in SC lungs, only one had a recognizable GC, whereas none of the LFs in the NSC lungs studied had a recognizable GC.

LF B-Cell BAFF Expression Correlated Indirectly with B-Cell Apoptosis and Directly with LF Size

BAFF-positive B cells were more numerous in LFs in GOLD stage IV versus I–II COPD, SC, and NSC lungs (Figures 2 and 3A; Figure E2). GOLD stages I–II COPD and SC pulmonary LFs had more active caspase-3–positive B cells than GOLD stage IV COPD pulmonary LFs (Figures 2 and 3B; Figure E2). There was a strong inverse correlation between the percentage BAFF-positive and active caspase-3–positive B cells in LFs in COPD lungs (Figure 3C), especially in the GOLD IV COPD group (Figure E3A). A weaker correlation was found in the GOLD I–II COPD group (Figure E3B). The percentage BAFF-positive B cells in LFs correlated directly with LF size in COPD lungs (Figure 3D) and all subject (not shown). In the patients with COPD, the percentage predicted FEV_1 correlated: (*1*) negatively (but weakly) with the % BAFF-positive B cells in LFs (not shown), and (*2*) positively with the percentage active caspase-3–positive B cells in LFs (Figure 3E). These correlations were not maintained when patients with GOLD

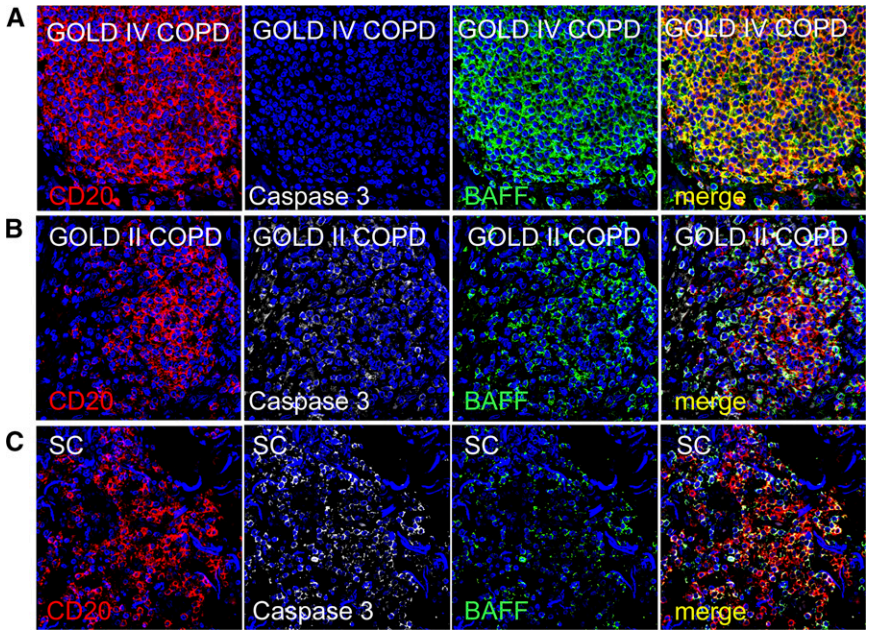

Figure 2. Lymphoid follicles (LFs) from patients with chronic obstructive pulmonary disease (COPD) with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage IV disease had more B cell–activating factor of tumor necrosis factor family (BAFF)-positive B cells and fewer apoptotic cells than patients with GOLD stage I–II COPD and smokers without COPD (SC). (*A–C*) Confocal images of triple-color immunofluorescence staining of representative pulmonary LFs from a patient with GOLD stage IV COPD (*A*), a patient with GOLD stage II COPD (*B*), and an SC (*C*). B cells were stained with a *red* fluorophore for CD20, BAFF-positive cells are identified with a *green* fluorophore, and active caspase-3–positive cells with a *gray* color. 4′,6-Diamidino-2-phenylindole (*blue*) was used to counterstain the nuclei. The images shown are representative of LFs in 5 to 10 subjects/group. Two out of 10 patients with GOLD stage IV COPD, 3 out of 9 patients with GOLD stages I–II COPD, 1 out of 5 SC, and 3 out of 5 nonsmoking control subjects had no LFs in the lung sections studied.

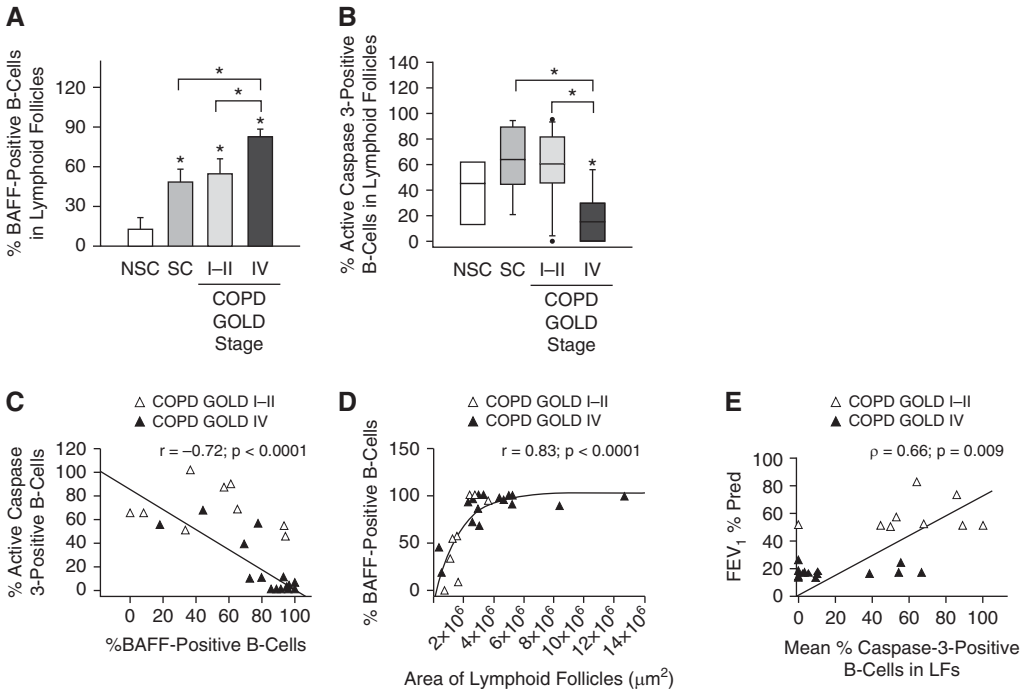

Figure 3. The percentage of B cell–activating factor of tumor necrosis factor family (BAFF)-positive B cells in pulmonary lymphoid follicles (LFs) in patients with chronic obstructive pulmonary disease (COPD) was correlated indirectly with the percentage of apoptotic B cells in LFs and directly with LF area. The percentages of BAFF-positive B cells (*A*) and active caspase-3–positive B cells (*B*) in LFs in the lungs of nonsmoking control subjects (NSC), smokers without COPD (SC), Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages I–II COPD, and GOLD stage IV COPD are shown. A Student's *t* test (*A*) and a Mann-Whitney test (*B*) were used to perform the statistical analysis of the groups. In *A*, data are mean + SEM. In *B*, the median and 5% and 95% confidence intervals are shown in the *box plots*, and the *error bars* in the *box plots* are SDs in *B*. In *A* and *B*, $^*P < 0.05$ versus NSC or versus the group indicated. In *C*, the correlation between percentage of BAFF-positive B cells and percentage of active caspase-3–positive B cells in LFs in the COPD group is shown. (*D*) Correlation between the percentage of BAFF-positive B cells and the area of pulmonary LFs in the COPD group. These results are nonlinear, and the best fit for the data was a three-parameter single exponential rise to maximum curve. (*E*) Correlation between percentage of active caspase-3–positive B cells and the FEV_1% predicted in patients with COPD is shown. The Pearson (*C* and *E*) or Durbin-Watson (*D*) statistical correlation tests were used to analyze the data. In *A–E*, a total of 29 subjects were analyzed; 4 LFs were analyzed in NSC, 9 LFs in SC, 10 LFs in GOLD stages I–II COPD, and 17 LFs in patients with GOLD stage IV COPD. In *C–E*, patients with COPD with GOLD I–II disease are represented by *open triangles*, and those with GOLD IV disease by *solid triangles*.

I–II and IV COPD were analyzed separately (not shown).

Type A and Type B LFs

Frequency histograms were used to assess the distribution of pulmonary LFs as measured by the percentage of BAFF-positive cells (mostly B cells) in the pulmonary LFs among our subject groups. In the SC and NSC lungs, LFs had few BAFF-positive B cells (Figure E4A) but relatively frequent apoptotic B cells (Figure E4B). In contrast, most of the LFs in GOLD stage IV COPD lungs had a high percentage of BAFF-positive cells but few apoptotic B cells. Patients with GOLD stages I–II COPD had an intermediate distribution of BAFF-positive cells between that of the subjects without COPD and those with GOLD stage IV COPD. Two main types of LFs (A and B) were identified in the study subjects' lungs based on their BAFF and active caspase-3 staining characteristics (Figure 2; Figure E2) after comparing different thresholds for positive staining for BAFF and active caspase-3 (Tables E4 and E5). The threshold for the percentage of BAFF-positive cells in LFs that yielded the greatest differences in the percentages of LFs labeled as "BAFF-positive" between the GOLD stage IV and GOLD stages I–II COPD and the control groups was 70% (Table E4). The threshold for the percentage of apoptotic cells in LFs that yielded the greatest differences in the percentages of LFs labeled as "apoptotic-positive" between the patients with GOLD stage IV COPD versus patients with GOLD stages I–II COPD or the No-COPD control group was 20% (Table E5). Thus, type A (apoptosis-high) LFs were characterized by the presence of greater than 20% active caspase-3–positive cells (mainly B cells but also some $CD4^+$ and $CD8^+$ T cells, and FDCs) and less than or equal to 70% BAFF-positive cells (mainly B cells; Figures 2B and 2C; Figures E2B and E2C). Type A LFs represented the majority of LFs in SC and GOLD stages I–II COPD lungs (Tables 2 and E4). Type B LFs (BAFF-high) were characterized by the presence of less than or equal to 20% active caspase-3–positive cells (mainly $CD4^+$ and $CD8^+$ T cells, and FDCs) and more than 70% BAFF-positive cells (mainly B cells and a few CD4 + and CD8 + T cells; Figure 2A, Figure E2A). Most LFs in GOLD stage IV COPD lungs were type B LFs (Table 2; Tables E4 and E5).

Markers of Cellular Activation and Proliferation, but Not BAFF-R Expression, Differed in Type A versus Type B LFs

Type A LFs had few B cells (CD20; Figure E5A), FDCs, and $CD4^+$ and $CD8^+$ T cells (Figure E5D or not shown) staining positively for BAFF (Figure E5B). Type A LFs also had variable percentages of B cells

Table 2. Distribution of Type A and Type B Pulmonary Lymphoid Follicles in Patients with COPD and Control Subjects without COPD

	No COPD*	GOLD Stages I–II COPD	GOLD Stage IV COPD	*P* Values for No COPD vs. GOLD Stage IV COPD	*P* Values for GOLD Stages I–II COPD vs. GOLD stage IV COPD	*P* Values for No COPD vs. GOLD Stages I–II COPD
Type A LFs[†], %	78 ± 10	69 ± 16	25 ± 15	0.001	0.018	0.9
Type B LFs[‡], %	0	8 ± 8	75 ± 15	0.001	0.027	0.9
Other LFs[§], %	22 ± 10	22 ± 16	6 ± 2	0.6	0.7	0.7

Definition of abbreviations: BAFF = B cell–activating factor of tumor necrosis factor family; COPD = chronic obstructive pulmonary disease; GOLD = Global Initiative for Chronic Obstructive Lung Disease; LF = lymphoid follicle; NSC = nonsmoker control subjects; SC = smoker control subjects.
The data show the mean ± SEM percentages of type A and type B LFs in the lungs of nonsmokers without COPD (No COPD; n = 10), patients with GOLD stages I–II COPD (n = 9), and patients with COPD with GOLD stage IV disease (n = 10). Among these subjects, no LFs were found in 10% of the patients with GOLD stage IV COPD, 40% of the patients with GOLD stages I–II COPD, 33% of the SC, and 67% of the NSC. Data for *P* values were analyzed using a *Z* test.
*No COPD group includes NSC and SC.
[†]Type A LFs were defined as having ⩽70% BAFF-positive cells and >20% active caspase-3–positive cells.
[‡]Type B LFs were defined as having >70% BAFF-positive cells and ⩽20% active caspase-3–positive cells.
[§]LFs classified as "other LFs" were defined as having either ⩽70% BAFF-positive cells and ⩽20% active caspase-3–positive cells or >70% BAFF-positive cells and >20% active caspase-3–positive cells.

and FDCs staining positively for markers of activation (CD83; Figure E5C) and proliferation (Ki67; Figure E5E). In contrast, type B LFs had more than 70% B cells (CD20; Figure E6A), FDCs, and $CD4^+$ and $CD8^+$ T cells (Figure E6D or not shown) staining positively for BAFF (Figure E6B). Type B LFs also had more than 20% of B cells and FDCs staining positively for markers of activation (CD83; Figure E6C) and proliferation (Ki67; Figure E6E). Type A LFs had no IgG-expressing B cells in any subject examined (Figure E5H), whereas greater than or equal to 5% of B cells in type B LFs stained for IgG (Figure E6H). BAFF-R expression did not differ in LFs in SC, GOLD I–II, and GOLD IV COPD lungs (Figure E6I).

Low CSE Concentrations Increased BAFF B-Cell Expression, Whereas High CSE Concentrations Increased B-Cell Apoptosis, Which Was Rescued by BAFF

Exposing blood B cells from NSC to 2.5 to 5% CSE increased BAFF expression in a time- and concentration-dependent manner (Figure 4A). Exposing human B cells to 10% CSE (Figures 4B, 4D, and 4F) or murine splenic B cells to 2% CSE (Figures 4C, 4E, and 4G) induced B-cell apoptosis (measured as intracellular active caspase-3 levels, positive TUNEL staining, and staining with PI). Adding recombinant human BAFF to human B cells or rmBAFF to murine B cells attenuated CSE-induced B-cell apoptosis (Figures 4C–4G). As BAFF signaling activates NF-κB in B cells to increase B-cell survival in other model systems (30, 31), we tested our hypothesis that BAFF rescues murine B cells from CSE-induced apoptosis by activating NF-κB in B cells. Contrary to our hypothesis, CSE-induced B-cell apoptosis was associated with increased NF-κB activation in B cells, which was inhibited by treating cells with rBAFF as assessed by electrophoretic mobility shift assays (Figures 5A and 5B). Immunostaining experiments demonstrated reduced ratio of nuclear localization of the NF-κB p65 subunit in murine B cells treated with CSE and BAFF (indicating reduced NF-κB activation) versus cells treated with CSE alone (Figures 5C and 5D).

NF-κB Nuclear Translocation Is Lower in LF B Cells in GOLD Stage IV COPD versus SC and GOLD I–II COPD Lungs

There was intense nuclear p65 staining in many LF B cells in SC lungs (Figure 6A), and this was greater than that in GOLD I–II stage COPD lungs (Figure 6B). Nuclear localization of p65 was less intense in LF B cells in GOLD IV COPD lungs (Figure 6C) and lowest in large LFs with high-level BAFF expression and vice versa (Figures E7A–E7C or not shown).

BAFF Expression Was Increased in COPD Blood and BAL B Cells

BAFF, but not BAFF-R, staining intensity per cell was higher in blood B cells from patients with COPD versus SC and NSC (Figures E8A–E8C). BAFF staining was also higher in COPD blood B cells when the percentage of BAFF-positive cells was analyzed (not shown). Blood B-cell BAFF expression was similar in current and former smokers with COPD (not shown). Patients with GOLD III–IV COPD had higher BAFF (but not BAFF-R) staining in B cells (but not in T cells) in BAL leukocyte cytocentrifuge preparations than patients with GOLD I–II COPD, SC, and NSC (Figure E8D). BAFF expression in blood T cells was similar in patients with COPD and control subjects (not shown). BAFF serum levels did not differ in patients with COPD versus control subjects (Figure E8E).

None of the subjects studied had a primary diagnosis of lung cancer. However, a secondary diagnosis of lung cancer was present in 3 out of the 14 patients with COPD in which the LFs were studied. When additional analysis was performed excluding the three patients with lung cancer, the results did not change (except for the loss of statistical significance for the comparison between the BAFF-positive B cells in GOLD stage IV and GOLD stages I–II COPD pulmonary LFs; Figure E9; $P = 0.1$). However, it is noteworthy that the power of this analysis was only 0.05, and likely statistical significance might be achieved with higher sample sizes.

Discussion

We report several novel findings linking BAFF to LF expansion in COPD lungs. First, GOLD stage IV COPD lungs had more

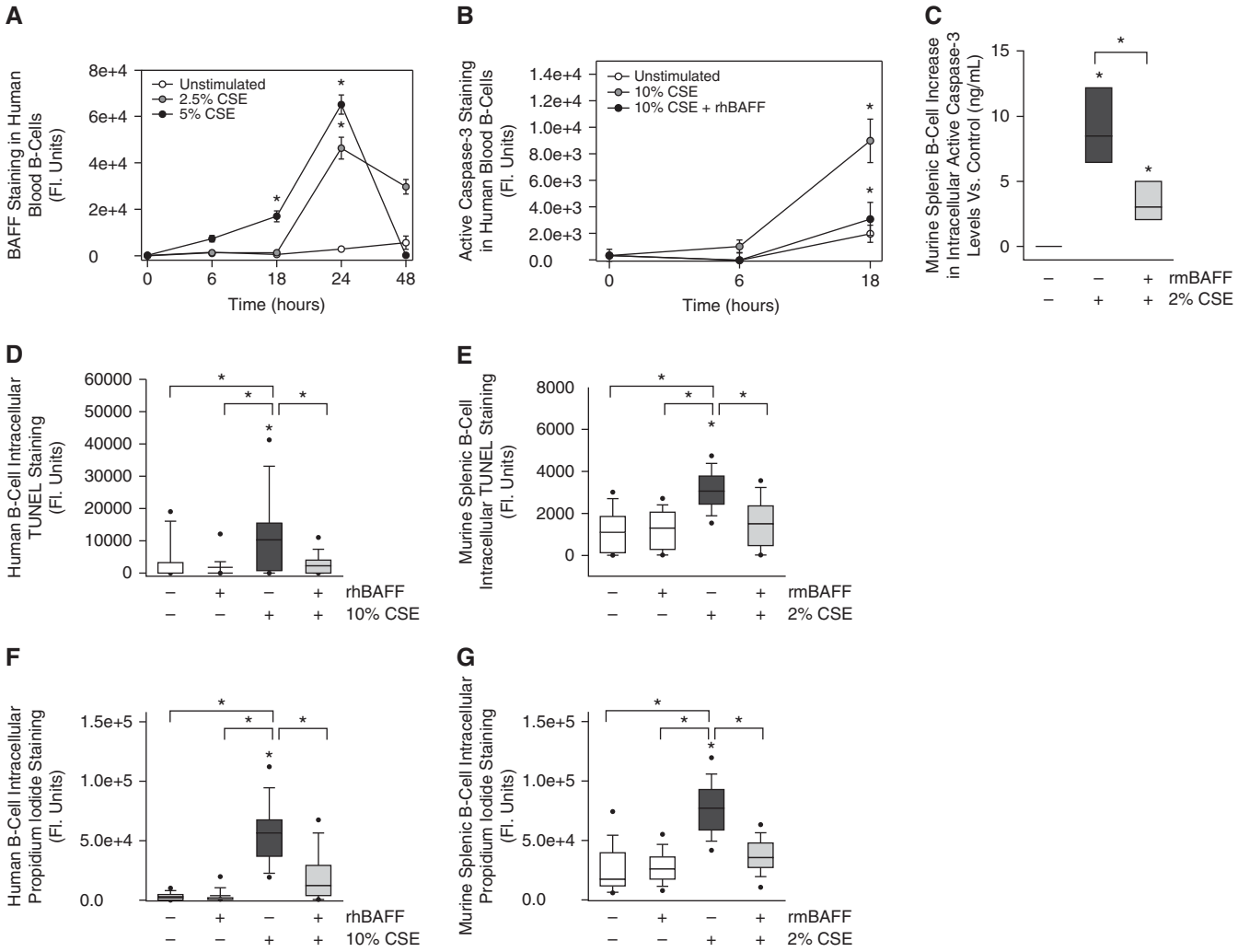

Figure 4. B cell–activating factor of tumor necrosis factor family (BAFF) was produced by B cells incubated with low concentrations of cigarette smoke extract (CSE), and exogenous recombinant BAFF rescues B cells from apoptosis induced by high concentrations of CSE in a nuclear factor-κB–dependent manner. (*A*) B cells were isolated from the blood of a healthy nonsmoker and incubated at 37°C with or without 2.5% or 5% CSE for 6, 18, 24, and 48 hours. Cells were then immunostained with Alexa-546 for CD20 and Alexa-488 for BAFF. BAFF staining in $CD20^+$ B cells was quantified as described in METHODS. Incubating human blood B cells with CSE concentrations greater than 5% did not increase B-cell BAFF expression (data not shown). (*B*, *D*, and *F*) Purified blood B cells from a healthy human nonsmoker were incubated at 37°C for up to 18 hours with or without 10% CSE and with or without 100 nM recombinant human BAFF (rhBAFF). In *B*, cells were immunostained with Alexa-546 for CD20 and Alexa-488 for active caspase-3. Active caspase-3 staining was quantified as described in METHODS. Human B cells incubated with or without CSE, BAFF, and CSE with BAFF as outlined above, and DNA fragmentation were assayed in the B cells using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (*D*), and the B cells were stained with propidium iodide (PI) (*F*). (*C*, *E*, and *G*) B cells were isolated from the spleens of unchallenged C57BL/6 wild-type mice, and equal numbers of B cells per experimental condition were incubated at 37°C with or without 2% CSE and with or without 100 nM recombinant murine BAFF (rmBAFF) for 12 hours. Increases in intracellular levels of active caspase-3 relative to cells incubated without BAFF or CSE were quantified in cell extracts using a quenched fluorogenic substrate specific for caspase-3 and assay standards of known concentrations of active caspase-3 (*C*), as described in the Methods section of the online supplement. Apoptosis of murine B cells was also measured by staining cells with either a TUNEL kit (*E*) or PI (*G*). Either Student's *t* tests (*A* and *B*) or Mann-Whitney tests (*C*–*G*) were used to analyze the data. Data are expressed as mean ± SEM (*A* and *B*) or presented as *box plots* showing medians and 5% and 95% confidence intervals, with *solid circles* indicating outliers (*C*–*G*). In *A*–*G*, $*P < 0.05$ versus baseline or versus the group indicated. The results shown in *A* and *B* are representative of six and three independent experiments, respectively.

BAFF-positive B cells and more numerous and larger LFs than GOLD stages I–II COPD and SC lungs. Second, we identified two main types of pulmonary LFs: (*1*) type A (the predominant type in GOLD stages I–II COPD and SC lungs), characterized by abundant apoptotic but few BAFF-positive B cells; and (*2*) type B (the main type in GOLD stage IV COPD lungs), characterized by abundant BAFF-positive but few apoptotic B cells. Third, low CSE concentrations increased B-cell BAFF expression, but high CSE concentrations induced B-cell apoptosis that was reduced by coincubating the cells with rBAFF. Surprisingly, BAFF may rescue B cells from CSE-induced apoptosis by inhibiting CSE-triggered NF-κB activation. Fourth, BAFF

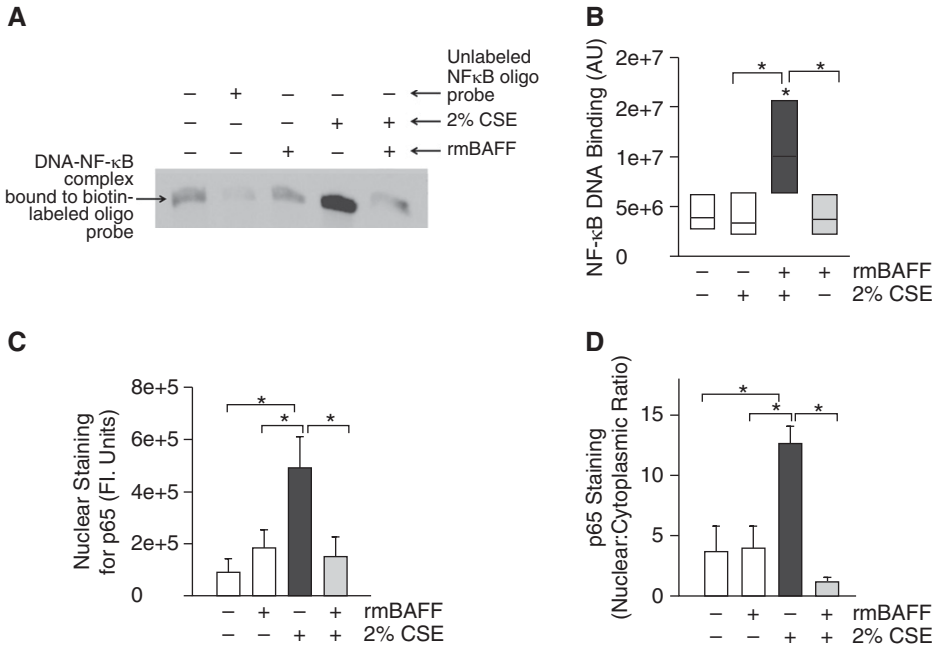

Figure 5. Exogenous recombinant B cell–activating factor of tumor necrosis factor family (BAFF) (rBAFF) rescues B cells from apoptosis induced by high concentrations of cigarette smoke extract (CSE) in a nuclear factor (NF)-κB–dependent manner. (*A* and *B*) Aliquots of B cells isolated from unchallenged wild-type mice were incubated with or without 2% CSE and with or without 100 nM rBAFF. Nuclear proteins were isolated, and equal amounts of protein (4 μg/sample) in the nuclear extracts were subjected to electrophoretic mobility shifts assays (EMSAs) using a labeled oligonucleotide probe containing the NF-κB consensus sequence. Assays were performed in the presence or absence of excess unlabeled probe to identify NF-κB protein bound specifically to the probe. *A* shows an image of an EMSA analysis of nuclear protein extracts from all experimental groups and is representative of the results from eight experiments (with two authors [M.L.-C. and P.T.] each performing four experiments independently). Note the marked reduction in signal of the band corresponding to the NF-κB–oligonucleotide complex when excess unlabeled probe is added, indicating specific binding of NF-κB present in nuclear extracts to the labeled oligonucleotide probe. In *B*, the intensities of the bands corresponding to NF-κB–oligonucleotide complexes were quantified using densitometry, and band intensities for all groups were normalized to signals in the B cells incubated without CSE or recombinant murine BAFF (rmBAFF). *Box plots* show the median values and the 5% and 95% confidence intervals. The *error bars* in the *box plots* (which are too small to be seen) are SDs; n = 8 independent experiments. In *B*, $^*P < 0.05$ compared with unstimulated cells or with the group indicated. In *C* and *D*, aliquots of B cells isolated from unchallenged wild-type mice were incubated with or without 2% CSE and with or without 100 nM rmBAFF. After 12 h, cells were immunostained with Alexa-546 for CD20 and Alexa-488 for the p65 subunit of NF-κB. P65 staining in the nuclear versus cytoplasmic areas of the cells was quantified as described in METHODS. *C* shows quantitation of staining for p65 in the nuclei of the cells. *D* shows quantitation of the ratio of nuclear:cytoplasmic staining for p65 in B cells. Either a Kruskall-Wallis one-way analysis of variance (ANOVA) by ranks test (*B*) or a one-way ANOVA test (*C* and *D*) was used to analyze the data. In *C* and *D*, $^*P < 0.05$ compared with the group indicated. Data are presented as box plots showing medians and 5% and 95% confidence intervals (*B*) or as mean + SEM (*C* and *D*). AU = arbitrary units.

expression was higher in blood and BAL B cells (but not T cells) from patients with COPD versus control subjects. Although serum BAFF levels did not differ between patients with COPD and control subjects, it is possible that blood leukocytes that are more numerous than B cells produce BAFF even when not activated by cigarette smoke (CS) or agonists generated in smoker and COPD blood and lung samples. Overall, our data support the hypothesis that B-cell BAFF expression creates a self-perpetuating loop contributing to COPD progression by promoting lung B-cell survival and LF expansion.

BAFF and LF Expansion in COPD

Emphysema-predominant GOLD stages III–IV COPD lungs are characterized by B-cell–containing LFs. However, the mechanism(s) by which LFs form and expand in COPD lungs have not been identified. Although BAFF contributes to autoimmunity (21–24, 34–36), BAFF has not been well studied in COPD. Increased BAFF expression occurs in macrophages in COPD lungs (29), but other cell types have not been studied. We now link BAFF to LF expansion in COPD, as BAFF expression in LF B cells correlated directly with LF size and number and indirectly with LF B-cell apoptosis in COPD lungs. We provide evidence that BAFF promotes LF expansion in severe COPD by inhibiting activation of NF-κB in LF B cells to promote B-cell survival as: (*1*) BAFF blocked both CSE-induced B-cell apoptosis and CSE-induced NF-κB activation *in vitro*, and (*2*) reduced activation of NF-κB was detected in LF B cells expressing high levels of BAFF in severe and very severe COPD lungs.

Our results indicate that LFs undergo dynamic changes (including expansion), especially in very severe COPD lungs, that hitherto have not been appreciated. Moreover, our data suggest that these dynamic changes in LF architecture are driven, at least in part, by LF B-cell BAFF expression that limits B-cell apoptosis and promotes B-cell survival, maturation, and proliferation (Figure 7). Although pulmonary infections can contribute to changes in LF size and number (37), none of the subjects from whom we obtained lung sections or BAL samples had clinical evidence of respiratory tract infection in the

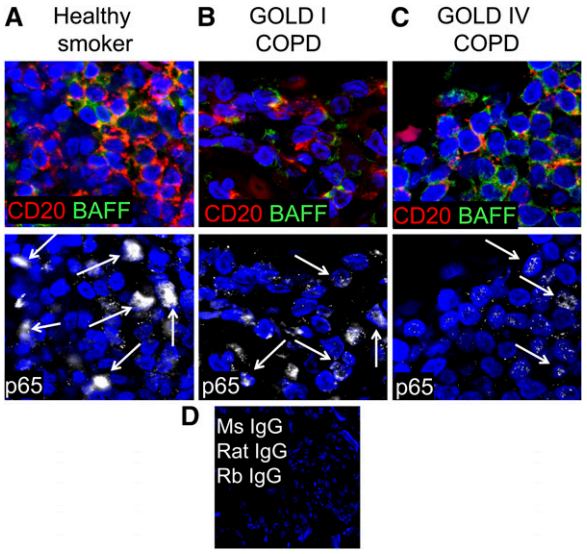

Figure 6. Nuclear localization of the p65 subunit of nuclear factor (NF)-κB is reduced in B cells in Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage IV chronic obstructive pulmonary disease (COPD) pulmonary lymphoid follicles (LFs) versus B cells in LFs of smokers without COPD (SC) and GOLD stage I–II COPD lungs. (*A–C*) Confocal images of triple-color immunofluorescence staining of pulmonary LFs from an SC (*A*), a patient with GOLD stage I COPD (*B*), and a patient with GOLD stage IV COPD (*C*). B cells were stained with a *red* fluorophore for CD20, a *green* fluorophore for B cell–activating factor of tumor necrosis factor family (BAFF), and a *gray* color for the p65 subunit of NF-κB. 4′,6-Diamidino-2-phenylindole (*blue*) was used to counterstain the nuclei. The images are representative of LFs in five to seven subjects/group. In *D*, a lung section from a patient with GOLD IV stage COPD was stained with isotype-matched nonimmune control antibodies. *White arrows* indicate nuclear localization of p65 staining in LF B cells, especially in the SC and patients with GOLD stage I COPD. Ms = murine; Rb = rabbit.

3 months before their surgery. Thus, it is unlikely that the differences in staining for BAFF, active caspase-3, or markers of activation or proliferation, or the differences in LF size or number that we detected in pulmonary LFs from patients with COPD, SC, or NSC, were due to the presence of bacterial infection in COPD but not SC lungs. However, we cannot exclude the possibility that infective events occurring at time points earlier than 3 months before the surgery contributed to some of the differences in BAFF expression and/or active caspase-3 staining that we detected between our subject groups.

Although data generated in human samples can only be correlative, it is noteworthy that the companion manuscript reports that antagonizing BAFF signaling by delivering a soluble fusion protein containing the BAFF-R and the Fc component of IgG to mice chronically exposed to CS prevents both LF formation and emphysema development (38). It is noteworthy that a prophylactic dosing strategy was substantially more effective at limiting LF generation and emphysema development in CS-exposed mice. Together, these results suggest that early BAFF-signaling events in murine lungs are crucial for the formation and expansion of pulmonary LFs (38) and that LF expansion contributes to emphysema development. These results also suggest that antagonizing BAFF signaling in the lungs may limit the progression of adaptive immune responses and emphysema progression in patients with COPD.

COPD Lungs Contain Two LF Types

Mature LFs contain germinal center B cells that proliferate to form $CD20^+$ plasma cells producing antibodies against pathogens or autoantigens (9). Large LFs in severe COPD, having abundant BAFF-positive B cells, might be the source of autoantibodies that are known to be present in the lungs of patients with COPD and are believed to contribute to disease progression (39). We only detected IgG-expressing B cells in LFs having abundant BAFF-expressing B cells. However, we did not assess whether these IgG-expressing B cells in type B LFs in COPD lungs are autoantibodies that contribute to disease progression by binding to autoantigens to induce complement activation and lung inflammation and destruction. These unknowns will be addressed by our future studies.

BAFF and B-Cell Survival

High CSE concentrations induced B-cell apoptosis associated with increased NF-κB activation. Both processes were attenuated by treating the cells with rBAFF. These *in vitro* results were consistent with our finding that B-cell BAFF expression was correlated inversely with B-cell apoptosis in pulmonary LFs and directly with markers of B-cell activation and proliferation and LF size in COPD lungs. Likely, in type A LFs in SC and patients with GOLD stage I–II COPD, the low-level BAFF expression leads to reduced B-cell survival and failure to undergo selection, thereby limiting the size and number of LFs in SC and GOLD stage I–II COPD lungs. In contrast in type B LFs, high-level BAFF expression by B cells promotes B-cell survival, maturation, and selection, thereby contributing to the expansion of B-cell–rich LFs in GOLD stage III–IV COPD lungs. It is noteworthy that BAFF promotes both T-cell–dependent and –independent response (11). In health, a combination of antigen–BCR binding, CD40 ligand on B cells binding to CD40 on T cells, and BAFF–BAFF-R interaction promotes B-cell activation and proliferation. However, when antigen–BCR signaling is suboptimal and BAFF levels are high, BAFF–BAFF-R interactions alone may permit B cells to survive and become autoreactive by responding to self-antigens (40). Whether the B cells in type B pulmonary LFs in patients with GOLD stage III–IV COPD that have high-level BAFF expression are autoreactive, and capable of T-cell–independent immune responses and autoantibody production, will be the focus of future studies in our laboratory.

Prior studies of other model systems have linked BAFF to B-cell survival, but these studies showed that BAFF activates the NF-κB pathway in B cells to increase B-cell survival (30, 31). However, in marked contrast to other models, the protective effects of BAFF on CSE-treated cells in our study were associated with reduced NF-κB activation, and we demonstrated reduced NF-κB activation (nuclear localization) in pulmonary LFs in patients with GOLD

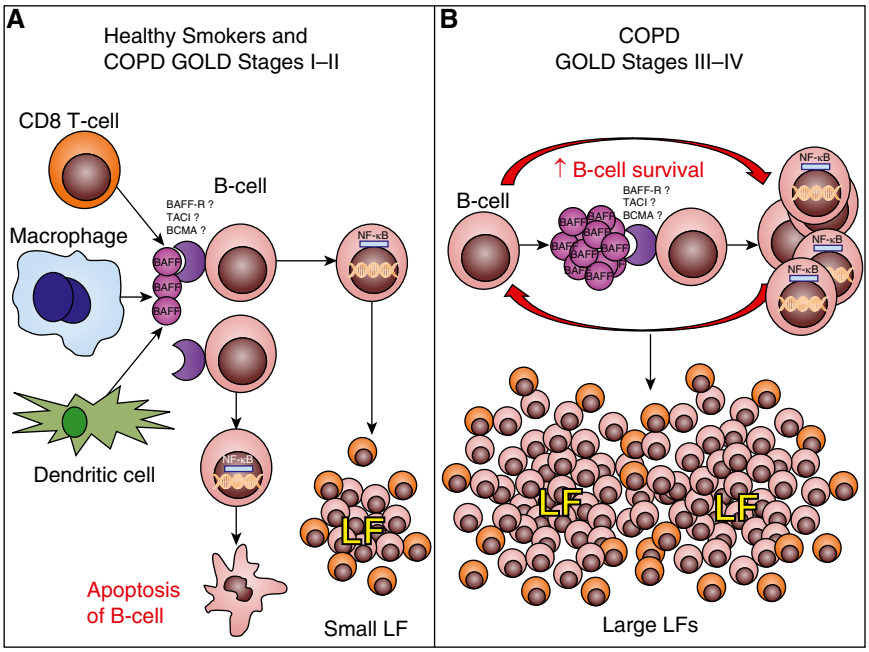

Figure 7. Cartoon illustrating potential roles for B cell–activating factor of tumor necrosis factor family (BAFF) in the regulation of B-cell homeostasis and lymphoid follicle (LF) expansion in chronic obstructive pulmonary disease (COPD) lungs. (*A*) Under mild chronic inflammatory conditions, such as those occurring in the lungs and other compartments of healthy smokers or patients with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages I and II COPD, limited quantities of BAFF are produced mainly by T cells, macrophages, and dendritic cells. Antigen recognition by the B-cell receptor (not shown) and engagement of CD19 on B cells with CD21 on dendritic cells (not shown) coupled with BAFF binding to its receptors on B cells promotes activation, proliferation, and differentiation of B cells. Limited BAFF signaling through its receptors on B cells is associated with increased nuclear factor (NF)-κB–induced apoptosis, leading to the formation of LFs that are limited in size and number. (*B*) Under severe chronic inflammatory conditions, such as those occurring in the severe stages of COPD, B cells themselves produce BAFF, and BAFF signaling via BAFF receptor (BAFF-R) in B cells inhibits nuclear translocation and activation of NF-κB, thereby preventing B cells in the LFs from undergoing apoptosis. Our data support the hypothesis that this process establishes a self-perpetuating loop of B-cell activation and increased B-cell survival, causing excessive expansion of the B-cell pool in the lung and especially within pulmonary LFs. These events may promote the growth of existing B-cell–rich LFs and the formation of additional LFs. BCMA = B-cell maturation antigen; TACI = transmembrane activator and calcium modulator and cytophilin ligand interactor.

stage III–IV COPD. It is noteworthy that NF-κB is a redox-sensitive transcription factor, and oxidants in CSE activate NF-κB in T lymphocytes (41). Although NF-κB activation often triggers prosurvival pathways, under conditions of cellular stress (e.g., hypoxia) NF-κB activation promotes apoptosis (42, 43). Thus, we hypothesize that in the CS-exposed lung, BAFF stimulation may promote B-cell survival by inhibiting oxidative stress–induced NF-κB activation.

Limitations

Our sample sizes for the LFs studies were small, and most of our SC were current smokers, whereas our patients with COPD were mostly former smokers. Although inhaling CS likely increases BAFF expression by B cells and other cells, our patients with COPD had higher BAFF expression in blood and lung B cells than SC. We also did not measure emphysema severity using high-resolution thoracic CT scans or autoantibody levels in patients with COPD and correlate them with BAFF B-cell expression and LF size and number to more directly link BAFF B-cell expression with disease pathogenesis. However, blocking BAFF–BAFF-R interactions using an anti–BAFF-R antibody reduces CS-induced LF formation and emphysema development in mice (38). We recognize that the sampling and assessment of the LFs may have been biased by our approach. Ideally, measures of LF dimension should be corrected for lung volume (44), but this is not feasible when analyzing human lungs. We did not study the other two BAFF receptors (transmembrane activator and calcium modulator and cytophilin ligand interactor [TACI] and B-cell maturation antigen [BCMA]). However, BAFF-R is the major mediator of BAFF-dependent costimulatory responses of B cells (45). Finally, although none of the subjects studied had clinical evidence of active respiratory infections, we cannot exclude the possibility that previous pulmonary infections, or lung microbiome changes, especially in patients with COPD with severe disease, contributed to the genesis or expansion of LFs.

Conclusions

Our data support our hypothesis that BAFF contributes to the formation and expansion of pulmonary LFs in the severe stages of COPD likely by promoting the survival and proliferation of LF B cells by inhibiting activation of NF-κB. Future studies will determine whether BAFF is a novel disease-modifying therapeutic target for COPD. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank Dr. Bernardino Barcelò, Biochemistry Department, Hospital Universitario Son Espases, Palma de Mallorca, Spain; Prof. Marco Cassatella, Pathology Department, University of Verona, Italy; and Dr. Raja Abdulnour, Medicine Department, Brigham and Women's Hospital, Boston, Massachusetts, for their helpful comments on the manuscript. They also thank Drs. Mark Perrella and Juan Osorio, Brigham and Women's Hospital, Boston, Massachusetts, for providing control lung tissue.

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