INFECTION AND IMMUNITY, Jan. 2010, p. 115–124 Vol. 78, No. 1
0019-9567/10/$12.00 doi:10.1128/IAI.00949-09

CD44 Deficiency Is Associated with Enhanced *Escherichia coli*-Induced Proinflammatory Cytokine and Chemokine Release by Peritoneal Macrophages[▽]

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Received 19 August 2009/Returned for modification 28 September 2009/Accepted 29 October 2009

CD44 is involved in several immune responses, such as cellular adhesion, migration, proliferation, and activation. Peritonitis is an important cause of sepsis, and *Escherichia coli* is one of the major pathogens involved therein. We sought to determine the role of CD44 in the host response to *E. coli*-induced abdominal sepsis and to assess the function of CD44 in the activation of primary peritoneal macrophages by *E. coli* or lipopolysaccharide (LPS) purified from this bacterium by using wild-type (WT) and CD44 knockout (KO) mice. CD44 KO mice already demonstrated enhanced CXC chemokine levels in peritoneal lavage fluid at 6 h after infection, whereas tumor necrosis factor alpha (TNF-α) and interleukin-6 levels were elevated at 20 h postinfection. In line with this, CD44 KO mouse peritoneal macrophages released more TNF-α and macrophage inflammatory protein 2 (MIP-2) than did WT cells upon stimulation with *E. coli* or LPS in the presence of autologous serum. In contrast, plasma TNF-α levels were lower in CD44 KO mice and CD44 KO blood leukocytes secreted similar amounts of TNF-α and MIP-2 upon *ex vivo* incubation with *E. coli* or LPS. The proinflammatory phenotype of CD44 KO macrophages was not associated with an altered expression of inhibitors of Toll-like receptor signaling, whereas it could be partially reversed by addition of WT serum. CD44 deficiency did not impact on leukocyte recruitment into the peritoneal cavity or organ failure. These data suggest that CD44 differentially influences cytokine and chemokine release by different leukocyte subsets.

Sepsis is the leading cause of death in critically ill patients in developed countries (14). Peritonitis is the second most common cause of sepsis (46), with *Escherichia coli* as one of the major pathogens involved (11). Upon infection, an immediate and adequate host defense is necessary to contain and kill the pathogen. Therefore, resident peritoneal macrophages release chemoattractant factors to recruit circulating neutrophils and monocytes to kill the pathogen (3).

CD44 is a transmembrane adhesion molecule that is present on a wide variety of cell types, including leukocytes and parenchymal cells. CD44 has several functions in innate and adaptive immune responses, such as involvement in cellular adhesion and migration, lymphocyte and monocyte activation and proliferation, and cell-mediated cytotoxicity in natural killer cells (15, 36, 37). Considering the wide expression pattern of CD44 on several cell types and the variety of functions that CD44 has during inflammation and infection, it is not surprising that the effect of CD44 deficiency differs extensively, depending on the pathogen species (or derivative) and the primary site of infection. For example, intratracheal instillation of 1 mg/kg *E. coli*-derived lipopolysaccharide (LPS), the major virulence factor of gram-negative bacteria (16), into CD44-deficient mice resulted in increased inflammatory cell influx and elevated chemokine levels in bronchoalveolar lavage fluid compared to those of control mice (27). On the other hand, an aerosol challenge with a lower LPS dose, as experienced by grain mill workers, resulted in attenuated macrophage recruitment into the bronchoalveolar space of CD44 knockout (KO) mice compared to that of control mice (13). In an *E. coli*-induced pneumonia model, CD44 deficiency resulted in enhanced neutrophil accumulation and elevated chemokine mRNA levels in the lungs, whereas no effect on pulmonary bacterial outgrowth was found (45). In contrast, in an *E. coli*-induced urinary tract infection model, CD44 deficiency influenced neither neutrophil accumulation nor cytokine and chemokine production, while the bacterial load in the kidneys was reduced in CD44 KO mice in this model (39).

We here sought to assess the function of CD44 in the host response to *E. coli*-induced abdominal sepsis and to determine the role of CD44 in the activation of primary peritoneal macrophages by *E. coli* or LPS purified from this bacterium. We focused on these gram-negative bacterial stimuli since these are most relevant for peritoneal macrophages (29, 34).

MATERIALS AND METHODS

Animals and design. The Institutional Animal Care and Use Committee approved all experiments. C57BL/6 wild-type (WT) mice were obtained from Harlan Sprague-Dawley Inc. (Horst, the Netherlands). CD44 KO mice on a C57BL/6 background (kindly provided by A. Berns, Netherlands Cancer Institute, Amsterdam, the Netherlands) (40) were bred in the animal facility of the Academic Medical Center (Amsterdam, the Netherlands). Nine- to 12-week-old male mice were used in all experiments. All experimental procedures, including induction of peritonitis, were done as described previously (25, 43). In brief, *E. coli* O18:K1 was cultured in Luria-Bertani medium at 37°C, harvested at mid-log phase, and washed twice before inoculation. Mice were injected intraperitoneally (i.p.) with 1×10^4 CFU of *E. coli* in 200 μl of sterile saline. The inoculum was plated on blood agar plates to determine viable counts. After 6 or 20 h, mice were sacrificed; the peritoneal cavity was lavaged with 5 ml of sterile 0.9% NaCl using

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▽ Published ahead of print on 9 November 2009.

an 18-gauge needle, and blood was drawn into heparin-containing tubes; organs were removed aseptically and homogenized in 5 volumes of sterile 0.9% NaCl using a tissue homogenizer (Biospec Products, Bartlesville, OK). CFU were determined from serial dilutions of peritoneal lavage fluid, blood, and organ homogenates and incubated at 37°C for 16 h before colonies were counted. Cell counts were determined for each peritoneal lavage sample in a hemocytometer (Beckman Coulter, Fullerton, CA), and differential cell counts were performed on cytospin preparations stained with Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland).

Assays. Interleukin-1β (IL-1β), keratinocyte-derived cytokine (KC), macrophage inflammatory protein 2 (MIP-2), and LPS-induced CXC chemokine (LIX) were measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Abingdon, United Kingdom). Monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor alpha (TNF-α), IL-6, IL-10, IL-12p70, and gamma interferon (IFN-γ) were measured by cytometric bead array multiplex assay in accordance with the manufacturer's recommendations (BD Biosciences, San Jose, CA). Urea, creatinine, aspartate aminotransferase (ASAT), and alanine aminotransferase (ALAT) were determined with commercially available kits (Sigma-Aldrich) by using a Hitachi analyzer (Roche) according to the manufacturer's instructions.

Histology. Lungs and livers were harvested 20 h after infection, fixed in 10% buffered formalin for 24 h, and embedded in paraffin. Hematoxylin-and-eosin-stained slides were coded and scored from 0 (absent) to 4 (severe). For lungs, the following parameters were scored: interstitial inflammation, endothelialitis, edema, and pleuritis. For livers, interstitial inflammation, endothelialitis, necrosis, and thrombus formation were scored. The total "inflammation score" was expressed as the sum of the scores for each parameter, the maximum being 16.

***Ex vivo* peritoneal macrophage stimulation.** Peritoneal macrophages from untreated CD44 KO and WT mice were harvested by peritoneal lavage, washed, and resuspended in RPMI 1640 medium (Gibco, Life Technologies, Rockville, MD) containing 2 mM L-glutamine, penicillin, streptomycin, and 5% autologous serum (i.e., pooled serum from mice of the same genotype: CD44 KO cells were incubated in the presence of serum obtained from CD44 KO mice, and WT cells were incubated with serum obtained from WT mice). Cells were then plated at a concentration of 1×10^5/100 μl per well in a 96-well flat-bottom microtiter plate, allowed to adhere for 2 h at 37°C in 5% CO_2, and then washed with medium to remove nonadherent cells. Next, the adherent monolayer cells were stimulated for 2, 6, or 18 h in medium containing 10% WT or CD44 KO serum with or without LPS from *E. coli* (200 ng/ml; serotype 0111:B4, Sigma, St. Louis, MO) or heat-killed *E. coli* (equivalent of 1×10^7 CFU/ml) at 37°C in 5% CO_2. Supernatants were taken and stored at −20°C until assayed. Cell pellets were immediately dissolved in RA1 buffer, and RNA was isolated as described by the manufacturer (Bioke, Leiden, the Netherlands) and reverse transcribed using oligo(dT) (Promega, Leiden, the Netherlands) and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Breda, the Netherlands). Reverse transcription-PCRs (RT-PCRs) were performed using LightCycler SYBR green I master mix (Roche, Mijdrecht, the Netherlands) and measured in a LightCycler 480 (Roche) apparatus under the following conditions: a 5-min 95°C hot start, followed by 40 cycles of amplification (95°C for 15 s, 60°C for 5 s, and 72°C for 20 s). For quantification, standard curves were constructed by PCR on serial dilutions of a concentrated cDNA, and data were analyzed using LightCycler software. Gene expression is presented as a ratio of the expression of the housekeeping gene β2-microglobulin (B2M) (30). The primers used were as follows: B2M, 5′-TGGTCTTTCTGGTGCTTGTCT-3′ and 5′-ATTTTTTTCCC GTTCTTCAGC-3′, A20, 5′-GGGACTCCAGAAAACAAGGG-3′ and 5′-TAC CCTTCAAACATGGTGCTT-3′, IL-1R-associated kinase M (IRAK-M), 5′-TG CCAGAAGAATACATCAGACAG-3′ and 5′-TCTAAGAAGGACAGGCAG GAGT-3′.

Whole blood stimulation. Heparinized blood from untreated CD44 KO and WT mice was stimulated with medium (RPMI 1640 medium containing 2 mM L-glutamine, penicillin, and streptomycin) with or without LPS from *E. coli* (80 ng/ml) or heat-killed *E. coli* (equivalent of 4×10^6 CFU/ml) for 6 or 24 h at 37°C in 5% CO_2. After centrifugation, supernatants were taken and stored at −20°C until assayed.

Statistical analysis. Differences between groups were analyzed by the Mann-Whitney U test using GraphPad Prism version 4.00 (GraphPad Software; San Diego, CA). Values are expressed as the mean ± the standard error of the mean (SEM). A value of $P < 0.05$ was considered to represent a statistically significant difference.

RESULTS

CD44 deficiency is associated with enhanced peritoneal TNF-α, IL-6, and chemokine release during *E. coli* peritonitis. To obtain a first insight into the role of CD44 in peritoneal cytokine and chemokine production during bacterial peritonitis, we injected CD44 KO and WT mice with live *E. coli* i.p. and measured TNF-α, IL-1β, IL-6 (proinflammatory cytokines), IL-10 (anti-inflammatory cytokine), MIP-2, KC, LIX (CXC chemokines), and MCP-1 in peritoneal lavage fluid collected 6 or 20 h thereafter (Fig. 1). Notably, 6 h after infection, CD44 KO mice had a modestly but significantly increased bacterial load in their peritoneal lavage fluid compared to WT mice ($2.12 \times 10^5 \pm 0.76 \times 10^5$ versus $6.29 \times 10^5 \pm 2.45 \times 10^5$ CFU/ml, respectively; $P < 0.05$). Bacterial loads in peritoneal lavage fluid did not differ between the two mouse strains 20 h after infection or in other body compartments (blood, lungs, and liver) at either 6 or 20 h postinfection (data not shown). At 6 h, peritoneal cytokine and MCP-1 levels did not differ between CD44 KO and WT mice; at 20 h, however, CD44 KO mice displayed markedly increased TNF-α and IL-6 concentrations ($P < 0.05$ versus WT mice, Fig. 1A and C), whereas the peritoneal levels of IL-1β, IL-10, and MCP-1 tended to be increased in these animals; however, these increased amounts did not reach statistical significance compared to WT levels (Fig. 1B, D, and H). With regard to CXC chemokine release, peritoneal MIP-2, KC, and LIX concentrations were all higher in CD44 KO mice at 6 h after infection (Fig. 1E to G; $P < 0.01$ to 0.05 versus WT mice). At 20 h, CXC chemokine levels had increased dramatically in both mouse strains; although these levels remained higher in CD44 KO mice, the difference from WT mice was only significant for MIP-2 ($P < 0.001$). In addition, we measured TNF-α, IL-1β, IL-6, IL-10, MIP-2, and MCP-1 in plasma from WT and CD44 KO mice at 6 and 20 h postinfection (Fig. 2). At 6 h, no differences were found, except for enhanced MIP-2 levels in CD44 KO mice compared to WT mice. At 20 h, however, in contrast to the peritoneal lavage fluid, TNF-α and MCP-1 concentrations were lower in CD44 KO mice than in WT mice. IL-12p70 and IFN-γ were not detectable or were very low at both time points, and in either peritoneal fluid or plasma, they did not differ between CD44 KO and WT mice (data not shown). Together, these data suggest that CD44 plays an inhibitory role with regard to the production of cytokines and chemokines in the peritoneal cavity upon infection with *E. coli*.

CD44 deficiency does not impact on cell recruitment during *E. coli* peritonitis. Controversial data exist on the role of CD44 in cell recruitment during inflammation incited by gram-negative stimuli: CD44 KO mice have been reported to respond with enhanced neutrophil and macrophage influx in response to intrapulmonary delivery of *E. coli* (17) or LPS (27), whereas another study found reduced influx of neutrophils and macrophages into bronchoalveolar lavage fluid of CD44 KO mice after LPS inhalation (13). CD44 KO mice were reported to have enhanced cell recruitment into the peritoneal cavity upon i.p. LPS injection (27). To the best of our knowledge, the role of CD44 in *E. coli*-induced cell recruitment into the peritoneal cavity has not been studied before. We found no differences in the number of neutrophils or macrophages at either 6 or 20 h after i.p. infection with *E. coli* (Table 1).

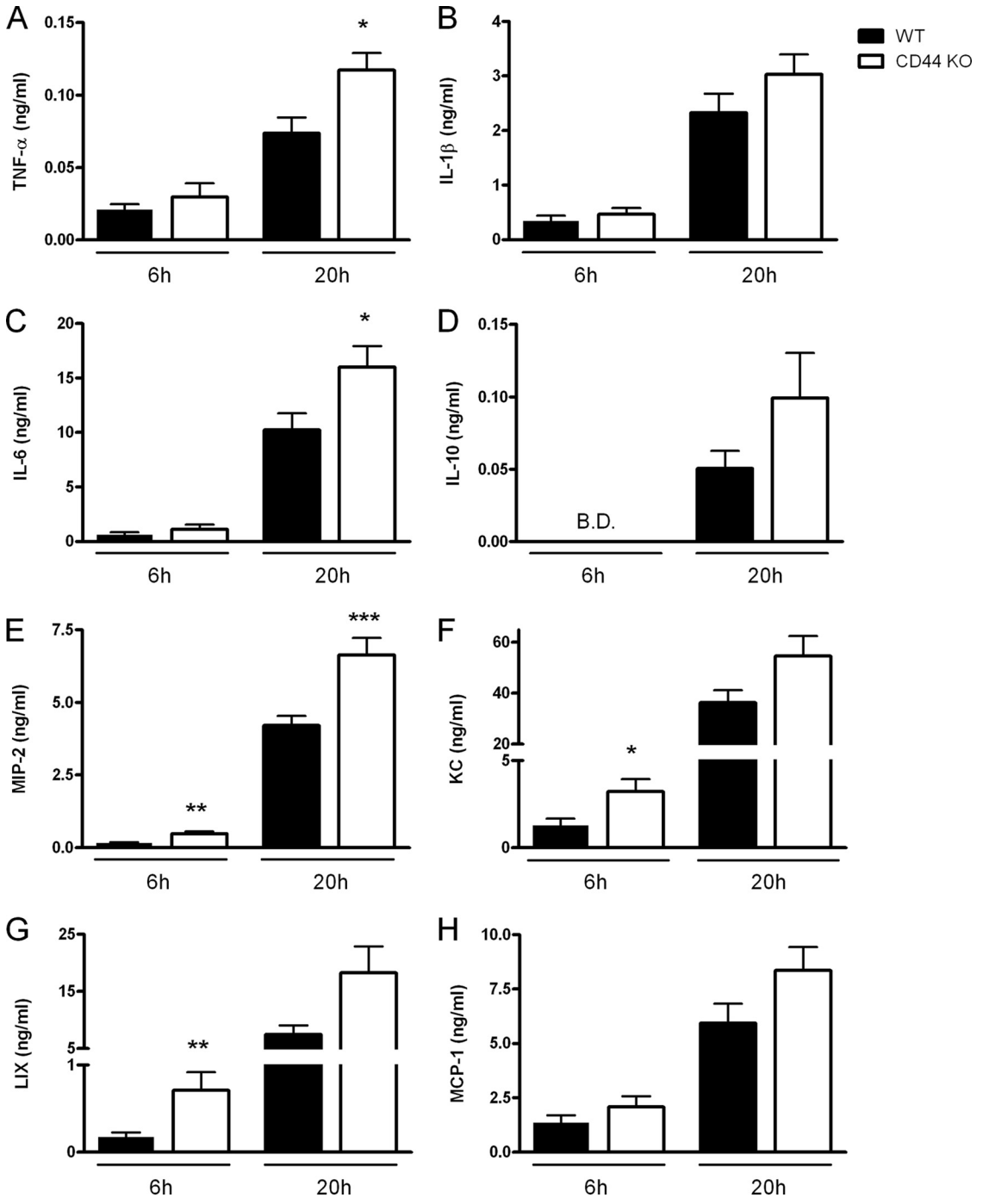

FIG. 1. CD44 KO mice demonstrate enhanced TNF-α, IL-6, and chemokine (MIP-2, KC, and LIX) production in peritoneal lavage fluid. Cytokine (TNF-α, IL-1β, IL-6, and IL-10) and chemokine (MIP-2, KC, LIX, and MCP-1) levels in peritoneal lavage fluid at 6 and 20 h after i.p. injection of 1×10^4 CFU of *E. coli* into WT (black bars) and CD44 KO (white bars) mice are shown. Data are expressed as means ± SEM; n = 8 mice/group/time point. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus WT mice at the same time point. B.D. means below detection.

CD44 does not impact on organ injury during abdominal sepsis. Our model of *E. coli* peritonitis is associated with organ injury (44). To evaluate the role of CD44 during abdominal sepsis, we determined lung and liver damage in WT and CD44 KO mice 20 h after infection. Lungs from both WT and CD44 KO mice showed clear signs of inflammation, as reflected by accumulation of leukocytes in the interstitium, around blood vessels, and in the pleura (Fig. 3A and B). The total histological scores were similar in the two groups (3.5 ± 0.3 in WT mice versus 2.9 ± 0.3 in CD44 KO mice). Both WT and CD44 KO mice also showed signs of inflammation in liver tissue, as characterized by influx of leukocytes into the hepatic parenchyma, liver necrosis, and thrombus formation (Fig. 3C and D). The extent of injury was similar in the two groups (3.5 ±

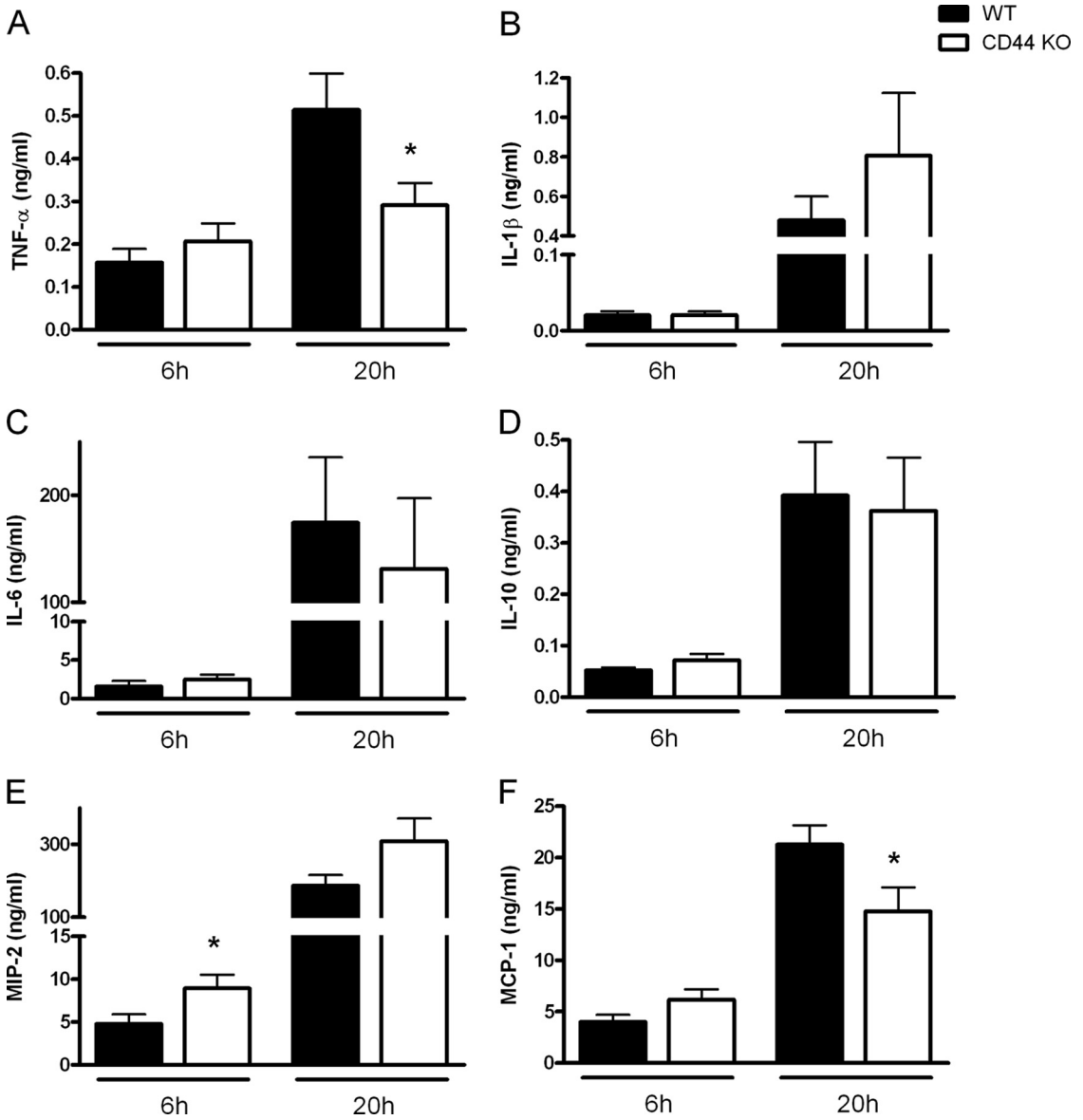

FIG. 2. CD44 KO mice demonstrate decreased TNF-α and MCP-1 levels in plasma. Cytokine (TNF-α, IL-1β, IL-6, and IL-10) and chemokine (MIP-2 and MCP-1) levels in plasma at 6 and 20 h after i.p. injection of 1×10^4 CFU of *E. coli* into WT (black bars) and CD44 KO (white bars) mice are shown. Data are expressed as means ± SEM; $n = 8$ mice/group/time point. *, $P < 0.05$ versus WT mice at the same time point.

TABLE 1. Similar cell influx into the peritoneal cavities of WT and CD44 KO mice upon *E. coli* infection

Time postinfection and strain	Mean no. ± SEM (10^4/ml PLF)[a] of:		
	Total cells	Neutrophils	Macrophages
6 h			
WT	127 ± 28	122 ± 28	5 ± 2
CD44 KO	118 ± 28	116 ± 31	6 ± 2
20 h			
WT	317 ± 24	295 ± 21	21 ± 4
CD44 KO	265 ± 31	235 ± 26	30 ± 5

[a] Total cell counts and neutrophil and macrophage numbers in peritoneal lavage fluid (PLF) at 6 and 20 h after injection of *E. coli* in WT and CD44 KO mice are shown. $n = 8$ mice/group/time point.

0.6 in WT mice versus 2.9 ± 0.7 in CD44 KO mice). This result was confirmed by clinical chemistry; although CD44 KO mice demonstrated lower levels of ASAT and ALAT, there was no significant difference from those of WT mice (Fig. 3E and F). Moreover, the extent of renal insufficiency was similar in the two mouse strains, as determined by the plasma levels of creatinine and urea (Fig. 3G and H). Taken together, these data indicate that CD44 does not affect the severity of organ injury induced by abdominal sepsis.

CD44-deficient peritoneal macrophages release more TNF-α and MIP-2 upon stimulation with *E. coli* or LPS. To investigate the role of peritoneal macrophage CD44 in the enhanced release of TNF-α and chemokines, we harvested primary macrophages from the peritoneal cavities of CD44 KO and WT mice and stimulated these with either *E. coli* or *E. coli* LPS for

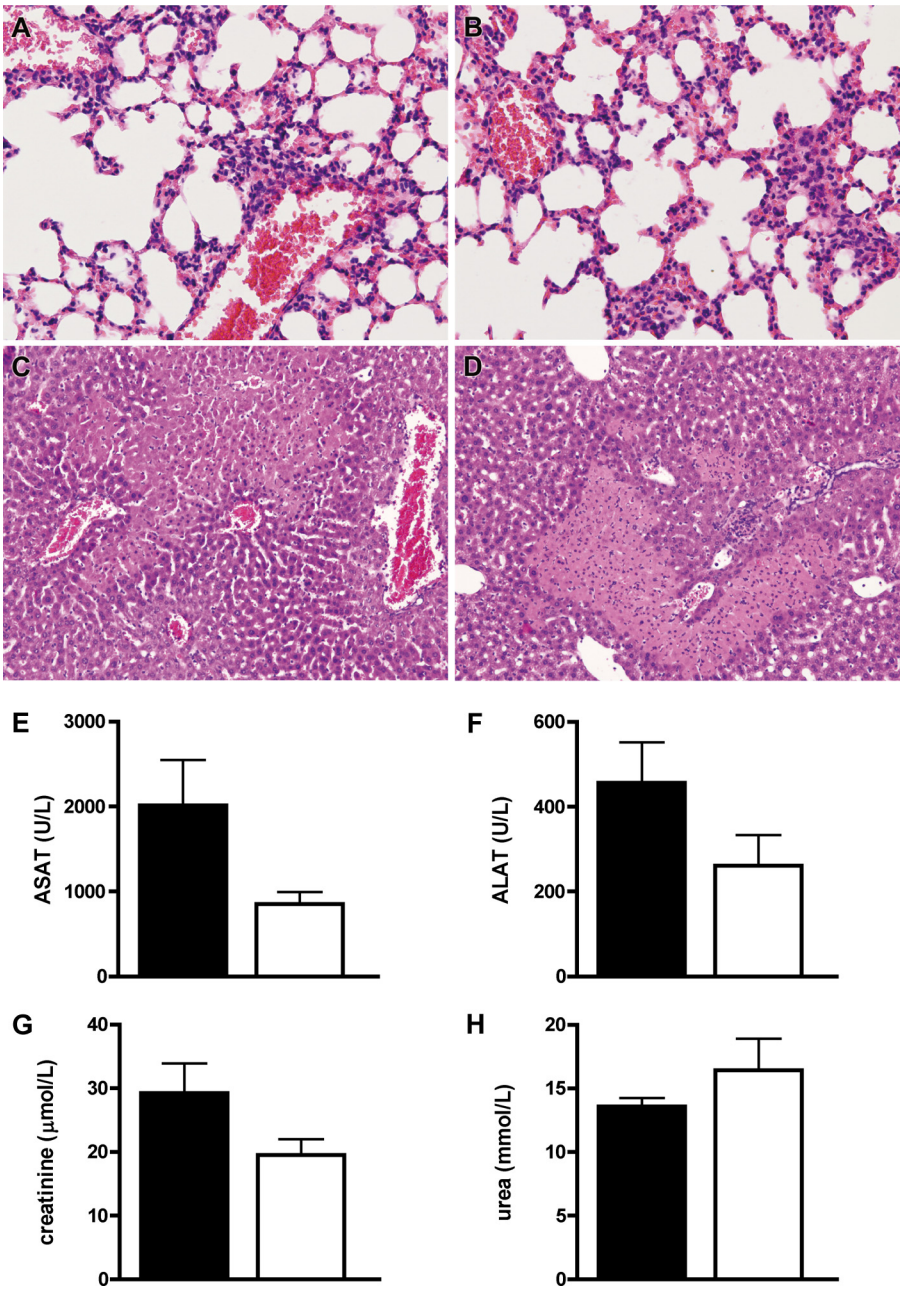

FIG. 3. CD44 does not impact on distant organ injury. Representative hematoxylin-and-eosin staining of lung (A and B) and liver (C and D) tissue at 20 h after i.p. injection of 1×10^4 CFU of *E. coli* into WT (A and C) and CD44 KO (B and D) mice is shown. Original magnifications: liver, ×10; lung, ×20. Plasma concentrations of ASAT (E), ALAT (F), creatinine (G), and urea (H) in WT (black bars) and CD44 KO (white bars) mice are also shown. Data are expressed as means ± SEM; $n = 8$ mice/group.

2, 6, or 18 h (Fig. 4). In these studies, we focused on TNF-α and MIP-2 since these mediators were most discriminative in the *in vivo* peritonitis model (Fig. 1). As CD44 is present in a soluble form in the extracellular environment in WT mice (21), we performed these stimulations in the presence of autologous (WT or CD44 KO) serum to mimic the *in vivo* situation as closely as possible. TNF-α release by WT macrophages already reached almost maximum levels at 2 h of LPS or *E. coli* stimulation, whereas TNF-α release by CD44 KO macrophages slightly increased from 2 to 18 h of stimulation and was significantly enhanced compared to that by WT macrophages upon LPS stimulation (2 h, $P < 0.01$; 6 and 18 h, $P < 0.05$; Fig. 4A) and *E. coli* stimulation (2 h, $P < 0.05$; 6 h, $P = 0.15$; 18 h, $P = 0.06$; Fig. 4B). MIP-2 levels released from WT and CD44 KO macrophages increased from 2 to 18 h of stimulation. Concentrations were not different between groups after 2 or 6 h of stimulation; however, at 18 h, macrophages from CD44 KO mice released significantly more MIP-2 than did those from WT mice when stimulated with *E. coli* or LPS ($P < 0.05$ for both stimuli; Fig. 4C and D). As plasma TNF-α results con-

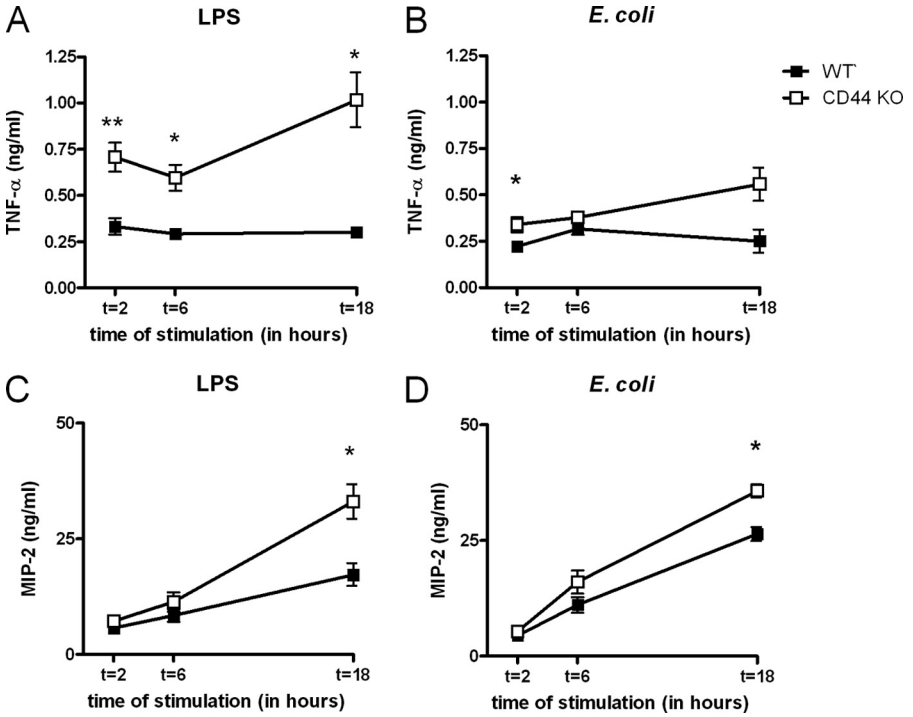

FIG. 4. CD44 KO peritoneal macrophages demonstrate increased TNF-α and MIP-2 release. TNF-α (A and B) and MIP-2 (C and D) release by WT (■) and CD44 KO (□) peritoneal macrophages after 2, 6, and 18 h of stimulation with 200 ng/ml LPS (A and C) or an equivalent of 1×10^7 CFU/ml heat-killed *E. coli* (B and D) is shown. Data are expressed as means ± SEM; $n = 5$ mice/group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus WT mice at the same time point.

trasted with the TNF-α peritoneal lavage fluid data, we additionally stimulated whole blood derived from WT and CD44 KO mice with *E. coli* or LPS for 6 or 24 h and measured TNF-α and MIP-2. Both mediators were similar in the two groups (Fig. 5). These data again suggest an inhibitory role for CD44 against cytokine and chemokine release by peritoneal macrophages but not by blood leukocytes.

CD44 deficiency does not impact on IRAK-M and A20 mRNA expression in peritoneal macrophages. Recently, it has been shown that CD44 KO lung macrophages display enhanced proinflammatory cytokine release upon LPS stimulation due to impaired expression of negative regulators of Toll-like receptor (TLR) signaling (27). Therefore, we examined the expression of the mRNAs for IRAK-M and A20, two important inhibitors of TLR signaling (28), after 0, 2, and 6 h of *E. coli* or LPS stimulation. IRAK-M mRNA was induced in both groups after 6 h of stimulation with either LPS or *E. coli* and was similar in the two groups at all time points (Fig. 6A and B). A20 mRNA expression levels already peaked at 2 h of stimulation and were also similar in WT and CD44 KO peritoneal macrophages (Fig. 6C and D).

WT serum inhibits TNF-α and MIP-2 release by CD44 KO peritoneal macrophages. Peritoneal macrophage stimulations were performed in medium containing autologous serum. As we could not find an explanation for the enhanced TNF-α and MIP-2 release due to differential mRNA expression of negative TLR regulators, we questioned whether soluble CD44 present in serum could explain our observations. Therefore, we repeated the 18-h peritoneal macrophage stimulations with a third group, i.e., CD44 KO macrophages stimulated in the presence of WT serum (Fig. 7). In stimulations performed with autologous serum, we again found significantly enhanced concentrations of TNF-α and MIP-2 released from CD44 KO macrophages compared to WT cells (TNF-α, $P < 0.001$; MIP-2, $P < 0.05$ [for both stimuli]). Strikingly, CD44 KO macrophages stimulated in the presence of WT serum released significantly lower levels of TNF-α than did CD44 KO macrophages stimulated in the presence of CD44 KO serum ($P < 0.01$ for LPS and $P < 0.001$ for *E. coli*). For MIP-2, a similar trend was found; however, differences did not reach statistical significance ($P = 0.08$ for LPS and $P = 0.14$ for *E. coli*).

DISCUSSION

CD44 is abundantly expressed and plays a role in developmental, physiological, and inflammatory processes (36, 37). Several studies demonstrated alterations in inflammatory cell recruitment and cytokine and chemokine responses in the absence of CD44 during inflammation and infection (13, 23, 26, 27, 39, 41, 45). The precise role of CD44 during inflammation, however, appeared to be diverse, varying with the infectious agent or derivative and the organs involved. In the present study, we focused on the role of CD44 in *E. coli*-induced abdominal sepsis, considering that peritonitis is the second most common cause of sepsis (46) and that *E. coli* is one of the major pathogens involved (11). The model employed has been demonstrated to be suitable to study the course of the bacterial infection by quantitative measurement of the bacterial loads in various body compartments, as well as the early innate immune response and the later development of organ injury (9, 24, 25,

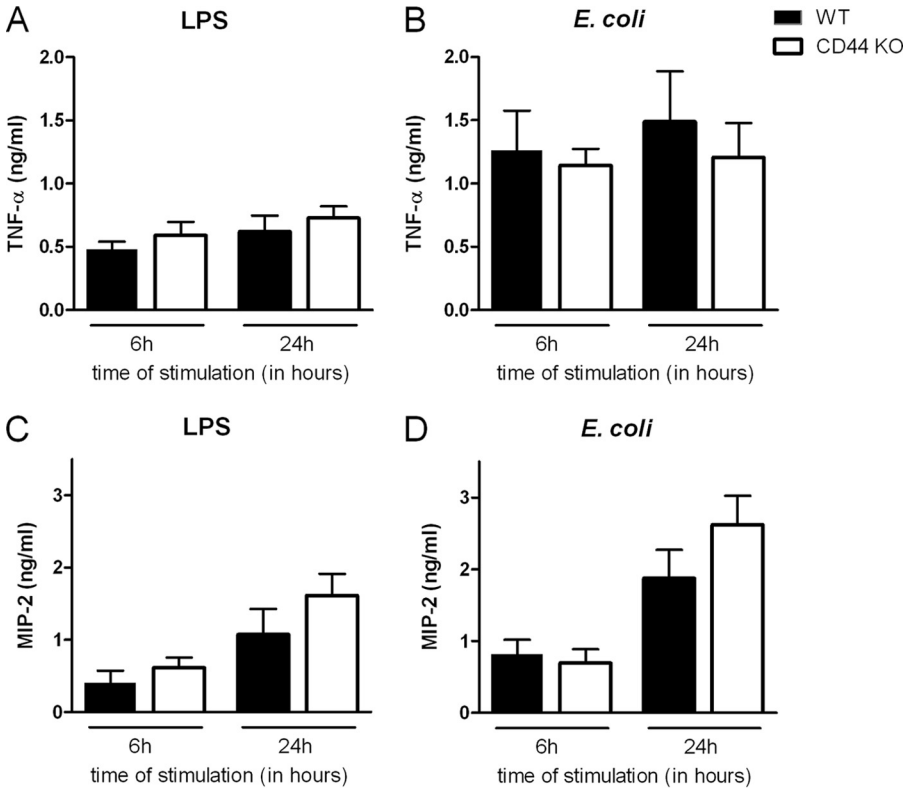

FIG. 5. CD44 deficiency does not influence TNF-α and MIP-2 release by whole blood. TNF-α (A and B) and MIP-2 (C and D) release by WT (black bars) and CD44 KO (white bars) whole blood after 6 and 24 h of stimulation with 80 ng/ml LPS (A and C) or an equivalent of 4×10^6 CFU/ml heat-killed *E. coli* (B and D) is shown. Data are expressed as means ± SEM; $n = 5$ mice/group.

38, 43, 44). Moreover, we determined the role of CD44 in the release of proinflammatory mediators by primary peritoneal macrophages in response to *E. coli* or LPS purified from this bacterium. We showed here that the peritoneal but not plasma concentrations of TNF-α, IL-6, and CXC chemokines were significantly increased in CD44 KO mice during *E. coli*-induced abdominal sepsis, whereas inflammatory cell recruitment and organ damage were unaffected. In line with this, CD44 KO peritoneal macrophages, but not blood leukocytes, released significantly more TNF-α and MIP-2 upon *ex vivo* LPS or *E. coli* stimulation, which was abolished when stimulation of CD44 KO cells was performed in the presence of WT serum.

Proinflammatory cytokine production and inflammatory cell recruitment to the site of infection are important events in the early response to bacteria (33). An earlier investigation studying CD44 KO and WT mice in a model of *E. coli*-induced pneumonia showed enhanced MIP-2 and KC mRNA expression in lung homogenates of the former mouse strain (45). In accordance, we found significantly elevated concentrations of TNF-α, IL-6, and the CXC chemokines KC, MIP-2, and LIX in peritoneal lavage fluid from CD44 KO mice infected with *E. coli* via the peritoneal route. The increased CXC chemokine levels, however, did not result in enhanced neutrophil recruitment into the peritoneal cavity. Although KC, MIP-2, and LIX are potent chemoattractants for neutrophils (31, 47), chemoattractive migration to gradients is a very complex process and might be dominated by exogenous molecules such as *N*-formyl-Met-Leu-Phe (fMLP), a chemoattractant released by *E. coli* (5, 6, 35), or by a prioritizing molecule such as PTEN (2, 12). Indeed, a recent study demonstrated impaired neutrophil recruitment in CD44 KO mice to i.p. injected fMLP (1). Moreover, infiltration of cells into the site of infection is not only mediated by chemoattractants but is a complex interplay of several processes, including adhesion and extravasation of inflammatory cells. Importantly, CD44 functions as an adhesion molecule on leukocytes, mediating rolling on E-selectin or hyaluronan and thereby extravasation of neutrophils and T lymphocytes (4, 10, 19, 32). Although CD44 and hyaluronan are both abundantly expressed (7, 36), the CD44-hyaluronan interaction is tightly regulated, as CD44 needs to be activated, e.g., by LPS or cytokines such as IL-1β and TNF-α to be able to bind hyaluronan and mediate leukocyte adhesion (8, 18, 36). The net result of the absence of CD44 for neutrophil extravasation might, therefore, depend on the stimulus and the organ or tissue involved. Therefore, our finding on similar cellular infiltration might be a net result of impaired extravasation compensated by enhanced chemoattractive molecules in the peritoneal cavities of CD44 KO mice.

Since peritoneal macrophages are the first cells to interact with bacteria delivered i.p., we studied proinflammatory cytokine release by those cells upon stimulation with LPS and *E. coli ex vivo* and found that CD44 KO macrophages release significantly more TNF-α and MIP-2 than WT cells do. Our results are in line with studies demonstrating that CD44 KO alveolar macrophages release more MIP-2 upon LPS stimulation (27) and that CD44 KO bone marrow-derived macro-

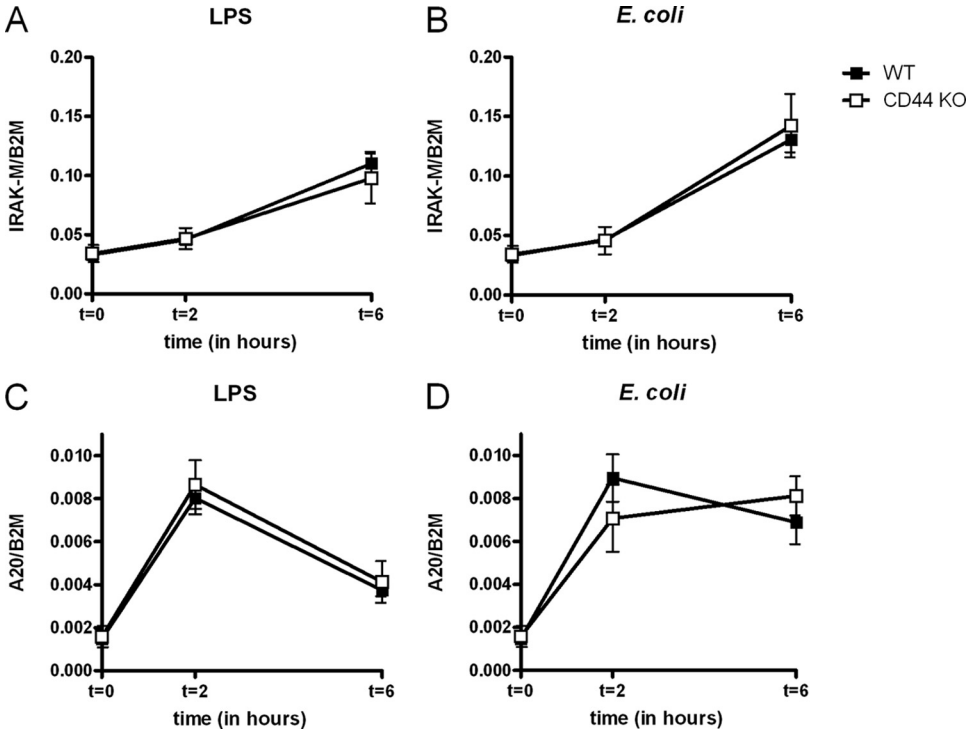

FIG. 6. CD44 deficiency does not influence IRAK-M and A20 mRNA expression in peritoneal macrophages. IRAK-M (A and B) and A20 (C and D) mRNA expression levels in WT (■) and CD44 KO (□) peritoneal macrophages corrected for B2M at 2 and 6 h after LPS (A and C) or heat-killed *E. coli* (B and D) stimulation are shown. Data are expressed as means ± SEM; $n = 5$ mice/group.

phages produce more TNF-α and IL-6 upon stimulation not only with LPS but also with ligands for TLR3, TLR5, TLR7, or TLR9 (23). Importantly, in lung macrophages, CD44 has been implicated as a negative regulator of TLR signaling. Indeed, in CD44 KO lung macrophages, reduced mRNA levels of negative regulators of TLR signaling, including A20, IRAK-M, and Tollip, appeared to be responsible for the proinflammatory phenotype, whereas for bone marrow-derived macrophages, it was reported that an association between the cytoplasmic domain of CD44 and the Toll/interleukin-1 receptor region may inhibit TLR signaling. We found no differences in IRAK-M and A20 mRNA levels between WT and CD44 KO peritoneal macrophages stimulated with either LPS or *E. coli*. Moreover, we demonstrate that plasma MIP-2 levels are enhanced after 6 h of infection; however, in contrast to those in peritoneal lavage fluid, the plasma TNF-α levels of CD44 KO mice appeared to be decreased at 20 h after infection. In addition, CD44 did not influence the release of TNF-α or MIP-2 by blood leukocytes stimulated *ex vivo* with *E. coli* or LPS. Together, these data indicate that CD44 exerts differential effects on cytokine/chemokine release by different leukocyte subsets and that the enhanced cytokine/chemokine release by CD44 KO peritoneal and lung macrophages is mediated by distinct mechanisms.

Our findings on enhanced TNF-α release by CD44 KO peritoneal macrophages contrast with a previous study reporting that CD44 KO peritoneal macrophages release less TNF-α than do WT macrophages upon LPS stimulation (13). However, several factors in that study were different from ours. For example, whereas we used freshly isolated macrophages, the earlier investigation used thioglycolate-elicited macrophages harvested 3 days after i.p. injection. The possibility cannot be excluded that this procedure affects the activation or inhibition of proinflammatory pathways in peritoneal macrophages. Furthermore, in that study, cells were stimulated in the presence of 10% fetal bovine serum, which likely contains soluble CD44, whereas we used autologous serum. In this context, it is important to note that we demonstrate here that WT serum components are, at least in part, responsible for inhibition of proinflammatory responses by peritoneal macrophages, as the enhanced TNF-α and MIP-2 release by CD44 KO peritoneal macrophages was abolished when stimulation of CD44 KO cells was performed in the presence of WT serum. As the presence of soluble CD44 is the most obvious difference between WT serum and CD44 KO serum, we attempted to confirm that soluble CD44 is the component in WT serum responsible for the inhibition. Therefore, we added the concentration of soluble CD44 that we measured in WT serum by ELISA (20, 21) to CD44 KO serum during stimulation. As murine soluble CD44 is not easily available, we used a CD44-immunoglobulin fusion protein (22). We did not find any effect of soluble CD44 on TNF-α or MIP-2 release under any stimulatory condition (data not shown). However, based on these data, we cannot exclude soluble CD44 as the component of WT serum that is responsible, as several aspects of this fusion protein might be different from soluble CD44 present in WT serum. For instance, the fusion of human immunoglobulin components to CD44 and the preparation in a human cell line might affect posttranslational modifications of CD44 such as glycosylation and acylation, which are important attributes for the adhesion of glycosaminoglycans (36, 42). Furthermore, this fusion pro-

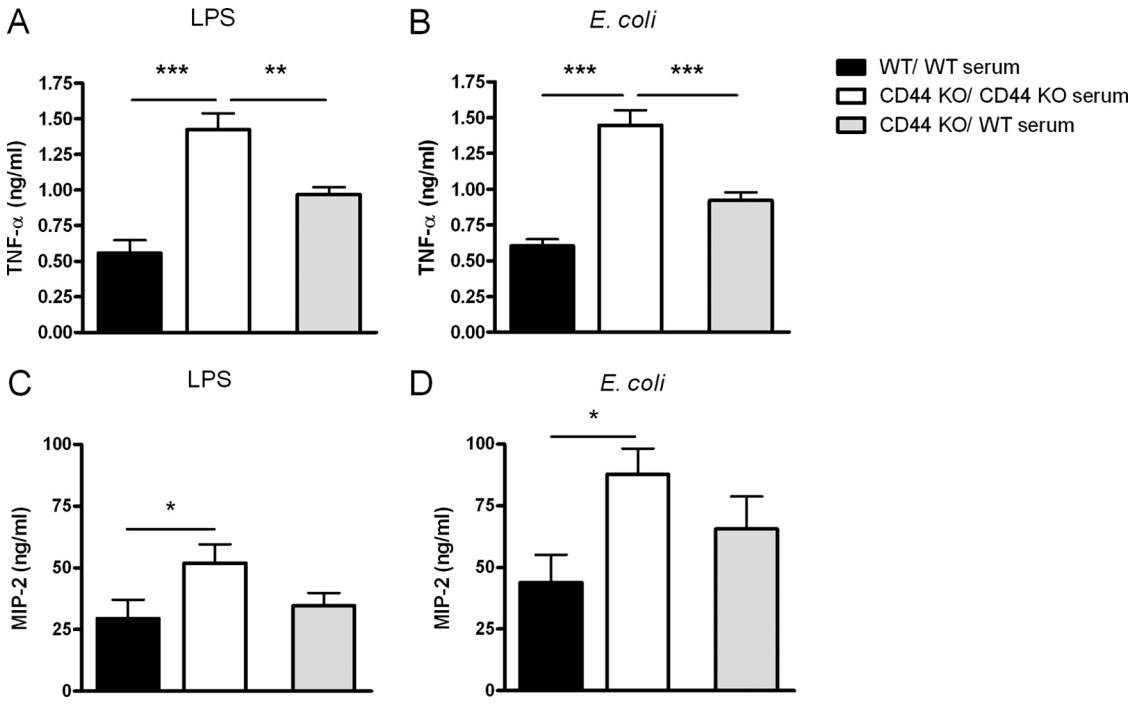

FIG. 7. WT serum abolishes increased TNF-α and MIP-2 release by CD44 KO peritoneal macrophages. TNF-α (A and B) and MIP-2 (C and D) release by WT (black bars, WT serum) and CD44 KO (white bars, CD44 KO serum; gray bars, WT serum) peritoneal macrophages after 18 h of stimulation with LPS (A and C) or heat-killed *E. coli* (B and D) is shown. Data are expressed as means ± SEM; $n = 5$ mice/group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus the indicated group.

tein contains the extracellular domain of murine hemopoietic CD44, thus only one isoform of CD44, whereas in WT serum a mixture of splice variants of CD44 might be present.

Two earlier studies, using different infection routes, reported on the role of CD44 in defense against *E. coli*. In a pneumonia model, CD44 KO and WT mice displayed similar bacterial numbers in their lungs 6 h after infection (45), whereas in a urinary tract infection model bacterial numbers were found to be significantly reduced in kidneys and blood from CD44 KO mice compared to those of WT mice (39). The latter phenotype appeared to result from the interaction of CD44 on tubular epithelial cells with hyaluronan bound to the bacteria, thereby facilitating the migration of *E. coli* through the epithelial layer. In our model, however, bacteria do not need to overcome any barrier to expand in the peritoneal cavity. We found modestly enhanced bacterial numbers in the peritoneal cavities of CD44 KO mice at 6 h after *E. coli* infection, whereas in all of the other body compartments investigated, the bacterial loads were similar for CD44 KO and WT mice. Moreover, at 20 h postinfection, bacterial numbers were similar in the two groups at all body sites. Furthermore, CD44 did not impact on lung, liver, and kidney injury induced in this model. Together, these data suggest that CD44 does not play an important role in antibacterial defense against *E. coli* and does not influence the severity of organ injury in this model of abdominal sepsis. Of note, however, our data do not exclude a role for CD44 in sepsis in general: further studies, for example, using the polymicrobial sepsis model of cecal ligation and puncture, are warranted to establish this.

In conclusion, we showed that CD44 KO mice display increased cytokine and chemokine levels in the peritoneal cavity upon intra-abdominal infection with *E. coli*. In line with this, CD44 KO mouse peritoneal macrophages demonstrated enhanced cytokine and chemokine release upon stimulation with *E. coli* or *E. coli*-derived LPS. Unlike the role of CD44 in the induction of negative TLR regulators in lung macrophages (27), CD44 KO peritoneal macrophages demonstrated unaltered expression of IRAK-M and A20, both in the resting state and after stimulation with *E. coli* or LPS. The presence of WT serum partially reversed the proinflammatory phenotype of CD44 KO peritoneal macrophages. Further studies are warranted to dissect the mechanisms underlying the differential effects of CD44 on the production of cytokines and chemokines by different leukocyte subsets.

ACKNOWLEDGMENTS

We thank Joost Daalhuisen, Marieke ten Brink, Anita de Boer, and Danielle Kruijswijk for expert technical assistance and Shigeki Katoh for providing CD44-Ig fusion protein.

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Editor: B. A. McCormick