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Role of Proapoptotic BAX in Propagation of *Chlamydia muridarum* (the Mouse Pneumonitis Strain of *Chlamydia trachomatis*) and the Host Inflammatory Response*

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The BCL-2 family member BAX plays a critical role in regulating apoptosis. Surprisingly, *bax*-deficient mice display limited phenotypic abnormalities. Here we investigate the effect of BAX on infection by the sexually transmitted pathogen, *Chlamydia muridarum* (the mouse pneumonitis strain of *Chlamydia trachomatis*). *Bax*$^{-/-}$ cells are relatively resistant to *Chlamydia*-induced apoptosis, and fewer bacteria are recovered after two infection cycles from *Bax*$^{-/-}$ cells than from wild-type cells. These results suggest that BAX-dependent apoptosis may be used to initiate a new round of infection, most likely by releasing *Chlamydia*-containing apoptotic bodies from infected cells that could be internalized by neighboring uninfected cells. Nonetheless, infected *Bax*$^{-/-}$ cells die through necrosis, which is normally associated with inflammation, more often than infected wild-type cells. These studies were confirmed in mice infected intravaginally with *C. muridarum*; since the infection disappears more quickly from *Bax*$^{-/-}$ mice than from wild-type mice, secretion of proinflammatory cytokines is increased in *Bax*$^{-/-}$ mice, and large granulomas are present in the genital tract of *Bax*$^{-/-}$ mice. Taken together, these data suggest that chlamydia-induced apoptosis via BAX contributes to bacterial propagation and decreases inflammation. *Bax* deficiency results in lower infection and an increased inflammatory cytokine response associated with more severe pathology.

Chlamydia species provoke serious infections of humans and animals worldwide, despite extensive work to better characterize the biology of the infection and develop effective vaccines (1–3). It is estimated that over 600 million persons are infected with *Chlamydia trachomatis*, whose strains include the most common sexually transmitted bacterial pathogen (4) as well as causative agents of conjunctivitis and trachoma. There are an estimated 4 million new cases annually of genital *C. trachomatis* infections of the male and female within the United States (5). In women, the most common consequence of chlamydial genital infection is salpingitis, which can lead to tubal obstruction and infertility (2).

An important element in the design of a vaccine for the prevention or control of chlamydial infections is a complete understanding of the immune response to infection. Little is known about the pathogenesis of human chlamydial infections, and most of our knowledge of acute infection has been obtained from animal models such as the mouse model with *Chlamydia muridarum* (the mouse pneumonitis (MoPn)[1] strain of *C. trachomatis*) (6, 7) and the guinea pig model with the *Chlamydia psittaci* guinea pig inclusion conjunctivitis strain (8). In controlled studies in guinea pigs and mice (9–11), bacteria are initially detected in the cervical epithelium, but the pathology ascends in most animals to the endometrium and the oviducts within 7–9 days after intravaginal inoculation. Most of the damage due to *Chlamydia* is not due to the infection itself but to the inflammation and fibrosis that follow the infection (2).

Polymorphonuclear leukocytes are typically observed in the cervix as early as 2 days after infection, and acute inflammation in the uterine horns and oviducts follows within 5–7 days (2). A number of inflammatory mediators are present during infection, including interleukin-1 (IL-1) and tumor necrosis factor (TNF-α), which have been detected in the Fallopian tubes from humans infected with *C. trachomatis* (12) and in secretions from *Chlamydia*-infected mice and guinea pigs (13–15). TNF-α and other inflammatory cytokines may aid in eradicating *Chlamydia* infection but also may promote long term tissue damage (14).

Contrasting with the epidemiological and pathological diversity of *Chlamydia* infections is the relative uniformity of the chlamydial infectious process. All *Chlamydia* sp. are thought to enter into, survive, and multiply within mucosal epithelial cells by conserved mechanisms involving a unique obligate intracellular developmental cycle, consisting of two phases (16). The extracellular form of *Chlamydia*, the elementary body (EB), is infectious and is thought to be metabolically inert. The EB are internalized into host epithelial cells into small vacuoles resembling endosomes, most of which avoid fusion with host cell

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[1] The abbreviations used are: MoPn, *C. trachomatis* mouse pneumonitis strain; EB, elementary body; IL, interleukin; TNF, tumor necrosis factor; PS, phosphatidylserine; PI, propidium iodide; PMN, polymorphonuclear neutrophils; IFN, interferon.

lysosomes. The EB differentiate within the entry vacuole into metabolically active reticulate bodies, which are presumably noninfectious (17). The reticulate bodies proliferate within the same membrane-bound vacuole and, after several divisions, differentiate back into EB. After 2–3 days, the EB are released from the infected cell through unknown mechanisms and begin a new cycle of infection (16, 17).

This biphasic developmental cycle allows for multiple sites of communication between the chlamydial pathogen and the host cell, many of which probably play a significant role in the pathogen-host cell relationship and thus strongly impact the outcome of the infection. An example of such a communication are the chlamydial signals that block and then later induce apoptosis of the host cell. Like mycobacteria, *Cryptosporidium parvum*, and the herpes virus (18–21), *Chlamydia* strains protect infected cells during early stages of the infection against apoptosis due to external stimuli (22–25) and induce apoptosis of the host cell during later stages of the infection cycle (26–29). The resistance to cell death may account for the observation that Fas- and perforin-dependent killer lymphocytes are not able to clear the infection in mice (30). Conversely, we had proposed that apoptosis due to the infection may be used by the chlamydiae to exit from infected cells and propagate within the host (29).

In mammalian cells, many of the morphological and biochemical features of apoptosis are due to activation of caspases, which can be initiated through engagement of cell surface receptors such as Fas (31) or following release from mitochondria of cytochrome *c*, which associates with the apoptosis regulator Apaf-1 and thereby activates caspase-9, which in turn activates caspase-3 (32). Both pathways are regulated by the BCL-2 family of proteins, which consists of antiapoptotic factors, such as BCL-2 and BCL-x_L, and proapoptotic proteins, such as BAX and BAK (33). BCL-2 proteins prevent apoptosis by preventing the release of cytochrome *c* from mitochondria, whereas BAX stimulates release of cytochrome *c* (34, 35). Nonetheless, caspase activation is not required for all types of cell death (36–38), and overexpression of BAX or BAK induces cell death without the involvement of caspases (37, 39), suggesting that factors other than caspases can also mediate apoptosis.

Apoptosis of *Chlamydia*-infected cells triggered with external ligands is blocked through inhibition of cytochrome *c* release and caspase-3 activation (22), whereas apoptosis induced by the infection itself is independent of known caspases (28, 29). We have found that BAX is activated and translocates from the cytosol to mitochondria in infected cells, and inhibition of BAX by overexpression of BAX inhibitor-1 or BCL-2 inhibits *Chlamydia*-induced apoptosis (28). Caspase-1 is not thought to be involved in apoptosis (40), except when targeted specifically by bacterial products secreted by *Shigella flexneri* or *Salmonella* sp. (41, 42). Nonetheless, caspase-1 is required for maturation and secretion of IL-1β and IL-18, and caspase-1 is activated during *Chlamydia* infection of monocytes and epithelial cells (29, 43).

The preferential target tissue of sexually transmitted chlamydial infections in females is the columnar epithelium of the cervix (2, 17), but monocytes and macrophages can also be infected (44) and may aid in disseminating the infection by certain serovars of *Chlamydia*. Since macrophages undergoing apoptosis secrete IL-1 (45), it is conceivable that apoptosis of these cells during *Chlamydia* infection may contribute to the inflammatory response. Conversely, cytokines such as TNF-α are able to induce apoptosis of some target cells (46), suggesting that the inflammation following *Chlamydia* infection may also directly trigger apoptosis.

Since BAX is activated during infection of an epithelial cell line *in vitro* (28), we here used *Bax*-deficient cells to evaluate the role of BAX in *Chlamydia*-induced apoptosis and to investigate the effect of BAX-dependent apoptosis on the yield of chlamydiae obtained from at least two infection cycles *in vitro*. The availability of *Bax*-deficient mice also allowed us to confirm a role for BAX during genital tract infection *in vivo* and to measure the host inflammatory response during infection of wild-type and *Bax*-deficient mice.

EXPERIMENTAL PROCEDURES

Cells and Bacteria—The mouse pneumonitis agent (MoPn) of *C. trachomatis* (*C. muridarum*) was from the ATCC (Manassas, VA). Bacteria were prepared, and cells were infected as previously described (29). The $Bax^{+/+}$ (wild type), $Bax^{-/-}$, $Bid^{+/+}$, and $Bid^{-/-}$ murine embryonic fibroblasts were described (47). All other cells and materials were described (28, 48).

Analysis of Cell Death—Murine embryonic fibroblasts were infected at a multiplicity of infection of 0.5. Cell death was measured by cytofluorimetry by staining detergent-permeabilized cells with PI (29, 49) or by double-staining unpermeabilized cells with PI and annexin V (48). Both adherent cells and cells in suspension were collected for analysis.

Effect of BAX on Infectious Activity of Chlamydia—Subconfluent $Bax^{+/+}$ and $Bax^{-/-}$ cells were infected at a multiplicity of infection of 0.1, and a 10-fold excess of uninfected $Bax^{+/+}$ cells was added after 24 h of infection. After an additional 2 days of infection, the cells and supernatant were centrifuged for 60 min at 12,000 rpm in a Sorvall type GSA rotor. The pellet was freeze-thawed three times and sonicated for 10 min in a bath sonicator at 4 °C to break cells and dissociate aggregates, giving the final suspension of chlamydiae used to measure bacterial yield. Serial dilutions of the chlamydial preparation were used to infect HeLa cells on cover slips for 48 h, and the chlamydial vacuoles were revealed with fluorescein isothiocyanate-conjugated anti-*Chlamydia* monoclonal antibody, as described (29). Samples were examined with a Zeiss fluorescence microscope attached to a cooled CCD camera. At least 10 separate fields containing an average of 200–300 HeLa cells were counted per sample, and the experiment was repeated on three separate occasions.

Animal Infections—Female $Bax^{+/+}$ and $Bax^{-/-}$ mice on a C57BL/6 background (Jackson Laboratories, Bar Harbor, MA) were infected intravaginally with 10^7 inclusion-forming units of *C. muridarum*. The course of infection was monitored by periodic cervico-vaginal swabbing of individual animals (50). Chlamydiae were isolated from swabs in tissue culture according to standard methods, and inclusions were visualized and enumerated by immunofluorescence (51). Results are expressed as mean and S.E. of inclusion-forming units per ml. Experiments were repeated once, and there were five animals per experimental group. Groups of mice were sacrificed at 7 and 24 days after primary infection or followed through day 70 and administered a challenge infection with 10^7 inclusion-forming units of MoPn on day 90, 7 days post-depo-provera treatment. Histopathology and cytokine secretion measurements were performed as described (50). Staining of cell surface antigens and qualitative evaluation of cell populations were performed as described by Morrison and Morrison (52). Vaginal secretions were assayed individually for cytokine or chemokine activity by enzyme-linked immunosorbent assay using commercial kits (R&D Systems, Minneapolis, MN). Antibody responses were measured in sera from mice and assayed by enzyme-linked immunosorbent assay as described (14).

All mice were given food *ad libitum* and maintained in environmentally controlled rooms with a 12/12-h light/dark cycle. All animal studies were approved by the University of Arkansas Medical Sciences' Institutional Animal Care and Use Committee.

Statistics—Statistical comparisons between the groups of mice for level of infection, antibody production, and cytokine production over the course of infection were made by a two-factor (days and murine strain) analysis of variance with the *post hoc* Tukey test as a multiple-comparison procedure. The Wilcoxon rank sum test was used to compare the duration of infection in the respective groups over time. One-way analysis of variance on ranks was used to determine differences in inflammatory cell populations among the groups. All experiments were repeated at least once.

RESULTS

Effect of BAX on Host Cell Death in Vitro—We have previously shown that BAX is activated in cells infected with *Chlamydia* (28). The effect of BAX activation on *Chlamydia*-

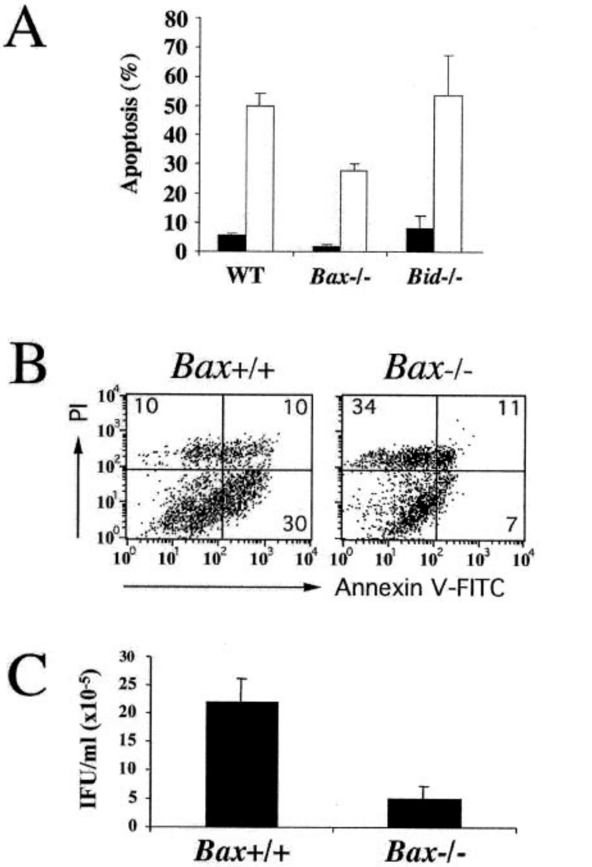

FIG. 1. **Effect of BAX on apoptosis and bacterial production *in vitro*.** *A*, apoptosis of wild-type, $Bax^{-/-}$ cells, and $Bid^{-/-}$ cells. Cells were infected with *C. muridarum* for 2 days, and apoptosis of PI-labeled detergent-permeabilized cells was measured by cytofluorimetry (see "Experimental Procedures"). *Black bar*, spontaneous apoptosis of uninfected cells; *white bar*, apoptosis of infected cells. *B*, necrosis of infected cells. $Bax^{+/+}$ and $Bax^{-/-}$ cells were infected for 48 h. Necrosis and apoptosis were quantified by double-labeling unpermeabilized cells incubated with PI and annexin V (see "Experimental Procedures"). The *numbers* in each *quadrant* refer to the percentage of cells in each quadrant. *C*, chlamydial production after at least two infection cycles. Subconfluent $Bax^{+/+}$ and $Bax^{-/-}$ cells were infected at a multiplicity of infection of 0.1, and a 10-fold excess of uninfected $Bax^{+/+}$ cells was added after 24 h of infection. After an additional 2 days of infection, the yield of chlamydiae from adherent cells and cells in suspension was measured by titrating on uninfected HeLa cells (see "Experimental Procedures"). *IFU*, inclusion-forming units.

induced apoptosis was therefore determined by infecting normal ($Bax^{+/+}$) and *Bax*-deficient cells. The infection led to a high level of apoptosis in $Bax^{+/+}$ cells, which was observed after 1 day of infection (Fig. 1*A*). At the same multiplicity of infection, the *Bax* deficiency resulted in a nearly 2-fold inhibition of apoptosis during infection (Fig. 1*A*), suggesting that this pathway of apoptosis requires, at least partially, BAX activation.

Engagement of surface death receptors such as Fas or TNFR1 results in cleavage of the BCL-2 family member BID, which triggers the oligomerization of proapoptotic family members BAK and BAX, leading to cell death (47). To determine whether BID cleavage may be required for BAX activation in infected cells, $Bid^{+/+}$ and $Bid^{-/-}$ cells were infected with *C. muridarum* for 2 days, and apoptosis was measured. No difference was observed in sensitivity to apoptosis of wild-type and *Bid*-deficient cells (Fig. 1*A*), suggesting that BAX activation is initiated within the interior of the infected cell.

Cells that are prevented from dying through apoptosis still manage to die, but they often succumb later, dying through necrosis (53–55). To determine quantitatively whether any infected cells may be necrotic, cells were infected for 2 days, and necrosis was measured by double-labeling the cells with PI and annexin V, which binds to phosphatidylserine (PS) that becomes exposed on the surface of dying cells. Cells labeled only with annexin V are considered to be apoptotic, whereas cells labeled only with PI, which have thus lost their plasma membrane integrity, are necrotic; cells labeled with both PI and annexin V are either necrotic or late apoptotic (48). Evaluation by cytofluorimetry showed that the $Bax^{-/-}$ cells were dying through necrosis more often than $Bax^{+/+}$ cells after a 2-day infection. Whereas 30% of the cells were apoptotic and 10% were necrotic in the $Bax^{+/+}$ population, 7% were apoptotic and 34% were necrotic in the $Bax^{-/-}$ population (Fig. 1*B*).

Effect of BAX on Bacterial Yield in Vitro—In order to distinguish between the possibility that apoptosis may be used by the bacteria to escape from the infected host cell, rather than by the host cell to eliminate bacteria, $Bax^{+/+}$ and $Bax^{-/-}$ cells were infected for 3 days, and the bacteria were harvested from supernatant and infected cells. The recovered bacteria were then used to reinfect wild-type cells, and the efficiency of infection was evaluated by immunofluorescence. A larger number of infectious chlamydiae were recovered from the $Bax^{+/+}$ than the $Bax^{-/-}$ cells (Fig. 1*C*), suggesting that the bacteria may use apoptosis to exit from cells at the end of the first infection cycle before beginning a new round of infection. To rule out the possibility that *Bax* deficiency may be inhibiting growth of intracellular chlamydiae, the number of infectious vacuoles was also measured after a 24-h infection, before any apoptosis is observed; the infection at 24 h was the same in either $Bax^{+/+}$ or $Bax^{-/-}$ cells (not shown). Since fibroblasts and epithelial cells express a PS receptor (56) that could be used to phagocytose *Chlamydia*-containing apoptotic bodies, these results suggest that *Chlamydia* may use apoptosis to release infectious bacteria from infected host cells in order to initiate a new infection cycle.

Effect of BAX on Bacterial Propagation during Genital Tract Infection—To confirm whether apoptosis has an effect on the yield of infectious bacteria *in vivo*, the infection was repeated with $Bax^{+/+}$ and $Bax^{-/-}$ mice. The mouse model of *C. muridarum* infection of the female genital tract mimics human infection (2, 9, 10) and is a useful model for *Chlamydia* infection and adaptive immunity to infection. *Bax*-deficient mice are also convenient for studies on *Chlamydia* infection, since the mice are healthy, the levels of the antiapoptotic molecules BCL2 and BCL-X_L are unaffected, and the distribution of different lymphocyte populations ($CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+$, and $CD8^+$ cells) are unaltered, compared with $Bax^{+/+}$ mice (57). The infection was less efficient and disappeared more quickly in the $Bax^{-/-}$ mice than in control $Bax^{+/+}$ mice (Fig. 2), consistent with a role for BAX-dependent apoptosis in the propagation of chlamydiae *in vivo*.

Effect of BAX on Cytokine Secretion during Genital Tract Infection—Prior studies in our laboratory have shown that murine chlamydial genital tract infection induces strong production of the proinflammatory cytokine, TNF-α, and of the murine CXC chemokine, macrophage inflammatory protein 2 (14, 50). These responses routinely peak during the first week of infection and decline toward base line during the second week. Enzyme-linked immunosorbent assay measurement of cytokines in genital tract secretions revealed similar kinetics in the $Bax^{+/+}$ and $Bax^{-/-}$ mice in this study (Fig. 3). However, the proinflammatory mediators were significantly increased during the first week of infection in the $Bax^{-/-}$ mice compared with the $Bax^{+/+}$ mice. Further, we detected extremely high levels of IFN-γ, a protein with marked antichlamydial effects, in the $Bax^{-/-}$ mice compared with $Bax^{+/+}$ mice during the first

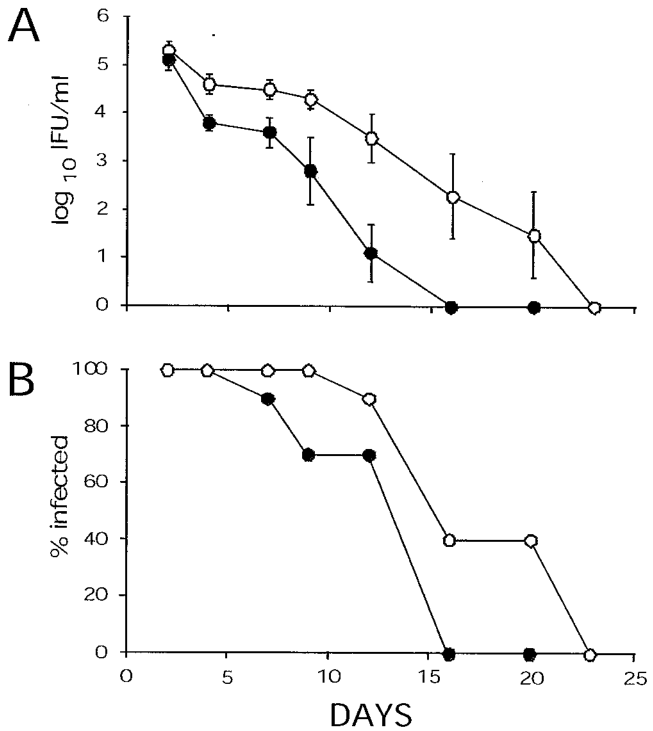

FIG. 2. **Chlamydial infection is decreased in *Bax*$^{-/-}$ mice.** *A*, intensity and duration of primary lower genital tract infection. Female *Bax*$^{+/+}$ (*open circles*) and *Bax*$^{-/-}$ (*closed circles*) mice were infected with *C. muridarum*, and the course of infection was monitored by cervicovaginal swabbing. $p < 0.001$ by two-way analysis of variance for *Bax*$^{+/+}$ *versus Bax*$^{-/-}$. *B*, elimination of chlamydiae from wild-type and *Bax*$^{-/-}$ mice. Results are expressed as the percentage of animals positive for infection over time. *Bax*$^{-/-}$ mice (*closed circles*) resolved the infection more rapidly than *Bax*$^{+/+}$ mice (*open circles*) with all of the *Bax*$^{-/-}$ mice being negative for infection by day 16. In contrast, 4 of 10 *Bax*$^{+/+}$ mice were still positive for infection on day 20. *IFU*, inclusion-forming units.

week of infection (Fig. 3). The detection of higher levels of inflammatory mediators in the *Bax*$^{-/-}$ mice compared with the *Bax*$^{+/+}$ mice is made more significant by the fact that the infection was much less efficient in the *Bax*$^{-/-}$ mice. These data are consistent with increased cell death by necrosis during chlamydial infection in the absence of BAX.

Pathology Associated with Bax Deficiency of Infected Mice—Histopathological and immunohistochemical examination of genital tract tissues from mice sacrificed on day 7 of primary infection revealed that the early inflammatory response was of similar quality and quantity in *Bax*$^{+/+}$ and *Bax*$^{-/-}$ mice. Moderate to severe inflammation was detected in the endocervix and uterine horns with a predominance of polymorphonuclear neutrophils (PMNs) but high numbers of lymphocytes also being seen. By immunohistochemical staining, the median inflammatory score for PMNs was 4 for *Bax*$^{-/-}$ and 3 for *Bax*$^{+/+}$ on day 7 ($p = 0.375$), and for lymphocytes it was 2.0 for *Bax*$^{-/-}$ and for *Bax*$^{+/+}$ ($p = 0.5$) (analysis of variance on ranks). Most of the lymphocytes were $CD4^+$ in both groups, with comparatively low numbers of $CD8^+$ cells being found (median score for $CD4^+$ T cells = 2.0 for both groups on day 7; $CD8^+$ T cells = 1). Mild to moderate inflammation was detected in the oviducts in both *Bax*$^{+/+}$ and *Bax*$^{-/-}$ mice, again with a predominance of PMNs being found. Tissues from mice sacrificed on day 24, at a time when infection had mostly resolved, revealed equal numbers of acute (PMNs) and chronic inflammatory cells (lymphocytes) in *Bax*$^{+/+}$ and *Bax*$^{-/-}$ mice. However, in 4 of 5 *Bax*$^{-/-}$ mice, large granulomatous nodules with marked central necrosis were found scattered throughout 9 of 10 uterine horns (Fig. 4). These nodules were seen in only 1 of 10 horns from

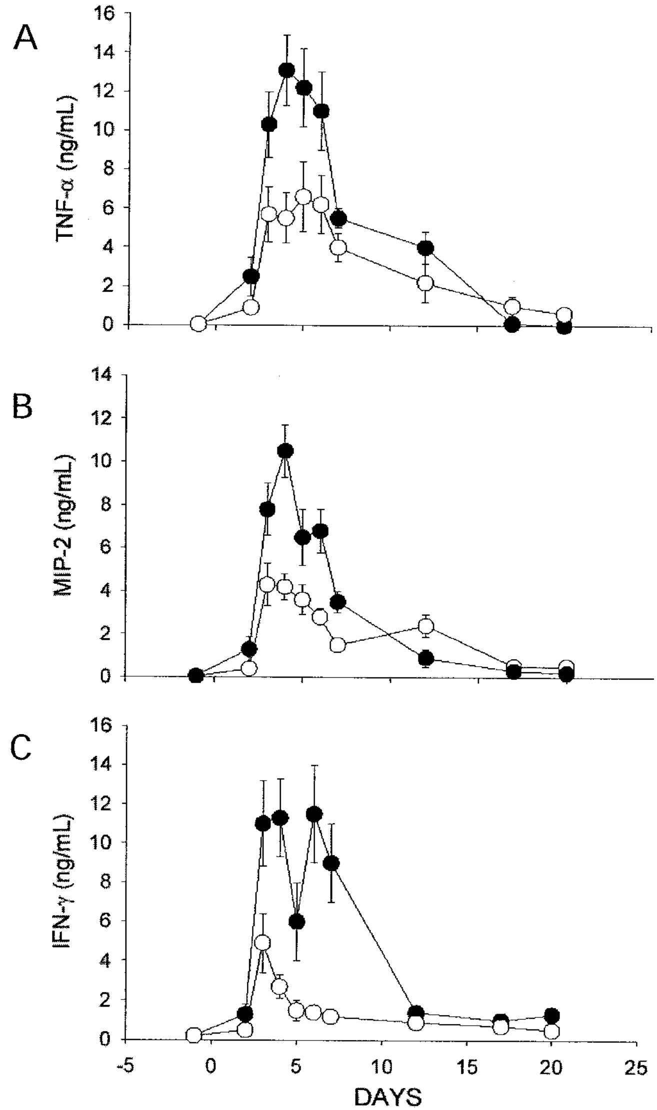

FIG. 3. **Secretion of inflammatory proteins is increased in *Bax*$^{-/-}$ mice.** TNF-α (*A*) macrophage-inflammatory protein 2 (*MIP-2*) (*B*), and IFN-γ (*C*) levels were significantly increased in the *Bax*$^{-/-}$ (*closed circles*) mice compared with *Bax*$^{+/+}$ (*open circles*) during the first week of infection. Genital tract secretions were eluted from vaginal sponges collected from individual animals before and after infection. Results are expressed as mean and S.E. of cytokine measurements from five animals.

Bax$^{+/+}$ mice; $p = 0.001$, Fisher exact test). Thus, although infection is less efficient in *Bax*$^{-/-}$ mice, it results in greater release of inflammatory mediators and increased chronic tissue pathology.

Acquired Immunity in Infected Wild-type and Bax-deficient Mice—The acquired immune response, as determined by antibody titers in serum and by resistance to reinfection, was similar in *Bax*$^{-/-}$ and *Bax*$^{+/+}$ mice. Both groups demonstrated high titers of IgG2a and low titers of IgG1 (Fig. 5), demonstrating that a T_H1 response was stimulated in both cases. Both *Bax*$^{+/+}$ and *Bax*$^{-/-}$ were also completely resistant to reinfection when challenged 70 days after primary vaginal inoculation (not shown). Thus, despite the increased release of inflammatory mediators and enhanced pathology after primary infection in *Bax*$^{-/-}$ mice, the absence of Bax did not affect the quality or magnitude of the acquired immune response.

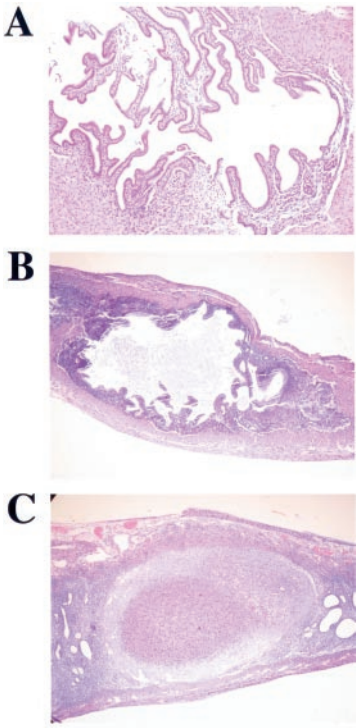

FIG. 4. **Large granulomas are prevalent in *C. muridarum*-infected *Bax*$^{-/-}$ mice.** *A*, histopathological examination of hematoxylin- and eosin-stained longitudinal sections from the uterine horns of mock-infected *Bax*$^{-/-}$ mice revealed normal endometrial type glands and an absence of inflammation. Uteri from mock-infected *Bax*$^{+/+}$ mice were also normal (not shown). *B*, whereas the uteri from *C. muridarum*-infected *Bax*$^{+/+}$ mice revealed a paucity of granulomas, the horns were dilated with PMNs within the lumen as well as the glandular epithelium and scattered lymphocytes and plasma cells in the stroma. *C*, the uterine horns from *C. muridarum*-infected *Bax*$^{-/-}$ mice revealed multiple areas of granulomatous inflammation with aggregates of large cells containing abundant eosinophilic cytoplasm consistent with histiocytes and scattered small lymphocytes.

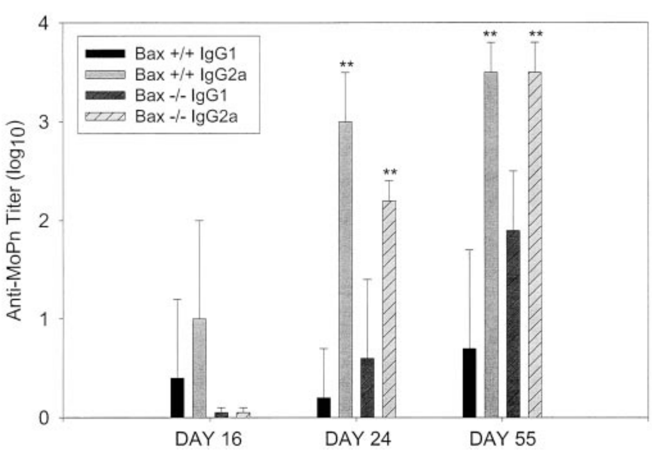

FIG. 5. **The antibody response in *Bax*$^{+/+}$ and *Bax*$^{-/-}$ mice.** The acquired immune response, as determined by antibody titers in serum, was similar in both groups of mice. *Black bar*, IgG1 in *Bax*$^{+/+}$; *gray bar*, IgG2a in *Bax*$^{+/+}$; *dark gray bar* with *diagonal lines*, IgG1 in *Bax*$^{-/-}$; *light gray bar* with *diagonal lines*, IgG2a in *Bax*$^{-/-}$. Data are expressed as the mean titer ($\log_{10}$) + S.E. for five mice at each time point. **, significantly higher titers of IgG2a compared with IgG1 for *Bax*$^{+/+}$ and *Bax*$^{-/-}$, $p > 0.005$. *Bax*$^{+/+}$ and *Bax*$^{-/-}$ titers were not significantly different at any time point.

DISCUSSION

We here show that *Bax*-deficient cells are more resistant to *Chlamydia*-induced apoptosis than wild-type cells. A biological role for BAX activation is suggested by the observation that the yield of chlamydiae after two infection cycles decreases in *Bax*-deficient cells compared with wild-type cells. BAX could therefore contribute to exit of chlamydiae from infected cells before initiation of a new infection cycle. The fact that *C. muridarum* infection of the genital tract disappears more rapidly in *Bax*$^{-/-}$ mice than in *Bax*$^{+/+}$ mice also reinforces the interpretation that BAX-dependent apoptosis could facilitate chlamydial propagation. Finally, *Bid*$^{-/-}$ cells are as sensitive to *Chlamydia*-induced apoptosis as *Bid*$^{+/+}$ cells. Ligation of the Fas or TNFR1 death receptors on the cell surface leads to cleavage of BID, which activates BAX (47). The lack of involvement of BID during *Chlamydia* infection suggests that BAX activation is initiated within the host cell. Activation could be related to infection-related metabolic stress (58), or it could be triggered by signals released from the chlamydial vacuole via type 3 secretion mechanisms (59–61). Activation of BAX is clearly advantageous for *Chlamydia*, and it is tempting to speculate that other intracellular microbes may use BAX-mediated apoptosis to enhance their propagation. These results thus reveal a novel function for a host cell proapoptotic protein, which until now has been known to promote apoptosis through induction of mictochondrial dysfunction and whose singular deficiency in mice results in only minor changes to the immune system (57).

Apoptotic cells and apoptotic bodies released from dying cells *in vivo* are cleared by professional scavengers such as macrophages, which express surface receptors that recognize apoptotic bodies and cells (62). Thus, PS exposed on the surface of dying cells interacts with PS receptors on human or murine macrophages, leading to phagocytosis of the corpses. However, the PS receptor is also expressed on the surface of fibroblasts and epithelial cell lines, including HeLa (derived from a carcinoma of the cervix) (56), and ubiquitously expressed molecules such as lectins or integrins could also participate in internalization of apoptotic bodies (63). Since an antibody against the PS receptor can block phagocytosis of apoptotic cells by fibroblasts and mammary epithelial cells (56), we propose that the PS receptor and/or similar receptors may be used to internalize *Chlamydia*-containing apoptotic cells and bodies by neighboring epithelial cells in the genital tract, thus beginning a new round of infection.

Despite the faster clearance of bacteria in *Bax*$^{-/-}$ mice, the secretion of inflammatory cytokines was higher in *Bax*$^{-/-}$ than in wild-type mice. The secretion of TNF-α, IFN-γ, and the murine equivalent of IL-8, macrophage inflammatory protein 2, have been previously reported during *C. muridarum* infection, but until now the extent of their secretion has always correlated with the intensity of infection (8, 13, 64). Whereas several interpretations of these data could be envisioned, we propose that apoptosis of infected cells in *Bax*$^{-/-}$ mice is postponed, causing the cells to die of necrosis more often than in *Bax*$^{+/+}$ mice. This explanation is consistent with the observation that more necrotic cells are observed when *Bax*-deficient cells are infected *in vitro* than when wild-type cells are infected. Phagocytosis of apoptotic cells by macrophages leads to secretion of anti-inflammatory cytokines such as IL-10 and transforming growth factor-β, but necrotic cells stimulate secretion of proinflammatory cytokines, including TNF-α, IL-1β, and IL-8 (65–67). Although these possibilities are not mutually exclusive, the resulting increase in IFN-γ observed in *Bax*$^{-/-}$ mice may also contribute to their faster resolution of infection. IFN-γ is a known inducer of aberrant forms of *Chlamydia in vitro*; the cytokine adversely affects normal growth and division of reticulate bodies and interrupts their redifferentiation into infectious EB (68). IFN-γ induction of aberrant, noninfectious forms of *Chlamydia* may thus contribute to reduced infection in the *Bax*$^{-/-}$ mice.

Most of the pathological damage observed during *Chlamydia* infection is thought to be due to the inflammatory response rather than to the microorganism itself (2, 8). The higher incidence of granulomatous nodules in the $Bax^{-/-}$ mice reinforces the notion that secretion of inflammatory cytokines by infected epithelial cells and neighboring macrophages may be responsible for the chronic tissue damage associated with *Chlamydia* infection. Although the hallmark of both ocular (trachoma) and urogenital chlamydial infections is the development of lymphoid follicles (69–72), granulomas have occasionally been reported in human (73), non-human primate (74), murine (75), and veterinary disease (76). Loss of function mutations in *Bax* have been reported in humans and may be associated with increased incidence and progression of cancer (77–80). Our data suggest that mutations in *Bax* might lead to an increase in the severity of chlamydial genital tract disease. This is the first report of the effect of *Bax* mutation in an infectious disease model.

Disordered cell death has been previously shown to have an impact on the immune system and human disease. Thus, reduced cell death and defective clearance of apoptotic material are thought to lead to autoimmune diseases, and macrophages secrete proinflammatory mediators following ingestion of cells undergoing secondary necrosis but not after ingestion of intact apoptotic cells (62, 81, 82). We find that defects within the core apoptotic program also lead to immunopathology. Whereas these diseases may share the common feature that more cells undergo necrosis when apoptosis is blocked, it is also conceivable that their pathogenesis may be multifactorial. However, they all demonstrate clearly that blocking the signaling pathways associated with apoptosis has consequences for antigens and infectious agents that are normally packaged into apoptotic bodies, with striking effects on host pathology.

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Role of Proapoptotic BAX in Propagation of *Chlamydia muridarum* (the Mouse Pneumonitis Strain of *Chlamydia trachomatis*) and the Host Inflammatory Response

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