Pivotal Involvement of Fcγ Receptor IIA in the Neutralization of Lipopolysaccharide Signaling via a Potent Novel Anti-TLR4 Monoclonal Antibody 15C1*

The mammalian Toll-like receptor (TLR) family has evolved to sense pathogens in the environment and protect the host against infection. TLR4 recognizes lipopolysaccharide (LPS) from Gram-negative bacteria and induces a signaling cascade that, when exaggerated, has been associated with severe sepsis. We have generated a TLR4-specific monoclonal antibody, 15C1, which neutralizes LPS-induced TLR4 activation in a dose-dependent manner. 15C1 potently blocks the effects of LPS on a panel of primary cells and cell lines in vitro. The binding of 15C1 was mapped to an epitope in the second portion of the extracellular region of TLR4, which has been shown previously to be functionally important in the recognition of LPS. Furthermore, we demonstrate a novel mechanism of inhibition, as the effects of 15C1 are partially Fc-dependent, involving the regulatory Fc receptor IIA (CD32A). In addition to introducing 15C1 as a potent clinical candidate for use in the treatment of LPS-mediated indications, our work demonstrates a newly discovered pathway whose manipulation is pivotal in achieving optimal neutralizing benefit.

In mammals, the innate immune system has evolved to principally distinguish between self and non-self and to sense a large spectrum of invading microbes. Recognition of pathogenic microorganisms evokes a proinflammatory response that is essential for the survival of the host organism and does not necessarily require previous exposure to the pathogen in question. Induction of an effective innate immune response requires recognition of conserved microbial ligands such as lipopolysaccharide (LPS), lipoproteins, lipoteichoic acids, flagellin, and CpG DNA. These ligands are collectively referred to as pattern-associated molecular patterns (1). Several receptor types have been shown to participate in the recognition of microbial ligands and are collectively known as pattern recognition receptors. One family that has been the focus of intense research over the past 10 years is the Toll-like receptor (TLR) family, originally identified based on their homology to the Drosophila Toll protein (2) and known to play a key role in the recognition of pathogens across a broad range of species. This family contains at least 10 mammalian homologs collectively recognizing a wide range of pattern-associated molecular patterns (3).

The TLRs have a common structure characterized by a leucine-rich repeat extracellular region and a cytoplasmic region essential for downstream signaling, which is homologous to the cytoplasmic domain of the human interleukin (IL)-1 receptor. This region is known as the Toll/IL-1R (TIR) domain and is the region sharing homology with Drosophila Toll (2, 4).

TLR4 is arguably the best characterized receptor of the mammalian TLR family and has been shown to recognize the major structural component of the cell wall of Gram-negative bacteria, LPS (5–7). Exposure of mammals to LPS as a result of bacterial infection leads to rapid cell activation via TLR4. The signaling pathways known to be involved to date exploit several adaptor proteins, including myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like protein (Mal), TIR-containing adaptor protein (TRIF), and TRIF-related adaptor molecule (TRAM) (8). It appears that the LPS response can be divided into an MyD88/Mal-dependent “early phase” NF-κB activation (characterized by the production of many proinflammatory cytokines such as TNF-α and IL-6) and a TRIF/TRAM-dependent phase characterized by “late phase” NF-κB activation and the interferon regulatory factor 3 (IRF3) activation (characterized by the production of interferon-β) (9). In certain cases, overactivation of the innate immune system via pattern recognition receptors such as TLR4, resulting in the systemic release of high levels of proinflammatory cytokines, is thought to trigger the majority of symptoms associated with acute inflammatory disorders such as septic shock (10–12).

In order for TLR4 to respond effectively to LPS and initiate signal transduction, contribution from the co-receptors myeloid differentiation protein 2 (MD-2) and CD14 is essential (13–15). TLR4, MD-2, and CD14 are expressed on many immune cells, including monocytes, macrophages, and dendritic cells. Innate immune recognition of LPS is initiated by its interaction with the serum protein LPS-binding protein (16), followed by a
rapid transfer to membrane-bound or -soluble CD14. This sequence of events is required for LPS recognition by the TLR4-MD-2 complex and signal transduction (17). MD-2 is a secreted glycoprotein that serves as an extracellular adaptor protein for TLR4 activation and itself is key for ligand recognition by TLR4. MD-2 must be bound to TLR4 on the cell surface for receptor activation to occur. LPS has been shown to directly bind MD-2, subsequently inducing an interaction with the leucine-rich repeats of TLR4 leading to TLR4 aggregation and downstream signal transduction (18). LPS stimulation of TLR4-MD-2 has been described to induce a micro-domain on the cell surface containing an “activation cluster” comprising several cell surface molecules, including CD14, Fcγ receptors (CD16, CD32, CD64), CD55, CD11b/CD18, CD36, and CD81 (19).

In this study we describe the generation of a TLR4 mAb antagonist. The mAb is highly specific for human TLR4 in the presence or absence of MD-2 and potently blocks the effects of LPS stimulation on a variety of isolated cell types and cells in human whole blood. 15C1 was found to bind to a functionally important region of the extracellular region of TLR4 known to be important for LPS recognition and receptor activation. Furthermore, we show that the potency of this mAb is enhanced on cells bearing the regulatory Fcγ receptor CD32A, and we demonstrate a functional contribution of the Fc portion of the mAb. These results suggest a cross-talk between the TLR4 and CD32A signaling pathways. We propose 15C1 as a potent novel inhibitor of TLR4 with potential therapeutic applications in acute inflammatory disorders such as septic shock driven by Gram-negative bacterial infection, and we identify the importance of addressing the role of CD32A to obtain maximal clinical efficacy.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Anti-human TLR2 mAb T2.5 (mouse IgG1 κ), anti-human TLR4 mAb HTA125 (mouse IgG2a κ), and anti-human CD14 mAb 28C5 (mouse IgG1 κ) were described previously (15, 20, 21). T2.5 and HTA125 were purchased from eBioscience. Anti-human TLR4 mAb 15C1 (mouse IgG1 κ) and the anti-human MD-2 mAb 18H10 (mouse IgG2b κ) were generated as described previously (22). Isotype controls were purchased from BD Biosciences; anti-human CD32 (AT10) was generated by modifying the human TLR4 vector by cloning at the unique AgeI and EcoRV restriction sites. Finally to generate MHMHM (i.e. containing mouse (M) TLR4 residues at position 23–291), human (H) TLR4, cloned into the mammalian expression vector pCDNA3.1(−)hygro (Invitrogen), was modified by introducing a novel Hpal site and destroying an existing Hpal site by site-directed mutagenesis (QuickChange™ kit, Stratagene). The N-terminal region of mouse TLR4 was PCR-amplified and used to replace the corresponding human DNA fragment in the Hpal-mutated human TLR4 vector by cloning at the unique NotI and Hpal restriction sites. HHHH (i.e. containing mouse TLR4 residues at position 487–629) was generated by PCR-amplifying the C-terminal region of mouse TLR4, which was used to replace the corresponding human DNA fragment in the human TLR4 vector by cloning at the unique EcoRV and Xhol restriction sites. MMHH (i.e. containing mouse TLR4 residues at position 23–371) was generated by modifying the MHMH construct (site-directed mutagenesis) to introduce a unique Agel restriction site into the TLR4 sequence. In parallel, an internal region of mouse TLR4 was PCR-amplified. This mouse DNA fragment replaced the corresponding human DNA fragment in the Agel-mutated MHHH vector by cloning at the unique Hpal and Agel restriction sites. Finally to generate MMHH (i.e. containing mouse residues at positions 23–291 and 371–487), an internal region of mouse TLR4 was PCR-amplified and used to replace the corresponding human DNA fragment in the Agel-mutated MHHH vector by cloning at the unique Agel and EcoRV restriction sites.

To generate MMHHs and MMHHb, PCR fragments were generated where mouse sequences were introduced in the non-hybridizing region of extended oligonucleotides. These fragments were cloned between the unique Hpal/Agel sites of MMHH. To generate site-specific TLR4 alanine mutants, site-directed mutagenesis with oligonucleotides was performed using the QuickChange™ kit following manufacturer’s guidelines (Stratagene).

**Cloning and Expression of Recombinant Antibody 15C1**—Total RNA extraction from the 15C1 hybridoma cell line and
cDNA synthesis was performed using standard techniques. The heavy (H) and light (L) chain variable regions coding for 15C1 mAb were determined using the PCR-based method described previously (24). The 15C1 L chain variable region was subcloned into the Lonza GS expression vectors containing the human or mouse κ constant region. The 15C1 H chain variable region was subcloned into the Lonza GS expression vectors containing the human γ-4, mouse γ-1, or mouse D265A γ-1 constant region. The genomic versions of the mouse γ-1 and κ constant regions were obtained directly from 15C1 hybridomas cells by nested PCR. The D265A mouse γ-1 mutant was obtained by site-directed mutagenesis using the QuikChange mutagenesis PCR protocol from Stratagene. The full-length 15C1 human IgG4 κ, mouse IgG1 κ, or mouse D265A IgG1 κ antibody was produced by transient transfection of PEAK™ cells. Cells were co-transfected with 0.75 μg of combinations of expression vectors encoding the appropriate full-length L and H chains using FuGENE 6™ transfection reagent (Roche Applied Science). Conditioned supernatants were collected 72 h post-transfection, and recombinant antibody was purified by protein G affinity column chromatography (GE Healthcare).

Transient Transfection of TLR4 and MD-2 in PEAK™ Cells—Cells were transfected with human or human/mouse hybrid TLR4 and/or human MD-2. PEAK™ cells were co-transfected with 0.75 μg of combinations of expression vectors encoding human TLR4-myc (N-terminal c-Myc tag), mouse MD-2-FLAG™ (C-terminal FLAG tag) human MD-2-FLAG™ (C-terminal FLAG tag) and chimeras of human and mouse TLR4-myc (N-terminal c-Myc tag, generated in house) using the Fugen6™ transfection reagent (Roche Applied Science). Cells were analyzed 48 h post-transfection.

Flow Cytometry—For HUVEC and iDC, 5 × 10^6 cells/ml were incubated in phosphate-buffered saline, 1% bovine serum albumin, and 10 μg/ml of the appropriate antibody. Cells were washed and incubated in the same buffer with allopurinol-conjugated goat anti-mouse IgG antibody (1:250 dilution; Molecular Probes). Cells were analyzed using a FACSCalibur® flow cytometer (BD Biosciences) in the FL-4 channel. For iDC, monoclonal mouse anti-human CD14-(R)-phycoerythrin (DAKO) was used, and cells were analyzed in the FL-2 channel. For circulating leukocytes, the appropriate antibodies (10 μg/ml) were added to human whole blood. Following two washes, cells were incubated with secondary antibody (allophycocyanin-conjugated anti-mouse IgG diluted 1:250) containing 100 μg/ml human IgG (Sigma) to prevent Fc-mediated interactions. Red blood cells were lysed, and the remaining cells were washed twice. Cells were analyzed as above. Different leukocyte populations were distinguished on the basis of forward and side light scatter.

HEK 293, HUVEC, and iDC Assays—Cells were plated in 96-well plates at 6 × 10^4 cells/well. HUVEC cells were cultured on gelatin-coated plates (Sigma). The medium was removed on the day of the experiment, and 30 μl of medium containing 5% heat-inactivated fetal calf serum (human serum for HUVEC) was added. mAbs were diluted in 30 μl of basal medium to the appropriate concentration and added to the cells for 1 h at 37 °C. LPS was diluted in 30 μl of medium, added to the cells, and left to incubate for 21 h at 37 °C. IL-6 (iDC) or IL-8 (HEK 293, HUVEC, and iDC) secretion in the culture supernatant was monitored by ELISA (Endogen). LPS concentrations were as follows: 100 ng/ml for HUVEC, 30 ng/ml for HEK 293 cells, and 2 ng/ml for iDC.

Whole Blood Assays—Fresh heparinized blood from healthy volunteers was obtained by venipuncture and diluted 1:2 with RPMI 1640 medium. Blood was plated at 60 μl/well in a 96-well plate and incubated for 15 min at 37 °C, and in some experiments anti-human CD32 (5 μg/ml final) was added in 60 μl of blood for 1 h. mAbs (15C1 or isotype control) were diluted in RPMI 1640 medium (30 μl final volume) and added to the blood. 1 h later, 30 μl of either E. coli K12 LPS (4 ng/ml final), Pam3CSK4 (100 ng/ml final), or heat-inactivated E. coli WT (107 cfu/ml final) was added to the blood and incubated for 6 h (24 h for heat-inactivated E. coli). These concentrations corresponded to the maximal response observed with these agonists. IL-6 levels were measured by ELISA. In some experiments, TNF-α, IL-8, and IP-10 were measured in parallel by ELISA (R & D Systems). All blood samples used in this study were obtained anonymously, and there is no reference to the donors within. Institutional review board approval for informed consent therefore was not required by our institution.

Screening of Healthy Individuals for Their CD32A Genotype at the Histidine or Arginine Polymorphism CD32A (Amino Acid 131)—To genotype the FcyRIIA R131H polymorphism, the method was partly obtained from that described by Carlsson et al. (25). Briefly, genomic DNA was isolated from sodium citrate-anticoagulated peripheral blood (Vacutainer Systems, BD Biosciences) using QIAamp DNA blood kit (Qiagen). The FcyRIIA-specific PCR amplification was carried out using the HotStar Taq DNA polymerase kit (Qiagen). One hundred and fifty nanograms of genomic DNA was added to 50-μl reaction mixtures, containing 1 × PCR buffer, 100 μg/ml bovine serum albumin, 2.75 mM MgCl₂, 0.25 mM of each dNTP, 0.4 mM each of P63 (5′-CAA GCC TCT GGT CAA GGT C) and P52 (5′-GAA GAG CTG CCC ATG CTG) primers, and 5 units of HotStar Taq (Qiagen). PCR conditions were as follows: 1 cycle at 95 °C for 15 min, 55 °C for 5 min, and 72 °C for 5 min. This was followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, ending with an extension step at 72 °C for 10 min. To analyze the polymorphic region, PCR products amplified by primers P63 and P52 were purified by GeneElute PCR Clean-Up kit (Sigma) and sequenced directly with a modified version of primer P13 (5′-AGG CTT CCA CCC CAC TCC TC). DNA sequencing was performed by Fasteris S.A. (Plan-les-Ouates, Switzerland).

All average results are presented as means ± S.D. Two-way analysis of variance computation combined with the Bonferroni test was used to analyze data using Prism version 5 (GraphPad). A probability level of 0.05 was considered significant.

RESULTS

Generation of a Panel of Anti-TLR4-MD-2 mAbs—Sera from BALB/c mice immunized with transfected CHO cells overexpressing TLR4-MD-2 were screened by FACS for TLR4-MD-2-specific titers. Positive mice were “hyper-boosted” intravenously with a soluble TLR4-MD-2 fusion protein and isolated splenocytes subjected to PEG-mediated fusion with the SP2/0
myeloma cell line as described previously (22). Supernatants from resulting hybridoma clones were screened for TLR4-MD-2 specific mAb binding on mock-transfected or TLR4-MD-2-transfected CHO cells. The HTA125 anti-TLR4 mAb was used as a positive control to confirm specificity. Supernatants from clones showing specific activity were evaluated for their ability to neutralize LPS-mediated IL-8 production in TLR4-MD-2-transfected HEK 293 cells (data not shown). Twelve clones showing neutralizing activity were expanded, and antibody was purified from supernatants by protein G chromatography. Specificity for the TLR4-MD-2 complex was determined using TLR4-MD-2-transfected PEAKTM cells as described previously (22). Neutralizing activity of these 12 antibodies was further evaluated in LPS-stimulated human whole blood using IL-6 production as a readout. 7 of the 12 antibodies were found to inhibit LPS in this assay (data not shown). Table 1 summarizes the characteristics of the TLR4-/MD-2 mAbs generated in this study.

**15C1 Binds TLR4 on Transfected and Primary Cells in Vitro**—We chose to further characterize mAb 15C1 due to its highly potent inhibition of LPS in whole blood. 15C1 produces a mouse IgG1 x immunoglobulin. 15C1 binds the TLR4-MD-2 complex on transfected CHO cells in a dose-dependent manner (Fig. 1A) and binds neither the mouse nor rabbit TLR4-MD-2 complexes (data not shown). FACs analysis of transiently transfected PEAKTM cells revealed that like the commercially available TLR4 mAb HTA125, 15C1 binds to TLR4 either in the presence or absence of MD-2 (Fig. 1B). 15C1 also binds to monocytes, granulocytes, iDC, and HUVEC (Fig. 1C).

**The Epitope for 15C1 Lies in a Functionally Important Region of the TLR4 Extracellular Domain**—Human-mouse hybrids were used to determine the region of 15C1 binding to TLR4. As 15C1 does not cross-react with mouse TLR4, expression constructs encoding human-mouse hybrid versions of TLR4 were generated to determine the precise region of human TLR4 containing the epitope recognized by 15C1. The extracellular region of TLR4 was nominally divided into four regions. We then constructed four human-mouse hybrids, MMHH, MMHH, MHHM, and MHHM (Fig. 2A). Each construct was co-transfected into PEAKTM cells along with human MD-2. 15C1 binding was not detected on cells expressing the MMHH construct (Fig. 2B). As cells expressing the MMHH construct were positive for 15C1 binding, we conclude that the epitope is at least partially contained within the 2nd region of human TLR4, corresponding to amino acids 289–375. MMHH was also negative for 15C1 binding. We were unable to draw conclusions from this observation, as MMHH was poorly expressed on the cell surface and was also negative for MD-2 binding, probably suggesting that the hybrid protein was misfolded or conformationally unrepresentative of TLR4. Investigation of the binding of other neutralizing and non-neutralizing mAbs generated in this study using the human-mouse TLR4 hybrids is summarized in Table 2. With one exception, all mAbs showing TLR4-neutralizing activity in human whole blood had epitopes at least partially located in the 2nd region of TLR4 (from the N to the C terminus). All four of a panel of non-neutralizing anti-TLR4 mAbs tested were located in the N-terminal region of TLR4.

MMHHa and MMHHb were constructed to further define the epitope for 15C1 binding (Fig. 2A). 15C1 bound MMHHa but failed to bind MMHHb, suggesting that the epitope was

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**TABLE 1**

Mouse monoclonal antibodies generated to the human TLR4/MD-2 complex

| Specificity | No. of hybridoma clones binding hTLR4-MD-2 CHO | No. of hybridoma clones neutralizing hTLR4-MD-2 HEK 293* | No. of hybridoma clones neutralizing whole blood* |
|------------|-----------------------------------------------|-------------------------------------------------|-----------------------------------------------|
| TLR4       | 106                                           | 6                                               | 4                                             |
| MD-2       | 4                                             | 4                                               | 1                                             |
| TLR4-MD-2  | 2                                             | 2                                               | 2                                             |

a Data are based on an inhibition of greater than 50% of LPS-induced IL-8 production.
b Data are based on an inhibition of greater than 50% of LPS-induced IL-6 production.
located in the C-terminal region of the TLR4 fragment spanning amino acids 289–375.

To determine amino acids involved in 15C1 binding to TLR4, alanine scanning of this region was performed. The human-mouse alignment for this region is shown in Fig. 2C. Twenty “blocks” of 2–4 amino acids were identified where differences were identified. Wherever complete amino acid mismatches occurred within these blocks, the human amino acid was converted to an alanine by site-directed mutagenesis (for example, block one sequence was converted from LDY to ADA). 15C1 binding to these mutants co-transfected with MD-2 in PEAKTM cells was analyzed by FACS. The anti-c-Myc and 18H10 mAbs detect the presence of WT/mutant TLR4 and MD-2 respectively. 15C1 binding to WT or mutant TLR4 is expressed as 15C1 mean fluorescence intensity compared with anti-c-Myc mean fluorescence intensity as determined by FACS. One representative experiment of three. Error bars show ± S.D.

FIGURE 2. The epitope for 15C1 lies in a functionally important region of the TLR4 extracellular domain. A, extracellular region of TLR4 was nominally divided into four regions. Four human-mouse hybrids were generated, MHHH, MMHH, MHHM, and MMHM, where H corresponds to a human fragment of TLR4 (dark gray) and M corresponds to a murine fragment of TLR4 (light gray). B, each construct was co-transfected into PEAKTM cells along with human MD-2. Flow cytometry quantification was performed for 15C1, anti-c-Myc (TLR4), and anti-FLAGTM for MD-2 binding. C, alignment of human and mouse TLR4 amino acid sequences from residues 289 to 375 (human sequence). The 20 regions of disparity identified from the alignment and subjected to alanine scanning are shown as annotated boxes within the human sequence. D, binding of 15C1 to alanine-scanning TLR4 mutants co-transfected in PEAKTM cells with human MD-2 analyzed by FACS. The anti-c-Myc and 18H10 mAbs detect the presence of WT/mutant TLR4 and MD-2 respectively. 15C1 binding to WT or mutant TLR4 is expressed as 15C1 mean fluorescence intensity compared with anti-c-Myc mean fluorescence intensity as determined by FACS. One representative experiment of three. Error bars show ± S.D.
CD32A Signaling Augments TLR4 Inhibition by 15C1

acids located within these blocks are essential for the interaction between 15C1 and human TLR4.

15C1 Potently Inhibits LPS-dependent TLR4 Induction on a Panel of Human Cells—mAb 15C1 was tested at a range of concentrations for inhibition of LPS-induced IL-8 production in TLR4-MD-2 transfected HEK 293 cells along with an irrelevant control mAb and the commercially available anti-human TLR4 mAb HTA 125. In contrast to control mAb and HTA 125, 15C1 showed a dose-dependent inhibition of LPS activity (Fig. 3A, left panel). Complete inhibition with 15C1 at 10 μg/ml was seen over a range of LPS concentrations from 0.1 ng/ml to 1 μg/ml (Fig. 3A, right panel).

In whole blood from two healthy human donors, 15C1 showed potent inhibition of LPS-induced IL-6 production, with almost complete inhibition being seen to concentrations of mAb at 100 ng/ml and below. An anti-CD14 neutralizing mAb (28C5) (26) was included in the experiment as a control for LPS inhibition. The control anti-TLR4 mAb HTA125 showed no inhibition of LPS under the conditions tested. An identical profile of inhibition was seen when measuring TNF-α (data not shown).

Both 15C1 and 28C5 blocked LPS-induced activation of HUVEC in a dose-dependent manner as measured by IL-8 production (Fig. 3C). In blood-derived iDC, LPS-induced IL-6 production was potently inhibited by 15C1 at all concentrations tested, from 10 to 0.1 μg/ml, whereas 28C5 showed no substantial blocking activity (Fig. 3D). Neither HUVEC (Fig. 1C) nor the iDC population (data not shown) used in this study express detectable levels of CD14 on their cell surface. In this case, it is probable that serum-derived soluble CD14 along with LPS-binding protein facilitates LPS recognition by TLR4-MD-2. The difference in activity of 28C5 between the two cell types tested can be explained by the fact that for HUVEC experiments human serum was used, and with iDC bovine serum was used. 28C5 is therefore blocking the effects of soluble human CD14 on LPS-mediated HUVEC activation, whereas it does not block the effects of soluble bovine CD14 LPS-mediated iDC activation, as it does not cross-react with this protein (23).

LPS activation of TLR4 can induce two different pathways based on activation of the adaptors MyD88 and TRIF. Activation of the MyD88 pathway typically results in the production of cytokines such as TNF-α, IL-6, and IL-8. On the other hand, activation of the TRIF pathway is typically characterized by the induction of type I interferons (α or β) and downstream chemokines such as IP-10. Therefore, to demonstrate inhibition of the TRIF pathway, we looked at levels of the interferon-inducible protein IP-10 following E. coli-induced TLR4 activation in a human whole blood experiment. Both 15C1 and the anti-CD14 mAb 28C5 efficiently inhibited IP-10 production compared with control IgG1 mAb (Fig. 3E).

15C1 Specifically Inhibits TLR4-dependent Activation of Human Whole Blood—To determine whether 15C1 showed specificity in its inhibition of TLR4, whole blood experiments were performed with both TLR4- and TLR2-specific ligands (LPS and Pam3CSK4, respectively). The anti-CD14 neutralizing mAb 28C5 (26), which blocks both TLR4 and TLR2 activation, was included along with the TLR2-specific neutralizing mAb T2.5 (20). Using LPS as the activating ligand, IL-6 produc-

![FIGURE 3. 15C1 potently inhibits LPS-dependent TLR4 induction on a panel of human cells. A, dose-dependent inhibition of mAb 15C1, by LPS (30 ng/ml)-induced IL-8 production in TLR4-MD-2 transfected HEK 293 cells along with an irrelevant control mAb, and the commercially available anti-human TLR4 mAb HTA 125 (left panel) and inhibition with 15C1 at 10 μg/ml over a range of LPS concentrations from 0.1 ng/ml to 1 μg/ml (right panel). One representative experiment of three. B, dose-dependent inhibition of mAb 15C1, by LPS-induced IL-6 production in whole blood assay of two healthy donors (left and right panels), along with an irrelevant control mAb, the anti-human TLR4 mAb HTA125, and the human anti-CD14 (28C5). Squares, isotype control; triangles, HTA125; circles, anti-CD14; diamonds, 15C1. n = 2. Error bars show ± S.D. C, dose-dependent inhibition of mAb 15C1, by LPS-induced IL-8 production in HUVEC cells along with an irrelevant control mAb, and the human anti-CD14 (28C5). One representative experiment of three. n = 2. Statistical comparison between antibody treatment and corresponding dose of isotype control. ***p < 0.001; *, p < 0.05; ns, not significant. Error bars show ± S.D. D, dose-dependent inhibition of mAb 15C1, by LPS-induced IL-6 production in blood-derived iDC. Checkered, 10 ng/ml; hatched bars, 1 μg/ml; filled bars, 0.1 μg/ml. One representative experiment of three. n = 2. Statistical comparison between antibody treatment and corresponding dose of isotype control. ***p < 0.001; **, p < 0.01; ns, not significant. Error bars show ± S.D. E, inhibition of mAb 15C1 by E. coli WT-induced IP-10 production on whole blood assay (24 h stimulation and 10⁷ cfu/ml). One representative experiment of three. n = 2. Statistical comparison between antibody treatment and corresponding dose of isotype control. ***p < 0.001. Error bars show ± S.D.
15C1 Inhibition of TLR4 Activation Is Partially Fc-mediated, through CD32 Binding—The initial selection of cell-based LPS activation assays performed with 15C1 revealed that the antibody showed the strongest dose-dependent inhibitory activity in whole blood and on iDC with inhibition on HUVEC- and HEK 293-transfected cells being noticeably less potent. As FcγR expression is present on many cell types in whole blood and on iDC, but absent on HEK 293 and HUVEC (see Ref. 27 and data not shown), we investigated whether the Fc portion of 15C1 and FcγR binding could be contributing to the inhibition of LPS signaling on such FcγR-bearing cell types.

15C1 Is a Mouse IgG1 Isotype—To reduce 15C1 binding to FcγR, we introduced a point mutation into the Fc region at amino acid 265 (D265A). This amino acid is known to be critical for the interaction between the Fc and the FcγR (28, 29). The mutated form of 15C1 was expressed in PEAK cells in parallel with the recombinant wild-type version, and both mAbs were purified by protein A affinity chromatography. The binding of both mAbs was compared with that of the original mAb derived from the 15C1 by FACS on hTLR4-MD-2-transfected CHO cells. All mAbs were seen to bind TLR4-MD-2 in an equivalent manner, demonstrating the integrity of the mAbs at the level of target binding (data not shown). The wild-type and mutated 15C1 were next compared for their activity to inhibit LPS activation of human whole blood. Fig. 5A shows the wild-type form of the mAb to be significantly more potent at inhibiting the effects of LPS than the Fc-mutated form over an equivalent dose range. This result indicates that Fc receptor binding plays a potential role in the neutralization of LPS-dependent TLR4 signaling by 15C1.

When aggregated, mouse IgG1 is known to have a relatively high binding to human CD32 compared with other human and mouse IgG isotypes (30). To demonstrate a role for CD32 binding in the neutralization of LPS by 15C1, an α-CD32 mAb (AT10), preventing binding of Fc to CD32, was introduced into the whole blood assay. Addition of the α-CD32 mAb dramatically reduced the potency of 15C1 to inhibit LPS activation (Fig. 5B). This observation was extended to other cell types bearing CD32 and responding to LPS, such as the monocyte cell line THP-1 and blood-derived iDC (Fig. 5, C and D, respectively). The α-CD32 mAb, on the other hand, had no significant effect on the activity of 15C1 in inhibiting LPS activation of either TLR4-MD-2-transfected HEK-293 cells (data not shown) or HUVEC (Fig. 5E), neither of which express CD32. The human whole blood assay was also used to demonstrate that the involvement of CD32 in 15C1 inhibition of LPS-specific TLR4 activation also extended to inhibition of the TRIF pathway, as determined by IP-10 secretion (Fig. 5F).

The CD32A Isoform Is Involved in 15C1-mediated Inhibition of TLR4 Activation—The signaling cascade that follows CD32A activation (ITAM) is very different from that which follows CD32B activation (ITIM). CD32A is expressed mainly on monocytes, macrophages, neutrophils, DCs, and platelets, whereas CD32B is principally expressed on B cells. Due to this expression pattern, we suspected an involvement of CD32A rather than CD32B in 15C1 neutralization of TLR4 activation. To confirm this, we first performed whole blood experiments with the blocking anti-CD32 mAb IV.3, which shows specificity for CD32A (31, 32). Fig. 6A shows that IV.3 reduces the potency of 15C1 in a similar fashion to AT10.

CD32A contains a polymorphism (histidine or arginine) in its extracellular domain at amino acid 131. The nature of this polymorphism has an influence on the binding of mouse IgG1 to CD32A, with arginine homozygous individuals having a much higher activity for mouse IgG1 than histidine homozygotes. Arginine/histidine heterozygotes have an intermediate binding capacity (33). We screened healthy individuals for their CD32A genotype at this polymorphism and tested 15C1-mediated blockade of LPS-dependent TLR4 activation in whole blood derived from homozygous and heterozygous individuals. For this experiment, 15C1 was produced in PEAK cells either in its original form (i.e. on a mouse IgG1 backbone) or as a chimeric mAb with the 15C1 variable region on a human IgG4 backbone. This format was chosen as human IgG4 is known to have a very poor activity for CD32 (33). Following protein A affinity chromatography purification, the integrity of both mAbs for TLR4-MD-2 binding on transfected CHO cells was confirmed and shown to be equivalent (data not shown). In whole blood LPS activation experiments, the mlgG1 version of 15C1 was significantly more potent at inhibiting TLR4 in Arg/
Arg and Arg/His donors than in His/His donors. In contrast, the hlgG4 15C1 mAb did not show significant differences in its capacity to inhibit different genotypes (Fig. 6B). Values obtained for each genotype at 156 ng/ml mAb are shown in Table 3. These values highlight the differences in potency between the hlgG1 and hlgG4 constructs for the different CD32 genotype donors. The hlgG1 construct shows a dramatic loss in potency from the Arg/Arg to the His/His donors. In contrast, the mIgG1 construct shows a dramatic loss in potency from the Arg/Arg to the His/His donors.

**DISCUSSION**

We have generated a panel of TLR4-MD-2-specific mAbs with a range of binding specificities for the complex and functional activities in terms of LPS activation of human cells, as summarized in Table 1. Interestingly, two of these mAbs showed agonistic activity on TLR4-MD-2-transfected HEK 293 cells (i.e. cellular activation in the absence of LPS) but did not reproduce this agonistic effect in human whole blood (data not shown). In a similar fashion, we identified several mAbs with significant LPS neutralizing activity on TLR4-MD-2-transfected HEK 293 cells, but were not antagonistic for LPS activity in human whole blood (Table 1), while maintaining their ability to bind to the TLR4-MD-2 complex in whole blood (data not shown). These results would point to a certain level of difference between the way that LPS functions on a TLR4-MD-2-transfected cell line and the way that it is processed and recognized by TLR4-MD-2 in a physiologically complete environment such as human whole blood.

15C1 is a highly potent LPS antagonist that binds TLR4 in the presence or absence of MD-2 on transfected cells, and neutralizes TLR4 activation by a variety of LPS species on a range of human cells and in human whole blood (23). Anti-TLR4 antibodies showing both agonistic and antagonistic properties have been described previously in the literature (15, 34–38). The anti-human TLR4 mAb HTA125 has been described to be capable of neutralizing LPS activity on certain cell types (15, 39). This mAb failed to display a significant neutralizing capacity using conditions under which 15C1 was a potent inhibitor on HEK 293 cells, THP-1 cells, and in human whole blood from several donors (Fig. 3A and B, and data not shown).

We therefore believe that 15C1 is at present a unique reagent in its ability to neutralize LPS-induced human TLR4 stimulation.

Human-mouse hybrid TLR4 variants and alanine-scanning mutagenesis allowed us to identify the precise region of 15C1 binding to TLR4. Interestingly, this region, shown to be highly variable across species (amino acids 287–368 in hTLR4), has been identified as mediating differential recognition of distinct structural variants of certain types of LPS (40). One conclusion from this study suggested that this region of TLR4 either binds LPS directly or contributes to the recognition of LPS through protein-protein interactions with itself or MD-2. We have not seen an effect of 15C1 binding to TLR4 following preincubation of the TLR4-MD-2 complex with LPS or vice versa (data not shown), possibly suggesting that this region itself is not involved in LPS binding to TLR4-MD-2 but rather in subsequent protein-protein interactions. Either way, it seems logical that a mAb targeting this functionally important region of TLR4 has antagonistic properties in terms of LPS activity. This is supported by the fact that 5 of the 6 neutralizing mAbs raised against TLR4 in our laboratory were found to bind to the same region. It is also noteworthy that one of the two functional polymorphisms identified for TLR4 (D299G), associated with endotoxin hyporesponsiveness, lies in this same hypervariable region of TLR4 (41). A recent study on these TLR4 polymor-
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We noted a marked increase in the potency of 15C1 when used to inhibit the effects of LPS on FcγR-bearing cells such as monocytes and iDC. A variant of 15C1 (15C1 AFεR), with an Fc portion having a markedly reduced ability to interact with human FcγRs showed a reduced potency on these cells when compared with wild-type (WT) 15C1. Using specific blocking mAbs and whole blood from polymorphic individuals, we were able to identify a contribution for the regulatory FcγR CD32A in the elevated potency of 15C1 for the inhibition of LPS activity. CD32A is located within the activation cluster induced upon LPS binding to the TLR4-MD-2 complex (19). It therefore seems reasonable that a high local concentration of both TLR4 and CD32A at defined locations on the cell surface following LPS binding would allow 15C1 to interact with both TLR4 via its Fv portion and CD32A via its Fc portion. An increased local concentration of 15C1 in the activation cluster would also result in a significantly increased binding of the Fc region of 15C1 to CD32A via an aggregation effect due to the proximity of these receptors within the cluster. Our model of 15C1 binding in the LPS activation cluster suggests that 15C1 is not only blocking LPS activity via TLR4 binding directly but also by inducing a signaling event downstream of CD32A activation which cross-talks with the TLR4 signaling pathway to inhibit subsequent downstream events, involving both the MyD88/Mal and TRIF/TRAM pathway activations (as demonstrated with IL-6 and IP-10, respectively; see Fig. 7).

We propose that this effect is dependent on the ligand-dependent (i.e. LPS) induction of the activation cluster and a subsequent proximity between TLR4 and CD32A. This is enforced by the fact that 15C1 is unable to inhibit activation of CD32A-expressing cells via the TLR2-specific ligand Pam3CSK4 (Fig. 4) and that TLR2 and TLR4 share overlapping signaling pathways upon activation. This model also supports our notion that 15C1 is not inhibiting TLR4-MD-2 function at the level of LPS binding, as we believe that this initial interaction is essential to induce TLR4/CD32A proximity, but rather at the level of subsequent protein/protein interaction, aggregation, and signal transduction. Further studies will be required to precisely elucidate the mode of action of 15C1 binding to TLR4 in inhibiting LPS activity.

CD32A is a so-called “activating” FcγR, characterized by its presence in its cytoplasmic domain of an ITAM. In contrast, CD32B is an “inhibitory” FcγR, containing a cytoplasmic ITIM (33). The effect of CD32A ligation by the Fc of the anti-TLR4 15C1 mAb is an inhibition of proinflammatory stimulation. Inhibitory effects mediated by FcγRIIA are unexpected because engagement of this receptor typically results in activation signals (43). However, there is precedence for inhibitory cross-talk between TLR and ITAM signaling pathways in the literature. DNAX-associated protein (DAP)-12 is a signaling adaptor that can associate with several different receptors, such as triggering receptor expressed on myeloid cells (TREM)-1 and TREM-2, on the plasma membrane (44). Recent work has demonstrated that mice deficient in DAP12 produced higher levels of inflammatory cytokines in response to TLR ligands and are more susceptible to endotoxic shock compared with wild-type mice. The

![Figure 6. CD32A is involved in 15C1-mediated inhibition of TLR4 activation.](image)

*For comparison of Arg/His and Arg/Arg with His/His polymorphism, p < 0.001 and NS indicates not significant.

**Figure 6.** CD32A is involved in 15C1-mediated inhibition of TLR4 activation. A, addition of either anti-CD32 mAb (AT10) or anti-CD32A mAb (IV.3) diminishes mAb 15C1 inhibition of LPS to a similar extent, as measured by IL-6 production in whole blood. Diamonds, mouse 15C1; triangles, mouse 15C1 + AT10; circles, mouse 15C1 + IV.3; squares, isotype control. One representative experiment of three. Statistical comparison between mouse 15C1 and corresponding dose of mouse 15C1 + anti-CD32. **,** p < 0.001; *, p < 0.05; ns, not significant. n = 2. Error bars show ± S.D.

**Table 3**

Average percentage of inhibition with 156 ng/ml mAb (two donors/group) for dose-dependent inhibition of LPS-dependent TLR4 activation in human whole blood from donors with different CD32A genotypes.

| Donor genotype | MlgG1 15C1 | hlG4 15C1 |
|----------------|------------|-----------|
| Arg/Arg        | 14.0 *     | 70.7 NS   |
| Arg/His        | 27.3 NS    | 71.6 NS   |
| His/His        | 61.9       | 78.1      |

ns, not significant.
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FIGURE 7. Illustration of the proposed mechanism of TLR4 inhibition by 15C1. LPS-induced formation of the activation cluster results in a proximal localization of TLR4 and CD32A on the cell membrane. 15C1 binds TLR4 and inhibits its activation via its Fab. A high local concentration of TLR4 results in mAb aggregation and subsequent engagement of CD32A via the Fc and triggers a signaling cascade, which acts to further inhibit TLR4-dependent cellular activation.

The conclusion of these studies is that certain DAP12-pairing receptors (such as TREM-2) negatively regulate TLR signaling using the conventional ITAM pathway (45–47). Inhibition of TLR signaling via the classical ITAM pathway is speculated to be at two distinct levels. As we have shown in this study, ITAM engagement leads to phosphatidylinositol 3-kinase phosphorylation. This modification leads to the subsequent activation of AKT3, which has been found to inhibit both NF-κB and mitogen-activated protein kinase activity, both of which are implicated in TLR signaling (early and/or late phase). Additionally, ITAM activation could interfere with the interaction between the TLR4 adaptor protein Mal and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) via activation of phospholipase Cγ1, a preferred substrate of PtdIns(4,5)P2. As Mal/PtdIns(4,5)P2 interaction is essential for the recruitment of MyD88 to the TLR signaling complex, activation of phospholipase Cγ1 could proximally inhibit TLR activation by reducing the amount of PtdIns(4,5)P2 available to facilitate recruitment of Mal/MyD88 by TLR. Such a mechanism would have the effect of reducing early phase TLR4 signaling. We also observed that 15C1 inhibited TRIF/TRAM signaling and that this inhibition was potentiated by CD32A activation. The precise mechanism of inhibition of TRIF/TRAM signaling via CD32A engagement is unclear at present based on our current understanding of this particular signaling cascade and shared elements with the MyD88/Mal pathway (8).

Previous studies have also demonstrated a cross-talk between FcγRs and TLRs (48). For example, activation of the novel human osteoclast-associated receptor OSCAR, which associates with and signals via the ITAM-bearing Fcγ chain, was shown to decrease the LPS-induced secretion of certain proinflammatory factors (IL-12p70 and IP-10) while increasing the anti-inflammatory cytokine IL-10 in monocyte-derived human DCs (49). The same receptor up-regulated LPS-induced IL-1β, TNF, and IL-8 in human monocytes when activated with an agonistic mAb (50).

One can speculate as to the physiological relevance of CD32A and ITAM activation in antibody-mediated down-regulation of TLR4 responses. It is plausible that a transition from innate immunity to acquired immunity is beneficial at a given stage of bacterial infection, by down-regulating potentially damaging prolonged acute responses and augmenting antigen-specific responses. The presence of protective natural anti-LPS antibodies in human serum during the course of the antibacterial infection is well documented and is protective (51–53). Binding of IgG-coated LPS or IgG-opsonized Gram-negative bacteria to TLR4-MD-2 could result in a bridging to CD32A (human IgG1, IgG2, and IgG3 all bind CD32 when aggregated (54, 55)), and a subsequent “switching off” of TLR4 signaling. As TLR4 signaling plays a role in germinal center responses, bridging between TLR4 and CD32B on germinal center B cells could trigger ITIM signaling and potentiate antibody responses.

In summary, we describe the characterization of a potent antagonistic anti-TLR4 mAb and reveal a novel Fc-mediated mechanism of action contributing to the functional activity of this mAb via CD32A. Several therapeutic strategies have been attempted for the treatment of acute inflammatory disorders such as sepsis induced by bacterial infection (56). Blockade of TLR4 activation is a promising approach currently being explored using synthetic TLR4 antagonists (57). A therapeutic product exploiting our potent neutralizing anti-TLR4 mAb (such as a humanized version of 15C1 with a modified Fc) to increase CD32A activity offers a potentially promising approach to the treatment of inflammatory diseases involving TLR4 stimulation.

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