Recognition of Double-stranded RNA by Human Toll-like Receptor 3 and Downstream Receptor Signaling Requires Multimerization and an Acidic pH*

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Studies involving Toll-like receptor 3 (TLR3)-deficient mice suggest that this receptor binds double-stranded RNA. In the present study, we analyzed ligand/receptor interactions and receptor-proximal events leading to TLR3 activation. The mutagenesis approach showed that certain cysteine residues and glycosylation in TLR3 amino-terminal leucine-rich repeats were necessary for ligand-induced signaling. Furthermore, inactive mutants had a dominant negative effect, suggesting that the signaling module is a multimer. We constructed a chimeric molecule fusing the amino-terminal ectodomain of TLR3 to the transmembrane and carboxyl terminal domains of CD32a containing an immunoreceptor tyrosine-based motif. Expression of TLR3-CD32 in HEK293T cells and the myeloid cell line U937 resulted in surface localization of the receptor, whereas the nonrecombinant molecule was intracellularly localized. The synthetic double-stranded RNAs poly(I-C) and poly(A-U) induced calcium mobilization in a TLR3-CD32 stably transfected U937 clone but not in control cells transfected with other constructs. An anti-TLR3 antibody also induced Ca2+ flux but only when cross-linked by a secondary anti-immunoglobulin antibody, confirming that multimerization by the ligand is a requirement for signaling. The inhibitors of lysosome maturation, bafilomycin and chloroquine, inhibited the poly(I-C)-induced biological response in immune cells, showing that TLR3 interacted with its ligand in acidic subcellular compartments. Furthermore, TLR3-CD32 activation with poly(I-C) was only observed within a narrow pH window (pH 5.7–6.7), whereas anti-TLR3-mediated Ca2+ flux was pH-insensitive. The importance of an acidic pH for TLR3-ligand interaction becomes critical when using oligomeric poly(I-C) (15–40-mers). These observations demonstrate that engagement of TLR3 by poly(I-C) at an acidic pH, probably in early phagosomal or endosomal, induces receptor aggregation leading to signaling.

Mammalian Toll-like receptors (TLRs)7 belong to a family of receptors that recognize pathogen-associated molecular patterns. TLRs play a key role in host defense during pathogen infection by regulating and linking the innate and adaptive immune responses (1–3). TLRs are expressed in dendritic cells (DC), sentinel of the immune system, endowing them with the capacity to sense pathogen-derived products and to alert the immune system (4). Members of the TLR family are also variably expressed on nonhematopoietic cells. TLR-deficient mice and transfected cell lines have been the keys to understanding TLR function. Ligand specificity has been elucidated for most TLRs; thus, TLR2 and TLR4 recognize Gram-positive and Gram-negative bacterial cell wall products, respectively. TLR5 recognizes a structural epitope of bacterial flagellin, and TLR7, TLR8, and TLR9 have been demonstrated to recognize different forms of microbial-derived nucleic acid (5). Host-derived ligands for the TLRs have also been identified; in particular, TLR4 recognizes heat shock proteins and pulmonary surfactant (6), and TLR9 recognizes chromatin-IgG complexes (7).

TLR3 has been extensively characterized to be a receptor for poly(I-C), a synthetic double-stranded RNA (dsRNA) mimic (8); recently, it has been shown to mediate responses to West Nile virus (9) as well as dsRNA derived from the helminth parasite Schistosoma (10). Additionally, host-derived mRNA has recently been shown to activate TLR3, suggesting that activation via TLR3 can occur in a variety of situations (11). Whereas most TLRs recruit myeloid differentiation factor 88 (12), with some variation in the signaling profile mediated by the additional recruitment of Toll/interleukin-1 receptor-containing adapter protein and TRIF-related adaptor molecule, TLR3 recruits only TRIF (13–15). TRIF can activate both NF-κB through TRAF6 and receptor-interacting protein-1 (16) and interferon-regulatory factor 3 through IkB kinase/TANK-binding kinase 1 (17) and phosphoinositide 3-kinase (18). Although the signaling pathway of TLRs is increasingly well characterized, the parameters controlling interactions between the receptors and the ligands remain poorly documented. A recent review (19) summarized the role of individual members of the TLR family or other surface antigens that either physically or functionally interact with TLRs and how the cumulative effects of these interactions instruct the nature and outcome of the immune response to a particular pathogen. For example, TLR2 recognizes specific ligands upon association with TLR1 or TLR6, and this heterodimerization is essential for signaling (20, 21). Equally, in most signaling assays involving recombinant CD4–TLR fusions, the CD4 moiety induces dimerization in order to produce constitutive signaling (22).

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7 The abbreviations used are: TLR, Toll-like receptor; DC, dendritic cell(s); HEK, human embryonic kidney; IL, interleukin; IFN, immunoreceptor tyrosine-based motif; LRR, leucine-rich repeat; Monocyte, monocyte-derived DC(s); PBMC, peripheral blood mononuclear cell(s); TRIF, Toll/interleukin-1 receptor domain-containing adapter protein; dsRNA, double-stranded RNA.
Whereas TLR2 and TLR4 are expressed at the cell surface, TLR7, -8, and -9 traffic between the endoplasmic reticulum and lysosomes (23–25). The recognition of CpG-containing DNA by TLR9 has been shown to occur in lysosomes (26), and optimal interaction was observed at an acidic pH (27). In addition, recognition of single-stranded RNA by TLR7 has been reported to require an acidic compartment (25, 28). In the case of TLR3, which is expressed on myeloid DC as well as on nonhematopoietic cells (29), cell surface expression has been described in both fibroblast and epithelial cells (30), whereas it is reported to be expressed in an intracellular compartment in monocyte-derived dendritic cells (31). Thus, to date, the exact subcellular compartment in which the interaction between TLR3 and its ligand occurs remains unknown.

The construction of chimeric molecules involving different TLRs or between TLR and other molecules (e.g. CD4) has been used mainly to decipher TLR signaling pathways (22, 32, 33). We thus decided to develop novel chimeric molecules to study interactions between TLR3 and its ligands. We constructed a recombinant molecule fusing the extracellular amino-terminal of TLR3 and the transmembrane and intracellular carboxyl terminus of CD32a. CD32a is a transmembrane molecule displaying an immunoreceptor tyrosine-based activation motif (ITAM) allowing recruitment of a cytosolic kinase complex, including phosphoinositide 3-kinase and phospholipase Cγ, leading to calcium mobilization from intracellular stores (34). Calcium flux assays are immediate, simple to perform, and highly sensitive and do not have the classical toxicity problems of molecules or solvent often found in reporter assays. The TLR3-CD32 construction was expressed in the myeloid cell line U937. We observed cell surface expression of the chimera and calcium mobilization upon TLR3 aggregation. Using this model as well as mutants that produced a dominant negative effect, we demonstrate that poly(I-C) mediates TLR3 cross-linking, and we observe a critical role of acidic pH in mediating this activation. Furthermore, we show that physiologically, in vivo immune cells, the TLR3/ligand interaction is dependent on the acidification of a subcellular compartment.

MATERIALS AND METHODS

Isolation of Immune Cells and Cell Culture—Human blood samples were obtained according to institutional guidelines. Peripheral blood mononuclear cells (PBMC) were purified by Ficoll-Hypaque centrifugation. For monocyte-derived DC (MonoDC) generation, monocytes were further purified as low density cells on a 52% Percoll gradient and plated for adherence. Nonadherent cells were removed, and complete RPMI (Invitrogen) supplemented with 200 ng/ml recombinant human granulocyte-macrophage colony-stimulating factor and 10 ng/ml recombinant human IL-4 (Schering-Plough Research Institute, Kenilworth, NJ) was added for 5–6 days (35). Cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Flow Laboratories, Irvine, UK), 2 mM l-glutamine, 100 μg/ml gentamicin (Schering-Plough, Levallois-Perret, France). HEK293T and HEK293 cell lines (ATCC, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium/F-12 with Glutamax (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum.

Activation of PBMC and DC and Detection of Cytokine Secretion—Freshly isolated PBMC and MonoDC were plated at a concentration of 10^6 cells/ml and 5 × 10^6 cells/ml, respectively, and activated with different TLR ligands for 18 h. The following activating factors were used at final concentrations of 10 μg/ml poly(I-C) (InvivoGen, San Diego, CA), 10 μg/ml poly(A-U) (Sigma), 5 μg/ml CpG D19 (synthesized by MWG (Courtaboeuf, France)), 1 or 10 μM R-848 (imidazoquinoline, resiquimod synthesized in our laboratory; Schering-Plough), and 1 μg/ml or 10 ng/ml Escherichia coli lipopolysaccharide (Sigma) for PBMC and MonoDC, respectively. Chloroquine and bafilomycin (Sigma) were added 45 min prior to the addition of activators. After 18 h, supernatants were collected and tested for IP-10/CXCL10 and IL-6 secretion by enzyme-linked immunosorbent assay (BD OptiEIA; BD Biosciences). Results are expressed as a mean of triplicates, and statistical analysis was performed using GraphPad Prism 3 (GraphPad Software, Inc., San Diego, CA).

Generation and Use of Small Poly(I-C) Fragments—Poly(I) (15-, 30-, and 40-mers) and poly(C) (15-, 30-, and 40-mers) (purchased from Ambion (Huntingdon, Cambridgeshire, UK) and Invitrogen) were mixed in equal proportions according to their size for 15 min at 95 °C. Independently, fragmentation of high molecular weight poly(I-C) was performed by metal-induced hydrolysis as recommended by Affymetrix (Affymetrix protocols for eukaryotic target preparation; Affymetrix, High Wycombe, UK). Briefly, 200 μg of poly(I-C) was incubated with 40 μl of 5 × RNA fragmentation buffer (200 mM Tris acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc) (Sigma) in a final volume of 200 μl for 35 min at 90 °C. Fragmented poly(I-C) was then allowed to reach room temperature before purification. The relative sizes of dsRNA fragments were examined on a 2% agarose gel (NuSieve® 3:1; Bio-Whittaker Molecular Applications, Walkersville, ME).

Mutagenesis of Human TLR3—Full-length human TLR3 was subcloned from the TLR3-pUNO plasmid (Invitrogen) into pMET7 (a gift of the DNAX Institute, Schering-Plough Research Institute, Palo Alto, CA), and TLR3-pMET7 was then used for mutagenesis. Generation of the TLR3 mutants was performed using site-directed mutagenesis of residues in the luminal domain of human TLR3. Mutations were inserted using the GeneEditor kit (Invitrogen) using oligonucleotides summarized below. Pairs of oligonucleotides were as follows: C95A, forward (5'-AGGCAGCCAGATTCGCCCAGAAACTTCCCC-3') and reverse (5'-AATTCTTGGCTCGTAATTGGTGTTGG-3'); C122A, forward (5'-GATAAAACTTCTGCTCCGACGATTTGA-3') and reverse (5'-GAAGGGCAAGTTTATCAAGAGGTG-3'); C226A, forward (5'-GAGTTTTTCTCAGGCCCCCTTCACGCAT-3') and reverse (5'-CCCCGCCGGAGCTTCTCAAGGCTCC-3'); C242A, forward (5'-GGCTTACAGAAAGTTGCTGGTTG-3') and reverse (5'-AGCCTTCTCAGGCTCCCGTCTACTTTT-3') and reverse (5'-GCCCTTACAGAAAGTTGCTGGTTG-3') and reverse (5'-AGCCTTCTCAGGCTCCCGTCTACTTTT-3') and reverse (5'-GCCCTTACAGAAAGTTGCTGGTTG-3') and reverse (5'-AGCCTTCTCAGGCTCCCGTCTACTTTT-3'). The inserted restriction site used to identify the mutation is underlined. After mutagenesis, the mutants were entirely sequenced, and no other mutations were detected. Double mutants (CC1A and NN3A) were created by a second round of mutagenesis on selected plasmids.

Transient Expression in HEK293T and U937—HEK293T cells were seeded into 24-well plates to give 50–70% confluence on the day of transfection. Cells were transfected with TLR3-pMET7 by incubating 3 μg of Fugene 6 (Roche Applied Science, Mannheim, Germany) with 1 μg of plasmid in 100 μl of Dulbecco’s modified Eagle’s medium/F-12 for 30 min. The mixture was divided to give a final transfection amount of 250 ng/well. After an 18-h incubation, cells were recovered and harvested for fluorescence-activated cell sorting analysis.

U937 cells were nucleofected using the Nucleofector™ technology (Amaxa Biosystems, Cologne, Germany) with the program T14 and the nucleofector solution V, as recommended by the manufacturer. Briefly,
6–8 × 10^6 cells were washed in PBS and resuspended extemporaneously in 100 μl of supplemented solution V at room temperature, and 6 μg of plasmid was added to the cell suspension and transferred in an appropriate cuvette for nucleoporation impulsion. After 18 h, cells were harvested for assays.

**NF-kB Reporter Assay**—Tissue culture plates (6-well) with HEK293 or HEK293T cells were transiently transfected using Fugene 6 as described above, with 500 ng of NF-kB luciferase reporter plasmid (Invitrogen) together with constant and increasing amounts of the wild type and mutant TLR3 expression vectors, respectively. Controls to test reporter activity of wild type and mutant TLR3 constructions were also tested in HEK293T cells. Cells were transiently transfected with 500 ng of NF-kB luciferase plasmid together with 250 ng of wild type or mutant TLR3 pMET7 vectors. In all transfections, the total DNA amounts were kept constant using empty vector (pMET7). Six hours post-transfection, cells from each transfection were divided into 96-well plates. The following day, cells were stimulated with poly(I-C) at 5 μg/ml for 6 h and analyzed for luciferase activity.

**Luciferase Assay**—Cells were analyzed for firefly luciferase activity using Promega steady-Glo reporter assay reagents as described by the manufacturer (Promega, Charbonnières, France). Luciferase units were measured using a Fusion α detection apparatus (Packard Instrument Co., Meriden, CT). Each experiment was performed in triplicate and was repeated three times; results generally deviated by less than 10% of the mean value.

**Western Blot of TLR3 Constructs**—HEK293T cells were transiently transfected using Fugene 6 as described above. 48 h post-transfection, transfected HEK293T and nontransfected HEK293 cells were lysed in mild lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1% aprotonin, 20 mM NaN3, and 0.3 mM ortho-sodium vanadate. In general, 30 μg of total cellular protein (determined by Bradford assay; Bio-Rad) was used for SDS-NuPAGE and immunoblotting (Invitrogen). After incubation with anti-TLR3 antibody (clone 40C1285; Imgenex, Sorrento Valley, CA), reactive proteins were detected with peroxidase-conjugated anti-mouse secondary antibodies (Jackson Immunoresearch Laboratories, Inc., Soham, Cambridgeshire, UK) and ECL detection apparatus (Packard Instrument Co., Meriden, CT). Each experiment was performed in triplicate and was repeated three times; results generally deviated by less than 10% of the mean value.

**Construction of the TLR3-CD32 Chimera and Cloning into the Expression Vector pMET7**—The luminal domain of human TLR3 was amplified from the TLR3-pUNO plasmid using the primers TLR3-Sall forward (5′-AGCGCTGACATCTAGAGACAGACTTTTGCC-3′) and TLR3-Asnl reverse (5′-TAGCATATAATGGTCAGAGGAGGCTACTGTGC-3′). The transmembrane and intracellular domains of CD32a were amplified from PMBC cDNA using the primers CD32a Sall forward (5′-ATTATAGGGGATCCTTTGGGC-3′) and CD32a Ncol reverse (5′-TAATGCGCGCTGCCGTAACGTTACTCTTGTAG-3′). For the construction, an Asnl restriction site was created between the luminal domain of TLR3 and methionine 215 of CD32a (Fig. 2A), leading to the insertion of a leucine at base pair 2113 of TLR3. The chimeric construction was cloned into the Sall and Ncol sites of the mammalian expression vector pMET7. Oligonucleotides designed for PCR amplification were derived from GenBank™ sequences NM003265 and M31932 for TLR3 and CD32a, respectively. PCR amplification was performed on a PerkinElmer Life Sciences 480 Thermal-Cycler.

**Stable Expression in U937 Cells**—U937 cells were nucleofected with plasmids at a ratio of one copy of pcDNA3.1 (+)/Hygromycin (Invitrogen), which contains an hygromycin resistance gene expressed in mammalian cells as a selective marker, to 10 copies of TLR3-CD32/pMET7, following the procedure described above. 24 h later, Hygromycin B (50 μg/ml; Sigma) was added at 150 μg/ml. After a 4-week culture period of selection, cloning was performed in round bottom plates, at approximately 1 cell/well. Clones were screened by fluorescence-activated cell sorting staining with the goat anti-human TLR3 antibody (R&D Systems, Minneapolis, MN).

**Fluorescence-activated Cell Sorting Analysis**—Cells (10^5/assay) were incubated with 2 μg of anti-TLR3 antibody (goat anti-human TLR3-specific IgG; R&D Systems) or a goat IgG1 control (Sigma). After washes, phytoerythrin-conjugated rabbit anti-goat antibody (Sigma) was added at a final dilution of 1:50. Before intracytoplasmic staining, permeabilization was performed using Cytofix/Cytoperm™ kit (BD Biosciences).

**Calcium Flux Assay**—Cells (10^5/ml) were loaded with Indo I at 3 μg/ml (Molecular Probes, Inc., Eugene, OR), in RPMI plus 10% fetal bovine serum for 40 min at 37 °C, washed twice, and resuspended at 10^7 cells/ml in Hanks’ balanced salt solution (0.44 mM potassium phosphate, 5.37 mM potassium chloride, 0.34 mM dibasic sodium phosphate, 136.89 mM sodium chloride, 5.55 mM d-glucose), 10 mM Hepes. For assays, 10^6 cells were diluted in 2 ml of Hanks’ balanced salt solution, 10 mM Hepes, supplemented with 1.6 mM CaCl_2 (complete Hanks’ balanced salt solution). Measurements were made in a temperature-controlled Deltascan spectrofluorometer (Photon Technology International, South Brunswick, NJ). Injections were performed when cells were completely stabilized in the cuvette at a volume of 20 μl. Results are expressed as a ratio of Indo I emission 405/485 nm (calcium-Indo I/free Indo I ratio). When not specified, the pH was kept at 6.6. Hanks’ balanced salt solution buffer adjusted to different pH in a gradient from 5.5 to 7.5 was prepared in advance by the addition of either HCl or NaOH, and cells were suspended in the buffer immediately before the calcium flux assay.

**Cross-linking and Desensitization Conditions**—Anti-CD32 (FcyRII) antibody (Stem Cell Technologies) was added at a final concentration of 0.5 μg/ml to the cells within the cuvette for 3–5 min. CD32 aggregation was effective by the addition of goat anti-mouse IgG (H + L) F(ab’)2 fragment, (Immunotech, Beckman Coulter, Marseille, France) at 10 μg/ml final concentration. For cells transfected with TLR3-CD32 chimer, either 0.5 μg/ml goat anti-human TLR3-specific IgG (R&D Systems) was added prior to the cross-linking performed with the addition of biotinylated rabbit anti-goat IgG (Dako, Glostrup, Denmark) at 1–10 μg/ml final concentration or mouse anti-TLR3.7 antibody (eBiosciences) at 5 μg/ml final concentration for the first incubation, followed by the addition of 10 μg/ml goat anti-mouse IgG (H + L) F(ab’)2 fragment (Immunotech; Beckman-Coulter). For experiments with the TLR2-CD32 chimera, cross-linking was performed with mouse anti-TLR2.1 (eBiosciences, San Diego, CA) at 2.5 μg/ml followed by the goat anti-mouse IgG.

Desensitization was performed as described above for cross-linking; briefly, a second injection was performed when the ratio 405/485 had retrieved the base line or 3 min after the latest injection, when no calcium flux had been induced.

**RESULTS**

Cysteine Residues and Glycosylation of TLR3 Leucine-rich Repeats Are Necessary for Ligand-induced Signaling—We compared the sequences of the TLR3 leucine-rich repeats in humans, mice, and rats. We noted that the TLR3 molecules contain two conserved pairs of cysteines (Cys^95^ and Cys^122^, Cys^256^ and Cys^242^) in adjacent leucine-rich repeats. The recently published crystal structures of TLR3 suggest an intramolecular disulfide bridge between Cys^95^ and Cys^122^ but do not...
allow us to predict a function for Cys$^{226}$ and Cys$^{242}$ (36, 37). Equally, we identified two conserved potential N-glycosylation (N$^X$(S/T)) sites (Asn$^{196}$ and Asn$^{247}$) that were of particular interest to us, since they were predicted to lie on the same side of the leucine-rich repeat solenoid as the cysteines. Site-specific substitution mutants of all of the four cysteines and these two N-glycosylation sites were produced (Fig. 1E).

Wild type and mutant TLR3 molecules were transfected into TLR3-negative HEK293T cells (Fig. 1A) together with a reporter plasmid containing consensus NF-$\kappa$B sites, which when activated directed the expression of firefly luciferase. Luciferase activity of TLR3 mutants was measured in response to stimulation with $5 \mu$g/ml poly(I-C). Loss of Function TLR3 Mutants Have a Dominant Negative Effect on TLR3 Signaling—Unlike the HEK293T cell line, HEK293 cells constitutively express TLR3 (Fig. 1A) and respond to poly(I-C). We performed experiments using TRIF small interfering RNA, which blocked NF-$\kappa$B signaling when HEK293 cells were transfected with poly(I-C), thereby demonstrating involvement of TLR3 (data not shown). We observed a nearly complete abrogation of poly(I-C)-induced luciferase activity for C95A, C122A, CC1A (C95A/C122A), N247R, and NN3A (N196G/N247R), in response to poly(I-C) at 25 $\mu$g/ml, suggesting that they have lost most of their ligand recognition activity, whereas mutants N196G, C226A, and C242A did not show significant loss of activity.

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activity in cells transfected with all nonfunctional mutants (Fig. 2, A and B), establishing that the loss of function was not due to an absence of expression. This dominant negative effect characteristic of di- or multimerization was confirmed by co-transfection of the mutants with wild type TLR3 at different ratios in TLR3-negative HEK293T cells (Fig. 2, C and D). Finally, nonspecific effects of the dominant negatives were excluded in HEK293 cells by assessing flagellin-mediated NF-κB activity through endogenous TLR5 (38) (data not shown).

These observations strongly suggested that TLR3 multimerization was a requirement for promoter induction and that this multimerization was dependent on the correct function of paired cysteine residues and/or certain glycosylation sites present on the native molecule.

Expression of the TLR3-CD32 Chimeric Receptor at the Surface of Transfected Cells—In order to study the interactions between TLR3 and its ligands, we constructed a TLR3-CD32 chimeric molecule. CD32a is a cell surface receptor that, upon cross-linking (multimerization) induced by interaction with its natural ligand multimeric IgG, causes a rapid rise in intracellular calcium levels (39, 40). CD32a signaling has been ascribed to a domain in the cytosolic portion of the molecule, containing a conserved motif called the ITAM (41) that allows recruitment of cytosolic kinases (40). We and others (31) have observed that TLR3 is not expressed at the cell surface in fibroblasts. Thus, in the TLR3-CD32 chimeric molecules, we conserved the transmembrane region of CD32a in order to favor cell surface expression (Fig. 3A). Transfection of the recombinant plasmid into the cell lines HEK293T and U937 indeed resulted in a high level of cell surface expression of the chimeric receptor as detected using anti-TLR3 antibodies (Fig. 3B, e and f). No signal was detected on the cell surface of nontransfected cells (not shown) or on cells transfected with the empty pMET7 vector (Fig. 3B, b and c) or in cells transfected with a plasmid encoding for a TLR2-CD32 construct (data not shown). The plasmid that contained the full-length TLR3 molecule induced TLR3 expression in the cytosol and not on the cell surface of transfected HEK293T cells (Fig. 3B, h and i). We produced stable clones of U937 cells transfected with TLR3-CD32. Clones were selected based on their cell surface expression level of TLR3 (e.g. clone 2H4) (Fig. 3B, g). These clones expanded very easily (up to 10^9 cells) and could be maintained for long periods in culture (several months) without loss of expression of the recombinant molecule.

Engagement of the TLR3-CD32 Chimera by Anti-TLR3 Antibody and dsRNAs Triggers Ca\(^{2+}\) Mobilization in U937 Cells—The hematopoietic cell line U937 was used in all Ca\(^{2+}\) mobilization assays. We observed that this cell line expresses native CD32, and we were able to show that ligation of this receptor by anti-CD32 antibodies followed by cross-linking with a secondary anti-mouse Ig antibody induces calcium mobilization in these cells (Fig. 4A). We investigated the effect of multimerization of the chimeric TLR3-CD32 receptor using anti-TLR3 antibodies. In the TLR3-CD32 stably transfected cell line 2H4 (Fig. 4, C–F), as well as in transient transfectants (not shown), anti-TLR3 antibodies alone induced no signal, but cross-linking with a secondary antibody induced a potent Ca\(^{2+}\) flux (Fig. 4, C and D). Similar results were
obtained with both a polyclonal goat anti-TLR3 (from R&D) (Fig. 4, C and E) and a mouse monoclonal anti-TLR3 (clone TLR3.7) (Fig. 4, D and F). No response to anti-TLR3 antibodies with or without cross-linking by a secondary antibody was seen on nontransfected U937 cells (Fig. 4 B) or on cells transfected with a plasmid encoding TLR2-CD32 (data not shown). In contrast, cross-linking of TLR2-CD32 with an anti-TLR2 and secondary antibody induced potent Ca\(^{2+}\)/H\(_{11001}\) flux (Fig. 5 B). These observations showed that, in the same manner as the endogenously expressed CD32, the TLR3-CD32 chimera required multimerization for signaling.

We used poly(I-C), a known ligand of TLR3, to test the response of the TLR3-CD32 chimeric receptor. Poly(I-C) produced a high intensity Ca\(^{2+}\)/H\(_{11001}\) flux on the U937 cells stably expressing the TLR3-CD32 chimera (Fig. 5, A and C) but not on U937 cells transfected with a plasmid encoding TLR2-CD32 (Fig. 5B). We could also produce a Ca\(^{2+}\)/H\(_{11001}\) flux of comparable intensity using poly(A-U), a molecule that shows comparable in vivo stimulation to poly(I-C) but to our knowledge has not previously been unequivocally shown to activate via TLR3 (Fig. 5D). We compared the dose response to poly(I-C) for activation of the chimeric receptor with that for IP-10/CXCL10 cytokine induction in PBMC (Fig. 3).
The optimal doses of poly(I-C) were 0.5–1 μg/ml for Ca²⁺ flux and around 25 μg/ml for the induction of cytokine secretion. This chimeric construction is therefore sensitive and specific and thus potentially provides an opportunity to characterize the receptor-proximal events linked to the interaction of TLR3 with its ligands. Interestingly, once the TLR3-CD32-transfected cells were stimulated with an optimal concentration of either poly(I-C) or poly(A-U), we were unable to induce a subsequent flux on the same cells either with poly(I-C) (Fig. 5, C and D) or with cross-linked anti-TLR3 antibodies (Fig. 4, E and F).

No signal was observed on empty vector-transfected cells even after cross-linking (B). Anti-TLR3 cross-linked response desensitized a secondary response to 25 μg of poly(I-C) (C and D) and vice versa (E and F).

**FIGURE 4.** Use of anti-TLR3 antibodies shows the requirement of cross-linking for TLR3-CD32 signalization. Ca²⁺ flux was induced by anti-CD32 antibody but only upon cross-linking with secondary reagent on myelomonocytic cell line U937 (A). Using the TLR3-CD32 chimera stably transfected U937, clone 2H4, a goat (C) or mouse (D) anti-TLR3 antibodies (0.5 and 2.5 μg/ml, respectively) were inactive alone but triggered Ca²⁺ flux upon cross-linking with their respective conjugate (10 μg/ml). No signal was observed on empty vector-transfected cells even after cross-linking (B). Anti-TLR3 cross-linked response desensitized a secondary response to 25 μg of poly(I-C) (C and D) and vice versa (E and F).

5, E and F). The optimal doses of poly(I-C) were 0.5–1 μg/ml for Ca²⁺ flux and around 25 μg/ml for the induction of cytokine secretion. This chimeric construction is therefore sensitive and specific and thus potentially provides an opportunity to characterize the receptor-proximal events linked to the interaction of TLR3 with its ligands. Interestingly, once the TLR3-CD32-transfected cells were stimulated with an optimal concentration of either poly(I-C) or poly(A-U), we were unable to induce a subsequent flux on the same cells either with poly(I-C) (Fig. 5, C and D) or with cross-linked anti-TLR3 antibodies (Fig. 4, E and F). Similarly, cross-linking of the anti-TLR3 mouse or goat antibodies desensitized the cells to a response to poly(I-C) (Fig. 4, C and D). This loss of secondary response, probably reflecting CD32a/ITAM physiology, strengthens the specificity of poly(I-C) and poly(A-U) for TLR3.

**The TLR3-CD32 Chimeric Receptor and Wild Type TLR3 Are Activated upon the Addition of Defined Length Poly(I-C) Oligomers**—The TLR3-CD32 molecule is expressed at the cell surface and mediates Ca²⁺ mobilization following the addition of ligands. The ability of poly(I-C) or poly(A-U) to mediate a potent Ca²⁺ mobilization comparable with that obtained with cross-linking of anti-TLR3 antibodies demonstrates that they can aggregate the luminal domain of TLR3. Agarose gel electrophoresis of poly(I-C) (InvivoGen) showed that the product was mainly composed of high molecular weight fragments (>2000 bp; data not shown). Since these fragments could aggregate the receptors by binding to several TLR3 molecules, we investigated the ability of defined small synthetic poly(I-C) fragments to induce Ca²⁺ flux on the TLR3-CD32 chimera. These fragments were produced by slow annealing of synthesized 15-, 30-, and 40-mers of poly(I) and poly(C). The length of the recovered double-stranded RNA fragments was verified on a 2% agarose 3:1 gel. As shown in Fig. 6A, all fragments were able to trigger Ca²⁺ mobilization in transfected U937 cells. However, 50-fold higher concentrations of these fragments (5–10 μg/ml) were required to trigger signals of an intensity comparable with that of high molecular weight poly(I-C) (Fig. 6A). Although differences in dose response between the different sizes of small poly(I-C) fragments could not be clearly delineated, in general, longer fragments trigger Ca²⁺ fluxes of higher intensity as illustrated by index values of the first signal versus the residual signal in response to poly(I-C) (Fig. 6, B and C). When an acidic fragmentation of poly(I-C) (yielding fragments of 100–200 base pairs)
was used (Fig. 6, A and D), no difference in potency was noted compared with high molecular weight poly(I-C) (>2000 bp). Finally, comparable Ca\textsuperscript{2+} mobilization dose responses were observed with poly(I-C) and poly(A-U) (Fig. 6A). Differences in potency are thus proportional to the ability of the different ligands to mediate TLR3 multimerization and suggest that even small oligomers of poly(I-C) can cause receptor aggregation and activation.

The Ability of Chloroquine and Bafilomycin to Block TLR3 Responses Suggests That TLR3 Functions in an Acidic Compartment—Human TLR3 has been described as a cytosol-localized receptor, at least in monocyte-derived DC, and interaction with its ligand may occur in subcellular compartments within the endocytic pathway (31). These intracellular compartments display an acidic pH, a demonstrated requirement for the interaction between TLR9 and CpG. Bafilomycin and chloroquine effectively blocked TLR9-mediated CpG response by human PBMC (IP-10/CXCL10 production) with only a minor effect on the lipopolysaccharide-induced response (IL-6 production by MonoDC or PBMC) (Fig. 7). Both drugs also blocked poly(I-C)-induced responses (IL-6 and IP-10/CXCL10 by human MonoDC and PBMC), suggesting that the TLR3-poly(I-C) interaction is occurring within an acidic subcellular vesicle. Surprisingly, the TLR8-mediated response induced by R-848 (IL-6 by MonoDC and PBMC) was blocked by bafilomycin but not by chloroquine (Fig. 7). This is in agreement with previous studies indicating that TLR8 was insensitive to chloroquine (25), possibly reflecting the ability of chloroquine to act through nonlysosomotropic mechanisms (42), unlike the more specific and potent proton pump inhibitor bafilomycin.

The Ability of Poly(I-C) to Induce Aggregation and Signaling through Cell Membrane-bound TLR3-CD32 Chimeric Receptor Is Dependent on an Acidic pH—We investigated the effect of the extracellular pH on the Ca\textsuperscript{2+} flux mediated from the cell surface by poly(I-C) via TLR3-CD32. Our initial observations showed a similar Ca\textsuperscript{2+} flux mediated by poly(I-C) or the cross-linked antibodies at pH 6.25 but showed that above pH 7 the poly(I-C) and poly(A-U) responses were abrogated, although the antibody response remained (Fig. 8A). We then examined the response over a range of pH values. Our observations (Fig. 8B)
Poly(I-C)-mediated TLR3 Cross-linking Is pH-sensitive

![Graphical representation of poly(I-C) mediated TLR3 cross-linking](image)

**Figure 6.** The intensity of the Ca$^{2+}$ response was proportional to the size of the synthetic dsRNA. Poly(I-C)s of defined size were tested on the TLR3-CD32 chimera stably transfected 9U37, clone 2H4. The concentration of small fragments (I-C)$_{15}$, (I-C)$_{30}$, and (I-C)$_{40}$ necessary to induce Ca$^{2+}$ flux is higher compared with high molecular weight poly(I-C) (ND, not done). 10 μg/ml small size poly(I-C) synthetic fragments (I-C)$_{15}$ (B) and (I-C)$_{40}$ (C) of 15 and 40 base pairs, respectively, induced a partial Ca$^{2+}$ flux, as seen by residual secondary response to 25 μg of poly(I-C). In contrast, 10 μg of 100–200-bp hydrolysis-fragmented poly(I-C) induced a complete Ca$^{2+}$ response (D).

showed that an acidic pH from 5.7 to 6.5 was required for an optimal response to poly(I-C), with a progressive decrease from pH 6.75 to 7.25 and that a pH of 7.5 prevented the response. In contrast, this range of pH had no influence on the signal mediated using anti-TLR3 antibodies (Fig. 8B) and did not induce detectable alteration of viability in this time frame (not shown). When oligomeric poly(I-C) fragments were used, the dependence on acidic pH was even more critical (Fig. 8C). As an example, a pH of 5.7 was required for full TLR3-CD32 activation by (I-C)$_{15}$. Full activation at optimal pH was characterized by a Ca$^{2+}$ flux of index $>$100 and the absence of a secondary response to high molecular weight poly(I-C), indicating complete occupancy and/or desensitization of the receptor. Between pH 6.25 and 6.6, the effect of (I-C)$_{15}$ was completely lost, whereas the response to high molecular weight poly(I-C) remained unaffected (Fig. 8D). Equally at this pH, (I-C)$_{15}$ did not engage TLR3, as evidenced by the lack of desensitization to a second poly(I-C) injection. These observations showed that pH has a strong impact on the interaction between poly(I-C) and TLR3 and suggested that under normal conditions, these interactions occur in the acidic milieu of subcellular compartments.

**DISCUSSION**

In the present study through a site-directed mutagenesis approach and construction of chimeric molecules, we show that dimerization or multimerization of the receptor is a requirement for TLR3 signaling and may thus have a mechanism of ligand recognition different to other TLRs. The disruption of this intrachain bridge altered the ability of the receptor to bind the ligand or, possibly, to prevent the conformational change needed for signal delivery. Mutation of a second pair of cysteines, Cys$_{226}$ and Cys$_{242}$, that do not appear to pair within the same TLR3 molecule had no effect on ligand binding on our assays. On the basis of their structure and glycosylation data, Choe et al. (36) proposed that, unlike other TLRs, the ligand dsRNA does not bind TLR3 in the inner concave part of the ectodomain that is occupied by carbohydrates and is not positively charged. Rather, they hypothesized that ligand binding would happen on the nonglycosylated face of the convex side in two positively charged patches, one of which was adjacent to the extended loop region of LRR 12. On the same face, a homodimer interface was identified, adjacent to the extended loop of LRR 20. However, we observed that a potential N-glycosylation (Asn$^{237}$) site, predicted to lie on the same side of the leucine-rich repeat solenoid as the cysteines, was critical for signaling. This residue was not glycosylated in either of the crystallographic studies; however, in these studies, not all of the potential sites were glycosylated, and major differences in the glycosylation sites between the two studies were observed. The discrepancies in N-linked glycosylation patterns between our study and those reported are probably due to the fact that Choe et al. (36) and Bell et al. (37) chose to express the TLR3 ectodomain in insect cells. Differences in the patterns of glycosylation between different cell lines have been already been noted (43). However, the potential glycosylation of Asn$_{146}$ and Asn$_{247}$ is fully compatible with the TLR3 structure models predicted through crystallography, since these two N-glycosylation sites are, like all of the other sites, not localized on the surface predicted to bind ligand. In the
Poly(I-C)-mediated TLR3 Cross-linking Is pH-sensitive

FIGURE 7. Effects of drugs blocking acidification on TLR activation. Cells were treated with 50, 100, or 200 nM bafilomycin (upper panels) or 2.5, 5, or 10 μM chloroquine (lower panels) 45 min prior to activation. The effects of the drugs on IL-6 secretion on MonoDC or PBMC and on IP-10/CXCL10 secretion by PBMC were analyzed. Whereas bafilomycin and chloroquine did not prevent lipopolysaccharide-mediated activation, they are highly effective in the inhibition of CpG-mediated IP-10/CXCL10 secretion. Poly(I-C) activation is very sensitive to drugs blocking acidification, in a dose-dependant manner (all panels). R-848 activation is more sensitive to bafilomycin than to chloroquine in both PBMC and MonoDC populations.

-case of TLR4 and myeloid differentiation factor-2, it has been demonstrated that N-linked glycosylation at specific residues is essential for the correct conformation, localization, and ligand recognition of these molecules (44). The fact that the double N-glycosylation mutant protein was present at a lower level in the cells than native TLR3 suggests that incompletely glycosylated TLR3 has a higher degradation rate and that glycosylation may be involved in TLR3 stability, as for other proteins (43). Interestingly, a recent report shows that N-linked glycosylation at a single residue of a viral glycoprotein is essential for protein folding and function (45), similarly to our observations with TLR3 mutants. However, we cannot rule out an alternative role for Asn247 in TLR3 function.

Additionally, we also made deletion mutants of the identified TLR3-specific loop regions in LRR 12 (Δ343–340) (adjacent to the hypothetical dsRNA binding site) and LRR 20 (Δ549–554). These deletion mutants continued to bind poly(I-C) and signal in HEK293T cells, and although we did not extensively characterize the mutants, in our hands they showed neither a significant alteration in activity in comparison with the wild type TLR3 nor a dominant negative effect (data not shown). Altogether, our results with mutants suggest that the conformation and glycosylation of the amino-terminal LRRs are critical for TLR3-poly(I-C) binding and signaling and need to be reconciled with the structural data and their interpretation for advancing our understanding of the ligand binding regions of TLR3.

Recent evidence suggests that the transmembrane and membrane-proximal regions are important for the cellular localization of individual TLR molecules (46, 47) in contrast to the ectodomain, which is involved in ligand interaction. Therefore, we took advantage of this modular nature of TLRs to create a chimeric molecule, coupling the TLR3 ectodomain to a cytosolic domain that would allow Ca2+ mobilization in an appropriate immune cell line. We chose the cytosolic domain of CD32a because this molecule has a well defined mode of action and also since it is known to be expressed and functional in the myelomonocytic cell line, U937. We have shown that the recombinant molecule is perfectly functional, since it recognizes the known and suspected TLR3 ligands, poly(I-C) and poly(A-U). Our construct is thus useful for the detection of TLR3 agonists or antagonists as well as to identify potential natural ligands from pathogens or endogenous ligands. The results presented in this study are restricted to TLR3, but it is expected that, using this type of approach, any TLR can be successfully coupled to Ca2+ mobilization. Indeed, a similar construction was made for TLR2 and calcium flux was also observed upon cross-linking of the chimera (data not shown). Although TLR2 is expressed at the cell surface and TLR3 in an intracellular compartment in MonoDC, both CD32 chimeras were expressed at the cell surface.

We used the TLR3-CD32 chimera to examine the nature of the interaction between TLR3 and its ligands. The signaling through the ITAM and the induction of Ca2+ flux are well known to depend on dimerization or multimerization of the ITAM carrying receptors or receptor-associated chains. We conclude that in a similar fashion to aggregation of the receptor via antibody cross-linking, the addition of poly(I-C) to cells expressing TLR3-CD32 causes aggregation of the receptor, and this multimerization leads to signaling of the chimera and Ca2+ flux. Commercially available poly(I-C) is formed of long double-stranded chains of up to 5000 bp, and it is thus not surprising that this molecule causes aggregation of the receptor. However, we demonstrated that shorter chains of poly(I-C) and even oligomeric (I-C)15 could induce receptor signaling. Thus, even very short dsRNA, smaller than microRNA or small interfering RNA, can potentially activate TLR3, either by directly cross-linking the receptor or by inducing conformational changes leading to dimerization and signaling, possibly similar to the situation observed with TLR7 and TLR8, which are activated by the small molecule resiquimod (R-848) mimicking the action of single chain GU-rich nucleic acid (48).

The need for TLR cross-linking to induce signaling has been demonstrated for other TLRs. It has been shown in Drosophila that one Spätzle dimer binds two Toll molecules, allowing signaling (49). Equally, using
Poly(I-C)-mediated TLR3 Cross-linking Is pH-sensitive

Although recent observations suggest that structural elements in the intracellular domain of TLRs are required for dimerization and the creation of a potential signaling complex (51), ligand-induced aggregation of TLR ectodomains is likely to be the strongest determinant in inducing signaling, as we demonstrate in this report. These results all tend to indicate that the proximity of at least two intracellular domains, followed by a conformational change generated by ligand binding leads to transduction of cellular activation signals. We have recently shown that TLR3 does not associate with myeloid differentiation factor-2 (52), and no evidence suggests the association of TLR3 with another TLR; thus, our data, as well as that of others, points to a simple association of a TLR3 dimer upon ligand binding. The dimers may be formed upon ligand binding or, as suggested by structural data, may be preformed, and signaling may be induced upon ligand binding by stabilization or conformational changes in the preformed dimers. Additionally, the possibility that receptor multimerization was associated with optimal signaling was suggested by the finding that mutants of TLR3 behaved as dominant negative molecules.

The observation that the interaction between dsRNAs and TLR3 is pH-dependent allows us to speculate on the nature and localization of the in vivo interaction. Under acidic conditions, adenosine and cytidine become protonated, which allows them to bind a water molecule and thus potentially bridge interactions with other molecules. Equally dsRNA starts to lose Watson-Crick base pairing at intermediate to low pH (53). One can thus hypothesize that under intermediate pH conditions, such as those which we investigated, the dsRNA is protonated and slightly dehybridized, and it is this form of the dsRNA that is recognized by the receptor. Alternatively, an RNA triplex structure may be recognized. The importance of pH has been recently reported for CpG/TLR9 interaction, with an optimal binding observed at pH 5.5–6.5 (27). The acidic pH required for an optimum poly(I-C)-mediated response suggests that the interaction with TLR3 also occurs in an acidic subcellular compartment. This is in agreement with the recent description in DC of TLR3 in subcellular compartments of the endocytic trafficking pathway (31). In line with this observation, we have shown that drugs that block subcellular compartment maturation and acidification suppress the poly(I-C) biological response in primary cells. The use of these drugs further suggests that either TLR3 and TLR8 are localized in alternate subcellular compartments, in agreement with previous studies (25, 54), or that chloroquine may act through nonlysomotropic mechanisms.

A recent study has also shown a role of TLR3 in the recognition of RNA released by dying or dead cells (11, 55, 56). In this case, it was demonstrated that secondary structure creating hairpin loops within the mRNA was responsible for TLR3 activation. The localization of TLR3 in the endosomal pathway in myeloid DC is consistent with its role in recognition of RNA from dead cells. The capacity of myeloid DC to capture antigens from apoptotic or necrotic cells is well documented (11, 55, 56). In this case, it was demonstrated that RNA was protonated, which allowed it to bind a water molecule and thus bridge interactions with other molecules. Similarly, dsRNA starts to lose Watson-Crick base pairing at intermediate to low pH (53). One can thus hypothesize that under intermediate pH conditions, such as those which we investigated, the dsRNA is protonated and slightly dehybridized, and it is this form of the dsRNA that is recognized by the receptor. Alternatively, an RNA triplex structure may be recognized. The importance of pH has been recently reported for CpG/TLR9 interaction, with an optimal binding observed at pH 5.5–6.5 (27). The acidic pH required for an optimum poly(I-C)-mediated response suggests that the interaction with TLR3 also occurs in an acidic subcellular compartment. This is in agreement with the recent description in DC of TLR3 in subcellular compartments of the endocytic trafficking pathway (31). In line with this observation, we have shown that drugs that block subcellular compartment maturation and acidification suppress the poly(I-C) biological response in primary cells. The use of these drugs further suggests that either TLR3 and TLR8 are localized in alternate subcellular compartments, in agreement with previous studies (25, 54), or that chloroquine may act through nonlysomotropic mechanisms.

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Recently, TLR3 has been shown to recognize double-stranded nucleic acid from Schistosoma mansoni and to be involved in the anti-parasite response (10). Although this is the only report, this suggests that RNA from many sources can activate TLR3 as long as it displays secondary structure, including double-stranded regions, and is present in the appropriate cellular vesicle.

TLR3 is a potent inducer of type I interferon, mainly interferon-β, in myeloid DC and nonhematopoietic cells. The fact that it induces type I interferon and recognizes dsRNAs has suggested a role in viral recogni-

**FIGURE 8.** Effect of pH on poly(I-C)-mediated TLR3 activation. Poly(I-C)-mediated TLR3 cross-linking is pH-sensitive. Variation in the appropriate cellular vesicle.
Poly(I-C)-mediated TLR3 Cross-linking Is pH-sensitive

...tion, but this remains to be demonstrated (62). A number of viruses have been shown to traffic through the endosomal compartments during cellular entry or to depend on the acidification of these compartments in order to replicate or assemble viral particles. Alphaviruses, such as semliki forest virus, enter into new cells by acid-triggered membrane fusion in endosomes (63). Late RNA synthesis in alphavirus-infected cells, generating plus-strand RNAs, has been shown to take place on cytoplasmic vacuoles, which are modified endosomes and lysosomes (64). Interestingly, retroviruses, such as human immunodeficiency virus, also replicate in and are assembled in lysosomes/endosomes (65) before budding off from infected cells. It has also been shown that infectious human immunodeficiency virus is enveloped by membrane derived from endosomes and still expressing endosomal markers (66). Herpesviruses such as human herpesvirus-8 use clathrin-mediated endocytosis and require a low pH intracellular environment in order to replicate in fibroblasts. Bafilomycin and chloroquine have been shown to block viral entry, showing a requirement of the acidification of intracellular compartments for human herpesvirus-8 infection. Bafilomycin and chloroquine were also active against the replication of prototype strain of minute virus of mice (MVMp). This shows a requirement for passage of the viral nucleic acid into a specific compartment is necessary to occur in order to activate TLR3 response pathways, suggesting that passage of the viral nucleic acid into a specific compartment is necessary for the response (9, 68, 69). Furthermore, recently, TLR3 has been reported to promote cross-presentation of virus-infected cells through engagement of virus-derived RNA (70). These studies, in conjuction with our demonstration that TLR3 is capable of ligand binding and signaling only at pH levels present in acidification-dependant vesicles, suggest that TLR3 shares cellular compartments often used for viral entry, replication, or detection and reinforce the notion that TLR3 is important in the early antiviral response.

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