Role of Thromboxane A\textsubscript{2} in the Induction of Apoptosis of Immature Thymocytes by Lipopolysaccharide

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Lipopolysaccharide (LPS) causes apoptotic deletion of CD4\textsuperscript{+} CD8\textsuperscript{+} thymocytes, a phenomenon that has been linked to immune dysfunction and poor survival during sepsis. Given the abundance of thromboxane-prostanoid (TP) receptors in CD4\textsuperscript{+} CD8\textsuperscript{+} thymocytes and in vitro evidence that thromboxane A\textsubscript{2} (TXA\textsubscript{2}) causes apoptosis of these cells, we tested whether enhanced generation of TXA\textsubscript{2} plays a role in LPS-induced thymocyte apoptosis. Mice injected with 50 μg of LPS intraperitoneally displayed a marked increase in generation of TXA\textsubscript{2} and prostaglandin E\textsubscript{2} in the thymus as well as apoptotic deletion of CD4\textsuperscript{+} CD8\textsuperscript{+} thymocytes. Administration of indomethacin or rofecoxib inhibited prostanoid synthesis but did not affect thymocyte death. In contrast, thymocyte apoptosis in response to LPS was significantly attenuated in TP-deficient mice. These studies indicate that TXA\textsubscript{2} mediates a portion of apoptotic thymocyte death caused by LPS. The absence of an effect of global inhibition of prostanoid synthesis suggests a complex role for prostanoids in this model.

Bacterial sepsis is associated with apoptotic cell death in a variety of organs and tissues, including the thymus (16, 19, 42). The LPS component of gram-negative bacteria appears to mediate most of these effects, since injection of LPS into mice is followed by a dramatic reduction in thymocyte numbers, simulating the situation during sepsis. LPS does not cause thymocyte apoptosis directly, as addition of LPS to in vitro cultures of thymocytes does not result in apoptosis of these cells (43). This result is not surprising, as thymocytes are not known to express the main LPS receptor, TLR4. Therefore, thymocyte apoptosis following LPS injection in vivo is likely mediated by soluble factors produced by TLR4-expressing cells of the innate immune system. The identification of such mediators could have implications for therapy, since recent evidence suggests that lymphocyte apoptosis during sepsis is deleterious because it might impair immune responses and contribute to mortality (20).

Prostanoids are produced by cells of the innate immune system exposed to LPS (27). TXA\textsubscript{2} is a prostanoid mediator derived by the sequential metabolism of cell membrane phospholipids by phospholipases, COX, and thromboxane synthase. During inflammatory states, this metabolic pathway is induced, resulting in enhanced TXA\textsubscript{2} production (39). TXA\textsubscript{2} has long been known for its vasoconstrictive and procoagulant properties, but a recent study revealed a novel and important role for this molecule in cell-mediated immunity (38). In addition, several lines of investigation point toward TXA\textsubscript{2} as a potential mediator of thymocyte apoptosis. First, Nusing et al. showed that the activity of TXA\textsubscript{2} synthase in dendritic cells of the thymic stroma is as high as it is in platelets (26). Second, Namba and coworkers demonstrated that the thymus is the organ with the highest concentration of TP receptors (24). In the thymus, immature CD4\textsuperscript{+} CD8\textsuperscript{+} thymocytes have the highest concentrations of TP receptors among cells; when they are exposed to a TXA\textsubscript{2} agonist, these cells undergo apoptosis in a concentration-dependent manner, a phenomenon that can be inhibited by a specific TP\textsubscript{a} antagonist (41). Finally, it has been shown that pharmacologic blockade of TP receptors in vivo abrogates donor-specific tolerance induced by thymic injection of alloantigen, suggesting that TXA\textsubscript{2} is involved in the apoptotic deletion of allogeneic thymocytes (30).

Given that LPS enhances the production of TXA\textsubscript{2} (and other prostanoids) (27) and the accumulating evidence that TXA\textsubscript{2} is involved in thymocyte apoptosis in vitro, we performed experiments to determine whether TXA\textsubscript{2} plays a role in LPS-induced thymocyte apoptosis in vivo. Our studies suggest that the generation of TXA\textsubscript{2} and the consequent activation of the TP receptor contribute to the loss of thymocytes following exposure to LPS.

MATERIALS AND METHODS

Abbreviations. BAD, Bcl-2 antagonist of cell death; BAK, Bcl-2 homologous antagonist/killer; BAX, Bcl-2-associated x protein; BCL-2, B-cell lymphoma/leukemia 2 protein; BCL-W, Bcl-2-like 2 protein; BCL-X, Bcl-2-like 1 protein; BFL, Bcl-2 family protein isolated from human fetal liver; Casp, caspase; COX, cyclooxygenase; DMEM, Dulbecco’s modified Eagle’s medium; FACS buffer, 2% fetal calf serum, 0.01% sodium azide in PBS; FADD, Fas-associated death domain; FAF, Fas-associated factor; FAS, Fas-associated protein; Fas, fas-related; FITC, fluorescein isothiocyanate; i.p., intraperitoneal; LPS, lipopolysaccharide; m-APO, mouse apoptosis; PBS, phosphate-buffered saline; PE, phycoerythrin; PGE\textsubscript{2}, prostaglandin E\textsubscript{2}; RIA, radioimmunoassay; RIP, receptor-interacting protein; Rofe, rofecoxib; RPA, RNase protection assay; SEM, standard error of the mean; TLR4, Toll-like receptor 4; TNF, tumor necrosis factor; TNF-α, tumor necrosis factor alpha;
of wild-type C57BL/6 mice 24 h after a single i.p. injection of 50 μg of LPS (n = 9) or a similar volume of PBS control (n = 5). LPS injection caused a dramatic reduction in total thymocyte count (from 62 ± 19 million to 9 ± 2 million cells; P < 0.001). The flow cytometry plots below each bar illustrate the thymocyte phenotype of animals treated with PBS (b) or LPS (c). The numbers inside the plot represent the contribution (%) of each thymocyte population to the total of gated cells: left lower quadrant, CD4⁺ CD8⁻ cells; left upper quadrant, CD4⁻ CD8⁺ cells; right upper quadrant, CD4⁺ CD8⁺ cells. These plots are representative of those from five experiments with control cells and nine experiments with thymocytes from LPS-injected animals. LPS-induced reduction in thymocyte count was due to a contraction of the CD4⁺ CD8⁻ population (from 88% ± 1% to 41% ± 5% of gated cells; P < 0.001).

Animals. Mice lacking TP receptors were generated by gene targeting, as reported previously (37). To eliminate the confounding effects of background genes in our studies, the TP mutation was backcrossed into C57BL/6 mice for more than 10 generations. The genotypes of individual mice were determined by Southern blotting, as described previously (37). All mice were bred and maintained in the Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal facility of the Durham Veterans Affairs Medical Center, according to the guidelines of the National Institutes of Health.

LPS-induced thymocyte deletion. Fifty micrograms of LPS (catalog no. L-4391; Sigma Chemical) dissolved in 100 μl of PBS (n = 9 per group) or a similar volume of PBS alone (n = 5 TP⁻/⁻ mice and n = 4 TP⁺/⁺ mice) was administered to mice by i.p. injection. Twenty-four hours after i.p. injection, the animals were killed by CO₂ asphyxiation and weighed; the thymuses and spleens were gently ground between glass slides, and the cell fragments were removed by allowing them to adhere to the walls of a 5-ml pipette. The cells were centrifuged at 1,200 rpm for 5 min at 4°C, resuspended in PBS, and any remaining tissue pellets were resuspended in PBS. The cells were then washed twice in PBS and fixed in 250 μl of 2% formalin in PBS. A FACSCalibur system scanner (Becton Dickinson, San Jose, CA) was used for analysis. Results were expressed as the mean percentage of gated cells ± SEM.

Separate groups of wild-type C57BL/6 animals were pretreated with indomethacin, rofecoxib, or vehicle control on the day prior to LPS or PBS injection. Both COX inhibitors were added to the drinking water at a concentration of 0.03 mg/ml, and the mice were left to drink ad libitum (28). Based on the spontaneous water consumption by the animals, the dose of COX inhibitor was approximately 7.5 mg/kg of body weight/day per 20 g mouse.

**RNA isolation from thymocytes.** Eight-week-old wild-type C57BL/6 mice were injected with 50 μg of LPS as described above. At the designated time points (3,
Detection of genes involved in apoptosis. To detect mRNA for a variety of apoptotic genes, commercially available multiple probe template sets (Riboquant; Pharmingen) were labeled with \( \text{[\text{-}32P\text{-}UTP} \) (Perkin-Elmer), according to the manufacturer’s instructions, and then diluted to a concentration of 300,000 cpm/\( \mu \)l of hybridization buffer. All reagents used in probe synthesis were obtained from Pharmingen (In Vitro Transcription kit, catalog no. 45004K). RNA samples were thawed on ice and passed through a 70-\( \mu \)m pore-size filter into a 50-ml conical tube, and 10 ml of PBS was immediately added to stop the lysis reaction. The cells were mixed, divided into three tubes (5 ml/tube), and centrifuged at 1,200 rpm and 4°C for 5 min. The supernatant was completely removed, and the RNA was extracted from the cell pellets with an RNaseasy mini kit (QIAGEN), according to the manufacturer’s instructions, and stored in RNase-free water at \( -70°C \).

**Detection of prostanoids in thymic supernatants.** Wild-type C57BL/6 mice (3-month-old littermates) were allocated to receive regular tap water, 0.03 mg/ml of indomethacin, or 0.03 mg/ml of rofecoxib. Twenty-four hours later, half of the mice receiving tap water alone were injected with PBS vehicle. The remaining animals were injected with LPS, as described above. Twenty-four hours after injection, the animals were killed with an overdose of pentobarbital (100 mg/kg), followed by cervical dislocation. Detection of prostanoid levels in thymic supernatants was performed as described previously (36). Briefly, the thymuses were harvested and placed inside a glass homogenizer containing 6 ml of Krebs-Henseleit medium. The thymuses were manually homogenized and the homogenates were placed in a 37°C water bath incubator in a 5% CO\(_2\) atmosphere. After 30 min of incubation, the homogenates were transferred to 5-ml polypro-
lymphocyte apoptosis. To document the effects of LPS on the thymus in vivo, wild-type C57BL/6 mice were treated with 50 μg of LPS intraperitoneally or a similar volume of PBS control. Twenty-four hours later, the animals were killed, the thymuses were harvested, the thymocytes were counted, and the cell surface phenotypes were determined by flow cytometry. At this dose of LPS, the animals developed diarrhea, piloerection, and reduced activity levels. However, this dose of LPS did not cause any mortality in this or any other experimental group. After LPS injection, there was a dramatic, ∼85% reduction in total thymocytes from (62 ± 19) × 10^6 to (9 ± 2) × 10^6 cells (Fig. 1a). As illustrated in Fig. 1b and c, the percentage of the population of CD4^+ CD8^+ immature T-cell precursors decreased by ∼53%, from 88% ± 1% to 41% ± 5% of total gated cells (P < 0.001).

This marked reduction in thymocyte numbers was accompanied by increased expression of a panel of apoptotic genes (Fig. 2). At the baseline, the expression of most genes involved in apoptosis was very low or undetectable. Three hours after an i.p. injection of 50 μg of LPS, there was a marked increase in the expression of caspases and genes of the mitochondrial and FAS pathways (Fig. 2a to c). For example, the expression of caspase-3, a cysteine protease that has a distinct role in the final induction of apoptosis, increased from 0.09 ± 0.01 at the baseline to 0.21 ± 0.03 at 3 h (P = 0.027; mean density of target band/mean density of housekeeping gene) (Fig. 2d). The expression of proapoptotic genes (BAX, BAK, BAD) and antiapoptotic genes (BCL-W, BCL-X, BLF-1, BCL-2) of the Bcl-2 family was also markedly increased in response to LPS. As depicted in Fig. 2e, expression of the prosurvival gene BCL-X increased from 0.12 ± 0.02 to 0.58 ± 0.01 (P < 0.001) 3 h after LPS injection. Genes of the FAS pathway were similarly induced by LPS administration; the expression of the death receptor FAS was undetectable at the baseline but increased rapidly after LPS injection, achieving peak levels (0.28 ± 0.02) at 6 h (Fig. 2f).

**RESULTS**

**LPS causes thymocyte apoptosis.** To document the effects of LPS on the thymus in vivo, wild-type C57BL/6 mice were treated with 50 μg of LPS intraperitoneally or a similar volume of PBS control. Twenty-four hours later, the animals were killed, the thymuses were harvested, the thymocytes were counted, and the cell surface phenotypes were determined by flow cytometry. At this dose of LPS, the animals developed diarrhea, piloerection, and reduced activity levels. However, this dose of LPS did not cause any mortality in this or any other experimental group. After LPS injection, there was a dramatic, ∼85% reduction in total thymocytes from (62 ± 19) × 10^6 to (9 ± 2) × 10^6 cells (Fig. 1a). As illustrated in Fig. 1b and c, the percentage of the population of CD4^+ CD8^+ immature T-cell precursors decreased by ∼53%, from 88% ± 1% to 41% ± 5% of total gated cells (P < 0.001).

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**LPS injection enhances prostanoid production in the thymus.** To test whether LPS injection into mice affects prostanoid production in the thymus, we measured the concentrations of PGE2 and of the stable thromboxane metabolite TXB2 in the supernatants of thymic homogenates 24 h after i.p. injection of 50 μg of LPS (n = 3) or vehicle (n = 3). As shown in Fig. 3, a single injection of LPS led to significant increases in prostanoid generation by the thymus. Twenty-four hours after LPS injection, PGE2 levels were increased almost twofold (28.25 ± 2.80 versus 53.18 ± 8.63 pg/μg protein; P = 0.02) (Fig. 3a). As shown in Fig. 3b, TXB2 levels were similarly increased (5.08 ± 0.31 versus 9.98 ± 1.24 pg/μg protein; P = 0.003).
COX inhibition does not protect against LPS-induced thymocyte apoptosis. To assess whether enhanced prostaglandin generation in the thymus modulates thymocyte apoptosis after LPS injection, we next tested the effect of COX inhibition. In these studies, wild-type C57BL/6 mice received the nonselective COX-1 and COX-2 inhibitor indomethacin (n = 7), rofecoxib (n = 5), or vehicle control (n = 12) in the drinking water beginning the day prior to LPS injection. Each experimental group had its own control, consisting of animals treated with indomethacin (n = 3), rofecoxib (n = 3), or vehicle control (n = 5) in the drinking water and injected with a similar volume of PBS. Twenty-four hours after injection, apoptotic thymocyte deletion was assessed as described in the text. The data are expressed as the percentage of the control thymocyte count (thymocyte count of experimental group divided by the mean thymocyte count of the respective PBS control and multiplied by 100). Neither indomethacin nor rofecoxib protected against the marked apoptotic thymocyte deletion induced by LPS administration.

Thromboxane A₂ contributes to LPS-induced thymocyte apoptosis in vivo. Although our studies with global COX inhibitors did not clearly reveal a role for prostaglandins in thymocyte depletion after LPS treatment, the various COX products may have distinct and opposing actions on thymocyte apoptosis. Because TXA₂ has been suggested to be a potential trigger for thymocyte apoptosis and TP receptors are highly expressed in the thymus, we assessed the effects of LPS injection in inbred C57BL/6 mice with targeted deletion of TP receptors. By comparison with animals treated with PBS, we found no differences in thymus weight, thymocyte count, or the percentage of CD4⁺ CD8⁺ cells between TP⁺/⁺ and TP⁻/⁻ mice. These results are similar to those from our initial characterization of the TP⁻/⁻ mouse line (37). After LPS injection, systemic manifestations of diarrhea, piloerection, and hypoactivity were similar in TP⁺/⁺ and TP⁻/⁻ animals. However, compared to the TP⁺/⁺ animals, the effects of LPS on thymocyte apoptosis in the TP⁻/⁻ mice were significantly attenuated. For example, LPS reduced the total thymocyte count by only ~68% in TP⁺/⁺ mice, from (56 ± 7) × 10⁶ to (18 ± 3) × 10⁶ cells. This reduction was less dramatic than the ~85% reduction seen in wild-type mice. Twenty-four hours after LPS injection, total thymocyte counts were two times higher in TP⁺/⁻ mice than in TP⁻/⁻ mice (18 ± 3 million versus 9 ± 2 million cells; P = 0.026) (Fig. 5a). Moreover, there was a significant preservation of CD4⁺ CD8⁺ cells in the TP-deficient animals. This cell compartment was reduced by only ~30%, from 88% ± 1% to 62% ± 6% of gated cells in TP⁻/⁻ mice (Fig. 2c), compared to the 53% reduction observed in the controls (Fig. 2b) (P = 0.02). Together, these data indicate an important role for TXA₂, which acts through the TP receptor to promote thymocyte apoptosis following exposure to LPS.

**DISCUSSION**

In our experiments, LPS administration to wild-type mice resulted in a dramatic reduction in thymocyte numbers, mostly in the CD4⁺ CD8⁻ compartment. This reduction in CD4⁺ CD8⁻ thymocytes was likely a result of apoptotic cell death, as previously demonstrated by others (25, 42, 43) and as suggested by our findings of up-regulation of proapoptotic genes of the Fas and mitochondrial pathways in thymocytes from mice exposed to LPS. The main findings of this study are that LPS exposure results in a significant increase in intrathymic levels of TXB₂ and that, when thromboxane signaling is absent (as in TP⁻/⁻ mice), the apoptotic response to LPS is significantly attenuated. Taken together, our data suggest that TXA₂...
might function as an effector molecule in LPS-induced thymocyte apoptosis.

Thymocytes harvested from mice 3 to 6 h after LPS administration demonstrated a marked up-regulation in the expression of pro- and antiapoptotic genes. Pro- and antiapoptotic family members can heterodimerize and seemingly titrate one another’s function, suggesting that their relative concentration may act as a rheostat for the suicide program. Thus, the delicate balance between these competing activities determines cell fate (1). In our study, the antiapoptotic protein BCL-X was strongly upregulated by LPS; this had been previously shown by others (11, 14). However, Fig. 2 shows that proapoptotic Bcl-2 family members, FAS-related genes, and caspases were also up-regulated by LPS. Our data clearly demonstrate that LPS administration induces a marked thymocyte loss, which indicates that proapoptotic stimuli prevail in this setting.

A apoptotic cell death is a prominent feature of sepsis (4, 5, 18, 29, 40). While parenchymal cell apoptosis is thought to be detrimental during sepsis (9, 10), the role of lymphocyte apoptosis is more controversial. Some propose that lymphocyte apoptosis during sepsis might be beneficial by down-regulating the immune response and decreasing the production of pathogenic proinflammatory cytokines (12). However, experimental data by Hotchkiss et al. indicate that lymphocyte apoptosis impairs host responses during sepsis and contributes to mortality (17, 20). Several mediators, such as TNF (42), adrenal steroids (22, 25), the complement split product C5a (15, 31), and nitric oxide (44), have been implicated in sepsis- or LPS-induced thymocyte apoptosis. However, the role of prostanoid inflammatory mediators in this process had not been previously investigated.

Prostanoids are key regulators of thymocyte physiology (34,
The enzymes involved in prostanoid biosynthesis are abundantly present in the thymus. COX-1 and COX-2 are constitutively expressed in all cell types; COX-1 is strongly expressed in the cortical cells, whereas COX-2 is highly expressed in the medulla. COX-2 is the inducible isofrom and is responsible for the surge in prostanoïd production in response to inflammatory stimuli and cytokines. PGE and thromboxane synthases are also present in thymic stromal cells and are responsible for the production of PGE₂ and TXA₂. In vitro data suggest that these molecules have opposite effects on CD4⁺ CD8⁺ thymocytes: PGE₂ appears to protect these cells from apoptosis, whereas TXA₂ appears to promote apoptosis of these cells (13). In our study, the intrathymic levels of both PGE₂ and TXA₂ were significantly increased after LPS injection. When only thromboxane signaling was blocked (as was the case in TP-/- mice), thymocyte apoptosis was attenuated. However, global inhibition of prostanoïd synthesis with indomethacin or rofecoxib did not confer similar protection. The difference in the outcomes of these experiments reflects distinct consequences of global inhibition of prostanoïd synthesis (i.e., COX inhibition) compared to inhibition of a single prostanoïd acting at its individual receptor. PGE₂ is known to protect against thymocyte apoptosis in vitro (13), and it is therefore likely that concomitant inhibition of PGE₂ synthesis (and perhaps other unmeasured protective prostanoïds) by indomethacin and rofecoxib negates the beneficial effects of TXA₂ inhibition in this setting. In patients with sepsis, treatment with ibuprofen reduces the levels of prostacyclin and thromboxane and decreases fever, tachycardia, oxygen consumption, and lactate acidosis; but it does not prevent the development of shock or the acute respiratory distress syndrome and does not improve survival (7). Perhaps future therapies aimed specifically at blocking TP signaling will produce better results.

Based on previous studies showing the potent actions of TP agonists in the induction of thymocyte apoptosis (41) and our findings of significant enhancement of TXA₂ generation by thymocytes after LPS administration, it is very likely that TP receptors promote LPS-induced thymocyte loss through direct effects in the thymus. Nonetheless, our studies cannot completely exclude the possibility that indirect, systemic actions of TP receptors might contribute to this effect. For example, it has been shown that abrogation of TP signaling during sepsis attenuates endotoxin-mediated acute renal failure (8) and intestinal ischemia (23), reduces TNF-α levels (3), and helps preserve pulmonary and circulatory function (21). In rats receiving an endotoxin challenge, administration of the TP receptor antagonist BAY U 3405 led to an increase in the survival rate at 48 h from 0% to 45% (2).

Several strategies to block inflammatory mediators have been attempted in patients with sepsis (32, 33), but only a few have proven effective (6). In animal models, recent interventions aimed at reducing apoptosis have proven effective in improving survival. Our data suggest that by attenuating thymocyte apoptosis in response to LPS, the TP receptor signaling blockade might constitute a potential therapeutic target in sepsis.

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