CD14 Works with Toll-Like Receptor 2 to Contribute to Recognition and Control of *Listeria monocytogenes* Infection

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Toll-like receptor 2 (TLR2) signaling has been shown to contribute to resistance to *Listeria monocytogenes* infection, as TLR2-deficient mice have a heightened susceptibility to infection with this organism. Because CD14 may associate with TLR2, we investigated the role of CD14 in *Listeria* responses. In both CD14-deficient and TLR2-deficient macrophages, nuclear factor κB translocation; CD40 and CD86; and the production of interleukin (IL)–12, IL-6, tumor necrosis factor, and nitric oxide are reduced. The absence of CD14 augmented susceptibility to *Listeria* infection, reduced survival, and diminished bacterial clearance, as observed in TLR2-deficient mice. Compared with C57BL/6 control mice, CD14-deficient mice were observed to have a greater number of hepatic microabscesses containing abundant neutrophils, these abscesses were larger in size, and there was reduced inducible nitric oxide synthase expression. Further, mice that are both CD14 deficient and TLR2 deficient display susceptibility to infection that is comparable to that of mice deficient in either CD14 or TLR2 alone. Therefore, the present data demonstrate the role of CD14 and TLR2 in the recognition and control of *Listeria* infection and host resistance.

Toll-like receptors (TLRs) recognize a wide range of microbial pathogens, and their products modulate the innate immune response that may lead to inflammation and altered host defense [1–3]. We previously reported that TLR2 signaling can be shown to contribute to resistance to *Listeria monocytogenes* infection because TLR2-deficient mice have a higher susceptibility to infection with this organism [4]. The involvement of the TLR signaling pathway was further confirmed by the heightened susceptibility of myeloid differentiation primary response gene (88) (MyD88)–deficient mice [5, 6]. Having tested several other TLRs, such as TLR3, TLR4, and TLR9, by using gene-deficient mice, we concluded that they play no role in *Listeria*-induced immune activation (unpublished data), which emphasizes the role of TLR2 in the immune response to *Listeria*. Because TLR2 may associate with other membrane receptors for pathogen recognition, we asked whether CD14 might contribute to the TLR2-mediated response to *Listeria*.

Together with TLR4 and MD2, CD14 is critical for the recognition of lipopolysaccharide (LPS) and/or endotoxin from gram-negative bacteria by different host cells that initiate cell activation and the release of proinflammatory cytokines [7, 8]. CD14 is not an obligatory coreceptor of TLR4. Indeed, in LPS-induced, TLR4-mediated acute lung injury, CD14 is required in response to low doses of LPS but is less critical at higher doses of LPS [9]. CD14 may participate in MyD88-independent, Toll/interleukin-1 receptor (TIR) domain-containing adaptor inducing interferon-β–mediated and/or TIR domain-containing adaptor inducing interferon-β–related adaptor molecule–mediated signalling of rough LPS or lipid A [10]. CD14 is also involved in corecognition by TLR2 of various TLR ligands, such as peptidoglycan from *Staphylococcus aureus* and *Streptococcus pneumonia* [11] and hu-

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man cytomegalovirus [12], and secreted microbial products from group B *Streptococcus* [13]; lipoteichoic acid–induced cell activation also depends on CD14 [14]. By using neutralizing antibodies to CD14 in TLR2-transfected CHO cells, the induction of tumor necrosis factor (TNF) production by killed *L. monocytogenes* was shown to depend, in part, on CD14 associated with TLR2 [15]. Typically, this is the case for the synthetic bacterial lipopeptide Pam3CSK4, signaling through heterodimers of TLR2 and TLR1, whereas diacylated Pam2CSK4, MALP2 or zymosan that signal through TLR2 and/or TLR6 require CD14 [10, 16]. CD14 may, however, participate to a lesser extent in signalling mediated by the TLR2-TLR6 complex than in signalling mediated by TLR4, because the TLR2-TLR6 complex was also shown to associate with CD36 [17]. Recently, activation of TLR3 has been shown to be enhanced by CD14, and an association of TLR3 and CD14 with the activating ligand has been demonstrated [18]. However, several TLR ligands recognize and activate cells independently of CD14.

In this study, we investigated whether CD14 participates in the host response to *Listeria* infection. There are a number of reasons to think that this is the case. First, CD14-deficient macrophages have a reduced proinflammatory response and reduced nitric oxide (NO) production. Second, CD14-deficient mice display diminished resistance to intravenous *Listeria* infection, similar to TLR2-deficient mice, and mice that are both TLR2 deficient and CD14 deficient are no more susceptible than mice deficient in either gene alone. Finally a role for TLR4 is excluded in this response. Therefore, the data suggest that CD14 is acting together with TLR2 to mount an efficient innate immune response to *Listeria*.

METHODS

Mice and infection. Mice 8–12 weeks old that were CD14 deficient (obtained from M.W Freeman [19]), TLR4 deficient [20], TLR2 deficient [21], TLR2 deficient and CD14 deficient (obtained from R. Landmann), or MyD88 deficient [22] and C57BL/6 control mice were used in this study. All mice were backcrossed for 10 generations onto the C57BL/6 background and bred under specific pathogen-free conditions at the Transgenose Institute (Centre National de la Recherche Scientifique). The animal experiments complied with the French government's ethical and animal experiment regulations. *L. monocytogenes* (L028 strain; Pasteur Institute) was cultured in LPS-free trypticase soy broth (soybean casein digest medium; Biovalley) and was aliquoted and stored in 30% glycerol at −80°C until use [4].

CD14-deficient mice, TLR2-deficient might, TLR4-deficient mice, CD14-deficient and TLR2-deficient mice, MyD88-deficient mice, and C57BL/6 control mice were injected in the caudal vein with 3×10^4 colony-forming units (cfu) per mouse. On days 2 and 3, livers and spleens were harvested. The organs were homogenized by use of a disposable homogenization system (Dispomix; Medictools AG), and the number of viable bacteria (colony-forming units) in the homogenates was determined by plating serial dilutions on trypticase soy broth agar plates (Biovalley) incubated at 37°C for 24 h as described elsewhere [4].

Primary macrophage culture. Murine bone marrow cells were isolated from femurs and differentiated into macrophages after culturing 10^6 cells/mL for 7 days in Dulbecco's modified Eagle medium (DMEM) (Sigma) supplemented with 20% horse serum and 30% L929 cell-conditioned medium (as a source of macrophage colony-stimulating factor) [23]. Three days after washing and reculturing in fresh medium, the cell preparation contained a homogenous population of macrophages (>97% $CD11b^+$ cells). Bone marrow–derived macrophages (BMDMs) were plated in 96-well microtiter culture plates (at 10^5 cells/well) by using serum-free DMEM (free of sCD14 and lipopolysaccharide-binding protein) with 100 U of interferon (IFN) γ and stimulated with LPS (LPS of *Escherichia coli*, serotype O111:B4 [Sigma], at 100 ng/mL), and *L. monocytogenes* (at MOI 1:2, e.g., 2×10^6 cfu/mL). The viability of the macrophages as tested by trypan blue exclusion was not affected. After 6 h and 18 h of stimulation, the supernatants were harvested for cytokine determination.

Cytokine and nitric oxide (NO) determination. TNF, interleukin (IL)–12p40, or IL-6 were quantified in cell-free supernatants from cell culture by use of commercial ELISA with a detection limit of 5 pg/mL (Duoset; R&D Systems). Nitrite (NO_2-; derived from NO breakdown) concentrations in supernatants from macrophages were determined by use of Griess reagent (1% sulphanilamide in 2.5% phosphoric acid, 0.1% n-1-napthylethylenediamide dichloric in 2.5% phosphoric acid [24]. After 30 min incubation at room temperature under agitation, the absorbance at 540 nm was measured. NO_2- was quantified using sodium nitrite ($NaNO_2$) as a standard.

Microscopic evaluation of liver tissue. Livers were fixed in 10% buffered formalin (Shandon). Tissues were dehydrated in ethanol and embedded in paraffin. Sections (3 μm) were cut and stained with haematoxylin and eosin for evaluation of pathologic changes. The number of microabscesses was quantified by counting in 20 microscopic fields at 100× magnification. The diameter of microabscesses was evaluated at 400× magnification by using an ocular grid, and 50 microabscesses were measured by 2 independent observers [4].

NO synthase type 2 (NOS2) expression in paraffin-embedded sections of liver tissue. Paraffin-blocked tissue slides were rehydrated through xylene, 100% ethanol, 96% ethanol, and 70% ethanol to water. To unmask epitopes, sections were placed in citrate buffer, heated for 10 min in a microwave oven, and fixed for 10 min in acetone. Endogenous peroxidase activity was blocked by 1%

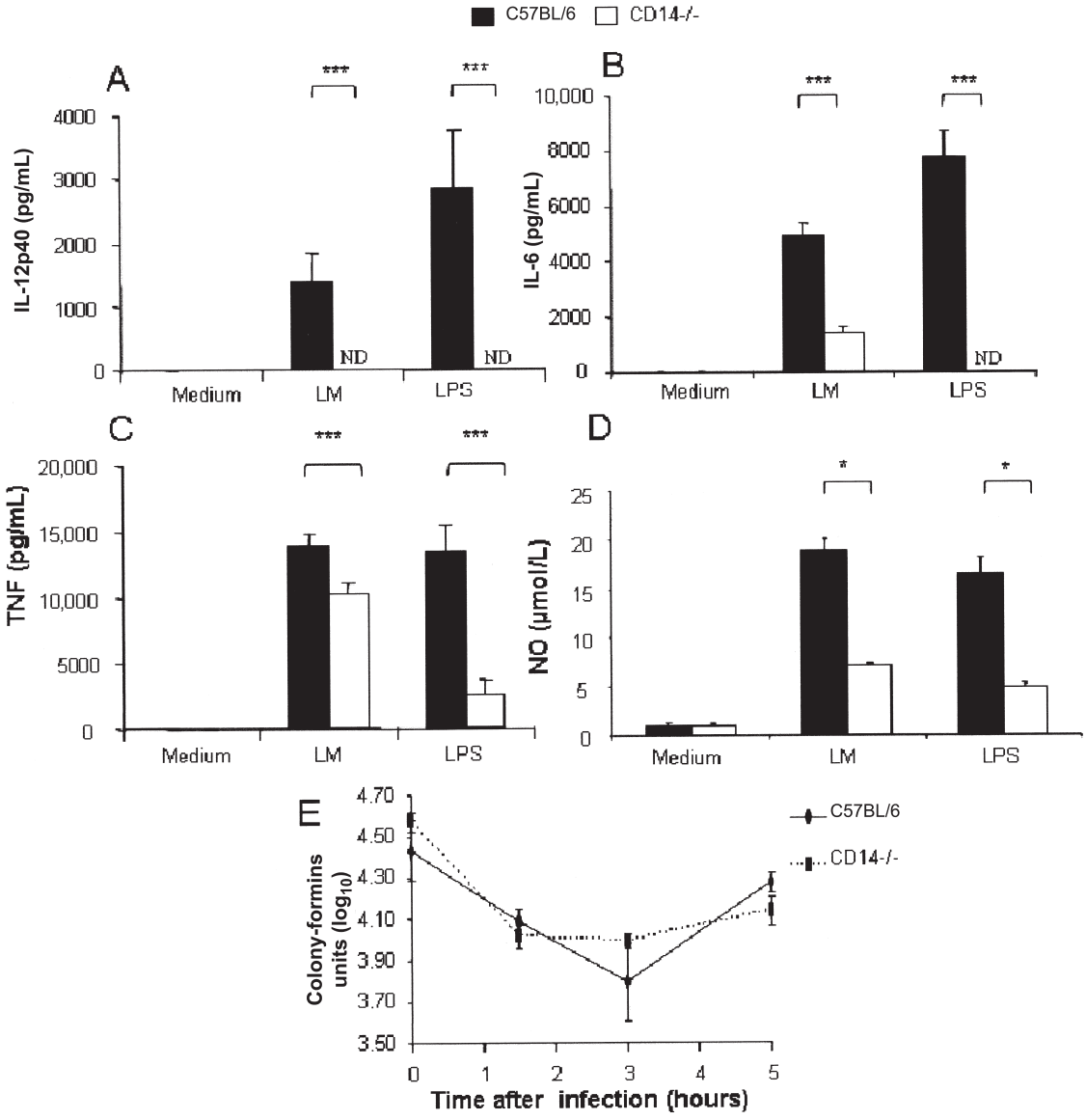

Figure 1. CD14-dependent production of proinflammatory cytokines in *Listeria monocytogenes* (LM)–infected bone marrow–derived macrophages (BMDMs). BMDMs from C57BL/6 control mice *(black bars)* and CD14-deficient (−/−) mice *(white bars)* were unstimulated (medium) or stimulated for 18 h with live *L. monocytogenes* (MOI, 1:2 [as described in the Methods section]) or lipopolysaccharide (LPS) (100 ng/mL). The concentrations of interleukin (IL)–12p40 *(A)*, IL6 *(B)*, tumor necrosis factor (TNF) *(C)* and nitric oxide (NO) *(D)* in the supernatant were determined by ELISA (for IL-12p40, IL-6, and TNF) and by the Griess method (for NO). Graphs show results of 1 experiment representative of 2 independent experiments, expressed as mean ± SD (*$P < .05$, ***$P < .001$). *E*, Bacteriocidal activity of macrophages from CD14−/− mice and C57BL/6 control mice assessed in vitro, as described in the Methods section. Graph shows results of 1 experiment representative of 2 independent experiments; number of viable bacilli are expressed as mean ± SD ($n = 4$). −/−, deficient; ND, not detectable.

H_2O_2 in methanol for 30 min, and endogenous biotin was blocked. Slides were incubated for 2 h at room temperature with the rabbit anti–mouse inducible nitric oxide synthase (NOS2) primary antibody (1:1000 dilution; supplied by J. Pfeilschifter), followed by incubation with biotinylated goat anti–rabbit antibody and revealed by use of the ABC Vector Kit for 30 min. Slides were washed and incubated for 10 min in fresh diaminobenzidine substrate. Sections were incubated for 2 min in 1% CuSO4, counterstained with haematoxylin, dehydrated through 70% ethanol, 96% ethanol, 100% ethanol to xylene and mounted in Eukitt (O.Kindler) for semiquantitative analysis by light microscopy.

Flow cytometry analysis of macrophages and cells obtained from liver tissue. After stimulation, macrophages were harvested, washed once in PBS that contained 0.5% bovine serum albumin (BSA), and incubated on ice at 10^5 cells/50 µL with primary antibodies (anti–CD40-PE [clone 3/23], anti–CD86–fluorescein isothiocyanate [FITC] [clone GL1], and anti–CD11b-PerCP Cy5.5 [clone M1/70]) for 20 min in the dark. All

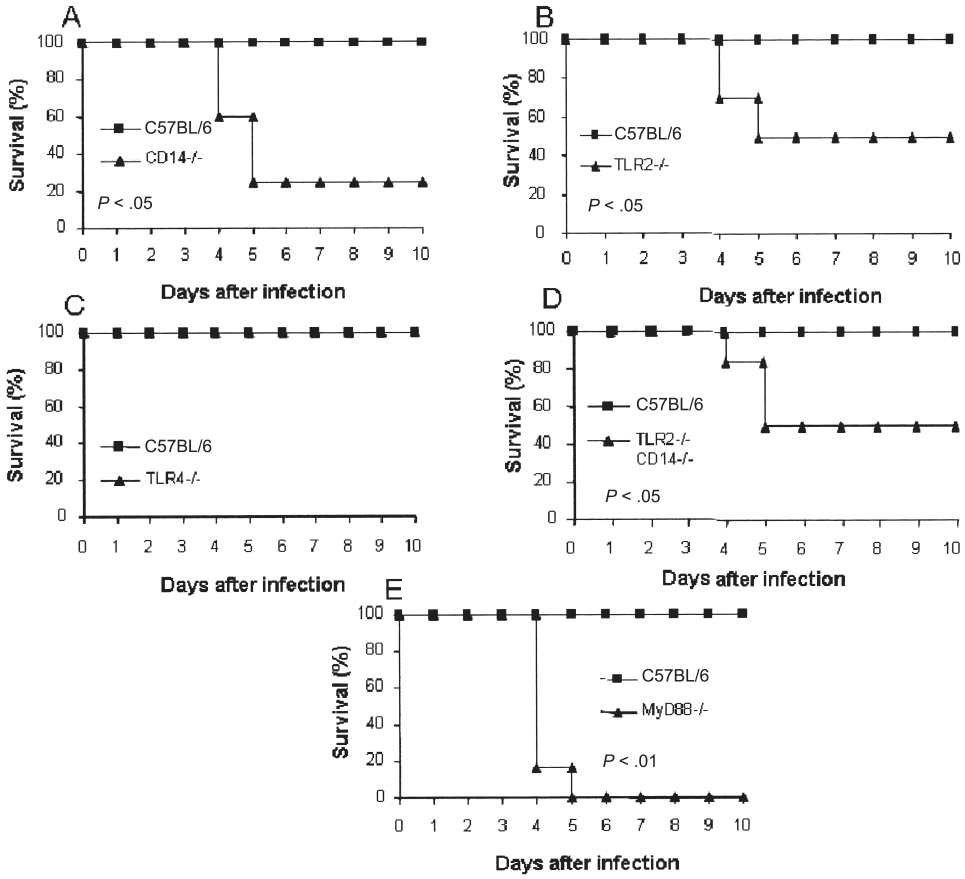

Figure 2. Reduced resistance of CD14-deficient (−/−) mice infected with *Listeria monocytogenes*. We injected 3×10^4 colony-forming units of *Listeria* intravenously into CD14−/− mice *(A)*, TLR2−/− mice *(B)*, TLR4−/− mice *(C)*, CD14−/− and TLR2−/− mice *(D)*, and MyD88−/− mice *(E)*. Survival rates were compared to that of C57BL/6-infected control mice during a 10-day period (for 6 mice per group). Graphs show results from 1 experiment representative of 2 independent experiments (*$P < .05$, **$P < .01$, determined by log rank test).

antibodies were obtained from BD Pharmingen. After washing with PBS that contained 0.5% BSA, cells were analyzed on a Becton Dickinson LSR analyzer.

For the flow cytometric analysis of inflammatory cells in the liver, mice were euthanized and the organs perfused with saline. Spleens and livers were minced with scissors, pressed through a nylon filter, resuspended in Percoll 33% gradient, and centrifuged at 1000 g for 20 min. Erythrocytes in the pellet were lysed with 155 mmol/L NH_4Cl, 10 mmol/L NaHCO3, and 0.1 mmol/L EDTA for 5 min on ice. Finally, the cells were resuspended in PBS that contained 0.5% BSA for fluorescence-activated cell sorter analysis using the protocol and antibodies described above.

Nuclear translocation of nuclear factor κB (NF-κB) in macrophages. BMDMs were grown on microscopic slides in the presence of 10 U IFNγ overnight and then incubated with *L. monocytogenes* (MOI, 1:2) for 1h , washed with PBS, and then fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Macrophages were incubated with goat anti–murine NF-κBp65 mAb for 1 h at room temperature, washed and incubated with swine anti–goat IgG FITC (Sigma). The nuclear translocation of NF-κB was assessed by fluorescence microscopy and 200 cells per group were counted. The nuclear translocation was confirmed and documented by confocal microscopy (Leica SP2).

Macrophage killing assay. The bactericidal activity of macrophages was determined as described elsewhere [25]. In brief, cells were plated in 24-well tissue culture plates (10^6 cells/well) in the presence of 100 U IFNγ overnight and then exposed to *L. monocytogenes* (MOI, 1:2 [e.g., 10^6 cells/mL per 2×10^6 cfu/mL]). After 30 min incubation at 37°C, gentamycin (10μg/mL) was added, and the cells were harvested immediately or after incubation periods of 1.5 h, 3 h, and 5 h and washed 3 times (each wash was with 1 mL of DMEM that contained 10% fetal calf serum). The supernatant was plated at 10-fold dilutions on trypticase soy broth (Biovalley), as described above. The plates were incubated at 37°C, and the number of colony-forming units was enumerated after 24 h.

Statistical analysis. The statistical evaluation of differences between the experimental groups was performed by the use of the log rank test for survival studies and the Student's t test.

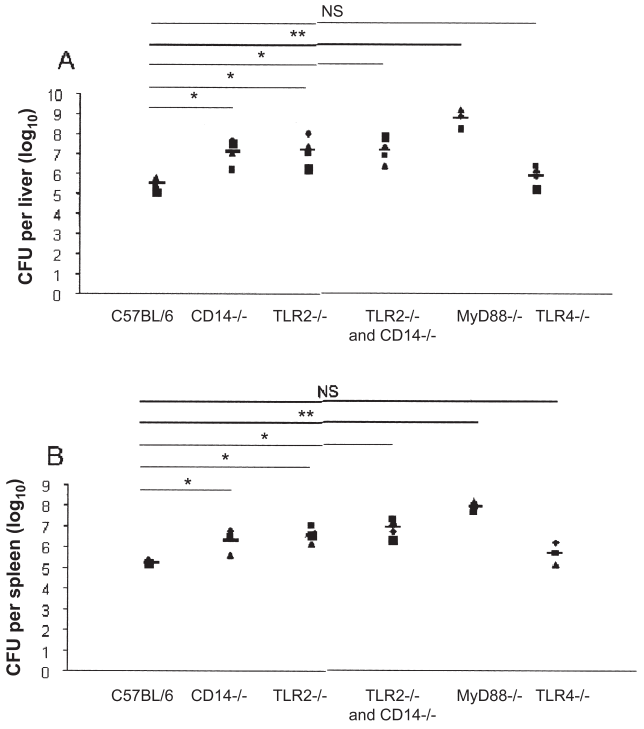

Figure 3. Reduced rate of bacterial clearance from the liver and spleen of CD14-deficient mice infected with *Listeria monocytogenes*. Bacterial load in the liver *(A)* and spleen *(B)* of CD14-deficient (−/−) mice, TLR2−/− mice, CD14 −/− and TLR2−/− mice, TLR4−/− mice, MyD88−/− mice, and C57BL/6 control mice 3 days after infection with 3×10^4 colony-forming units of *L. monocytogenes* (4 per group). Each symbol indicates the liver or spleen of 1 mouse. Results are from 1 experiment representative for 2 independent experiments and are expressed as mean ± SD (*$P < .05$, **$P < .01$. NS, not significant.

RESULTS

***Reduced production of IL-12p40, IL-6, TNF, and NO in CD14–deficient macrophages stimulated by* Listeria.** To characterize the role of CD14 for proinflammatory cytokine production in response to *L. monocytogenes*, primary bone marrow–derived macrophages from CD14-deficient mice and C57BL/6 control mice were stimulated with *L. monocytogenes*. The production of IL-12p40, IL-6, and, to a lesser extent, TNF was significantly reduced after *L. monocytogenes* activation of CD14-deficient macrophages at 6 h (not shown) and 18 h (figures 1*A*–1*C*), compared with cells from control mice, without affecting the cells' viability. As expected, the cytokine responses to low doses of LPS were abrogated in CD14-deficient macrophages (figure 1), as was also seen in TLR4-deficient macrophages (not shown). A similar reduction in cytokine production in response to *L. monocytogenes* infection is seen in TLR2-deficient and MyD88-deficient macrophages, as reported elsewhere [4].To explore the potential mechanisms of a bactericidal effect, we measured NO production. Macrophages from CD14-deficient mice produced less NO in response to *L. monocytogenes* infection, compared with macrophages from control mice (figure 1*D*) ($P < .05$). Despite diminished NO production, there was no significant reduction of bacterial killing by macrophages from CD14-deficient mice (figure 1*E*) ($P > .05$).

Therefore, the data suggest that CD14 is involved in *L. monocytogenes*–induced macrophage activation. In view of the reduced proinflammatory cytokine and NO production, we hypothesized that host resistance to infection with this intracellular pathogen may be diminished in CD14-deficient mice.

Reduced resistance and bacterial clearance in* Listeria*-infected, CD14-deficient mice. Because *L. monocytogenes* activates macrophages in a CD14-dependent and TLR2-dependent manner, we asked whether the CD14 engagement contributes to TLR2-dependent resistance to an in vivo *L. monocytogenes* infection. We compared the resistance of C57BL/6 control mice and CD14-deficient mice to a systemic injection of *L. monocytogenes*. Intravenous infection with 3×10^4 cfu per mouse caused death in 60% of CD14-deficient mice within 5 days, whereas all control mice survived (figure 2*A*). TLR2-deficient mice display a similar susceptibility to infection, whereas TLR4-deficient mice are resistant (figures 2*B* and 2*C*). To test whether there was an additive effect when both CD14 and TLR2 genes were inactivated, we used mice that were both CD14 deficient and TLR2 deficient; their susceptibility to *L. monocytogenes* infection was comparable to that of single gene–deficient mice (figure 2*D*). In contrast, we confirm that MyD88-deficient mice are highly sensitive to *L. monocytogenes* infection (figure 2*E*), as we reported before [6]. Therefore, these data clearly show that CD14-deficient mice have heightened susceptibility to *L. monocytogenes* infection.

In view of their increased sensitivity to infection with this organism, we asked whether the rate of bacterial clearance might be reduced in CD14-deficient mice. The bacterial load in spleen and liver tissue from control and CD14-deficient mice that had been infected with 3×10^4 cfu of *L. monocytogenes* per mouse was analyzed. Three days after infection, CD14-deficient mice displayed ~2 log higher levels of colony-forming units in the liver and 1 log higher levels in the spleen, compared with wild-type control mice, and MyD88-deficient mice had significantly higher levels than control mice (figures 3*A* and 3*B*). TLR2-deficient mice and mice deficient in both CD14 and TLR2 displayed an increase in the number of viable bacteria in the liver and spleen that was comparable to that seen in mice that lacked CD14 expression alone, without evidence of an additive effect (figure 3). Therefore, reduced host resistance in the absence of CD14 and/or TLR2 is associated with a significant increase of bacilli in the liver. As consequence of the higher bacterial load, augmented inflammation with an increased number of hepatic microabscesses was expected.

Augmented hepatic inflammatory response in* Listeria*-infected, CD14-deficient mice. Microscopic examination of liver tissue at low magnification revealed distinct differences in

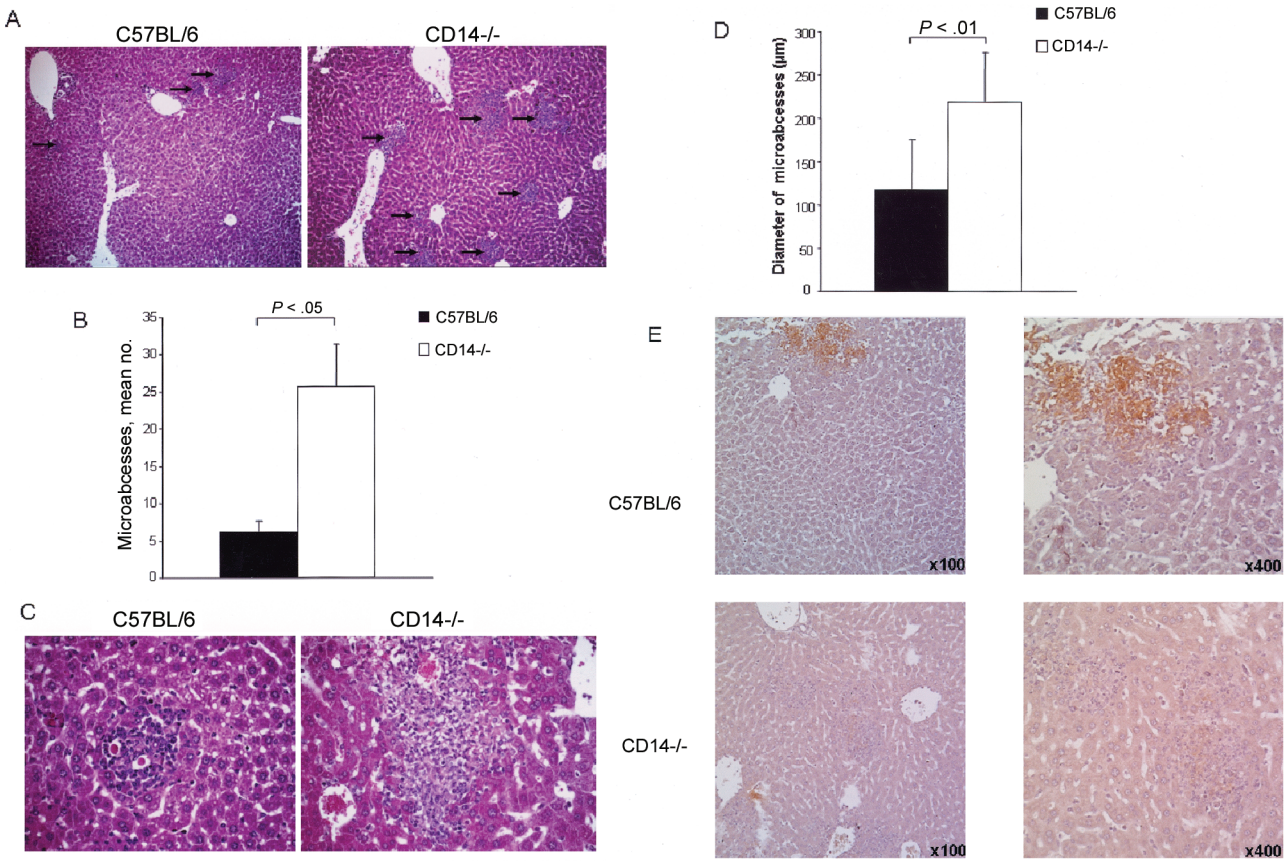

Figure 4. More abundant and larger hepatic microabscesses observed in CD14-deficient (−/−) mice. C57BL/6 and CD14−/− mice were infected with 3×10^4 colony-forming units of *Listeria monocytogenes* and liver tissue was histologically examined 3 days after infection (4 mice per group). Sections of liver tissue showing small, confined microabscesses in wild-type mice and spreading infection in CD14−/− mice *(A)* (hematoxylin and eosin staining, ×100). Increased numbers of microabscesses in CD14−/− mice, compared with C57BL/6 controls *(B).* Histological sections of liver tissue showing small, confined microabscesses in wild-type mice and spreading infection in CD14−/− mice *(C)* (hematoxylin and eosin staining, ×400). *D,* Mean diameter of microabscesses in millimeters. Graphs show results from 1 experiment representative of 2 independent experiments expressed as mean ± SD (*$P < .05$, **$P < .01$). *E,* Reduced expression of inducible nitric oxide synthase in the liver of CD14−/− mice. Immunostaining was performed at 3 days after infection in C57BL/6 control mice and CD14−/− mice by immunohistochemical analysis (×100 and ×400) ($n = 4$).

the hepatic microabscesses observed in CD14-deficient mice, compared with those observed in C57BL/6 control mice (figure 4*A*) 3 days after infection. The hepatic microabscesses in CD14-deficient mice were generally larger, less confined, and partially confluent. The number of abscesses was significantly increased in CD14-deficient mice (figure 4*B*). Moreover, the microabscesses in these mice had less-defined boundaries, contained more abundant neutrophils and necrotic tissue (figure 4*C*), and were larger (figure 4*D*), compared with the abscesses observed in control mice. Further, as an indicator of reduced phagocyte activation and bactericidal activity in vivo, NOS2 expression in infected liver tissue was clearly reduced in CD14-deficient mice, compared with control mice (figure 4*E*). Therefore, the absence of CD14 is associated with reduced NOS2 expression, enhanced neutrophil recruitment, and formation of large microabscesses in the liver.

Flow cytometric analysis of the inflammatory cells in the liver at 2 days after infection, when the bacterial loads are comparable between the experimental groups (data not shown), revealed decreased expression of CD40 by $CD11b^+$ cells from CD14-deficient mice, compared with cells from C57BL/6 control mice, which suggested reduced activation of monocytes and/or macrophages in the liver (figure 5). In contrast, the number of $Gr1^+$ neutrophils is slightly increased at the same time point (not shown), as shown by microscopic examination of the tissue sections.

CD14- and TLR2-dependent activation of NF-κB and costimulatory molecules. To further dissect potential crosstalk between the TLR2 and CD14 receptor pathways, we assessed the translocation of cytosolic NF-κB into the nucleus in BMDMs at several time points after *L. monocytogenes* infection. Although uninfected macrophages from control mice did not show any significant nuclear staining, incubation with *L. monocytogenes* induced rapid nuclear translocation of NF-κB in macrophages from C57BL/6 mice, which reached a maximum at 1 h with 64% of macrophages showing nuclear staining. In contrast, nuclear

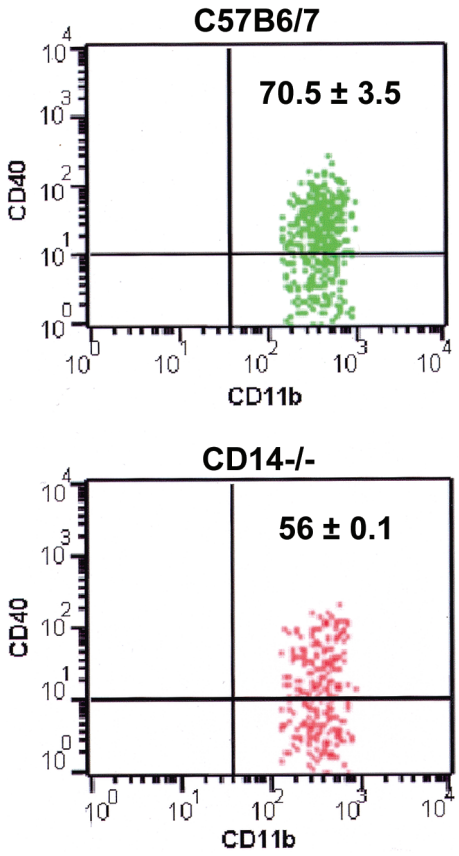

Figure 5. CD14-dependent recruitment and activation of macrophages in the liver. CD14-deficient mice and control mice were infected with 3×10^4 colony-forming units of *Listeria monocytogenes,* and mononuclear cells from the liver were isolated 2 days after infection (4 mice per group). The cells were stained with CD11b and CD40 and analyzed by flow cytometry. Numbers are the percentage of double-positive cells in the mononuclear cell population. Graphs show results from 1 experiment that are representative of 2 independent experiments, expressed as mean ± SD ($n = 2$).

translocation was absent in macrophages from CD14-deficient or TLR2-deficient mice after *L. monocytogenes* infection (figure 6*A*). Therefore, *L. monocytogenes* induced NF-κB activation through CD14 and TLR2 with similar kinetics and magnitude. Further, reduced NF-κB activation was found to be associated with diminished upregulation of CD40 and CD86 expression in macrophages from CD14-deficient mice and TLR2-deficient mice infected with *L. monocytogenes,* compared with macrophages from control mice (figure 6*B*). Therefore, both NF-κB and costimulatory molecule expression are reduced to a similar extent in macrophages from CD14-deficient mice or TLR2-deficient mice. The data suggest that CD14 and TLR2 sense molecular patterns expressed by *L. monocytogenes* in concert. Reduced macrophage activation, cytokine production, and NO production may explain reduced in vivo host responses with diminished rates of bacterial clearance and enhanced inflammation observed in CD14-deficient mice and TLR2-deficient mice.

DISCUSSION

The present data demonstrate for the first time, to our knowledge, that CD14-deficient mice have an increased sensitivity to *L. monocytogenes* infection with reduced survival rates due to impaired activation and killing of phagocytes that results in reduced rates of bacterial clearance and augmented inflammatory pathology. In fact, in macrophages stimulated by *L. monocytogenes,* the production of IL-12p40, IL-6, NO, and, to a lesser extent, TNF that depends in part on TLR2 signaling, but not on TLR4 signaling, is reduced. These results are in agreement with those of Flo et al., who demonstrated that CD14 neutralization reduced TNF production in TLR2-transfected CHO cells activated by heat killed *L. monocytogenes* [15]. In the present study, we show that cytokine and NO production by primary macrophages stimulated with live *L. monocytogenes* is dependent on CD14 and TLR2 [4]. In addition, the nuclear translocation of NF-κB and expression of the costimulatory molecules CD40 and CD86 are diminished in the absence of either of CD14 or TLR2. In conformity with our results, reduced cytokine production in macrophages from TLR2-deficient mice has been shown [26] and reduced serum levels of IFNγ, TNF, and IL-12 have been reported in these mice [5]. Furthermore, we extend this observation and demonstrate a role for CD14 in in vivo *Listeria* infection. Reduced resistance to *Listeria* infection is seen in both CD14-deficient and TLR2-deficient mice. Moreover, mice deficient in CD14 or TLR2 alone and mice deficient in both CD14 and TLR2 display comparable reduction in host resistance, with increased of bacterial load and enhanced acute hepatic inflammation. The discrepancy between the susceptibility reported previously in TLR2-deficient mice [6, 27] and our data here may be explained by different routes of infection (intraperitoneal versus intravenous), the number of backcrosses of the mice, and the strains of *Listeria* used.

The involvement of CD14 agrees well with our unpublished data that suggest *L. monocytogenes* signals through TLR2-TLR6 complexes. Indeed, ligands of TLR2-TLR6 heterodimers, such as Pam2CSK4, MALP2 or zymosan, seem to require CD14, whereas ligands of TLR2-TLR1 heterodimers, such as Pam3CSK4, bind and signal independently of CD14 [1, 28, 29]. It should be emphasized that the MyD88-deficient mice are distinctly more susceptible to *L. monocytogenes* infection than TLR2-deficient or CD14-deficient mice (figure 2*D*). Previously, a high sensitivity to *Listeria* infection was reported for MyD88-deficient mice, but interestingly, an adaptive immune response with protective immunity was obtained in the absence of MyD88 [30]. MyD88 is further involved in crosstalk with other pathways such as the focal adhesion kinase [31] that may play a role in the

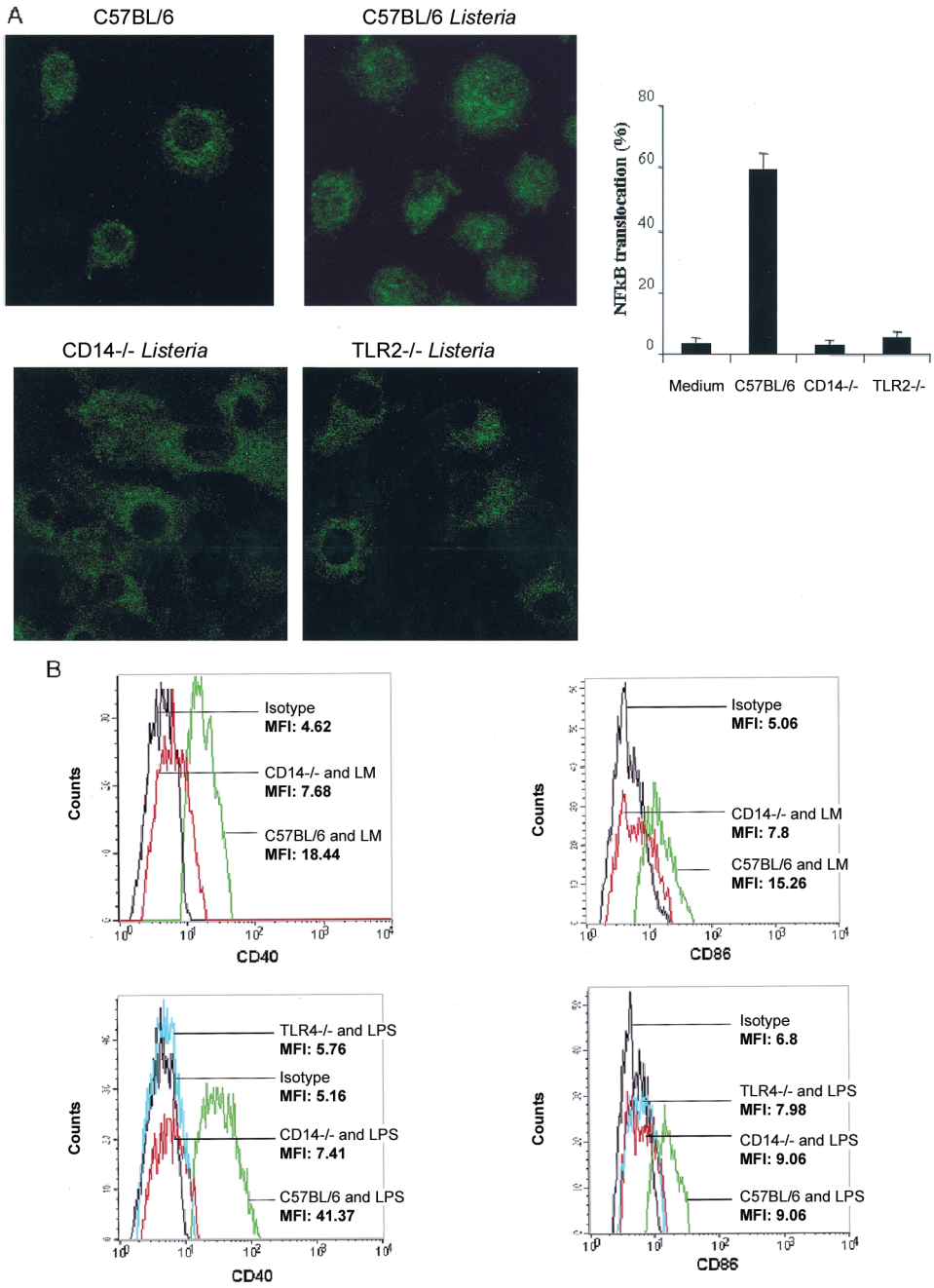

Figure 6. CD14-dependent nuclear factor κB (NF-κB) translocation and upregulation of CD40 and CD86 expression in *Listeria monocytogenes* (LM)–infected bone marrow–derived macrophages (BMDMs). NF-κB translocation in *L. monocytogenes*–infected macrophages *(A).* BMDMs from C57BL/6 mice and CD14-deficient mice were stimulated for 1h with *L. monocytogenes* (MOI, 1:2), and NF-κB staining was assessed. A quantitative evaluation of the percentage of cells showing NF-κB translocation is presented here as a bar graph. Reduced upregulation of CD40 and CD86 expression *(B)* in CD14-deficient macrophages after *L. monocytogenes* or lipopolysaccharide (LPS) stimulation (2 mice per group). Graphs show results from 1 experiment representative of 3 independent experiments. Counts represent the relative cell number. MFI, mean fluorescence intensity.

process of spreading *Listeria* infection. CD14 has so far not been reported to be involved in receptor complexes other than TLR4 and TLR2, with the exception of TLR3. A TLR3 ligand polyinositine-cytosine analogue has been shown to activate antigen-presenting cells in a TLR3- CD14 dependent manner, and a ligand- TLR3-CD14 complex has been demonstrated [18].

CD14 seems to be involved in regulating neutrophil influx, because CD14-deficient mice show increased expression of CXCR2, MIP2, and neutrophil transmigration in pneumococcal infection [32], whereas neutrophil migration was delayed in CD14-deficient mice early after peritoneal infection with *Salmonella,* acting through TLR4 [33]. Here, neutrophil recruitment

after *L. monocytogenes* infection is enhanced, resulting in large, diffuse microabscesses in the liver of CD14-deficient mice. However, the data suggest that macrophages and neutrophils from CD14-deficient mice and TLR2-deficient mice may be less effective in killing in vivo, as indicated by reduced iNOS expression in the liver, although the macrophage mediated killing was not reduced in vitro. Reactive nitrogen is a key bactericidal mechanism for mycobacteria [34] and *Listeria* [35]. However, the killing of *Listeria* is further supported by reactive oxygen produced by phagocytes oxidase ($pg91^{phox}$). In fact, mice that are double-deficient for $pg91^{phox}$ and NOS2 are more susceptible to *Listeria* infection [35] than single-deficient mice, but the existence of alternative mechanisms of killing is discussed [36].

Although we showed reduced resistance in TLR2-deficient mice [5, 6] and CD14-deficient mice, the dramatically heightened susceptibility of MyD88-deficient mice that has bee described before [5, 6] cannot be solely ascribed to defective TLR sensing of *L. monocytogenes*. We have tested most of the TLRs by using single-gene deficient mice (data not shown), suggesting that TLR2-CD14 may contribute to this heightened susceptibility, along with other non-TLR pathogen recognition receptors on the membrane, which are yet to be identified.

Previous investigations demonstrated the induction of MCP-1 in macrophages by *L. monocytogenes* and CCR2-dependent recruitment and activation of monocytes [27]. The induction of MCP-1 was found to be MyD88-dependent and TLR2-independent. Edelson et al. [6] offer data that reinforce the MyD88-dependent control of *L. monocytogenes* infection, suggesting a contribution by IL-1 and IL-18 receptor pathways, which depend on TLR signaling on the MyD88 adapter protein. This hypothesis is further reinforced by reduced IL-1 production in *L. monocytogenes*–infected, ASC-deficient macrophages [26]. Therefore, TLR2-CD14 and additional, undefined pattern recognition receptors induce IL-1 and IL-18, which are required, together with IFNγ, IL-1, and TNF, to control *L. monocytogenes* infection. However, a recent report demonstrated that IL-1 signaling is dispensable for the control of systemic *Listeria* infection, but required to control cerebral listeriosis [37].

In conclusion, *Listeria* activates macrophages in vitro in a CD14-dependent and/or TLR2-dependent manner. CD14 expression is required for host resistance to *L. monocytogenes* infection in vivo; reduced CD14 expression results in bacterial persistence, reduced NOS2 expression in the liver, and heightened mortality. However, there is no additive effect with TLR2; mice deficient in both CD14 and TLR2 have comparably heightened susceptibility to *Listeria* infection.

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