

Organ-Specific Role of MyD88 for Gene Regulation during Polymicrobial Peritonitis†

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Sepsis leads to the rapid induction of proinflammatory signaling cascades by activation of the innate immune system through Toll-like receptors (TLR). To characterize the role of TLR signaling through MyD88 for sepsis-induced transcriptional activation, we investigated gene expression during polymicrobial septic peritonitis by microarray analysis. Comparison of gene expression profiles for spleens and livers from septic wild-type and MyD88-deficient mice revealed striking organ-specific differences. Whereas MyD88 deficiency strongly reduced sepsis-induced gene expression in the liver, gene expression in the spleen was largely independent of MyD88, indicating organ-specific transcriptional regulation during polymicrobial sepsis. In addition to genes regulated by MyD88 in an organ-dependent manner, we also identified genes that exhibited an organ-independent influence of MyD88 and mostly encoded cytokines and chemokines. Notably, the expression of interferon (IFN)-regulated genes was markedly increased in septic MyD88-deficient mice compared to that in septic wild-type controls. Expression of IFN-regulated genes was dependent on the adapter protein TRIF. These results suggest that the influence of MyD88 on gene expression during sepsis strongly depends on the organ compartment affected by inflammation and that the lack of MyD88 may lead to disbalance of the expression of IFN-regulated genes.

The induction of septic shock is initiated by massive activation of immune cells by bacterial components such as endotoxin or other pathogen-associated molecular patterns recognized by Toll-like receptors (TLRs) (4). Subsequently, signaling cascades are activated, inducing profound changes in gene expression. Several biological mediators play a central role in the pathophysiology of sepsis, including microbial signal molecules, complement activation products, coagulation factors, cell adhesion molecules, and cyto- and chemokines (37). The production of cyto- and chemokines can contribute to protective immune responses but can also lead to organ failure and death (23, 37).

The engagement of TLRs by conserved microbial structures is critical for the induction of innate immune responses against polymicrobial sepsis. Ten mammalian TLRs have been identified, and each receptor exhibits a unique specificity for microbial components (4, 35). MyD88 is the central adaptor protein for signal transduction of most TLRs and the interleukin-1 (IL-1) receptor family (1). MyD88 interacts with TLRs and recruits IL-1 receptor-associated kinases (IRAKs) (30, 31, 43). Subsequently, IRAKs associate with TNF receptor-activated factor 6 (TRAF6), which leads to the activation of mitogen-activated protein (MAP) kinase pathways and NF- κ B, causing the expression of proinflammatory mediators such as cytokines and chemokines. MyD88 deficiency leads to delayed activation

of NF- κ B and MAP kinases after TLR activation (25). In addition to this common pathway, TLR4 and TLR3 utilize a MyD88-independent signaling pathway, using the adapter protein TRIF/TICAM-1 (33, 44), that results in the activation of IRF-3, the induction of beta interferon (IFN- β), and the expression of IFN-dependent genes. Both MyD88 and TRIF contribute to gene expression induced by the TLR4 ligand lipopolysaccharide (LPS). MyD88-deficient macrophages show attenuated production of several proinflammatory cytokines and chemokines (1, 5, 20, 25), whereas TRIF deficiency inhibits the upregulation of costimulatory molecules on dendritic cells (22) as well as the production of several cyto- and chemokines and expression of type I IFN-regulated genes in macrophages and dendritic cells (20, 21, 41, 44). In macrophages, dependency on either TRIF or MyD88 could be demonstrated for some genes, including those encoding IL-1 α and IL-6 (20), indicating a complex regulation of MyD88- and TRIF-induced pathways after TLR4 triggering.

Previous work from our group showed that a genetic deficiency of MyD88 partially protected mice from the lethal effects of polymicrobial septic peritonitis and that the hyperinflammatory response to sepsis was strongly attenuated in the absence of MyD88 (42). In contrast, recruitment of neutrophils to the infected peritoneal cavity and bacterial clearance were not altered in MyD88-deficient mice, suggesting that the MyD88-dependent signaling pathway contributes to immunopathology but is dispensable for the antibacterial defense during sepsis.

To better understand the distinct functions of MyD88-dependent signaling processes for the regulation of immune responses to sepsis, the present study analyzed local gene ex-

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pression profiles induced by polymicrobial peritonitis in the spleen and liver. Microarray analysis revealed a striking organ-specific contribution of MyD88 to gene expression. In livers of wild-type (WT) mice, a large number of genes were upregulated, whereas gene induction was mostly abrogated in livers of MyD88-deficient mice. In contrast, gene induction in spleens of MyD88-deficient mice was comparable to that in wild-type mice, suggesting the involvement of other signaling pathways independent of MyD88. In addition, MyD88 deficiency was associated with a marked upregulation of IFN-regulated genes during sepsis, suggesting a disproportionate activation of MyD88-independent signaling pathways.

MATERIALS AND METHODS

Mouse strains and CASP model of polymicrobial septic peritonitis. MyD88-deficient mice backcrossed eight times to the C57BL/6 background were kindly provided by S. Akira (1). TRIF^{lps2/lps2} mice were kindly provided by B. Beutler (Scripps Research Institute, La Jolla, Calif.). Mice at 8 to 12 weeks of age were used for all experiments. The colon ascendens stent peritonitis (CASP) procedure used for induction of septic peritonitis was described in detail previously (47). Wild-type control mice that underwent sham surgery served as controls.

Affymetrix gene chip analysis. Spleens and livers of mice were collected 6 h after CASP or sham surgery. Total RNA was prepared using a QIAGEN RNeasy kit according to the recommendations of the manufacturer (QIAGEN, Hilden, Germany). RNAs from three individual mice were pooled and processed, and 15 μ g of labeled RNA was hybridized to mouse expression gene chip arrays (murine genome array U74Av2) according to Affymetrix protocols (Santa Clara, CA). Gene chips were scanned and analyzed using Affymetrix Microarray Suite 5.0 software (MAS 5.0). Gene expression data are the results of three individual experiments.

Microarray data analysis. Affymetrix CEL files were normalized with dCHIP 1.3 (<http://biosun1.harvard.edu/complab/dchip/>) (27). Expression values and present calls were generated using the same program and the PM/MM model. Probe sets with fewer than two present calls were excluded from further analysis (separately for each organ). This resulted in lists of 7,089 (56.77%) and 8,060 (64.54%) probe sets for livers and spleens of septic WT and MyD88^{-/-} mice, respectively.

Significance analyses between sham-operated control mice and septic wild-type mice, sham-operated control mice and septic MyD88-deficient mice, and septic wild-type mice and septic MyD88-deficient mice were performed with the SAM two-class paired algorithm (<http://www-stat.stanford.edu/~tibs/SAM/>) (36). Genes that were differentially expressed among the experimental groups were identified by setting the threshold level at a twofold change and the lowest significance level for both organs at 8%. Differentially expressed genes of sham-operated versus wild-type versus MyD88-deficient mice were hierarchically clustered. Hierarchical clustering of the significant genes was done using the program Genesis (release 1.1) (34), with average linkages and euclidean distances as similarity distance measurements.

Analysis of cytokine and chemokine production. Livers and spleens of sham-operated mice and septic wild-type and MyD88-deficient mice were prepared 12 h after surgery as described previously (42). Cytokine and chemokine concentrations were measured by enzyme-linked immunosorbent assays (ELISAs) specific for IL-1 β , IL-6, colony-stimulating factor 3 (CSF-3), CXCL9, CXCL10, CCL4, and CCL5 (all from R&D Systems, Minneapolis, MN). Cytokine levels in peripheral organs were normalized against the protein concentration in each organ extract. Protein concentrations were determined by the bicinchoninic acid method according to the manufacturer's instructions (Pierce, Rockford, IL).

Real-time PCR analysis. Spleens and livers were collected at different time points after sepsis induction and stored in RNA-later buffer (QIAGEN, Hilden, Germany). RNA extractions were carried out using an RNeasy mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. First-strand cDNAs were synthesized from 1 μ g of total RNA, using a mixture of oligo(dT)₁₂₋₁₈ random hexamer primers, and Superscript reverse transcriptase (Invitrogen, Karlsruhe, Germany). The reaction mixture was incubated for 60 min at 42°C, and the reaction was terminated by being heated to 95°C for 5 min. SYBR green master mix was used to detect the accumulation of PCR products during cycling on an SDS7700 cycler (Applied Biosystems, Foster City, CA). RNA expression levels in samples from septic animals were normalized to β -actin levels and displayed as *x*-fold changes relative to those in samples from control mice, used as the calibrator (set to 1). Primers for β -actin and IFN- β were designed using

PrimerExpress software (Applied Biosystems, Foster City, CA). Primers for amplification of specific cDNA fragments were as follows: β -actin sense, 5'-AC CCACACTGTGCCCATCTAC-3'; β -actin antisense, 5'-AGCCAAGTCCAGACGCAGG-3'; CXCL-10 sense, 5'-CCTATCCTGCCACGTGTG-3'; and CXCL-10 antisense, 5'-CGCACCTCCACATAGCTTACA-3'. Primers for interferon-induced gene with tetratricopeptide repeats 1 (ift1), interferon-induced gene with tetratricopeptide repeats 3 (ift3), and viral hemorrhagic septicemia virus-induced gene (*vig-1* pending [also known as Viperin and cig 5]; referred to hereafter as *vig-1*) were detected with published primer sets (5). Data were derived from four to six individual animals.

Flow cytometry analyses. Spleens of wild-type and MyD88-deficient mice were removed 6 h after sepsis induction. Single-cell suspensions were stained with antibodies against B220 (RA3-6B2), CD90.2 (53-2.1), CD11b (M1/70), CD11c (HL30), CD49b/pan-NK (DX5), Ly-6G/Gr-1 (RB6-8C5), and F4/80 (CI:A3-1), using appropriate isotype-matched controls (all from BD Pharmingen, San Diego, CA). Percentages of B cells (B220⁺ CD11b⁻), T cells (CD90.2⁺ CD11b⁻), macrophages (CD11b⁺ F4/80⁺), neutrophils (CD11b⁺ Gr1⁺), conventional dendritic cells (CD11b⁺ CD11c⁺), and NK cells (CD11b⁺ CD49b⁺) were calculated. Data were derived from four to six individual mice.

RESULTS

Organ-specific influence of MyD88 on gene expression during septic peritonitis. During septic peritonitis, the release of bacterial pathogen-associated molecular patterns activates TLR-induced signaling pathways, leading to the induction of a systemic hyperinflammatory response. We described previously that mice deficient in the central TLR adapter protein MyD88 have a survival advantage in a model of polymicrobial septic peritonitis, with MyD88 deficiency strongly attenuating the systemic inflammatory response (42). To analyze sepsis-induced transcriptional changes and the contribution of MyD88-dependent gene induction in different organ compartments (liver and spleen), a genome-wide transcriptome analysis was performed 6 h after sepsis induction. This time point was chosen to provide information about primary and secondary immune response genes that are described to be directly induced after TLR engagement (12). Furthermore, 6 h after sepsis induction, signs of inflammation, such as cytokine induction and a bacterial load at the site of infection, are measurable (17).

We used an algorithm combining statistical testing and filtering to identify genes that were differentially regulated at least twofold, comparing wild-type and MyD88-deficient mice and mice that underwent sham surgery. Sepsis induction in mice by the CASP procedure resulted in 140 and 191 differentially expressed genes in the liver and the spleen, respectively (Fig. 1A; see the supplemental material). Differential gene expression included a small number of genes which were downregulated, while most of the genes were upregulated in the liver and the spleen during septic peritonitis (Fig. 1A). Hierarchical cluster analysis revealed that gene expression profiles for septic spleens of wild-type and MyD88-deficient mice were closely related but differed markedly from the expression profile for control mice that underwent sham surgery. In the liver, gene expression of septic MyD88-deficient mice more closely resembled the gene expression of control mice. Gene expression in septic wild-type livers, however, substantially differed from that in MyD88-deficient and control livers, as shown in the dendrogram (Fig. 1A). Thus, hierarchical cluster analysis revealed differential contributions of MyD88 to sepsis-induced gene expression in the liver and the spleen. To further analyze gene induction during septic peritonitis, we focused on

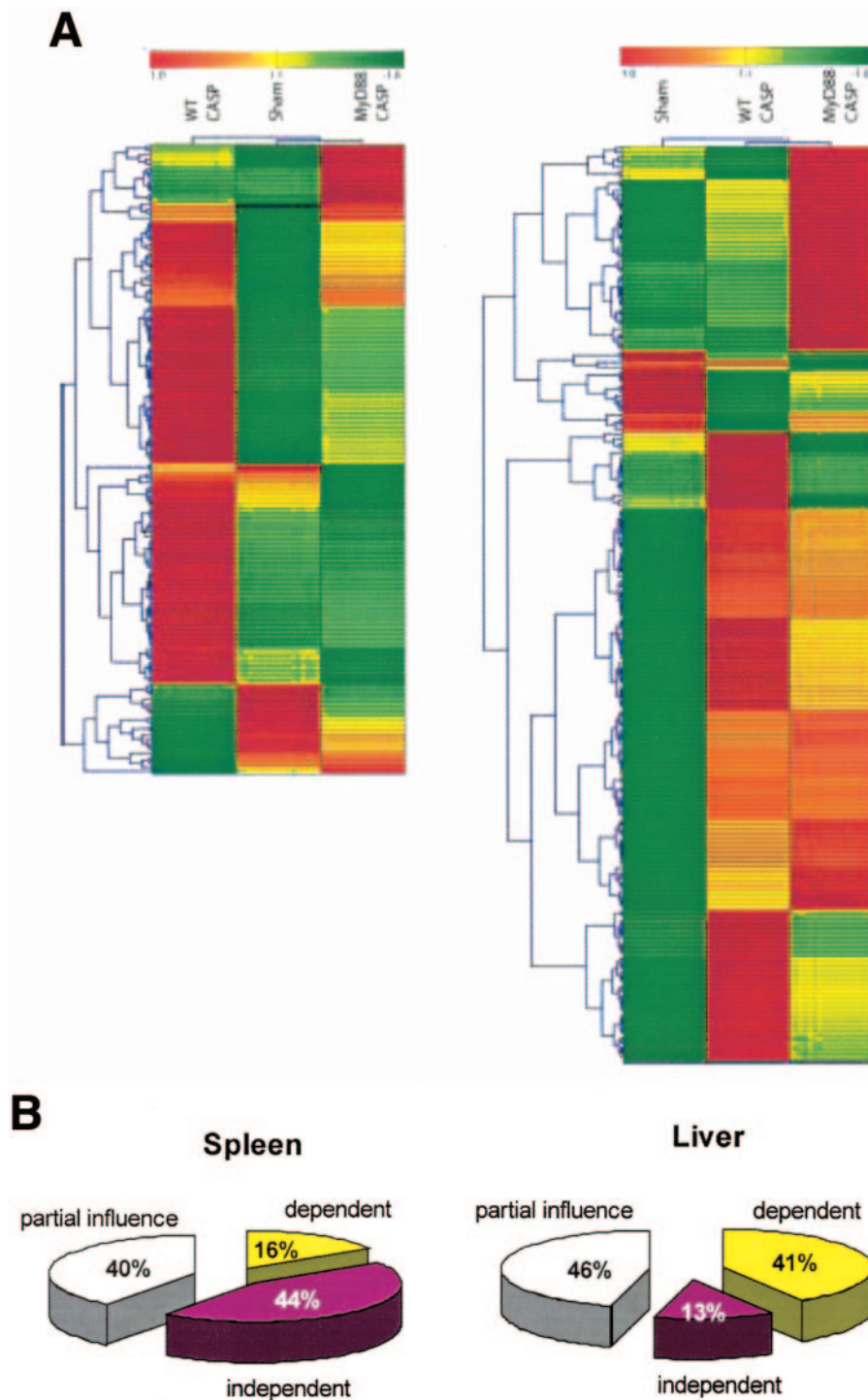


FIG. 1. Gene expression profiling in livers and spleens of septic MyD88-deficient and wild-type mice. (A) Wild-type (CASP) and MyD88-deficient mice (MyD88) underwent CASP surgery. Wild-type mice undergoing sham surgery (sham) served as controls. Spleens and livers were removed 6 h after sepsis induction. RNAs were prepared and processed for hybridization to Affymetrix U74Av2 mouse expression arrays and analyzed as described in Materials and Methods. For hierarchical cluster analysis of the 140 and 191 significantly regulated genes (liver and spleen, respectively), average expression values were normalized gene-wise, using Z scores. Clustering was done using average linkages and euclidean distances as similarity measurements. Gene expression is described as the mean intensity for three independent experiments. Each experiment was carried out with three animals per group. RNAs from the individual mice were pooled before being labeled. (B) Amounts of MyD88-dependent (yellow), MyD88-independent (red), and partially MyD88-dependent (white) genes were compared between the spleen and the liver.

TABLE 1. Gene regulation in the spleen during septic peritonitis

Regulatory category	No. (%) of genes in WT mice	No. (%) of genes in MyD88 ^{-/-} mice
Downregulated genes	15	4
Upregulated genes	117	107
>2-fold	82 (69.8)	79 (73.8)
>3-fold	18 (15.5)	16 (14.9)
>4-fold	17 (14.6)	12 (11.2)

the analysis of those genes that were upregulated in either wild-type or MyD88-deficient mice after sepsis induction based on the statistical analysis described above. Genes were considered upregulated compared to those in sham controls when they showed an increase of at least twofold (Tables 1 and 2). In spleens and livers of wild-type mice, most of the genes were upregulated compared to those of sham-operated mice (117 genes in the spleen and 86 genes in the liver). Detailed analysis revealed that 82 genes (69.8% of the 117 upregulated genes) were upregulated two- to threefold, 18 genes (15.5%) were increased three- to fourfold, and 17 genes (14.6%) were upregulated more than fourfold in wild-type spleens. In wild-type livers, 61 (70.5%) genes were upregulated two- to threefold, 9 (10.5%) genes were induced three- to fourfold, and 22 genes (18.8%) were upregulated more than fourfold. In MyD88-deficient spleens, 107 genes were upregulated. Of those, 79 genes (73.8%) were induced two- to threefold, 16 genes (14.9%) were upregulated three- to fourfold, and 12 genes (11.2%) were upregulated more than fourfold. In MyD88-deficient livers, only 35 genes were upregulated, of which 20 genes (57.1%) were induced two- to threefold, 6 genes (17.1%) were induced three- to fourfold, and 9 genes (25.7%) were upregulated more than fourfold (Tables 1 and 2).

The absolute numbers of genes that were upregulated in spleens and livers of septic wild-type mice were comparable. In contrast, in septic MyD88-deficient mice, gene induction seemed to differ markedly between the liver and the spleen. In the spleen, a large number of genes was upregulated during septic peritonitis in the absence of MyD88, with numbers comparable to those in the wild-type situation. In the liver, however, only a small number of genes could be observed to be upregulated during septic peritonitis in the absence of MyD88, indicating a strong dependency on MyD88 in this organ.

Next, we examined the MyD88 dependency of genes that were upregulated during septic peritonitis in wild-type mice to elucidate the contribution of MyD88-dependent signaling events to gene induction early after sepsis induction. Genes were considered MyD88 dependent when the expression levels in wild-type mice exceeded the expression levels in MyD88-deficient mice more than twofold. Genes that were considered

TABLE 2. Gene regulation in the liver during septic peritonitis

Regulatory category	No. (%) of genes in WT mice	No. (%) of genes in MyD88 ^{-/-} mice
Downregulated genes	20	11
Upregulated genes	86	35
>2-fold	61 (70.5)	20 (57.1)
>3-fold	9 (10.5)	6 (17.1)
>4-fold	22 (18.8)	9 (25.7)

TABLE 3. Cell populations in the spleen 6 h after sepsis induction

Cell population	% of cells in WT mice (mean ± SD)	% of cells in MyD88 ^{-/-} mice (mean ± SD)	P value
B220	58.95 ± 5.6	55.1 ± 7.2	0.303
CD90.2	32.56 ± 11.8	32.32 ± 12.57	0.96
CD11b ⁺ F4/80 ⁺	1.95 ± 0.15	3.03 ± 1.64	0.18
CD11b ⁺ Ly6Gr1 ⁺	1.96 ± 1.08	1.35 ± 0.73	0.332
CD11b ⁺ CD11c ⁺	1.51 ± 0.53	1.58 ± 1.12	0.27
CD11b ⁺ pan-NK ⁺	1.54 ± 0.41	1.04 ± 0.11	0.29

MyD88 independent showed a <1.2-fold difference between septic MyD88-null and wild-type mice (Fig. 1B). Genes that were regulated <2-fold and >1.2-fold were considered partially influenced by MyD88.

In the spleen, 18 genes (15.5% of 117 genes that were upregulated during sepsis in wild-type mice) were dependent on MyD88, whereas the majority of genes (52 genes, corresponding to 44.8% of the 117 upregulated genes) were MyD88 independent. In the liver, however, 35 genes (41.1% of 86 genes that were upregulated during sepsis in wild-type mice) were MyD88 dependent, whereas only 11 genes (12.9%) were MyD88 independent. In both organs, genes that showed a minor influence of MyD88 (46 genes in the spleen and 39 genes in the liver [39.6% and 45.8%, respectively]) could be detected.

These data demonstrate that in the spleen, a large number of the genes that were upregulated during septic peritonitis were regulated independently of MyD88, whereas in the liver, most of the genes were regulated in a MyD88-dependent manner. We concluded that MyD88 is a major regulator of sepsis-induced gene expression in the liver, whereas in the spleen, MyD88-independent mechanisms may be important.

To determine whether an altered distribution of cell populations, which may be caused by differences in inflammatory cell recruitment or cell death, may contribute to the differential gene expression in MyD88-deficient mice during sepsis, we analyzed the proportions of B cells (B220⁺ CD11b⁻), T cells (CD90.2⁺ CD11b⁻), macrophages (CD11b⁺ F4/80⁺), neutrophils (CD11b⁺ Gr1⁺), conventional dendritic cells (CD11b⁺ CD11c⁺), and NK cells (CD11b⁺ CD49b⁺) in the spleen 6 h after sepsis induction. Flow cytometry analyses revealed no significant differences in the distributions of the analyzed cell populations (Table 3). Thus, altered cellular composition may not be responsible for the differential gene expression in spleens of MyD88-deficient mice.

Distinct roles of MyD88 in expression of cyto- and chemokines in the spleen and the liver. Sepsis leads to the induction of large amounts of cyto- and chemokines, which are critically involved in the systemic hyperinflammation reaction and organ failure (23, 37). A deficiency of MyD88 substantially reduces systemic cytokine levels, preventing septic hyperinflammation (42). The examination of gene expression during septic peritonitis revealed that in both wild-type and MyD88-deficient mice, a large number of the genes upregulated in the spleen and the liver could be functionally assigned to inflammatory processes (see the supplemental material). Thus, during the early phase of septic peritonitis, a mainly inflammation-biased gene activation program is initiated.

A large number of chemokines and cytokines were upregu-

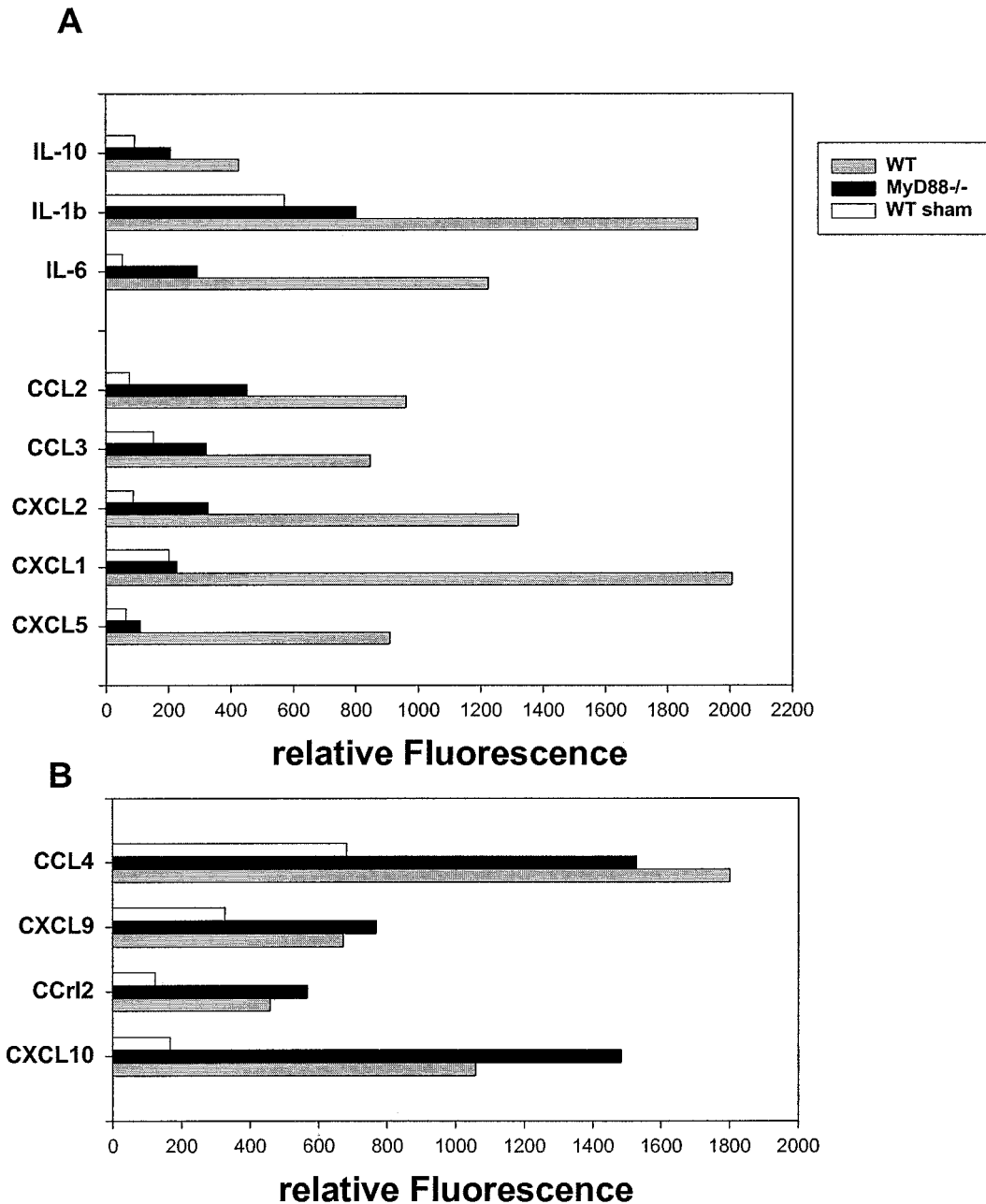


FIG. 2. Cyto- and chemokine mRNA induction in the spleen during septic peritonitis. RNAs from septic wild-type (gray bars) and MyD88-deficient (black bars) mice and control mice after sham surgery (white bars) were prepared for hybridization to Affymetrix U74Av2 mouse expression arrays as described in Materials and Methods. The expression of MyD88-dependent (A) and MyD88-independent (B) cyto- and chemokines is shown.

lated in the spleen during septic peritonitis. The expression of CXCL5, CXCL1, CXCL2, CCL3, CCL2, IL-6, IL-1 β , and IL-10 was found to be upregulated in septic wild-type spleens (more than twofold upregulation compared to mice undergoing sham surgery) (Fig. 2). Whereas CXCL5, CXCL1, and IL-1 β were marginally induced in MyD88-deficient spleens (1.7-fold, 1.1-fold, and 1.4-fold, respectively), CXCL2, CCL3, CCL2, IL-6, and IL-10 were induced more than twofold in MyD88-deficient spleens compared to sham-treated controls. However, compared to wild-type spleens, their induction

levels were more than twofold lower, classifying these genes as MyD88 dependent (Fig. 2). The expression levels of CXCL10, CCL2, CXCL9, and CCL4 were comparable between wild-type and MyD88-deficient mice and could therefore be considered to be regulated independently of MyD88. Notably, the expression levels of CXCL10 in septic MyD88^{-/-} spleens exceeded the expression levels in wild-type spleens (8.9-fold induction versus 6.3-fold induction relative to sham surgery).

To corroborate these findings, whole-organ protein extracts of septic wild-type and MyD88^{-/-} spleens were analyzed by

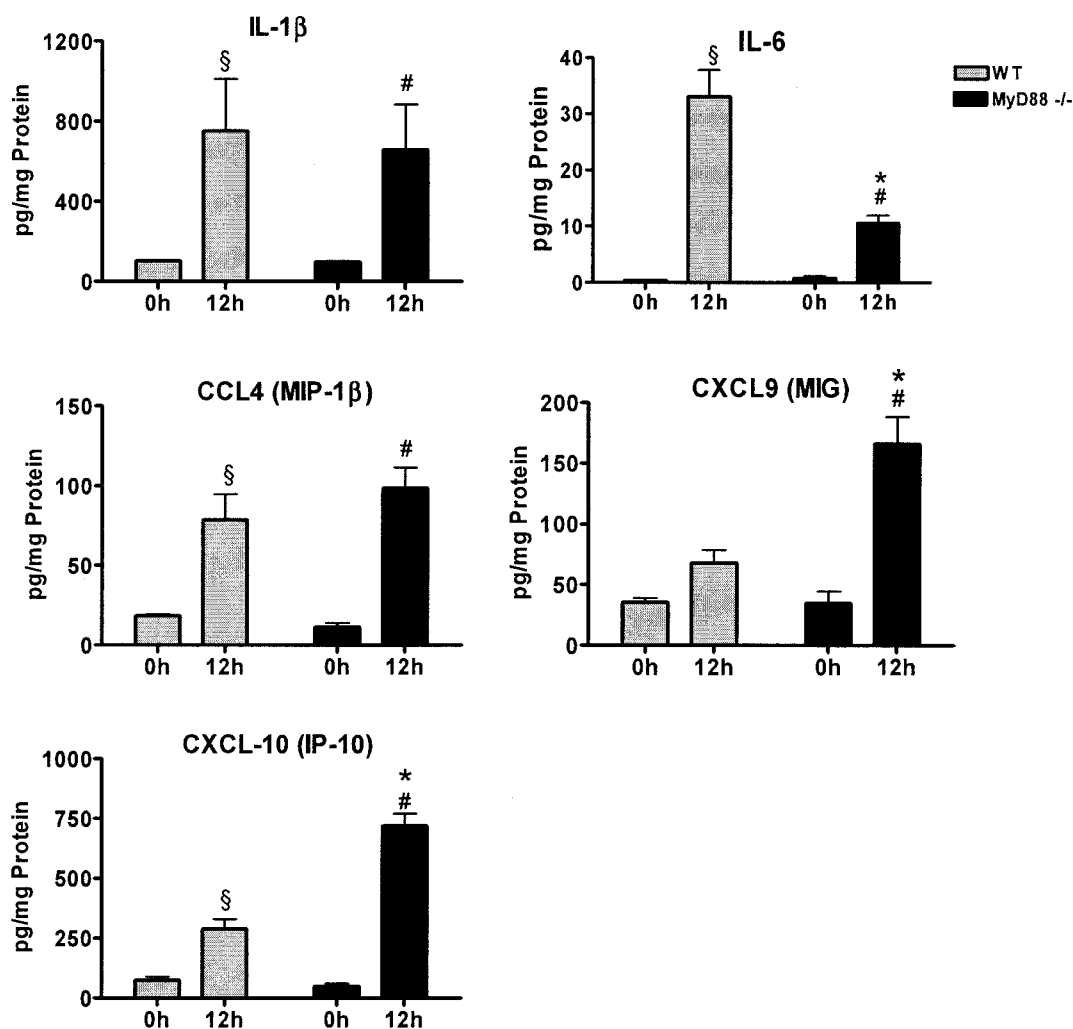


FIG. 3. Cyto- and chemokine protein expression in the spleen during septic peritonitis. Spleens were removed from MyD88-deficient (black bars) and wild-type (gray bars) mice before (0 h) or 12 h after CASP. Organs were homogenized, and protein extracts were prepared. Cytokine concentrations were determined by ELISA and normalized against the total protein concentration. Results were derived from four mice per group and time point. *, $P < 0.05$ (MyD88^{-/-} versus C57BL/6 mice); #, $P < 0.05$ (MyD88^{-/-} mice at 0 h versus 12 h); §, $P < 0.05$ (C57BL/6 mice at 0 h versus 12 h).

ELISA for mediator expression 12 h after the onset of sepsis (Fig. 3). Protein levels of CXCL10, CXCL9, CCL4, and IL-6 correlated with the results obtained by microarray analysis. IL-6 was regulated MyD88 dependently, while CXCL10, CXCL9, and CCL4 showed MyD88-independent regulation. Protein levels of CXCL10 and CXCL9 were significantly higher in MyD88-deficient than in wild-type spleens. The expression levels of CXCL2, CCL3, and CCL2 were consistent with protein data we described previously (42). The protein levels of IL-1 β and IL-10, however, differed from the microarray data (Fig. 3) (42). Whereas the mRNA levels indicated MyD88-dependent expression, protein levels 12 h after sepsis induction were comparable for MyD88-deficient and wild-type mice.

In septic wild-type livers, mRNA induction of CCL2, CCL3, CXCL5, CXCL2, CXCL9, IL-10, IL-6, IL-1 β , and CSF-3 could be observed (Fig. 4). Except for CSF-3, these genes were also significantly induced in septic spleens, suggesting an important role of these cyto- and chemokines during septic peritonitis.

Comparing the expression levels of sepsis-induced cyto- and chemokines in wild-type and MyD88^{-/-} livers revealed that CXCL5, CXCL9, IL-1 β , IL-6, and CSF-3 were only weakly induced (less than twofold) in MyD88-deficient livers (Fig. 4), indicating MyD88-dependent expression. Although CCL2, CCL3, CXCL2, and IL-10 were induced >2-fold in MyD88-deficient livers, induction levels of these mediators were >2-fold greater in wild-type than in MyD88^{-/-} livers. Thus, the genes for CCL2, CCL3, CXCL2, and IL-10 were considered MyD88-dependent genes. The expression of CCL5 was strongly induced in septic wild-type as well as MyD88-deficient livers, indicating MyD88-independent regulation. As observed for MyD88-independent expression of CXCL10 and CXCL9 in the spleen, the expression levels of CCL5 were higher in septic MyD88-deficient than in septic wild-type livers (8.2-fold induction versus 4.7-fold induction relative to sham surgery).

To validate these data, we determined the protein levels of several cyto- and chemokines in the liver 12 h after CASP

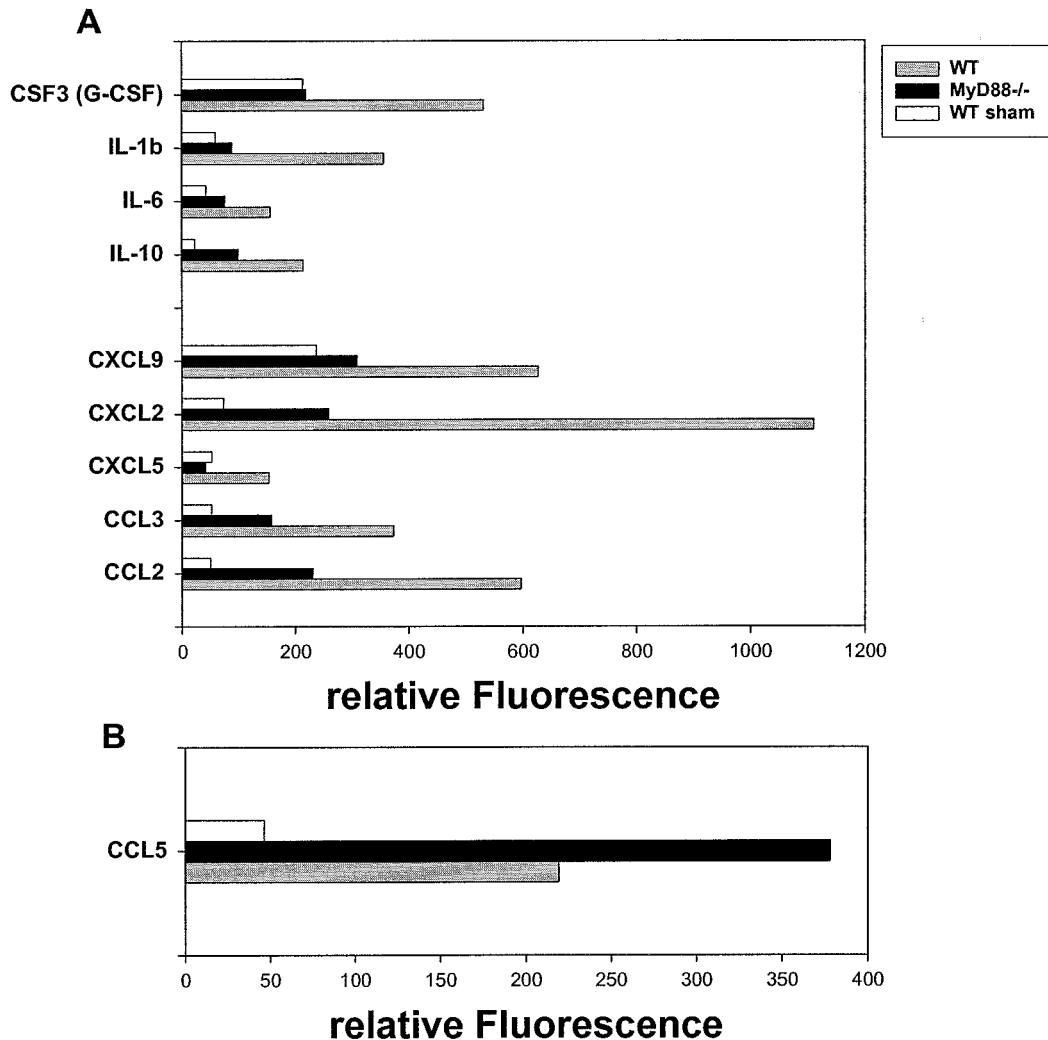


FIG. 4. Cyto- and chemokine mRNA induction in the liver during septic peritonitis. RNAs from septic wild-type (gray bars) and MyD88-deficient (black bars) mice and control mice after sham surgery (white bars) were prepared for hybridization to Affymetrix U74Av2 mouse expression arrays as described in Materials and Methods. The expression of MyD88-dependent (A) and MyD88-independent (B) cyto- and chemokines is shown.

induction by using ELISAs (Fig. 5). The expression levels of IL-6 correlated with the microarray data (Fig. 5), showing MyD88 dependency. Consistent with the mRNA data, our previous work revealed MyD88-dependent expression for IL-10, CCL2, CXCL2, and CCL3 (42). CSF-3 protein expression was partially MyD88 dependent, thereby correlating with the microarray data, which indicated fourfold lower expression in MyD88^{-/-} than in wild-type livers. The expression of CCL5 was found to be MyD88 independent, as shown by both mRNA and protein expression data. Thus, except for IL-1 β and IL-10 expression in the spleen, the vast majority of protein data were consistent with the mRNA expression levels.

Common and specific gene expression in the liver and the spleen. Organ-related expression profiles of genes that were upregulated during septic peritonitis were analyzed further. The majority of genes induced were specific for either the spleen or the liver. In the spleen and the liver, 100 of 116 genes and 69 of 85 genes, respectively, were induced in an organ-

specific manner (Table 4). Comparing spleens and livers from septic wild-type mice, we found 16 genes to be induced in both organs (Table 4), which identified them as common genes. Further analysis showed that 8 of the 16 common genes were cyto- and chemokine genes. Specifically, the chemokines CXCL2, CCL3, CCL2, CXCL5, and CXCL9 and the cytokines IL-1 β , IL-6, and IL-10 were significantly upregulated in both organs during septic peritonitis, suggesting an important role for these genes during sepsis. The MyD88 dependency of these genes is described above. In the spleen, 9 of these 16 common genes were regulated MyD88 dependently (>2-fold induction in wild-type mice compared to MyD88-deficient mice), while 5 of them were regulated MyD88 independently (<1.2-fold induction in wild-type mice compared to MyD88-deficient mice) and 2 showed a minor influence of MyD88. In the liver, however, all common genes were regulated MyD88 dependently, again pointing to an organ-specific influence of MyD88 signaling (Table 5).

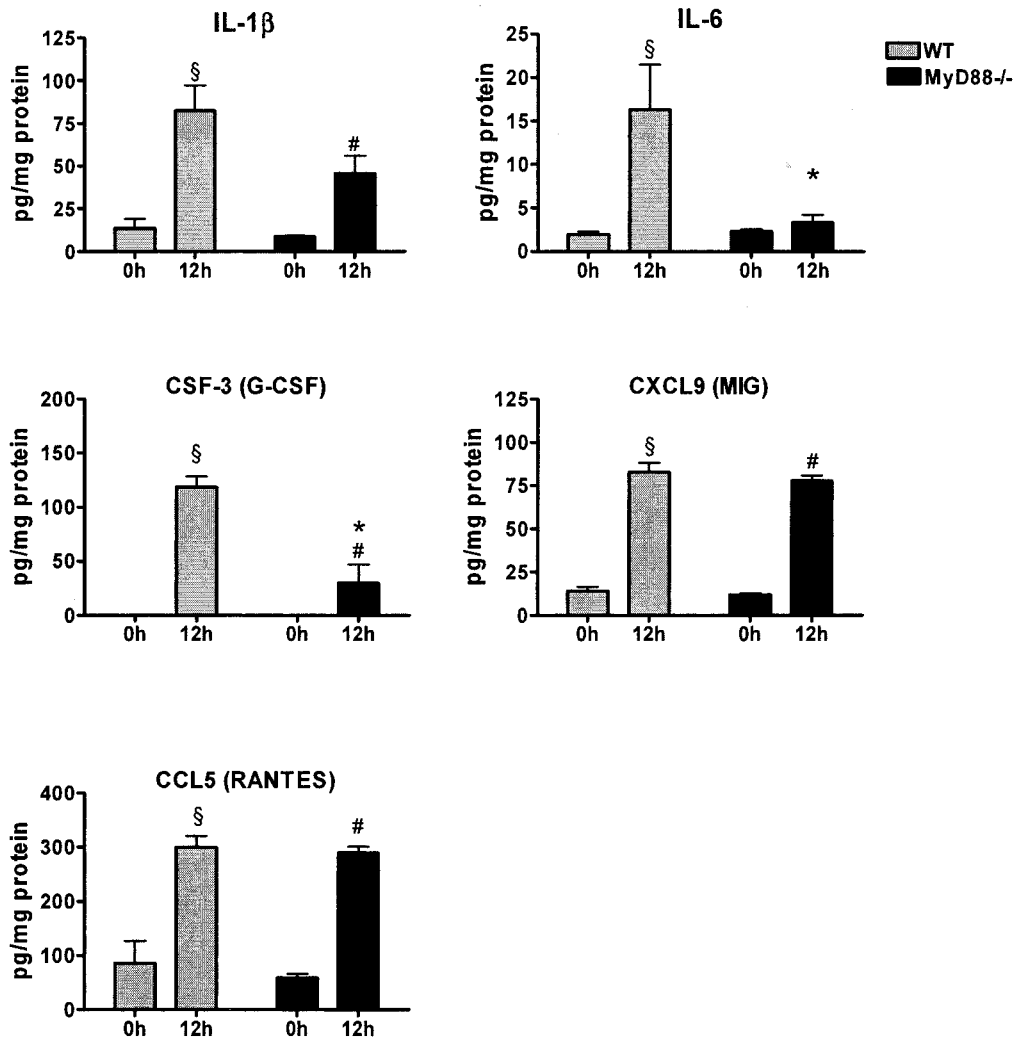


FIG. 5. Cyto- and chemokine protein expression in the liver during septic peritonitis. Livers were removed from MyD88-deficient (black bars) and wild-type (gray bars) mice before (0 h) or 12 h after CASP. Organs were homogenized, and protein extracts were prepared. Cytokine concentrations were determined by ELISA and normalized against the total protein concentration. Results were derived from four mice per group and time point. *, $P < 0.05$ (MyD88^{-/-} versus C57BL/6 mice); #, $P < 0.05$ (MyD88^{-/-} mice at 0 h versus 12 h); §, $P < 0.05$ (C57BL/6 mice at 0 h versus 12 h).

Several common genes were found to encode signaling-associated proteins (Fig. 6). Map3k8 (Tpl) and IκBζ (a molecule possessing ankyrin repeats induced by lipopolysaccharide) were identified as significantly upregulated in both organs in

wild-type mice. Their expression was marginally induced in MyD88-deficient spleens and livers, showing MyD88-dependent regulation. The genes for the signaling adapter proteins SOCS-1 and SOCS-3, as well as the transcription factor ATF-3, the inflammatory mediator adrenomedullin, c-Myc, and Dmbt1 (deleted in malignant brain tumors 1), were also identified as common genes. Map3k8, IκBζ, SOCS-1, SOCS-3, ATF-3, and adrenomedullin have been implicated in the regulation of cytokine expression (2, 13, 14, 45). All of the signaling-associated common genes were MyD88-dependently regulated in the liver. In the spleen, only a partial dependency on MyD88 could be observed for the expression levels of SOCS-3 and Dmbt1. The SOCS-1, ATF3, c-Myc, and adrenomedullin genes, however, were expressed MyD88 independently in the spleen. These data indicate that besides the regulation of chemo- and cytokines, several signaling-associated proteins are induced during septic peritonitis, and their expression partially depends on MyD88, possibly contributing to organ-specific gene expression profiles.

TABLE 4. Common and specific genes expressed in the spleen and liver and their dependency on MyD88

Category	No. of genes expressed in the spleen		No. of genes expressed in the liver	
	Common	Specific	Common	Specific
CASP-induced genes	16	100	16	69
Genes expressed more in WT than in MyD88 ^{-/-} mice (≥2-fold)	9	9	16	19
Genes expressed equally in WT and MyD88 ^{-/-} (≤1.2-fold)	5	47	0	11
Partially influenced genes	2	44	0	39

TABLE 5. Genes regulated in both the liver and the spleen during septic peritonitis

Array ID	Gene product	Expression level ^a			Ratio (WT/MyD88 ^{-/-} expression)	Expression level			Ratio (WT/MyD88 ^{-/-} expression)
		WT liver	MyD88-deficient liver	Control liver		WT spleen	MyD88-deficient spleen	Control spleen	
100319_at	IL-10	214.2	99.1	22.4	2.2	425.0	207.2	92.1	2.1
102218_at	IL-6	156.1	75.6	42.6	2.1	1,222.8	293.1	52.9	4.2
103486_at	IL-1 β	355.6	87.9	58.9	4.0	1,895.1	801.4	571.3	2.4
102736_at	CCL2	596.6	230.4	50.2	2.6	961.2	451.7	73.9	2.1
102424_at	CCL3	372.8	157.5	52.2	2.4	845.5	321.4	151.4	2.6
98772_at	CXCL5	153.2	40.9	52.6	3.7	909.0	108.3	63.0	8.4
101160_at	CXCL2	1,110.0	258.7	73.2	4.3	1,318.9	326.0	86.1	4.0
101436_at	CXCL3	626.3	308.7	236.8	2.0	671.6	767.6	326.6	0.9
104155_f_at	ATF3	377.5	185.3	96.4	2.0	306.6	317.8	104.4	1.0
104712_at	C-MYC	130.3	63.5	44.7	2.1	900.7	946.5	428.3	1.0
99479_at	DMBT-1	257.8	69.4	87.8	3.7	793.1	548.4	354.8	1.4
162206_f_at	SOCS3	1,204.2	512.2	548.7	2.4	2,355.5	1,494.1	647.7	1.6
98988_at	Mail (pending)	438.3	77.3	150.1	5.7	847.3	192.2	248.5	4.4
102798_at	ADM	138.9	36.9	33.0	3.8	85.5	100.0	42.5	0.9
97106_at	MAP3K8	143.7	44.6	43.4	3.2	499.7	201.3	183.8	2.5
92832_at	SOCS1	449.9	222.9	178.2	2.0	803.6	779.1	348.5	1.0

^a Values for expression level are expressed in relative fluorescence units.

Upregulation of IFN-regulated genes in MyD88-deficient mice. Interestingly, an analysis of genes that were upregulated in MyD88^{-/-} organs revealed that the expression of a subset of genes was >2-fold greater in MyD88^{-/-} than in wild-type organs, indicating a positive influence of MyD88 deficiency (Fig. 1). In MyD88-deficient spleens, the expression of 16 genes (14.9% of 107 upregulated genes) was upregulated more than twofold compared to that for wild-type mice, while in MyD88-deficient livers, 9 genes (25.7% of 35 upregulated genes) showed this expression pattern. Importantly, in the clusters of genes which were overexpressed in septic MyD88-deficient organs compared to wild-type organs, interferon-regulated genes were highly represented (Fig. 7). Moreover, overexpression of interferon-regulated genes in septic MyD88-deficient mice was also observed at the protein level, as was shown for CXCL9 and CXCL10 (Fig. 3).

The expression of IFN-regulated genes was validated by real-time PCR for interferon-induced gene with tetratricopeptide repeats 1 (ifit1), interferon-induced gene with tetratricopeptide repeats 3 (ifit3), viral hemorrhagic septicemia virus-induced gene (*vig-1*), and CXCL-10, which were shown to be upregulated in MyD88-deficient mice by microarray analyses. The results in Fig. 8A reveal upregulation of ifit1, ifit3, and *vig* in the spleen and the liver, which is consistent with the microarray data. Also in accordance with the microarray analyses, CXCL-10 mRNA was found to be upregulated in spleens of septic MyD88-deficient mice compared to those of wild-type mice (Fig. 8A and 2B).

To analyze whether the upregulation of IFN-regulated genes is constitutive or dependent on sepsis induction, we compared the baseline expression levels of the IFN-regulated genes by real-time PCR. As depicted in Fig. 8B, the baseline expression levels of ifit1, ifit3, *vig*, and CXCL10 were comparable between wild-type and MyD88^{-/-} mice, except for a weak basal elevation of *vig* expression in livers of MyD88-deficient mice. However, this difference in basal expression of *vig* cannot explain the difference observed after sepsis induction. These data

therefore indicate that the upregulation of IFN-dependent genes in MyD88-deficient mice is sepsis related.

These data therefore suggest that MyD88 deficiency leads to a disproportionate induction of the MyD88-independent pathway and indicate interference of different adaptor-driven pathways during septic peritonitis.

Role of TRIF in upregulation of IFN-regulated genes in MyD88-deficient mice. Toll-like receptor signaling through the adapter protein TRIF stimulates the production of type I interferons and the expression of IFN-regulated genes (33, 44). To analyze the contribution of TRIF to sepsis-induced gene expression in the spleen, we analyzed the expression of IFN-regulated genes in TRIF^{1ps2/1ps2} mice 6 h after sepsis induction by real-time PCR. The results in Fig. 9 demonstrate that the expression of ifit1, ifit3, and IP-10 was significantly attenuated in TRIF^{1ps2/1ps2} spleens, whereas the expression of *vig* was only weakly reduced.

Thus, TRIF is involved in sepsis-induced gene expression during polymicrobial sepsis and contributes to MyD88-independent gene regulation.

DISCUSSION

Global transcriptional changes during sepsis were investigated by gene array studies using a model of polymicrobial peritonitis in mice. To determine the contribution of TLR-mediated pathways to sepsis-induced gene transcription, we included MyD88-deficient mice in the study. Previously, we showed that the expression of some sepsis-induced cyto- and chemokines is differentially regulated in the spleen and the liver during MyD88 deficiency (42). Using microarray analysis, we now demonstrate organ-specific gene regulation by the MyD88 pathway at the level of genome-wide expression analysis. In addition, we identified common and specific genes induced during sepsis in the liver and the spleen. Notably, MyD88 deficiency was also associated with a disproportionately increased induction of IFN-regulated genes.

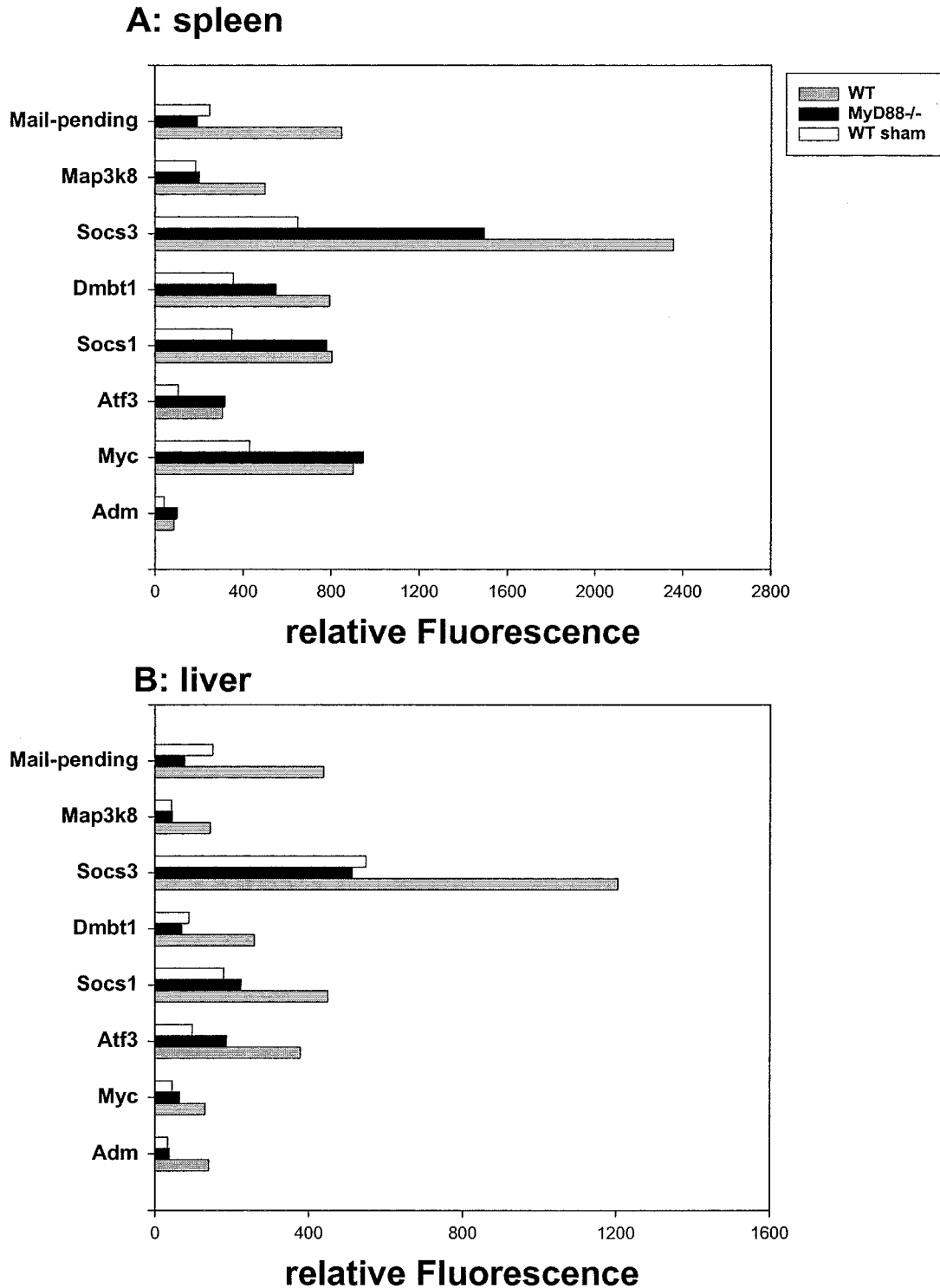


FIG. 6. Common genes induced by septic peritonitis. RNAs from septic wild-type (gray bars) and MyD88-deficient (black bars) mice and control mice after sham surgery (white bars) were prepared for hybridization to Affymetrix U74Av2 mouse expression arrays as described in Materials and Methods. Gene expression in the spleen (A) and the liver (B) is shown.

Comparing gene expression in wild-type livers and spleens to that in MyD88-deficient organs, gene induction in septic MyD88-deficient livers was strongly attenuated compared to that in wild-type mice, indicating a strong MyD88 dependence

of transcription in the liver. In the spleen, however, gene expression was only marginally affected by the lack of MyD88, indicating that gene expression in the spleen is largely MyD88 independent. These data indicate that gene expression during

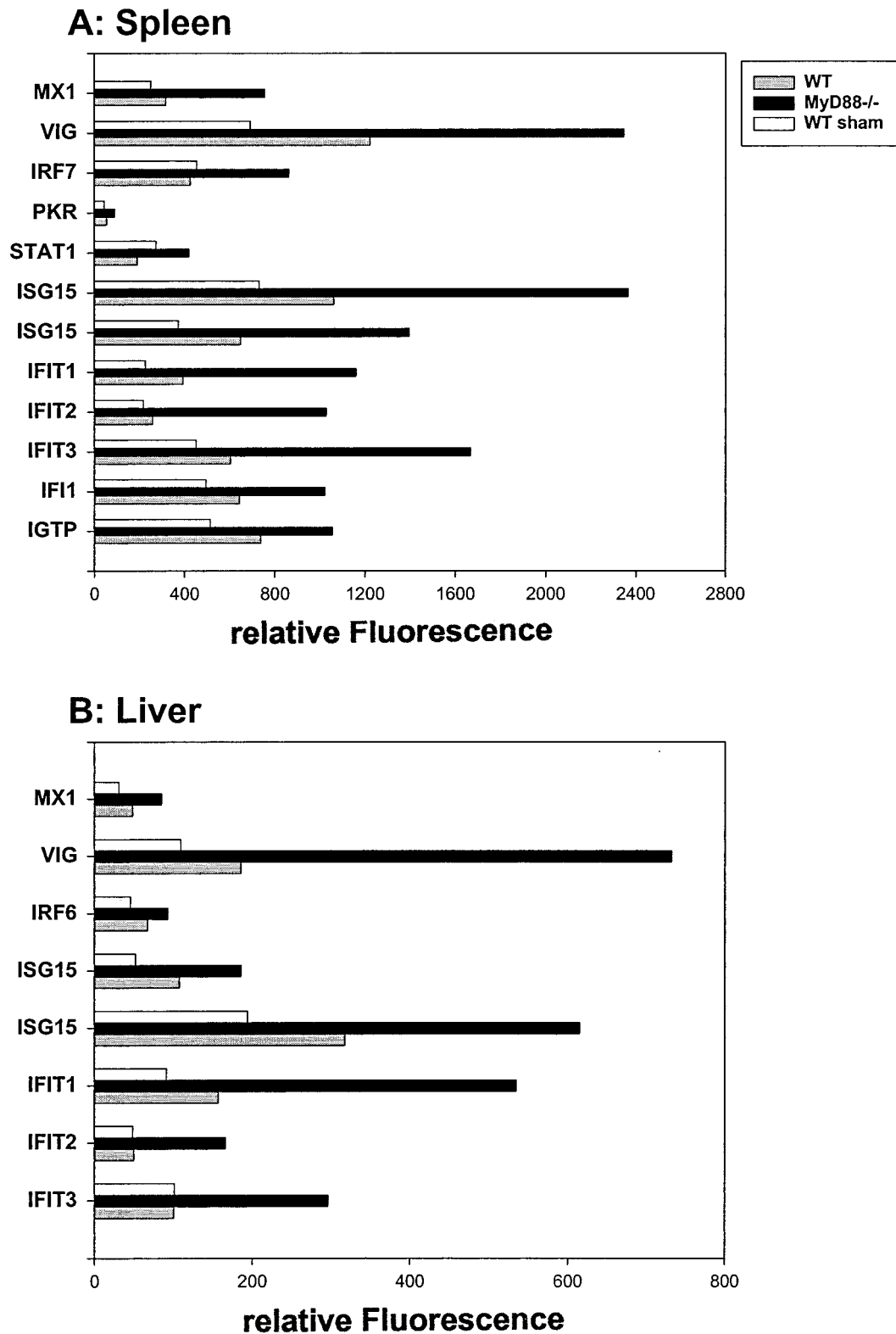


FIG. 7. MyD88 deficiency leads to overexpression of IFN-regulated genes. RNAs from septic wild-type (gray bars) and MyD88-deficient (black bars) mice and control mice after sham surgery (white bars) were prepared for hybridization to Affymetrix U74Av2 mouse expression arrays as described in Materials and Methods. Gene expression in the spleen (A) and the liver (B) is shown.

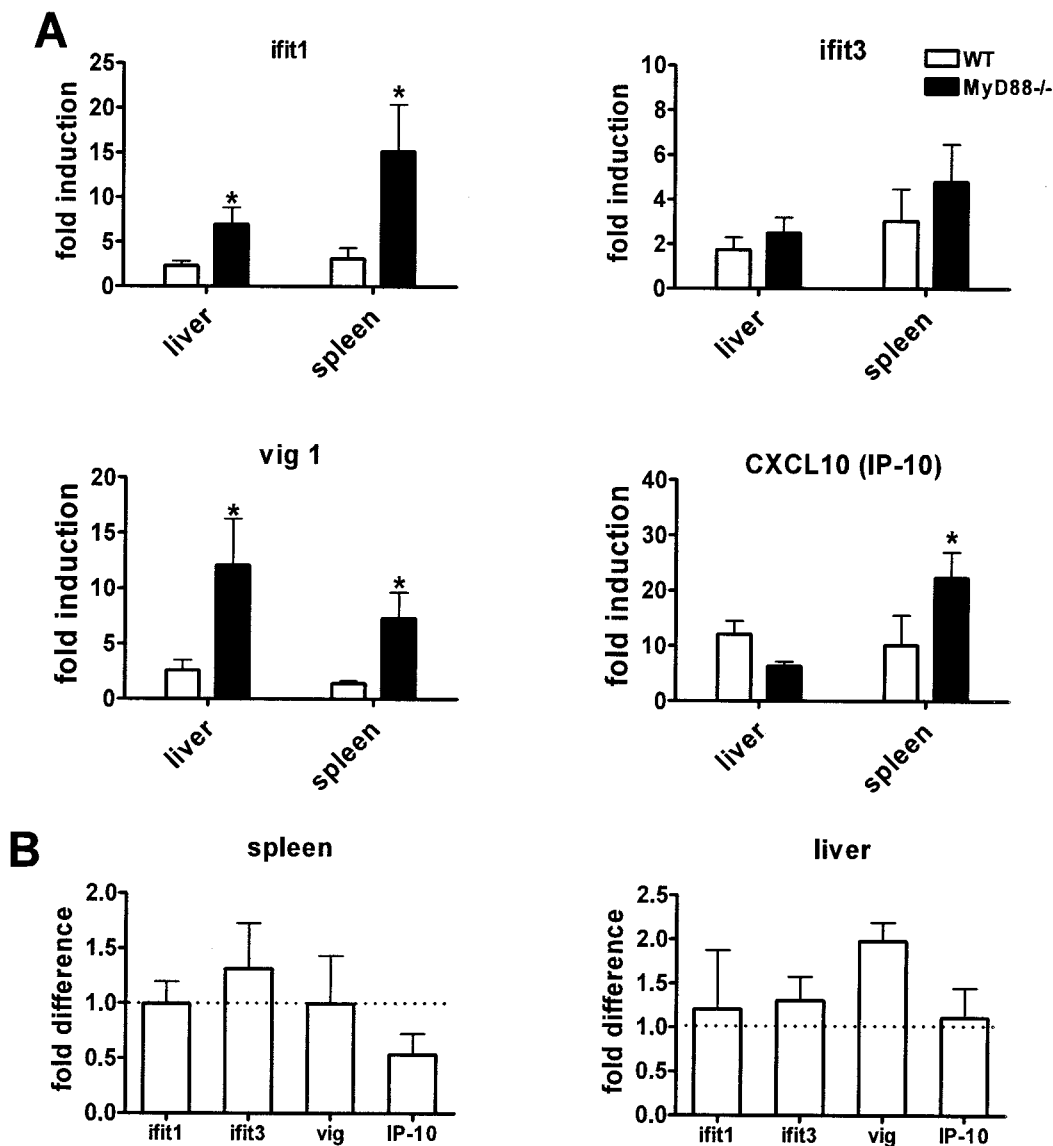


FIG. 8. Overexpression of IFN-regulated genes. RNAs were extracted from spleens and livers of wild-type (white bars) and MyD88-deficient (black bars) mice before and 6 h after CASP. Expression levels for ifit1, ifit3, vig, and CXCL10 were determined by quantitative real-time PCR. (A) Relative expression was calculated, using expression levels before CASP as a calibrator. (B) To compare baseline expression levels in MyD88-deficient and wild-type mice, relative expression was calculated, using expression levels of wild-type mice as a calibrator. Results were derived from four to six mice per group and time point. *, $P < 0.05$ (MyD88^{-/-} versus C57BL/6 mice).

polymicrobial sepsis is regulated by MyD88 in an organ-specific manner. At the molecular level, an organ-specific regulatory role of MyD88 could be due to differential expression of TLR adapter proteins (38) or inhibitors of TLR signaling (29), such as MyD88s (24). MyD88s is a splice variant lacking the intermediate domain between the TIR and the death domain. MyD88s lacks the ability to recruit IRAK-4 and therefore acts as a dominant-negative inhibitor of MyD88 (7). Since MyD88s is predominantly expressed in the spleen, it is conceivable that gene expression in the spleen might be selective for a predominance of MyD88-independent mechanisms. In addition, differences in the cellular compositions of the organs studied as well as cell type-specific usage of signaling pathways (38) may contribute to the organ-specific role of MyD88 in the regula-

tion of the immune response to sepsis. However, analyzing the distribution of B cells, T cells, macrophages, conventional dendritic cells, NK cells, and granulocytes by flow cytometry revealed no significant difference between wild-type and MyD88-deficient mice, indicating that cellular composition may not be responsible for the differential gene expression in spleens of MyD88-deficient mice.

Previous work has indicated that MyD88-deficient mice exhibit a survival advantage in a model of septic peritonitis (42). Compartmentalized gene regulation and residual MyD88-independent gene expression may provide a possible explanation for this finding. Due to these processes, MyD88 deficiency would not result in complete ablation of the inflammatory response and impairment of the host defense. Instead, the lack

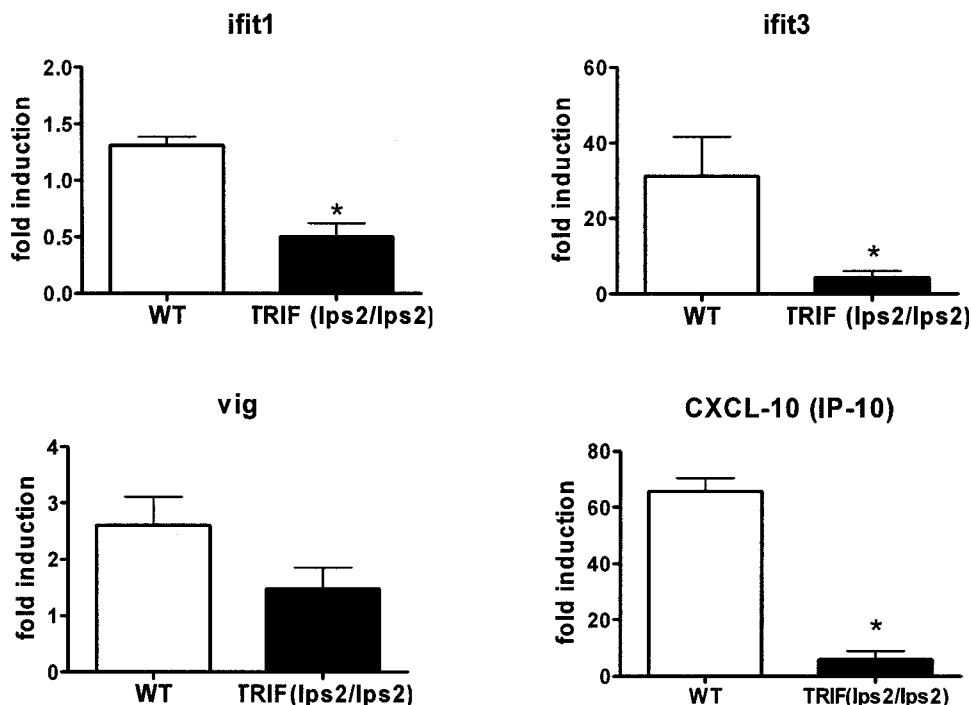


FIG. 9. TRIF-dependent production of IFN-regulated genes during septic peritonitis. RNAs were extracted from spleens of wild-type and TRIF^{lps2/lps2} mice before and 6 h after CASP. Expression levels for ifit1, ifit3, vig, and CXCL-10 were determined by quantitative real-time PCR. Relative expression was calculated, using expression levels before CASP as a calibrator. Results were derived from four mice per group and time point. *, $P < 0.05$ (TRIF^{lps2/lps2} versus C57BL/6 mice).

of MyD88 would strongly attenuate inflammation in organs typically damaged during sepsis, such as the liver, while preserving a strong immune response in a lymphoid organ such as the spleen. As a consequence, MyD88-dependent gene expression in the liver may reduce inflammatory organ injury, whereas MyD88-independent gene expression in the spleen may lead to the production of a distinct amount of inflammatory mediators which may contribute to a protective immune response. MyD88 is not only part of the TLR-induced signaling pathway but is also involved in the IL-1 and IL-18 receptor signaling pathway. Both cytokines are upregulated by the CASP procedure (5, 16, 40) and may therefore contribute in an autocrine manner to the sepsis-induced cytokine burst. It is therefore conceivable that MyD88 deficiency may also influence IL-1 and IL-18 functions during sepsis in addition to direct TLR-induced signaling.

The activation of immune responses involves the upregulation of inflammatory mediators, such as cyto- and chemokines. When analyzing genes that were upregulated during polymicrobial peritonitis, we detected a large number of inflammation-related cyto- and chemokines. Consistent with our findings with the CASP model, the induction of CXCL2, CCL4, IL-1 β , and IL-10 was also observed after sepsis induction by cecal ligation and puncture (9). To corroborate the microarray data, the expression of inflammatory mediators was also measured in whole-organ protein extracts. Importantly, the influence of MyD88 on gene expression, as determined by microarray analysis, was confirmed at the protein level for most genes examined in the present study (e.g., IL-6, CCL4, CCL5, CXCL9, CXCL10, and granulocyte CSF) or in previous work

(42). Notable exceptions, however, were IL-1 β and CXCL9, which exhibited differences between mRNA and protein expression. These findings suggest that, at least for some genes, posttranscriptional mechanisms may be crucial for the regulation of expression during polymicrobial sepsis. For example, IL-1 β expression is also regulated posttranscriptionally through processing by ICE protease, which is regulated in a MyD88-independent manner (28). Thus, the differences between IL-1 β protein and mRNA levels could result from intracellular accumulation of IL-1 β (8, 11). In addition, the mere presence of IL-1 β or CXCL9 in organ extracts does not allow for conclusions about its site of production. Thus, it is also conceivable that IL-1 β or CXCL9 released into the systemic circulation at a distant location could be trapped by specific receptors in the organ investigated.

In septic livers, a large number of cyto- and chemokines were induced. As in the spleen, IL-1 β , IL-10, IL-6, CXCL9, CXCL2, CCL3, and CCL2 were induced, thus identifying them as common genes and pointing to an important role of these mediators during sepsis. The induction levels of most of the cyto- and chemokines were higher in wild-type than in MyD88-deficient livers. Only CCL2 and CCL3 showed significant upregulation in septic MyD88-deficient livers compared to controls (from sham surgery), but their expression levels were still significantly lower than those in wild-type livers, indicating largely MyD88-dependent expression. The expression of CSF-3, IL-1 β , IL-6, IL-10, CXCL9, CXCL2, and CXCL5 was strongly dependent on MyD88. mRNA data could be validated for the majority of genes by ELISA measurements of whole-organ protein extracts. CCL5 was MyD88-independently regulated in

septic livers, consistent with the reported dependency on the adaptor proteins TRAM and TRIF (18).

Besides chemo- and cytokines, several signaling-related molecules were identified as common genes, suggesting that they might play a role in the regulation of the proinflammatory response during septic peritonitis. SOCS-1 and SOCS-3 were found to be induced in both organs in wild-type mice. Both molecules were implicated in TLR signaling (2, 3, 10, 32) and may regulate cytokine expression or downstream effects (26, 46) in inflammatory processes such as septic peritonitis. SOCS-3 expression was completely MyD88 dependent in the liver but was induced in MyD88-deficient septic spleens. ATF-3 belongs to the family of ATF/Creb transcription factors (19) and was also shown to be induced after LPS stimulation in macrophages (6, 13), suggesting a role in inflammatory processes. Adrenomedullin, which we found to be upregulated in both organs after sepsis induction, was shown to be of importance during sepsis (39). I κ B ζ (45) and Map3k8 (14, 15) are also involved in TLR-induced signaling. The expression of both molecules was MyD88 dependent in the liver and the spleen, suggesting that they might be involved in MyD88-dependent cytokine regulation. The expression of SOCS-1, SOCS-3, ATF-3, and adrenomedullin was differentially regulated in MyD88-deficient spleens and livers, further emphasizing organ-specific effects during polymicrobial peritonitis.

While the expression of a large number of genes was attenuated during MyD88 deficiency, some genes were expressed at elevated levels in MyD88-deficient compared to wild-type organs. Further analyses revealed that these genes were IFN-regulated. Analysis of baseline expression levels by real-time PCR revealed that the upregulation of IFN-regulated genes in MyD88-deficient mice was not constitutive but was dependent on peritonitis induction. Enhanced expression of Vig1, ifit3, and thymidylate kinase as well as CXCL-10 and interferon-regulated factor 1 (IRF1) could also be demonstrated in LPS-stimulated MyD88-deficient macrophages (5). TLR-induced signaling also involves the adaptor protein TRIF, which leads to the induction of type I IFN and the expression of IFN-induced genes. Thus, MyD88-independent signaling may involve the adaptor TRIF during sepsis. Analysis of gene expression in TRIF^{lps2/lps2} defective mice showed TRIF-dependent expression of IFN-regulated genes in septic spleens, thereby demonstrating a contribution of TRIF to sepsis-induced MyD88-independent gene regulation. Gene expression data for LPS-activated dendritic cells (41) and macrophages (20) revealed TRIF-dependent ifit1, ifit2, and *vig-1* expression. These data demonstrate that the deficiency of MyD88 not only attenuates the expression of some genes but also leads to the upregulation of several TRIF/IFN-induced genes, indicating a delicate balance between MyD88- and TRIF-controlled pathways. Expression levels of IFN- β , which is induced by TLR/TRIF-dependent signaling, were only weak and were not statistically significant in either the liver or the spleen (data not shown). Nevertheless, enhanced induction of STAT1 and IRF7 in MyD88^{-/-} compared to wild-type spleens could point to amplified activation of type I IFN-induced signaling and to a significant biological activity of IFN- β even at very low concentrations.

Besides organ compartmentalization of the immune response during septic peritonitis, the overexpression of IFN-regulated

genes might contribute to the improved survival of MyD88-deficient mice. Although the role of some IFN-regulated genes in host defense has been well established with infection models using viral, parasitic, or intracellular bacterial pathogens, further studies will be required to elucidate the function of this set of genes during the immune response against polymicrobial sepsis. Nevertheless, it appears important to consider the possibility that an overrepresentation of the TRIF/IFN pathway may also have contributed to the phenotype of MyD88-deficient mice and cells observed in previous studies.

In summary, we showed that during the early phase of septic peritonitis, a mainly inflammation-biased gene expression program is activated in the liver and the spleen. Whereas gene expression in the liver during sepsis is widely attenuated by MyD88 deficiency, gene expression in the spleen is largely MyD88 independent, indicating an organ-specific regulation of MyD88 signaling. Furthermore, the increased expression of a set of IFN- and TRIF-induced genes in MyD88^{-/-} organs indicates a critical balance between MyD88 and TRIF pathways.

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