Surviving sepsis: bcl-2 overexpression modulates splenocyte transcriptional responses in vivo

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Wagner TH, Drewry AM, MacMillan S, Dunne WM, Chang KC, Karl IE, Hotchkiss RS, Cobb JP. Surviving sepsis: bcl-2 overexpression modulates splenocyte transcriptional responses in vivo. Am J Physiol Regul Integr Comp Physiol 292: R1751–R1759, 2007. First published January 18, 2007; doi:10.1152/ajpregu.00656.2006.—We hypothesized that spleen microarray gene expression profiles analyzed with contemporary pathway analysis software would provide molecular pathways of interest and target genes that might help explain the effect of bcl-2 on improving survival during sepsis. Two mouse models of sepsis, cecal ligation and puncture and tracheal instillation of Pseudomonas aeruginosa, were tested in both wild-type mice and mice that overexpress bcl-2. Whole spleens were obtained 6 h after septic injury. DNA microarray transcriptional profiles were obtained using the Affymetrix 430A GeneChip, containing 22,690 elements. Ingenuity Pathway Analysis software was used to construct hypothetical transcriptional networks that changed in response to sepsis and expression of the bcl-2 transgene. A conservative approach was used wherein only changes induced by both abdominal and pulmonary sepsis were studied. At 6 h, sepsis induced alterations in the abundance of hundreds of spleen genes, including a number of proinflammatory mediators (e.g., interleukin-6). These sepsis-induced alterations were blocked by expression of the bcl-2 transgene. Network analysis implicated a number of bcl-2-related apoptosis genes, including bcl2L1 (bim), bcl-2L2 (bcl-w), bmf, and mcl-1. Sepsis in bcl-2 transgenic animals resulted in alteration of RNA abundance for only a single gene, ceacam1. These findings are consistent with sepsis-induced alterations in the balance of pro- and anti-apoptotic transcriptional networks. In addition, our data suggest that the ability of bcl-2 overexpression to improve survival in sepsis in this model is related in part to prevention of sepsis-induced alterations in spleen transcriptional responses.

The bcl-2 family of proteins currently includes over 15 members that function either in an anti-apoptotic or a pro-apoptotic fashion (14). The founding member of this family, bcl-2, was discovered by serendipity during investigation of patients with follicular non-Hodgkin’s B cell lymphoma in which chromosomal translocations activate the bcl-2 gene (5, 42). A number of investigators have demonstrated that overexpression of bcl-2 can prevent apoptotic death in many types of cells from an array of insults (growth factor withdrawal, radiation, hypoxia, ionomycin, glucocorticoids, etc.; see Refs. 29, 30, 32, 35, 40). Importantly, bcl-2 has proven capable of blocking cell death not only in vitro but also in vivo. For example, transgenic mice in which bcl-2 has been selectively overexpressed in the cell of cecal ligation and puncture; principal components analysis

interest are protected against brain ischemia, neurotoxins, fas-mediated hepatic necrosis, and other injuries (3, 29, 30, 32).

A number of laboratories, including our own, demonstrated that overexpression of the anti-apoptotic protein bcl-2 in lymphocytes abrogated sepsis-induced lymphocyte apoptosis and caused a dramatic increase in survival (17, 25). In addition, Iwata and associates (26) showed in mice that overexpression of bcl-2 in myeloid-derived cells (circulating neutrophils and monocytes) also demonstrated improved survival in sepsis. Although bcl-2 is postulated to block apoptosis by preventing loss of mitochondrial membrane potential, subsequent investigations have implicated other possible anti-apoptotic mechanisms, including binding/inactivating pro-apoptotic bcl-2 family members and regulation of endoplasmic reticulum calcium release (28).

To explore the potential anti-apoptotic mechanisms responsible for the beneficial effects of bcl-2 in sepsis, we performed a survey of transcriptional changes induced by sepsis in mouse spleens (a site of a high degree of apoptotic cell death). We hypothesized that spleen microarray gene expression profiles coupled with contemporary pathway analysis software could reduce the genome-wide space of potential gene-gene interactions (2), providing novel molecular insight and direction for future investigations. To increase confidence in the analysis, two clinically relevant mouse models of sepsis (cecal ligation and puncture and pneumonia) were tested in both wild-type mice and mice that overexpress bcl-2. In silico network analysis was used to identify informational genes, the importance of which was validated in subsequent studies (3, 22).

METHODS

Animals. Mice that were heterozygous for the H2K-Bcl-2 transgene on a C57BL/Ka Thy-1.1, CD45.2 background were kindly provided by Dr. Irving Weissman (8, 9). The H2K promoter induces expression of human bcl-2 in hematopoietic stem cells with resultant expression in both lymphoid and myeloid-derived cells. Thus human bcl-2 is expressed in lymphocytes, monocytes, and granulocytes. These mice were backcrossed seven generations onto a C57BL6 background. Male heterozygous mice (5–15 wk old) were used in all studies to avoid confounding effects of changes occurring during the estrus cycle. Flow cytometry and immunostaining for human bcl-2 of circulating peripheral blood cells was used to identify mice heterozygous for the H2K-Bcl-2 transgene (data not shown). Control wild-type mice for CLP were either male C57BL6 mice purchased from Jackson laboratories.

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bcl-2 MODULATES SPLEEN TRANSCRIPTIONAL RESPONSES

(Bar Harbor, ME) or nontransgenic littermates. Control animals for the pneumonia experiments were all nontransgenic littermates.

**Sepsis models.** Animal care and use protocols were approved by the Animal Studies Committee at Washington University School of Medicine. The cecal ligation and puncture (CLP) murine model that reproduces many of the clinical features of sepsis in patients was used to induce intra-abdominal peritonitis (1). As reported previously, blood cultures are positive for numerous aerobic and anaerobic gram-positive and gram-negative bacteria in CLP but are not positive in sham operated mice (19). Six mice were anesthetized with halothane, and a midline abdominal incision was made. The cecum was mobilized, ligated below the ileocecal valve, and punctured two times with a 23-gauge needle. The abdomen was closed in two layers, and the mice were injected subcutaneously with 2.0 ml of 0.9% saline. Six sham-operated mice were handled in the same manner, except that the cecum was not ligated or punctured.

The ATCC 27853 strain of *Pseudomonas aeruginosa* was used to induce pneumonia as described previously (21). In brief, organisms were grown overnight in trypticase soy broth with constant shaking. A 10-ml volume of the culture medium was placed in a 50-ml conical tube, and the cells were harvested by centrifugation for 10 min at 6,000 g. The final density of the inoculum was adjusted to 0.3 absorbance units at 600 nm, corresponding to a cell density ranging between 5 × 10^8 and 1 × 10^9 colony-forming units/ml as determined by serial dilution and colony counts. Pneumonia was induced in five animals by intratracheal injection of 40 μl of the bacterial suspension via a midline cervical incision under halothane anesthesia. Five other animals treated with the same volume (40 μl) of intratracheal normal saline served as controls. Immediately after injection, the mice were held vertically to enhance delivery of the injected material in the lung. Animals received 1 ml normal saline subcutaneously. In an additional cohort of two animals, livers and spleens harvested 24 h after bacterial injection were sent for bacterial cultures.

**Harvesting of spleens.** Bcl-2 transgenic mice and C57BL6 wild-type mice underwent surgery as described above. At 6 h after surgery, mice were killed by cervical dislocation, and spleens were rapidly harvested and placed in liquid nitrogen. The 6-h time point was chosen for tissue sampling because there is no change in cell phenotype demonstrated by flow cytometry (Ref. 33 and Hotchkiss RS, unpublished observations), and the host response to sepsis has started (10, 38).

**Experimental design.** To determine the splenic response in these lethal models of CLP and *Pseudomonas* pneumonia, microarray analysis was performed on each spleen harvested from wild-type animals 6 h after CLP or tracheal instillation of bacteria. The responses of the CLP or *Pseudomonas* spleens were compared concurrently with those of the wild-type controls, sham laparotomy, and tracheal instillation of saline, respectively. This study was repeated in animals overexpressing bcl-2. Thus the splenocyte effect of sepsis secondary to CLP (n = 6) or *Pseudomonas* pneumonia (n = 5) could be determined compared with their controls (n = 6 and 5, respectively), and the effect of bcl-2 overexpression in turn also could be determined in both CLP (n = 5) and pneumonia models (n = 5) compared with controls (n = 5 and 5, respectively, see experimental groups and comparisons in Fig. 1).

**Preparation of mRNA.** Splenic total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) followed by cleanup on RNeasy spin columns (QIAGEN, Valencia, CA). Quality and yield of RNA were determined using the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Using previously reported protocols,(7) cRNA was prepared and hybridized with the Mouse Expression Array 430A GeneChip (Affymetrix, Santa Clara, CA) to measure relative changes in RNA abundance. GeneChip data were deposited in the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) with accession no. GSE5811.

**GeneChip analysis.** Normalized, perfect-match model-based expression values were calculated from all 42 GeneChip.cel files using GeneChip software. Changes in mRNA expression were considered significant if the absolute value of the log-ratio was greater than 1.0. Multiple expression analyses were conducted using the above parameters.

![Fig. 1. Sepsis-induced alterations in spleen RNA abundance. Microarray expression profile analysis indicated that a large number of probe sets changes in abundance in response to sepsis (see also Table 1). A: comparison of spleen RNA abundance after cecal ligation and puncture vs. sham laparotomy in bcl-2 transgenic mice compared with wild-type animals. B: comparison of spleen RNA abundance after *Pseudomonas* vs. saline tracheal instillation in bcl-2 transgenic mice compared with wild-type animals. C: common changes to sepsis, whether because of cecal ligation and puncture or pneumonia, in bcl-2 transgenic mice compared with wild-type animals. CLP, cecal ligation and puncture; WT, wild type.](http://ajpregu.physiology.org/doi/10.1152/ajpregu.00451.2006)
DNA chip analyzer (dChip) software (31). Principal components analysis (PCA) was performed using all genes to explore microarray expression profile differences induced by sham laparotomy or CLP on splenocytes from C57BL/6 wild-type littermates compared with purchased C57BL6 mice (Partek Pro; Partek, St. Charles, MO). Differential RNA abundance was determined using a one-way ANOVA with a Bonferroni correction for multiple test groups for CLP or pneumonia. The four anticipated, pairwise contrasts expected pre hoc were sham laparotomy vs. CLP in wild-type mice, sham vs. CLP in bcl-2 transgenic mice, intratracheal saline treatment vs. intratracheal P. aeruginosa treatment in wild-type mice, and saline vs. Pseudomonas in bcl-2 transgenic mice. Lists of genes with significant differences in the above contrasts were created using a false discovery rate (FDR, step-up method) of 0.01, and the intersections of resultant gene lists were examined. Gene lists were uploaded using the Ingenuity Pathway Analysis program (Ingenuity Systems, Mountain View, CA) to explore transcriptional profiles and to identify potential functional modules altered by CLP and pneumonia in the model. The Ingenuity program creates interaction maps based on gene list-based queries of a large database of protein-protein (gene-gene) interactions reported in the literature (2).

RESULTS

Hybridization analysis. Default dChip settings flagged one out of the 42 GeneChip hybridizations as an outlier (containing >15% probe pair outliers). Data from this WT Sham microarray were excluded from further analysis. PCA indicated that the expression profiles of treated nontransgenic littermates clustered with their respective Sham or CLP counterparts (data not shown). Thus data from nontransgenic littermates and similarly treated purchased C57BL/6 mice were combined for further analysis.

Effect of bcl-2 overexpression and experimental procedures in control animals. The effects of the bcl-2 transgene and control procedures on splenocyte expression profiles were gauged by comparing differences between control groups, specifically the effect of sham laparotomy or the effect of saline tracheal infection in wild-type and bcl-2 transgenic animals. Hundreds of probe sets differed in response to sham and saline treatment manipulations in either wild-type or bcl-2 transgenic animals (affect of general anesthesia and surgery; Table 1). Specifically, bcl-2 overexpression altered 230 probe sets in the sham surgical group and 417 probe sets in the saline control group. Of these, RNA abundance for 27 probe sets was altered comparing wild-type with bcl-2 transgenic controls, regardless of whether the treatment was sham laparotomy or tracheal instillation of saline (that is, the effect of bcl-2 overexpression in control animals; Fig. 1C). As expected, the abundance of bcl-2 transcript was increased in bcl-2 transgenic animals ~3.6-fold. The only gene demonstrating increased abundance was a gene associated with neuroblastomas, v-myc (myelocytomatosis viral-related oncogene), up ~7.7-fold in bcl-2 transgenics compared with wild-type animals. RNA abundance of the other genes identified was decreased by 2.5-fold or less. Pathway analysis software did not identify network interactions among these probe sets.

Effect of sepsis secondary to CLP and Pseudomonas pneumonia. Bacterial sepsis secondary to CLP or pneumonia induced marked differences in splenocyte relative mRNA abundance at 6 h compared with their respective controls (Table 1). Regardless of genetic background, hundreds to thousands of probe sets were altered using an FDR of 0.01. In each treatment-control comparison, the majority of changes in expression profiles were decreases in relative RNA abundance. To determine the common effects of sepsis on splenocytes regardless of infection source or genotype, the gene lists from Table 1 were compared (Fig. 1).

To increase confidence in the functional analysis of these comparisons, a conservative approach was used wherein only alterations in RNA abundance that occurred in both the CLP and pneumonia models were studied (Fig. 1C). Of note, there were only 88 probe sets that were altered by CLP and pneumonia in those animals overexpressing bcl-2 (Fig. 1C). This represented 5.7% of the total number of probe sets that were altered by CLP, pneumonia, or both in bcl-2 transgensics. In contrast, there were 409 probe sets (8.2%) altered by CLP and pneumonia in wild-type animals (Fig. 1C). Of the 88 probe sets in bcl-2 transgensics and the 409 probe sets in wild-type mice, 85 were commonly altered by CLP or pneumonia regardless of genotype: 36 were increased in abundance and 49 were decreased in abundance. This set of 85 probe sets was used to derive a hypothetical transcriptional network of 102 genes with mapped interactions based on literature reports (Fig. 2). Nuclear factor (NF)-κB and NF-κBIA (IkB-α) interactions play a central role in this network. Metallothione (up ~10-fold), suppressor of cytokine signaling 3 (SOCS3, up ~5-fold), and solute carrier family 40, member 1 (iron-regulated transporter, down ~3-fold) were the probe sets most altered by sepsis. The gene function annotations most strongly associated with the list of 85 probe sets common to sepsis were cell death and cellular assembly and organization, based in part on three subnetworks of genes responsible for cell movement, cell-to-cell signaling and interaction, immune and lymphatic system development, cell cycle progression, and amino acid metabolism, among others. Canonical pathways that were associated with this network included apoptosis signaling (Bcl2A1, NF-κBIA), integrin signaling (ITGα6, ITGα9, ITGB7, RALA), and JAK/STAT signaling (SOCS3), among others.

Table 1. Significant differences in spleen RNA abundance altered by bacterial sepsis or bcl-2 overexpression

<table>
<thead>
<tr>
<th>Comparison</th>
<th>P Value Cutoff*</th>
<th>No. of Probe Sets</th>
<th>Probe Sets Increased</th>
<th>Probe Sets Decreased</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT sham vs. WT CLP</td>
<td>0.00141472</td>
<td>3,210</td>
<td>1,295</td>
<td>1,915</td>
</tr>
<tr>
<td>bcl-2 Sham vs. bcl-2 CLP</td>
<td>0.000171</td>
<td>388</td>
<td>149</td>
<td>239</td>
</tr>
<tr>
<td>bcl-2 Sham vs. WT Sham</td>
<td>0.0000101366</td>
<td>230</td>
<td>57</td>
<td>173</td>
</tr>
<tr>
<td>WT saline vs. WT pneumonia</td>
<td>0.0000782283</td>
<td>1,775</td>
<td>677</td>
<td>1,098</td>
</tr>
<tr>
<td>bcl-2 Saline vs. bcl-2 pneumonia</td>
<td>0.0000514764</td>
<td>1,168</td>
<td>342</td>
<td>826</td>
</tr>
<tr>
<td>bcl-2 Saline vs. WT saline</td>
<td>0.0000183781</td>
<td>417</td>
<td>135</td>
<td>282</td>
</tr>
</tbody>
</table>

WT, wild type; CLP, cecal ligation and puncture. *Based on a false discovery rate with significance value: 0.01.
Interaction of sepsis and bcl-2. Comparison of the diagrams in Fig. 1 demonstrates that there were 311 probe sets that responded uniquely to both varieties of sepsis in wild-type mice and showed no response in bcl-2 overexpressors (Fig. 1C). Of these probe sets, 169 demonstrated increased RNA abundance, whereas 142 showed decreased abundance. Conversely, only two probes sets responded uniquely (with decreases) to both varieties of sepsis in transgenic mice (Fig. 1C). The sequences for these two probe sets are from the same gene, CEA-related cell adhesion molecule 1 (ceacam-1), a member of the immunoglobulin superfamily.

Functional analysis indicated that the 311 genes altered by sepsis in wild-type but not bcl-2 transgenics were most strongly associated with the cellular processes of development, morphology, and death. Canonical pathways associated with these genes include ketone body metabolism (ACAT1, HMGCS1), interferon signaling (JAK2, SOCS1), and interleukin-6 signaling (IL-6, HSPB1, IL-1β, MAP2K3). Three genes in this group of 311 probe sets demonstrated a marked increase in RNA abundance: chemokine ligand 1 (CXCL-1, 20-fold), IL-6 (20-fold), and prostaglandin-endoperoxide synthase 2 (COX-2, 14-fold). Pathway analysis linked these three genes.
into a common network of 206 genes constituting five overlapping subnetworks, integrating signaling pathways for extracellular signal-regulated kinase/mitogen-activated protein kinase, IL-6, apoptosis, B cell receptors, and estrogen receptors, among others (Fig. 3). Detailed analysis of subnetwork 5, which includes \textit{bcl}-2, revealed a large number of interactions among apoptosis genes that were altered by \textit{bcl}-2 overexpression, including \textit{bcl2L11} (\textit{bim}), \textit{mcl-1}, \textit{bcl2L2} (\textit{bcl-w}), and \textit{bmf} (Fig. 4 and Table 2). These findings in turn directed subsequent experiments aimed at better elucidating mechanism, as reported elsewhere [e.g., targeted gene deletion of \textit{bcl2L11} (\textit{bim}) and administration of TAT-\textit{Bcl-xL} constructs (3a, 22)].

DISCUSSION

Sepsis induces extensive lymphocyte and dendritic cell apoptosis that alters immune responsiveness by the following three mechanisms: 1) profound depletion of immune effector cells, 2) uptake of apoptotic cells causing anti-inflammatory/immunosuppressive effect on surviving cells, and 3) decreased clearance of invading organisms (19, 44, 45). The molecular mechanisms by which \textit{bcl}-2 alters cell survival in sepsis remain controversial (18, 26, 36). We hypothesized that microarray analysis coupled with contemporary network analysis would help identify transcriptional pathways responsible for the pro-

Fig. 3. Hypothetical network of genes altered by sepsis in wild-type mice but not \textit{bcl}-2 transgenic animals. See Fig. 2 for an explanation of gene symbol colors. The \textit{bcl}-2 subnetwork is shown in Fig. 4 in more detail.
Protective effect of bcl-2 against sepsis-induced apoptosis. This strategy was reported recently to be useful in narrowing the number of potential target genes in a genomewide study of the host inflammatory response (2). In the current study, two complementary, well-accepted mouse models of sepsis were employed: polymicrobial peritonitis and gram-negative pneumonia. By examining effects of bcl-2 overexpression on changes in RNA abundance that were common to both sepsis models, we hoped to identify functional modules and regulatory nodes by which bcl-2 protects against death from sepsis. Our analysis suggests the novel hypothesis that bcl-2 exerts its protective effects in sepsis by “preventing” changes in transcriptional networks induced by sepsis, rather than by itself inducing transcriptional changes that alter the septic phenotype. Specifically, our analysis indicates that overexpression of bcl-2 in septic animals altered only one gene (2 probe sets) but prevented alteration in 311 probe sets. In addition, a number of cell death molecular pathways were directly implicated in transcriptional networks, mapped de novo using in silico analysis. Finally, this unbiased analytical approach suggested gene targets for future study, including bcl2L11 (bim, see discussion below), mycn, and ceacam1. The latter is a member of the

Table 2. Changes in relative gene expression of bcl-2 family members in sepsis that do not occur when bcl-2 is overexpressed

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Protein Activity</th>
<th>WT Sepsis</th>
<th>bcl-2 Sepsis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mcl-1</td>
<td>Myeloid cell leukemia sequence 1</td>
<td>Antiapoptotic</td>
<td>Decreased</td>
<td>No change</td>
</tr>
<tr>
<td>Bim</td>
<td>Bcl-2-like 11</td>
<td>Proapoptotic</td>
<td>Increased</td>
<td>No change</td>
</tr>
<tr>
<td>Bcl-w</td>
<td>Bcl-2-like 2</td>
<td>Antiapoptotic</td>
<td>Increased</td>
<td>No change</td>
</tr>
<tr>
<td>Bmf</td>
<td>Bcl-2-modifying factor</td>
<td>Proapoptotic</td>
<td>Increased</td>
<td>No change</td>
</tr>
</tbody>
</table>
immunoglobulin superfamily that mediates cell-cell adhesion. *Ceacam1*, the single gene altered by sepsis in *bcl-2* transgenics but not wild-type animals, has been demonstrated to play key roles in T cell and neutrophil responses, including direct effects on immune regulation, cell growth, proliferation, and survival (15).

Transcriptional analysis of the response to sepsis in these two models demonstrated distinct differences in the expression of pro- and anti-apoptotic Bcl-2 family members in wild-type compared with Bcl-2 transgenic mice (Fig. 4 and Table 2). As expected, the abundance of *bcl-2* message was increased in *bcl-2* transgenic mice. The significance of the observed increase of *mycn* in the *bcl-2* transgenic animals is not known. Of interest, however, a recent report using a mouse model of autoimmune diabetes linked *bcl-2* and *mycn* at the center of gene networks based on the opposing biological process of cell death (*bcl-2*) and cell proliferation (*mycn*) (13). Three other *bcl-2* family members, *mcl-1*, *bcl2L11* (*bim*), and *bmf*, showed altered RNA abundance consistent with increased apoptosis in septic compared with control wild-type animals (Fig. 2). However, these genes showed no microarray evidence of altered RNA abundance in septic *bcl-2* transgenic animals, consistent with the overall effect of *bcl-2* to modulate sepsis-induced transcriptional changes, as described above. *Mcl-1* has potent anti-apoptotic activity that is critical for the development and maintenance of B and T lymphocytes (12, 16). Although *mcl-1* deficiency results in embryonic lethality, induced deletion of *mcl-1* that is restricted to peripheral T and B cells demonstrated that *mcl-1* is essential for maintenance of mature lymphocytes (34). *Bim* is a member of the proapoptotic Bcl-2 homology3-only protein family that binds to and inhibits selected members of the anti-apoptotic *bcl-2* family (37, 41). In addition to binding and inhibiting anti-apoptotic Bcl-2 proteins, there is evidence that *bim* may directly induce apoptosis by binding to and activating the proapoptotic Bcl-2 proteins Bax and/or Bak (19). The close interaction between the various members of the pro- and anti-apoptotic *bcl-2* family is further highlighted by the fact that *mcl-1* binds to *bim* and may thereby inhibit *bim*'s proapoptotic activity. Based upon these results and the network depicted in Fig. 4, we investigated subsequently the effect of sepsis on *bim* null mice (3a). Lymphocytes from *bim* null mice were protected from sepsis-induced apoptotic cell death (3a). Moreover, these mice had a greater than threefold improvement in sepsis survival (*P < 0.001*). Importantly, even loss of a single allele of *bim* provided remarkable protection against sepsis-induced lymphocyte apoptosis (unpublished findings).

One of the more striking observations made when reviewing the microarray results globally was the effect of the *bcl-2* transgene on RNA abundance of *IL-6*, *COX-2*, and *CXCL1* in *bcl-2* transgenic animals. The data indicate that the increase in RNA abundance of these three genes induced by sepsis in wild-type animals was blocked by *bcl-2* overexpression during sepsis, whether caused by peritonitis or pneumonia. The effects of sepsis on these genes are well described, indicating significant pathology induced by each. IL-6 is produced by a broad range of cells, including T and B cells, macrophages, and fibroblasts. Work from Remick et al. (38) and others has shown that markedly elevated circulating concentrations of IL-6 at 6 h after CLP or pneumonia predict mortality in murine sepsis. In addition, Riedemann and colleagues (39) reported that administration of anti-IL-6 antibodies could improve survival in the mouse CLP model of sepsis. IL-6 plays an important role in stimulating B cell differentiation and inducing hepatocyte production of acute-phase proteins (43). Thus IL-6 is similar to a number of other cytokines in that its effects on sepsis survival are a function of time, concentration, and the severity of the model. To investigate if the increase in mRNA for IL-6 in wild-type compared with Bcl-2 transgenic mice resulted in differences in circulating IL-6 protein concentrations, we measured plasma concentrations of IL-6 from wild-type and Bcl-2 transgenic mice 8 h after CLP, but no difference was found (3a). In addition to IL-6, *COX2* message also was decreased in septic animals with the *bcl-2* transgene compared with septic wild-type mice. *COX-2* is an inducible enzyme that is upregulated by endotoxin and cytokines and acts upon arachidonic acid to generate prostaglandins, other mediators of inflammation. *COX-2*-deficient mice are resistant to endotoxin-induced inflammation and have improved survival (11). Activity of *COX-2* during sepsis likely mirrors that of *IL-6* in that adaptive alterations in enzyme activity are beneficial during sepsis, but excessive activation may have adverse effects. Finally, *CXCL1*, a cytokine-induced neutrophil chemoattractant, has been implicated in adhesion formation in a rat model of abdominal sepsis (4). These data collectively suggest that one possible mechanism for the improved survival in the *bcl-2* transgenic mice may be a modulation of the pathogenic effects of sepsis mediators, such as IL-6, COX2, and CXCL1.

There are a number of limitations to this study. Most important, the tissue samples consisted of whole spleen. We were thus unable to account for the potentially confounding effect of sepsis-induced difference in splenocyte populations. It is important to note, however, that we found no differences in splenocyte cellular populations (such as CD4+ T cells and B cells) between sham and CLP mice at the same 6-h time point (Ref. 33 and unpublished observations). Given this caveat, we were deliberately conservative in our approach, basing conclusions on similarities across two models of mouse sepsis. We consider the data presented herein to be useful in identifying potential gene targets and regulatory networks, reducing hypothesis space from the 20,000 or so mouse genes that potentially could be involved in the splenic response to sepsis to the few hundred RNA species identified. The large number of genes altered by sepsis and *bcl-2* overexpression does not allow for comprehensive validation of the results using other molecular techniques. Instead, we successfully used contemporary network analysis software to identify functional modules of interest and direct our focus toward validating therapeutic targets, including *bcl2L11* (*bim*), as described elsewhere (3a).

In conclusion, our data are consistent with alterations in the balance of pro- and antiapoptotic transcriptional networks in spleen 6 h after septic challenge, with the net result being increased splenocyte apoptosis (23, 24). In addition, these findings suggest that the ability of *bcl-2* overexpression to improve outcome from sepsis in these models is associated with prevention of sepsis-induced alterations in spleen transcriptional responses, including but not limited to, decreased RNA abundance of proinflammatory mediators such as IL-6, COX2, and CXCL1. The microarray data from these experiments are available as a resource for the community at the Gene Expression Omnibus (accession nos. in Preparation of mRNA). Pathway analysis of these data sets suggested impor-
tant molecular targets for future study, such as \textit{bcl2L11} (\textit{bim}; see Ref. 3a), \textit{mycn}, and \textit{ceacam1L}. Ongoing work using enriched splenocyte populations (such as CD4+ T cells) will elucidate more clearly the molecular underpinnings and dynamics of these responses (33).

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