Selective Antibody Intervention of Toll-like Receptor 4 Activation through Fc γ Receptor Tethering

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Background: Dysregulated leukocyte activation via Toll-like receptor 4 (TLR4) is central to numerous inflammatory disorders.

Results: A novel mechanism of action involving Fc γ receptor tethering allows anti-TLR4 blocking antibodies to achieve increased potency on inflammatory leukocytes.

Conclusion: This novel mechanism of action allows selective targeting of TLR4 activation during inflammation.

Significance: The data provide a novel mechanism to dampen TLR4-mediated inflammatory disorders.

Inflammation is mediated mainly by leukocytes that express both Toll-like receptor 4 (TLR4) and Fc γ receptors (FcyR). Dysregulated activation of leukocytes via exogenous and endogenous ligands of TLR4 results in a large number of inflammatory disorders that underlie a variety of human diseases. Thus, differentially blocking inflammatory cells while sparing structural cells, which are FcyR-negative, represents an elegant strategy when targeting the underlying causes of human diseases. Here, we report a novel tethering mechanism of the Fv and Fc portions of anti-TLR4 blocking antibodies that achieves increased potency on inflammatory cells. In the presence of ligand (e.g. lipopolysaccharide (LPS)), TLR4 traffics into glycolipoprotein microdomains, forming concentrated protein platforms that include FcyRs. This clustering produces a microenvironment allowing anti-TLR4 antibodies to co-engage TLR4 and FcyRs, increasing their avidity and thus substantially increasing their inhibitory potency. Tethering of antibodies to both TLR4 and FcyRs proves valuable in ameliorating inflammation in vivo. This novel mechanism of action therefore has the potential to enable selective intervention of relevant cell types in TLR4-driven diseases.

TLR4 and FcγRs are part of a broad range of sensors forming the basis of our immune system’s response against infectious pathogens

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6 The abbreviations used are: TLR, Toll-like receptor; FcγR, Fc γ receptor; ITAMi, inhibitory immunoreceptor tyrosine-based activation motif; BAL, bronchoalveolar lavage; CDR, complementarity determining regions; GM1, ganglioside M1.
of inflammatory mediators, and antibody-dependent cellular cytotoxicity (12). The human FcγR family includes FcγRI (CD64), FcγRIIA (CD32A), FcγRIIB (CD32B), FcγRIIC (CD32C), FcγRIIIA (CD16A), and FcγRIIIB (CD16B). Thus, FcγR biology represents a complex effector function system that has evolved to be exploited mainly by cells of the immune system (13).

We previously reported the characterization of a mouse IgG1 anti-human TLR4 antibody, 15C1, that exploits a novel FcγRIIA-binding mechanism (14). In the current study, we demonstrate that a humanized version (of the original mouse antibody) with an engineered human IgG1κ backbone (Hu 15C1) engages both FcγRI and FcγRIIA, but not FcγRIII, increasing its inhibitory potency on inflammatory cells to block TLR4 signaling. The contribution of Fc engagement to the increased effect is dependent on the clustering of TLR4 with FcγRs in lipid rafts following agonist ligation and is independent of signaling through FcγRs. The FcγR contribution also increases the duration of inhibition of TLR4 activity. The benefit of this mechanism of action involving TLR4-FcγR co-engagement is demonstrated in vivo in a murine model of inflammation.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Recombinant antibodies were produced in house using the Lonza CHO-GS mammalian expression system (Lonza Biologics, Slough, UK). Anti-human FcγRIIA mAb IV.3 was purchased from StemCell Technologies. Ultrapure LPS from *Salmonella minnesota* R595 (Re) and Ultrapure LPS from *Escherichia coli* 055:B5 were obtained from List Laboratories. The antibodies used for FRET studies were as follows. Anti- *Escherichia coli* LPS (CD32C), FcγRIIA, CD16A, and retained high affinity binding to FcγRIIB from commercial sources (R&D Systems or MyBioSource). The neonatal Fc receptor (FcRn) was produced in house as described previously (16). FcγRs were diluted in 10 mM acetate buffer at the appropriate pH according to the pH scouting and then coupled to a CM5 or CM4 sensor chip by amine coupling, following the manufacturer’s instructions (GE Healthcare). An 800–1600-reference unit immobilization signal was reached, depending on the ligand. The FcRn was biotinylated in vivo and captured on a streptavidin-coated CM5 chip for orientated immobilization of the ligand (16). Five concentrations of Hu 15C1 were injected, in duplicate and randomly, on immobilized receptors. The concentration range was adapted to the expected *K_*0 for the different ligands. Data were collected at 25 °C in HBS EP buffer (GE Healthcare) at a flow rate of 30 μl/min. A regeneration scouting was performed when necessary, and a 10 mM glycine, pH 3.0, solution was used for FcγRI and FcγRIIA 131R receptors. Because IgG binding to FcRn occurs in a pH-dependent manner, the HBS EP running buffer was adjusted to pH 6.0 for Hu 15C1 dilution and baseline, association, and dissociation steps and to pH 8.0 for regeneration. According to the kinetic profiles, the steady state model was used for low affinity interactions (FcγRIIA and FcγRIIB), whereas the 1:1 Langmuir binding model was applied to higher affinities (FcγRI and FcRn). Data were evaluated using BIAevaluation version 4.1.1 software. A double referencing was applied for analysis to subtract buffer signal drift on coating surface and nonspecific background signal on a reference channel. All experiments were performed in duplicate.

For TLR4 binding, after dilution in 10 mM acetate, pH 4.5, the recombinant receptor was immobilized on a CM5 sensor chip. IgGs were injected at five concentrations starting from 667 nM, randomly and in duplicate. Hu 15C1 was diluted in HBS EP running buffer and injected at 25 °C at a flow rate of 50 μl/min. Regeneration was assessed using a 10 mM glycine, pH 3.0 solution. Data were analyzed, after double subtraction, using the 1:1 Langmuir fitting model on the BIAevaluation software.

**FACS-based Binding Assay**—Antibodies (Mu 15C1 or Hu 15C1) were incubated at different concentrations with CHO cells expressing human TLR4 and MD-2. After a washing step, fluorescent anti-15C1 idiotype was added to the cells. Finally, cells were washed and then acquired on a FACSCalibur (BD Biosciences).

**Human Macrophage Assay with LPS and Nickel**—Monocytes—Monocytes obtained from human blood were cultured with 100 ng/ml M-CSF (Peprotech) for macrophage differentiation. Five days later, the differentiated macrophages were incubated with a dose range of Hu 15C1 together with ultrapure LPS from *S. minnesota* (1, 10, or 100 ng/ml; List Biological Laboratories) or nickel chloride (0.5 mM; Sigma) for 24 h. Supernatants were harvested for IL-6 ELISA (eBioscience).

**U937 Cell Assays**—U937 cells were differentiated with medium containing 25 nM phorbol myristate acetate for 48 h. After differentiation, the cells were pretreated with Hu 15C1 in the presence or absence of 50 μg/ml IVIG and/or 50 μg/ml anti-FcγRIIA antibody IV.3 for 30 min. The cells were subse-
quently incubated with 1 ng/ml ultrapure LPS from *S. minnesota* for 20 h, and IL-6 in the supernatants was quantified using the Quantikine ELISA kit (R&D Systems).

**Confocal Microscopy and FRET Analysis**—THP-1 cells either untreated or treated with ultrapure LPS from *E. coli* (100 ng/ml) in the presence or absence of different concentrations (0.1, 1, or 10 μg/ml) of Hu 15C1 (or 15C1 D265A), for 30 min or 1 h, were rinsed twice in PBS, 0.02% BSA. The cells were fixed and stained with specific detection Fab fragments for TLR4 and FcyRs directly conjugated to fluorophores, prior to imaging on a Carl Zeiss, Inc. LSM510 META confocal microscope (with an Axiovert 200 fluorescent microscope) using a 1.4 numerical aperture ×63 Zeiss objective. The images were analyzed using LSM 2.5 image analysis software (Carl Zeiss, Inc.). For FRET analysis, THP-1 cells were labeled with 100 μl of a mixture of donor Cy3-conjugated Fab and acceptor-conjugated Cy5 Fab against the molecules of interest. FRET was measured using a method described previously (8).

**THP-1 Blue Prolonged Blockade Assay**—THP-1 Blue cells (Invivogen) were pretreated with 10 μg/ml Hu 15C1 or 15C1 D265A for 30 min. Excess antibody was removed by washing cells twice in PBS. The cells were resuspended in fresh medium and incubated for 0, 1, or 24 h prior to stimulation with 5 ng/ml ultrapure LPS from *S. minnesota* for 24 h. NF-κB reporter gene activation, represented by secreted embryonic alkaline phosphatase activity in the cell culture supernatant, was determined by colorimetric analysis using photospectrometry at λ650 nm.

**LPS Lung Instillation**—C57BL/6 mice (female, 8 weeks old) from Charles River Laboratories were injected with the indicated antibodies intravenously. One hour later, mice were anesthetized and instilled intranasally (*i.n.*) with 10 μg of ultrapure LPS from *S. minnesota*. 24 h post-LPS challenge, mice were sacrificed, and bronchoalveolar lavage (BAL) was performed with a volume of 500 μl of PBS/BSA (0.2%). Lumines and ELISA were performed to measure cytokine and antibody concentrations in BAL fluid. Antibody concentration in BAL fluid was measured by an in house sandwich ELISA using anti-5E3 idiotype antibody (DA4).

**RESULTS**

**Fc Engagement Promotes the Potency of Hu 15C1**—We have previously described a mouse anti-human TLR4 mAb, 15C1, with which the observation was made that the engagement of the mouse Fc with human FcRIIA added neutralization benefits (14). To further explore FcyR assistance, the antibody was humanized. Following conventional CDR grafting of 15C1 into a human IgG1 backbone, the Fc portion was altered by inserting N32SS and L328F substitutions within the CH2 domain to eliminate FcyRII binding. The modification, although abolishing this binding, maintained high affinity for FcyRI and resulted in intermediate affinities for FcyRII (Table 1). FcRn binding was unaffected by the Fc engineering (Table 1). The engineered antibody was designated Hu 15C1. Humanization did not modify the binding to human TLR4 because Hu 15C1 and the parental murine antibody (Mu 15C1) demonstrated overlapping binding profiles for human TLR4/MD-2 expressed on CHO cells (Fig. 1A). This maintenance of TLR4 engagement capacity aligned with the results of surface plasmon resonance analysis, in which the dissociation constant (KD) of the parental Mu 15C1 was 3.2 nm, whereas that of Hu 15C1 was 2.1 nm for human TLR4.

When comparing the ability of Hu 15C1 versus the parental Mu 15C1 to inhibit TLR4 activation on human myeloid cells, Hu 15C1 was more potent (Fig. 1B). The IC50 of Hu 15C1 was 6 ng/ml, whereas that of Mu 15C1 was 29 ng/ml. To then investigate whether FcyR engagement played a role in enhancing the activity of Hu 15C1, experiments were designed in which the receptors were blocked individually or in combination. For FcyRI, a polyclonal preparation of human IgG (IVIG), and for FcyRIIA, the antibody, IV.3, were used. Adding both reagents allowed the establishment of baseline TLR4 blockade without FcyR interaction, which resulted in an IC50 of 2200 ng/ml. The occurrence of either FcyR interaction (when IVIG or IV.3 was added) increased the potency of Hu 15C1 ~100-fold, with a slightly larger contribution from FcyRI than FcyRIIA (IC50 of 30 ng/ml in the presence of IVIG alone versus 18 ng/ml in the presence of IV.3 alone) (Fig. 1C). The availability of both FcyRs further increased the potency of Hu 15C1 and restored the IC50 to 6 ng/ml (Fig. 1C), demonstrating the contribution of both FcyRs in enhancing TLR4 inhibition.

**Hu 15C1 Inhibits TLR4 Activation by Interfering with Receptor Dimerization**—Dissecting the mechanism of action, it has been shown that the epitope of 15C1 is made up of at least three sites on human TLR4 (14) between amino acid residues 289 and 367 (Fig. 2A). This stretch is part of the interface between the two TLR4 molecules that interact upon ligand-induced dimerization (17), an event required for TLR4 activation. Thus, we hypothesized that Hu 15C1 interferes with receptor dimerization and therefore would present a non-competitive profile. To evaluate this, the TLR4 agonists, nickel, which directly triggers receptor dimerization (18), and LPS were used. As shown in Fig. 2B, Hu 15C1 inhibited nickel-induced IL-6 production in a dose-dependent manner. When assessing a range of LPS concentrations (i.e. 1–100 ng/ml), overlapping inhibitory profiles were observed (Fig. 2C). The IC50 values for Hu 15C1 were similar when using either nickel or LPS to activate TLR4 (Fig. 2). Taken together, these data demonstrate that the mechanism of action by which Hu 15C1 interferes with TLR4 signaling is ligand type- and concentration-independent, as would be expected for a molecule blocking receptor dimerization.

**Tailoring TLR4 Inhibition**

| Analyte   | FcyRI | FcyRIIA 13I8 | FcyRIIA 13I1 | FcyRIIB | FcyRIIA 15R | FcyRIIB | FcRn       |
|-----------|-------|--------------|--------------|---------|--------------|---------|------------|
| Hu 15C1   | 4.7E–8 | 1.5E–7       | 3.1E–7       | 8.7E–7  | No binding   | No binding | 1.82E–9  |
|           | ± 0.13| ± 0.15       | ± 0.37       | ± 0.38  | No binding   | No binding | ± 0.09    |

The KD values (in μM) represent the average mean ± S.E. from at least three measurements.

**TABLE 1**

Affinity of Hu15C1 to human Fc Receptors

The KD values (in μM) represent the average mean ± S.E. from at least three measurements.
Tailoring TLR4 Inhibition

Blockade of Receptor Dimerization Abrogates Association of TLR4 with Lipid Rafts—Following ligand-induced activation, TLR4 migrates to lipid rafts, where it clusters with several membrane proteins, including FcγRI and FcγRIIA (8, 19). However, it is not clear whether receptor dimerization is required for migration to lipid rafts or whether dimerization occurs postmigration. Using confocal microscopy in conjunction with FRET, we investigated ligand-dependent TLR4 trafficking to lipid rafts and the subsequent association of TLR4 and FcγRs in the presence of varying concentrations of Hu 15C1 (Fig. 3). At 10 μg/ml, Hu 15C1 blocked LPS-induced TLR4 clustering (Fig. 3, A–D). Hu 15C1 at this concentration also blocked the migration of TLR4 to lipid rafts (Fig. 3F), and full inhibition of LPS-stimulated IL-6 secretion was observed (Fig. 3E). The clustering data together with the FRET data suggest that TLR4 clustered in lipid rafts. At 0.1 μg/ml, although only partially inhibiting TLR4 clustering (Fig. 3C) and migration to lipid rafts (Fig. 3F), Hu 15C1 completely abrogated LPS-stimulated IL-6 secretion (Fig. 3E). At 0.01 μg/ml, Hu 15C1 only reduced the migration of TLR4 to lipid rafts by ~30% (Fig. 3F), but this concentration was still sufficient to fully block IL-6 secretion (Fig. 3E). Further investigating the potential of trimeric interactions of the antibody with TLR4 and FcγRs within the lipid rafts, the FcγR-non-binding variant of Hu 15C1, designated 15C1 D265A, which has a point mutation in the Fc portion abrogating interaction with FcγRs (20), was employed. When assessing this variant, at 0.01 and 0.1 μg/ml, again only partial inhibition of TLR4 association with lipid rafts was measured, similar to that observed with Hu 15C1 (Fig. 3F). In contrast, 15C1 D265A was not able to block TLR4 signaling (Fig. 3E). When a higher dose (i.e. 10 μg/ml of 15C1 D265A) was used, both TLR4 association with lipid rafts (Fig. 3F) and TLR4 signaling (Fig. 3E) were completely impaired. These results demonstrate that, depending on the dose, the co-engagement of Hu 15C1 with TLR4 and FcγRs renders the antibody capable of inhibiting TLR4 activation even following ligand-dependent TLR4 migration to lipid rafts. Because both variants of Hu 15C1, which interfere with TLR4 dimerization, abrogate TLR4 association with lipid rafts at higher concentrations, these results suggest that receptor dimerization occurs prior to TLR4 transfer to lipid rafts.

Blockade of TLR4 Dimerization Abrogates Ligand-dependent Association between FcγRs and TLR4 in Lipid Rafts—To further dissect the interplay between FcγRs, TLR4, and lipid rafts on the cell surface, we investigated their association in the presence or absence of LPS. FRET analysis revealed FcγRI to be constitutively associated with lipid rafts, independently of the presence of LPS, Hu 15C1, or 15C1 D265A (Fig. 4A). FcγRI was not associated with TLR4 under resting conditions, and neither Hu 15C1 nor 15C1 D265A induced association between TLR4

FIGURE 1. Manipulating the FcγRI binding capacity of anti-human TLR4 mAb, 15C1, does not affect its binding to human TLR4 but increases its potency in blocking TLR4 activation. A, 15C1 humanization does not affect binding to TLR4. CHO cells expressing human TLR4/MD-2 were used to assess Mu and Hu 15C1 binding to TLR4 by flow cytometry. B, 15C1 on an engineered human IgG1 backbone (Hu 15C1) is more potent than on mouse IgG1 backbone (Mu 15C1) in blocking IL-6 secretion by U937 cells stimulated with LPS (1 ng/ml). C, both FcγRIIA and FcγRI are involved in Hu 15C1-mediated inhibition of LPS (1 ng/ml)-induced IL-6 secretion. IV.3 is an FcγRIIA-specific blocking antibody. IVIG is a polyvalent IgG preparation saturating high affinity FcγRI. The data shown are representative of four independent experiments. An F test was used to compare the fitted curves of different groups. ***, p < 0.001; **, p < 0.01; ns, not significant.

FIGURE 2. 15C1 binds to an epitope involved in TLR4 dimerization and blocks TLR4 activation via different ligands. A, epitope of 15C1 on human TLR4. The area in red depicts the epitope of 15C1 on human TLR4. The area in green represents the MD-2 binding domain. B and C, inhibition by Hu 15C1 of nickel (0.5 mM)-stimulated (B) and LPS (1, 10, and 100 ng/ml)-stimulated (C) IL-6 secretion from human macrophages. Samples were analyzed in triplicate and repeated five(C) or nine(C) independent healthy blood donors. Data are presented as the mean ± S.E. (error bars). An F test was used to compare the fitted curves of different groups. ns, not significant.
and FcγRI. However, association between FcγRI and TLR4 occurred upon LPS stimulation, which was blocked by the addition of Hu 15C1 or 15C1 D265A in a dose-dependent manner (Fig. 4, A and C). FcγRIIA was not constitutively associated with lipid rafts (Fig. 4, B). LPS stimulation induced the association between FcγRIIA, TLR4, and lipid rafts, whereas either antibody alone did not. Interestingly, neither antibody affected LPS-dependent association of FcγRIIA and lipid rafts, yet both antibodies blocked FcγRIIA and TLR4 interaction in a dose-dependent manner with no significant difference between the two antibodies (Fig. 4, B and D). Thus, TLR4 and FcγRs cluster in lipid rafts upon LPS exposure, producing a microenvironment whereby anti-TLR4 antibodies, such as Hu 15C1, co-engage TLR4 and FcγRs.

Next we determined whether engagement by Hu 15C1 stimulates signaling through FcγRs. To this end, THP-1 cells were incubated with Hu 15C1 for 30 or 60 min, and the inhibitory immunoreceptor tyrosine-based activation motif (ITAMi) pathway (21) was investigated. The Fc-engineered antibody Hu 15C1 failed to induce phosphorylation of SHP-1 (Fig. 5). In contrast, the positive control, anti-CD89 (22), stimulated SHP1 phosphorylation at both 30 and 60 min. Thus, activation of the ITAMi pathway (21) is not implicated in the mechanism of action of Hu 15C1.

Co-engagement of TLR4 and FcγRs Enhances the Potency and Prolongs the Inhibitory Duration of Hu 15C1 on FcγR-bearing Cells—As demonstrated in Fig. 1C and Fig. 3E, engagement of FcγRs increased the potency of Hu 15C1 on FcγR-bearing cells. This mechanism of action suggests that Hu 15C1 would have higher potency on FcγR-bearing cells than on FcγR-negative cells. Consistent with this prediction, Hu 15C1 had a much higher potency on HEK293 cells transfected with TLR4/H18528MD-2/H18528FcγRIIA than on those transfected only with TLR4/H18528MD-2 (Fig. 6A). Furthermore, Hu 15C1 and its Fc-null variant, 15C1 D265A, had a similar IC50 on FcγR-negative human umbilical vein endothelial cells (Fig. 6B), confirming the lack of Fc contribution to Hu 15C1 potency on these FcγR-negative cells. The beneficial effect afforded by the novel mechanism of action of Hu 15C1 was further explored. The FcγR-bearing THP-1 Blue reporter cell line was incubated with a dose range of either Hu 15C1 or 15C1 D265A for 30 min. After washing to remove unbound antibody, cells were cultured in fresh medium for various lengths of time before being subjected to LPS stimulation (Fig. 6C). When LPS was added immediately after anti-

![FIGURE 3. Blockade of TLR4 dimerization abrogates LPS-dependent TLR4 clustering in lipid rafts. A–D, Hu 15C1 blocks LPS-induced TLR4 clustering on THP-1 cells. Photomicrographs depict TLR4 expression patterns under resting conditions (A) or post-LPS (100 ng/ml) stimulation alone (B) and in the presence of 0.1 μg/ml (C) or 10 μg/ml (D) of Hu 15C1. E, blockade of LPS-stimulated IL-6 secretion by Hu 15C1 and 15C1 D265A from THP-1 cells. Data shown are representative of two independent experiments, with each point assessed in duplicate. An F test was used to compare different groups. ***, p < 0.001, FRET analysis of LPS-dependent TLR4 association with lipid rafts in the presence of Hu 15C1 (0.01, 0.1, 1, and 10 μg/ml), 15C1 D265A (0.01, 0.1, 1, and 10 μg/ml), or a human IgG1, isotype control (10 μg/ml). Cells were incubated in the presence or absence of LPS (100 ng/ml) and mAbs for 1 h before being subjected to FRET analysis. The assessment of the proximity of TLR4 to GM1 was used as a marker for clustering in lipid rafts, whereas that of TLR4 to MHC class I served as a baseline control to provide values for molecular non-association. Data shown represent the mean ± S.D. (error bars) of four independent experiments. The data were analyzed using Student’s t test. The statistical difference between the LPS group and the LPS + mAb groups is shown on individual bars. No difference was observed between the Hu 15C1 and 15C1 D265A groups at the concentrations tested. ***, p < 0.001; ns, not significant.](image-url)
Tailoring TLR4 Inhibition

Body incubation, a 10 μg/ml concentration of the Fc-null mutant, 15C1 D265A, produced maximal blockade, whereas the effect was reduced at lower concentrations (Fig. 6D). When LPS was added 1 h after removing unbound antibody, the inhibitory effect seen at 10 μg/ml was reduced to less than 40% and reduced to less than 20% when LPS was added at 24 h (Fig. 6D). In contrast, a substantially lower concentration of Hu 15C1 (i.e. 0.1 μg/ml) produced maximal blockade when LPS was added immediately after antibody incubation. The inhibitory effect was maintained at more than 80% when LPS was added 1 h later and more than 70% when LPS was added 24 h after removing unbound antibody. Even at the lowest concentration of Hu 15C1 tested (0.01 μg/ml), inhibition was ~40% when LPS was.
added 24 h after antibody removal. These results demonstrate that the Fc-dependent mechanism of action of Hu 15C1 on FcγR-bearing cells not only enhances its inhibitory potency but also substantially increases the duration of its inhibitory effect.

**FcγR Engagement Also Enhances in Vivo Efficacy**—To explore whether the FcγR-dependent mechanism of action afforded additional benefit in TLR4-driven experimental models of inflammation, the previously described rat anti-mouse TLR4 monoclonal antibody (15), 5E3, was reengineered. To ensure FcγR engagement on mouse cells, the antibody's Fv portion was reconstructed onto a mouse IgG2a, FcγRIIA backbone (5E3 mIgG2a), providing high affinity for mouse FcγRI (13). A D265A variant of 5E3 mIgG2a, which does not bind to any FcγR, was also generated.

In vitro, 5E3 IgG2a was found to be 100-fold more potent than 5E3 D265A in blocking LPS-stimulated TLR4 activation, demonstrating the potential to exploit this mechanism of action involving FcγR co-engagement in a murine system (Fig. 7A).

Next, to investigate beneficial effects of FcγR engagement in vivo, these mAbs were used in a mouse model of LPS-induced acute lung inflammation. Intranasal administration of LPS induced inflammatory cytokines (i.e. IL-6 and TNFα) in the bronchoalveolar lavage (BAL) fluid (Fig. 7, B and C). Both 5E3 mIgG2a and its D265A variant blocked IL-6 and TNFα in a dose-dependent manner. However, 5E3 mIgG2a demonstrated a greater potency in blocking the cytokines as compared with the FcγR-null mutant, 5E3 D265A (Fig. 7, B and C). Significant differences were observed between the two antibodies at all three doses, with 5E3 IgG2a being 10-fold more potent than the D265A mutant. To confirm that the Fc mutation had not affected the ability of the 5E3 variant to reach the alveolar space, antibody concentrations in the BAL fluid were measured by ELISA. At all doses tested, both mAbs (5E3 mIgG2a and the D265A mutant) reached the lung compartment in a dose-dependent manner, and no difference in BAL fluid concentrations was detected (Fig. 7D). These results indicate that the difference in potency between the two 5E3 variants is due to their differential ability to engage FcγRs rather than differences in their in vivo distribution.

**DISCUSSION**

In this study, we have demonstrated that the anti-human TLR4 antibody, Hu 15C1, exploits both FcγRI and FcγRIIA to increase its potency on FcγR-bearing inflammatory cells. This
Tailoring TLR4 Inhibition

**A**

![Graph A](image)

**B**

IL-6 in BAL

**C**

TNFα in BAL

**D**

mAb concentration in BAL

FIGURE 7. Inhibitory effect of anti-mouse TLR4 antibody 5E3 and its Fc variant, 5E3 D265A, on LPS-induced cytokine secretion both in vitro and in vivo. 

A, 5E3 was more potent than its Fc variant 5E3 D265A in inhibiting LPS-induced IL-6 secretion from RAW264.7 cells. RAW264.7 cells were stimulated with 10 ng/ml LPS in the presence of different concentrations of 5E3 or 5E3 D265A, and the levels of IL-6 in the supernatants were measured by ELISA. The results were analyzed using an F test. The data shown represent three independent experiments. **,** \( p < 0.001 \). B and C, 5E3 was more potent than its Fc variant 5E3 D265A in inhibiting LPS-induced IL-6 (B) and TNFα (C) secretion into the BAL fluid in the mouse LPS instillation model. Cytokines in the BAL fluid were measured 24 h after LPS instillation. Data in the figure are shown as the average mean ± S.E. (error bars) (n = 10/group). The data were analyzed with Student’s t test (*, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \)). D, concentrations of 5E3 and 5E3 D265A in BAL fluid from mice administered with 5E3 or 5E3 D265A. Data shown represent three independent experiments.

Contribution from FcγRs required ligand-dependent clustering of TLR4 with FcγRs in lipid rafts (Fig. 8). Lower concentrations of Hu 15C1 (i.e. below 1 μg/ml) were not sufficient to completely prevent LPS-induced clustering of TLR4 with FcγRs. We consider, therefore, that binding to FcγRs in the lipid raft under these conditions increases the local concentration of Hu 15C1 and renders it capable of interacting with incoming TLR4 dimers. Our data suggest that binding of Hu 15C1 to TLR4 in the lipid raft either disrupts the TLR4 dimers or blocks further aggregation among TLR4 dimers and thus blocks TLR4 signaling. The co-engagement of Hu 15C1 to TLR4 and FcγRs in the lipid raft subsequently increases the avidity of Hu 15C1 binding to the cell surface and thus further increases its ability to block TLR4 activation. At higher Hu 15C1 concentrations (i.e. >1 μg/ml), the clustering of TLR4 with FcγRs was totally blocked. Because TLR4 is not in the proximity of FcγRs under these conditions, Hu 15C1 is not able to co-engage TLR4 and FcγRs. Thus, FcγR engagement is not expected to contribute to the inhibitory effect of Hu 15C1 at higher antibody concentrations. The ability of Hu 15C1 to engage FcγRs also enables the antibody to block TLR4 activation for a longer duration. The relatively high FcγRI and FcγRIIA densities on the surface of inflammatory cells compared with TLR4 may allow preferential Hu 15C1 binding at lower antibody concentrations. We therefore speculate that the interaction with FcγRI and possibly FcγRIIA provides another means for Hu 15C1 to remain on cell surfaces when free antibody in the solution is removed, directly translating into a longer period of inhibition.

Beyond increasing the avidity of Hu 15C1 to the cell surface, it is plausible that FcγR engagement could also induce anti-inflammatory signaling cascades capable of down-regulating TLR4 activation, hence increasing the potency of Hu 15C1 on FcγR-bearing cells. Indeed, monovalent ligation of Fc receptors has been demonstrated to induce ITAMi activation, which subsequently induces the phosphorylation of SHP1 and down-regulates proinflammatory responses (21). However, no increase in SHP1 phosphorylation was detected when THP-1 cells were treated with Hu 15C1, either in the presence or absence of LPS. These results are consistent with our hypothesis that increased local receptor concentration and subsequent antibody binding avidity, rather than Fc-induced signaling events, are solely responsible for the increased potency of Hu 15C1 upon FcγR engagement.

The FcγR-dependent mechanism of action was also observed with an anti-mouse TLR4 antibody, 5E3. Because the epitope of 5E3 is also located within a region involved in TLR4 dimerization (15), this finding suggests that the mechanism could be widely applicable to TLR4 blocking antibodies directly interfering with receptor dimerization or even more generally to all anti-TLR4 blocking antibodies. Two other anti-human TLR4 antibodies, H4 and H52, were reported to block TLR4 activation via an FcγR-independent mechanism (23). Nevertheless, high antibody concentrations (10 μg/ml) were used in the above report, which, from our own experience, may mask any potential contribution from FcγR engagement. Indeed, anti-receptor antibodies with mechanisms of action involving beneficial FcγR engagement have previously been reported. For example, FcγRIIB engagement has been reported to enhance the function of an anti-CD40 agonistic antibody targeting cells co-expressing FcγRIIB and CD40 (24). The underlying mechanism was not clarified in the report. We speculate that the anti-CD40 antibody may use a similar mechanism as described here for TLR4 to enhance its effects. Additionally, an Fc contribution was recently demonstrated in the mechanism of action of an anti-CD115 mAb (25).

The clustering of TLR4 upon stimulation has been reported previously (8, 19) and confirmed in this study. In addition to its association with apparent activation partners upon agonist stimulation, TLR4 also clusters in lipid rafts with other proteins not directly associated with the stimuli, such as FcγRs (19). Because FcγRI is constitutively present in lipid rafts, it is anticipated that TLR4 will cluster with FcγRI upon migration of TLR4 to lipid rafts. In contrast, no association between FcγRIIA and lipid rafts or between FcγRIIA and TLR4 was detected under resting conditions, whereas the association was triggered following LPS stimulation. Interestingly, blockade of TLR4 dimerization did not affect LPS-induced association between FcγRIIA and lipid raft structures but totally abrogated the asso-
The fact that TLR4 clusters with FcγRII and FcγRIIa in inflammatory cells at lower concentrations. This suggests that the association between FcγRII and lipid rafts requires LPS but not TLR4. Further studies are necessary in order to elucidate the precise mechanism by which LPS induces FcγRII migration to lipid rafts independently of TLR4.

The fact that TLR4 clusters with FcγRs upon activation suggests that TLR4 activation may provide a platform to facilitate FcγR activation. Indeed, the ability of IgG immune complexes to induce cytokine release in neutrophils and macrophages was lost in TLR4 mutant mice (26), confirming a role for TLR4 in FcγR activation. Furthermore, signaling through FcγRIII was shown to modulate TLR4 signaling pathways in human monocytes (27). Due to the lack of FcγRIII binding by Hu 15C1, potential cross-talk was not tested in the current study. On the other hand, such clustering may also enable FcγRs to modulate innate immune responses, which may play a role in preventing the detrimental effects of uncontrolled inflammation. In support of this, TLR4 responses have been reported to be enhanced in dendritic cells from Fc receptor γ chain-deficient mice (28), and high avidity ligation of FcγRs has been shown to dampen TLR signaling under certain circumstances (29). Both reports demonstrated a negative role for FcγRs in TLR signaling. Although the clustering between TLR4 and FcγRs has been established, it is not clear whether the activation of other TLRs induces similar clustering, and as such, this represents a topic for future investigational work.

It has been established that TLR4 dimerization and clustering in lipid rafts are critical events in TLR4 signaling. However, the relationship between dimerization and clustering has not been well elucidated. LPS-triggered TLR4 dimerization was reported to be only detected in lipid rafts, and such dimerization was abrogated by a lipid raft disruptor (30). On the other hand, artificially forced TLR4 dimerization is by itself sufficient to trigger TLR4 signaling (31, 32). We demonstrate here that blockade of TLR4 dimerization with Hu 15C1 totally abrogates TLR4 clustering in lipid rafts, which disrupts the TLR4 dimer or the further aggregation of TLR4 dimers.

The mechanism of action of Hu 15C1 demonstrates that the association between FcγRIIa and TLR4 in lipid rafts, which disrupts the TLR4 dimer in a lipid raft. This suggests that Hu 15C1 may be able to disrupt established TLR4 dimers. Alternatively, further aggregation of TLR4 in lipid rafts is required after ligand-induced dimerization for maximal TLR4 activation, and Hu 15C1 is able to interfere with this amplification event. Supporting these concepts, we found that Hu 15C1 could efficiently block TLR4 activation when added several hours after ligand exposure.

Dysregulated activation of TLR4 has been implicated in numerous acute inflammatory, chronic inflammatory, and autoimmune disorders, such as infection, asthma, acute lung injury, ischemia/reperfusion injury, inflammatory bowel disease, graft rejection, transplantation, rheumatoid arthritis, and multiple sclerosis (33). Because uncontrolled leukocyte activation plays a pivotal role in the maintenance of chronic inflammation (34), blocking the activation of these cells is considered beneficial to such diseases. On the other hand, TLR4 signaling on epithelial cells has been demonstrated to play a positive role in tissue homeostasis (11). A prolonged total shutdown of TLR4 signaling on epithelial cells may therefore be detrimental to tissue repair. Because inflammatory cells express FcγRs, whereas epithelial cells do not, the ability to engage FcγRs bestows a higher and longer lasting potency upon Hu 15C1 toward inflammatory cells but a lower and shorter lived potency toward epithelial cells. This feature provides the prospect for Hu 15C1 to preferentially block the dysregulated activation of TLR4 on inflammatory cells while sparing some beneficial TLR4 signaling on structural cells. Furthermore, this mechanism highlights events that may occur with other antibodies that target cell surface receptors that traffic to lipid rafts.

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