Conserved Features in the Extracellular Domain of Human Toll-like Receptor 8 Are Essential for pH-dependent Signaling*

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Toll-like receptor (TLR) 8 has an important role in initiating immune responses to viral single-stranded RNA and the antiviral compound resiquimod. Together with TLR3, -7, and -9, it forms a subgroup of the TLRs that are localized intracellularly and signal in response to pathogen-derived nucleic acids. In this work, we have used site-directed mutagenesis to identify regions of the TLR8 extracellular domain that are required for stimulus-induced signal transduction. We have shown that a cysteine-rich sequence predicted to form a loop projecting from the selenoidal ectodomain structure at leucine-rich repeat 8 is essential for signaling in response to both single-stranded RNA and resiquimod. A second region, centered on an aspartic acid residue in leucine-rich repeat 17, is also required for TLR8 function. The corresponding residue in TLR9 is known to be important for pH-dependent binding and signaling in response to unmethylated CpG DNA, suggesting that the TLR7/8/9 subgroups share a common signaling mechanism. We have also shown that TLR8 is localized predominantly in the endoplasmic reticulum but that signaling is completely abolished by an inhibitor of vesicle-type H\(^+\) ATPases. This indicates that TLR8 is present at low levels in an acidified compartment and that a lowered pH is required for receptor function. We propose that pH-dependent changes in the ligand facilitate activation of the receptor. The protonated form of resiquimod, a cell-permeable weak base, is likely to concentrate significantly (~100×) in acidified compartments, and this may potentiate low affinity interactions with either the receptor or a specific binding protein.

Toll-like receptors (TLRs) are a vital part of the immune response to viruses and respond to a wide variety of pathogens. They have a shared modular structure with an extracellular domain consisting mainly of leucine-rich repeats (LRRs), a single transmembrane helix, and a cytoplasmic Toll/interleukin-1 identity region. The role of Toll-like receptors is to elicit an innate immune response and to regulate the development of adaptive immunity. The initial signaling complex leads to the activation of NF-κB, which induces transcription of tumor necrosis factor-α, interleukin-12, and a large number of other genes. Some TLRs also activate the interferon response factor family of transcription factors that induce genes involved in anti-viral responses, such as interferon-α (see Ref. 1 for a review). Humans have 10 functional TLRs, and these have evolved to recognize a variety of components derived from pathogenic or commensal microorganisms, principally bacteria and viruses. These molecules include lipids, such as lipopolysaccharide, from Gram-negative bacteria (TLR4) (3), proteins (e.g. flagellin, TLR5 (4)), and nucleic acids. Nucleic acids and small molecules, such as imidazoquinolines and immunostimulatory guanosine nucleotides, are recognized by a subgroup of the TLRs (5–8). It is noticeable that these subgroups are more closely related to each other than the rest of the TLR family and that they have conserved structural features. The nucleic acid subfamily comprises TLRs -3, -7, -8, and -9. TLR3 responds to double-stranded RNA and TLR9 to unmethylated CpG DNA sequences (9). TLR7 and TLR8 are activated by imidazoquinolines, a family of small molecule drugs that elicit powerful anti-viral responses (see Fig. 3A) (6, 10). Imidazoquinolines have been identified that stimulate either TLR7 or TLR8, or both TLRs 7 and 8 (11). These compounds are only slightly different in structure, and it remains unclear how this specificity is achieved. It is likely that single-stranded RNA (ssRNA) from a viral origin is the natural agonist for human TLR8 and mouse TLR7 (5, 12).

The TLR3/7/8/9 subgroups also share a common cellular localization. Rather than being secreted to the cell surface, they are retained in intracellular compartments such as the endoplasmic reticulum (ER), and this subcellular localization is thought to provide specificity and to prevent activation by self-nucleic acid (13). Studies on TLR9 activation have shown that, in the resting state, the receptor resides in the endoplasmic reticulum (14). Following stimulation, there is a rapid translocation of the receptor into lysosomes. In the case of viral nucleic acid, lysosomal proteases are required for the degradation of viral capsid and therefore release the viral nucleic acid into this intracellular compartment (13).
The only structure of a TLR extracellular domain solved to date is that of TLR3. The 23 LRRs form a horseshoe-shaped solenoid substituted with a high density of N-linked glycans (15, 16). However, one face of the LRR solenoid is free from glycosylation, and positively charged patches on this surface may associate with double-stranded RNA. There are also two inserts outside the LRR framework that extend from the non-glycosylated face of the receptor and could provide binding sites for double-stranded RNA, one in LRR12 and another in LRR20. In addition, a recent mutagenesis study has identified several cysteine and asparagine residues in the ectodomain that are required for activation of TLR3 (17). TLR7–9 are more closely associated with double-stranded RNA. There are also two inserts in LRR8, a sequence that has four conserved cysteine residues, and a linker region of 16 amino acids at LRR14 that may subdivide the LRRs into two structurally independent blocks (18). A study (19) with purified TLR9 extracellular domain shows specific and pH-dependent binding of DNA containing unmethylated CpG dinucleotides, and a dissociation constant of 200 nM has been measured for this interaction. The binding activity was mapped to a region on the convex surface of LRR17, and this work provides evidence for direct interaction of nucleic acid-derived stimuli with TLR ectodomains.

In this paper, we have studied the requirement of the cysteine-rich insert in LRR8 and an acidic residue in LRR17 for signaling by TLR8. TLR8 is unusual, as it appears to be redundant in the mouse but plays a critical role in humans. For example, it is the only TLR that is active in neonates (20) and is important for virally induced maturation of certain human dendritic cell subtypes (11). We report here that the short insert sequence in LRR8 is critical for TLR8-mediated signaling in response to both imidazoquinolines and single-stranded RNA and that all four cysteine residues are absolutely required for function. We also show that oligonucleotides enhance signaling by human TLR8 in response to imidazoquinolines but not by TLR7.

MATERIALS AND METHODS

Cell Culture and Biological Reagents—Osteosarcoma (U20S) and human embryonic kidney (HEK)293 cells were from stocks maintained by GlaxoSmithKline (Stevenage, UK). The cells were cultured in Dulbecco’s modified Eagle’s culture medium (Invitrogen) supplemented with 10% fetal calf serum, glutamine, non-essential amino acids, and where appropriate, penicillin/streptomycin. The cells were incubated at 37 °C with 5% carbon dioxide. U20S cells stably expressing TLR8 were produced by GlaxoSmithKline and were maintained as for transformed U20S cells, including 500 µg/ml G418 for selection.

pCDNA3hTLR8 and pCDNA3hTLR7 vectors were supplied by GlaxoSmithKline. pEFPN1 and pECFPN1 were from Clontech. Human TLR8 isoform 2 was used (GenBankTM reference NP_61954). U20S and HEK293 cells do not endogenously express TLRs 7–9.

Resiquimod was synthesized by GlaxoSmithKline. CpG DNA 2006 (stimulatory, 5′-TGCTGCTTTTGTGCTTTTGTGTT-3′ with phosphothioate linkages) and GpC 2006 (non-stimulatory, 5′-TCGTCGTTTTGTCGTTTTGTCGTT-3′ with phosphothioate linkages) were obtained from MWG Biotech. ssRNA40 (5′-GCCCGUCUGUGUGUGACUC-3′ with phosphothioate linkages) was purchased from Metabion. Bafilomycin A1 from Streptomyces griseus was purchased from Sigma.

Mutagenesis—The primers used to mutate TLR8 are detailed in supplemental Fig. S1. The QuikChange mutagenesis kit (Stratagene) was used, and two rounds of mutagenesis were performed where necessary to create all the desired mutations. Successfully mutated colonies were back-cloned into non-mutated pcDNA3 backbone. All constructs were fully sequenced to verify only the required mutation had been introduced.

NF-κB Luciferase Assay—Cells were seeded in 6-well plates so as to reach ~95% confluency after 18 h. The cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer’s guidelines. For one well, DNA mixtures of 2 μg of TLR (or empty vector), 1 μg of NF-κB-luc reporter, and 0.1 μg of pRLTK (Promega) were used. 24 h after transfection, the cells were seeded into 96-well plates and stimulated with compound. After incubation with stimulating compound for 18 h, the Dual Glo luciferase assay system (Promega) was performed according to the manufacturer’s instructions. Firefly luciferase readings (created from the NF-κB promoter system) were normalized to the Renilla luciferase readings (under the control of the thymidine kinase-constitutive promoter) for the corresponding wells to correct for any variation in cell number. Thereafter, data were expressed as a fold induction of NF-κB when comparing stimulated with non-stimulated (medium-only) wells. Means and standard deviations were obtained from quadruplicate readings. Results are from one experiment and are representative of at least three separate assays. Signaling assays were performed in U20S cells, including a stably expressing TLR8 cell line, and in HEK293 cells. Similar results were obtained in both cell types. Interleukin-1β at 50 nM was used as a positive control for NF-κB activation. When using ssRNA40 as a stimulating compound, RNA-Dotap complexes were first created using a ratio of 1 μg of RNA to 3 μl of Dotap (Roche Applied Science). Dotap was also complexed in the same way to either CpG DNA or resiquimod to see whether this delivery system enhanced NF-κB activation.

Western Blotting—Transfected cells were harvested 48 h after transfection. The cells were lysed in lysis buffer and then centrifuged to remove debris. Samples were then run on a 4–12% BisTris gel (Invitrogen) and blotted onto a polyvinylidene difluoride membrane. Mouse anti-GFP antibody (Clontech) was used at a 1:10000 dilution as the primary antibody. Horseradish peroxidase-conjugated anti-mouse antibody was used as the secondary antibody and then developed using the ECL plus reagent (Amersham Biosciences).

Fluorescence Confocal Microscopy—Fluorescence confocal microscopy was performed on the Leica TCS SP2 AOPS system or the Leica DM IRBE with two-photon titanium:sapphire laser in the Multi-Imaging Centre, University of Cambridge. Co-localization studies were performed using the blue-white ER-tracker (Invitrogen). CFP was excited at 405 nm and the emission collected at 450–490 nm. YFP was excited at 514 nm and the emission collected at 540–600 nm. Using a two-photon
FIGURE 1. Residues in leucine-rich repeats 8 and 17 are required for signal transduction by TLR8. A, schematic diagram of human TLR8 highlighting the two blocks of LRRs. The first block contains LRR8 with its extended LRR structure containing the cysteine motif. The second block contains residues known to be important for CpG DNA binding in TLR9 (19). B, basic LRR structure highlighting the LRR consensus residues and showing the site of the insert in LRR8. The diagram was generated using PyMol with TLR3 LRR16 as an example. C, sequence alignment of LRR8 in TLRs 7–9. Mutated cysteines are highlighted in red. D, NF-κB luciferase assay to determine the effect of the single point cysteine to alanine mutations upon stimulation. U20S were transfected with wild-type or single point mutation constructs and responses to stimulation tested with the NF-κB assay. Data are presented as fold induction as compared with non-stimulated (medium-only) cells. E, double mutants are also defective in NF-κB activation. Each possible combination of cysteine pairs were mutated, transfected into U20S cells, and stimulated with resiquimod or ssRNA40 to determine NF-κB activation. F, NF-κB luciferase assay using transfected U20S cells. U20S cells were transfected with wild-type or mutant receptors, and the response to TLR8 agonists was determined. Wild-type TLR8 induces NF-κB activation upon stimulation with either resiquimod or ssRNA40. Both a truncated receptor (8uD, with a deletion between LRR8–15) and a mutant receptor (8uA, where all four cysteines of LRR8 are mutated to alanines) abolish NF-κB activation upon stimulation with resiquimod or ssRNA40.
TLR8 Signaling Mechanism

A) TLR8 full length (1-1041)
   6 aa linker
   YFP(1-238)

B) U2OS transfected with non-tagged and tagged constructs

C) TLR 8 fluorescent fusion protein

D) YFP
   ER-tracker
   Merge

HEK 293
TLR 8YFP / ER-tracker

E) HEK 293
TLR 8u2YFP / ER-tracker

F) HEK 293
TLR 8C FP (green)/
TLR 8u5Y FP (ed)
system, ER-tracker was excited using 808 nm and emission collected at 400–600 nm. Live cells were used for the work with ER-tracker. The cells were fixed in 4% formaldehyde for 15 min for the CFP and YFP co-localization studies. Images were generated using the Leica LCS software.

Acidification Inhibitor Experiments—Experiments were performed as for the NF-κB luciferase assay. Before stimulation with the required compound, cells were incubated at 37 °C for 30 min with lysosomal acidification inhibitors. Either 10 or 100 nM bafilomycin A1 was diluted in cell culture medium. The cells were then stimulated for 18 h at 37 °C before performing the luciferase assay.

RESULTS

The Extracellular Domain of TLR8 Contains Regions That Are Critical for NF-κB Activation upon Stimulation with Resiquimod or ssRNA—As outlined above, the TLR7/8/9 subgroups have features that may confer specificity for nucleic acid-derived stimuli (Fig. 1A). In particular, the 16-amino-acid insert in LRR8 is unlikely to be accommodated within the LRR framework (Fig. 1B) and probably forms a loop structure protruding from the convex surface of the solenoid. The consensus of the loop is CARCXxxxxPCXXX, suggesting that the four cysteine residues and the basic arginine are of particular importance (Fig. 1C). We therefore carried out a systematic mutagenesis of these cysteine residues in TLR8 and assayed the mutant receptors for signaling activity to NF-κB in response to both resiquimod and ssRNA. We found that changing any of these four cysteine residues to alanine completely abolished signaling by TLR8 when stimulated by both resiquimod and ssRNA (Fig. 1D). Likewise, mutation of aspartate 543, a conserved residue shown previously to be important for TLR9 function, also resulted in an inactive receptor. To eliminate the possibility that the loss of function we observed was a secondary consequence of introducing unpaired cysteine residues into the structure, we generated all six combinations of double cysteine mutation and found that these were also unable to signal (Fig. 1E). In addition, mutant receptors that had either a deletion of LRRs 8–15 or all four cysteine residues changed to alanine were inactive (Fig. 1F).

Wild-type and Mutant TLR8 Receptors Are Expressed at Similar Levels and Localize Predominantly to the Endoplasmic Reticulum—We next tested whether the signaling defects of the mutant receptors might be caused by misexpression or mislocalization. Full-length constructs of both wild-type TLR8 and the single cysteine mutants were prepared, fused at the C terminus with either CFP or YFP (cyan or yellow fluorescent proteins) (Fig. 2A). The tagged constructs were tested in the NF-κB assay to determine whether the activity of the receptor was retained. The tagged wild-type receptors were able to activate NF-κB upon stimulation, although at somewhat reduced levels as compared with the untagged receptor (Fig. 2B). By contrast, all four single cysteine mutants were unable to signal. Western blotting of cell lysates with an anti-GFP antibody showed that mutant and non-mutant receptors were expressed in equivalent amounts (Fig. 2C). Thus, the mutant receptors were no more sensitive to degradation than the wild-type receptor. We then examined the subcellular localization of the receptors in living HEK293 cells with fluorescent microscopy. Wild-type TLR8 visualized by fluorescence from the YFP tag is observed in the endoplasmic reticulum, as it has the same distribution as an ER-specific marker dye (Fig. 2D). The localization of the single cysteine mutant u2 is indistinguishable (Fig. 2E). Finally, co-transfection of wild-type TLR8 tagged with CFP and the Asp-543 (u5) mutant tagged with YFP confirms that the inactive mutants are expressed in the same pattern as the functional wild-type receptors (Fig. 2F).

Signaling by TLR8 Is Dependent on Vesicle Acidification and Is Modulated by Oligodeoxynucleotides—Next, we tested the effect on signaling of bafilomycin A1, an inhibitor of v-type ATPases (21) that are required for acidification of Golgi, late endosomes, and lysosomes. We found that signaling in response to both resiquimod and ssRNA was profoundly sensitive to bafilomycin A1 treatment. We then studied the effects of co-stimulation with TLR9 agonists on the TLR8 pathway. As expected, ssRNA40 activated TLR8, whereas stimulation with either CpG or GpC DNA had no effect. However, when both ssRNA and CpG DNA were used to co-stimulate the cells, there was a 40% reduction in NF-κB activation, although this inhibition was not observed with GpC DNA (Fig. 3B). We then examined the effect on NF-κB activation of co-stimulating either TLR7- or TLR8-expressing cells with resiquimod and CpG DNA. U20S cells transiently transfected with TLR7 or TLR8 were stimulated by treatment with resiquimod and DNA oligonucleotides or combinations of the two. In the case of TLR7, as previously reported (22), there is a slight decrease in NF-κB activation upon stimulation with resiquimod and both CpG and GpC DNA (Fig. 3C). However, in the case of TLR8, stimulation with resiquimod and DNA caused an increase in signaling by both stimulatory and non-stimulatory oligonucleotides. These experiments were repeated using a stably expressing TLR8 U20S cell line with stimulation in the presence or absence of Dotap, which enhances endocytic uptake of DNA (23). Again, there was an increase in NF-κB activation when both resiquimod and CpG DNA were used for stimulation, and this effect was enhanced when Dotap was included in the assay. Both the stimulatory and the non-stimulatory forms of DNA

**FIGURE 2. Biosynthesis and localization of mutant and wild-type TLR8.** A, a schematic diagram of full-length C-terminally, fluorescently tagged TLR8. Both CFP and YFP constructs were generated. B, the fluorescently tagged TLR8 constructs behave in the same way as their non-tagged counterparts in the activation of NF-κB. Constructs were transfected into HEK293 cells, and the cells were stimulated with resiquimod, flagellen, or interleukin-1 as indicated. Refer to the Fig. 1 legend for the identity of the mutants. C, anti-GFP Western blot shows that both full-length and mutated tagged proteins are expressed to comparable levels in HEK293 cells. D, live cell confocal microscopy of TLR8YFP (green), ER-tracker (red), and a merge of the two. HEK293 cells transiently transfected with TLR8YFP show that TLR8 appears to co-localize with the ER-tracker. E, live cell confocal microscopy of TLR8u2YFP (green), ER-tracker (red), and a merge of the two. HEK293 cells transiently transfected with TLR8u2YFP show that TLR8u2 co-localizes with the ER-tracker. F, HEK293 cells were transfected with TLR8CFP and TLR8u5YFP. The cells were fixed and viewed by confocal microscopy. Sequential scanning was performed to generate images, with CFP in green and YFP in red. Co-localization of the two receptors can be seen, and similar co-localization patterns were observed for the other mutant receptors (supplemental Fig. S2 and data not shown). N, non-transfected, M, markers.
can cause this effect, and bafilomycin A1 abolished all signaling other than that mediated by the positive control interleukin-1.

**DISCUSSION**

The mechanism of signaling by all Toll and Toll-like receptors is likely to involve dimerization of the ectodomains, but this is achieved in remarkably diverse ways by different stimuli. For example, Drosophila Toll is activated by a cytokine ligand, Spatzle, whereas TLR4 signals in response to lipopolysaccharide indirectly through the co-receptor protein MD-2 (24–26). By contrast, the closely related subgroup TLR7–9 is stimulated by non-self nucleic acids or synthetic compounds structurally related to purine nucleosides and are thus likely to have related mechanisms of activation. In this paper, we have probed, using site-directed mutagenesis, the role played by two distinct sequences that are highly conserved in the TLR7/8/9 subgroups but absent in other family members. The first sequence was a short insert in LRR8 containing four cysteine residues. Bell et al. (18) have proposed previously that the insert is homologous to a short sequence in a cytoplasmic CpG-binding protein. However, the LRR8 insertion sequence was not identified as significantly similar to a cytoplasmic CpG-binding protein in homology searches (not shown); therefore, the significance of this similarity is unclear, especially as the extracellular cysteines of
TLR8 may be oxidized to form disulfide bonds. Nevertheless, it was clear from our results that the integrity of the LRR8 loop is critical for the signaling function of TLR8, as mutating the four cysteine residues in any combination abolished the capacity of the receptor to signal. We have also shown that a second site (Asp-543) in LRR17 was critical for receptor function. This residue was conserved in TLR7–9 but not in TLR3 (see Fig. 4A). Mutation of the corresponding residue in TLR9 abolishes specific binding of the ectodomain to CpG DNA in vitro (19).

We have also shown that, similar to TLR9, TLR8 is localized predominantly in the endoplasmic reticulum. In the case of TLR9, stimulation of dendritic cells with CpG DNA causes a profound reorganization of the ER membrane system, with projections moving out and potentially fusing with the plasma membrane (14). By contrast, our studies suggest that treatment of TLR8-expressing cells with resiquimod causes no gross perturbations of the ER (supplemental Fig. S2). Nevertheless, functional TLR8 must be present at some level in an acidified compartment, such as the late endosome or lysosome, as signaling is completely abolished by bafilomycin A1. The pH in the ER was 7.2 and was not acidified by v-type H^+ ATPases. On the other hand, bafilomycin A1 prevents acidification of late endosomes and lysosomes, which normally have a pH of ~5, and the Golgi, which has a pH of 6.5 (27), but does not affect vesicle trafficking (21). These findings raise the question of how TLR8 is trafficked into an acidified compartment. This could involve constitutive cycling of ER-derived vesicles through the Golgi from where they can be targeted to late endosomes and lysosomes. Alternatively, a mechanism similar to that proposed for TLR9 might operate in which projections of the ER are thought to fuse with the plasma membrane and then transferred receptors are internalized by endocytosis (14). Irrespective of the mechanism of delivery, the amount of receptor in the late endosome or lysosome must be a small fraction of the total (Fig. 2). It is unlikely that treatment with resiquimod induces trafficking of TLR8 to lysosomes, as no difference in TLR8 localization is seen between stimulated and unstimulated cells (supplemental Fig. S2). If the amount of TLR8 in acidified compartments is low, this could explain the enhancement we observed when cells were co-stimulated with resiquimod and oligodeoxynucleotides. The level of signaling would be directly proportional to the receptor number at low concentrations of TLR8, and CpG or GpC oligonucleotides would induce transfer of the ER membrane to acidified compartments, thereby increasing the TLR8 receptor number (14). On the basis of our results, it is also likely that TLR8 is unable to function in the ER, as resiquimod is a neutral molecule and cell-permeable. Although it is possible that all of the receptors seen in the ER are non-functional due to misfolding, it is more likely that low pH is a necessary prerequisite for signaling to occur. Certainly, as our signaling assay was dependent on vesicle acidification, a fraction of wild-type TLR8 in the ER must have been correctly biosynthesized and trafficked to the late endosome or lysosome. The observed pH dependence of signaling could operate at two levels. First, there could be conformational changes in the receptor ectodomains, presumably involving the ionization of histidine side chains. This kind of mechanism operates in the low density lipoprotein receptor, which binds tightly to low density lipoprotein at the cell surface.
but releases it in the late endosome. This occurs because a histidine-rich pseudoligand in the ectodomain becomes ionized and displaces the bound low density lipoprotein (28). Such a mechanism could contribute to pH dependence in the case of TLR8, as there are a number histidine residues in the ectodomain, although only one (His-575 in LRR18; see Fig. 4A) is conserved between TLRs 7–9. A second possibility is that the ligands themselves are activated as a consequence of lowered pH. In this regard, it is significant that the binding of CpG DNA to the TLR9 ectodomain is strongly pH-dependent, and binding is abolished by mutation of the residue corresponding to Asp-543 in TLR9 (19). This suggests that specific binding may be dependent on the ionization state of the CpG DNA or resiquimod and ssRNA in the case of TLR8. There are a number of ionizable groups in both polynucleotides and resiquimod. For example, cytidine has a pK_a of 4.2, although this value would be significantly higher when the base is substituted with phosphate in the nucleotide and polynucleotide forms. Thus, a significant fraction of the CpG DNA delivered to the lysosome would carry a positive charge but would be largely unprotonated in the cytoplasm or the ER. In a recent paper (13), a chimeric TLR9-TLR4 receptor has been shown to be expressed on the cell surface and to signal in response to CpG DNA, findings that appear to argue against such a mechanism. However, in those experiments, a high concentration of CpG DNA (3 μM) was used, and at the pH of the experiments, a subpopulation in the protonated form may have been sufficient to activate the surface-exposed receptor. CpG oligonucleotides are inefficiently taken up by cells, and the use of agents such as Dotap to stimulate uptake enhances signaling by up to 50-fold (23). Thus the overall concentration of CpG in the endosome or lysosome is likely to be much lower than that in the culture medium, but a much higher proportion would be in the conjugate acid form because of the lower pH.

In the case of the imidazoquinolines, a pK_a value of 6.9 has been measured for the imidazo group of imiquimod (29) and 7.2 for resiquimod.³ Thus, at pH 5, these molecules are likely to be present largely as the positively charged protonated species of the conjugate acid. In addition, resiquimod is a membrane-permeable weak base and, as such, the protonated form will accumulate in acidic compartments, in principle being 100× more concentrated in the late endosomes and lysosomes than in the ER. If the conjugate acid form of resiquimod accumulates in the acidified compartment, this would stabilize low affinity interactions and favor receptor activation. Thus, a plausible model is that activation of the TLR7–9 subgroup involves binding to negatively charged regions in the receptor ectodomain by ligands that develop a positive charge at pH 5 in the late endosome and lysosome. In fact, there are several conserved acidic residues in LRR17 and 18 in addition to Asp-543 located on both concave and convex surfaces of the solenoid (see Fig. 4A). In conclusion, our results argue that the nucleic acid-type ligands of TLR7–9 are activated by low pH and induce homodimerization of the receptor ectodomains, for example, by neutralizing surface charges and promoting hydrophobic aggregation (Fig. 4C). Alternatively, the ligands may bind to a co-receptor protein, an indirect mechanism similar to that used by TLR4 (24) (Fig. 4D).

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