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The Scavenger Receptor CD36 Downmodulates the Early Inflammatory Response while Enhancing Bacterial Phagocytosis during Pneumococcal Pneumonia

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CD36 is a scavenger receptor that exhibits pleiotropic functions, including adhesion to thrombospondin, inhibition of angiogenesis, transport of long-chain fatty acids, and clearance of apoptotic cells. In addition, it has been implicated in the host immune response because it acts as a coreceptor for TLR2 and plays a role in Staphylococcus aureus infection. However, its role in other Gram-positive bacterial infections is unclear. In this study, using mice deficient in CD36, we sought to examine the role of CD36 in pneumococcal pneumonia, a major cause of morbidity and mortality worldwide. We show that CD36 is expressed on both alveolar macrophages and respiratory epithelial cells. Early in infection, CD36^−/− mice have an exaggerated inflammatory response compared with wild-type littermate controls. In vitro studies using CD36^−/− primary cells confirm the enhanced early inflammation in response to S. pneumoniae and its lipoteichoic acid, demonstrate that S. pneumoniae binds to cells via its phosphocholine residues, and suggest a role for CD36 in reducing inflammation induced by the phosphocholine residues of pneumococcal lipoteichoic acid. Later in infection, although CD36^−/− mice exhibit impaired bacterial clearance, owing to a decreased capacity of CD36^−/− macrophages to phagocyte S. pneumoniae, minor effects on mortality occur, in comparison with those in wild-type littermate control mice. These data show that CD36 contributes to the pulmonary host response during S. pneumoniae infection by virtue of its ability to act as a phagocytic receptor and as a modulator of the early innate immune response. The Journal of Immunology, 2013, 190: 000–000.

S. pneumoniae is the main cause of community-acquired pneumonia worldwide. Despite many scientific efforts, such as the advent of antibiotics and vaccines against pneumococci, there remain invasive and virulent serotypes that are resistant to such therapeutics, stressing the importance of a deeper understanding of the host defense mechanisms involved in pneumococcal pneumonia (1).

Scavenger receptor CD36 mediates diverse biological processes, such as angiogenesis, atherosclerosis, metabolism, and sensory perception (2). The ability of CD36 to bind to a wide variety of ligands, including oxidized low-density lipoprotein (OxLDL), long-chain fatty acids, and apoptotic cells, is reflective of its multiple cellular functions (2). In addition, a number of reports implicate this receptor in innate immunity to bacteria, particularly Gram-positive pathogens. Overexpression of CD36 in nonphagocytic HEK 293 cells confers binding and uptake of Staphylococcus aureus, suggesting a role for CD36 in the phagocytosis of Gram-positive pathogens (3). CD36-deficient macrophages display impaired phagocytosis of S. aureus, and CD36^−/− mice exhibit enhanced mortality compared with their wild-type (WT) counterparts following i.v. S. aureus challenge (3, 4). CD36 also cooperates with other pattern recognition receptors (PRRs), such as members of the TLR family. TLR2 is the major PRR for Gram-positive pathogens and can recognize the cell wall components lipoteichoic acid (LTA) and lipoproteins and thus initiate inflammatory responses (5, 6). CD36 cooperates with a TLR2/6 heterodimer in the recognition of LTA and diacylglycerides (4). The exact mechanism behind this cooperation is debatable, with some authors indicating that the C-terminal domain of CD36 is critical for the internalization of LTA and proinflammatory responses, whereas others demonstrate an exclusive role for the ectodomain of CD36 in ligand recognition and subsequent inflammation (3, 7). CD36 also cooperates with a TLR4/6 heterodimer, but this occurs following sterile inflammation, such as that mediated by OxLDL (8).

Evidence indicates that molecular mimicry exists between the Gram-positive pathogen S. pneumoniae and OxLDL (9). T15 clonospecific natural IgM Abs, which are protective during S. pneumoniae infection (10), bind to the phosphocholine (PC) moiety of S. pneumoniae, a component that is unique to the LTA of this pathogen, but not other Gram-positive bacteria, such as S. aureus (11). Significantly, PC is the major epitope of oxidized phospholipids (OxPL) that binds to CD36 (12). Altogether, these
studies suggest that S. pneumoniae could bind to CD36, possibly via its PC, but no reports conclusively demonstrate this.

Current knowledge regarding the role of CD36 during clinically relevant respiratory tract infections is limited. An exception to this are studies that have addressed the role of CD36 during Mycobacterium tuberculosis infection and come up with contradictory results, with one study suggesting that CD36 deficiency confers resistance to mycobacteria and the other suggesting that CD36 plays no role in this infection (13, 14). In this study, we sought to examine the role of CD36 in the innate immune response to pneumococcal pneumonia.

Materials and Methods

Abs and reagents

S. aureus LTA and S. pneumoniae LTA (R6 strain) were kind gifts from Sonja von Aulock (University of Konstanz, Konstanz, Germany); purified EO6 (15), KLH (isotype control) Ab, and PC-BSA were kindly provided by Christoph Binder (Research Center for Molecular Medicine of the Austrian Academy of Sciences and Medical University of Vienna, Vienna, Austria). CD36 Abs used for FACS, immunohistochemistry (IHC), and blocking experiments were from Millipore (clone 63), Novus Biologicals, and Cayman Chemical (clone JC63.1). CD45-V500 (clone 30-F11) was from BioLegend. As a secondary reagent for CD36 testing by FACS, CD45 (eBioscience) and 50% positive cells at 4°C) were used. Approval before all experimentation was obtained from the Animal Care and Use Committee of the Medical University of Vienna and the Austrian Ministry of Sciences.

Animals

CD36−/− mice have previously been described and were obtained from Bruce Beutler (Scripps Research Institute, La Jolla, CA) via the Mutant Mouse Regional Resources Center (4). For all in vivo experiments, we used age-matched (8–10 wk old, female) CD36−/− and WT littermate controls. Approval before all experimentation was obtained from the Animal Care and Use Committee of the Medical University of Vienna and the Austrian Ministry of Sciences.

Mouse model of pneumococcal pneumonia and determination of bacterial load

S. pneumoniae serotype 3 obtained from American Type Culture Collection (ATCC 6303) were grown for 6 h to midlogarithmic phase at 37°C in Todd–Hewitt broth (Difco), harvested by centrifugation at 4000 rpm for 15 min at 4°C, and washed twice in sterile saline. Bacteria were diluted in sterile saline to obtain an estimated CFU concentration per 50 μl for intranasal inoculation of mice. The true concentration was determined by growing serial 10-fold dilutions on sheep blood agar plates overnight. Mice were lightly anesthetized with isoflurane, and pneumonia was induced by intranasal administration of the bacterial suspension of S. pneumoniae, as described earlier (16, 17). Eight or 44 h post infection, mice were anesthetized with ketamine (Pfizer) and sacrificed. Whole lungs were homogenized at 4°C, using a tissue homogenizer (Biospec Products), after which serial 10-fold dilutions in sterile saline were plated on blood agar plates, left at 37°C, and CFU were counted 16 h later. Bronchoalveolar lavage (BAL) was performed 8 h post infection by exposing the trachea of mice through a midline incision, cannulating it with a sterile 18-gauge Venflon (BD Biosciences), and instilling two 500-μl aliquots of sterile saline. Approximately, 0.9 ml was retrieved per mouse. Total cell numbers were counted using a hemocytometer (Turck chamber); differential cell counts were done on cytospin preparations stained with Giemsa. BAL fluid supernatant was stored at −20°C for cytokine measurements. In some experiments, to assess survival, mice were intranasally infected with S. pneumoniae, and survival was monitored twice a day for 8 d.

Alveolar macrophage and bone marrow–derived macrophage cell isolation

Alveolar macrophages (AMs) were obtained by BAL from healthy WT and CD36−/− mice and resuspended in RPMI 1640 containing 1% penicillin/streptomycin (pen/strep) and 10% FCS and plated at the appropriate density. Bone marrow–derived macrophages (BMDMs) obtained from the tibia and femur of WT and CD36−/− mice were differentiated in RPMI 1640 supplemented with 1% pen/strep, 10% FCS, and 10% L929-conditioned medium for 7 d. Differentiated macrophages were harvested and plated at the appropriate density.

MH-S and MLE-12 cell culture

MH-S cells (ATCC) were cultured in RPMI 1640 containing 1 mM pyruvate, 2 mM l-glutamine, penicillin, streptomycin, and 10% FCS at 37°C, 5% CO2. MLE-12 cells were cultured in HITES medium, which is RPMI 1640 containing 2 mM l-glutamine, penicillin, streptomycin, 2% FCS, 5 mM L-glutamine (Sigma-Aldrich), 10 mM β-estradiol (Sigma-Aldrich), 100 μg/ml transferrin (Sigma-Aldrich), 30 mM (5 pg/ml) sodium selenite (Sigma-Aldrich), at 37°C, 5% CO2.

Isolation of primary lung epithelial cells

Primary lung epithelial cells were isolated as previously described, with some slight modifications (18). Lungs from WT and CD36−/− mice were perfused with 20 ml PBS, injected into the heart ventricle to clear the lungs of blood. Then 3 ml dispase 1 U/ml (Sigma-Aldrich) was instilled into the lung, using an 18-gauge Venflon (BD Biosciences). Immediately thereafter, 0.5 ml low-melting-point agarose (Sigma-Aldrich) was instilled into the lungs; the lungs were removed 2 min post instillation and placed in 1 ml dispase for 45 min at room temperature for enzymatic digestion. Lungs were separated, using forceps, into small pieces in the presence of 7 ml DMEM supplemented with 0.01% DNase I (Sigma-Aldrich) in a 10-cm tissue culture dish. The lung suspension was filtered successively through 70 and 40-μm filters (BD Biosciences). The cell isolate obtained was centrifuged at 1100 rpm for 12 min at 4°C and resuspended in 10 ml DMEM supplemented with 10% FCS, 1% pen/strep. Cells were plated onto 10-cm tissue culture dishes coated with 50 μg rat anti-mouse anti-CD45 (eBioscience) and 50 μg rat anti-mouse anti-CD16/32 (eBioscience), diluted in 5 ml PBS and incubated for 2 h at 37°C in order for attachment of leukocytes, monocytes, and dendritic cells to take place. The unattached cells were collected and centrifuged as described above. The cell isolate was resuspended in HITES medium. Viability was determined by trypan blue exclusion before plating. Cells were plated at 104/ml in HITES media onto 96-well plates that were coated beforehand with 10 μg/ml collagen IV (Sigma-Aldrich) dissolved in glacial acetic acid. Before plating of cells, the collagen-coated plates were washed twice with PBS to remove the acetic acid. The following day, media were removed, and cells were supplemented with fresh HITES media containing the appropriate stimulus.

Cell treatments and cytokine and chemokine ELISAs

Cells were plated at 105/ml in 96-well dishes in RPMI supplemented with 3% heat-inactivated FCS and 1% pen/strep and stimulated with 2 × 106/ml heat-killed S. pneumoniae (ATCC 6303) or 10 μg/ml S. aureus or S. pneumoniae LTA or 1 μg/ml LPS for 6 h. For CD36-blocking experiments, cells were pretreated with blocking Ab (clone JC63.1) or isotype control for 30 min, prior to stimulation with S. pneumoniae (ATCC 6303) for 6 h. For experiments involving PC-BSA, cells were pretreated with PC-BSA or BSA for 1 h, prior to stimulation with S. pneumoniae (ATCC 6303) for 6 h. For experiments studying the effects of EO6 on cytokine synthesis, heat-killed S. pneumoniae (ATCC 6303) or E. coli were incubated with 25 μg/ml EO6 or isotype control Ab for 1 h prior to addition to cells at 2 × 104/ml for 6 h. Following 6 h of culture treatment, supernatants were stored at −20°C until assays were performed. TNF-α, keratinocyte-derived chemokine (KC), and MIP-2 were measured using specific ELISAs (R&D Systems) according to the manufacturers’ instructions. For lung homogenate cytokine measurements, homogenates were diluted 1:2 in lys buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl2, 2 mM CaCl2, 1% Triton X-100, and Pepsatin A, Leupeptin, and Aprotinin (all 20 mg/ml; pH 7.4; Sigma-Aldrich) and incubated at 4°C for 30 min. Homogenates were centrifuged at 1500 × g at 4°C for 15 min, and supernatants were stored at −20°C until assays were performed. The detection limits were 31 pg/ml for TNF-α, 12 pg/ml for KC, and 94 pg/ml for MIP-2.

Phagocytosis and binding assays

AMs from WT and CD36−/− mice were plated at 0.5 × 106 per well in 12-well microtiter plates, allowed to adhere, and following washing steps, FITC-labeled heat-killed S. pneumoniae (ATCC 6303) were added in the presence of RPMI 1640 for 1 h (multiplicity of infection (MOI) 10) at 37°C or 4°C (as a negative control), respectively. Cells were treated with proteinase K at 50 μg/ml for 15 min at room temperature to remove adherent but not internalized bacteria and subsequently placed on ice, washed, and analyzed using a FACS Calibur (Becton Dickinson) (19, 20). The phagocytosis index of each sample was calculated: (mean fluorescence × % positive cells at 37°C)/(mean fluorescence × % positive cells at 4°C) − (mean fluorescence × % positive cells unstained). In experiments to determine the
effects of EO6 on bacterial binding. FITC-labeled heat-killed *S. pneumoniae* (ATCC 6303) or *E. coli* were incubated with 25 μg/ml EO6 [T15 idiotype (21)] or isotype control Ab for 1 h prior to addition to cells at 4°C.

**Immunohistochemistry**

Paraffin-embedded lungs from WT and CD36−/− mice were deparaffinized in xylene and ethanol and subjected to Ag retrieval using 10 mM citrate buffer (pH 6.0). Thereafter, endogenous peroxidase activity was blocked with 1.6% H2O2 in TBS. Following washing and blocking steps using 10% goat serum (Vector Laboratories) in TBS, sections were incubated overnight at 4°C with biotinylated CD36 Ab (Novus Biologicals) in TBS/1% BSA. After washing twice with TBS/0.025% Triton, binding was visualized using the VECTASTAIN ABC Kit (Vector Laboratories). Sections were counterstained with hematoxylin, dehydrated, and subjected to microscopy.

**RT-PCR**

RNA isolation and RT-PCR were conducted as previously described (17). CD36 gene-specific primers were 5′-TGGAGCACCTGGTGGATGGTT-3′ (5′ to 3′ sense), 5′-TTTCTACGTGGCCCGGTTC-3′ (5′ to 3′ antisense). Hypoxanthine phosphoribosyltransferase gene-specific primers were 5′-GTTAAGCAGTACAGCCCCAAAATG-3′ (5′ to 3′ sense), 5′-AAATCC-AACAAAGTCTGGCCTGTA-3′ (5′ to 3′ antisense).

**Statistical analysis**

Data are presented as the mean ± SEM. Comparison between groups was assessed using either the Mann–Whitney *t* test or the one-way ANOVA followed by the Tukey or Bonferroni multiple comparisons analysis, where appropriate. Survival data were analyzed by the log rank (Mantel–Cox) test using GraphPad Prism Software. Differences were considered significant if *p* < 0.05.

**Results**

**Pulmonary CD36-expressing cells and the effect of *S. pneumoniae* herein**

Knowing that TLRs, including TLR2 and 4, are expressed on both respiratory epithelial cells and AMs (22–24) and that CD36 is a coreceptor for TLR2 (3, 4, 7), we hypothesized that CD36 would also be expressed on these cell types. In a first attempt using flow cytometry, we found CD36 was expressed on both MH-S and MLE-12 cells (Fig. 1A, 1B), routinely used immortalized AM and alveolar epithelial cell lines (25, 26). IHC for CD36 in lungs of healthy WT and CD36−/− mice verified that CD36 was also expressed on primary respiratory epithelium (Fig. 1C). Strong

![FIGURE 1.](http://www.jimmunol.org/)
CD36 expression in WT lungs and no expression in the lungs of CD36\(^{-/-}\) mice demonstrated specificity for our CD36 Ab. We next set out to determine the effects of \textit{S. pneumoniae} on pulmonary CD36 expression in vivo, as well as on these cell types. Infecting mice with \textit{S. pneumoniae} revealed that pulmonary CD36 transcript levels steadily increased over time, with the highest and most significant increases in CD36 transcription 24 h post infection (Fig. 1D). This response was mimicked when ex vivo isolated AMs were challenged with \textit{S. pneumoniae} (Fig. 1E). No change was noted in CD36 transcription in primary respiratory epithelial cells following \textit{S. pneumoniae} treatment (Fig. 1F). To discover whether \textit{S. pneumoniae} induced changes in CD36 protein levels, we measured CD36 levels via FACS following in vitro challenge of AM and respiratory epithelial cells. We observed a time-dependent upregulation of CD36 surface expression on AMs, with significant increases 24 h and 48 h post \textit{S. pneumoniae} treatment (Fig. 1G–H). Although CD36 was expressed at baseline on primary respiratory epithelial cells, its expression was not significantly changed following \textit{S. pneumoniae} treatment (Fig. 1I, 1J). We therefore hypothesized that the increases observed in pulmonary CD36 transcription following \textit{S. pneumoniae} infection (Fig. 1D) reflected the effects of this pathogen on AMs. Indeed, infecting mice with \textit{S. pneumoniae} for 24 h and 48 h, followed by FACS analysis of BAL cells, revealed that CD36 expression increased on AMs (CD45\(^+\) F4/80\(^+\) CD11c\(^+\) cells) 48 h post infection (Fig. 1K, 1L). Together, these data demonstrate that CD36 is present in AMs and the respiratory epithelium of na"ive mice and that its expression increases in vivo on AMs following \textit{S. pneumoniae} encounter.

Paradoxical effects of CD36 on inflammatory responses following \textit{S. aureus} LTA or \textit{S. pneumoniae} challenge

We next determined the effects of CD36 on \textit{S. pneumoniae}–mediated inflammation in these pulmonary CD36-expressing cell types. Respiratory epithelial cells were pretreated with a CD36-blocking Ab prior to \textit{S. pneumoniae} treatment, and levels of proinflammatory cytokines were evaluated. Blocking CD36 in a dose-dependent manner significantly enhanced \textit{S. pneumoniae}–induced IL-6, KC, and MIP-2 responses by primary respiratory epithelial cells (Fig. 2A). Although this response was significant, the absolute levels of cytokines were < 2-fold enhanced. In contrast, respiratory epithelial cells pretreated with the isotype control Ab displayed similar levels of cytokine release to those cells that were treated with \textit{S. pneumoniae} alone (Fig. 2A). These data suggested that CD36 suppressed \textit{S. pneumoniae}–induced inflammation in respiratory epithelial cells. Having established that 2 \(\mu\)g/ml of CD36-blocking Ab is sufficient to augment \textit{S. pneumoniae}–induced MIP-2 levels in lung epithelial cells (Fig. 2A), we next treated AMs with this dose of blocking or isotype control Ab and evaluated levels of \textit{S. pneumoniae}–induced MIP-2 and TNF-\(\alpha\). Blocking CD36 in AMs led to increased levels of \textit{S. pneumoniae}–induced TNF-\(\alpha\) and MIP-2, compared with the isotype control Ab, demonstrating that the suppressive effects of CD36 on \textit{S. pneumoniae}–induced inflammation were not limited...
to respiratory epithelium (Fig. 2B). We next set out to corroborate these observations, using AMs deficient in CD36. For these experiments, we used AMs from CD36-/-mice, which were shown to be deficient in cell surface expression of CD36 (4), a finding additionally confirmed by us (data not shown). CD36-/- AMs that were challenged with S. pneumoniae produced elevated levels of TNF-α and MIP-2, compared with AMs from WT littermate control mice, providing genetic evidence that CD36 did indeed suppress S. pneumoniae-induced inflammation (Fig. 2C). These findings were rather surprising, as CD36 has been described as a coreceptor for TLR2/TLR6 and important for LTA- and diacylated lipoprotein-induced inflammation (4, 7). Given that a major pathogen-associated molecular pattern of S. pneumoniae is LTA (1, 27), we had hypothesized that CD36-/- cells would exhibit a blunted inflammatory response following S. pneumoniae encounter. To establish whether CD36 exerted paradoxical effects on S. pneumoniae- and LTA-induced inflammation, we isolated AMs from CD36-/- and WT littermate mice, treated these with S. pneumoniae and S. aureus LTA, and subsequently measured levels of TNF-α. CD36-/- AMs exhibited a blunted TNF-α response to S. aureus LTA, compared with WT controls, as previously described (4, 7). Importantly, S. pneumoniae-induced TNF-α levels were higher in CD36-/- AMs compared with WT controls (Fig. 2D). Altogether, our blocking Ab data, along with our data from cells isolated from CD36-/- mice, proved that CD36 exerted suppressive effects on inflammation mediated by S. pneumoniae. Furthermore, these data demonstrated that CD36 could have paradoxical effects on inflammation, depending on the stimulus—i.e., S. aureus LTA versus S. pneumoniae.

Inflammatory role of the PC of S. pneumoniae

The opposite effects of CD36 on S. pneumoniae and S. aureus LTA-mediated inflammation led us to hypothesize that differences in these stimuli could account for these observations. In contrast to S. aureus LTA, S. pneumoniae LTA contains covalently bound PC residues, and PC has previously been shown to bind to CD36 (11, 12). Thus, we considered it possible that in the absence of CD36, the PC of S. pneumoniae LTA would not be able to bind to cells, leading to a decreased capacity for CD36 to scavenge these bacteria, consequently resulting in enhanced inflammation. To first determine whether the PC of S. pneumoniae could indeed bind to cells, we incubated FITC-labeled S. pneumoniae with EO6 Ab, an IgM that binds PC (9), and performed a FACS-based binding assay. To exclude the possibility of opsonization effects via complement, we performed our assays in serum-free media at 4°C. We used a nonspecific IgM raised against KLH (isotype control) for the specificity of the EO6 Ab and additionally used E. coli as a control in our binding experiments, as E. coli does not contain a PC in its cell wall. Thus, blocking PC with use of the EO6 Ab should have no effect on E. coli binding. As shown in Fig. 3A, S. pneumoniae in which the PC epitope was blocked by EO6 exhibited a decreased capacity to bind to cells, compared with the isotype control. This response was specific, as it was not observed with E. coli (Fig. 3A; representative FACS plots shown in Supplemental Fig. 1). Agglutination tests demonstrated that at the concentration of EO6 used in our binding assays, no effect of EO6 on bacterial agglutination was seen (data not shown). These observations suggested that S. pneumoniae could bind to cells via its PC, but did not demonstrate a role for CD36 in this process, particularly because blocking the PC of S. pneumoniae could affect other interactions between cells and S. pneumoniae, such as the platelet-activating factor receptor. We next incubated macrophages from WT and CD36-/- mice with S. pneumoniae and compared bacterial binding to S. pneumoniae whose PC epitopes were blocked by EO6. Again, S. pneumoniae preincubated with EO6 exhibited a decreased binding capacity to WT cells (Fig. 3B; representative FACS plots shown in Supplemental Fig. 1). Significantly, decreased bacterial binding was also observed in

FIGURE 3. S. pneumoniae binds to CD36 via its PC, which suppresses inflammation. (A) WT BMDMs seeded at 1.6 × 10^6/ml were incubated with S. pneumoniae or E. coli (MOI 100) that were pretreated with either isotype (KLH) or EO6 Ab for 2 h at 4°C, and bacterial binding was assessed using FACS. (B) WT or CD36-/- BMDMs seeded at 1.6 × 10^6/ml were incubated with FITC-labeled S. pneumoniae that had been pretreated with EO6 Ab (MOI 100) for 2 h at 4°C, and bacterial binding was assessed using FACS. (C) WT AMs seeded at 10^6/ml were incubated with either EO6 Ab and incubated with 2 × 10^7 CFU/ml heat-killed S. pneumoniae or E. coli for 6 h, and levels of MIP-2 were evaluated in the supernatant. (D) WT or CD36-/- AMs seeded at 10^6/ml were pretreated with either PC-BSA or BSA and incubated with 2 × 10^7 CFU/ml heat-killed S. pneumoniae for 6 h, and levels of TNF-α were evaluated in the supernatant. (E) WT and CD36-/- BMDMs were incubated with heat-killed S. pneumoniae, 10 µg/ml S. pneumoniae LTA (R6 strain), or 1 µg/ml LPS for 6 h, and levels of MIP-2 were evaluated in the supernatant. All data presented are mean ± SEM and representative of duplicate experiments (A, B, E). *p < 0.05 versus isotype (A, C) or S. pneumoniae (B).
CD36<sup>−/−</sup> macrophages, demonstrating that <i>S. pneumoniae</i> did bind to cells in a CD36-dependent manner, possibly via its PC residue (Fig. 3B; representative FACS plots shown in Supplemental Fig. 1). Blocking the PC residues of <i>S. pneumoniae</i> did not result in a further decreased bacterial binding to CD36<sup>−/−</sup> macrophages, suggesting that bacterial binding was dependent on the recognition of PC via CD36. Having established a role for the PC epitopes of <i>S. pneumoniae</i> in bacterial binding to cells, we next examined whether this resulted in altered inflammation. We treated AMs with <i>S. pneumoniae</i> or <i>E. coli</i> that had previously been treated with the PC-blocking Ab EO6 or isotype control, and measured levels of MIP-2 in the supernatant. Elevated MIP-2 levels were observed in AMs that were treated with <i>S. pneumoniae</i> whose PC epitopes were blocked (but not with <i>E. coli</i>), suggesting a role for these epitopes in suppressing <i>S. pneumoniae</i>–induced inflammation (Fig. 3C). To determine the potential role of CD36 in this response, we performed a competition assay and added the CD36 ligand PC-conjugated BSA to WT and CD36<sup>−/−</sup> AMs, treated these cells with <i>S. pneumoniae</i>, and measured levels of TNF-α in the supernatant. In agreement with Fig. 2, CD36<sup>−/−</sup> AMs pretreated with BSA and subsequently stimulated with <i>S. pneumoniae</i> released augmented levels of TNF-α, compared with WT AMs (Fig. 3D). Significantly, WT cells that were pretreated with PC-BSA, thereby blocking potential CD36 binding sites for bacterial PC epitopes, exhibited enhanced inflammation, compared with AMs treated with BSA alone. These data demonstrate that blocking binding sites of CD36, using PC-BSA–enhanced levels of <i>S. pneumoniae</i>, induced inflammation (Fig. 3D). No effect of PC-BSA on pneumococcal-induced inflammation in CD36<sup>−/−</sup> AMs was noted (Fig. 3D). Blocking the binding sites for CD36 using PC-BSA also led to enhanced <i>S. pneumoniae</i>–mediated MIP-2 release, but in this experimental setup the effects of PC-BSA on pneumococcal-induced TNF-α secretion were stronger (data not shown). Thus, blocking the PC residue of <i>S. pneumoniae</i> resulted in augmented levels of inflammation (Fig. 3C), a response mimicked by exogenous administration of PC-BSA prior to pneumococcal encounter in a CD36-dependent manner (Fig 3D). Altogether, these data show that <i>S. pneumoniae</i> binds to cells via its PC residue and suggest a role for this epitope in suppressing <i>S. pneumoniae</i>–mediated inflammation in a CD36-dependent manner. To confirm this hypothesis, we next treated WT and CD36<sup>−/−</sup> BMDMs with <i>S. pneumoniae</i>, <i>S. pneumoniae</i> LTA, and LPS. Challenge of CD36-deficient cells with <i>S. pneumoniae</i> LTA led to enhanced MIP-2 release, mimicking the effect of the whole bacterium (Fig. 3E). We observed no effect of CD36 deficiency on LPS-mediated MIP-2 synthesis. We conclude that covalently bound PC residues of pneumococcal LTA contribute to the suppressive effects of CD36 on <i>S. pneumoniae</i>–induced inflammation.

**CD36 contributes to <i>S. pneumoniae</i>–induced inflammation in vivo**

To determine the contribution of CD36 to the early inflammatory response during pneumococcal pneumonia in vivo, we next intranasally inoculated CD36<sup>−/−</sup> or WT littermate control mice with <i>S. pneumoniae</i> and measured levels of proinflammatory cytokines in the lungs as well as cell influx 8 h post infection. CD36<sup>−/−</sup> mice, compared with WT mice, exhibited elevated pulmonary levels of TNF-α and MIP-2 (Fig 4A, 4B). No differences were observed in KC as well as other cytokines, including IL-1β and IL-6.
IL-6 (Fig. 4C and data not shown). As we had observed, elevated S. pneumoniae induced KC secretion in ex vivo isolated CD36−/− cells, but not in vivo (Fig. 2A versus Fig. 4C); these data show that not all effects of CD36 on S. pneumoniae–mediated inflammation observed in vitro are translatable to the in vivo situation. Augmented TNF-α and MIP-2 levels at this time of infection were independent of pulmonary bacterial load (Fig. 4D and 4E). No significant differences were observed in the influx of neutrophils or macrophages into the lungs between the two strains of mice at this time point, nor were differences noted in chemokine or IL-6 levels in the BAL (Fig. 4F, 4G; and data not shown). Altogether, these data show that CD36 partially suppresses early S. pneumoniae–mediated inflammation in vivo, and corroborates the observed contribution of CD36 to pneumococci-induced inflammation—namely, TNF-α and MIP-2 release in vitro (Fig. 2) —in vivo. Furthermore, the increased levels of TNF-α and MIP-2 observed 8 h post S. pneumoniae infection in CD36−/− mice relative to their WT counterparts are similar to those observed in vitro, that is, < 2-fold enhanced (Fig. 2).

**CD36 contributes to S. pneumoniae clearance but has minor effects on mortality**

To determine the contribution of CD36 to bacterial clearance during pneumococcal pneumonia, we next intranasally inoculated CD36−/− or WT littermate control mice with S. pneumoniae and measured bacterial loads 44 h post infection. CD36−/− mice, compared with WT mice, exhibited elevated pneumococcal burden in their lungs (Fig 5A). This response was reproducible and also seen using lower doses of bacteria (data not shown). As we had observed decreased binding of S. pneumoniae in CD36−/− macrophages (Fig. 3B) and CD36 has been described as an important phagocytic receptor for S. aureus (3), we hypothesized that the enhanced bacterial loads observed late during pneumococcal pneumonia were due to a decreased capacity of CD36−/− AMs to phagocytose pneumococci. Thus, we next incubated WT and CD36−/− AMs with FITC-labeled S. pneumoniae and measured phagocytosis using FACS. CD36−/− AMs did indeed exhibit lower levels of S. pneumoniae phagocytosis compared with WT AMs (Fig. 5B, representative FACS plots are shown in Supplemental Fig. 2). The enhanced bacterial loads and decreased phagocytosis led us to examine the effects of CD36 on mortality during pneumococcal pneumonia. CD36−/− mice, compared with WT controls, exhibited a tendency toward increased mortality (Fig. 5C). As mice in this experiment were given a relatively high dose of pneumococci (10⁵ CFU/ml), we hypothesized that the high dose could overwhelm the ability of CD36 to control phagocytosis in vivo, and so inoculated mice next with a lower dose of pneumococci. At low doses of pneumococci, the first few mice to succumb to infection were CD36−/− mice, but eventually the WT mice died at the same rate, demonstrating again that CD36 had no significant effects on mortality during pneumococcal pneumonia (Fig. 5D).

**Discussion**

In this article, we have examined the contribution of CD36 to the innate immune response during pneumococcal pneumonia. We show that CD36 is expressed on both respiratory epithelial cells and AMs and that S. pneumoniae binds to CD36. Using CD36−/− mice and cells, we demonstrate that this receptor surprisingly suppresses S. pneumoniae–mediated inflammation both in vitro and in vivo and is required for the phagocytosis of this bacterium. In vivo, the requirement of CD36 for phagocytosis translates into increased bacterial burden in the lungs of CD36−/− mice compared with their WT counterparts. Ultimately, however, this receptor has minor effects on mortality, using infectious doses ranging from lethal to nonlethal. In sum, we demonstrate that CD36 has a complex role during pneumococcal pneumonia, as it is important for both the early inflammatory response and in bacterial defense.

The expression of CD36 on lung epithelial cells is somewhat controversial. Some studies report that CD36 is not expressed on lung epithelium (28), whereas others report expression in both primary human respiratory epithelial cells and mouse trachea (29, 30). These differences could be due to differences in both species: that is, mouse versus human and primary versus immortalized cell lines, as the authors of the aforementioned study used human BEAS-2B cell lines, whereas we used primary mouse epithelial cells as well as the immortalized MLE-12 cell line. Our observation of CD36 expression in AMs is, however, consistent with previous studies, which show that CD36 is expressed on both human and mouse AMs (31–33). We demonstrate that following S. pneumoniae challenge in vivo, pulmonary CD36 transcript levels are upregulated. This response can be recapitulated by ex vivo challenge of AMs, but not respiratory epithelial cells, with S.
pneumoniae, suggesting that the enhanced S. pneumoniae-induced CD36 expression observed in vivo reflects the effects of this pathogen on AMs but not on respiratory epithelium. Flow cytometry for CD36 following S. pneumoniae challenge, in vivo and in vitro, confirmed that CD36 expression was upregulated on AMs following pneumococcal challenge. Regardless of the differential effects of S. pneumoniae on CD36 expression in AMs versus respiratory epithelial cells, it plays a similar role in both cell types, suppressing S. pneumoniae–induced inflammation.

The suppressive effects of CD36 on S. pneumoniae–mediated inflammation are surprising. TNF-α release is attenuated in CD36−/− macrophages following challenge with S. aureus LTA, suggesting CD36 is required for inflammatory responses to cell wall components of Gram-positive pathogens (3, 4, 7, 34). Given that LTA is a major component of the cell wall of S. pneumoniae, we would have expected to observe the same phenotype. Further, the requirement of CD36 for S. pneumoniae–induced inflammation would be consistent with the dogma of CD36 acting as a coreceptor for TLR2 and with previous observations from our laboratory of TLR2 being indispensable for the proinflammatory response to S. pneumoniae (16). We used a variety of approaches to demonstrate the suppressive effects of CD36 on S. pneumoniae–induced inflammation, including blocking CD36 with the use of Abs as well as knockout mice and cells. Moreover, we can confirm the requirement of CD36 in S. aureus LTA–mediated inflammation and show that in the same experiment CD36−/− macrophages display opposite responses to S. aureus LTA and S. pneumoniae. As the LTA of these differ with respect to PC content, we would like to suggest that the PC residue of S. pneumoniae LTA is responsible for these divergent effects.

CD36 is a type III transmembrane receptor that consists of two transmembrane domains, an extracellular loop and two short intracellular tails. The extracellular domain contains binding sites for the CD36 ligands, including thrombospondin, a Plasmodium falciparum protein called PfEMP-1, and OxLDL (35). OxLDL binding has been reported at several sites of the protein, including amino acids 155–183, 28–93, and possibly 120–155 (36, 37). Notably, in our Ab-blocking experiments, we used a clone of CD36-blocking Ab, JC63.1, that is directed against one of the OxLDL binding sites of CD36 (38). The PC headgroup of the oxidation products of OxLDL, such as 1-palmitoyl-2-(5′-oxovaleryl)-sn-glycero-3-phosphocholine, binds to CD36 (12). Given that our blocking Ab was directed against the OxLDL binding site and that this site binds to PC, which is found only in LTA of S. pneumoniae but not on other Gram-positive bacteria such as S. aureus (11), these experiments suggested a role for the OxLDL binding epitope of CD36 in suppressing S. pneumoniae–induced inflammation. Indeed, in our binding experiments, we could show that blocking the PC epitopes of S. pneumoniae, using EO6 Abs, displayed lower binding to cells and that this occurred in a CD36-dependent manner, directly implicating the PC of S. pneumoniae in binding to cells through CD36. To our knowledge, this is the first evidence demonstrating this role of PC, expanding on previous studies suggesting that the platelet-activating factor receptor binds to the PC of S. pneumoniae (39). Cells that were challenged with S. pneumoniae in which the PC residues had been blocked also displayed augmented inflammation, similar to those cells in which the OxLDL binding site of CD36 was blocked, implicating a role for S. pneumoniae PC in this phenotype. Experiments using pneumococcal LTA further supported the concept that covalently bound PC epitopes of pneumococcal LTA were mediating the suppressive effects of CD36 on S. pneumoniae–induced inflammation, as pneumococcal LTA mimicked the effects of the whole bacterium. Our experiments support the concept of molecular mimicry between epitopes of OxLDL and S. pneumoniae. Indeed, vaccination of LDLR−/− mice with S. pneumoniae increases circulating levels of OxLDL–specific IgM Abs that are cross reactive with pneumococcal determinants and decreases atherosclerotic lesion formation (9). Furthermore, our results are consistent with effects seen by Greenberg and colleagues (40), who show that different LTA structures have different specificities for scavenger receptor A, depending on their negative charge and distribution.

In vivo, early in infection, we could recapitulate the suppressive effects of CD36 on S. pneumoniae–induced pulmonary inflammation that we observed in vitro. These effects are independent of bacterial load. At later times of infection, CD36−/− mice display enhanced bacterial burden, at both low and high doses of S. pneumoniae. This observation, we believe, is due to the ability of AM-expressed CD36 to act as a phagocytic receptor for this bacterium, a hypothesis supported by our phagocytosis assays. Furthermore, this observation is consistent with previous ones, which describe CD36 as a phagocytic receptor for S. aureus (3). Ultimately, however, although CD36−/− mice tend to exhibit enhanced mortality, at low and high doses of S. pneumoniae this effect is not significant. Of interest, although Stuart and colleagues (3) used an i.v. S. aureus model, 20% of CD36−/− mice died when given encapsulated S. aureus, whereas 100% of WT mice survived. Although this effect was statistically significant, the authors argue that alternative phagocytic receptors such as SRA-I or opsonins could compensate to protect the host from S. aureus–induced lethality. A variety of other phagocytic receptors, including MARCO and SRA-I, have been shown to be beneficial during pneumococcal pneumonia (41, 42). Therefore, we propose that similar to S. aureus, other phagocytic receptors could compensate for effects on mortality. This hypothesis is also consistent with observations of CD36 playing a redundant role with other PRRs during M. tuberculosis infection (13). Alternatively, during pneumococcal pneumonia, an early pulmonary proinflammatory is beneficial (17, 43, 44). Given that CD36−/− mice exhibit enhanced early lung inflammation, compared with their WT counterparts, it is plausible that exaggerated inflammation early during infection in CD36−/− mice compensates for the detrimental effects of CD36 on bacterial clearance, producing a net outcome in which mortality is unchanged between the genotypes of mice. Finally, minor effects of CD36 on mortality could also be explained by an unknown interplay between the multiple ligands of CD36 found in vivo with the PC of S. pneumoniae, or by protective effects of anti-PC IgM during S. pneumoniae infection, that would still be observed in a CD36-deficient situation (10).

Of note, a previous study indicates that the thiazolidinedione ciglitazone reduces bacterial outgrowth during S. pneumoniae pneumonia in mice, while having minor effects on mortality (45). Thiadizolidinediones are well-known peroxisome proliferator–activated receptor γ (PPAR-γ) agonists, which initially showed promise in the treatment of type II diabetes, but their use is currently limited owing to serious cardiovascular side effects (46). Given that CD36 is activated by PPAR-γ (32, 35) and we show that CD36 has an impact on bacterial clearance during pneumonia, while having minor effects on mortality, it will be interesting in the future to examine the effects of next-generation PPAR-γ agonists on the course of pneumonia infection and the role of CD36 herein.

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The scavenger receptor CD36 downmodulates the early inflammatory response while enhancing bacterial phagocytosis during pneumococcal pneumonia

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Fig. S1

A

Unstained  KLH S. pneum.  EO6 S. pneum.  KLH E. coli  EO6 E. coli

B

Unstained  S. pneum. WT  EO6 WT  S. pneum. CD36−/−  EO6 CD36−/−

Supplemental Figure legends

Fig S1. Representative FACS plots showing S. pneumoniae binds to cells via its phosphocholine residues and that binding is decreased in CD36−/− cells.

These FACS plots are representative of the data shown in Fig 3A and B of the manuscript. (A) WT BMDM seeded at 1.6 x 10^6/ml were incubated with S. pneumoniae or E. coli (MOI 100) that were either pre-treated with isotype (KLH) or EO6 antibody for 2h at 4°C and bacterial binding...
was assessed using FACS. (B) WT or CD36<sup>−/−</sup> BMDM seeded at 1.6 x 10<sup>6</sup>/ml were incubated with FITC-labeled <i>S. pneumoniae</i> that had been pre-treated with EO6 antibody (MOI 100) for 2h at 4°C and bacterial binding was assessed using FACS.

**Fig. S2.** Representative FACS plots showing phagocytosis is decreased in CD36<sup>−/−</sup> AM

These FACS plots are representative of the data shown in Fig 5B of the manuscript. WT or CD36<sup>−/−</sup> AM seeded at 1.6 x 10<sup>6</sup>/ml were incubated with FITC labeled <i>S. pneumoniae</i> (MOI 100) and phagocytosis was assessed using FACS. Shown are the 4°C as well as 37°C samples.