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Divergent Response to LPS and Bacteria in CD14-Deficient Murine Macrophages

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Gram-negative bacteria and the LPS constituent of their outer membranes stimulate the release of inflammatory mediators believed to be responsible for the clinical manifestations of septic shock. The GPI-linked membrane protein, CD14, initiates the signaling cascade responsible for the induction of this inflammatory response by LPS. In this paper, we report the generation and characterization of CD14-null mice in which the entire coding region of CD14 was deleted. As expected, LPS failed to elicit TNF-α and IL-6 production in macrophages taken from these animals, and this loss in responsiveness is associated with impaired activation of both the NF-κB and the c-Jun N-terminal mitogen-activated protein kinase pathways. The binding and uptake of heat-killed Escherichia coli, measured by FACS analysis, did not differ between CD14-null and wild-type macrophages. However, in contrast to the findings with LPS, whole E. coli stimulated similar levels of TNF-α release from CD14-null and wild-type macrophages at a dose of 10 bioparticles per cell. This effect was dose dependent, and at lower bacterial concentrations CD14-deficient macrophages produced significantly less TNF-α than wild type. Approximately half of this CD14-independent response appeared to be mediated by CD11b/CD18, as demonstrated by receptor blockade using neutrophil inhibitory factor. An inhibitor of phagocytosis, cytochalasin B, abrogated the induction of TNF-α in CD14-deficient macrophages by E. coli. These data indicate that CD14 is essential for macrophage responses to free LPS, whereas other receptors, including CD11b/CD18, can compensate for the loss of CD14 in response to whole bacteria. The Journal of Immunology, 2000, 165: 4272–4280.

Lipopolysaccharide, the major constituent of the outer membrane of Gram-negative bacteria (1, 2), plays a crucial role in mediating host responses to Gram-negative infections by stimulating the release of inflammatory mediators, including cytokines, from various target cells. LPS activation of these mediators is thought to be responsible for the clinical manifestations of septic shock (3) and it may also play a role in the pathophysiology of chronic inflammatory disorders such as asthma, pelvic inflammatory disease, and inflammatory bowel disease. Although the targets of LPS activation include a broad array of cells, including polymorphonuclear cells and epithelial and endothelial cells, macrophages are especially responsive to LPS. Exposure of macrophages to nanogram quantities of LPS results in the rapid synthesis and release of myriad cytokines, such as IL-1, -6, -8, and TNF-α, as well as proinflammatory eicosanoids and NO (1).

Three cloned families of molecules on the surface of leukocytes are known to bind the toxic lipid A moiety of LPS. These include CD14, the macrophage scavenger receptors (SR-A family), and the β2, or CD11/CD18 leukocyte integrins (4). LPS binding to CD14 is enhanced by serum factors; these serum components include the acute phase reactant LPS binding protein (LBP) and soluble CD14 (sCD14). When expressed on the surface of cells, CD14 has no intrinsic signaling capabilities, but is postulated to present LPS to its high affinity signal transducer, Toll-like receptor (TLR) 4 (5–8). CD14 also enhances the responses of phagocytes to bacterial products that activate cells via TLR2 (e.g., peptidoglycan and lipopeptide), although the exact contribution of TLR2 to LPS responses remains to be clarified. Although all of the above receptors are components of the host response to endotoxin, the extraordinary sensitivity of the macrophage response to LPS appears to depend upon the presence of either membrane-bound or soluble CD14.

The presence of sCD14 in serum and the expression of membrane-bound CD14 on myeloid cells have complicated the analysis of alternative receptors involved in activation by bacterial products. This is particularly true of the β2 integrins (CD11/CD18), which bind LPS, especially when LPS is presented to phagocytes as a component of a membrane (e.g., whole bacteria; Ref. 9). Although phagocytes bind the LPS moiety of Gram-negative bacteria via CD11/CD18, this interaction was felt initially to be biologically insignificant because children with congenital CD11/CD18 deficiency responded normally to LPS (10). In retrospect, this observation might have been predicted because the cells that were harvested from these children expressed CD14. When expressed in a cell line that does not coexpress CD14, the β2 integrins function as low affinity LPS receptors (11–13). Although the ED50 for CD11/CD18 engagement is higher than that for CD14, few qualitative differences in LPS activation by these two distinct receptor systems have been described (14). Like CD14, CD11/CD18 can activate cells in the absence of cytoplasmic signaling residues, as demonstrated by experiments that showed equivalent LPS responses to a

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4 Abbreviations used in this paper: LBP, LPS binding protein; TLR, Toll-like receptor; NIF, neutrophil inhibitory factor; ES, embryonic stem; JNK, c-Jun N-terminal kinase; sCD14, soluble CD14.
full length and “tailless” integrin (12). It seems likely that other, less well-characterized, cell surface proteins that bind bacteria may also activate phagocytes, but these will be challenging to identify as long as the highly sensitive CD14 activation pathway can be engaged.

To explore the contributions of both CD14-dependent and independent signaling mechanisms to LPS and bacterial stimulation, we chose to generate mice lacking this receptor. Macrophages from our CD14-null mice proved to be unresponsive to LPS stimulation over an LPS dosage range of two log orders (1–100 ng/ml), confirming similar observations Haziot et al. previously made in CD14-null mice generated with a different gene-targeting strategy (15). In this work, we further confirm that the lack of cytokine responsiveness in LPS-stimulated CD14-null macrophages is associated with impaired activation of both the NF-κB and the c-Jun N-terminal mitogen-activated protein kinase (MAPK) pathways (16, 17). In contrast with their unresponsiveness to LPS, CD14-null macrophages retained cytokine responses to whole bacterial particle stimulation. On exposure to 10 heat-killed *Escherichia coli*, per cell or greater, CD14-null and wild-type macrophages produced similar amounts of TNF-α. At 1 *E. coli* per cell, CD14-null macrophage cytokine responses were definitely impaired but not lost. This CD14-independent cytokine production was substantially reduced when neutrophil inhibitory factor (NIF), a competitive inhibitor of the β2 integrin CD11B, was included in the experiments. Inhibition of phagocytosis by cytochalasins completely abolished CD14-independent responses. These data indicate that CD14-null mice will be of considerable value in dissecting the contributions of the multiple response pathways macrophages use to combat bacterial infections.

Materials and Methods

**Reagents**

Protein-free LPS derived from *E. coli* K235 was provided by Dr. S. Vogel (Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD). Recombinant CD14 was purified from the conditioned medium of Chinese hamster ovary/CD14 cells as described elsewhere (18). The *Ancylostoma caninium* (hook-worm) protein, NIF, was provided by Dr. Matthew Moyle (Corvas International, San Diego, CA) (19). Endotoxin-free cell culture media and additives were purchased from Life Technologies (Grand Island, NY).

**Generation of CD14-deficient mice**

A P1 clone with ~60 kb of mouse genomic DNA, including the gene encoding CD14, was obtained from Genome Systems (St. Louis, MO) after screening a genomic library using a combination of PCR and Southern hybridization as previously described (20). The genomic structure of the CD14 locus was mapped by a combination of restriction digest and Southern blotting. A targeting vector, KO3CD14 (Fig. 1A), was generated that removed the entire coding sequence of the CD14 gene. KO3CD14 contained the 4 kb of sequence immediately upstream of the start site of translation of the CD14 coding sequence, an intervening neo’ cassette derived from PGKneoA, which replaced all CD14 coding sequences, and the 2.5 kb of sequence immediately downstream of the coding region. A thymidine kinase gene was also incorporated into the plasmid as a negative selectable marker to reduce the frequency of nonhomologous recombinant transformants. The linearized vector was electroporated into J1 embryonic stem (ES) cells derived from 129/SvEv mice, and 200 G418/gancyclovir resistant colonies were selected. Genomic DNA from selected colonies was digested with SacI and analyzed by Southern blotting. Southern blots were developed with 17-kb HindIII digested fragments as a probe. This probe recognized a 17-kb fragment in the targeted locus, and the 6-kb fragment in the homologous locus. A 1.7-kb SacI fragment at the endogenous locus was not detected.

**FIGURE 1.** Targeting of the mouse CD14 gene. A. Schematic diagram of the targeting vector KO3CD14, the endogenous CD14 locus, and the targeted CD14 locus. KO3CD14 contains 4 kb of 5’ sequence immediately upstream of the initiator methionine codon in the CD14 gene, followed by a neo’ cassette derived from PGKneoA, and 2.5 kb of sequence immediately 3’ of the coding region. Homologous recombination results in the replacement of the entire coding region of CD14 with the neo’ cassette. The targeting vector also contains a thymidine kinase gene that acts as a negative selectable marker to eliminate nonhomologous recombinants. Screening of selected ES cells was performed by Southern blot analysis of SacI-digested DNA using the indicated NheI/NcoI fragment located outside of the targeting vector as a probe. This probe recognizes a 6-kb fragment in the endogenous locus and a 17-kb fragment in the targeted locus as indicated. B. Southern blot analysis of F1 CD14 heterozygote intercross mating. Mouse tail DNA from the intercross of F1 CD14 heterozygotes was digested with SacI and analyzed by Southern blot as described above. A band of 6 kb is detected in wild-type (+/+), bands of 6 and 17 kb in heterozygous (+/−) mice, and a single band of 17 kb in homozygous mutant (−/−) mice. C. Western blot confirming the absence of CD14 protein in CD14-deficient mice. Cell lysates from peritoneal macrophages derived from CD14+/+, CD14+/−, and CD14−/− mice (n = 2) were analyzed by Western blot for CD14 and the macrophage marker F4/80. CD14 protein was detected in CD14-deficient mice confirming the deletion of this gene. However, the macrophage-specific F4/80 Ag was detected in cell lysates from all genotypes (+/+, +/−, and −/−).

Backcrossing of the CD14-deficient mice into the C57BL/6J strain has been continued for four generations in a pathogen-free facility, but the macrophages used for the experiments described in this work were derived from animals that were backcrossed for one or two generations. For experiments requiring autologous serum, CD14 wild-type, -hemizygous, and -null mice were bled by retro-orbital puncture using heparinized microcapillary tubes. Blood was pooled within genotypes and centrifuged at 5000 rpm for 10 min, then serum was isolated.

**Isolation of peritoneal macrophages**

Peritoneal macrophages were isolated from 12-wk-old mice 4 days following i.p. injection of 1 ml of 3% thioglycollate broth (Difco, Detroit, MI) as...
we previously described (21). Unless otherwise stated, wild-type and hemizygous controls for CD14-null mice were F2 littersmates. Mice were anesthetized using Metofane (Union, NJ) and sacrificed by cervical dislocation. Peritoneal lavage was performed with 6 ml of HBSS (Life Technologies) containing 0.5 mM EDTA. Collected peritoneal cells were treated with 0.17 M ammonium chloride to disrupt contaminating RBC, washed twice in HBSS, and resuspended in DMEM (Life Technologies) containing 100 U/ml penicillin and 100 μg/ml streptomycin and supplemented with 2.5% autologous mouse serum. Peritoneal cells were allowed to adhere to tissue culture plastic for 2 h, after which nonadherent cells were removed by rinsing the cell monolayer with media. Adherent cells were typically >97% positive for the macrophage-specific F4/80 Ag as determined by FACS analysis.

Western analysis

For Western analysis, peritoneal macrophages were seeded at 3×10^6 cells per well in six-well plates. Cells were lysed in TNET buffer (50 mM Tris, pH 8, 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100) and centrifuged for 5 min at 16,000 rcf to pellet debris. To detect CD14 and phosphorylated or total ε-Jun N-terminal kinase (JNK), equivalent amounts of protein were heat denatured in the presence of Laemmlı sample buffer containing 5% 2-ME and electrophoresed on 10% SDS-PAGE gels. To detect F4/80, samples were run on 8% polyacrylamide gels under nonreducing conditions. Proteins were transferred to Immobilon-polyvinylidene difluoride membrane (Millipore, Bedford, MA) and blocked in TBS-T containing 5% nonfat milk for CD14 and F4/80, or 1% BSA in TBS-T for phosphoJNK. Primary Abs used were anti-murine CD14 rat monoclonal Ab (catalog no. 09471D; PharMingen, San Diego, CA), anti-F4/80 cell culture supernatant (1:5 dilution) from the F4/80 hybridoma cell line (American Type Culture Collection, Manassas, VA), rabbit anti-phosphoJNK Ab (Promega, Madison, WI), and rabbit antistress-activated protein kinase/JNK (New England Biolabs, Beverly, MA). Blots were incubated with HRP-conjugated secondary Ab (Sigma, St. Louis, MO) against the appropriate species and developed using an enhanced chemiluminescence detection system.

Detection of nuclear NF-κB

EMSAs for nuclear NF-κB were performed as previously described (22). Briefly, 4 μg of crude nuclear protein was incubated with a ^32P-labeled oligonucleotide containing the consensus sequence for NF-κB binding from the murine IgG light chain enhancer. The resulting complexes were analyzed by non-denaturing PAGE. The gels were transferred to filter paper, dried, and exposed to x-ray film. Scanning densitometry of the autoradiographs was performed using Molecular Analyst software.

Cytokine ELISAs

Peritoneal macrophages were seeded in six-well plates at 5×10^5 cells per well in 0.6 ml of medium. Peritoneal macrophages were stimulated with LPS from E. coli K235 or heat-inactivated E. coli K12 bioparticles (Molecular Probes), and cell supernatants were harvested at the indicated times. Supernatants were centrifuged for 5 min to remove cellular debris, transferred to new tubes, and stored at −70°C until analysis. TNF-α and IL-6 in cell supernatants were measured by ELISA using matched pair Abs from Endogen (Cambridge, MA).

Binding assays

The binding of bacterial membrane particles to peritoneal macrophages was assessed as previously described (14). BODIPY-labeled E. coli K12 bioparticles (Molecular Probes) were added to CD14 wild-type or -null peritoneal macrophages (ratio of bacteria to cells was 100:1 or 10:1) in media containing 2.5% autologous serum for 5, 15, and 30 min at 37°C. To assess binding and uptake, cells were washed in PBS and assessed by FACs analysis. To assess uptake alone, cells were incubated in 2% trypsin blue for 1 min to quench extracellular fluorescence, washed in PBS, and assessed by FACs analysis. The ability of trypsin blue to quench BODIPY-labeled particles has been previously established in cells lines that cannot engage in CR3-mediated phagocytosis due to the expression of mutant “tailless” CD11b/CD18 (12). Ten thousand gated events were recorded for each condition, excluding nonbound bacteria and cellular debris on the basis of forward and side light scatter. A second gate was established separating bright from nonfluorescent macrophages. Results are expressed as a percentage of cells that fall within this gate.

Blocking studies

Cells were preincubated with 4 μg of NIF protein/ml to inhibit CD11b/CD18 binding, or with 10 μM cytochalasin B to inhibit phagocytosis, for 15 min before stimulation with E. coli bioparticles. Inhibitors were maintained in culture media for the duration of the experiment (4 h).

Results

Characterization of CD14-deficient mice

Screening of ES cell clones by Southern blotting, as described in Fig. 1A, revealed the correct disruption of the CD14 allele in 2 of 100 clones. Although both ES cell clones were injected into blastocysts, only one line produced high percentage chimeras. Male chimeras from this line were backcrossed to C57BL/6J females and offspring were screened for germline transmission of the targeted allele using tail DNA. F1 hemizygotes were intercrossed, and screening revealed the expected Mendelian ratio of CD14-null, -hemizygous, and wild-type offspring. A representative Southern blot depicting the three genotypes is shown in Fig. 1B. The wild-type allele is 6 kb and the targeted allele is 17 kb. CD14-null mice appeared to be phenotypically normal. The deletion had no obvious adverse effect on fertility or litter size.

Western blot analysis of peritoneal macrophages from CD14-deficient mice showed no expression of CD14 (Fig. 1C). As expected, CD14 was detected in peritoneal macrophages from hemizygous and wild-type mice. Western blot analysis of the same lysates for the macrophage-specific Ag, F4/80, revealed similar levels of this protein in CD14-null, -hemizygous, and wild-type macrophages, eliminating nonspecific protein degradation as an explanation for the absence of CD14 in the lysates of the null animals. These results established that the CD14-null mice were homozygous for the targeted genomic allele and that they were therefore unable to produce any CD14 protein.

Impaired responses of CD14-deficient macrophages to LPS

We evaluated pretranscriptional events, such as NF-κB translocation and JNK kinase activation, as well as cytokine production, to determine the consequences of the absence of CD14 on macrophage responses to endotoxin. A loss of NF-κB activation in response to LPS was observed in CD14-null peritoneal macrophages as assessed by an EMSA (Fig. 2A). Wild-type macrophages exhibited dose-dependent activation of NF-κB in response to as little as 1 ng/ml of LPS. In contrast, doses of up to 100 ng of LPS/ml did not induce NF-κB activity in CD14-null macrophages. However, IL-1β, which acts independently of CD14, induced the same level of NF-κB translocation in CD14-null and wild-type macrophages, indicating that the loss of LPS-induced NF-κB translocation in the null mice was a specific consequence of the CD14 deletion.

In addition to NF-κB translocation, LPS induces the rapid activation of several MAPKs, including the c-Jun N-terminal MAPK (17). Western analysis revealed the accumulation of phosphorylated JNK in wild-type macrophages within 30 min of LPS or E. coli stimulation (Fig. 2B). In contrast, LPS and E. coli activation of JNK was reduced in CD14-deficient macrophages. However, IL-1β, which stimulates JNK activation independently of CD14, rapidly induced JNK phosphorylation in both CD14-null and wild-type macrophages. These results indicate that engagement of CD14 by either LPS or whole E. coli contributes to JNK activation.

Corresponding with the loss of the early steps in LPS-induced signal transduction, CD14-null macrophages exhibited a loss of cytokine production in response to LPS (Fig. 3). In wild-type macrophages, a 5-h incubation with LPS resulted in a dose-dependent increase of both TNF-α and IL-6 in culture supernatants. However, no TNF-α or IL-6 were detected in culture supernatants from CD14-null macrophages stimulated with up to 100 ng/ml of LPS from E. coli K235. Similar results were obtained using LPS from

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Salmonella minnesota R595 (data not shown). Although the precise level of CD14 expression required for sensitive responses to LPS is unknown, an intermediate phenotype in cytokine production was observed in LPS-stimulated hemizygous macrophages. The reduction of TNF-α (Fig. 3A) and IL-6 (Fig. 4A) production in CD14-hemizygous macrophages, as compared with wild-type cells, clearly demonstrates a CD14 gene dosage effect. Levels of TNF-α and IL-6 were undetectable in unstimulated macrophages from mice of all genotypes. Taken together, these data demonstrate that CD14 appears to be critical to the activation of several signal transduction pathways involved in macrophage activation by LPS.

Contribution of serum-sCD14 to macrophage responses to LPS
sCD14 is believed to enhance delivery of LPS monomers to membrane-bound CD14 at the cell surface, as well as to enable delivery of LPS to additional components of the LPS signal transduction apparatus in cells lacking surface CD14 (23). Therefore, macrophages and sera from CD14-hemizygous and -null mice were used to clarify the relative contributions of sCD14 and membrane CD14 to cytokine responses to LPS. Both CD14-null and -hemizygous macrophage cytokine production levels were restored to wild-type values by the addition of sCD14 to the medium (Fig. 4A). These results are similar to those found by Haziot et al. (15) despite our use of concentrations of sCD14 that more closely mimic the physiologic levels of that protein (~2 μg/ml) as opposed to the 10-fold higher concentration (20 μg/ml) used in the previous study. Using autologous and CD14-null serum, we demonstrate the important contribution of serum sCD14 in mediating signaling in response to low doses of LPS. At 1 ng/ml of LPS, serum sCD14 concentration significantly enhanced cytokine production by membrane CD14-bearing cells (Fig. 4B). Wild-type macrophages produced 10-fold more TNF-α when cultured in 2.5% autologous serum than in 2.5% CD14-deficient serum. A similar effect was observed when CD14-hemizygous macrophages were cultured in autologous serum vs CD14-deficient serum. The degree of potency of sCD14 proved to be unexpected; simply culturing cells from knockout mice in FCS for several days was sufficient to restore near normal LPS responses even after these cells were extensively washed in serum-free medium (data not shown). This implies that residual levels of sCD14, originally provided by culture in 5% FCS, were capable of restoring LPS responsiveness to CD14-null macrophages. These data
confirm that circulating sCD14 contributes significantly to macrophage responses to LPS, presumably by enhancing delivery of LPS to membrane-bound CD14 or by directly enabling LPS delivery to the TLRs.

E. coli binding and uptake is not altered in CD14-deficient macrophages

As CD14 can bind whole bacteria as well as its component LPS, we examined the impact of CD14 deficiency on macrophage responses to E. coli. CD14 has been reported to mediate the binding of bacteria to macrophages as well as to function as a phagocytic receptor for Gram-negative organisms, despite its lack of physical connections to the cytoskeleton (24, 25). First, we used flow cytometry to measure binding and uptake of BODIPY-labeled E. coli bioparticles, a commercial preparation of fluorescently labeled heat-killed bacteria. This assay takes advantage of the ability of trypan blue to quench bound extracellular, but not intracellular, fluorescent particles (14, 25). No significant differences in E. coli binding and uptake were observed in CD14-deficient macrophages, as compared with wild type. As shown in Fig. 5, binding was evaluated using 100:1 E. coli per macrophage at 5-, 15-, and 30-min time points. The percentage of binding was similar in CD14-deficient and wild-type macrophages under all conditions. Similar results were obtained using 10:1 E. coli per macrophage (data not shown). No differences were observed when the presence of extracellular organisms was quenched with trypan blue (data not shown). In some experiments, a modest reduction in binding was observed in CD14-null macrophages compared with wild-type cells when bacteria were preincubated in heat-inactivated (complement-deficient) serum (data not shown). However, when data from five individual experiments were analyzed together, this decrease was not statistically significant. Furthermore, bacterial binding was not increased by sCD14 in either wild-type or CD14-null macrophages. Incubation of CD14-null macrophages in medium with 2% wild-type mouse serum containing endogenous sCD14 did not increase bacterial binding, nor did the addition of recombinant sCD14 to either wild-type or null macrophages (data not shown). These findings suggest that CD14 is not essential for bacterial uptake and that, in the absence of CD14, other bacterial binding receptors (e.g., CD11b/CD18, SR-A, Fcγ receptor, or mannose receptor) can compensate.

E. coli outer membranes induce cytokine production in CD14-deficient macrophages

We next examined cytokine production from CD14-deficient peritoneal macrophages stimulated with heat-killed E. coli to determine the significance of the observation that bacterial particles can bind and enter macrophages via alternate receptors to CD14. In contrast to our findings with LPS, CD14-deficient macrophages stimulated with 10 heat-killed E. coli per cell for 6 h elaborated both TNF-α (Fig. 6A) and IL-6 (data not shown) in amounts equivalent to those produced by wild-type peritoneal macrophages. This effect was dose dependent and at bacterial concentrations <10 E. coli per cell, TNF-α production by CD14-null macrophages was significantly reduced compared with wild-type macrophages (Fig. 6B). Although wild-type macrophages produced TNF in response to as little as 0.1 E. coli/macrophage, no cytokine production was observed at <1 E. coli/macrophage in CD14-null macrophages. This result establishes that CD14 can also enhance the sensitivity of macrophage responses to bacterial particles, but that this enhancement is observed over a narrower range than that found with LPS. Although we did not detect cytokine responses to LPS over a dosage range of 0.1–100 ng/ml in our CD14-null animals, Haziot et al. did find some LPS responsiveness in their animals at dosages of 1 μg/ml and higher. The concentration of bacteria that gave half maximal stimulation in CD14-null macrophages was 5 E. coli/macrophage. Using current estimates of the amount of LPS present in the bacterial cell wall of Gram-negative organisms (106 LPS molecules/bacterium), this dose represents ~40 ng/ml of LPS in our experiments. This dose of LPS could not induce cytokine production in CD14-null macrophages, suggesting that other factors, either intrinsic to the bacterium or to the host cell, may contribute to the better preserved response of CD14-null macrophages to whole organisms.

Interestingly, a strain-dependent difference was observed in the amount of TNF-α produced in response to 10:1 heat-killed E. coli. When CD14-null macrophages were taken from animals that were 129Sv/C57BL/6J hybrids and exposed to E. coli, their TNF-α production was intermediate between that produced by macrophages taken from purebred mice of the two parental strains (Fig. 6A).
CD14-null mice used in this experiment had been back-bred two generations to C57BL/6J and thus were 75% C57BL/6J. These data indicate that genetic variation at other murine loci contribute to the macrophage cytokine response to bacterial stimulation and that caution must be used in interpreting in vivo differences using CD14-null mice that have not been back-bred to strain homogeneity.

CD11b/CD18 mediates bacterial signaling in the absence of CD14

The CD11/CD18 family of leukocyte integrins can bind whole Gram-negative bacteria as a result of the recognition of the lipid A moiety in the outer membrane (9, 26). Like CD14, CD11/CD18 integrins have been reported to initiate signal transduction (11–14) and are believed to enable LPS responses via the same downstream signaling molecules as CD14. Unlike CD14, CD11/CD18 integrins do not have a high affinity for monomeric LPS (27), but avidly interact with larger aggregates of LPS, including whole bacteria. In addition to its ability to bind directly to the lipid A moiety of LPS as a constituent of the bacterial membrane, CD11b/CD18 is also a receptor for complement (iC3b) and LBP-coated bacteria (28).

Although all of the β2 integrins appear to be capable of mediating responses to LPS (13), mouse peritoneal macrophages express CD11b/CD18 as the predominant β2 integrin (29). The hookworm protein NIF has been demonstrated previously to inhibit CD11b/CD18 binding to its ligands by interfering with the function of the integrin metal ion-dependent adhesion site domain (19). We used NIF to block CD11b/CD18 binding to Gram-negative bacteria to quantitate that portion of the E. coli-induced cytokine response that occurred via this receptor in CD14-null macrophages. In a rosetting assay, NIF pretreatment inhibited binding of iC3b-coated sheep RBC to CD14-null peritoneal macrophages, confirming its ability to inhibit binding to CD11b/CD18 in these cells (data not shown). Pretreatment of CD14-null macrophages with NIF reduced TNF-α production by 50% in response to 5 E. coli per macrophage, a dose that was determined to give half maximal TNF-α production (Fig. 7). In contrast, no difference was observed in TNF-α production by wild-type macrophages treated with NIF (Fig. 7, inset). These data imply that bacterial internalization via CD11b/CD18 accounts for a substantial fraction of the induction of cytokine signaling that occurs in the absence of CD14.

Bacterial internalization is required for induction of cytokine production by CD14-deficient macrophages

Because the concentration of LPS contained in the doses of E. coli bioparticles used in our study is unable to initiate signal transduction in CD14-null macrophages alone, we hypothesized that the

**FIGURE 5.** E. coli binding and uptake is not altered in CD14-deficient macrophages. Binding of heat-inactivated, BODIPY-labeled E. coli K12 bioparticles to CD14 wild-type or -null peritoneal macrophages from F2 littermate mice was assessed by FACS analysis. Fluorescent bioparticles were added to cells (100:1) in media containing autologous serum (+/+ or −/−) for 5, 15, and 30 min at 37°C and removed by washing in PBS; then cell fluorescence was assessed. A gate was set for fluorescence above background (R2) and 10,000 events were recorded for each condition. Data are presented as dot plots with the percentage of positive cells in the upper right corner, and are representative of five separate experiments.
internalization of intact bioparticles was essential for presentation of the inflammatory components of the bacterial membrane to the signaling apparatus. To test whether phagocytosis of E. coli was required for TNF-α production, we pretreated CD14-null and wild-type macrophages with cytochalasin B. Cytochalasin B inhibited TNF-α production by CD14-deficient, but not CD14-intact, macrophages stimulated with 1–5 E. coli per cell for 4 h (Fig. 8). Inhibition of phagocytosis in both wild-type and CD14-null macrophages was confirmed by examining the uptake of fluorescently labeled E. coli bioparticles in cytochalasin B-treated cells (data not shown). These data imply that the initiation of cytokine signaling by alternative bacterial receptors requires internalization of the whole bacterium in the absence of CD14 on the cell surface.

Discussion
The invasion of Gram-negative bacteria and the subsequent widespread activation of the innate immune system has long been presumed to be due to the interactions of bacterial LPS with mammalian cells. The study of LPS biology has been based, in part, upon the assumption that purified preparations of LPS are valid surrogates for Gram-negative bacteria. The identification of CD14 as the binding receptor for LPS implied that CD14 was involved in the pathogenesis of invasive Gram-negative infections. However, the presence of soluble and membrane-bound CD14 complicate the study of other receptors involved in macrophage responses to whole bacteria. Thus, we generated mice in which the entire coding region of the CD14 gene was deleted. This strategy, unlike the gene interruption technique used in the generation of the previous CD14-null mouse, ensures that no CD14 gene product could be made (15). Despite this difference in our gene-targeting strategy, like Haziot et al. (15), we found the phenotype of our CD14-null mouse to be characterized by profound hyporesponsiveness to purified endotoxin. No response of CD14-null macrophages was observed at 100 ng of LPS per milliliter, whereas this concentration gave a maximal response in wild-type cells. Addition of physiologic amounts of sCD14 restored LPS responsiveness to the null macrophages. Additionally, we confirm previous observations that two major downstream effectors of LPS activation, NF-κB translocation and JNK

FIGURE 6. CD14-deficient macrophages elaborate cytokines in response to E. coli stimulation. A, CD14-deficient macrophages stimulated with 10:1 E. coli produce equivalent TNF-α as compared with wild-type macrophages. Macrophages derived from the 129Sv mouse strain are more responsive to E. coli than C57BL/6J macrophages. Peritoneal macrophages derived from wild-type129Sv and C57BL/6J strain or the hybrid CD14-null mouse strain were stimulated with E. coli bioparticles (10:1), and cell culture supernatants were harvested at 2, 4, and 6 h poststimulation. TNF-α in culture supernatants was analyzed by ELISA. Data are expressed as the mean of triplicate samples ± SD. B, TNF-α production by CD14-deficient macrophages stimulated with E. coli bioparticles is dose dependent. To evaluate the effect of bacterial concentration on cytokine production, macrophages obtained from CD14-null and wild-type littermate mice were stimulated with 0.01–10 E. coli bioparticles per cell, and cell supernatants were harvested at 4.5 h poststimulation. TNF-α in culture supernatants was analyzed by ELISA. Data are expressed as the mean of triplicate samples ± SD.

FIGURE 7. Inhibition of CD11b/CD18 reduces TNF-α production by CD14-deficient macrophages. CD14-null or wild-type (inset) peritoneal macrophages were preincubated with 4 μg/ml NIF protein for 15 min before stimulation with E. coli bioparticles (1:1 or 5:1), and NIF was maintained in cultures throughout the experiment. Cell culture supernatants were harvested at 4 h poststimulation and TNF-α was analyzed by ELISA. Data are expressed as the mean of triplicate samples ± SD.
phagolysosome, in the absence of CD14. However, the preservation of CD14-null macrophage cytokine stimulation in response to whole E. coli, despite the failure to phosphorylate JNK, suggests that JNK-independent signal transduction pathways are activated by bacteria that are not engaged by LPS alone. Alternatively, other proinflammatory molecules in E. coli membranes, such as lipoproteins and peptidoglycan, may account for part of the residual responses observed in the CD14-null mice. It is well established that these molecules have immunostimulatory capabilities, (33–40); however, in vitro studies have indicated that, like LPS, activation by these alternative bacterial products is also enabled by CD14 (36, 40–43).

Our hypothesis that E. coli internalized via alternate receptors engage a signaling pathway within the phagolysosome is supported by our finding that inhibition of bacterial phagocytosis in CD14-null macrophages completely abrogates cytokine production. One receptor that mediates bacterial signaling in the absence of CD14 appears to be CD11b/CD18. Using NIF to block this receptor, we show that CD11b/CD18 accounts for approximately half of the CD14-independent TNF-α production. CD11b/CD18 has been shown to selectively modulate LPS-induced gene expression, qualitatively influencing inflammatory responses to bacterial endotoxin (29). As a number of studies have provided evidence for direct interactions between GPI-linked proteins, Fc receptors, and the integrins (44–49), it seems likely that the relationships between phagocytosis receptors and downstream signaling pathways will be complex.

The studies presented in this report suggest that macrophages rely heavily on CD14 to activate their arsenal of host defenses when confronting an early infection characterized by low pathogen number. We demonstrate that at low bacterial inocula (1 E. coli per cell), the inflammatory response is severely compromised in the absence of CD14. One prediction of this work is that animals inoculated with low dosages of pathogens might be especially susceptible to infection in the absence of CD14. For example, Jack et al. observed that LBP-null mice, when infected with small numbers of Salmonella typhimurium, were hypersusceptible to infection and thus succumbed to sepsis (50). In an animal model of human infection where a high inoculum is used, the outcome might be expected to more closely resemble endotoxin challenge. This might explain the contradictory findings of Haziot et al., who observed that CD14 knockout mice were resistant to Gram-negative infection (15) when they used very large numbers of bacteria that are not engaged by LPS alone. Alternatively, other JNK-independent signal transduction pathways are activated by bacteria that are not engaged by LPS alone. Perhaps just as importantly, there is a growing number of diseases that are thought to use the signal transduction pathways by LPS, including asthma, atherosclerosis, arthritis, and inflammatory bowel disease. These diseases should also benefit from the opportunity to use genetically engineered mice that serve as models of those diseases and that can now be rendered null for a critical component of the LPS signal transduction pathway.

References
Bacterial stimulation of CD14-null macrophages


