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*J Immunol* 2012; 188:5833-5837; Prepublished online 14 May 2012; doi: 10.4049/jimmunol.1200038

http://www.jimmunol.org/content/188/12/5833
Cutting Edge: Divergent Cell-Specific Functions of MyD88 for Inflammatory Responses and Organ Injury in Septic Peritonitis

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Although global MyD88 deficiency attenuates lethal inflammation in sepsis, cell-specific functions of MyD88 remain largely unknown. Using mice with selective expression of MyD88 in myeloid cells (Myd88<sup>MYEL</sup>), we show that, during polymicrobial septic peritonitis, both myeloid and nonmyeloid cells contribute to systemic inflammation, whereas myeloid cell MyD88 was sufficient to fully establish the peritoneal cytokine response. Importantly, Myd88<sup>MYEL</sup> mice developed markedly aggravated liver injury that was linked to impaired upregulation of cellular inhibitor of apoptosis protein 2 and an excessive production of TNF-α. Uptregulation of inducible cAMP early repressor (ICER), a known transcriptional repressor of the Tnfα gene, was impaired in Myd88<sup>MYEL</sup> mice. Moreover, Myd88<sup>MYEL</sup> mice showed enhanced transcription of the Tnfα gene and an excessive production of CCL3, which is also negatively regulated by ICER, but they had normal levels of CXCL1, which is expressed in an ICER-independent manner. Together, these findings suggest a novel protective role for nonmyeloid cell MyD88 in attenuating liver injury during septic peritonitis. The Journal of Immunology, 2012, 188: 5833–5837.

Signaling through TLRs is considered important for the induction of innate immune responses against microbial pathogens, but it may also cause inflammatory organ damage (1). TLR signaling involves recruitment of adapter proteins, such as MyD88, to the cytoplasmic receptor interfaces, leading to activation of MAPKs and transcription factors, such as NF-κB and IRF proteins, and inflammatory gene expression. Global gene deficiency of Myd88 protects mice from LPS-induced septic shock (2) and ameliorates immune pathology caused by polymicrobial septic peritonitis (3, 4). Although Myd88 is ubiquitously expressed, it may exhibit cell-specific functions in sepsis. For example, global Myd88 deficiency severely impaired gene expression in liver, but it had only minor influence in spleen (5).

To elucidate cell-specific functions of MyD88 in polymicrobial septic peritonitis, a mouse strain exhibiting MyD88 expression exclusively in myeloid cells was generated. We found that Myd88 contributes to systemic inflammation by acting both in myeloid and nonmyeloid cells. Myd88 expression in myeloid cells was sufficient to establish a normal inflammatory response in the peritoneal cavity and the lung. Surprisingly, myeloid cell-specific expression of Myd88 resulted in markedly enhanced liver injury that was associated with an excessive production of TNF-α and an impaired expression of the antiapoptotic protein cellular inhibitor of apoptosis protein (c-IAP)2 and the transcriptional repressor inducible cAMP early repressor (ICER).

Materials and Methods

Colon ascendens stent peritonitis model of polymicrobial septic peritonitis

Female Myd88<sup>LSL/LSL</sup>, Myd88<sup>MYEL</sup>, and wild-type (WT) C57BL/6 mice were used at 8–12 wk of age. Mice were kept according to national guidelines for animal care in a specified pathogen-free animal facility. All Myd88 mutant mice used in this study were backcrossed to the C57BL/6 background for 8–10 generations.

The colon ascendens stent peritonitis (CASP) procedure for the induction of polymicrobial septic peritonitis was described in detail previously (6). Briefly, an 18-gauge venous catheter was inserted into the antimesenteric part of the colon ascendens. Stool was milked into the colon ascendens until a small drop appeared at the end of the stent. Fluid resuscitation of mice was performed by i.p. administration of 0.5 ml saline. Tissue samples were obtained 6 or 12 h after the induction of septic peritonitis by the CASP procedure. Cytokine production and organ injury were previously shown to reach plateau levels during this time period (6, 7).
Cytokine protein levels

Protein concentrations of cytokines and chemokines in blood plasma, peritoneal lavage fluid, and tissue culture supernatants were determined using ELISA kits from R&D Systems.

Organ injury

For determination of lung myeloperoxidase activity, tissue samples were weighed, frozen in liquid nitrogen, and stored at −70°C. Tissues were homogenized in 50 mM potassium phosphate buffer (pH 6) containing 0.5% (w/v) hexadecylammonium bromide (Sigma). The homogenates were shaken frozen in liquid nitrogen, thawed rapidly, and centrifuged at 14,000 × g for 7 min. Serial dilutions of the supernatants were added to the tetramethylbenzidine substrate solution for determination of myeloperoxidase activity. The reaction was stopped by the addition of H2SO4, and the absorption was determined at 450 nm. Myeloperoxidase from human leukocytes (Sigma) served as the standard.

To examine the development of liver injury during septic peritonitis, plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by standardized protocols at the Institute of Clinical Chemistry, Technische Universität München.

Determination of mRNA and primary transcript levels

RNA was prepared from liver samples using the RNeasy mini kit (QIAGEN). First-strand cDNA was synthesized from 1 μg total RNA using the QuantiTect Reverse Transcription Kit (QIAGEN). Quantitative RT-PCR analyses were performed using the Universal ProbeLibrary (Roche Diagnostics). mRNA expression levels were normalized to those of β-actin and were displayed as fold change relative to liver samples of untreated mice used as calibrator.

Statistical analysis

ANOVA, followed by the Bonferroni post hoc test, was used for analysis of differences among experimental groups. The p values < 0.05 were considered significant.

Results and Discussion

Generation of a mutant mouse strain allowing for conditional expression of MyD88

Global Myd88 deficiency attenuates systemic hyperinflammation and lethality in murine sepsis models (2, 3), but the cell-type-specific functions of MyD88 in sepsis remain largely unknown. In this study, we generated a conditional mutant mouse strain (Myd88SL/LSL) by inserting a floxed transcriptional termination element between exons 1 and 2 of the Myd88 gene (Supplemental Fig. 1A). Myd88MYEL mice were generated by intercrossing Myd88SL/LSL mice with LysMcre mice (9). Using bone marrow-derived macrophages, we found that Myd88SL/LSL cells lacked detectable MyD88 protein, whereas cells from Myd88MYEL mice expressed WT levels of MyD88 protein and showed a complete loss of the transcriptional termination element (Supplemental Fig. 1B, 1C).

Following stimulation with the TLR2 agonist Pam3Cys, phosphorylation of p38 and JNK kinases, degradation of IκBα, and production of IL-6 and TNF-α were comparable between WT and Myd88MYEL macrophages but were absent in macrophages from Myd88SL/LSL mice (Supplemental Fig. 1C, 1D). Thus, Myd88SL/LSL mice harbor a conditional null allele of Myd88, whereas MyD88 expression and function are fully recovered in macrophages of Myd88MYEL mice.

MyD88 expression in both myeloid and nonmyeloid cells is required for systemic hyperinflammation in septic peritonitis

Using the CASP model of septic peritonitis (6), we found that blood plasma levels of IL-12p40, IL-10, IL-6, and CXCL1, all of which are known to be released in a MyD88-dependent manner (3, 5), were strongly reduced in Myd88SL/LSL mice compared with WT controls (Fig. 1), confirming inactivation of the Myd88 gene. Importantly, selective expression of MyD88 in myeloid cells was sufficient to reconstitute WT levels of systemic IL-10 and IL-12p40, whereas plasma levels of IL-6 and CXCL1 did not differ significantly between myeloid cell-reconstituted Myd88MYEL and Myd88-deficient Myd88SL/LSL mice (Fig. 1). Consistent with their regulation through the TRIF-signaling pathway of TLRs, plasma levels of RANTES and CXCL10 were not affected by the absence or myeloid cell expression of MyD88.

The results indicate that the MyD88-dependent systemic inflammatory response in sepsis is mediated, in part, by myeloid cells, revealing an important contribution of nonmyeloid cells. Endothelial cells serve as an interface between the host and blood-borne pathogens and secrete cytokines, such as IL-6 and IL-8, upon TLR engagement (10, 11). Therefore, it appears conceivable that, during septic peritonitis, endothelial cells contribute to systemic IL-6 and CXCL1 levels, whereas IL-10 and IL-12p40 may be released into the circulation by myeloid cells. Consistent with this notion, previous work showed that hepatic Kupffer cells are the major source of systemic IL-10 in murine septic peritonitis (12), and Kupffer cell density was found to be comparable among WT, Myd88SL/LSL, and Myd88MYEL mice (Supplemental Fig. 1E).

Compartment-dependent influence of myeloid cell MyD88 on inflammatory responses

Consistent with their plasma levels, peritoneal concentrations of IL-6, IL-10, CXCL1, and CXCL2 were strongly reduced in Myd88SL/LSL mice compared with WT mice (Fig. 2). Production of RANTES and CXCL10 was not altered by the lack of MyD88 (Fig. 2). In contrast, expression of MyD88 in myeloid cells was sufficient to fully reconstitute not only peritoneal levels of IL-10 and CXCL2 but also those of IL-6 and CXCL1 (Fig. 2). Furthermore, peritoneal CXCL10 production was significantly reduced in Myd88MYEL mice.
compared with Myd88SL/SL mice. Control experiments showed that untreated WT, Myd88SL/SL, and Myd88MYEL mice had comparable numbers of peritoneal macrophages (Supplemental Fig. 1F).

These results suggest that the relative contribution of myeloid and nonmyeloid cell MyD88 to the inflammatory response in septic peritonitis is dependent on the anatomic compartment involved and is consistent with a previous studies revealing organ-specific activities of MyD88 (3, 5). Although human intestinal epithelial and peritoneal mesothelial cells were shown to produce various cytokines upon in vitro stimulation (13–16), our studies do not support a role for these cells in murine septic peritonitis. Species-specific differences, different conditions of stimulation, and a potentially more potent cytokine-producing activity of macrophages compared with epithelial or mesothelial cells may provide possible explanations.

**Expression of MyD88 in myeloid cells promotes organ injury in septic peritonitis**

To elucidate the role of myeloid cell-specific MyD88 expression in septic lung inflammation, myeloperoxidase activity was measured in organ extracts as a marker of neutrophil accumulation, which is considered to play a key role in organ damage (17). We found that septic peritonitis caused a comparable increase in lung myeloperoxidase activity in WT and Myd88MYEL mice (Fig. 3A). In contrast, lung myeloperoxidase activity was only weakly elevated in septic Myd88SL/SL mice (Fig. 3A). Thus, myeloid cell-specific expression of MyD88 is sufficient to fully induce lung inflammation during polymicrobial septic peritonitis.

In additional experiments, the development of liver injury was examined. The results in Fig. 3B show that septic WT mice had significantly elevated plasma AST and ALT levels compared with untreated controls and that the sepsis-induced increase in AST and ALT levels was attenuated in Myd88SL/SL mice. Surprisingly, in septic Myd88MYEL mice, both AST and ALT levels were strongly increased compared with WT mice (Fig. 3B). In addition, AST and ALT levels were significantly higher in Myd88MYEL mice than in Myd88SL/SL mice. Therefore, these results indicate that myeloid cell-specific expression of MyD88 markedly aggravates liver injury during septic peritonitis.

**Aggravated liver injury in septic Myd88MYEL mice is associated with enhanced TNF-α production**

To uncover potential mechanisms underlying the enhanced liver injury in Myd88MYEL mice, the expression of genes encoding anti- or proapoptotic proteins was examined. The results show that mRNA levels of XIAP, Bcl-2, Bcl-w, Bcl-XL, and cFLIP were not significantly regulated in liver samples of WT, Myd88SL/SL, and Myd88MYEL mice (Supplemental Fig. 2A). In contrast, c-IAP1 and c-IAP2 were elevated at both 6 and 12 h after sepsis induction (Fig. 4A, Supplemental Fig. 2A). Although expression levels did not differ significantly among the three mouse strains for c-IAP1, induction of c-IAP2 was significantly higher in Myd88MYEL mice than in Myd88SL/SL mice. Therefore, these results indicate that myeloid cell-specific expression of MyD88 markedly aggravates liver injury during septic peritonitis.
c-IAP2 was significantly enhanced in WT mice compared with Myd88LSL/LSL and Myd88MYEL mice at the 6-h, but not the 12-h, time point (Fig. 4A). Thus, the early and transient upregulation of c-IAP2 during septic peritonitis appears to require expression of MyD88 in nonmyeloid cells.

Analysis of genes encoding proapoptotic proteins revealed that liver mRNA levels of the death receptor ligands FasL and TRAIL, as well as of BH3-only members Noxa, Bim, and Puma, were not significantly different among WT, Myd88LSL/LSL, and Myd88MYEL mice (Supplemental Fig. 2B). Hepatic TNF-α mRNA levels were elevated to a comparable extent in the three mouse strains, when analyzed 6 h after sepsis induction. However, at the 12-h time point, TNF-α mRNA levels further increased in liver samples from Myd88MYEL mice but not WT or Myd88LSL/LSL mice (Fig. 4B). Protein concentrations of TNF-α in blood plasma paralleled hepatic mRNA levels and were substantially increased in Myd88MYEL mice compared with WT and Myd88LSL/LSL mice at the 12-h, but not the 6-h, time point (Fig. 4C). Thus, selective expression of MyD88 in myeloid cells substantially impairs the control of both hepatic and systemic TNF-α production.

TNF-α is also known to mediate inflammatory liver injury during systemic inflammation that is elicited following administration of Con A or upon injection of LPS or bacterial superantigens into mice sensitized with D-galactosamine (18–20). Interestingly, c-IAP proteins are known to suppress the formation of cell death-inducing signaling complexes upon engagement of TNFR1, and overexpression of c-IAP2 inhibits apoptosis of rat hepatocytes (21). Therefore, it is conceivable that excessive TNF-α production is crucial for mediating enhanced liver injury in Myd88MYEL mice and that the lack of early c-IAP2 upregulation aggravates these effects.

Expression of MyD88 in nonmyeloid cells is required for upregulation of the transcriptional repressor ICER

The results indicate that, during septic peritonitis, MyD88 in nonmyeloid cells is important to control the release of TNF-α by a paracrine mechanism. TNF-α production may be inhibited by multiple pathways, including IL-10, and agents that increase cellular cAMP levels. However, we found that both local and systemic IL-10 levels are fully reconstituted in Myd88MYEL mice (Figs. 1, 2), suggesting an IL-10–independent mechanism.

Macrophage TNF-α production may be regulated by pathways involving TGF-β1, PGE2, or NO (22–24). However, hepatic mRNA levels of NO synthase 2, PG-endoperoxide synthase 2, and TGF-β isoforms did not differ significantly among WT, Myd88LSL/LSL, and Myd88MYEL mice, with the exception of a moderate increase in TGF-β3 expression in Myd88MYEL mice 6 h after sepsis induction (Supplemental Fig. 2C). However, it appears unlikely that the lack of transiently increased TGF-β3 expression may explain enhanced liver injury in Myd88MYEL mice, because transgenic overexpression of TGF-β was reported to promote, rather than to inhibit, TNF-α levels (24).

Previous studies showed that elevation of cellular cAMP levels may induce expression of ICER, which represses Tnfa gene expression by competing with transactivating transcription factors, such as ATF2, for promoter binding (8, 25). In liver samples from WT mice, ICER mRNA levels were strongly elevated 6 h after induction of septic peritonitis and declined thereafter (Fig. 4D). In marked contrast, the early induction of ICER mRNA was completely absent in liver samples from Myd88LSL/LSL mice and, importantly, also from those of Myd88MYEL mice (Fig. 4D). The transient upregulation of ICER may be explained by previous observations showing that it represses the activity of its own promoter in an autoregulatory manner (26).

To obtain evidence for an involvement of ICER in the regulation of TNF-α expression during septic peritonitis, we first examined the transcription rate of the Tnfa gene. As shown in Fig. 4E, primary transcript levels of TNF-α increased to a comparable extent in liver samples from WT, Myd88LSL/LSL, and Myd88MYEL mice 6 h after sepsis induction. However, at the 12-h time point, primary transcript levels of TNF-α were significantly increased in Myd88MYEL mice compared with WT and Myd88LSL/LSL mice, suggesting that the transcriptional control of the Tnfa gene is impaired during this phase of septic peritonitis in Myd88MYEL mice. In addition, the expression of CCL3, which is repressed by ICER (27), was investigated. CCL3 mRNA levels were markedly upregulated in Myd88MYEL mice compared with WT and Myd88LSL/LSL mice 12 h after the induction of septic peritonitis, whereas CCL3 expression did not significantly differ between Myd88MYEL and WT mice at the 6-h time point (Fig. 4F). Notably, plasma and peritoneal levels of CXCL1, which is not a target of ICER (25), were not enhanced in Myd88MYEL mice (Figs. 1, 2). Thus, the expression pattern of inflammatory mediators is consistent with a regulatory role for ICER.

The findings of the current study suggest that MyD88 expression in both myeloid and nonmyeloid cells contributes to systemic inflammation and organ injury in septic peritonitis. Importantly, the lack of MyD88 expression in nonmyeloid cells leads to enhanced liver injury, which is associated with an impaired upregulation of the apoptosis inhibitor c-IAP2 and an impaired control of TNF-α production. Our results further suggest that activation of nonmyeloid cells through MyD88 contributes to the control of TNF-α production in septic peritonitis by induction of the transcriptional repressor ICER. Consistent with this interpretation, the protective effects of calcitonin gene-related peptide on the development of liver injury caused by injection of LPS into d-galactosamine–sensitized mice correlated with the induced expression of ICER (28).

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure Legends

Supplemental Figure 1: Generation of Myd88\textsuperscript{MYEL} mice.

\textbf{(A)} Targeting strategy for switch-on mutagenesis of Myd88. Schematic maps of the Myd88 wildtype locus (\textit{MyD88\textsuperscript{WT}}), the targeting vector, the conditionally inactivated Myd88 allele (\textit{Myd88\textsuperscript{LSL}}), and the Myd88 allele after excision of the intron gene trap cassette by Cre-mediated recombination (\textit{MyD88\textsuperscript{lox}}) are shown. The targeting vector was constructed using homologous fragments of the \textit{Myd88} locus with an intron gene trap cassette (Ref. 1) placed between exons 1 and 2 of the \textit{Myd88} gene. The expected fragment sizes after hybridization of Xba I-digested genomic DNA with the 3' flanking probe (3'FP) are indicated.

\textit{En-2/stp}, engrailed-2 splice acceptor site followed by stop codons in all reading frames;

\textit{IRES}, internal ribosomal recognition site; \textit{lacZ}, β-galactosidase expression cassette;

\textit{DSE}, downstream element; \textit{neo}, neomycin resistance cassette; \textit{TK}, herpes virus 1 thymidine kinase expression cassette.

\textbf{(B)} Southern blot analysis of BMM. Correctly targeted E14.1 embryonic stem cell clones were injected into C57BL/6 blastocysts and chimeric mice were intercrossed to C57BL/6 mice. For generation of \textit{Myd88\textsuperscript{MYEL}} mice, \textit{Myd88\textsuperscript{LSL/LSL}} mice were intercrossed with LysMcre mice. Genomic DNA of BMM from the indicated mouse strains was digested with Xba I and hybridized with the 3' FP as depicted in Suppl. Fig. 1A. Bands reflecting different \textit{Myd88} alleles are indicated.

\textbf{(C)} Reconstitution of MyD88 expression and signaling in BMM of \textit{Myd88\textsuperscript{MYEL}} mice. BMM were generated by differentiation of bone marrow cells in 15 % L cell-conditioned medium as a source of M-CSF for 5–7 d. BMM from \textit{Myd88\textsuperscript{LSL/LSL}} (LSL), \textit{Myd88\textsuperscript{MYEL}} (MYEL) and wildtype (WT) mice were stimulated stimulated with 1 μg/ml (S)-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-N-
palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4-OH (P3C) for the indicated time periods and analyzed by Western blotting for expression of MyD88 and induction of TLR2 signaling. Antibodies specific for phospho-p38 MAPK, phospho-SAPK/JNK, IκBα (all from Cell Signaling Technology) and MyD88 (Stressgen) or α-tubulin (Calbiochem) were used.

**D** Reconstitution of MyD88 function in BMM of Myd88MYEL mice. BMM were generated from Myd88LSL/LSL (LSL), Myd88MYEL (MYEL) and wildtype (WT) mice. Cells were stimulated with P3C for 16 h. Secretion of IL-6 and TNFα into culture supernatants was analyzed by ELISA. Results are representative of at least 3 independent experiments.

**E** Kupffer cell density is independent of MyD88 expression. Histologic sections of livers from Myd88LSL/LSL (LSL), Myd88MYEL (MYEL) and wildtype (WT) mice were stained with mAb F4/80 to visualize Kupffer cells or an isotype matched IgG control. Results of IgG controls for LSL and MYEL mice are similar to those of WT mice (not shown).

**F** Peritoneal macrophage numbers are independent of MyD88 expression. Peritoneal lavage cells were collected from Myd88LSL/LSL (LSL), Myd88MYEL (MYEL) and wildtype (WT) mice and analyzed by flow cytometry. Absolute numbers of CD11bhi F4/80+ cells are indicated. Values are not significantly different.

**Supplemental Figure 2: Gene expression in liver.**

RNA was extracted from liver samples of Myd88LSL/LSL (LSL), Myd88MYEL (MYEL) and wildtype (WT) mice 6 h or 12 h after CASP. Expression of genes encoding various anti-apoptotic (A) and pro-apoptotic (B) proteins as well as potential regulators of TNFα expression or liver injury (C) was determined by quantitative real-time PCR. Relative mRNA expression was calculated using samples of untreated mice as calibrator. Differences in Bcl-2 expression (12
h time point) were not statistically significant (p = 0.470) as calculated using ANOVA and Bonferroni multiple comparison test. n = 5-8 mice per group; ns, not significant.

Supplemental References
