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CD44 Is a Negative Regulator of Acute Pulmonary Inflammation and Lipopolysaccharide-TLR Signaling in Mouse Macrophages

Jiurong Liang,* Dianhua Jiang,* Jason Griffith,† Shuang Yu,‡ Juan Fan,‡ Xiaojian Zhao,‡ Richard Bucala,† and Paul W. Noble2* 

CD44 is a transmembrane adhesion molecule and a transmembrane adhesion molecule and hematopoietic. CD44 has an essential role in hyaluronan clearance and in the regulation of LPS-TRL-signaling. Following intratracheally LPS treatment, CD44−/− mice demonstrated an exaggerated inflammatory response characterized by increased inflammatory cell recruitment, elevated chemokine expression in bronchoalveolar lavage fluid, and a marked increase in NF-κB DNA-binding activity in lung tissue in vivo and in macrophages in vitro. Furthermore, CD44−/− mice were more susceptible to LPS-induced shock. Reconstitution of hematopoietic CD44 reversed the inflammatory phenotype. We further found that the induction of the negative regulators of TLR signaling IL-1R-associated kinase-M, Toll-interacting protein, and A20 by intratracheal LPS in vivo and in macrophages in vitro was significantly reduced in CD44−/− mice. Collectively, these data suggest CD44 plays a previously unrecognized role in preventing exaggerated inflammatory responses to LPS by promoting the expression of negative regulators of TLR-4 signaling. The Journal of Immunology, 2007, 178: 2469–2475.

Inflammation is characterized by increased vascular permeability, inflammatory cell infiltration, release of inflammatory mediators by infiltrating leukocytes and parenchymal cells, and accumulation and turnover of extracellular matrix. These inflammatory responses are the primary host defense against pathogenic or noninfectious insults. CD44 is a transmembrane adhesion molecule and the major receptor for hyaluronan, a major extracellular matrix component (1). It has been demonstrated that CD44 plays an important role in local clearance of hyaluronan (2) and mediates cell-matrix interactions involved in tumor cells metastasis, tumor formation, T cell extravasation (3–5), and in various inflammatory diseases such as pneumonia (6), arthritis, vascular leak syndrome, hepatitis, and Mycobacterium tuberculosis (4). The work from our laboratory has demonstrated that CD44-deficient (CD44−/−) mice (5) succumb to unremitting inflammation in a bleomycin-induced lung injury model, characterized by impaired clearance of apoptotic neutrophils, persistent accumulation of inflammatory hyaluronan fragments at the site of tissue injury, and impaired activation of TGF-β1 (2). CD44−/− mice were found to have enhanced neutrophil accumulation and edema in Escherichia coli-induced pneumonia (6). Leemans et al. (7) reported that as a new macrophage binding site for M. tuberculosis CD44 mediates mycobacterial phagocytosis and macrophage recruitment. LPS is a major component of the Gram-negative bacteria cell wall and is largely responsible for recognition by the host innate immune system. LPS binds to and signals through a receptor complex, which consists of TLR-4, CD14, and MD2 (8, 9). Activation of TLR4 promotes NF-κB-mediated production of proinflammatory cytokines in many cell types (reviews, Refs. 10–12). Recent studies have also started to decipher the mechanisms of the negative regulation of TLR signaling (13), under which the host attenuates TLR signaling to avoid detrimental effects of inflammatory responses. Several intracellular proteins including IL-1R-associated kinase M (IRAK-M)3 (14), Toll-interacting protein (Tollip) (13, 15), suppressor of cytokine signaling 1 (SOCS-1) (16, 17), TNF-α-induced protein 3 (also called A20) (18, 19), and PI3K (20) have been identified as negative regulators of TLR signaling, although the mechanisms differ. Targeted deletion of IRAK-M led to enhanced macrophage responsiveness to LPS (14). A20-deficient mice developed severe inflammation and cachexia, were hyporesponsive to both LPS and TNF, and died prematurely, due to the failure to regulate TNF-induced NF-κB activation (18, 19).

The role that CD44 plays during the initiation of pulmonary inflammation remains unclear. Our current study examined the role of CD44 in regulating the host response to intratracheal LPS using CD44-deficient mice (5) and elucidated possible mechanisms. Our data suggest that CD44 plays a previously unrecognized role in preventing exaggerated inflammatory responses to LPS by promoting the expression of the negative regulators of TLR-4 signaling in macrophages.

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3 Abbreviations used in this paper: IRAK-M, IL-1R-associated kinase M; Toll-interacting protein; SOCS, suppressor of cytokine signaling; BAL, bronchoalveolar lavage; bHABP, biotinylated hyaluronan-binding protein; PMN, polymorphonuclear neutrophil; HA, hyaluronan.
CD44 NEGATIVELY REGULATES LPS-INDUCED INFLAMMATION

Materials and Methods

CD44-deficient mice, LPS administration, and bronchoalveolar lavage (BAL)

CD44-deficient (CD44−/−) mice were backcrossed onto C57BL/6j background for more than six generations. CD44+/− mice lack all known CD44 isoforms and manifest no overt developmental phenotype (2, 5). Eight- to 10-week-old male and female animals were used for experiments. Age- and sex-matched wild-type C57BL/6j mice were obtained from The Jackson Laboratory. LPS (from E. coli 0127:BS; Sigma-Aldrich) were dissolved in 0.9% sodium chloride solution at the concentration of 0.2 mg/ml. Under anesthesia, 1 mg/kg LPS was instilled into the lung through an angled feeding needle (22 gauge; Popper) inserted into the trachea via the mouth. Control animals received saline alone. The mice were allowed to recover until the time of bronchoalveolar lavage. Mice were anesthetized and the lungs and heart were surgically exposed. The tracheas were cannulated and the lungs were lavaged two times with 0.8 ml of PBS. BAL sample was centrifuged, and the supernatants were stored at −70°C until use. The live cells were recovered and counted using a hemocytometer. Cytospin preparations of BAL cells were stained with Protocol HEMA3 (Biochemical Sciences) and differential cell counting was performed. All animal experiments were conducted under the protocol approved by the Yale Institutional Animal Care and Use Committee.

mRNA analysis

RNA was extracted from lung tissues or cultured macrophages using TRIzol Reagent (Invitrogen Life Technologies) following the manufacturer’s instruction. For Northern analysis, 15 μg of total RNA was ethanol-precipitated on a formaldehyde agarose gel and transferred to Nytran membrane (Schleicher & Schuell). RNA was cross-linked to the membrane by UV cross-linking (Stratagene), and the blots were hybridized overnight with 106 cpm/ml [32P]DNA synthesized by the random prime method (New England Biolabs). Following hybridization, blots were washed and autoradiographies were performed. Equal loadings of RNA were documented by hybridizing selected blots with [32P]DNA for aldolase. For real-time RT-PCR analysis, 1–3 μg of total RNA were reverse-transcribed using SuperScript II RNase H−Reverse Transcriptase (Invitrogen Life Technologies) with 0.5 μg/μl oligo(dt)17 primers. One microfitter of the resultant cDNAs were subjected to real-time PCR (qPCR). Core kit for SYBR Green; Eurogentec) using the ABI Prism 7500 Detection system (Applied Biosystems). The specific primers were designed based on cdNA sequences deposited in the GenBank database. Primers for A20 (NM_000397) were CATCCACCAAGAACCTTATTGGACA (sense), and GAGGTGTCGTAGC TTTTCCCGCTTG (antisense); primers for IRAK-M (NM_028679), GCCAGA AGAATACATCAGACAGG (sense), GTCTAAGAAGGACAGGCAGGAGT (antisense); primers for Tollip (NM_023764), ATTATGGCATTGACTCG TATTGAGG (sense), CTATACACTGCTCTTCCTTGG (antisense); and primers for GAPDH (NM_00100130) ATCATCTCCGCCCCTTCTG (sense) and GGTCATGAGCCCTTCCACAAT. The primers were all cDNA-specific, not amplifying genomic DNA. Contamination of genomic DNA was negligible. The conditions for real-time PCR were as following: one cycle at 95°C for 1 min, 34 cycles at 95°C for 15 s and 60°C for 1 min, one cycle at 25°C for 2 min, no reverse transcription levels of each gene were determined against GAPDH levels in the samples.

Cytokine production

MIP-1α, MIP-2, TNF-α, CXCL10, and IL-1β protein levels in BAL were measured immunologically using commercial ELISA kits per the manufacturer’s instructions (R&D Systems).

Western blot analysis

Fresh tissues were homogenized and centrifuged and soluble supernatants were taken as whole tissue lysates. For cultured macrophages, total protein was isolated from by resuspension of cells in 62.5 mM Tris-HCl (pH 7.5) with protease inhibitors. Equal amounts of protein (10–20 μg) were separated on 4–20% gradient SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Western blots were performed with Abs against IRAK-M (Cell Signaling Technology) and visualized using enhanced chemiluminescence (Amersham Biosciences).

Hyaluronan quantitation

Hyaluronan concentrations in BAL fluid were measured with a competitive ELISA using biotinylated hyaluronan-binding protein (bHABP; Seikagaku America) as described previously (2). Briefly, samples and bHABP were incubated for 1 h. The sample-bHABP mixtures were linked onto hyaluronan coated microtiter CovaLink NH modules (Nunc). Bound bHABP were measured with a colorimetric reaction. Sample concentrations were calculated from a standard curve that was generated using hyaluronan standards of known concentration (range 0–2000 ng/ml).

Bone marrow transplantation

Six- to 8-week-old mice were irradiated with 1000 rad (137Cs irradiator) followed by i.v. injection of 5 million bone marrow cells. The mice were allowed to recover and survive for 8 wk before experiment. Successful reconstitution of CD44−/− alveolar macrophages and polymorphonuclear neutrophils (PMNs) was demonstrated by FACS analysis as described previously (2). Eight weeks after reconstitution, the mice were challenged with intratracheal LPS and the mouse lungs were lavaged after 3 h or 3 days. Total inflammatory cells and hyaluronan (HA) contents in BAL were determined. MIP-2 protein expression was measured with an ELISA kit.

Flow cytometric analysis of BAL cells

After washing with PBS, BAL cells were incubated with Mac3, Gr-1, and KMB1 Abs in PBS containing 1% BSA/0.02% sodium azide. PE-conjugated Mac-3 and Gr-1 Ab were obtained from BD Pharmingen. Anti-CD44 Ab KM1 was isolated from culture medium of hybridoma cells obtained from American Type Cell Collection. FITC-labeled secondary Ab was from Sigma-Aldrich. Samples were washed with PBS twice and then fixed with 3.7% formaldehyde. Flow cytometry was performed using a FACS-Calibur analytical flow cytometer (BD Biosciences). KMB1 binding of the cells was analyzed after gating on the macrophage and neutrophil populations using CellQuestPro software (BD Biosciences).

Isolation of lung macrophages

Alveolar macrophages were isolated from BAL of untreated mice by adherence to plastic tissue culture dishes in RPMI 1640 medium with 10% FBS (21).

Endotoxin shock and peritoneal macrophage isolation

Eight- to 10-week-old female CD44−/− and wild-type mice were injected i.p. with LPS at a dose of 20 mg/kg in 0.9% saline. Survival was monitored every 2–3 h.

Age- and sex-matched wild-type and CD44−/− mice were injected i.p. with 10 mg/kg LPS in 0.9% saline. Six hours after injection, mice were euthanized and their peritoneum was washed with 10 ml of cold PBS. The cell number was then counted in a hemocytometer. For in vitro IL-6 production by isolated peritoneal macrophages 6 h following 10 mg/kg LPS, 1 × 106 cells/well in a 24-well cell culture plate were plated in DMEM with 10% FCS. Twenty-four hour conditioned media were harvested and assayed for IL-6 cytokine production by ELISA (Bioscience).

Statistics

Differences in measured variables between genetically altered mice and control groups were assessed using the Student t test and the differences between survival curves were determined by the Wilcoxon test. Data are expressed as the mean ± SEM where applicable. Statistical difference was accepted at p < 0.05.
Results

Increased inflammatory cell transepithelial cell migration into alveolar spaces in LPS-challenged CD44-deficient mice

CD44−/− and wild-type mice all survived the acute inflammation induced by intratracheal LPS at the dose of 1 mg/kg and recovered by day 7. Examination of the inflammatory response to intratracheal LPS revealed a significant increase in total inflammatory cell numbers in the BAL fluid from CD44−/− mice over a time course from 3 h to 7 days after LPS treatment (Fig. 1A). Differential cell counts of the BAL cells revealed an increase in the cell numbers of macrophage, neutrophils, and lymphocytes (Fig. 1B). The cell infiltration peaked at day 3 after the LPS challenge and the inflammatory cells were cleared by day 7 in both CD44−/− mice and wild-type mice. Interestingly, an earlier neutrophil influx was observed in CD44−/− mice. The earliest, significant increase of neutrophil percentage in BAL cells of CD44−/− mice appeared 3 h after LPS challenge, compared with 6 h after LPS challenge in wild-type mice (Fig. 1C). Thus, CD44 appears to have a role in regulating the magnitude and the onset of the host response to LPS challenge.

We examined the role of CD44 in regulating extracellular matrix turnover during acute lung inflammation. Hyaluronan concentration was measured in BAL of LPS-treated CD44−/− and wild-type mice over a time course of 7 days. The hyaluronan content in the BAL of CD44−/− mice was 2-fold higher compared with that of wild-type mice (Fig. 1D). Hyaluronan levels in BAL of both CD44−/− and wild-type mice peaked at day 3 and declined to close to baseline at day 7 after LPS treatment (Fig. 1D).

Increased inflammatory gene expression in lung tissue of LPS-treated CD44-deficient mice

To begin to understand the mechanisms that regulate the inflammatory phenotype in CD44 deficiency, we first examined the profile and time course of cytokines and chemokines produced in response to LPS. Northern blot analysis of total RNA from LPS-challenged lung tissues revealed a pronounced induction of Cxcl2 (which encodes MIP-2), Ccl3 (which encodes MIP-1α), and Cxcl10 (which encodes IFN-γ-inducible protein 10) mRNA expression 1 and 3 h after LPS instillation in CD44−/− mice relative to wild-type mice (Fig. 2A). The induction of these chemokines occurred much earlier in CD44−/− mice, as evidenced at the 1 h time point. The inflammatory gene expression levels were diminished after 6 h in both CD44-deficient mice and wild-type mice (data not shown).

Similarly, the protein levels of chemokines and cytokines in BAL of the LPS-challenged CD44-deficient mice were also dramatically elevated in the first 6 h after LPS treatment. We found that MIP-1α, MIP-2, TNF-α, CXCL10, IL-1β, and IL-6 protein levels in BAL of CD44−/− mice were 2- to 3-fold increased relative to wild-type mice (Fig. 2, B and C, and data not shown). More dramatic differences for MIP-1α, MIP-2, CXCL10, IL-1β, and TNF-α were seen at 3 h and 6 h following LPS challenge.
Chimeric mice as well as reconstituted wild-type and CD44 (KM81) binding using flow cytometric analysis (Fig. 3, phages and neutrophils was demonstrated by anti-CD44 Ab. These are representative from two similar experiments. MIP-2 in BAL collected 3 h after LPS were measured (\(**, p < 0.05; n = 4–6\)). These are representative from two similar experiments. WT→CD44+/−; wild-type bone marrow to irradiated CD44−/− mice; CD44−/−→CD44−/−; CD44−/− bone marrow to irradiated CD44−/− mice; and WT→WT: wild-type bone marrow to irradiated WT mice.

**Reversal of the inflammatory phenotype by reconstitution of hemopoietic CD44 in CD44-deficient mice**

CD44 is expressed on the cell surface of both hemopoietic and parenchymal cells. To determine whether hemopoietic CD44 was required to regulate the inflammatory response to LPS, we reconstituted irradiated CD44−/− mice with bone marrow from wild-type mice. Successful reconstitution of CD44+ alveolar macrophages and neutrophils was demonstrated by anti-CD44 Ab (KM81) binding using flow cytometric analysis (Fig. 3, A and B). Chimeric mice as well as reconstituted wild-type and CD44−/− mice were challenged with intratracheal LPS and the inflammatory cell accumulation, hyaluronan content in BAL, and inflammatory protein levels in BAL were evaluated. The phenotype of enhanced inflammatory cell infiltration of CD44−/− mice after LPS treatment was significantly reversed in chimeric mice (Fig. 3C). MIP-2 protein production in BAL of chimeric mice returned to the levels of reconstituted wild-type mice (Fig. 3C).

MIP-2 protein after LPS treatment. Equal numbers of lung alveolar macrophages isolated from BAL of untreated wild-type (WT) and CD44−/− mice were plated onto 24-well plates overnight before stimulation with LPS at the indicated concentrations. MIP-2 protein in 24-h conditioned medium was measured by ELISA (\(*, p < 0.01; **, p < 0.05; n = 3\)). ND, not detectable. These are representative from two similar experiments.

**Enhanced NF-κB translocation in CD44-deficient mice**

Previous studies have shown that LPS induces nuclear translocation of NF-κB in murine lungs (23, 24). To determine whether CD44-deficient alveolar macrophages have enhanced responses to LPS in vitro

To further determine the cell types that may play a role in the enhanced responsiveness to LPS challenge in CD44−/− mice, alveolar macrophages were isolated from BAL of unchallenged wild-type and CD44−/− mice and stimulated with LPS in vitro. We found that CD44−/− alveolar macrophages produced significantly more MIP-2 protein after LPS stimulation than wild-type macrophages in a dose-dependent manner (Fig. 4).

**NF-κB DNA-binding activity in CD44 deficiency after LPS challenge**

A. Wild-type (WT) and CD44−/− mice were treated with LPS for 45 min, 1.5 h, and 3 h. Nuclear proteins were isolated from the lung tissue of the treated mice and were subject to EMSA. NF-κB complexes were indicated. B. Lung macrophages were isolated from WT and CD44−/− mice by flow cytometric sorting and treated with 50 ng/ml LPS for 20 min in vitro. NF-κB activity in nuclear proteins was assessed with EMSA.
CD44 plays a role in regulating LPS-induced NF-κB translocation in vivo, nuclear proteins from lung tissues of CD44−/− mice were extracted and EMSA was performed. LPS-induced NF-κB DNA-binding activity was evident 6 h after LPS in wild-type mice (Fig. 5A). A marked shift to the left in time course of NF-κB DNA-binding activity was observed in CD44−/− mice. In fact, LPS-induced NF-κB DNA-binding activity was apparent as early as 45 min after LPS treatment. More significantly, CD44 appears to have a role in the early response to sustain mouse survival against lethal LPS-induced shock (Fig. 6A). CD44−/− mice had a significant decrease in mean survival (25 h) compared with wild-type mice (43 h) upon systemic LPS treatment. More significantly, CD44 appears to have a role in the early response to sustain mouse survival against lethal LPS-induced shock (Fig. 6A).

To determine whether CD44 has an important role in the systemic response to LPS as well as local responses in the lung, we tested the impact of CD44 deficiency in a standard model of LPS-induced shock (9, 16, 17, 25–28). In accord with previous observations, CD44−/− mice were more susceptible to LPS-induced shock (Fig. 6A). CD44−/− mice had a significant decrease in mean survival time (25 h) compared with wild-type mice (43 h) upon systemic LPS treatment. More significantly, CD44 appears to have a role in the early response to sustain mouse survival against lethal LPS-induced shock (Fig. 6A).

**Figure 6.** CD44-deficient mice showed more rapid susceptibility to LPS-induced shock. A, Mean time to death of CD44−/− mice was decreased compared with wild-type mice. Survival was monitored every 2–3 h. Time to death was statistically significant by Wilcoxon test (p < 0.05, n = 9/group). Note that the loss of CD44 appeared to have a more rapid susceptibility to lethal LPS. B, Total cells were recovered from the peritoneum of CD44−/− and wild-type after administration of LPS 10 mg/kg i.p. (p = 0.001, n = 4). C, Peritoneal macrophages isolated from CD44−/− and wild-type mice after LPS injection for 6 h were cultured in vitro for 24 h. IL-6 in the conditioned medium was measured by ELISA (p < 0.0001, n = 4).

CD44 limits endotoxin shock

To determine whether CD44 has an important role in the systemic response to LPS as well as local responses in the lung, we tested the impact of CD44 deficiency in a standard model of LPS-induced shock (9, 16, 17, 25–28). In accord with previous observations, CD44−/− mice were more susceptible to LPS-induced shock (Fig. 6A). CD44−/− mice had a significant decrease in mean survival time (25 h) compared with wild-type mice (43 h) upon systemic LPS treatment. More significantly, CD44 appears to have a role in the early response to sustain mouse survival against lethal LPS-induced shock (Fig. 6A). Examination of the peritoneum revealed considerably more inflammatory cells in CD44−/− mice compared with wild-type mice (Fig. 6B), as well as production of IL-6 by peritoneal macrophages isolated from LPS-treated mice, which is a useful marker of the acute cytokine response (Fig. 6C). Thus, CD44 appears to have a significant role in the systemic host response to bacterial LPS.

**CD44 deficiency leads to impaired expression of negative regulators of TLR signaling**

The finding that there was a dramatic effect of CD44 on NF-κB activation led us to consider that CD44 might function as a negative regulator of TLR signaling. Several intracellular proteins including IRAK-M, Tollip, SOCS-1, TNF-α-induced protein 3 (also called A20), and PI3K, have been implicated in inhibiting TLR4 signaling (13, 15, 18, 19). Targeted deletion of these molecules has shown that unregulated TLR signaling can have profound effects on inflammatory responses (14, 16–19). We explored the possibility that the inflammatory phenotype in CD44 deficiency is due to an effect on negative regulators of TLR4 signaling. We found that LPS induced IRAK-M, Tollip, and A20 mRNA expression in the lung tissue of wild-type mice in vivo, and the induction of all

**Figure 7.** Decreased induction of IRAK-M, Tollip, and A20 in CD44−/− mice after LPS treatment in vivo and in vitro. A, CD44−/− and wild-type (WT) mice were treated with LPS intratracheally for 1 and 3 h in vivo. Expression of A20, IRAK-M, and Tollip mRNA was analyzed with real-time RT-PCR. Fold induction over untreated was expressed (⁎, p < 0.05, n = 4). B, IRAK-M protein expression of mice treated with LPS in vivo was analyzed with Western blot. β-tubulin was used as an internal control. C, Lung macrophages were isolated from CD44−/− and WT mice by flow cytometry and treated with 50 ng/ml LPS for 1 and 3 h in vitro. Expression of A20, IRAK-M, and Tollip mRNA was analyzed with real-time RT-PCR. Fold induction over untreated was expressed (⁎, p < 0.01, n = 3). D, Lung macrophages isolated from CD44−/− and WT mice by flow cytometry were treated with 50 ng/ml LPS for 1 and 3 h in vitro. IRAK-M protein expression was analyzed with Western blot.
three of these genes were significantly decreased in CD44−/− mice (Fig. 7A). Similarly, the induction of IRAK-M protein expression by LPS in CD44−/− mice in vivo was decreased compared with that in wild-type mice (Fig. 7B). Furthermore, we found that the induction of IRAK-M, Tollip, and A20 mRNA (Fig. 7C) and IRAK-M protein (Fig. 7D) by LPS in CD44−/− macrophages in vitro was significantly decreased compared with that of wild-type macrophages. These data suggested that CD44, especially CD44 on macrophages, may regulate the expression of several intracellular signaling molecules such as Tollip, IRAK-M, and A20, that negatively regulate NF-κB activation.

Discussion

CD44 plays a role in development, tissue homeostasis, tumor metastasis, T cell activation, and in various inflammatory diseases (4, 6, 29, 30). We have previously demonstrated that in a bleomycin-induced lung injury model, CD44-deficient mice succumb to unremitting inflammation, characterized by impaired clearance of apoptotic neutrophils, persistent accumulation of proinflammatory hyaluronan fragments at the site of tissue injury, and impaired activation of TGF-β1 (2). An intriguing observation in this study was the finding that early neutrophil recruitment appeared to be selectively enhanced in the absence of CD44. The main goal of this study has been to further elucidate the mechanisms by which CD44 regulates inflammatory responses. The major finding in this study is that CD44 has a profound effect on the host response to LPS. We found that the total inflammatory cell infiltration was significantly increased relative to wild-type mice. The hyperresponsiveness of CD44−/− mice to LPS challenge manifested not only as a greater magnitude of inflammatory cell accumulation but also, strikingly, as a more rapid influx of inflammatory cells. CD44−/− mice released significantly more chemokines and cytokines into BAL upon LPS treatment compared with wild-type mice at earlier time points. In addition to the local effect in the lung, we also found that CD44 deficiency led to more rapid susceptibility to LPS-induced lethal shock. These data identify a previously unrecognized role for CD44 in limiting LPS-induced acute inflammation.

The inflammatory phenotype was dependent on hematopoietic CD44 because we were able to reverse the phenotype by reconstitution of CD44−/− mice with bone marrow from CD44+ mice. In vitro experiments demonstrated that alveolar macrophages from CD44−/− mice produced significantly more MIP-2 protein than that of wild-type mice suggesting that alveolar macrophages play a dominant role in the exaggerated responses to LPS in CD44−/− mice. To determine whether the increased accumulation of chemokines in the BAL was due to release of preformed protein or augmented gene expression, we analyzed the time course for mRNA expression and found a marked difference from wild-type mice with increased expression of chemokine mRNAs as early as 1 h post-LPS treatment in vivo in CD44−/− mice. These data suggest that CD44 limits macrophage responses to LPS at the level of gene expression.

The observation that CD44−/− mice demonstrated enhanced chemokine gene expression in response to LPS led us to determine whether there might be differences in NF-κB activation in the absence of CD44. We found a dramatic shift in the time course of NF-κB activation in CD44−/− mice that can be seen as early as 45 min after LPS treatment in vivo. LPS induces a myriad of inflammatory mediators in the lung (31). The transcription of a number of these inflammatory genes such as MIP-2, TNF-α, and matrix metalloproteinase 9 is mediated by NF-κB (32–36). The time course for NF-κB activation upon LPS treatment in CD44−/− mice strongly suggests that the burst of chemokine and MMP-9 production (data not shown) is due to this critical regulator of host defense. The finding that a number of important inflammatory mediators are dysregulated in the absence of CD44 suggests that the mechanism of regulation is at the level of a fundamental host response factor such as NF-κB.

When encountering pathogenic or noninfectious insults, the host must be able to provoke a defense response including recruitment of inflammatory cells and release of chemokines and cytokines to resolve inflammation and restore tissue integrity. In contrast, the host must also be capable of activating responses to curb overwhelming inflammatory responses to prevent detrimental tissue destruction. Several cell surface or soluble receptors have been suggested to play a role in the negative regulation of host responses to LPS by interfering with TLR signaling (37–40). These receptors either directly interact with TLRs (37, 40) and/or act as intracellular decoys by trapping key components of the signal transduction cascade such as TRAF6 and IRAK (37). Recent studies have identified several intracellular proteins including IRAK-M (14), Tollip (13, 15), SOCS-1 (16, 17), A20 (18, 19), and P65 (20) as negative regulators of TLR signaling, although different mechanisms have been proposed for their respective functions. Targeted deletion of these negative regulators of TLR signaling resulted in enhanced inflammatory responses to LPS (14, 16–19). We made the unexpected observation that the absence of CD44 leads to the suboptimal expression of IRAK-M, Tollip, and A20 in response to LPS in vivo and in macrophages in vitro. None of these three genes were completely abolished but all three were substantially diminished, suggesting that the inflammatory phenotype is a result of impaired expression of several negative regulators of TLR4 signaling rather than a single mediator. These data suggest that CD44 has a profound regulatory effect on the LPS-TLR4 signaling pathway and acts very proximal in the pathway that leads to the expression of negative regulators of TLR signaling. Reconstitution of any single one of these signaling molecules may not be sufficient to rescue the enhanced LPS responses caused by CD44 deficiency. We recently reported that hyaluronan fragments induce MIP-2 in a TLR4- and TLR2-dependent manner in macrophages (22). It has also been suggested that hyaluronan may up-regulate IRAK-M in macrophages in a CD44-dependent manner (41). Collectively, these data suggest that CD44 has evolved on the macrophage cell surface to have critical roles in regulating inflammatory responses. CD44 is important in removing extracellular matrix from sites of tissue injury (2) and we now suggest that CD44 also is important in providing a brake for innate immune inflammatory responses by promoting the expression and function of important negative regulators of macrophage responses to invading pathogens to prevent tissue injury from excessive inflammation. Elucidating the mechanisms by which CD44 regulates inflammation to prevent tissue destruction could lead to new therapeutic approaches to inflammatory diseases.

Disclosures

The authors have no financial conflict of interest.

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