

Joseph Pierce, Jules Rir-Sima-Ah, Isaac Estrada, Julie Wilder, Andreas Strasser and Yohannes Tesfaigzi

Am J Physiol Lung Cell Mol Physiol 291:862-870, 2006. First published Jun 16, 2006;
doi:10.1152/ajplung.00516.2005

You might find this additional information useful...

This article cites 57 articles, 30 of which you can access free at:

<http://ajplung.physiology.org/cgi/content/full/291/5/L862#BIBL>

This article has been cited by 3 other HighWire hosted articles:

The BH3-only protein Bik/Blk/Nbk inhibits nuclear translocation of activated ERK1/2 to mediate IFN{gamma}-induced cell death

Y. A. Mebratu, B. F. Dickey, C. Evans and Y. Tesfaigzi
J. Cell Biol., November 3, 2008; 183 (3): 429-439.

[\[Abstract\]](#) [\[Full Text\]](#) [\[PDF\]](#)

Expression of the proapoptotic protein Bax is reduced in bronchial mucous cells of asthmatic subjects

K. Schwalm, J. F. Stevens, Z. Jiang, M. R. Schuyler, R. Schrader, S. H. Randell, F. H. Y. Green and Y. Tesfaigzi

Am J Physiol Lung Cell Mol Physiol, June 1, 2008; 294 (6): L1102-L1109.

[\[Abstract\]](#) [\[Full Text\]](#) [\[PDF\]](#)

IL-9 and IL-13 Induce Mucous Cell Metaplasia That Is Reduced by IFN- γ in a Bax-Mediated Pathway

J. Xiang, J. Rir-Sim-Ah and Y. Tesfaigzi

Am. J. Respir. Cell Mol. Biol., March 1, 2008; 38 (3): 310-317.

[\[Abstract\]](#) [\[Full Text\]](#) [\[PDF\]](#)

Updated information and services including high-resolution figures, can be found at:

<http://ajplung.physiology.org/cgi/content/full/291/5/L862>

Additional material and information about *AJP - Lung Cellular and Molecular Physiology* can be found at:

<http://www.the-aps.org/publications/ajplung>

This information is current as of September 28, 2009 .

Loss of pro-apoptotic Bim promotes accumulation of pulmonary T lymphocytes and enhances allergen-induced goblet cell metaplasia

Joseph Pierce,¹ Jules Rir-Sima-Ah,¹ Isaac Estrada,¹
Julie Wilder,¹ Andreas Strasser,² and Yohannes Tesfaigzi¹

¹Lovelace Respiratory Research Institute, Albuquerque, New Mexico; and

²The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

Submitted 7 December 2005; accepted in final form 25 May 2006

Pierce, Joseph, Jules Rir-Sima-Ah, Isaac Estrada, Julie Wilder, Andreas Strasser, and Yohannes Tesfaigzi. Loss of pro-apoptotic Bim promotes accumulation of pulmonary T lymphocytes and enhances allergen-induced goblet cell metaplasia. *Am J Physiol Lung Cell Mol Physiol* 291: L862–L870, 2006. First published June 16, 2006; doi:10.1152/ajplung.00516.2005.—Immunological tolerance during prolonged exposure to allergen is accompanied by a shift in the lymphocyte content and a reduction of goblet cell metaplasia (GCM). Bim initiates negative selection of autoreactive T and B cells and shut down of T cell immune responses *in vivo*. The present study investigated whether Bim plays a role in the resolution of GCM during prolonged exposure to allergen. Loss of Bim increased T lymphocyte numbers in the bronchoalveolar lavage at 4 and 15 days of allergen exposure. The numbers of pulmonary CD4⁺8⁺, CD4⁺8⁺, and $\gamma\delta$ T cells were significantly higher in naive and allergen-challenged *bim*^{−/−} mice compared with wild-type (WT) littermates. When activated, pulmonary *bim*^{−/−} T cells produced increased levels of IFN γ compared with *bim*^{+/+} T cells. No differences were noted in the total numbers of epithelial cells per millimeter of basal lamina between *bim*^{+/+} and *bim*^{−/−} mice, and the rate of resolution over 15 days of exposure was similar in both groups of mice. However, GCM was significantly enhanced and expression of IL-13R α 2 was reduced in *bim*^{−/−} mice compared with WT mice at 4 days. Furthermore, treatment of bronchiolar explant cultures with increasing IFN γ levels reduced immunostaining for IL-13R α 2. Collectively, these studies suggest that, during prolonged exposure to allergen, Bim plays no role in the resolution of GCM, but increased IFN γ levels in *bim*^{−/−} mice may be responsible for reduced expression of IL-13R α 2 and enhanced GCM despite similar levels of IL-13 in *bim*^{+/+} and *bim*^{−/−} mice.

asthma; interferon- γ and tolerance; apoptosis; IL-13R α 2; mucous cell metaplasia

ASTHMA IS AN increasingly common disease that remains poorly understood and difficult to manage. Allergic asthma is a T helper type 2 (Th2) lymphocyte-mediated inflammatory airway disease characterized by airway eosinophilia, increased mucus production caused by goblet cell metaplasia (GCM), and structural remodeling of the airway wall (9, 52). Many studies have shown that, in WT mice, prolonged exposure to allergen causes the removal of Th2 cells, eosinophils, and GCM (27, 41, 54). This transition from antigen sensitization to immunological tolerance is accompanied by a shift in the lymphocyte content in the lung tissue and bronchial lavage fluid (BALF) (6, 54). Antigen-specific regulatory T cells are believed to produce IL-10 transiently and to thereby inhibit the asthma phenotype during the development of tolerance (2, 43). IL-4, a Th2

cytokine, enhances type I dendritic cell maturation and kills type II dendritic cells, a mechanism of mature Th2 cells to selectively inhibit vigorous Th2 responses and enhance Th1 responses by regulating the selective survival of dendritic cell subsets (2, 43). It is, however, not clear whether regulators of apoptosis play a role in the survival or death of regulatory T cells or in the reduction of T cell numbers during prolonged exposure to allergen and the development of tolerance.

The Bcl-2 family of proteins are critical regulators of apoptotic cell death. These proteins contain variable numbers of four conserved Bcl-2 homology (BH) domains, all of which include α -helical segments (29). Anti-apoptotic members, such as Bcl-2 and Bcl-xL, contain all BH domains. The pro-apoptotic proteins are subdivided into multidomain members such as Bax and Bak (containing BH1, -2, and -3) and the BH3-only members such as Bid and Bim, which share only the BH3 region with their relatives. These so-called BH3-only proteins bind via their α -helical BH3 domain to a groove formed by the BH1, -2, and -3 domains on the surface of pro-survival Bcl-2 family members, and this interaction is required for their ability to kill cells (21). Bim, 1 of 10 mammalian BH3-only proteins, is expressed as many isoforms, which are generated by alternative splicing (28), (1) and is found in many cell types including lymphocytes, myeloid cells, epithelial cells, neurons, and germ cells (30). The pro-apoptotic activity of Bim can be regulated by a range of mechanisms such as transcriptional induction by FOXO3A (12, 44), sequestration of the protein to the microtubular dynein motor complex by interaction with dynein-like chain LC8 (32), and Erk-mediated phosphorylation leading to ubiquitination and proteasomal degradation (24). The pro-apoptotic function of Bim can also be inhibited by anti-apoptotic players, such as Bcl-2 or Bcl-xL (5). Deletion of the *bim* gene in mice produced animals with abnormally elevated numbers of T as well as B cells in all hemopoietic organs (3). In culture, these lymphocytes displayed abnormal resistance to certain apoptotic stimuli, including cytokine deprivation or calcium flux, but they had normal or only slightly reduced sensitivity to other death stimuli such as phorbol ester and DNA damage that could be countered by expression of a Bcl-2 transgene (3, 20). Further studies *in vivo* demonstrated that Bim is an essential initiator of apoptosis in negative selection of autoreactive thymocytes (4), mature T cells (7), and B cells (11) in shut down of T cell immune responses to a herpes simplex virus infection (31) or an injected superantigen (20).

Address for reprint requests and other correspondence: Y. Tesfaigzi, Lovelace Respiratory Research Institute, 2425 Ridgecrest Dr., SE, Albuquerque, NM 87108 (e-mail: ytesfaigz@lrrri.org).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We had observed that allergen-induced GCM is reduced by apoptotic mechanisms during prolonged exposures to allergen (45). Because Bim has been shown to be expressed in epithelial cells (30), the present study investigated whether Bim has a role in the cell death process of metaplastic mucous cells and other airway epithelial cells during the resolution of inflammation after prolonged exposure to allergen. While the resolution of GCM was not affected by the loss of Bim, we found that Bim enhanced allergen-induced GCM because of increased levels of IFN γ that downregulated IL-13R α 2 expression.

MATERIALS AND METHODS

Animals. Male specific pathogen-free wildtype (WT) C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in isolated cages under specific pathogen-free conditions. After a 14-day quarantine period, mice were acclimatized for 8 days and entered into the experimental protocol at 8–10 wk of age. The *bim*^{-/-} mice were made available by Dr. Philippe Bouillet (Walter and Eliza Hall Institute); gene targeting was performed in 129Sv embryonic stem (ES) cells, and, before our experiments, mice had been backcrossed for >10 generations with C57Bl/6 mice. We used *bim*^{+/+} and *bim*^{-/-} littermates that were bred from *bim*^{+/+} mice at the Lovelace Respiratory Research Institute (LRRI) under specific pathogen-free conditions. Mice were genotyped by PCR as described (4). All experiments were approved by the Institutional Animal Care and Use Committee and were carried out at LRRI, a facility approved by the Association for the Assessment and Accreditation for Laboratory Animal Care International.

Sensitization and allergen challenge protocols. Mice, 6–8 wk of age, were housed in whole body exposure chambers (H1000; Hazelton Systems, Aberdeen, MD) in shoebox-type plastic cages with hardwood chip bedding and were given food and water ad libitum inside the chambers. Chamber temperatures were maintained at 26 $\pm$ 2°C, and lights were on a 12:12-h on-off cycle. Mice were sensitized by injection (ip) with 10 μ g of ova albumin (OVA, grade V; Sigma-Aldrich, St. Louis, MO) adsorbed to 2 μ g of Alhydrogel (Superfos Bisector) in 0.5 ml of sterile water. A booster injection was given on day 7, using the same reagents. Seven days later, mice were exposed 6 h/day to OVA aerosols. OVA exposures were generated by aerosolizing (6 h/day, 5 days/wk) 1% heat-aggregated OVA (chicken egg, grade V; Sigma), diluted with filtered air, and then delivered to whole body exposure chambers. The total mass concentration of OVA was determined by gravimetric analysis of filter samples taken every 2 h during exposure. The mass concentration of OVA was 2.3 mg/m³. Mice were exposed to allergen for 4 or 15 consecutive days and euthanized immediately after the last exposure. Naive mice that were and not sensitized served as 0 time point.

Collection of BALF and cell counting. Mice were injected (ip) with 150 units of heparin (ICN Biomedical, Aurora, OH) 10 min before euthanization with 0.2 ml of 1:10 Euthasol (penytoin sodium; Delmarva Laboratories, Midlothian, VA) and exsanguination via the renal artery. The thoracic content was exposed, and the lungs were perfused by cardiac puncture with 0.9% saline (wt/vol; McGraw, Irvine, CA). The trachea was cannulated with a 23-gauge blunt needle tipped with surgical tubing, the lungs were lavaged three times with 0.5 ml of ice-cold PBS, and the BALF was collected. The cells recovered by lavage were counted using a hemacytometer. Cytological preparations were prepared and stained with Wright Giemsa solution (Sigma-Aldrich) to determine the different types of cells present in the BALF. At least 400 cells were counted from each slide, and the distribution of macrophages, neutrophils, eosinophils, and lymphocytes was determined.

Isolation of lung cells. The lung cells were isolated using a procedure adapted from Lovchik et al. (25). Briefly, the lavaged lungs

were minced and incubated with collagenase A (0.7 mg/ml; Roche Applied Science, Indianapolis, IN) in RPMI-1640 (Gibco, Grand Island, NY) with 5% FBS (Hyclone, Logan, UT) and DNase I (0.03 mg/ml; Sigma-Aldrich) for 90 min at 37°C with agitation. Digested lungs were passed through a wire mesh cup. Larger particles were removed from the digested lung by gravitational flow through a syringe column plugged with loose nylon wool. Cells in suspension were pelleted and resuspended in RPMI-1640 with 5% FBS, and red blood cells were lysed by incubating the homogenate with red blood cell lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃, and 0.1 mM Na₂EDTA, pH to 7.4 with 1 N HCl) at room temperature for 1 min. Cells were pelleted and resuspended in RPMI-1640 with 5% FBS. Cellular debris was removed by spinning the homogenate through a 30% Percoll-PBS solution (Amersham Biosciences) and removing the supernatant. The cell pellet was resuspended in RPMI-1640 with 5% FCS, and live cells were stained with Trypan blue and counted with the use of a hemacytometer. To obtain a sufficient number of lung cells for analyses of secreted cytokines and for immunophenotyping of pulmonary T cells, isolated lung cells from three to four mice had to be pooled. We have, therefore, 10–15 mice/group for cell counts and cytokine assays within the BALF but only 3–6/group for immunophenotyping and for analyses of secreted cytokines from isolated lung cells.

Cytokine secretion and ELISA measurements. For cytokine secretion studies, a portion of the isolated lung cells was incubated on 100 $\times$ 20 mm tissue culture plates for 2 h at 37°C, 5% CO₂, to remove adherent cells. Nonadherent cells were recovered and resuspended in lung cell medium (2 mM L-glutamine, 100 U/ml penicillin-streptomycin, 1 mM MEM-sodium pyruvate, and 1 $\times$ MEM nonessential amino acids; all from Gibco) with 5 $\times$ 10⁻⁵ M β -mercaptoethanol (Sigma-Aldrich), 1 μ g/ml indomethacin (Sigma-Aldrich), 250 U/ml catalase (Sigma-Aldrich), and 10% FCS in RPMI-1640. Cells were either left unstimulated or were stimulated with concanavalin A (5 μ g/ml; Sigma-Aldrich) for 72 h at 37°C, 5% CO₂. Culture supernatants were collected, frozen at -80°C, and analyzed for cytokine content at a later time.

ELISA plates (Greiner Bio-Ore, USA Scientific) were coated with capture antibodies diluted in 0.1 M Na₂HPO₄ overnight at 4°C, washed, and blocked with 1% BSA in PBS. Samples were added to the plates after subsequent washes and incubated overnight at 4°C. Detection proceeded the following day by adding biotinylated monoclonal antibodies, streptavidin-horseradish peroxidase (1 μ g/ml), and ABTS substrate, and the light absorption was read at optical density (OD)_{405 nm}. Pairs of monoclonal antibodies against IFN γ (R46A2 and biotin-XMG1.2) were purchased from BD Biosciences Pharmingen, San Diego, CA. Antibodies specific for IL-13 (38213.11 and biotin-BAF413) were purchased from R&D Systems, Minneapolis, MN. Cytokines were quantified by comparison with standard curves generated using recombinant cytokines (BD Biosciences Pharmingen). Detection limits were assigned as the lowest concentration in the linear portion of the standard curve (IFN γ at 190 pg/ml, IL-13 at 20 pg/ml).

Immunophenotyping by flow cytometry. A portion of the isolated lung cells were subjected to direct immunofluorescence staining for T-lymphocyte markers and then counted by three-colored flow cytometric analysis. All conjugated monoclonal antibodies (Ab) were purchased from BD Biosciences Pharmingen. T cells (1 $\times$ 10⁶) from each sample were washed with 1% FCS-PBS, resuspended in 0.0125 mg/ml Mouse BD Fc Block, and allowed to incubate at 4°C for 10 min. Samples were washed and resuspended in 10 μ g/ml FITC-conjugated hamster anti-mouse γ δ T-cell receptor Ab, 8 μ g/ml R-phycoerthrin-conjugated rat anti-mouse CD8a (Ly-2) Ab, and 4 μ g/ml PerCP-conjugated rat anti-mouse CD4 (L3T4) Ab solution in 1% FCS-PBS and allowed to incubate at 4°C for 30 min. Incubation with these primary antibodies was followed by three washes in 1% FCS-PBS and fixation with 0.5% paraformaldehyde (Sigma-Aldrich) at 4°C. All cells were analyzed in a Becton Dickinson FACSCalibur

(BD Biosciences Pharmingen), and the data were analyzed using the BD CellQuestPro v.4.0.1 program.

Histological evaluation. Airway perfusion for whole lung fixation for histopathological examinations was performed as described previously (41). Briefly, lungs were inflated with $1 \times$ zinc formalin (3.7% zinc formalin; Anatech, Battlecreek, MI) at a hydrostatic pressure of 25 cm for 1 h and immersed in a large volume of the same fixative for 1 day.

A stratified random-sampling scheme was used to cut the fixed left lung into cross-sectional slices, each ~ 0.4 -cm thick. Tissue slices were prepared, embedded in paraffin, and sectioned at 5- μ m thickness for histochemical staining. Tissue sections were stained with Alcian blue and periodic acid-Schiff (AB/PAS) or hematoxylin and eosin as described (10, 42). The number of AB-positive cells per millimeter basal lamina (BL) was quantified using an Olympus BH-2 light microscope equipped with the NIH Image analysis system (National Institutes of Health, Bethesda, MD) by counting the number of mucous cells and dividing by the length of the BL. The volume of stored mucosubstances in airway epithelia was analyzed by procedures as described (18). Briefly, the volume (in nl) of mucus per unit area (in mm^2) of basement membrane (V_s) was determined from the area of AB/PAS-stained material in the epithelium using the equation $V_s = (\text{area of mucous in } \text{mm}^2 \times 1,000)/(\text{length of BL in mm} \times 1.27)$. Regions of epithelium lining the bronchus-associated lymphoid tissue are not representative of the epithelium in the remainder of an airway and were, therefore, excluded from all morphometric analyses. Morphometry in all sections was carried out by a person unaware of the exposure history of mice from which the airway tissues were taken.

Microdissected airway cultures. Distal airway bronchioles were removed by microdissection and placed in culture essentially as described (50). Briefly, after removal from mice, lungs were inflated with 1% SeaPlaque low-melting temperature agarose (FMC Bioproducts, Rockland, ME) in $2 \times$ DMEM (Life Technologies, Grand Island, NY), and distal airway branches were dissected starting from the branch point. Up to 15 bronchioles were obtained from each mouse, and 3 bronchioles were used for each treatment. Bronchioles from at least three mice were prepared for each treatment to normalize for mouse-to-mouse variability. Distal airways (3–5/well) were transferred to 0.4- μ m pore size Transwell (Costar, Pleasanton, CA) mesh inserts in 12-well plates. Four hundred milliliters of media were added to the bottom of the Transwell system and 60 μ l to the top chamber with the bronchioles. Culture media, previously described (50), were changed 24 h after placing the organ cultures and every other day thereafter. Bronchioles were kept in culture at the air-liquid interface for 7 days while being treated with IL-13 at 1 ng/ml and IFN γ at 0, 1, 10, or 50 ng/ml. Explants were fixed in zinc formalin for 24 h and preembedded in 1% agarose before processing for paraffin embedding. Tissue sections (5 μ m) were then prepared and subjected to immunostaining procedures.

Immunohistochemistry. Endogenous peroxidase activity was blocked by incubating sections in 2% hydrogen peroxide-methanol for 1 min. Slides were washed in deionized water, and all subsequent washes consisted of 0.05% Brij-Dulbecco's PBS (pH 7.4). Proteins were unmasked by incubation of tissue sections with a trypsin solution (Zymed, San Francisco, CA) at 37°C for 10 min as described by the manufacturer. Slides were first incubated for blocking in 1% normal goat serum in 2% BSA-0.1% Triton X-100 before the Bim (Stressgen Biotechnologies, San Diego, CA; at 1:500 dilution) or the IL-13R α 2 (R&D Systems, Minneapolis, MN; at 5 μ g/ml) antibodies were applied. After an overnight incubation at room temperature, the bound antibodies were visualized using biotinylated goat-anti-rabbit antibody, VECTASTAIN ABC reagent, and the peroxidase substrate diaminobenzidine (DAB; Vector Laboratories, Burlingame, CA) as described by the manufacturer. Epithelial cells with mucosubstances were detected by counter-staining tissue sections with AB (19).

Immunoblot detection of proteins. Protein extracts were prepared from the entire right lung of mice by homogenization in radioimmu-

noprecipitation assay (RIPA) buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 5 mM EDTA) supplemented with the protease inhibitors PMSF (1 mM), pepstatin (10 μ g/ml), aprotinin (2 μ g/ml), and benzamidine (2 μ g/ml) and analyzed by Western blotting as described (46). The polyclonal rabbit anti-Bim (human, rat, mouse antibody; Stressgen) that detects the three isoforms, Bim_{EL}, Bim_L, and Bim_S, was used at 1:500 dilution. For detection of IFN γ , the biotinylated rat anti-mouse IFN γ (BD Biosciences Pharmingen) was used at 1:100 dilution (5 μ g/ml).

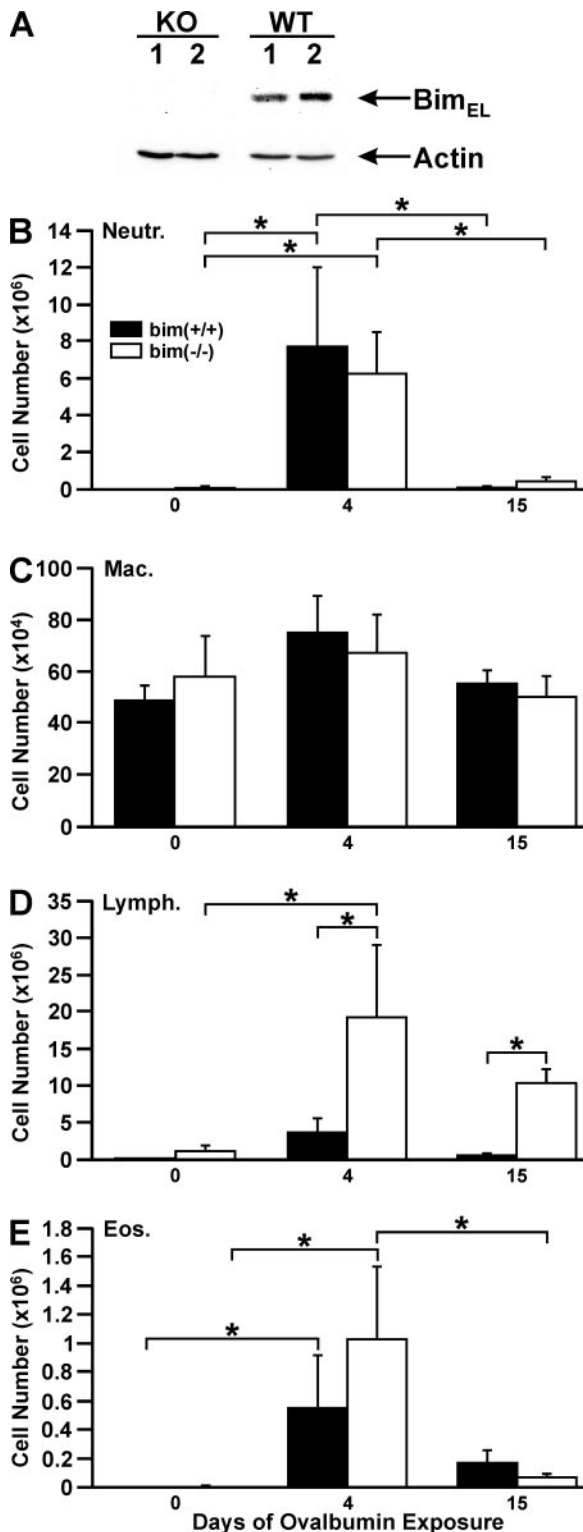
Statistical analysis. Grouped results from at least four different mice were expressed as means $\pm$ SE. Data were analyzed using Statistical Analysis Software (SAS) from the SAS Institute (Cary, NC). Results grouped by time point and genotype were analyzed using two-way analysis of variance (ANOVA). When significant main effects were detected ($P < 0.05$), Fishers least significant difference test was used to determine differences between groups. A P value of < 0.05 was considered to indicate statistical significance.

RESULTS

Exposure of mice and rats to prolonged aerosol challenge with allergen is typified by a significant decrease in airway inflammation (54), airway hyperresponsiveness (16, 22), and GCM (41) from an initial peak response level. To study whether cell death and apoptosis regulatory proteins are involved in the decrease of GCM and epithelial cell numbers during prolonged exposures to allergen, we exposed *bim*^{+/+} and *bim*^{-/-} littermates to allergen for 0, 4, or 15 days. Expression of Bim_{EL} protein was detected in the lungs from *bim*^{+/+} but not from *bim*^{-/-} mice, while other Bim isoforms were not detected (Fig. 1A). In general, inflammatory cell numbers that were recovered by bronchoalveolar lavage reached maximum levels at 4 days and were reduced by 15 days postexposure (Fig. 1, B–E). No significant differences between *bim*^{+/+} and *bim*^{-/-} mice were observed for the number of neutrophils, macrophages, and eosinophils (Fig. 1). However, significant numbers of lymphocytes were present in the BALF of naive *bim*^{-/-} but absent in *bim*^{+/+} mice (Fig. 1D). The numbers of lymphocytes were significantly increased (~ 5 -fold) in *bim*^{-/-} compared with *bim*^{+/+} littermates at 4 days as well as at 15 days of exposure. Furthermore, while the number of lymphocytes were increased from 0 to 4 days in *bim*^{-/-} mice, a statistically nonsignificant increase in lymphocytes was found in *bim*^{+/+} mice at 4 days that disappeared to background levels at 15 days (Fig. 1D). In *bim*^{+/+} mice, IL-13 levels significantly increased from 0 to 4 days and decreased from 4 to 15 days, whereas IL-13 levels in *bim*^{-/-} mice remained unchanged overall (Fig. 2A). No differences in IFN γ levels were observed between *bim*^{+/+} and *bim*^{-/-} at any time point (Fig. 2B).

Loss of Bim leads to increased numbers of T cells in nonimmunized Bim-deficient mice (3) and inhibits shut down of T-cell immune responses in the spleen (20, 31). However, the role of Bim in peripheral T-cell subsets during prolonged exposure to allergen has not been characterized. The numbers of CD4⁺8⁻, CD4⁺8⁺, and $\gamma\delta$ T cells in the lungs were significantly increased in naive *bim*^{-/-} mice compared with naive *bim*^{+/+} mice (Fig. 3). Although the numbers of CD4⁺8⁻ and CD4⁺8⁺ cells were not different between *bim*^{-/-} and *bim*^{+/+} mice at 4 days of allergen exposure, their numbers were statistically different at 15 days of exposure because of an apparent decrease from 4 to 15 days in *bim*^{+/+} mice. No apparent difference was observed at 4 days between *bim*^{-/-} and *bim*^{+/+} mice because of a statistically nonsignificant

increase in the numbers of $CD4^+8^-$ and $CD4^+8^+$ T cells in $bim^{+/+}$ mice (Fig. 3, A and B). Most strikingly, there was an approximate fivefold increase in the numbers of $\gamma\delta$ T cells in $bim^{-/-}$ mice compared with $bim^{+/+}$ mice in nonexposed and allergen-exposed mice. The number of $\gamma\delta$ T cells remained unchanged throughout the exposure period in both $bim^{+/+}$ and $bim^{-/-}$ mice (Fig. 3C).



We next analyzed the Th1 and Th2 cytokine profiles of $bim^{+/+}$ and $bim^{-/-}$ pulmonary nonadherent lung cells to determine the state of T-cell differentiation. Significantly increased levels of IL-13 were produced by cultured T cells from both $bim^{+/+}$ and $bim^{-/-}$ mice at 4 days of allergen exposure compared with their naive counterparts (Fig. 4A). Interestingly, at all time points, cultured T cells from the lungs of $bim^{-/-}$ mice produced three- to fourfold more IFN γ compared with T cells from $bim^{+/+}$ mice, although at 4 days, this difference was only marginally significant (Fig. 4B). Cells that were not treated with concanavalin A showed a similar pattern of cytokine secretion; however, the levels of IL-13 and IFN γ were in the range of concentration of 0.2–0.4 ng/ml. This finding was confirmed by analyzing the protein extracts from $bim^{+/+}$ and $bim^{-/-}$ lung and finding that IFN γ levels were significantly higher in $bim^{-/-}$ compared with $bim^{+/+}$ lungs (Fig. 4C). Densitometric analysis of the bands representing Bim normalized to the actin band showed twofold increased IFN γ levels in $bim^{-/-}$ compared with $bim^{+/+}$ lungs (data not shown).

To investigate whether Bim has any role in the resolution of airway epithelial cell hyperplasia, the numbers of total epithelial cells per millimeter BL were counted in naive and allergen-exposed $bim^{+/+}$ and $bim^{-/-}$ mice (Fig. 5). A significant increase from ~80 to ~130 cells/mm BL from 0 to 4 days was followed by an apparent decrease to ~100 cells/mm BL from day 4 to day 15 of allergen exposure. No significant differences were observed between $bim^{+/+}$ and $bim^{-/-}$ mice, suggesting that Bim does not play a role in the resolution of epithelial cell hyperplasia during prolonged exposure to allergen.

We have previously shown that the resolution of the GCM during prolonged exposure to allergen is in part dependent on the Bax protein (45). Because BH3-only proteins can initiate the cell death pathway upstream of Bax/Bak (33), we tested whether Bim plays a role in the cell death of metaplastic goblet cells in mice exposed to allergen. Interestingly, both the intraepithelial-stored mucosubstances [measured as nl/mm² epithelium (Vs)] and the numbers of mucous cells per millimeter BL increased, resulting in a two- to threefold higher amount of stored mucosubstances as well as mucous cell numbers in $bim^{-/-}$ compared with $bim^{+/+}$ littermates at 4 days postallergen exposure (Fig. 6, A and B); see representative photomicrographs (Fig. 6, E and F). There was an enhanced decrease of both volume density (Vs) of stored intraepithelial mucosubstances and mucous cell numbers per millimeter BL in $bim^{-/-}$ mice from 4 to 15 days of allergen exposure compared with $bim^{+/+}$ mice (Fig. 6, A and B, and representative photomicrographs in Fig. 5, C–G, for $bim^{+/+}$ and Fig. 5, D–H, for $bim^{-/-}$ mice). Although the parameters for GCM were still elevated in $bim^{-/-}$ mice at 15 days, the rate of resolution of mucous cells appeared to be similar in $bim^{+/+}$ and $bim^{+/+}$ mice.

Fig. 1. Bim is essential for the deletion of lymphocytes during prolonged exposure to allergen. After exposure to allergen for 4 and 15 days, increased nos. of T lymphocytes are found in the bronchial lavage fluid (BALF) of $bim^{-/-}$ mice compared with $bim^{+/+}$ littermates. A: representative immunoblot showing that Bim_{EL} was detected in the lungs of $bim^{+/+}$ mice but absent in lungs of $bim^{-/-}$ mice. Western blot with 2 representative samples from $bim^{-/-}$ (knockout, KO; lanes 1 and 2) and $bim^{+/+}$ (wild-type, WT; lanes 1 and 2). Nos. of neutrophils (B), macrophages (C), lymphocytes (D), and eosinophils (E) in the BALF of $bim^{+/+}$ and $bim^{-/-}$ mice were determined in naive mice and mice exposed to allergen for 4 and 15 days. Bars = group means $\pm$ SE ($n = 11$ –13/group). *Significantly different as indicated by bars; $P < 0.05$.

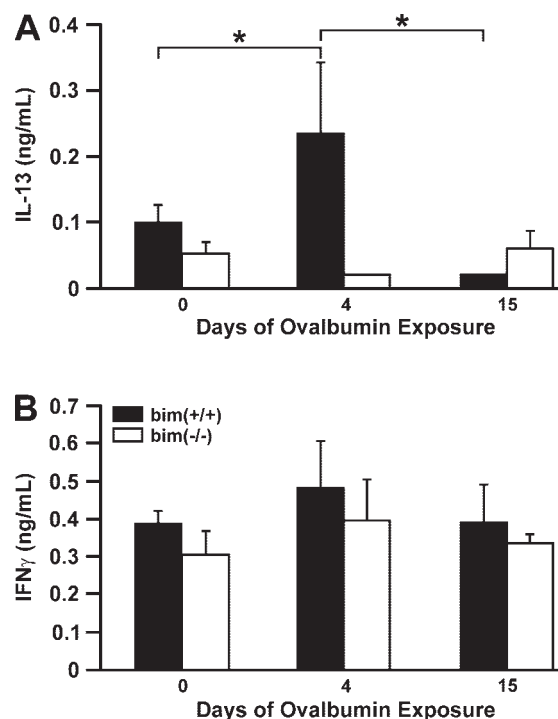


Fig. 2. In the BALF of *bim*^{+/+} mice, IL-13 levels are increased at 4 days and decreased at 15 days of allergen exposure. While statistically no different from *bim*^{+/+} mice, IL-13 levels remained unchanged in *bim*^{-/-} mice at all time points. No differences were observed for IFN γ at any time point. Mouse lungs were lavaged, and IL-13 (A) and IFN γ (B) were detected by ELISA. Bars = group means $\pm$ SE ($n = 10$ –15/group). *Significantly different as indicated by bars; $P < 0.05$.

GCM was significantly higher in *bim*^{-/-} mice compared with *bim*^{+/+} mice, despite IL-13 levels being lower in the BALF of *bim*^{-/-} compared with *bim*^{+/+} mice and *bim*^{+/+} and *bim*^{-/-} pulmonary T cells mice producing similar levels of IL-13. Therefore, we wanted to test the hypothesis that higher levels of IL-13R α 2 expression in *bim*^{+/+} mice inhibit the effect of IL-13. IL-13R α 2 functions as an inhibitor of IL-13 signaling and Stat 6-responsive gene expression, likely by functioning as a decoy receptor (14, 34). T cells showed no immunostaining for IL-13R α 2 in both *bim*^{+/+} and *bim*^{-/-} mice (not shown). However, while immunoreactivity to IL-13R α 2 was present in airway epithelia from both *bim*^{+/+} and *bim*^{-/-} mice that were devoid of GCM, little to no immunostaining was observed with IL-13R α 2 antibodies in *bim*^{-/-} mice in areas with extensive GCM (Fig. 6, I and J).

To further investigate whether increased presence of IFN γ may have affected expression of IL-13R α 2, we treated explant cultures of mouse bronchioles from WT mice with 1 ng/ml IL-13 combined with 0, 1, 10, or 50 ng/ml IFN γ . Immunohistochemical staining showed that expression of IL-13R α 2 decreased with increasing IFN γ levels in the culture medium (Fig. 7, A–D).

DISCUSSION

The present study shows that *bim*^{-/-} mice develop significantly greater GCM in response to allergen exposure compared with *bim*^{+/+} mice without any evidence of increased IL-13 levels in the BALF or synthesis from activated pulmonary T

lymphocytes. However, Bim was not required for the resolution of metaplastic mucous cells during prolonged exposure to allergen.

The number of neutrophils, macrophages, and eosinophils increased over 4 days of allergen exposure and then decreased to background levels from day 4 to day 15 of exposure in both *bim*^{+/+} and *bim*^{-/-} mice, suggesting that the death of neutrophils and eosinophils is not Bim dependent. We have previously reported that the decrease in number of eosinophils during prolonged exposure to allergen is not affected by loss of Bax (45). Others have shown that eosinophils are cleared by apoptosis through the Fas-FasL-dependent pathway (48). The death of neutrophils has been reported to be initiated by reactive oxygen species, which then activate autocrine and paracrine death receptor signaling (38). However, whereas the numbers of lymphocytes were increased $\sim$ 2-fold in *bim*^{+/+} mice from 0 to 4 days of exposure, their numbers increased by approximately $\sim$ 10-fold in *bim*^{-/-} mice during this time. This

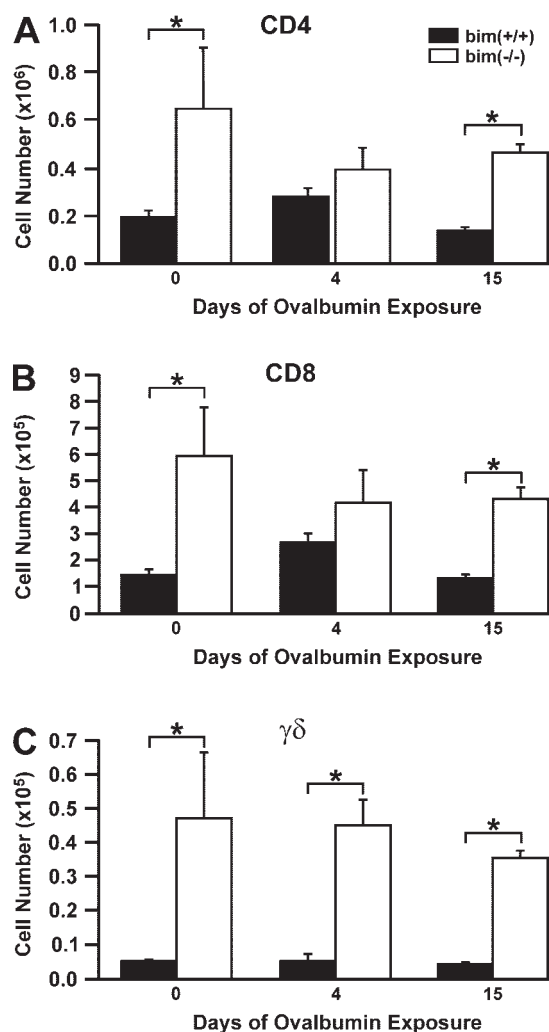


Fig. 3. Increased nos. of CD4⁺8⁻, CD4⁻8⁺, and $\gamma\delta$ T cells are found in the lungs of *bim*^{-/-} mice compared with *bim*^{+/+} littermates. Lung tissues from *bim*^{+/+} and *bim*^{-/-} mice were harvested, and T cells were isolated after digestion of the lung with collagen. Nos. of CD4⁺8⁻ (A), CD4⁻8⁺ (B), and $\gamma\delta$ (C) T cells were determined by cell counting and flow cytometry analysis. Bars = group means $\pm$ SE ($n = 3$ –6/group). *Significantly different as indicated by bars; $P < 0.05$.

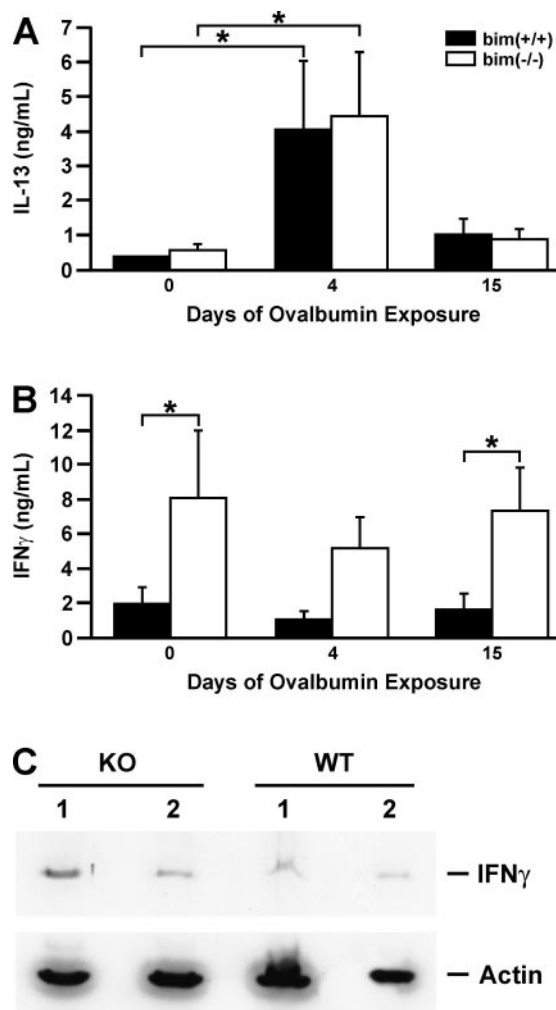


Fig. 4. T cells isolated from lungs of *bim*^{-/-} mice produce abnormally increased levels of IFN γ . Isolated pulmonary T cells were stimulated with concavalin A for 72 h at 37°C. Levels of IL-13 (A) and IFN γ (B) were determined by ELISA in cell culture supernatants. Bars = group means $\pm$ SE ($n = 3$ –6/group). *Significantly different from WT controls; $P < 0.05$. C: immunoblot showing that IFN γ levels are higher in lung extracts from *bim*^{-/-} mice compared with *bim*^{+/+} mice. Western blot with 2 representative samples from *bim*^{-/-} (KO, lanes 1 and 2) and *bim*^{+/+} (WT, lanes 1 and 2).

observation suggests that at least some T cells may undergo a Bim-dependent apoptosis in WT mice during early time points of allergen exposure. However, this increase in T cell numbers may also be due to increased numbers of precursor T cells recruited to the lung or altered T-cell proliferation in *bim*^{-/-} mice. Furthermore, whereas T-cell numbers in the BALF decreased from 4 to 15 days in *bim*^{+/+} mice, their numbers remained at high levels in *bim*^{-/-} mice, suggesting that Bim is essential for the removal of T cells during prolonged allergen exposures.

Bim can be present in at least three isoforms, Bim_S, Bim_L, and Bim_{EL}, generated by alternative splicing (28, 30). Some studies have found numerous additional isoforms, all generated by alternative splicing (1, 26, 49). However, only the Bim_{EL} form was detected in the lungs of allergen-exposed *bim*^{+/+} mice; the Bim_L, Bim_S, or other isoform was either present at below detection levels or absent. The antibody used detected

all three isoforms, Bim_{EL}, Bim_L, and Bim_S, in lung extracts from rats (data not shown).

As expected, *bim*^{-/-} mice had increased levels of pulmonary CD4⁺8⁺, CD4⁺8⁺, and $\gamma\delta$ T cells. This finding is in agreement with previous reports demonstrating that both the CD4⁺8⁺ pro-T cells and the mature T cells (CD4⁺8⁺ and CD4⁺8⁺) were two- to threefold higher in *bim*^{-/-} compared with *bim*^{+/+} littermates (3). Bim is required for deletion of autoreactive thymocytes (4) as well as mature CD4⁺8⁺ T cells (7) and of T-cell receptor/CD3-induced killing of semimature (CD4⁺8⁺HSA⁺) thymocytes (51). In addition, Bim was shown to be required for the shut down of T-cell immune responses in vivo (20, 31).

No differences in IFN γ levels were observed in the BALF of *bim*^{+/+} and *bim*^{-/-} mice, but T cells isolated from the lung tissues of both naive and allergen-exposed *bim*^{-/-} mice had a capacity to produce approximately fourfold more IFN γ compared with those from *bim*^{+/+} mice. Furthermore, immunoblotting of lung extracts showed that IFN γ levels are higher in *bim*^{+/+} compared with *bim*^{-/-} lungs. These results suggest that Bim deficiency preferentially sustains $\gamma\delta$ T cells that are known to produce IFN γ , indicating that *bim*^{-/-} T cells are biased toward Th1 cytokine production and that most of the IFN γ in the BALF may be derived from cell types other than T cells, natural killer cells being likely candidates.

The numbers of airway epithelial cells did not differ significantly between *bim*^{+/+} and *bim*^{-/-} mice; however, exposure to allergen did increase the numbers of total epithelial cells per millimeter BL at 4 days of allergen exposure in both sets of animals. No increase in epithelial cells was observed after a single antigen challenge in BALB/c mice (13). Reader et al. (36) reported that after three allergen challenges, each 3 days apart, 10% of mucous cells proliferated. In those studies, the increase in the numbers of mucous cells was accompanied by a compensatory decrease in Clara and ciliated cell numbers (36). The increase in total cell number observed in our study may be due to the repeated daily challenge with allergen for 6 h/day over 4 days, which may cause significant injury to the lung.

While exposure to allergen for 15 days did not completely reverse this increase, the reduction of airway epithelial cells from 4 to 15 days was similar in *bim*^{+/+} and *bim*^{-/-} mice, suggesting that the absence of Bim has no effect on the resolution of airway epithelial cell hyperplasia in allergen-

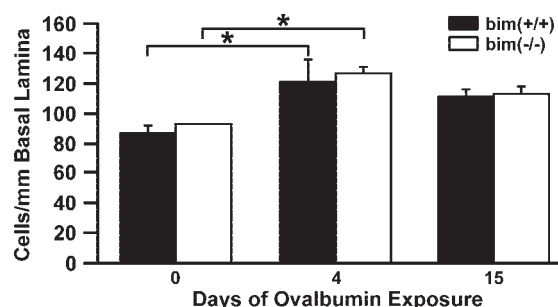


Fig. 5. No differences are observed in no. of total epithelial cells per mm basal lamina (BL) in naive and allergen-exposed *bim*^{+/+} and *bim*^{-/-} mice. Lung tissue sections were stained with Alcian blue/hematoxylin and eosin, and no. of nuclei/mm BL was counted in the axial airways. Bars = group means $\pm$ SE ($n = 4$ –6/group). *Significantly different as indicated by bars; $P < 0.05$.

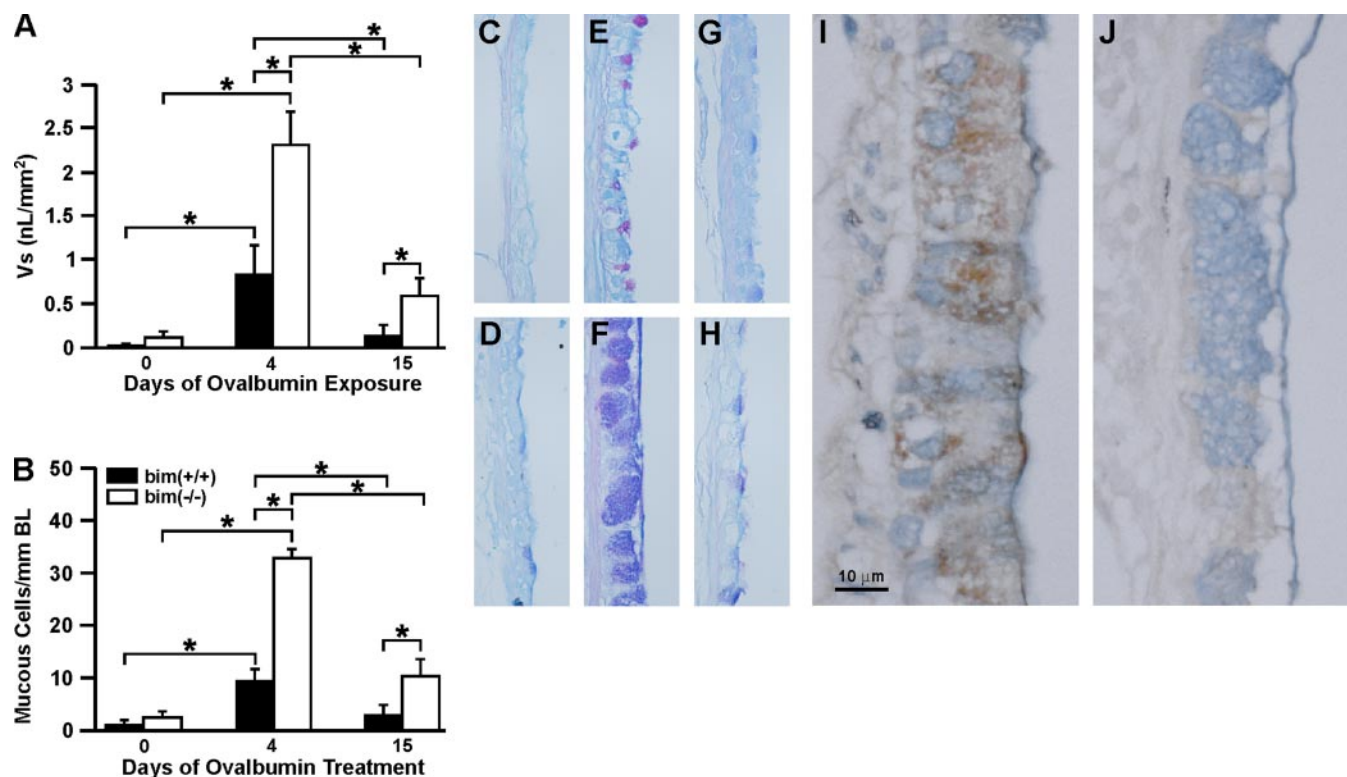


Fig. 6. In *bim*^{-/-} mice, intraepithelial-stored mucosubstances and the nos. of mucous cells/mm BL are increased during the first 4 days of allergen challenge, but they decline at a rate similar to that seen in *bim*^{+/+} mice. Tissue sections prepared from the lungs of *bim*^{+/+} and *bim*^{-/-} mice were stained with Alcian blue and periodic acid-Schiff (AB/PAS) for quantification of volume of mucus per unit area of basement membrane (Vs; A) and counting the nos. of mucous cells/mm BL (B). Bars = group means $\pm$ SE ($n = 4-6$ /group). *Significantly different as indicated by bars; $P < 0.05$. Representative photomicrographs show goblet cell metaplasia (GCM) in naive mice (C and D) and after 4 days (E and F) and 15 days (G and H) of allergen exposure in *bim*^{+/+} (C, E, and G) and *bim*^{-/-} (D, F, and H) mice. Tissue sections from *bim*^{+/+} (I) and *bim*^{-/-} (J) mice were immunostained for IL-13Rα2 and AB to detect mucous cells.

exposed mice. A previous study reported low to moderate levels of Bim expression in ciliated epithelia of the trachea and bronchi, but no Bim was found in the alveoli of the lung (30). In this study, we were able to detect Bim in macrophages but not in airway epithelial cells (data not shown). The absence of Bim in epithelial cells is consistent with the observation that Bim deficiency had no effect on the resolution of airway epithelial cells after allergen exposure.

As previously described (41), GCM was significantly increased in WT mice at 4 days and significantly decreased from 4 to 15 days of allergen exposure. Both quantification of intraepithelial-stored mucosubstances (Vs) and the number of

goblet cells per millimeter BL showed similar results, indicating that increases were due to GCM and not only enlargement of existing mucous cells. The question of whether increased number of T cells together with increased GCM in *bim*^{-/-} mice will result in more severe airway hyperresponsiveness could be studied in the future. Interestingly, the number of goblet cells and the total epithelial cell numbers increased by ~ 30 in *bim*^{-/-} mice at 4 days of allergen challenge, suggesting that all proliferating cells differentiated into mucus-producing cells. Similarly, we have previously shown that proliferating cells differentiate into mucous cells after an intratracheal instillation of rats with LPS (47).

In general, immunoreactivity for IL-13Rα2 was dramatically reduced or absent in epithelia of *bim*^{-/-} mice, where GCM was extensive compared with what was observed in *bim*^{+/+} mice. IL-13Rα2 functions as an inhibitor of IL-13 signaling and Stat 6-responsive gene expression, likely by functioning as a decoy receptor (14, 34). Both IL-13α1 and IL-13Rα2 receptors are expressed in lung epithelial cells (8, 55). However, because IL-13Rα2 binds IL-13 with 50- to 100-fold higher affinity than IL-13Rα1 (8), it is possible that, despite similar IL-13 levels in *bim*^{+/+} and *bim*^{-/-} mice, reduced expression of IL-13Rα2 allows IL-13 to cause increased GCM in *bim*^{-/-} mice.

The abnormal increase in the numbers of mucous cells in *bim*^{-/-} mice at 4 days of allergen challenge may result from the presence of abnormally increased levels of IFNγ, which

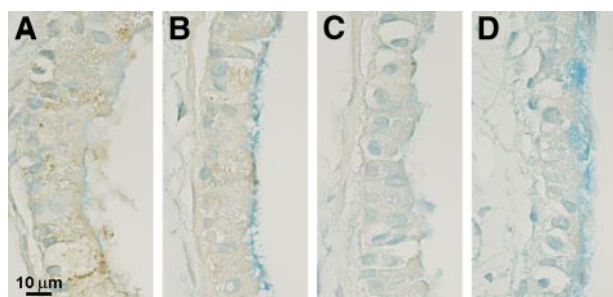


Fig. 7. IL-13Rα2 levels are decreased in IL-13-treated bronchiolar explant cultures treated with increasing levels of IFNγ treatment. Explant cultures were maintained in air-liquid interface cultures and treated with IL-13 at 1 ng/ml together with 0 (A), 1 (B), 10 (C), or 50 (D) ng/ml IFNγ for 7 days. Tissue sections were immunostained with antibodies to IL-13Rα2.

may enhance GCM induced by the Th2 cytokine IL-13, which is the main cytokine to induce GCM in airway epithelia (23, 53, 56, 57). This hypothesis is supported by our finding that expression of IL-13R α 2 was reduced in IL-13-treated organ cultures when explants were co-treated with increasing concentrations of IFN γ . The reduction of IL-13R α 2 expression suggests that, in *bim*^{-/-} mice, high IFN γ levels may have downregulated expression of this decoy receptor, thereby enhancing the effect of IL-13 to induce increased GCM. Zheng et al. (55) have reported that IL-13R α 2 levels are increased in both IL-13- and IFN γ -overexpressing transgenic mice. However, they did not study the effect of combined treatment with IL-13 and IFN γ over a long period, as our study did. We have previously reported that IFN γ suppresses allergen-induced GCM (41). Such a double-sided effect of IFN γ (inhibiting some, potentiating others) on IL-13-induced changes in the lungs has been reported by various groups (15, 17, 35).

The mechanism underlying the development of tolerance after prolonged exposure to allergen has been reported to be both dependent (27) and independent (39, 40) of IFN γ and $\gamma\delta$ T cells. Results from our studies suggest that, while the deletion of pulmonary T cells is Bim dependent, the mechanisms involved in the death of dendritic and regulatory T cells (37) that may be responsible for inhibiting prolonged Th2 responses are probably independent of Bim.

ACKNOWLEDGMENTS

We thank Yoneko Knighton for preparing tissue samples for light microscopic analyses and Dr. James Aden for carrying out the statistical analyses.

GRANTS

This research was supported by grants from National Institute of Environmental Health Sciences (NIEHS; ES-09237) and National Heart, Lung, and Blood Institute (HL-68111) and by the NIEHS Center (P30-ES-012072), the National Health and Medical Research Council (NHMRC, Canberra, Australia), and the Leukemia and Lymphoma Society of America (SCOR Grant).

REFERENCES

- Adachi M, Zhao X, and Imai K. Nomenclature of dynein light chain-linked BH3-only protein Bim isoforms. *Cell Death Differ* 12: 192–193, 2005.
- Akbari O, Freeman GJ, Meyer EH, Greenfield EA, Chang TT, Sharpe AH, Berry G, DeKruyff RH, and Umetsu DT. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperactivity. *Nat Med* 8: 1024–1032, 2002.
- Bouillet P, Metcalf D, Huang DC, Tarlinton DM, Kay TW, Kontgen F, Adams JM, and Strasser A. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* 286: 1735–1738, 1999.
- Bouillet P, Purton JF, Godfrey DI, Zhang LC, Coultas L, Puthalakath H, Pellegrini M, Cory S, Adams JM, and Strasser A. BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. *Nature* 415: 922–926, 2002.
- Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T, and Korsmeyer SJ. BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol Cell* 8: 705–711, 2001.
- Cui ZH, Joetham A, Aydinug MK, Hahn YS, Born WK, and Gelfand EW. Reversal of allergic airway hyperactivity after long-term allergen challenge depends on gammadelta T cells. *Am J Respir Crit Care Med* 168: 1324–1332, 2003.
- Davey GM, Kurts C, Miller JF, Bouillet P, Strasser A, Brooks AG, Carbone FR, and Heath WR. Peripheral deletion of autoreactive CD8 T cells by cross presentation of self-antigen occurs by a Bcl-2-inhibitable pathway mediated by Bim. *J Exp Med* 196: 947–955, 2002.
- Donaldson DD, Whitters MJ, Fitz LJ, Neben TY, Finnerty H, Henderson SL, O'Hara RM Jr, Beier DR, Turner KJ, Wood CR, and Collins M. The murine IL-13 receptor alpha 2: molecular cloning, characterization, and comparison with murine IL-13 receptor alpha 1. *J Immunol* 161: 2317–2324, 1998.
- Elias JA, Zhu Z, Chupp G, and Homer RJ. Airway remodeling in asthma. *J Clin Invest* 104: 1001–1006, 1999.
- El-Zimaity HM, Ota H, Scott S, Killen DE, and Graham DY. A new triple stain for *Helicobacter pylori* suitable for the autostainer: carbol fuchsin/Alcian blue/hematoxylin-eosin. *Arch Pathol Lab Med* 122: 732–736, 1998.
- Enders A, Bouillet P, Puthalakath H, Xu Y, Tarlinton DM, and Strasser A. Loss of the pro-apoptotic BH3-only Bcl-2 family member Bim inhibits BCR stimulation-induced apoptosis and deletion of autoreactive B cells. *J Exp Med* 198: 1119–1126, 2003.
- Essafi A, Fernandez de Mattos S, Hassen YA, Soeiro I, Mufti GJ, Thomas NS, Medema RH, and Lam EW. Direct transcriptional regulation of Bim by FoxO3a mediates STI571-induced apoptosis in Bcr-Abl-expressing cells. *Oncogene* 24: 2317–2329, 2005.
- Evans CM, Williams OW, Tuvim MJ, Nigam R, Mixides GP, Blackburn MR, DeMayo FJ, Burns AR, Smith C, Reynolds SD, Stripp BR, and Dickey BF. Mucin is produced by clara cells in the proximal airways of antigen-challenged mice. *Am J Respir Cell Mol Biol* 31: 382–394, 2004.
- Feng N, Lugli SM, Schnyder B, Gauchat JF, Graber P, Schlagenhauf E, Schnarr B, Wiederkehr-Adam M, Duschl A, Heim MH, Lutz RA, and Moser R. The interleukin-4/interleukin-13 receptor of human synovial fibroblasts: overexpression of the non-signaling interleukin-13 receptor alpha2. *Lab Invest* 78: 591–602, 1998.
- Ford JG, Rennick D, Donaldson DD, Venkayya R, McArthur C, Hansell E, Kurup VP, Warnock M, and Grunig G. IL-13 and IFN-gamma: interactions in lung inflammation. *J Immunol* 167: 1769–1777, 2001.
- Haczku A, Moqbel R, Elwood W, Sun J, Kay AB, Barnes PJ, and Chung KF. Effects of prolonged repeated exposure to ovalbumin in sensitized Brown Norway rats. *Am J Respir Crit Care Med* 150: 23–27, 1994.
- Hansen G, Berry G, DeKruyff RH, and Umetsu DT. Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperactivity but cause severe airway inflammation. *J Clin Invest* 103: 175–183, 1999.
- Harkema JR and Hotchkiss JA. In vivo effects of endotoxin on nasal epithelial mucosubstances: quantitative histochemistry. *Exp Lung Res* 17: 743–761, 1991.
- Harkema JR and Hotchkiss JA. Ozone-induced proliferative and metaplastic lesions in nasal transitional and respiratory epithelium: comparative pathology. *Inhal Toxicol* 6: 187–204, 1994.
- Hildeman DA, Zhu Y, Mitchell TC, Bouillet P, Strasser A, Kappler J, and Marrack P. Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim. *Immunity* 16: 759–767, 2002.
- Huang DC and Strasser A. BH3-only proteins-essential initiators of apoptotic cell death. *Cell* 103: 839–842, 2000.
- Kips JC, Cuvelier CA, and Pauwels RA. Effect of acute and chronic antigen inhalation on airway morphology and responsiveness in actively sensitized rats. *Am Rev Respir Dis* 145: 1306–1310, 1992.
- Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, Elias JA, Sheppard D, and Erle DJ. Direct effects of interleukin-13 on epithelial cells cause airway hyperactivity and mucus overproduction in asthma. *Nat Med* 8: 885–889, 2002.
- Ley R, Hadfield K, Howes E, and Cook SJ. Identification of a DEF-type docking domain for extracellular signal-regulated kinases 1/2 that directs phosphorylation and turnover of the BH3-only protein BimEL. *J Biol Chem* 280: 17657–17663, 2005.
- Lovchik JA, Wilder JA, Huffnagle GB, Riblet R, Lyons CR, and Lipscomb MF. Ig heavy chain complex-linked genes influence the immune response in a murine cryptococcal infection. *J Immunol* 163: 3907–3913, 1999.
- Marani M, Tenev T, Hancock D, Downward J, and Lemoine NR. Identification of novel isoforms of the BH3 domain protein Bim which directly activate Bax to trigger apoptosis. *Mol Cell Biol* 22: 3577–3589, 2002.
- McMenamin C, McKersy M, Kuhnlein P, Hunig T, and Holt PG. Gamma delta T cells down-regulate primary IgE responses in rats to inhaled soluble protein antigens. *J Immunol* 154: 4390–4394, 1995.
- O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S, and Huang DC. Bim: a novel member of the Bcl-2 family that promotes apoptosis. *EMBO J* 17: 384–395, 1998.

29. Opferman JT and Korsmeyer SJ. Apoptosis in the development and maintenance of the immune system. *Nat Immun* 4: 410–415, 2003.
30. O'Reilly LA, Cullen L, Visvader J, Lindeman GJ, Print C, Bath ML, Huang DC, and Strasser A. The proapoptotic BH3-only protein bim is expressed in hematopoietic, epithelial, neuronal, and germ cells. *Am J Pathol* 157: 449–461, 2000.
31. Pellegrini M, Bouillet P, Robati M, Belz GT, Davey GM, and Strasser A. Loss of Bim increases T cell production and function in interleukin 7 receptor-deficient mice. *J Exp Med* 200: 1189–1195, 2004.
32. Puthalakath H, Huang DC, O'Reilly LA, King SM, and Strasser A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol Cell* 3: 287–296, 1999.
33. Puthalakath H and Strasser A. Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. *Cell Death Differ* 9: 505–512, 2002.
34. Rahaman SO, Sharma P, Harbor PC, Aman MJ, Vogelbaum MA, and Haque SJ. IL-13R(alpha)2, a decoy receptor for IL-13 acts as an inhibitor of IL-4-dependent signal transduction in glioblastoma cells. *Cancer Res* 62: 1103–1109, 2002.
35. Randolph DA, Stephens R, Carruthers CJ, and Chaplin DD. Cooperation between Th1 and Th2 cells in a murine model of eosinophilic airway inflammation. *J Clin Invest* 104: 1021–1029, 1999.
36. Reader JR, Tepper JS, Schelegle ES, Aldrich MC, Putney LF, Pfeiffer JW, and Hyde DM. Pathogenesis of mucous cell metaplasia in a murine asthma model. *Am J Pathol* 162: 2069–2078, 2003.
37. Rissoan MC, Soumelis V, Kadowaki N, Grouard G, Briere F, de Waal Malefyt R, and Liu YJ. Reciprocal control of T helper cell and dendritic cell differentiation. *Science* 283: 1183–1186, 1999.
38. Scheel-Toellner D, Wang K, Craddock R, Webb PR, McGettrick HM, Assi LK, Parkes N, Clough LE, Gulbins E, Salmon M, and Lord JM. Reactive oxygen species limit neutrophil life span by activating death receptor signaling. *Blood* 104: 2557–2564, 2004.
39. Schramm CM, Puddington L, Wu C, Guernsey L, Gharaee-Kermani M, Phan SH, and Thrall RS. Chronic inhaled ovalbumin exposure induces antigen-dependent but not antigen-specific inhalational tolerance in a murine model of allergic airway disease. *Am J Pathol* 164: 295–304, 2004.
40. Seymour BW, Gershwin LJ, and Coffman RL. Aerosol-induced immunoglobulin (Ig)-E unresponsiveness to ovalbumin does not require CD8+ or T cell receptor (TCR)-gamma/delta+ T cells or interferon (IFN)-gamma in a murine model of allergen sensitization. *J Exp Med* 187: 721–731, 1998.
41. Shi ZQ, Fischer MJ, De Sanctis GT, Schuyler M, and Tesfagzi Y. IFN γ but not Fas mediates reduction of allergen-induced mucous cell metaplasia by inducing apoptosis. *J Immunol* 168: 4764–4771, 2002.
42. Spicer SS, Chakrin LW, Wardell JR Jr, and Kendrick W. Histochemistry of mucosubstances in the canine and human respiratory tract. *Lab Invest* 25: 483–490, 1971.
43. Stock P, Akbari O, Berry G, Freeman GJ, Dekruyff RH, and Umetsu DT. Induction of T helper type 1-like regulatory cells that express Foxp3 and protect against airway hyper-reactivity. *Nat Immun* 5: 1149–1156, 2004.
44. Sunter A, Fernandez de Mattos S, Stahl M, Brosens JJ, Zoumpoulidou G, Saunders CA, Coffey PJ, Medema RH, Coombs RC, and Lam EW. FoxO3a transcriptional regulation of Bim controls apoptosis in paclitaxel-treated breast cancer cell lines. *J Biol Chem* 278: 49795–49805, 2003.
45. Tesfagzi Y, Fischer MJ, Green FHY, De Sanctis GT, and Wilder JA. Bax is crucial for IFN γ -induced resolution of allergen-induced mucous cell metaplasia. *J Immunol* 169: 5919–5925, 2002.
46. Tesfagzi Y, Fischer MJ, Martin AJ, and Seagrave J. Bcl-2 in LPS- and allergen-induced hyperplastic mucous cells in airway epithelia of Brown Norway rats. *Am J Physiol Lung Cell Mol Physiol* 279: L1210–L1217, 2000.
47. Tesfagzi Y, Harris JF, Hotchkiss JA, and Harkema JR. DNA synthesis and Bcl-2 expression during the development of mucous cell metaplasia in airway epithelium of rats exposed to LPS. *Am J Physiol Lung Cell Mol Physiol* 286: L268–L274, 2004.
48. Tsuyuki S, Bertrand C, Erard F, Trifileff A, Tsuyuki J, Wesp M, Anderson GP, and Coyle AJ. Activation of the Fas receptor on lung eosinophils leads to apoptosis and the resolution of eosinophilic inflammation of the airways. *J Clin Invest* 96: 2924–2931, 1995.
49. U M, Miyashita T, Shikama Y, Tadokoro K, and Yamada M. Molecular cloning and characterization of six novel isoforms of human Bim, a member of the proapoptotic Bcl-2 family. *FEBS Lett* 509: 135–141, 2001.
50. Van Winkle LS, Buckpitt AR, and Plopper CG. Maintenance of differentiated murine Clara cells in microdissected airway cultures. *Am J Respir Cell Mol Biol* 14: 586–598, 1996.
51. Villunger A, Scott C, Bouillet P, and Strasser A. Essential role for the BH3-only protein Bim but redundant roles for Bax, Bcl-2, and Bcl-w in the control of granulocyte survival. *Blood* 101: 2393–2400, 2003.
52. Watanabe A, Mishima H, Renzi PM, Xu LJ, Hamid Q, and Martin JG. Transfer of allergic airway responses with antigen-primed CD4+ but not CD8+ T cells in brown Norway rats. *J Clin Invest* 96: 1303–1310, 1995.
53. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, and Donaldson DD. Interleukin-13: central mediator of allergic asthma. *Science* 282: 2258–2261, 1998.
54. Yiamouyiannis CA, Schramm CM, Puddington L, Stengel P, Baradaran-Hosseini E, Wolyniec WW, Whiteley HE, and Thrall RS. Shifts in lung lymphocyte profiles correlate with the sequential development of acute allergic and chronic tolerant stages in a murine asthma model. *Am J Pathol* 154: 1911–1921, 1999.
55. Zheng T, Zhu Z, Liu W, Lee CG, Chen Q, Homer RJ, and Elias JA. Cytokine regulation of IL-13Ralpha2 and IL-13Ralpha1 in vivo and in vitro. *J Allergy Clin Immunol* 111: 720–728, 2003.
56. Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, Zhang Y, and Elias JA. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* 103: 779–788, 1999.
57. Zuhdi Alimam M, Piazza FM, Selby DM, Letwin N, Huang L, and Rose MC. Muc-5/5ac mucin messenger RNA and protein expression is a marker of goblet cell metaplasia in murine airways. *Am J Respir Cell Mol Biol* 22: 253–260, 2000.