

A Role for Bid in Eosinophil Apoptosis and in Allergic Airway Reaction¹

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Bid, a proapoptotic member of Bcl-2 family, is involved in Fas receptor signaling. Fas activation promotes human eosinophil cell death and is believed to accelerate the resolution of pulmonary Th2-driven allergic reaction in mice. We hypothesized that Bid would regulate eosinophil apoptosis and Ag-induced airway inflammation, particularly eosinophilia. C57BL/6 Bid^{-/-} and wild-type mice were immunized and repeatedly challenged with OVA, and bronchoalveolar lavage (BAL) fluid, lung, and spleen were collected 4–240 h after the final challenge. Cultured BAL eosinophils from Bid-deficient mice showed resistance to Fas-mediated apoptotic DNA fragmentation, phosphatidylserine exposure, mitochondria depolarization, and caspase-3 activity. In addition, OVA-challenged Bid^{-/-} mice had higher BAL eosinophilia and a lower proportion of BAL apoptotic eosinophils than Bid^{+/+} mice. This was accompanied by augmented BAL levels of the eosinophil-tactic cytokine, IL-5, and of the eosinophil-associated mediators, TGF- β 1 and fibronectin. Finally, cultured OVA-stimulated lung mononuclear cells and splenocytes from Bid-deficient mice showed increased release of the Th2-type cytokines, IL-4 and IL-5, but no change in cell number. We conclude that Bid modulates BAL eosinophilia by regulating both eosinophil apoptosis and Th2-type cytokine production. *The Journal of Immunology*, 2009, 182: 5740–5747.

Asthma is a chronic inflammatory disease associated with predominant Th2 response, IgE synthesis, airway infiltration by inflammatory cells, particularly eosinophils, and bronchial remodeling (1, 2). In particular, activated eosinophils play a major effector role in asthma pathogenesis by releasing proinflammatory lipid mediators, cytokines, cytotoxic cationic proteins such as eosinophil peroxidase (EPO),⁵ and fibrogenic growth factors including TGF- β 1. Eosinophil-derived mediators entertain airway inflammation and promote tissue injury and features of airway remodeling, such as subepithelial fibrosis and mucus overproduction (3–8).

Increased survival and decreased apoptotic death are believed to participate in the accumulation and the persistence of eosinophils in the asthmatic airways (9–12). Moreover, the rate of apoptosis of cul-

tured lung eosinophils correlates with the mouse strain-dependent resistance to develop asthma-related responses (13). Accordingly, in vivo maneuvers aimed at directly promoting apoptosis within the airways, such as Fas receptor ligation or glucocorticosteroid administration, have been shown to facilitate the resolution of eosinophilic inflammation (14–18). In addition, Fas deficiency delays the resolution of airway inflammation in mouse models of asthma (19, 20).

Of note, Fas triggering and glucocorticosteroids reduce eosinophilic inflammation by acting directly or indirectly on eosinophils. For instance, in addition to inducing eosinophil cell death, glucocorticosteroid also inhibit the release of chemoattractant cytokines and survival factors by lymphocytes and enhance the phagocytic potential of macrophages and epithelial cells (21). Concerning Fas activation, it has been postulated that direct engagement of Fas on infiltrating eosinophils is involved in their removal from inflamed tissues because eosinophils are highly sensitive to Fas-mediated apoptosis (12, 19, 22). It has also been reported that Fas-positive lymphocytes are essential in the resolution of airway inflammation in a murine model of asthma (20).

Fas ligation induces two main intracellular apoptotic pathways (23). Indeed, in type I cells, Fas triggering leads to the recruitment to its intracellular domain of the protease caspase-8 and to caspase-8 activation. This is followed by the direct cleavage of the downstream caspase-3 that in turn hydrolyzes different substrates responsible for DNA fragmentation and phosphatidylserine exposure. In type II cells, activated caspase-8 cleaves a proapoptotic member of Bcl-2 family, namely Bid, that translocates to mitochondria where it provokes membrane depolarization and cytochrome *c* release into the cytoplasm. Cytochrome *c* activates caspase-9 that in turn cleaves caspase-3. The signaling pathway engaged in Fas-mediated eosinophil apoptosis has been partly elucidated (12). Hence, incubation of human eosinophils with activator anti-Fas Ab induces caspase-8 and -3 activation, mitochondria depolarization, and Bid cleavage, suggesting that eosinophils are type II cells (24, 25).

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⁵ Abbreviations used in this paper: EPO, eosinophil peroxidase; WT, wild type; BAL, bronchoalveolar lavage; PI, propidium iodide; DioC₆(3), 3,3'-dihexyloxycarbocyanine; DEVDfmk, aspartate glutamate valine aspartate fluoromethylketone.

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Together, these findings led us to propose the hypothesis that Bid would be an important endogenous modulator of eosinophil apoptosis and eosinophilic inflammation in asthma. The present study was therefore aimed at determining whether a deficiency in Bid expression would alter the onset and the resolution of pulmonary allergic reaction via an increased apoptotic death of eosinophils. To address this question, we examined the role of Bid in apoptotic cell death of cultured lung-derived eosinophils and in eosinophilic inflammation, Th2 responses, IgE synthesis, and airway remodeling in a model of OVA-mediated pulmonary allergic reaction in Bid^{-/-} mice.

Materials and Methods

Mice immunization and challenges

C57BL/6 Bid^{-/-} mice (26) were a gift from Dr. S. J. Korsmeyer (Dana-Farber Cancer Institute, Harvard Medical School, Cambridge, MA). Male Bid^{-/-} aged 8 wk and age- and sex-matched C57BL/6 wild-type (WT) mice (Centre d'Élevage R. Janvier, Le Genest-St-Isle, France) were sensitized by two i.p. injections, 1 wk apart, of 0.9% NaCl (saline) containing 10 µg OVA adsorbed in aluminum hydroxide (27). Seven days after the second immunization, mice were challenged four times, at 24-h intervals, by intranasal instillations of 20 µg OVA in saline (OVA mice). Control mice received the same volume of saline (Saline mice). Mice were used 4–240 h after the final saline or OVA challenge. Animal procedures were conducted according to policies of the French Department of Agriculture.

Tissue collection and processing

Mice were terminally anesthetized by an i.p. injection of 1.4 g/kg ethyl-carbamate, and blood was collected from the abdominal aorta. Serum was stored at -80°C until IgE level quantitation.

Bronchoalveolar lavage (BAL) fluid was harvested by injecting and recovering four × 0.5 ml saline through a tracheal catheter. Total cell number was evaluated using a hemocytometer and BAL cells, spun down on a slide and stained, were differentially counted (27). BAL fluid was centrifuged (5 min, 4°C, 400 × g), and aliquots of supernatant were stored at -80°C until cytokine measurements. Cell pellet was resuspended in culture medium and immediately processed for cell culture.

Lungs were thoroughly washed through the pulmonary vasculature by injecting 10 ml saline in the right ventricle. The left lung lobe was homogenized with Ultrathurax (IKA-Labortechnik) in 2 ml of cold 0.05 M Tris (pH 8) and 0.1% Triton X-100, centrifuged (15 min, 4°C, 10,000 × g) and the supernatant was immediately used for EPO activity determination. Alternatively, the left lobe was incubated overnight at room temperature in PBS containing 4% paraformaldehyde, embedded in paraffin, and sliced in 5-µm sections that were stored at 4°C until histological stainings. In some experiments, saline-washed whole lungs were inflated with 1 ml of a solution of PBS/optimal cutting temperature compound (v/v), the main left bronchus was ligatured, and the left lobe was removed. It was covered by optimal cutting temperature compound, frozen, and sliced in 5-µm sections that were fixed in acetone, air dried, and stored at -20°C until EPO staining.

Finally, saline-washed whole lungs, thymus, and spleen were collected, immersed in culture medium, and immediately processed for cell isolation and culture.

BAL cell, thymocyte, lung mononuclear cell, and splenocyte preparation and culture

BAL cells, thymus, whole lungs, and spleen were collected 24–120 h after the final OVA challenge. BAL cells (75 ± 2% eosinophils, 23 ± 2% mononuclear cells in which 3/4 are macrophages, *n* = 34) were resuspended at 1 × 10⁶ cells/ml in basal culture medium, i.e., RPMI 1640 containing 10% FCS. Cells were incubated for 12–18 h in the presence of 1–100 ng/ml of the activator anti-mouse Fas mAb (clone Jo-2; BD Biosciences), or 100 ng/ml control isotype, with or without 50 µM Z valine alanine aspartate fluoromethylketone (Calbiochem-VWR International), or its vehicle, i.e., 0.1% DMSO (15, 24). Cells were also treated for 18–36 h with the medium alone, or supplemented with 1 µM dexamethasone (12). Expression of Bid and cell death were then analyzed.

Thymus was dispersed using a Dounce glass homogenizer, and erythrocytes were lysed using the Mouse Erythrocyte Lysing kit (R&D Systems). Thymocytes were isolated by centrifugation (30 min, 20°C, 800 ×

g) in the Mouse Nycoprep 1.077A separation medium (Axis Shield), and resuspended at 1.875 × 10⁶ cells/ml in basal medium supplemented with 2-ME. Cells were incubated for 18 h with a combination (26) of 10 µg/ml cycloheximide and 10–100 ng/ml activator anti-Fas mAb or 100 ng/ml control isotype, and apoptotic cells were quantified.

Lung was digested with 2 mg/ml collagenase (Worthington Biochemical Corporation) and 0.02 mg/ml DNase, and cells were filtered through a 40 µm cell strainer (28). In parallel, spleen was dispersed as described for thymus, and erythrocytes were lysed. Lung mononuclear cells and splenocytes were further isolated in a 1.077A separation medium. Cells (1.875 × 10⁶ cells/ml) in basal medium containing 2-ME were incubated for 72 h (for cytokine determination and cell proliferation evaluation) or 11 days (for total IgE measurement) with or without 1 mg/ml OVA (13, 29).

Assessment of Bid expression and cleavage in BAL cells

Cultured BAL cells were lysed as described (24) and the presence of Bid in protein extracts was evaluated by Western blot using an anti-mouse Bid mAb (clone 91508, R&D Systems). This Ab recognizes full length Bid (22 kDa) and its active cleavage product (16 kDa). The expression of Bid was normalized using β-actin (24).

Evaluation of apoptosis in cultured cells and in freshly collected BAL fluids

Cultured BAL cells and thymocytes were permeabilized and then stained with propidium iodide (PI), and the percentage of apoptotic, i.e., hypodiploid, cells was evaluated by flow cytometry (24).

Cultured BAL cells were also stained using the FITC-labeled annexin V/PI kit from Beckman Coulter. The percentages of dead, i.e., PI permeable, cells and of cells with exposed phosphatidylserines were calculated as the ratios of PI-positive cell number to total cell number, and of PI-negative and annexin V-positive cell number to overall PI-negative cell number, respectively.

Cultured BAL cells were stained with 3,3'-dihexyloxycarbocyanine iodide (DiOC₆(3); Molecular Probes-Invitrogen) and the proportion of low DiOC₆(3)-stained cell, i.e., cells with depolarized mitochondria, was calculated (24).

Caspase-3 activity in cultured BAL cells was quantified using the Caspase-3 Activity Detection kit (Calbiochem-VWR), based on the binding to active caspase-3 of the FITC-labeled caspase-3 inhibitor aspartate glutamate valine aspartate fluoromethylketone (DEVDfmk). Five microliters PI, at 250 µg/ml, were added to 500 µl cell suspension immediately before flow cytometry analysis, and the proportion of active caspase-3 containing, i.e., DEVDfmk-FITC positive, cells was determined within the population of PI-negative cells.

All the apoptotic features above-mentioned were analyzed by flow cytometry in BAL cells, after gating the population of eosinophils in the forward scatter vs side scatter plot.

Apoptotic eosinophils were also counted in BAL fluids previously spun down on slides and stained with Diff-Quick dye (IMEB). Apoptotic eosinophils were defined as cells showing shrinkage of their eosin-stained cytoplasm and condensed chromatin and were enumerated over a total number >1000 BAL cells per cytospin.

Determination of the number of viable splenocytes (30)

Splenocyte-containing plates were centrifuged (5 min, 4°C, 400 × g). The supernatants were stored at -80°C until cytokine measurements. Cell pellets were resuspended in medium supplemented with 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (Sigma-Aldrich) and incubated for 4 h at 37°C in a 5% CO₂ humidified atmosphere. Half of the supernatant was replaced by isopropanol containing 0.04 mol/l HCl. After vigorous stirring, OD was read at 550 nm and the number of viable cells was calculated of ODs measured in serial dilutions of freshly isolated splenocytes.

Measurement of IgE, IL-4, IL-5, TGF-β1, and fibronectin

The levels of serum OVA-specific IgEs and of total IgEs in splenocyte supernatants were measured by specific ELISAs (29, 31), using an anti-mouse IgE mAb (clone R35-72; BD Biosciences). IL-4 was measured by a specific ELISA (27) using mouse rIL-4 (R&D Systems) to generate standard curves. IL-5 and TGF-β1 were assayed using Quantikine mouse kits (R&D Systems). Sensitivities were of 7.5, 15.6, and 31.2 pg/ml for IL-4, IL-5, and TGF-β1, respectively.

Fibronectin was assessed by enzyme immunometric assay (32) with sensitivity of 0.5 µg/ml. BAL samples (100 µl) were incubated with an

anti-mouse cellular fibronectin Ab (1/5,000; Biogenesis) and then transferred to a plate precoated with 2 mg/ml mouse fibronectin (Biogenesis). After washings, excess Ab, that did not react with soluble fibronectin, was revealed by sequentially adding a biotinylated anti-rabbit IgG and ExtrAvidine peroxidase solution (Sigma-Aldrich). A ready-to-use solution of 3,3',5,5'-tetramethyl-benzidine (Sigma-Aldrich) was added for 15 min. After addition of 20% H₂SO₄, OD was measured at 450 nm.

Assessment of lung inflammation, fibrosis, and mucus production

Paraformaldehyde-fixed and paraffin-embedded lung sections were stained with H&E and inflammation was evaluated in a random blinded fashion by two observers using a scoring 0–3+ system (20). To specifically detect eosinophils, acetone-fixed frozen-lung sections were stained with the peroxidase substrate 3,3' diaminobenzidine in the presence of cyanide (33). Finally, total lung EPO activity, expressed as OD, was measured in lung homogenates (34).

Fibrosis and mucus production were assessed by a scoring 0–3+ system (35) on Masson's trichrome, red picosirius, periodic acid/Schiff, or Alcian blue-stained sections from paraformaldehyde-fixed and paraffin-embedded lungs.

Statistical analyses

Log-transformed data were analyzed statistically using Graph Pad Prism 3.02 software for Windows (GraphPad Software). Significance among groups was calculated using two-way ANOVA followed by regular unpaired *t* test, or *t* test with Welch correction in case of unequal variance. *p* < 0.05 was considered significant. The results are expressed as means ± SEM of the indicated number of mice or in vitro experiments.

Results

Cleavage of Bid in Fas-activated BAL cells

Bid full length was detected in BAL cell and spleen extracts from Bid^{+/+} mice but not from Bid^{-/-} animals (Fig. 1A). Bid full length expression decreased while that of its cleavage product increased in cells treated for 20 h with 100 ng/ml anti-Fas mAb, as compared with those incubated with the control isotype (Fig. 1A).

Involvement of Bid in eosinophil, but not thymocyte, apoptosis

Almost one-fifth of BAL eosinophils and thymocytes from Bid^{+/+} mice incubated for 18 h with control hamster IgG or with the medium alone died spontaneously (Fig. 1B). Stimulation with 1–100 ng/ml activator anti-Fas mAb dose-dependently increased eosinophil and thymocyte apoptosis at 18 h and 1 μM dexamethasone promoted a similar effect at 18 and 36 h in eosinophils (Fig. 1B). Spontaneous-, Fas-, and dexamethasone-mediated eosinophil apoptosis was reduced by 30, 50, and 25%, respectively, in BAL eosinophils from Bid^{-/-} mice, as compared with WT cells (Fig. 1B). In contrast, Bid deficiency had no effect on thymocyte cell death (Fig. 1B).

Eosinophil apoptosis, as evidenced by hypodiploidy assessment (Fig. 1B), was accompanied by augmented plasmic membrane permeability (Fig. 1C), mitochondria depolarization (Fig. 1D), phosphatidylserine exposure (Fig. 1E), and caspase-3 activity (Fig. 1F). To verify that DEVDfmk-FITC-positive cells were those containing active caspase, eosinophils were incubated with anti-Fas mAb in the absence or in the presence of Z valine alanine aspartate fluoromethylketone. This caspase inhibitor suppressed Fas-mediated increment in the proportion of DEVDfmk-FITC-positive cells (data not shown).

Similar percentages of permeable cells, DioC₆(3)-low stained cells, annexin-V-positive, and DEVDfmk-positive cells were found in IgG-treated eosinophils from Bid^{+/+} and Bid^{-/-} mice (Fig. 1, C–F). After anti-Fas mAb treatment, the proportion of permeable cells, DioC₆(3)-low stained cells, annexin-V-positive, and DEVDfmk-positive cells was reduced by 66% in Bid deficient eosinophils as compared with WT cells (Fig. 1, C–F).

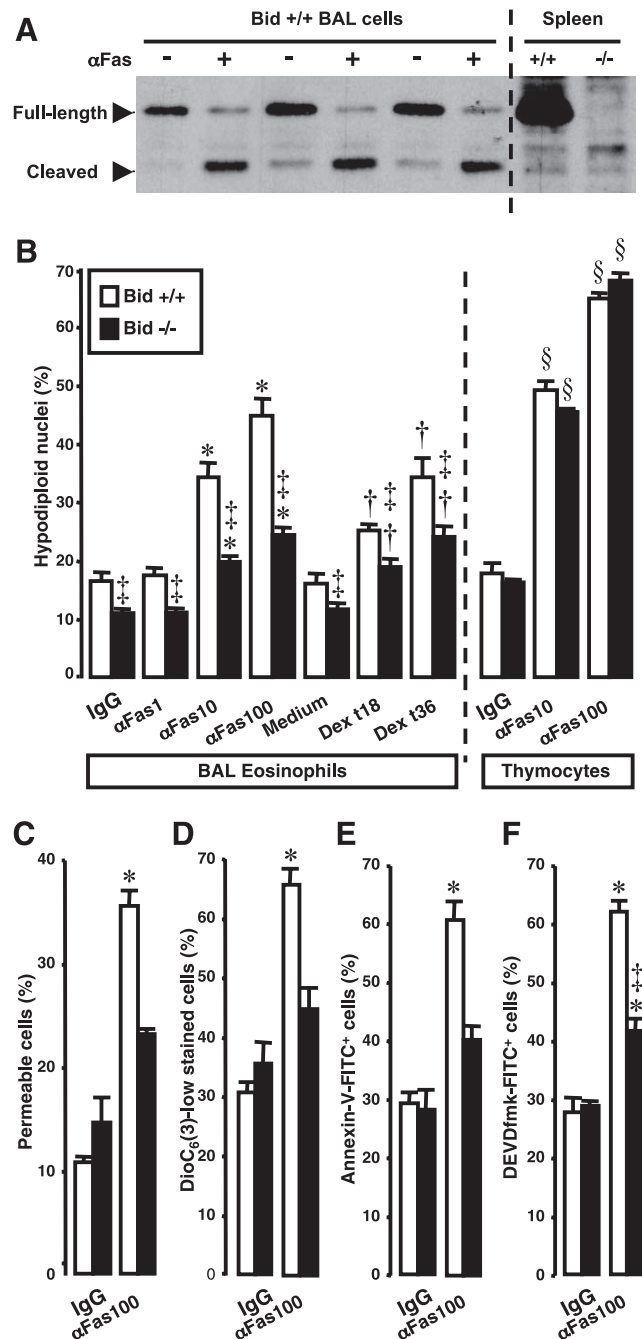


FIGURE 1. Bid is involved in in vitro eosinophil, but not thymocyte, apoptosis. **A**, Expression of Bid full length (22 kDa) and its cleavage product (16 kDa) assessed by Western blot in protein extracts from Bid^{+/+} BAL cells (10 μg) cultured for 20 h with 100 ng/ml control IgG (αFas –; *n* = 3) or anti-Fas Ab (αFas +; *n* = 3) or from Bid^{+/+} or Bid^{-/-} spleen (25 μg). The expression of β-actin (data not shown) was similar in all the samples. **B**, Percentages of apoptotic BAL eosinophils and thymocytes cultured for 18 h with 100 ng/ml control isotype, or 1–100 ng/ml anti-Fas Ab (αFas) or incubated for 18–36 h with medium alone or 1 μM dexamethasone (Dex). **C–F**, Percentages of dead eosinophils (**C**), of eosinophils containing depolarized mitochondria (**D**), expressing phosphatidylserine on their surface (**E**), or containing active caspase-3 (**F**). BAL cells were incubated for 12–18 h with 100 ng/ml control IgG or anti-Fas Ab. Results are expressed as means ± SEM of *n* = 3–9 cell preparations. *, *p* < 0.05, anti-Fas-activated as compared with IgG-treated eosinophils; †, *p* < 0.05, dexamethasone- as compared with medium-treated eosinophils; ‡, *p* < 0.05, Bid^{-/-} as compared with Bid^{+/+} eosinophils; §, *p* < 0.05, anti-Fas-activated as compared with IgG-treated thymocytes.

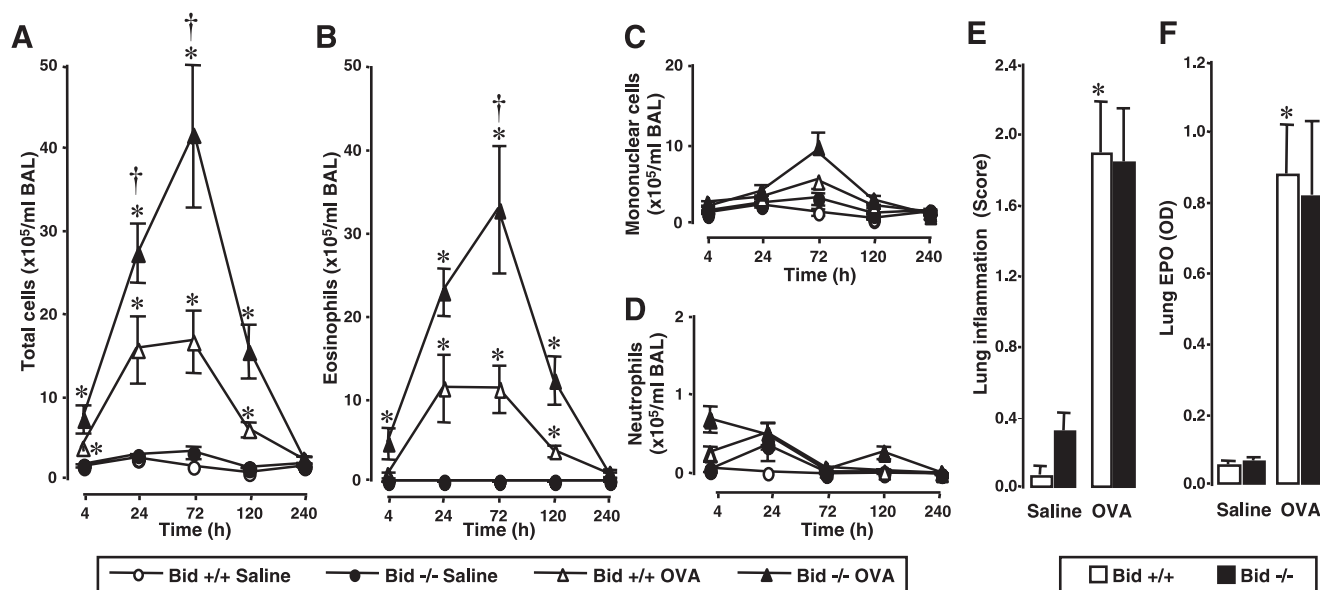


FIGURE 2. Increased eosinophilia in BAL fluids, but not in lungs, of OVA Bid^{-/-} mice. A–D, BAL fluids were collected 4–240 h after the final intranasal challenge in Saline or OVA mice and total cells (A), eosinophils (B), mononuclear cells (C), neutrophils (D) were enumerated. Peribronchial and perivascular inflammation (E) was quantified using a scoring 0–3+ system on H&E stained lung sections, and EPO activity (F) was evaluated on lung homogenates. Results are expressed as means $\pm$ SEM of $n = 3$ –8 mice (or $n = 13$ –14 mice in the case of BAL fluids at 72 h). *, $p < 0.05$, OVA as compared with Saline mice; †, $p < 0.05$, Bid^{-/-} as compared with Bid^{+/+} mice.

Effect of Bid deficiency on allergic pulmonary inflammation

At 4–120 h, OVA challenges induced BAL eosinophilia in Bid^{+/+} mice that was further increased in Bid^{-/-} animals (Fig. 2, A and B). In OVA Bid^{+/+} and Bid^{-/-} mice, BAL eosinophilia resolved at 240 h (Fig. 2, A and B). In both strains, OVA challenges failed to modulate the number of BAL neutrophils and mononuclear cells (Fig. 2, C and D).

At 24–120 h, BAL eosinophilia was accompanied in both strains by perivascular and peribronchial inflammation, as assessed by H&E staining of lung tissue sections, and by augmented EPO activity in whole lung homogenates (Fig. 2, E and F for 72 h, and data not shown for the other time-points). Perivascular and peribronchial eosinophilia, as detected by EPO staining, greatly accounted for pulmonary inflam-

mation (data not shown). EPO-positive cells were also found in lung parenchyma and alveolar lumen (data not shown). Bid deficiency failed to modify the degree of lung inflammation and tissue eosinophilia, i.e., total lung EPO activity (Fig. 2, E and F).

The absence of Bid affected none of the parameters measured in BAL fluids and in lungs in Saline mice (Fig. 2).

Apoptosis in Bid-deficient OVA mice

A few apoptotic eosinophils (mean $\pm$ SEM $1.1 \pm 0.4\%$ apoptotic eosinophils, $n = 7$) were identified on cytospin preparations from BAL cells of OVA Bid^{+/+}, but not Bid^{-/-}, mice collected 72 h after the final challenge. At the same time-point, BAL fluids of Saline mice contained no apoptotic cells.

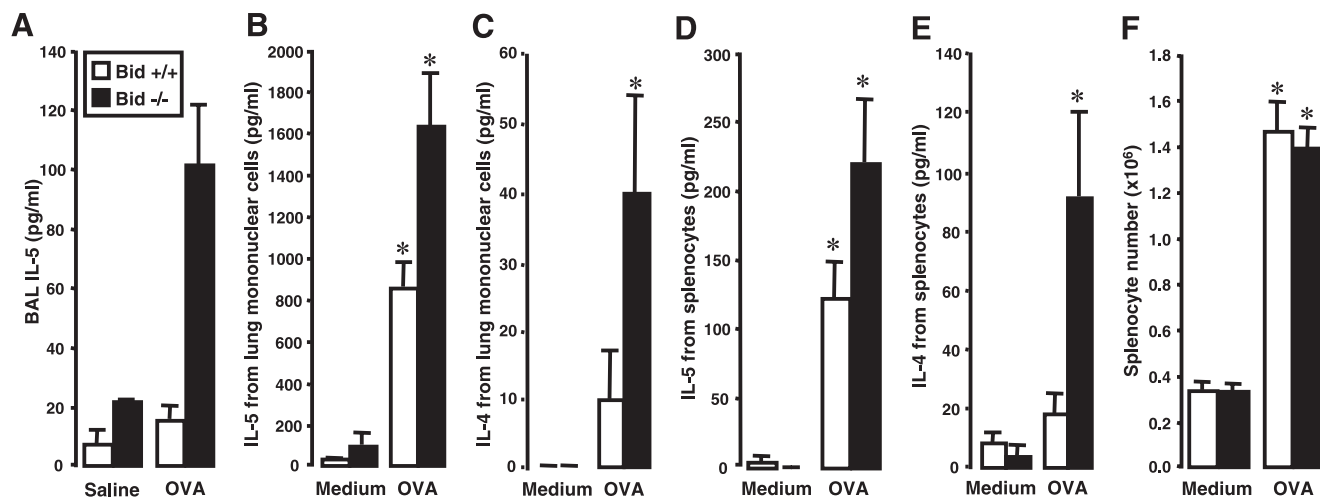


FIGURE 3. Bid deficiency results in augmented Th2 cytokine production in vivo and in vitro. A, IL-5 levels were measured in BAL fluids collected 4 h after the final challenge of Saline or OVA Bid^{+/+} or Bid^{-/-} mice. B–F, Lung mononuclear cells (B and C) and splenocytes (D and E) from OVA Bid^{+/+} or Bid^{-/-} mice were cultured for 72 h in the presence of the medium alone or of 1 mg/ml OVA. The levels of IL-5 (B and D) and IL-4 (C and E) were measured in culture supernatants and cell pellets were used to evaluate the number of viable cells (F). Results are means $\pm$ SEM of $n = 3$ –6 BAL fluids or $n = 7$ –12 cell preparations. *, $p < 0.05$, OVA-activated as compared with medium-treated cells.

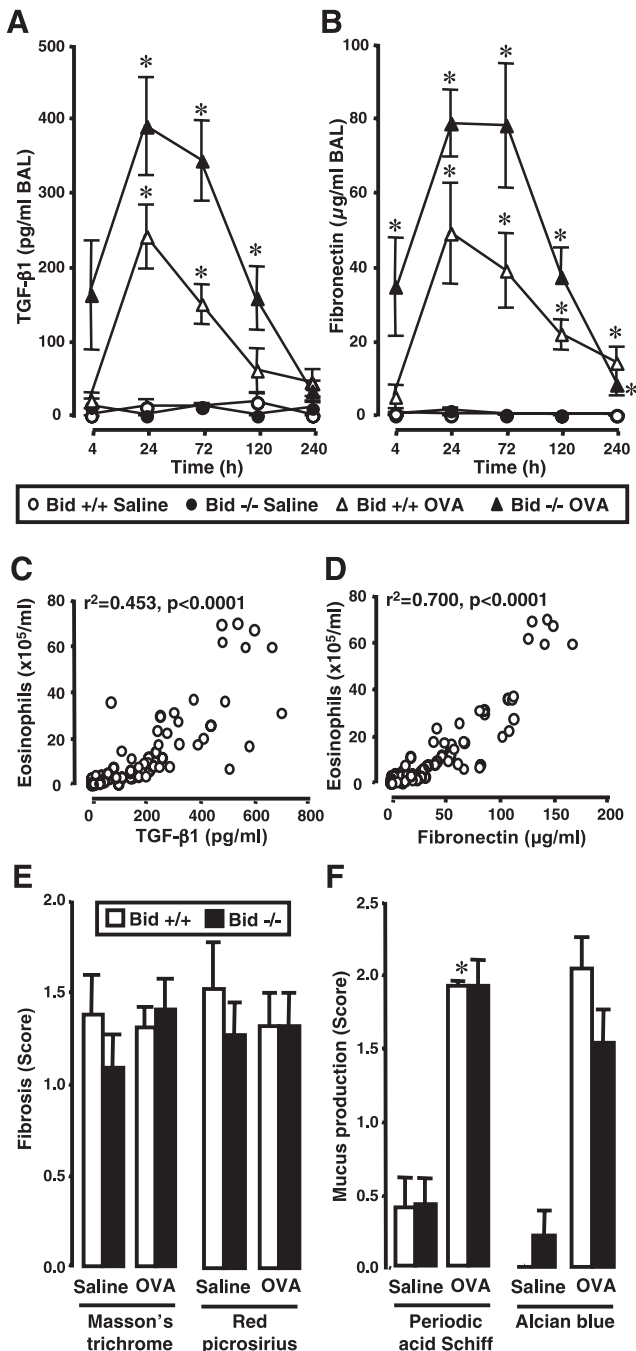


FIGURE 4. Bid deficiency results in higher TGF- β 1 and fibronectin production in BAL fluids without altering OVA-mediated lung remodeling. *A* and *B*, BAL fluids from Saline or OVA Bid^{+/+} or Bid^{-/-} mice were collected 4–240 h after the final challenge and TGF- β 1 (*A*) and fibronectin (*B*) levels were evaluated. *C* and *D*, Regression between BAL eosinophilia (evaluated as in Fig. 2) and TGF- β 1 (*C*) or fibronectin levels (*D*). *E* and *F*, Lung fibrosis (*E*) and mucus production (*F*) evaluated at 240 h by a scoring 0–3+ system on Masson's trichrome-, red picosirius-, periodic acid Schiff-, or Alcian blue-stained lung sections. Results are means $\pm$ SEM of $n = 3$ –8 mice (or $n = 13$ –14 OVA Bid^{+/+} or Bid^{-/-} mice in the case of BAL fluids at 72 h). *, $p < 0.05$, OVA as compared with Saline mice.

Involvement of Bid in OVA-induced Th2 responses

Bid deficiency had no effect on the in vivo or in vitro production of IgE. Indeed, OVA challenges in both strains resulted in a 1.5-fold increase in the level of serum OVA-specific IgE, and,

irrespective of the strain, OVA increased by 3.5-fold the production of total IgE in cultured splenocytes.

IL-5 production was augmented in BAL fluids of OVA Bid^{-/-} mice, as compared with their saline counterparts or with OVA Bid^{+/+} mice (Fig. 3*A*). The levels of IL-5 in BAL fluids collected 24 h and later on after the final OVA challenge and those of IL-4 at any time-points were below the threshold of the sensitivity of the ELISA in all animal groups (data not shown).

To determine more precisely whether Bid regulated Th2-type cytokine production, IL-4 and IL-5 levels were measured in culture supernatant of isolated lung mononuclear cells and splenocytes from Bid^{+/+} and Bid^{-/-} mice. OVA-stimulated cells from Bid^{-/-} mice produced more IL-4 and IL-5 than those from Bid^{+/+} mice (Fig. 3, *B–E*). Bid deficiency had no effect on the number of viable splenocytes (Fig. 3*F*).

OVA-induced airway remodeling in Bid^{+/+} and Bid^{-/-} mice

OVA Bid^{+/+} mice had higher levels of TGF- β 1 and fibronectin in BAL fluids at 24–120 h than Saline mice (Fig. 4, *A* and *B*). These levels were further increased in OVA Bid^{-/-} mice (Fig. 4, *A* and *B*). Regardless of the mouse strain, TGF- β 1 and fibronectin concentrations in BAL fluids of OVA mice correlated positively with the number of BAL eosinophils (Fig. 4, *C* and *D*) and between each other ($r^2 = 0.537, p < 0.0001$).

The degree of lung fibrosis at 24–240 h was similar in saline and OVA Bid^{+/+} and Bid^{-/-} mice (Fig. 4*E* for 240 h, and data not shown for the other time-points). The production of neutral or acid glycoprotein-containing mucus was then assessed using periodic acid/Schiff staining or Alcian blue, respectively. OVA challenges increased the production of mucus to the same extent in Bid^{+/+} and Bid^{-/-} mice (Fig. 4*F* for 240 h, and data not shown for 24–120 h).

Discussion

To evaluate the role of Bid in eosinophil apoptosis, we took advantage of genetically engineered mice and of animal model of asthma, as sources of large numbers of Bid-deficient eosinophils (13, 15, 26). The present study extends previous observations (12, 13, 15, 21, 36) showing that culture of human blood or mouse lung eosinophils in the absence of stimuli that prolong their survival results in their apoptosis and that addition of an agonistic anti-Fas mAb or of dexamethasone provokes Bid cleavage, thus corroborating previous observations on human eosinophils (25), and augments cell death. Based on cell viability, hypodiploidy, and phosphatidylserine exposure, we also demonstrate that Bid is responsible for 20–30% of spontaneous and glucocorticosteroid-mediated cell death, and for 50–70% of Fas-mediated eosinophil apoptosis, but is not involved in thymocyte apoptosis (26).

Of note, cultured BAL cells are not pure eosinophils. Mononuclear cells, mainly macrophages, contaminated preparations. Macrophages are known to produce cytokines, i.e., IL-8, TNF- α , CXCL-1/KC, and CXCL-2/MIP-2, after Fas activation (37, 38). It is thus possible that Fas-activated contaminant macrophages produce cytokines, such as IL-3, IL-5, GM-CSF, IFN- γ , that at a high concentration of 1 ng/ml prevent Fas-induced eosinophil apoptosis (15, 24). To rule out any influence of Bid^{-/-} macrophages on the resistance of Bid deficient eosinophils to cell death, we attempted at purifying BAL eosinophils. The attempts were as follows: depletion of macrophages by making them adhere to plastic, by loading BAL cells onto a density gradient, by immunomagnetic depletion using anti-F4/80 Ab, and BAL sampling. The four techniques we used failed (M. Maret and A. Druilhe, data not shown). We also performed immunohistochemistry on BAL cells to determine

whether mononuclear cells express Bid. Unfortunately, the two commercial Abs tested gave only nonspecific stainings (J. Marchal, M. Maret, and A. Druilhe, data not shown). Finally, we incubated 0.2×10^6 pure naive alveolar macrophages with anti-Fas mAb for 24 h and measured by ELISA the concentration of IL-3, IL-5, GM-CSF, and IFN- γ in the culture medium. IL-5, GM-CSF and IFN- γ were below the detection threshold (15 pg/ml) while IL-3 was detectable. However, the same concentration (300 ng/ml) of this cytokine was found in the culture medium of IgG or anti-Fas treated Bid^{+/+} and Bid^{-/-} macrophages. Although we cannot definitely exclude that contaminant macrophages affected eosinophil behavior, our results argue against a major influence of contaminant BAL cells in the readout of in vitro experiments.

The resistance of Bid^{-/-} eosinophils to Fas-induced apoptosis together with the role of Bid in depolarization of mitochondria and in caspase-3 activity, presently reported, argues for an involvement of the intrinsic, i.e., mitochondria/cytochrome *c*/caspase-9 (23), pathway in eosinophil apoptosis. It also suggests that eosinophils are type II cells. However, resistance of Bid^{-/-} eosinophils to apoptosis is partial, indicating either that Bid-dependent and -independent pathways are involved in cell death in this cell type, or that Bid participates in an amplification loop rather than in the main intracellular apoptotic pathway.

In line with the involvement of Bid in apoptosis of cultured eosinophils, we demonstrate that Bid deficiency leads to a decreased proportion of apoptotic eosinophils in BAL fluids of OVA mice paralleled to an augmented BAL eosinophilia.

Of note, the scarcity of BAL apoptotic eosinophils is in agreement with previously published reports (11, 18, 19, 39) and unpublished observations (M. Maret and A. Druilhe), showing that very few apoptotic (TUNEL-positive) cells were found in the lung of OVA mice during normal resolution of airway inflammation. In situ apoptosis may be underestimated because clearance of apoptotic inflammatory cells through macrophage and epithelial cell phagocytosis is likely to occur very rapidly (21, 40). In addition, phagocytosis has been shown to precede DNA fragmentation (the feature detected using TUNEL assay), and digestion of apoptotic cells by macrophages to be accompanied by a loss of TUNEL staining (41–44). Alternatively, the rarity of detectable apoptotic cells, particularly eosinophils, may reflect the existence of other mechanisms of tissue clearance of inflammatory cells, such as luminal entry, systemic recirculation or lymph node drainage (45, 46).

Although Bid deficiency up-regulates OVA-induced accumulation of eosinophils in BAL, it does not alter their infiltration around vessels and bronchioles and in the lung parenchyma (a normal feature of allergic reaction in C57BL/6 mice, Ref. 47), suggesting that Bid proapoptotic effect is restricted to BAL cells. Interestingly, our results are reminiscent of those previously obtained in Fas^{-/-} mice (19). This indicates that the main function of Bid during allergic airway inflammation may be to mediate eosinophil apoptosis triggered only by Fas activation and that Fas-induced cell death is minimally involved in the resolution of tissue eosinophilia. In addition, epithelium is the major site of Fas-ligand expression in the bronchial wall (9, 48). Collectively, these observations suggest that Bid is involved exclusively in the apoptosis of eosinophils induced by Fas/Fas-ligand interaction during their transmigration across the epithelial barrier, and that this phenomenon results in an up-regulation of BAL, but not of lung, eosinophilia.

Of interest, BAL eosinophilia correlates with levels of BAL TGF- β 1 and fibronectin. This result extends previously published data showing that eosinophilia controls TGF- β 1 and fibronectin production in mouse models of asthma (6–8, 49). Paradoxically,

augmented production of TGF- β 1 is not accompanied by lung fibrosis even at late time-points after OVA challenge. To explain this discrepancy three complementary hypotheses could be raised. The first hypothesis is that TGF- β 1 released in the BAL fluid remains in the lumen and is not effective on resident lung cells such as fibroblasts. The second is that metalloproteases originating from eosinophils or other cells contribute to the clearance of newly formed collagen by TGF- β 1-activated fibroblasts. Therefore, eosinophils, via TGF- β 1 production, initiate fibroblast proliferation and activation, yet collagen accumulation in the extracellular matrix, i.e., fibrosis, starts only long after eosinophilia has resolved (50). The third hypothesis is that Bid deficiency prevents TGF- β 1-induced fibrogenesis, as recently demonstrated in mice overexpressing TGF- β 1 under epithelial Clara cell-specific promoter and *null* Bid (51).

Increased BAL eosinophilia in Bid^{-/-} is neither accompanied by lung fibrosis nor by overproduction of mucus, as we expected based on published data (4). Of note, the key effector function of eosinophils in mouse lung remodeling, particularly in mucus production, is still a matter of debate because two independent studies demonstrated that Ag-induced mucus production either was down-regulated (4) or was not altered (5) in genetically engineered eosinophil-deficient mice.

In vivo experiments were completed by the analysis of the role of Bid on Th2-type responses, that, like airway remodeling, accompany allergen-driven lung eosinophilia. The weak Th2-type responses of C57BL/6 strain, which we observed and that has been previously reported (13, 35, 47, 52), led us to use cultured lung mononuclear cells and splenocytes to determine precisely the consequence of Bid deletion on IgE, IL-4, and IL-5 production. We demonstrate that Bid deficiency does not affect IgE production, yet it augments IL-4 and IL-5 cytokine production by OVA-stimulated splenocytes and lung mononuclear cells, without altering the number of viable cells, i.e., cell proliferation and apoptosis. Bid deficiency tends also to augment allergen-driven IL-5 production in vivo. These results suggest a novel function for Bid as a regulator of Th2-type cytokine synthesis by T-lymphocytes. Although the direct role for Bid in cell activation has never been documented, our observations are consistent with the previously reported cleavage of Bid in anti-CD3 Ab-stimulated CD4⁺ T cells (53) and with the increase in Bid expression observed during IL-4-mediated initial stages of Th2 cell differentiation (54). Of interest, other proteins, such as caspases, or the ligands of death receptors, Fas-ligand and TRAIL, have been shown to regulate a variety of cell functions other than apoptotic cell death, including lymphocyte activation (55–58). In this respect, in vivo pharmacological inhibition of caspases modulates anti-CD3-induced IL-4 and IL-5 release in splenocytes from OVA-immunized and -challenged mice (59).

In conclusion, using Bid^{-/-} mice and derived cells, we demonstrate that Bid plays a crucial role in both spontaneous and in Fas- or dexamethasone-mediated in vitro eosinophil apoptosis, and limits in vitro lymphocyte activation, especially the production of Th2 cytokines. Bid also regulates in vivo allergen-induced BAL eosinophilia, eosinophil apoptosis, and production of TGF- β 1 and fibronectin and of IL-5, a potent chemoattractant, activator, and survival factor for eosinophils (15, 60), but is not involved in systemic IgE synthesis, lung fibrosis and mucus production. Collectively, these results support the concept that Bid deficiency in OVA mice results in increased BAL eosinophilia and TGF- β 1 and fibronectin levels as a consequence of augmented IL-5 production by lymphocytes, eosinophil transmigration, and activation and decreased eosinophil apoptosis.

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Disclosures

The authors have no financial conflict of interest.

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