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*J Immunol* published online 14 February 2014
http://www.jimmunol.org/content/early/2014/02/14/jimmunol.1300157

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2014/02/14/jimmunol.1300157.DCSupplemental

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A Key Regulatory Role for Vav1 in Controlling Lipopolysaccharide Endotoxemia via Macrophage-Derived IL-6

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Macrophages are centrally involved in the pathogenesis of acute inflammatory diseases, peritonitis, endotoxemia, and septic shock. However, the molecular mechanisms controlling such macrophage activation are incompletely understood. In this article, we provide evidence that Vav1, a member of the RhoGEF family, plays a crucial role in macrophage activation and septic endotoxemia. Vav1-deficient mice demonstrated a significantly increased susceptibility for LPS endotoxemia that could be abrogated by anti–IL-6R Ab treatment. Subsequent studies showed that Vav1-deficient macrophages display augmented production of the proinflammatory cytokine IL-6. Nuclear Vav1 was identified as a key negative regulator of macrophage-derived IL-6 production. In fact, Vav1 formed a nuclear DNA-binding complex with heat shock transcription factor 1 at the HSE2 region of the IL-6 promoter to suppress IL-6 gene transcription in macrophages. These findings provide new insights into the pathogenesis of endotoxemia and suggest new avenues for therapy. The Journal of Immunology, 2014, 192: 000–000.

Sepsis develops as a life-threatening escalation of bacterial and bacterial-driven endotoxemia and still represents a key challenge in the field of intensive care medicine. Advanced septic organ manifestations are primarily based on an uncontrolled exposure of immune cells to immunogenic bacterial components, such as LPS (1). Because of their ability to sense these bacterial motifs through pattern recognition receptors, innate immune cells and, in particular, macrophages are centrally involved in the initiation of septic disease (2, 3). By releasing proinflammatory mediators, activated macrophages are able to drive direct tissue damage. A pathological overproduction of IL-6 was demonstrated in patients with sepsis and correlated with disease mortality (4, 5). To improve the clinical outcome of sepsis patients, it would be elementary to dampen specifically this overwhelming burst of innate immune cells without completely abolishing their capacity to control bacterial invasion. However, future identification of appropriate molecular target structures requires a more detailed understanding of intracellular signaling transduction.

Vav1 represents the first member of the Rho/Rac guanine nucleotide exchange factor family, is exclusively expressed in hematopoietic cells (6), and broadly impacts the intracellular signaling-transduction machinery, sometimes independent from its GDP/GTP-exchange activity (7–9). Findings from Vav1-deficient mice revealed a pivotal role for Vav1 in T cell immunity (10). Vav1 relevantly impacts T cell proliferation, apoptosis, and cytoskeletal reorganization (7, 9, 11, 12). In contrast to the precise description of Vav1 in lymphocytes (9, 13), its role in the innate immune response remains less well defined. In accordance with its impact on cytoskeleton remodeling, Vav1 was shown to control dendritic cell migration, neutrophil invasion, and macrophage phagocytosis (14–16). However, until now, data regarding the functional involvement of Vav1 in the downstream-signaling cascade of TLR have been incomplete. LPS-initiated TLR-4 signaling resulted in MyD88-dependent phosphorylation of Vav proteins, and a combined Vav1/Vav2/Vav3 deficiency was associated with decreased production of reactive oxygen intermediates, as well as TNF-α, in LPS-stimulated macrophages (17). On a molecular level, the intracellular link between LPS-initiated Vav1 activation and induction of proinflammatory mediators remained undefined.

In this study, we took advantage of Vav1-deficient mice (10) and analyzed the modulating effect of Vav1 in LPS-induced endotoxemia. We identified an unexpected protective function of Vav1 and uncovered its capacity to suppress LPS-induced IL-6 gene transcription in macrophages.

Materials and Methods

Animals

Vav1-deficient (Vav1−/−) mice were described earlier (10) and were kindly provided by V.L.J.T. Unless otherwise indicated, the genetic background of Vav1−/− and WT control animals was BALB/c. Cross-breeding allowed
Vav1 CONTROLS LPS ENDOTOXEMIA VIA MACROPHAGE-DERIVED IL-6

Confocal microscopy was performed with a Leica SPS laser scanning confocal microscope. Images were acquired using a 63× 1.3 NA objective.

Immunofluorescence staining of liver cryosections was performed using anti-myeloperoxidase Ab (Abcam), followed by Cy3 donkey anti-rabbit secondary Ab (Biogen) and Hoechst 3342 staining of nuclei. Quantification of positive cells was performed by fluorescence microscopy (Olympus) using a 10× objective.

Western blot
Western blotting was performed as described previously (12). Abs directed against Vav1, phospho–NF-κB p65, phospho–SAPK/JNK, β-actin (Cell Signaling Technology), or HSF1 (Santa Cruz Biotechnology) were used. HRP-conjugated anti-rabbit IgG Ab (Cell Signaling Technology) combined with the ECL Western blotting system (Thermo Scientific) allowed signal detection.

Immunoprecipitation
Using whole-cell extracts, immunoprecipitation was performed with HSF1 Abs (Santa Cruz Biotechnology) or an Ab directed against EGFP (Clontech) and the Catch and Release Reversible Immunoprecipitation System (Merck). Normal rabbit IgG (Santa Cruz Biotechnology) was included as control. Immunoprecipitates were analyzed for Vav1 (Cell Signaling Technology) or HSF1 (Thermo Scientific) by Western blot technology.

Chromatin immunoprecipitation
Specific protein–DNA complexes were detected by chromatin immunoprecipitation (ChIP) assay (Millipore). Cells were fixed, lysed, and sheared by sonification. Protein–DNA complexes were immunoprecipitated with Vav1 or HSF1 Abs or normal rabbit IgG (Santa Cruz Biotechnology) and protein A–agarose. Specific detection of the HSE2 region of the murine IL-6 promoter was performed by PCR (primers: 5′-GCA ACT CTC ACA GAG ACT AAA GG-3′ and 5′-GGG CAC CAG CACA GTATA GTG TGT C-3′). The pMACS FactorFinder Kit (Miltenyi Biotec) was used for identification of HSE-binding proteins. Whole-cell lysates were incubated with a biotinylated DNA probe containing an ideal HSE sequence (21): 5′-TTC ACC CAC TTT ACC CTA GAA GCT TCT TGT AGC AGC TTC ACC TAG CAA ATT TGG AGG-3′ (ideal HSE sequence is underscored). A scrambled biotinylated DNA probe was used as negative control: 5′-GAT GAT TAA CTC CTT AAT TCA CGG GTA TCT AAT CCA CCG AGT CCT GCG ATA GAA-3′. Probe-bound proteins were selected via Streptavidin Microbeads and were analyzed by Western blotting.

Plasmid constructs and site-directed mutagenesis
The murine IL-6 promoter fragment (NT_16294.3; 4014963–4016554) was amplified from WT BALB/c genomic DNA (primers: 5′-AAG CAC ACG GCA GGG AAT AG-3′ and 5′-GTT GGC TCC TGA AGC GAA GTA GG-3′), subcloned (StrataClone Blunt PCR Cloning Kit; Agilent Technologies), and inserted into the pGL4.10[luc2] vector (Promega). A deletion mutation (ΔHSE2 IL-6 promoter), which lacked the HSE2 region, was generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene). EGFP-Vav1 constructs were generated (22) and kindly provided by V. L. J. T. EGFP-Vav1 coding fragments were PCR amplified from pMSCV-EGFP-Vav1 vectors using primers introducing an NheI site and Kozak sequence at the 5′ end and a XhoI site at the 3′ end. Amplified EGFP-Vav1 constructs were cloned into NheI and XhoI cut pcDNA3.1 vector (Invitrogen).

Transfection and luciferase reporter assay
Roti-Fect (Roth) was used for transient transfection of J774A.1 cells. An Amaza Mouse Macrophage Nucleofector Kit (Lonza) was used for transient transfection of primary macrophages. The Dual-Luciferase Reporter Assay System (Promega) was used. Relative promoter activity was defined as the ratio of firefly luciferase/Renilla luciferase activity and was normalized to WT IL-6 promoter activity.

Statistical analysis
Statistical significance of differences was determined using the Student t test (Excel; Microsoft) or log-rank test (GraphPad Prism).

Results
Hypersensitivity of Vav1+/− mice to LPS exposure
Although numerous ex vivo studies (17, 22–24) described a crucial involvement of Vav1 in adoptive and innate immunity, until now...
only limited data existed about the in vivo relevance of Vav1 in the context of acute inflammatory diseases. To address this aspect, we analyzed Vav1<sup>−/−</sup> mice in the experimental model of LPS-induced endotoxemia. Interestingly, Vav1 deficiency resulted in disease exacerbation. Vav1<sup>−/−</sup> mice showed significantly decreased survival rates, with a median survival of 24 h compared with 42 h for WT mice (Fig. 1A). Even when the LPS dosage was reduced to levels that resulted in a milder course of disease (60% of WT mice survived), Vav1 deficiency was associated with earlier and increased mortality (Supplemental Fig. 1A). Furthermore, significantly increased serum levels of GLDH, AST, and creatinine in Vav1<sup>−/−</sup> mice indicated more pronounced organ damage (Fig. 1B). Additional analysis of Vav1-deficient mice in two bacterial-driven sepsis models confirmed the protective role of Vav1 in the context of septic endotoxemia (Supplemental Fig. 1B).

Vav1<sup>−/−</sup> mice are known to have reduced numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes in the periphery (10, 25), a failure of TCR-signaling transduction (23), and a subsequently diminished alloreactivity (26). To exclude the possibility that the lack of Vav1 in lymphocytes plays a role in the observed hypersensitivity of Vav1<sup>−/−</sup> mice to LPS endotoxemia, Vav1-deficient and -proficient Rag2<sup>−/−</sup> mice were subjected to LPS treatment. Indeed, even in the absence of B and T lymphocytes and NKT cells in Rag2<sup>−/−</sup> mice, deficiency of Vav1 resulted in significantly higher mortality (Fig. 1C), suggesting a predominant role for Vav1-deficient innate immune cells. To further characterize involved innate immune cell populations, we found that tissue infiltration of neutrophil granulocytes remained unchanged in the absence of Vav1 (Supplemental Figs. 2A, 2B).

**FIGURE 1.** LPS hypersensitivity of Vav1<sup>−/−</sup> mice. (A) Kaplan–Meier survival rate analysis of WT (n = 30) and Vav1<sup>−/−</sup> (n = 30) mice after i.p. administration of LPS (20 mg/kg). Summarized data from three independent experiments. (B) Two hours after LPS administration, serum concentrations of GLDH, AST, and creatinine were quantified (n ≥ 4). *p < 0.05. (C) Survival of LPS (10 mg/kg)-exposed Rag2<sup>−/−</sup> Vav1<sup>+/+</sup> (n = 9) and Rag2<sup>−/−</sup> Vav1<sup>−/−</sup> (n = 5) mice was monitored. Summarized data from two independent experiments. (D) Macrophage-depleted WT mice (Clod−lip−WT) were reconstituted with WT or Vav1<sup>−/−</sup> macropahes (n = 7) before LPS injection (20 mg/kg) and monitored for survival. Combined results of two independent experiments are shown.

Vav1 regulates LPS-induced IL-6 expression in macrophages

More detailed in vitro analysis of the regulatory function of Vav1 in activated macrophages revealed that Vav1<sup>−/−</sup> peritoneal macrophages show a significantly increased expression of the proinflammatory cytokine IL-6 in response to LPS stimulation, whereas the absence of Vav1 did not affect the expression of TNF-α (Fig. 2A, 2B). Furthermore, transient Vav1 restoration in knockout macrophages resulted in a significantly decreased production of IL-6 after LPS stimulation (Fig. 2C). Because Vav1-deficient T lymphocytes showed unaltered IL-6 production in response to TCR stimulation (Supplemental Fig. 2C), a selective impact of Vav1 on LPS-initiated IL-6 production in activated macrophages was suggested.

The capacity of Vav1 to control LPS-induced expression of IL-6 also was confirmed in vivo. Significantly increased levels of IL-6 were detected in the serum, as well as in the peritoneal lavage, of LPS-exposed Vav1<sup>−/−</sup> mice compared with WT mice, whereas the concentration of TNF-α or IL-10 did not differ markedly (Fig. 2D, 2E, Supplemental Fig. 3). Macrophages promote septic organ damage through the overwhelming release of proinflammatory cytokines, but they likewise participate crucially in the early defense against invading bacteria (28). Notably, augmented IL-6 secretion in the absence of Vav1 could not be explained by alterations in the overall percentage of macrophages within blood or peritoneal cells (Fig. 2F), but rather resulted from increased IL-6 expression per single cell (Fig. 2G). The functional relevance of IL-6 for Vav1 functions was demonstrated by the fact that Ab-mediated in vivo neutralization of IL-6R signaling abrogated hypersensitivity of Vav1<sup>−/−</sup> mice to LPS endotoxemia. Moreover, LPS-exposed WT mice revealed lower mortality upon blockade of IL-6R signaling, although the effects were less pronounced compared with Vav1-deficient animals (Fig. 2H). Therefore, our data suggest that Vav1 plays a crucial role in endotoxemia by controlling IL-6 expression in macrophages.

**Nuclear Vav1 controls IL-6 promoter activity**

The observation that WT and Vav1<sup>−/−</sup> macrophages produced equally low levels of IL-6 in the presence of Actinomycin D (data not shown) indicated that the regulatory role of Vav1 depends on a de novo induction of IL-6 gene transcription. However, in agreement with previous findings in Vav<sup>−/−</sup> macrophages (17), analysis of NF-kB, p38 MAPK, JNK, and SOCS as key regulators of IL-6 expression revealed no differences between WT and Vav1<sup>−/−</sup> cells (Fig. 3). In addition to these signaling molecules, HSF1 recently was shown to be essential for optimized induction of IL-6 gene transcription in macrophages. Mechanistically, binding of HSF1 to the IL-6 promoter induces opening of the chromatin structure and subsequently enables the recruitment of additional activators of transcription (29). Therefore, we assessed...
potential regulation of HSF1 activity by Vav1. Peritoneal macrophages from WT or Vav1−/− mice were isolated and stimulated with LPS ex vivo. (A) At 30 min after LPS stimulation, mRNA expression of IL-6 and TNF-α was analyzed by real-time PCR. Mean values ± SEM of six independent experiments. (B) At 2 h after LPS stimulation, concentrations of IL-6 and TNF-α in supernatants were quantified by ELISA. Cytokine secretion of stimulated WT macrophages was defined to be 100%. Mean values ± SEM of five independent experiments. (C) Vav1−/− peritoneal macrophages were transiently transfected with pcDNA3.1 expression vector encoding EGFP-Vav1. At 4 h posttransfection, macrophages were exposed to LPS for 1–2 h, and secreted IL-6 in supernatants was quantified by ELISA. Vav1−/− macrophages transfected with empty pcDNA3.1 vector served as control and accordant IL-6 production was set to 100%. Mean values ± SEM of three experiments. At 2 h after in vivo LPS administration, cytokine concentrations in serum (n = 6) (D) and peritoneal lavage (n = 8) (E) of WT or Vav1−/− mice were measured (mean values ± SEM of four independent experiments) and blood and peritoneal lavage cells were characterized by flow cytometry (*p < 0.05). (F) Gated CD11b+ cells were analyzed for F4/80 and Ly6C expression. The percentage of CD11b+ F4/80+ Ly6C− cells is indicated. (G) CD11b+ F4/80+ Ly6C− macrophages from WT and Vav1−/− mice were analyzed for intracellular IL-6 expression. Control IgG isotype staining was included. One representative experiment of three independent experiments is shown. (H) Vav1−/− and WT mice were challenged with LPS (20 mg/kg) and treated with anti–IL-6R Abs (WT ○, n = 3; Vav1−/− ▼, n = 3) after 2 and 3 h. LPS-challenged Vav1−/− mice (▼, n = 3) and WT mice (▲, n = 3) were included as control group. Survival was monitored. Summarized results of two independent experiments are shown (p = 0.02).

FIGURE 2. LPS hypersensitivity of Vav1−/− mice is driven by macrophage-derived IL-6. Peritoneal macrophages from WT or Vav1−/− mice were isolated and stimulated with LPS ex vivo. (A) At 30 min after LPS stimulation, mRNA expression of IL-6 and TNF-α was analyzed by real-time PCR. Mean values ± SEM of six independent experiments. (B) At 2 h after LPS stimulation, concentrations of IL-6 and TNF-α in supernatants were quantified by ELISA. Cytokine secretion of stimulated WT macrophages was defined to be 100%. Mean values ± SEM of five independent experiments. (C) Vav1−/− peritoneal macrophages were transiently transfected with pcDNA3.1 expression vector encoding EGFP-Vav1. At 4 h posttransfection, macrophages were exposed to LPS for 1–2 h, and secreted IL-6 in supernatants was quantified by ELISA. Vav1−/− macrophages transfected with empty pcDNA3.1 vector served as control and accordant IL-6 production was set to 100%. Mean values ± SEM of three experiments. At 2 h after in vivo LPS administration, cytokine concentrations in serum (n = 6) (D) and peritoneal lavage (n = 8) (E) of WT or Vav1−/− mice were measured (mean values ± SEM of four independent experiments) and blood and peritoneal lavage cells were characterized by flow cytometry (*p < 0.05). (F) Gated CD11b+ cells were analyzed for F4/80 and Ly6C expression. The percentage of CD11b+ F4/80+ Ly6C− cells is indicated. (G) CD11b+ F4/80+ Ly6C− macrophages from WT and Vav1−/− mice were analyzed for intracellular IL-6 expression. Control IgG isotype staining was included. One representative experiment of three independent experiments is shown. (H) Vav1−/− and WT mice were challenged with LPS (20 mg/kg) and treated with anti–IL-6R Abs (WT ○, n = 3; Vav1−/− ▼, n = 3) after 2 and 3 h. LPS-challenged Vav1−/− mice (▼, n = 3) and WT mice (▲, n = 3) were included as control group. Survival was monitored. Summarized results of two independent experiments are shown (p = 0.02).
significantly in the absence of Vav1, whereas a lack of the HSE2 binding site completely abrogated differences between WT and Vav1−/− macrophages (Fig. 5C).

In summary, the interaction among Vav1, HSF1, and the HSE2 binding site could be identified as a crucial event in the regulation of IL-6 promoter activity in macrophages (Fig. 5D). This finding significantly complements former reports about a potential function of Vav1 within transcriptional active complexes in the cell nucleus (8, 9). To our knowledge, this is the first time that the involvement of Vav1 in DNA-binding complexes could be linked to a particular in vivo consequence.

Discussion
In the present study, we took advantage of Vav1-deficient mice to uncover an unexpected role for Vav1 in the LPS-triggered immune response by controlling macrophage-derived IL-6 production. Vav1 deficiency resulted in augmented IL-6 production in macrophages and in a subsequently increased susceptibility of Vav1−/− mice to LPS endotoxemia. Regarding the underlying molecular mechanism, we identified the capacity of Vav1 to participate in nuclear DNA-binding protein complexes in the HSE2 region of the IL-6 promoter and, thereby, to prevent overwhelming IL-6 promoter activity in LPS-stimulated macrophages. These findings provide important new insights into the molecular pathogenesis of LPS endotoxemia and introduce the Vav1/HSF1/HSE2 axis as a potential therapeutic target.

The observation that Ab-mediated blockade of IL-6 signaling was able to efficiently reverse the LPS hypersensitivity of Vav1−/− mice in our experimental setting once again underlined the critical relevance of the proinflammatory cytokine IL-6 in the pathogenesis of endotoxemia. Beneficial preventive effects of IL-6 small interfering RNA pretreatment (30) and a significant therapeutic capacity of sgp130Fc-mediated blockade of IL-6 trans-signaling (31) were demonstrated in the CLP model of sepsis. Findings from
HSF1 on TLR-4–triggered IL-6 expression. HSF1 on the IL-6 promoter and, thereby, dampens the supportive function of this cytokine in multiple intercellular processes (40) and, finally, lead to the conclusion that therapeutic neutralization of complete IL-6 signaling may not be beneficial under all circumstances. Along those lines, it is interesting to note that Vav1 targeting Vav1-regulating microRNAs to prevent downregulation of function of Vav1. In this context, Vav1-regulating microRNAs (41) might represent potential therapeutic target structures. Because our data do not exclude the existence of other nuclear-binding partners of Vav1, in addition to HSF1, through which Vav1 might impact on macrophage biology, this issue, as well as how Vav1 is able to interfere with transcription factors, such as NFAT and NF-κB, in T lymphocytes. Because our data do not exclude the existence of the described oncogenic potential of Vav proteins (7), even described an unaltered overall mortality of IL-6–deficient mice in the CLP model (37). These difficulties in defining the exact functional contribution of IL-6 in the pathogenesis of sepsis (38, 39) probably arise primarily from the pluripotent function of this cytokine in multiple intercellular processes (40) and, finally, lead to the conclusion that therapeutic neutralization of complete IL-6 signaling may not be beneficial under all circumstances. Along those lines, it is interesting to note that Vav1 deficiency augmented IL-6 production in macrophages only, whereas T lymphocyte–derived IL-6 production remained unaffected. This selective induction of IL-6 production was associated with significantly decreased survival rates for Vav1-deficient mice. Because the observed LPS hypersensitivity of Vav1−/− mice was not associated with an altered expression of TNF-α or IL-10, and it turned out to be mediated primarily by Vav1-deficient macrophages, our data suggested that the endotoxic phase of septic disease might be controlled by a selective regulation of IL-6 expression in macrophages.

Macrophages exert pleiotropic functions in the pathogenesis of sepsis (2, 27). However, the functional role of these cells was underlined by studies demonstrating that mice with reduced numbers of monocytes had an impaired bacterial clearance but, at the same time, significantly milder levels of septic tissue destruction (28). In this study, we identified Vav1 as a key protective player in controlling the overwhelming LPS-induced IL-6 cytokine response of these innate immune cells by targeting HSF1 in the nucleus. Structural studies revealed that the NLS of Vav1 protein is essential to mediate cellular Vav1/HSF1 complex formation. This finding allowed two potential interpretations: either HSF1 interacts directly with the NLS-containing PH domain of Vav1 or a stable formation of the Vav1/HSF1 complex depends on the capacity of Vav1 to translocate into the cell nucleus. Because the SH3-SH2-SH3 cassette, rather than the PH domain, was reported to allow Vav1 to interact directly with a number of well-described protein-binding partners (7, 22), our findings suggest a key role for nuclear Vav1 localization in Vav1/HSF1 complex formation. Via its capacity to dampen the HSF1-triggered excessive IL-6 production in LPS activated macrophages (Fig. 5D), Vav1 suppressed lethality in experimental endotoxemia. Previous reports (8, 9) described a relevant nuclear interaction between Vav1 and transcription factors, such as NFKB and NF-κB, in T lymphocytes. Because our data do not exclude the existence of the described oncogenic potential of Vav proteins (7), even described an unaltered overall mortality of IL-6–deficient mice in the CLP model (37). These difficulties in defining the exact functional contribution of IL-6 in the pathogenesis of sepsis (38, 39) probably arise primarily from the pluripotent function of this cytokine in multiple intercellular processes (40) and, finally, lead to the conclusion that therapeutic neutralization of complete IL-6 signaling may not be beneficial under all circumstances. Along those lines, it is interesting to note that Vav1 expression of complete IL-6 signaling may not be beneficial under all circumstances. Along those lines, it is interesting to note that Vav1 deficiency augmented IL-6 production in macrophages only, whereas T lymphocyte–derived IL-6 production remained unaffected. This selective induction of IL-6 production was associated with significantly decreased survival rates for Vav1-deficient mice. Because the observed LPS hypersensitivity of Vav1−/− mice was not associated with an altered expression of TNF-α or IL-10, and it turned out to be mediated primarily by Vav1-deficient macrophages, our data suggested that the endotoxic phase of septic disease might be controlled by a selective regulation of IL-6 expression in macrophages.

In the clinical context of septic shock, the described nuclear mechanism of action of Vav1 might provide relevant information for future therapeutic approaches. Our data suggest that augmented nuclear translocation of Vav1 and the stable proximity of Vav1 to the HSE2 region of the IL-6 promoter might prevent overwhelming IL-6 production in septic patients. Therefore, these findings add a new perspective to the field of therapeutic cytokine blockade in sepsis. Future studies aiming at therapeutically applicable ways to increase the overall expression of Vav1 in macrophages and, in particular, to promote the enrichment of Vav1 within the cell nucleus will be challenging, but they might benefit from recently gained insights into the regulation, structure, and function of Vav1. In this context, Vav1-regulating microRNAs (41) might represent potential therapeutic target structures. Because of the described oncogenic potential of Vav proteins (7), targeting Vav1-regulating microRNAs to prevent downregulation of cellular Vav1 levels might be a more attractive option than a therapeutically induced overexpression of Vav1.

Most of the cytokine-based clinical efforts concentrated on anti–TNF-α Ab treatment of septic patients and were able to achieve a modest survival benefit (42). However, the failure of Ab therapy to induce more extensive clinical effects in sepsis might be due to the fact that a global TNF-α blockade leads to a potent systemic immunosuppression and, thereby, weakens the control of pathogen invasion (43). Therefore, mimicking the dampening function of Vav1 in the cell nucleus could provide a more sophisticated

FIGURE 5. Nuclear Vav1 impacts IL-6 promoter activity. Macrophages were isolated from WT or Vav1−/− mice and stimulated with LPS ex vivo. (A) Vav1- or HSF1-bound DNA fragments were precipitated by ChIP assay from WT and Vav1−/− macrophages and analyzed by PCR for the presence of the HSE2 region of the IL-6 promoter. (B) Cellular extracts from unstimulated or LPS-treated WT macrophages were incubated with biotinylated HSE probe or scrambled control probe. Vav1- or HSF1-containing DNA-protein complexes were identified by Western blot. One representative experiment of three independent experiments is shown. (C) WT and Vav1−/− macrophages were transfigured with reporter plasmids: IL-6 promoter (−1831 to +18) or ΔHSE2 IL-6 promoter. Promoter activity was measured by luciferase reporter assay after 60 min of LPS stimulation. Mean values ± SEM from three independent experiments are shown. (D) Mechanistic model: Vav1 participates in the DNA-binding complex of HSF1 on the IL-6 promoter and, thereby, dampens the supportive function of HSF1 on TLR-4–triggered IL-6 expression.
strategy to specifically target the exacerbated IL-6 expression in activated macrophages without affecting further aspects of the antimicrobial immune response.

Acknowledgments

We are pleased to use Cloc-lips, produced and provided by Nico van Rooijen, Department of Molecular Cell Biology, Free University Medical Center, Amsterdam, The Netherlands. We thank T.K., Yoshihui Oshugi, and Masahiko Mihara for providing MR16-1 (Chugai Pharmaceuticals, Shizuoka, Japan).

Disclosures

M.F.N. has provided expert scientific advice or received funding from Giuliani Pharma, Schering-Plough, Essex, UCB, Abbott Laboratories, and Pentax. The other authors have no financial conflicts of interest.

References


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Supplement Figure 1
Hypersensitivity of Vav1 deficient mice to septic stimuli.
(A) Kaplan-Meier survival rate analysis of WT (closed triangle; n=10) and Vav1<sup>−/−</sup> (open triangle; n=8) mice after i.p. administration of LPS (15 mg/kg). (B) Survival analysis of WT (closed triangle) and Vav1<sup>−/−</sup> (open triangle) mice in the experimental model of cecal ligation and puncture model (n=3 per group; upper graph) and cecal content injection (n=8 per group; summarized results from 2 experiments; lower graph).
Supplement Figure 2

LPS Hypersensitivity of Vav1 deficient mice can not be explained by neutrophil infiltration, NK cell activation or T cell derived cytokine production.

(A) Quantification of liver infiltrating neutrophils by immunofluorescence microscopy. Liver cryosections of LPS exposed WT and Vav1−/− mice were stained with anti-MPO antibody (Cy3) and Hoechst 3342. Representative images and summarized quantification (MPO+ cells per image; mean ± SEM of 3 animals per group). Scale bars represent 25 µm. (B) NK cell depletion of Vav1−/− (n=3) and WT (n=4) C57BL/6 mice was performed by i.p. injection of anti-NK1.1 antibodies at day-3 and day-1. At day 0, NK cell depleted mice were exposed to LPS (25 mg/kg i.p.) and survival was monitored. (C) Splenic CD4+ T lymphocytes from WT and Vav1−/− mice were stimulated for 24 hours with anti-CD3 and anti-CD28 antibodies. Concentrations of IL-6 and TNF-α were quantified by ELISA.
Supplement Figure 3
IL-10 serum levels in LPS exposed Vav1 deficient mice.
2 hours after \textit{in vivo} LPS administration, IL-10 cytokine concentrations in serum of WT or Vav1\textsuperscript{-/-} mice were measured by ELISA. Mean values \pm SEM with n=6.
Supplement Figure 4
Schematic view of the murine IL-6 promoter.
HSE2 region, HSE1 region and the binding sites of HSF1 within HSE2 of the IL-6 promoter (-1831 to +18) are indicated.