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MODULATION OF THE BCL-2 FAMILY BLOCKS SEPSIS-INDUCED DEPLETION OF DENDRITIC CELLS AND MACROPHAGES

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Abstract

This study examined the fate of dendritic cells (DCs) and macrophages (MΦ) *in vivo* in a murine model of sepsis. Wild-type, knockout, and transgenic mice were used to examine the role of Bcl-2 family members on the regulation of splenic DCs and MΦ survival. Bim knockout (Bim^{-/-}) mice and mice overexpressing Bcl-2 in selected hematopoietic cells were used: (a) overexpression of Bcl-2 in all hematopoietic cells using a vav promoter (Vav-Bcl-2) and (b) overexpression of Bcl-2 in all MHC class I cells (H-2K-Bcl-2). Mice underwent sham surgery or cecal ligation and puncture, and absolute numbers of splenic DCs and MΦ were determined. Importantly, two distinct MΦ populations, that is, well-differentiated “mature” MΦ population and a less differentiated “immature,” “monocyte-like” (IMΦ) population were identified that demonstrated differential susceptibility to apoptosis. In wild-type mice, sepsis induced a 64% ± 7% and a 77% ± 3% decrease in absolute cell numbers of splenic DCs and IMΦ, respectively (n = 7, P < 0.05). Mature MΦ were not depleted in sepsis. No significant cell depletion was evident in Vav-Bcl-2, H-2K-Bcl-2, or Bim^{-/-} mice. We conclude that sepsis induces a major depletion of developing MΦ as well as DCs, and this depletion may be an important mechanism of immune suppression in sepsis.

Keywords

Apoptosis; cytokine; cecal ligation and puncture

INTRODUCTION

Despite highly specialized delivery of intensive care, ~250,000 critically ill patients die of sepsis annually (1–3). Studies elucidating the mechanisms of this complex disease are essential

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for the development of a more effective therapy (4). Macrophages (MΦ) and dendritic cells (DCs) are key components of the innate immune system and are essential in orchestrating an effective host response. These cells present antigen to T cells and produce a myriad of cytokines that activate other effector cells (5). Dendritic cells are the most efficient antigen-presenting cells and can help direct the immune response to a proinflammatory or anti-inflammatory phenotype (6). Macrophages and immature DCs also engage in phagocytosis, thereby providing a frontline defense against microorganisms and ensuing efficient bacterial clearance. Yet, in sepsis, this response is severely impaired (6–8).

A key pathophysiological event in sepsis is the widespread apoptosis of immune effector cells. Studies by numerous independent laboratories have shown that sepsis induces profound apoptosis of CD4⁺ T cells and B220⁺ B cells (8–11). A great deal less is known regarding the fate of MΦ and DCs in sepsis because of the difficulty in distinguishing apoptotic MΦ and DCs from live cells that have ingested apoptotic cells. Therefore, many diagnostic tests for apoptosis have a high false-positive rate when applied to phagocytic cells because of the apoptotic debris that is present within these cells (9,12). Despite these technical difficulties, several groups have reported that MΦ and selected subsets of DCs undergo apoptosis in sepsis (13–15). Although numerous studies have characterized *in vitro* monocyte (MO) and MΦ dysfunction (13,16–19) during sepsis, few studies have examined the *in vivo* propensity of MΦ and DCs to undergo apoptosis.

The Bcl-2 family consists of both antiapoptotic and proapoptotic proteins that regulate cell survival in a number of pathological conditions including I/R injury, hypoxia, and toxin exposure. A number of studies demonstrated that overexpression of Bcl-2 prevented apoptotic cell death of lymphocytes during sepsis (20–23). Ding et al. (14) have reported that myeloid-restricted overexpression of Bcl-2 only minimally protected splenic DCs (CD11b⁺) from sepsis-induced cell depletion. However, few studies have examined whether the deletion of proapoptotic or the overexpression of antiapoptotic proteins (hematopoietic cell or lymphocyte-restricted) prevents splenic MΦ or DC death in sepsis. In this study, we characterized the effect of sepsis on splenic MΦ and DCs and identified a potential mechanism for this cell loss. Based on known changes in gene expression and cellular function that occur with maturation, we hypothesized that different MΦ subsets may have distinct propensities to undergo sepsis-induced apoptosis. We evaluated the role of the Bcl-2 family members in sepsis-induced apoptosis using both transgenic and knockout animals. Cell loss was measured indirectly by determining absolute cell counts via flow cytometry. The intracellular expression of Bcl-2 and Bim was also examined to investigate whether significant changes in these death modulators correlated with cell loss and, ultimately, apoptosis. Finally, we evaluated the *in vivo* functional capacity of these cells to produce the multifunctional cytokine, IL-6, because several studies have reported that high IL-6 concentrations correlated with higher sepsis-induced mortality (24,25).

MATERIALS AND METHODS

Mice

C57BL/6 mice (6 – 8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, Me). Vav-Bcl-2 mice, which overexpress human Bcl-2 on all nucleated cells of hematopoietic tissue (controlled by the vav promoter), were kindly provided by Dr Jerry Adams (WEHI Institutes) (26). H-2K-Bcl-2 mice, which overexpress human Bcl-2 on all MHC class I cells (controlled by the H-2K promoter), were kindly provided by Dr Irving Weissmann (Stanford University, Palo Alto, Calif) (27). Bim^{-/-} mice were generated by Drs Philippe Bouillet and Andreas Strasser (28) (Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria, Australia) and were kindly provided by Dr David Holtzman (Washington University School of Medicine, St Louis, Mo). All transgenic and knockout male mice (6 – 8 weeks old) are on

a C57/BL6 background and have been backcrossed for at least 10 generations. Each group of mice was compared with their respective age- and sex-matched controls. Wild-type (WT) littermates or WT “in-house” controls (C57/BL6 mice born and bred in the Washington University Animal Housing facility) were used as controls.

Polymicrobial intra-abdominal sepsis

Mice were housed for a minimum of 1 week before operation. The modified cecal ligation and puncture (CLP) model of polymicrobial intra-abdominal sepsis was used to examine sepsis-induced innate cell loss (29). Animals were prepared for surgery, abdominal hair was shaved, and povidone-iodine (Betadine) was applied to the area. Isoflurane-anesthetized animals underwent a 1-cm abdominal incision. The cecum was ligated below the ileocecal valve with 4-0 silk thread and punctured twice with a 27-gauge needle. Sham mice were treated in an identical fashion, but the cecum was neither ligated nor punctured. The abdomen was closed in two layers with both wound glue and 4-0 silk sutures. After surgery, the animals also received 1 mL of 0.9% saline/buprenorphine (Buprenex; 0.75 mg/kg) subcutaneously. They were allowed water and standard chow *ad libitum*. Animal experimentation and care were approved by the Animal Studies Committee at Washington University School of Medicine and were in accordance with the National Institute of Health animal guidelines.

Flow cytometric detection of different splenocyte populations—phenotype markers and forward- and side-scatter properties

To determine if the putative sepsis-induced loss in splenic DCs and MΦ was due to apoptosis, mice with selective overexpression of Bcl-2 or deletion of Bim were studied. Previous studies have shown that Bcl-2 overexpression or Bim deletion confers marked protection against sepsis-induced lymphocyte apoptosis (20–23). Twenty hours after sham or CLP injury, WT, transgenic, and knockout spleens were harvested and ground between sterile frosted glass slides. Cells were washed and resuspended in 3 mL/spleen of RPMI-1640–10% fetal calf serum (supplemented with 500 ng/mL collagenase type 2 (Worthington Biochemicals, Freehold, NJ) and 0.02 µg/mL DNase I (Sigma, St Louis, Mo) to break adherence and splenic stromal interactions and aid in optimal recovery of DCs and MΦ. Forty-five minutes after continuous shaking at 37°C, erythrocytes were lysed with hypotonic saline, and cells were washed with RPMI 1640–10% fetal calf serum. To diminish nonspecific binding of immunoglobulins to FcγIII and FcγII receptors, cells were incubated for 10 min with purified anti-mouse CD16/CD32 (e-Bioscience, San Diego, Calif). As previously described (30), the cells were then stained for 30 min in the dark with the following appropriate isotype controls and cocktails that were obtained from e-Bioscience unless otherwise noted and include fluorescein isothiocyanate, PE, PE-Cy5, PerCP5.5, APC, Alexa 750, and biotin cell surface markers: anti-mouse CD11b (clone M1/70), CD11c (clone N418), CD3 (clone 145-2C11), CD4 (clone RM4-5), CD8 (clone 53-6.7), CD19 (clone 1D3), CD45R/B220 (clone RA3-6B2), CD68 (clone FA-11; AbD Serotec), F480 (clone BM8), CD49b (clone DX5), Gr-1 (clone RB6-8C5), Ly6.C (clone HK1.4; Abcam, Cambridge, Mass), MHCII (clone M5/114.15.2; BD Pharmingen, San Jose, Calif), NK1.1 (clone PK136). Twenty hours after sham or CLP injury, splenic cell populations were identified via specific cell surface phenotypes. Characteristic forward- and side-scatter properties of different cell types, that is, lymphocytes, neutrophils, MΦ, or DCs were used as confirmatory indicators of cell subsets and to exclude dead cells.

Flow cytometry quantification of splenic cell loss

Cells were analyzed (100,000 – 150,000 events per sample) via FACScan flow cytometer (BD Biosciences, San Jose, Calif) (upgraded to five colors by Cytek, Freemont, Calif), which used both CELL Quest and Rainbow (Cytek) software for data acquisition. Flow Jo (TreeStar, Ashland, Ore) software was used to further analyze the data to quantitate cell loss. Forward-

and side-scatter parameters were used to define specific cell populations (T and B lymphocytes, granulocytes, and natural killer cells) and eliminate those cell types from further analysis).

The decision not to use conventional markers of apoptosis, such as TUNEL, active caspase 3, propidium iodide, or annexin V, was warranted in our *in vivo* CLP model because phagocytic cells can stain false positive. Apoptotic cells ingested by MΦ can transfer their membrane to the surface. Thus, the MΦ can stain falsely positive for annexin V (9,12). Because MΦ are highly phagocytic, which may cause them to stain falsely positive for TUNEL or active caspase 3, apoptosis was measured indirectly by a decrease in absolute cell counts. Total cell counts per spleen were determined via the Vi-Cell counter (Beckman Coulter), which was used as 100% for further calculation of subset populations of total cell counts. Flow cytometric analysis provided the percentages for the various splenic populations, that is, CD4⁺ T cells, CD11b⁺, F4/80⁺, MΦ, and so on. The cell counts for each splenic subset population were calculated using the following formula: cell counts of subpopulations = total cell counts [determined by Vi-Cell counter] × subset population percentage [determined by flow cytometer] / 100.

Determination of Bcl-2 and Bim expression in splenic MΦ

Wild-type cells were prepared and identified using fluorescently labeled surface markers as described above. Cells were permeabilized with 1× Perm Wash, washed, and stained with PE-labeled anti-Bcl-2 (e-Bioscience) overnight at 4°C. An additional group of cells was examined for Bim expression using a primary rabbit anti-Bim antibody and a PE-labeled donkey anti-rabbit secondary antibody (Cell Signaling Technology).

Intracellular cytokine production

The percentage of intracellular IL-6–positive cells was examined *in vivo* 6 h after the onset of sepsis (CLP). Mice underwent sham or CLP and, 90 min after operation, received brefeldin A (250 μg/500 μL) via i.v. injection (tail vein). The brefeldin A regimen was modified from Liu and Whitton (31), and the animals were given 500 μL instead of 400 μL of the 250-μg solution. Brefeldin A is a Golgi transport inhibitor that allows the accumulation of cytokines, which can be detected via flow cytometry. Approximately 6 h after brefeldin A injection, splenocytes were harvested. The cells were permeabilized and labeled with fluorescein isothiocyanate–anti-IL-6 (e-Bioscience) for 3 h at 4°C. The cells were washed with PBS and analyzed (150,000–300,000 events per sample) via a FacScan flow cytometer to determine intracellular mediator production.

Statistical analysis

Data are reported as the mean ± SEM. The Student *t* test was used in analyzing data involving only two groups and the one-way ANOVA with Tukey multiple-comparisons test was used to analyze data with more than two groups (nonparametric) using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, Calif; www.graphpad.com). Significance was reported at *P* < 0.05.

RESULTS

Sepsis-induced differential MΦ cell loss from the spleen

Two distinct splenic MΦ populations (Fig. 1, A and B) were identified in WT sham and CLP-operated mice. Splenic MΦ were categorized as either immature MΦ (IMΦ) or mature MΦ (MMΦ) based on their expressions of CD11b and F4/80. F4/80 is expressed on mostly resident tissue MΦ in the red pulp of the spleen and is significantly lower on less mature precursors of tissue MΦ (32, 33). Although both MΦ populations stained positive for intracellular macrophage markers (murine CD68, a heavily glycosylated transmembrane protein that is specifically

expressed by tissue MΦ) (data not shown), they differed in CD11b and F4/80 cell surface expression. The less differentiated IMΦ stained CD11b^{hi}F4/80^{inter}, whereas the well-differentiated MMΦ stained F4/80^{hi}CD11b^{inter}. The MΦ population stained positive for CD68, CD11b, and F4/80 and negative for CD11c, Gr-1, CD3, CD4, CD8, B220, and NK1.1, thereby confirming that these cells were in fact MΦ and not DCs, granulocytes, T cells, B cells, or natural killer cells. Interestingly, compared with WT sham mice, WT CLP mice exhibited a $77\% \pm 3\%$ decrease in total cell counts in the IMΦ population ($n = 8 - 14$ animals per group, $P < 0.05$; Fig. 1E).

Sepsis-induced DC loss from the spleen

A DC population was also identified that stained highly positive for both CD11c and MHCII (Fig. 1, C and D). Interestingly, compared with WT sham mice, WT CLP mice exhibited a $64\% \pm 7\%$ decrease in total cell counts in the DC population ($n = 8 - 14$ animals per group, $P < 0.05$; Fig. 1E). In contrast to the findings in the IMΦ and DCs, the absolute cell counts of MMΦ were not significantly decreased in sepsis in any mouse model (Fig. 1E).

Bcl-2 overexpression prevented sepsis-induced IMΦ and DC depletion

To determine whether the depletion of IMΦ was indicative of apoptosis, the effects of overexpression of human Bcl-2 was investigated in both sham and CLP injury in mice whose cells had selected overexpression of antiapoptotic proteins. Interestingly, overexpression of human Bcl-2 broadly in all hematopoietic-derived cells (Vav-Bcl-2 mice) and on all MHC class I cells (H-2K-Bcl-2 mice) did confer protection during sepsis to the splenic IMΦ population. IMΦ from Vav-Bcl-2 septic mice exhibited $14\% \pm 12\%$ cell loss compared with the $76\% \pm 9\%$ IMΦ loss in the WT septic mice (Fig. 2A). Similar to the septic Vav-Bcl-2 mice, septic H-2K-Bcl-2 mice conferred protection upon the IMΦ population, and minimal cell loss occurred ($n = 3 - 6$ per group, $P < 0.05$; Fig. 2B). Similar to the protection exhibited in the IMΦ population during sepsis, the DC population exhibited minimal depletion in septic Vav-Bcl-2 mice compared with septic WT mice. Dendritic cells from Vav-Bcl-2 septic mice exhibited only a $6\% \pm 2\%$ cell loss compared with the WT septic mice that exhibited a pronounced $72\% \pm 6\%$ cell loss ($n = 3 - 6$ per group, $P < 0.05$; Fig. 2A).

Bim^{-/-} mice exhibited significantly less sepsis-induced total cell loss in the splenic IMΦ and DC population

To confirm that IMΦ and DC cell loss was secondary to apoptosis, a second apoptotic strategy using Bim null mice was used. Wild-type sham and Bim^{-/-} sham mice did not display depletion in either the splenic IMΦ or the DC population. The sepsis-induced depletion of IMΦ and DCs in WT mice was attenuated in septic Bim^{-/-} mice. Only $22\% \pm 2\%$ of IMΦ in septic Bim^{-/-} mice were lost compared with $62\% \pm 8\%$ in the septic WT mice. Similarly, DCs in septic Bim^{-/-} mice showed an $11\% \pm 5\%$ loss compared with a $58\% \pm 3\%$ loss in septic WT animals ($n = 8 - 14$ per group, $P < 0.05$; Fig. 3).

The expression of Bcl-2 was significantly decreased whereas Bim was increased in septic splenic IMΦ

To determine potential mechanism or pathways responsible for cell depletion during sepsis, intracellular content of Bcl-2 and Bim was examined in MΦ. The ratio of Bcl-2 to Bim has been shown to be a critical factor in cell survival (34,35). Previous results from our laboratory have shown that lymphocytes that overexpressed Bcl-2 or have a deletion of Bim are resistant to sepsis-induced apoptosis (22,36). Our data show that overexpression of Bcl-2 or deletion of Bim prevented IMΦ and DC cell loss during sepsis. We sought to determine if differences in Bcl-2 and Bim expression may be involved in the different sensitivity to undergo apoptosis exhibited by these two cell populations. We analyzed intracellular expression of Bcl-2 and Bim

in the two MΦ populations in WT mice. Septic IMΦ exhibited a significant reduction in Bcl-2 expression (mean fluorescence intensity) compared with MMΦ (22 ± 4 vs. 67 ± 7 , $n = 7$ per group, $P < 0.05$), respectively (Fig. 4A). Conversely, sepsis did not cause a significant difference in Bim expression between the IMΦ and MΦ populations (43 ± 9 vs. 38 ± 14 , $n = 7$ per group, $P < 0.05$), respectively (Fig. 4B).

The ratio of Bcl-2 to Bim expression in WT sham animals was not significantly different between the IMΦ and MMΦ (2.1 ± 0.2 vs. 2.3 ± 0.1). Not surprising, in septic WT mice, the ratio of Bcl-2 to Bim was significantly decreased in the IMΦ compared with the MMΦ, 0.5 ± 0.1 vs. 1.7 ± 0.3 , respectively ($n = 7$ per group, $P < 0.05$).

Differential *in vivo* intracellular IL-6 mediator production

Studies have implicated IL-6 production as a predictor of sepsis-induced mortality in the CLP model of sepsis (24,25). To characterize potential differences in the two MΦ subtypes in regard to cytokine production, we analyzed the percentage of cells obtained from sham or septic mice that stained positive for IL-6. Ninety minutes after sham or CLP injury, mice received brefeldin A via i.v. injection. Brefeldin A prevents Golgi transport, thus inducing the accumulation of intracellular cytokines, which can be detected by flow cytometry (31). Six hours later, minimal to no intracellular IL-6-producing cells were seen in sham mice. In contrast, in CLP mice, $34\% \pm 4\%$ of MMΦ and $16\% \pm 2\%$ of IMΦ were IL-6-positive ($n = 6$, $P < 0.05$; Fig. 5).

DISCUSSION

Previous studies have shown that systemic apoptosis occurs diffusely in lymphoid tissue and correlates with sepsis-induced morbidity and mortality (9,10). Few studies have examined whether MΦ undergo programmed cell death in sepsis because of difficulties in the specificity of the apoptotic assays in these phagocytic cells. Macrophages that have engulfed apoptotic cells will be falsely positive when evaluated by most tests for apoptosis. Consequently, we elected to evaluate apoptosis by quantifying absolute cell counts in WT mice and mice resistant to apoptosis because of modulation of the Bcl-2 family. The current study demonstrates that sepsis also induced depletion of a selective subset of splenic MΦ and DCs. The fact that IMΦ and DC depletion was inhibited by overexpression of the antiapoptotic protein, Bcl-2, or deletion of the proapoptotic protein, Bim, strongly supports the contention that the cells that were being lost were dying of apoptosis. The splenic MΦ population is heterogeneous. Developmental, functional, and phenotypic differences exist among the diverse MΦ population and may, in part, be responsible for the differential cell loss and *in vivo* cytokine production demonstrated in the IMΦ versus the more differentiated MMΦ noted in the present study (37–39). Loss of the IMΦ and DC populations may have important consequences on host immune function and may contribute to impaired bacterial phagocytosis and clearance. This loss of MΦ and DCs will also have detrimental effects on efficient antigen presentation to lymphocytes (40).

One of the interesting findings in the present study is the differential susceptibility of the IMΦ versus the MMΦ to undergo apoptosis. Unlike the MMΦ that were resistant to cell loss, IMΦ exhibited a marked decrease in absolute cell counts at 20 h after the onset of sepsis. The present study demonstrates for the first time that, in the CLP model of sepsis, there was a significant decrease in absolute cell counts of splenic IMΦ ($CD11b^{high}/F480^{inter}$), whereas the highly differentiated MMΦ ($F480^{high}/CD11b^{inter}$) were unaffected. The data suggest that sepsis induced differential cellular apoptosis in MΦ. We also demonstrated that splenic DCs ($CD11c^{high}/MHCII^{high}$) underwent sepsis-induced depletion. It has been reported that sepsis induced a significant decrease in the percentage of DCs that stain single positive for CD11c but not DCs that stain double positive for CD11c and MHCII (14). This discrepancy may be

due to differences in the mouse strain, CLP injury, DC harvest methodology, and/or flow cytometry cell-gating strategies.

Several antiapoptotic and proapoptotic protein transgenic and knockout mouse models have been established and shown to abolish sepsis-induced lymphocyte apoptosis (20,36,41). The present study extends these findings by demonstrating that modulation of antiapoptotic and proapoptotic Bcl-2 family members prevents sepsis-induced loss of MΦ and DCs as well. Sepsis-induced depletion of splenic IMΦ was attenuated by overexpression of the human antiapoptotic protein Bcl-2 on all nucleated cells of hematopoietic tissues (Vav-Bcl-2 mice) and all MHC class I cells (H-2K-Bcl-2 mice).

The vulnerability of cells to undergo apoptosis has been reported to be dependent on the ratio of antiapoptotic versus proapoptotic Bcl-2 family members (34,35). Given previous work from the laboratory demonstrating the key roles of Bcl-2 and Bim in sepsis-induced apoptosis, we examined the Bcl-2/Bim ratio as an index of apoptotic propensity. In the septic IMΦ population, there was a marked decrease in intracellular antiapoptotic Bcl-2 expression coupled with a significant increase in intracellular proapoptotic Bim expression. Importantly, the Bcl-2/Bim ratio was significantly decreased in the IMΦ compared with the MMΦ population, and this finding is consistent with the increased cell loss in the former compared with the latter. Along the same lines, it has been reported that staphylococcal enterotoxin A-activated T cells undergoing apoptosis exhibited a ~50% decrease in their expression of Bcl-2 compared with resting T cells (42,43). Furthermore, our studies showing decreased IMΦ Bcl-2 concentration are consistent with the data by Adrie et al. (44), who noted that blood MOs from septic nonsurvivors exhibit significantly less intracellular Bcl-2 expression compared with septic survivors.

Finally, the data demonstrating differential susceptibility to apoptosis in IMΦ versus MMΦ are paralleled by studies in differentiated MOs. Perlman et al. (45) have reported that, during MO differentiation into MΦ, the expression of FLICE-inhibitory protein (Flip) prevented the activation of Fas-mediated apoptosis in MΦ. Thus, highly differentiated MΦ were more resistant to apoptosis than the less differentiated MO. The present data demonstrate that immune effector cells from the Bcl-2 transgenic and Bim null mice are not lost in sepsis. This finding establishes that cell depletion in sepsis is secondary to programmed cell death and suggests potential therapeutic implications. Previous work from Wesche-Soldato et al. (46) as well as that from our group (47) has shown that siRNAs directed against the caspase family or the proapoptotic Bcl-2 family members prevented apoptotic death and improved survival in sepsis. Macrophages and DCs are able to “take up” siRNAs, and thus, anti-Bim siRNA therapy may be one method to prevent loss of these critical cells during sepsis.

The splenic MΦ populations differed not only in their phenotypic cell surface marker expression, Bcl-2/Bim ratios, and propensity to undergo sepsis-induced apoptosis, but also in their capacity to produce IL-6. The ability to investigate *in vivo* intracellular mediator production allows one to detect tissue cell-specific cytokine responses. Because MΦ are a major source of IL-6, we investigated the effects of sepsis on MΦ production of IL-6. The 6-h time point was examined because the absolute cell counts of the splenic IMΦ population are comparable between both sham and CLP animals. IL-6 elicits both proinflammatory and anti-inflammatory responses (48) and is upregulated in the plasma for up to 24 h after CLP (25). *In vivo*, 6 h after the onset of sepsis, a significantly higher percentage of MMΦ (34% ± 4%) was IL-6-positive compared with the percentage of IMΦ (16% ± 2%). However, the former has an inherently higher IL-6-producing capacity. The differential production of IL-6 may in part be due to the maturation of the populations and the efficiency of downstream IL-6 receptor signaling. It may be of relevance to further characterize mediator production in both MΦ subsets during the progression of sepsis.

In summary, sepsis-induced extensive depletion of IM Φ and DCs and the loss in these important immune effector cells may seriously impair host antimicrobial defenses. The ability to prevent cell loss through the modulation of Bcl-2 family members such as overexpression of antiapoptotic proteins or deletion of proapoptotic proteins suggests that these cell populations undergo sepsis-induced apoptosis *in vivo*. The decreased Bcl-2/Bim ratio may be an important mechanism of sepsis-induced depletion of IM Φ . Antiapoptotic therapies targeting these cell populations may marshal the host response and decrease sepsis-induced morbidity and mortality.

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Fig. 1.

Sepsis induces differential splenic cell loss. A and B, Representative dot plot of splenic populations identified in sham and CLP mice ($n = 1$ mouse per sham or CLP group). Two distinct MΦ populations, that is, a “mature” macrophage (MMΦ-F480^{hi}CD11b^{inter}) population and an “immature” MO-like (IMΦ-CD11b^{hi}F480^{inter}) population, were identified based on differential cell surface receptor expression. C and D, Representative dot plot of splenic DC (CD11c^{hi}MHCII^{hi}) population identified in sham and CLP mice ($n = 1$ mouse per sham or CLP group). E, IMΦ and DCs were significantly decreased in total cell counts in septic animals. * $P < 0.05$ vs. corresponding sham group. In contrast, the MMΦ total cell counts were not significantly altered. Total cell counts of subpopulations are equal to the total splenic cell count multiplied by the subset population divided by 100 ($n = 8 - 14$ /group for WT mice).

Fig. 2.

Overexpression of human Bcl-2 attenuates sepsis-induced cell loss. Both septic Vav-Bcl-2 (A) and H-2K-Bcl-2 (B) mice exhibited significantly less total cell loss in the IMΦ and DC populations compared with septic WT mice. * $P < 0.05$ vs. corresponding sham group ($n = 10$ each for Vav-Bcl-2 mice, H-2K-Bcl-2, and WT mice).

Fig. 3. Bim^{-/-} mice exhibit attenuated sepsis-induced total cell loss. Minimal IMΦ and DC total cell loss was exhibited in the septic Bim^{-/-} mice compared with the septic WT mice. **P* < 0.05 vs. corresponding sham group (n = 10/group for Bim^{-/-} mice and n = 10/group for WT mice).

Fig. 4.

Differential Bcl-2 and Bim expression is exhibited in the splenic MΦ. Intracellular Bcl-2 and Bim (MFI) expression was examined in MMΦ and IMΦ populations obtained from sham and septic animals. A, Intracellular Bcl-2 (MFI) expression was comparable between the MMΦ and IMΦ populations from sham animals. However, in the IMΦ population, sepsis induced a significant decrease in intracellular Bcl-2 (MFI) expression. B, Intracellular Bim (MFI) expression was significantly increased in the (n = 7 per group; * $P < 0.05$).

Fig. 5.

Flow cytometry analysis of *in vivo* intracellular IL-6 expression in splenic MΦ from WT mice that underwent sham or CLP injury. In the CLP group, a higher percentage of both MMΦ and IMΦ expressed IL-6 compared with sham. Overall, there was a higher percentage of IL-6–

positive septic MMΦ compared with the percentage of IL-6–positive IMΦ 6 h after CLP (* $P < 0.05$ vs. corresponding sham group, $n = 6$ mice/group). A, Representative dot plot of unstained, anti-IL-6, isotype control. B, Representative dot plot of sham and CLP groups positive for IL-6 ($n = 1$ for each flow cytogram).