The covalent modification and regulation of TLR8 in HEK-293 cells stimulated with imidazoquinoline agonists

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The mammalian TLRs (Toll-like receptors) mediate the rapid initial immune response to pathogens through recognition of pathogen-associated molecular patterns. The pathogen pattern to which TLR8 responds is ssRNA (single-stranded RNA) commonly associated with ssRNA viruses. TLR8 also responds to small, purine-like molecules including the imidazoquinoline IRMs (immune-response modifiers). The IRMs include molecules that selectively activate TLR7, selectively activate TLR8 or non-selectively activate both TLR7 and TLR8. Using HEK-293 cells (human embryonic kidney cells) stably expressing an NF-kB (nuclear factor κB)/luciferase promoter-reporter system as a model system, we have examined the regulation of TLR8 using the non-selective TLR7/8 agonist, 3M-003. Using conservative tyrosine to phenylalanine site-directed mutation, we show that of the 13 tyrosine residues resident in the cytosolic domain of TLR8, only three appear to be critical to TLR8 signalling.

Two of these, Tyr^{209} and Tyr^{304}, reside in the Box 1 motif and the third, Tyr^{294}, lies in a YXXM putative p85-binding motif. TLR8 is tyrosine-phosphorylated following 3M-003 treatment and TLR8 signalling is inhibited by tyrosine kinase inhibitors. Treatment with 3M-003 results in the association of the p85 regulatory subunit of PI3K (phosphoinositide 3-kinase) with TLR8 and this association is inhibited by tyrosine to phenylalanine mutation of either the YXXM or Box 1 motifs. As a further consequence of activation by 3M-003, TLR8 is modified to yield both higher and lower molecular mass species. These species include a monoubiquitinated form as deduced from ubiquitin peptide sequencing by HPLC/MS/MS (tandem MS).

Key words: covalent modification, immune response modifier, Toll/interleukin-1 receptor (TIR) domain, Toll-like receptor (TLR), tyrosine phosphorylation, ubiquitination.

INTRODUCTION

The survival of vertebrate species is dependent in part on their ability to mount a rapid defence to the sea of potential pathogens to which they are exposed. One evolutionary survival strategy has been for host species to reduce the complexity of the microbial world to recognition of a series of far more simple molecular patterns, which are recapitulated across multiple types of pathogens. This process of reductive defence is mediated through a family of receptors for such PAMPs (pathogen-associated molecular patterns) [1].

The mammalian TLRs (Toll-like receptors), homologues of the Drosophila Toll receptor, are among the PAMP receptors of the innate immune system. The ten different hTLRs (human TLRs) (TLR1–10) identified are type I transmembrane proteins. They are composed of an N-terminal extracellular domain with leucine-rich repeats involved in PAMP recognition, a single transmembrane domain, and a cytoplasmic domain largely made up of the TIR (Toll/interleukin-1 receptor) homology domain required for downstream signalling. TLR1, TLR2, TLR4, TLR5 and TLR6 are involved in recognizing PAMPs associated with bacterial membranes. TLR3, TLR7, TLR8 and TLR9 recognize oligonucleotide (RNA and DNA)-based molecular patterns from both bacteria and viruses. TLR7 and TLR8 also respond to 3M Pharmaceuticals imidazoquinoline IRMs (immune-response modifiers). The role of TLR10 has not been established and its corresponding PAMP has not been identified [1].

Much has been learned of the signalling pathways for the TLRs and the understanding of these pathways has been broadened beyond the initial assumptions that the TLRs shared a common signalling pathway, both with the IL-1R [IL-1 (interleukin-1) receptor] and with each other. It is now appreciated that there exists a great deal of heterogeneity among the individual TLR signalling pathways [2]. This heterogeneity includes the spectrum of adaptor proteins and downstream effector proteins utilized to access either the pathways to pro-inflammatory cytokine or those to type 1 IFN (interferon) (IFNκB) synthesis. Whereas the TLR3 and TLR4 type 1 IFN synthetic pathways are TRIF (TIR domain-containing adaptor protein inducing IFNβ)-dependent [TLR4 requires TRAM (TRIF-related adaptor molecule) as well], TLR7- and TLR9-stimulated IFNα is, instead, MyD88 (myeloid differentiation factor 88)-dependent. While TLR3 and TLR4 stimulation of type 1 IFN is mediated through a TBK1 [TANK (tumour-necrosis-factor-receptor-associated factor-associated factor-associated nuclear factor κB activator)-binding kinase 1]–IKKi (IkB [inhibitor of NF-κB (nuclear factor κB)] kinase i)–IRF3 (IFN regulatory factor-3) effector pathway, TLR7 and TLR9 stimulation of IFNα occurs through an IRAK1

Abbreviations used: Akt, thymoma viral proto-oncogene; ERK, extracellular-signal-regulated kinase; HA, haemagglutinin; HEK-293 cells, human embryonic kidney cells; Hrs, hepatocyte growth factor-regulated tyrosine kinase substrate; TLR, Toll-like receptor; hTLR, human TLR; IFN, interferon; NF-κB, nuclear factor κB; IRAK, IL-1 receptor-associated kinase; IRF, IFN regulatory factor; IRM, immune-response modifier; ITIM, immunoreceptor tyrosine-based inhibitory motif; JNK, c-Jun N-terminal kinase; LC, liquid chromatography; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MS/MS, tandem MS; MyD88, myeloid differentiation factor 88; nDHI, nuclear DNA helicase II; NFAT, nuclear factor of activated T-cells; PAMP, pathogen-associated molecular pattern; PBMC, peripheral blood mononuclear cell; PISK, phosphoinositide 3-kinase; PTK, protein tyrosine kinase; RhoGAP, Rho GTPase-activating protein; RT, reverse transcriptase; SH domain, Src homology domain; SIGIRR, single immunoglobulin domain-containing IL-1R-related protein; ssRNA, single-stranded RNA; ST2, suppressor of tumorigenicity 2; TAK1, TGF (transforming growth factor)-β-activated kinase 1; TIR, Toll/IL-1R; TNF, tumour necrosis factor; TRAF, TNF-receptor-associated factor; TRIF, TIR domain-containing adaptor protein inducing IFNβ; UIM, ubiquitin interaction motif; wt, wild-type.

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TLR8 activation of NF-κB has been reported. Although IRM-stimulated generating TLR8 gene-disrupted mice. Incomplete. Obviously, there has been limited value placed on experimental information on its regulation is, by comparison, to agonists otherwise known to be effective against hTLR8 [5], TRAF3 and TRAF6 [4].

By comparison, less is known about the regulation of TLR8 signalling. Since the murine form of this receptor is not responsive to agonists otherwise known to be effective against hTLR8 [5], experimental information on its regulation is, by comparison, incomplete. Obviously, there has been limited value placed on generating TLR8 gene-disrupted mice.

Recently, however, apparent anomalies in the downstream effectors of hTLR8 have been reported. Although IRM-stimulated TLR8 activation of NF-κB and JNK (c-Jun N-terminal kinase) is IRAK1-dependent, IRAK1 does not become modified, as seen downstream of other TLRs and of the IL-1R [6]. Similarly, IL-1R/TLR stimulation commonly results in TRAF6-mediated activation of IKK through a kinase complex centred on TAK1 [TGF (transforming growth factor)-β-activated kinase 1]. However, TLR8 signalling to the IKK complex is not dependent on TAK1, but is dependent, instead, on MEKK3 [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase kinase 3]. While both IkBα phosphorylation and degradation are common downstream consequences of TLR activation, TLR8 activation results in IkBα phosphorylation but, curiously, not IkBβ degradation [6]. It has been recently reported that the activation of TLR8 by IRMs can be dramatically enhanced by the inclusion of DNA oligonucleotides. This occurs in a manner that is wholly independent of TLR9 and appears unique to TLR8 [7]. In addition, co-expression of TLR8 with either TLR7 or TLR9 in HEK-293 cells (human embryonic kidney cells) suppresses the function of either. In turn, TLR8 function is not modulated by the co-expression of either TLR7 or TLR9, suggesting that this effect is not due to simple competition for a common adaptor [8].

Given these unanticipated properties of the TLR8 signalling pathway, we have sought to study the properties of TLR8 at rest and after activation by small molecule IRM agonists in transfected HEK-293 cells. Differences in the utilization of downstream adaptors and its interactions with other TLRs might logically be related to elements of structure and its modification.

Our attention was first drawn to the number and pattern of tyrosine residues within the TLR8 cytosolic TIR domain (Figure 1). Unique to all TLRs, TLR8 has 13 tyrosine residues in its cytosolic domain. Some of these tyrosine residues are arranged in patterns previously characterized in other proteins as interactive motifs. Among these are two potential ITIM (immunoreceptor tyrosine-based inhibitory motif; \{SILV\}XYXX\{LV\}) found at residues 869–874 and 1005–1010 [9,10]. Also found is a YXXM motif positioned at the C-terminus at residues 1048–1051 [11]. Both PI3K (phosphoinositide 3-kinase) binding and endosomal sorting functions have been attributed to this motif [11]. Another feature of TLR8, unique among the TLRs, is the tyrosine composition found in and adjacent to the Box 1 segment. This conserved segment, \{YF\}XX\{YF\}XX\{YF\}, is, again, unique in TLR8 in that tyrosine is found at each of the three positions (Figure 1B).

In order to determine the importance of the tyrosine residues in the cytosolic domain of TLR8, we have utilized a model system in HEK-293 cells transiently expressing wt (wild-type)- or sequence-modified TLR8. We have mutated the cytosolic tyrosine residues conservatively to phenylalanine residues individually or in combination. We demonstrate that certain of these tyrosine residues are critical for signalling activity and that TLR8 is significantly modified following stimulation with either a small molecule IRM agonist or ssRNA (single-stranded RNA). Modifications in addition to tyrosine phosphorylation include association with the p85 regulatory subunit of PI3K, the generation of ubiquitinated species migrating in SDS gels slower than the parent protein, as well as a third faster migrating TLR8 species. Immunoprecipitation of TLR8 following IRM treatment also reveals its association with the ‘DEAD/H’ (Asp-Glu-Ala-Asp/His) family member, nDHII (nuclear DNA helicase II).

**EXPERIMENTAL**

**Cells, reagents and plasmids**

NF-κB stable HEK-293 cell line was maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 units/ml penicillin and 50 μg/ml streptomycin. Monoclonal and polyclonal anti-HA (haemagglutinin) antibodies were purchased from Santa Cruz Biotechnology and Novus Biologicals respectively. Monoclonal anti-phosphotyrosine antibody (4G10) was obtained from Upstate Biotechnology, the monoclonal anti-PI3K, p85, was from BD Biosciences and polyclonal antibodies to native or phosphorylated ERK1/ERK2 and SAPK (stress-activated protein kinase)/JNK were purchased from Cell Signaling Technology. Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit IgG was purchased from Pierce Biotechnology. PTK (protein tyrosine kinase) inhibitors were obtained from EMD Biosciences and Biocon.

The TLR8 cDNA constructs were made by cloning hTLR8 isoform 1 fragment into pcDNA3 vector (Promega). The vectors with various N-terminal epitope tags (FLAG, HA, c-Myc and 6 × His) were constructed by inserting the tags after the predicted signal peptide sequence (after 45 residues for TLR8 isoform 1) in pcDNA3-hTLR8 clone. All the tyrosine mutants of TLR8 were generated either by PCR or using a QuikChange® site-directed mutagenesis kit (Stratagene) using TLR8 plasmids as the template. All mutations were confirmed by sequencing.

**NF-κB luciferase transient transfection assay**

Transient transfections were performed as described previously [12]. NF-κB stable HEK-293 cells were seeded at 1 × 10⁵ cells/well in a 6-well tissue culture plate (Becton Dickinson) and transfected with 1 μg of wt-TLR8 or mutant TLR8 expression vectors using 3 μl of FuGENETM 6 transfection reagent (Roche) following the manufacturer’s instructions. Transfection efficiency was monitored by immune blotting of TLR8 proteins using anti-HA antibody. The plates were incubated at 37°C in 5% CO₂ for 40 h. The cells were harvested and plated at 50 000 cells/well on to a white 96-well plate (Nunc), allowed to adhere for approx. 4 h and then stimulated with various concentrations of small molecule TLR7 and TLR8 agonists in DMEM. The plates were incubated for an additional 16–18 h. NF-κB luciferase assay was performed by lysing the cells with reporter lysis buffer (Steadylite HTS; PerkinElmer) and the lysate was assayed for luciferase activity by using a Lmax luminometer (Molecular Devices). For each experiment, luciferase assays were performed in triplicate, and each experiment was repeated at least four times. Fold induction of NF-κB luciferase was calculated and averaged against uninduced controls.

**Effect of PTK inhibitors on TLR8 response**

A variety of PTK inhibitors were tested for their effect on IRM NF-κB response in HEK-293 cells transfected with TLR8.
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Figure 1  TIR-domain amino acid sequence of hTLRs

(A) Alignment of the amino acid sequence using ClustalW and Boxshade. The three conserved regions (boxes 1–3) across all TIR domains are shown. (B) Amino acid sequence of cytosolic domain of hTLR8 depicting putative ITIM motifs (underlined), PI3K motif (double underlined) and the Box 1, 2 and 3 domains (boxed).

40 h of transfection, the cells were plated at 50000 cells/well (in 100 µl of medium) on to a white 96-well plate and allowed to adhere for approx. 4 h. The cells were then treated with 1 µl of various dilutions of the inhibitors in DMSO for 30 min to 1 h, followed by addition of IRM (3 or 10 µM). The cells were incubated for an additional 16–18 h and NF-κB luciferase assay was performed as described above. For each experiment, luciferase assays were performed in triplicate, and each experiment was repeated at least three times.

Detection of phosphotyrosine residues in TLR8
NF-κB stable HEK-293 cells transfected with wt-HA–TLR8 or mutants in the putative PI3K motif were seeded at 2 × 10^5 cells/well and treated with 20 µM IRM in the presence of 200 µM vanadate to inhibit dephosphorylation. The cells were lysed in 1× PBS (pH 7.4) (Biosource) containing 1% (v/v) Triton X-100, 0.5% Igepal CA-630, 3 mM Na3VO4 (sodium orthovanadate) and 1× protease and phosphatase inhibitors (protease inhibitor cocktail III and phosphatase inhibitor cocktail II; EMD Biosciences). Lysates containing 1 mg of protein were immunoprecipitated with 2 µg of anti-HA antibody (Novus Biologicals) pre-absorbed to 15 ml of Dynabeads Protein G (Invitrogen) for 4 h at 4°C. Beads were washed twice with lysis buffer and isolated using a Dynal MPC-S magnet. Immunoprecipitates were boiled in 1.25× LDS (lithium dodecyl sulfate) sample buffer for 10 min and resolved on 4–12% Bis/Tris gels. Lysates containing 1 mg of protein were immunoprecipitated with 2 µg of anti-HA antibody (Novus Biologicals) pre-absorbed to 15 ml of Dynabeads Protein G (Invitrogen) for 4 h at 40°C. Beads were washed twice with lysis buffer and isolated using a Dynal MPC-S magnet. Immunoprecipitates were boiled in 1.25× LDS (lithium dodecyl sulfate) sample buffer for 10 min and resolved on 4–12% Bis/Tris gels (Bio-Rad). Tyrosine phosphorylation was detected by Western blotting with 4G10 antibody (Upstate Biotechnology). Blots were stripped...
and also reprobed with specific antibodies for detecting HA–TLR8, ubiquitin or p85, the regulatory subunit of PI3K using p85 antibody. Loading uniformity was confirmed by monitoring consistency of otherwise irrelevant non-specific bands in the blots.

MAPK activation assay
At 40 h post transfection, NF-κB stable HEK-293 cells expressing either wt or mutant HA–TLR8 (1 × 10⁵ cells/well) were washed twice with serum-free medium and incubated in serum-free medium 1 h prior to stimulation. At various time points following drug treatment, the medium was removed and the cells were lysed with ice-cold lysis buffer (PBS, pH 7.4, 1% Triton X-100, 0.5% Igepal CA-630, 3 mM Na₂VO₄ and 1×protease/phosphatase inhibitor cocktails). Lysates were centrifuged at 20°C for 6 min (25 000 g), and supernatants containing equal amounts of protein were used for Western blotting.

Protein sequencing
Protein sequencing was performed by Protein Research Services (Ann Arbor, MI, U.S.A.). An Investigator ProPic robot was used to excise selected protein spots from polyacrylamide gels. The samples were prepared by in-gel trypsin digestion of proteins, performed robotically using Investigator ProGest. Peptides were analysed by nano-LC (liquid chromatography)/MS/MS (tandem MS) on a Micromass Q-TOF2 MS/MS spectrometer interfaced with a nano-LC system. The MS/MS data were searched using a local copy of MASCOT (http://www.matrixscience.com). An identical procedure was utilized on samples submitted to 3M Corporate Analytical Laboratory.

RESULTS
Tyrosine residues in the cytosolic domain of TLR8
Depiction of a ClustalW alignment of the TIR domains of the ten hTLRs illustrates elements of structure that are common to this family of receptors (Figure 1A). These common elements include the Box 1 domain, the BB loop or Box 2 domain, and the Box 3 domain. Previous reports have indicated that mutation of residues in either Box 1 or Box 2 is inhibitory to the downstream signalling of the receptor [14]. Review of Figure 1(B) reveals in detail the 13 tyrosine residues found in the cytosolic domain of TLR8, the conserved Box 1, 2 and 3 domains, and the putative ITIM and the YXXM PI3K binding or endosomal sorting motifs. TLRs 1, 2, 5 and 6 also contain YXXM putative PI3K-binding motifs in their TIR domains.

Tyrosine residues are not found in the transmembrane domain of TLR8, but Tyr²⁷¹, included in one of the putative ITIM motifs, is very closely positioned near the transmembrane domain. This would not, however, be typical of a functional ITIM as these commonly appear near the cytoplasmic tail of their host protein [9,10]. The Box 1 sequence of TLR8 is unique to all TLRs in that all three of the aromatic residues are tyrosine, Tyr²⁹⁸, Tyr³⁰¹ and Tyr³⁰⁴ (YXXYXXY). The TIR domain has four additional residues, Tyr²⁷¹, Tyr³⁰⁶, Tyr³⁷⁹ and Tyr¹⁰⁰⁷. Three additional tyrosine residues, Tyr¹⁰⁴₈, Tyr¹⁰⁵₂ and Tyr¹⁰⁵⁹ are clustered near or at the C-terminus, and Tyr¹⁰⁴₈ forms part of the putative PI3K or endosomal-sorting motif (YXXM/Φ).

It is of interest to note that three of the TLR receptors have a phenylalanine residue as the first aromatic residue in their Box 1 domain: TLR1, TLR6 and TLR10 (Figure 1A). None of these three are known to signal independently, although TLR1 and TLR6 have co-receptor functions with TLR2. A recent report indicates that neither TLR1 nor TLR6 is able to bind MyD88. However, conservative mutation of the N-terminal phenylalanine to tyrosine imparts MyD88 binding to these receptors [14]. Such reports underscore the importance of this tyrosine residue in the Box 1 domain. Perhaps a failure to identify a ligand for TLR10 relates to it acting in a co-receptor or modulatory function, which has yet to be elucidated [13,15].

Identification of cytosolic tyrosine residues critical to TLR8 signalling in HEK-293 cells
Prior to the conduct of these studies, the activities of the wt-TLR8 and wt-HA–TLR8 were compared for their responsiveness to the TLR7/8 IRM agonist 3M-003. These were shown to be equivalent with respect to the amount of DNA transfected and the magnitude of the NF-κB/luciferase response stimulated with 3M-003 in the HEK-293 cell model system that we employed (results not shown).

We have examined the roles of the tyrosine residues in TLR8 signalling by mutating them conservatively to phenylalanine residues individually or in combination. For this purpose, stably expressing NF-κB/luciferase HEK-293 cells were transiently transfected with an expression vector of wt- or mutant-TLR8. Luciferase expression levels were measured after treatment with 3M-003.

As shown in Figure 2, conservative mutation of only four of the 13 tyrosine residues resulted in a statistically significant change in IRM-induced TLR8 activation of NF-κB. The TLR8 response to 3M-003 was significantly lower (P < 0.01) when any of the residues Tyr²⁹⁸, Tyr³⁰⁴ and Tyr¹⁰⁴₈ were mutated to phenylalanine. The response to changes of residues Tyr³⁰¹, Tyr³⁰⁶, Tyr³⁷⁹, Tyr³⁰¹, Tyr³⁰⁶, Tyr³⁰⁷, Tyr³⁰⁷, Tyr³⁰⁷, Tyr³⁰⁷ and Tyr³⁰⁹⁹ was not statistically significant and was similar to that observed in the wt. The triple mutation of Tyr³⁰⁴₈, Tyr³⁰⁷ and Tyr³⁰⁹⁹ had the same effect as Y1048F alone. Interestingly, the mutation of residue Tyr³⁰⁹⁹ significantly enhanced the TLR8 response (P < 0.01). Tyr³⁰⁹⁹ is not a conserved residue, nor does it reside in a highly conserved region among the TLR TIRs. Tyrosine mutations in the putative ITIM motifs were without effect, and we conclude that these are without apparent inhibitory function. In all cases, similar results were also obtained with the TLR8-selective IRM, 3M-002, and with ssRNA (results not shown).

Effects of inhibitors of tyrosine kinases on TLR8-mediated activation of NF-κB
Tyrosine residues play important roles in various aspects of signal transduction and are prominent in protein–protein interaction sites [16]. The tyrosine side chain has both hydrophobic and hydrophilic properties in its aromatic ring and hydroxy group respectively. As such, tyrosine can participate in hydrophobic, amino-aromatic and van der Waals interactions, via its aromatic ring and hydrogen-bonding through its hydroxy group. Of these, Tyr → Phe mutation would affect only the latter [17].

Nevertheless, having observed that of the three tyrosine residues of Box 1, only two were shown, individually, to be critical to activity, we asked first whether the importance of these tyrosine residues related to their being potential sites for phosphorylation by tyrosine kinases. This question was first approached by screening known inhibitors of various tyrosine kinases and determining their effects on IRM/TLR8 activation of the NF-κB reporter in transfected HEK-293 cells.

Table 1 lists the spectrum of model tyrosine kinase inhibitors evaluated and their apparent IC₅₀ values in TLR8-transfected
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Figure 2  Requirement of tyrosine residues in the cytoplasmic domain of hTLR8 for NF-κB induction by IRMs

Stable NF-κB luciferase HEK-293 cells were transfected with wt-TLR8 or TLR8 tyrosine mutants. After 40 h of transfection, the cells were treated with 10 µM of the IRM 3M-003, and after 16 h, luciferase levels were measured. Results are expressed as fold change relative to vehicle (DMSO) control and results shown are representative of at least four independent experiments (**P < 0.01).

Table 1  Effects of kinase inhibitors on IRM-induced responses in human PBMCs and in NF-κB stable HEK-293 cells transfected with either TLR7 or TLR8

Human PBMCs were pretreated for 30 min with different concentrations of the kinase inhibitors listed or with DMSO (control). The cells were then stimulated overnight with 5 µM TLR7-selective 3M-001, 3 µM TLR8-selective 3M-002 or 10 µM non-selective 3M-003. IFNα and TNFα measured in the supernatants by ELISA. Experiments using HEK-293 cells were conducted as described in the Experimental section. Although the inhibitors show broader activity, their nominal selectivities are listed. Mean IC50 values (+ S.D., n = 3) are shown for each compound tested. Where values are greater than 33.3 µM the compound was deemed to be inactive. Abbreviations: NKS, nominal kinase selectivity; PDGFR, platelet-derived-growth-factor receptor; Stim., stimulation.

<table>
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<th>Inhibitor</th>
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<th>Stimulus . . .</th>
<th>IC50 (µM)</th>
<th>Stimulated cell types</th>
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HEK-293 cells stimulated with 3M-003. Herbimycin A, SU11562 and typhostin 9 are potent inhibitors of TLR8 activation of NF-κB in this system and show similar inhibition of IFNα and TNFα in 3M-003-stimulated human PBMCs (peripheral blood mononuclear cells). Other general tyrosine kinase inhibitors, such as PP1 {4-amino-5-(4-methylphenyl)-7-(t-butyl)-pyrazolo[3,4-d]pyrimidine}, PP2 {4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine} and genistein, in addition to SYK (spleen tyrosine kinase) inhibitor, were active in the PBMC, but failed to inhibit IRM-stimulation of NF-κB in HEK-293 cells.

The rest of the compounds were tested as part of TLR8 signalling characterization and are shown to be active only in the PBMC. Wortmannin inhibition of 3M-001-induced IFNα
stabilization suggests that the differences between these IRMs goes beyond selectivity of the former for TLR7 and dual TLR7–
TLR8 selectivity for the latter. Comparison of the PI3K inhibitors, wortmannin and LY294002, show further the pitfalls of reliance on inhibitor data to define signalling pathways.

While inhibitor data in this instance are by no means definitive, these data may suggest that the role for critical tyrosine residues revealed by conservative Tyr → Phe mutation may be related in some cases to their being sites of tyrosine phosphorylation. Furthermore, comparison of the data generated in the human PBMC with that in the recombinant HEK-293 system demonstrates the limitations of the latter as a model for primary human immune cells.

**IRM-induced phosphorylation of TLR8 and p85 association**

Since few discrete tyrosine residues of TLR8 were critical for the IRM response and some tyrosine kinase inhibitors inhibited the 3M-003 response in the HEK-293 system, we next determined whether TLR8 was in fact tyrosine-phosphorylated following IRM stimulation. As shown in Figure 3, tyrosine phosphorylation of HA–TLR8 is observed in response to IRM. An HA-labelled phosphotyrosine-specific protein band is seen most prominently in IRM-treated samples. Although we routinely observed the earliest phosphorylation to occur at approx. 30 min, there is a late phosphorylation of TLR8 beginning at 2 h. Also observed is an increase in HA–TLR8 protein expression, which corresponds temporally to heavier phosphorylation. This increase in TLR8 phosphorylation is inhibited directly (Figure 5).

**Figure 3 Tyrosine phosphorylation of wt-TLR8 in response to IRM**

Stable NF-κB luciferase HEK-293 cells expressing wt-HA–TLR8 were treated with 20 μM of TLR7/8 (mixed) IRM, 3M-003 or DMSO (vehicle control) for various time points. In addition, stable NF-κB luciferase HEK-293 cells transfected with vector (pCINEO) were treated with 3M-003. Cell lysates were immunoprecipitated with anti-HA antibody and Western blotted with anti-HA antibody (A) or anti-phosphotyrosine antibody (B). These results are typical of three independent experiments.

Inhibition of TLR8 activation of the NF-κB response by the tyrosine kinase inhibitors (Table 1) could be attributed to effects on signalling components downstream of TLR8 itself. In the cases of tyrphostin 9 and herbimycin, however, TLR8 tyrosine phosphorylation is inhibited directly (Figure 5).

A reciprocal blot using the 4G10 anti-phosphotyrosine antibody for immunoprecipitation and anti-HA antibody for immunoblotting showed identical results (results not shown). These results clearly indicate that TLR8 undergoes tyrosine phosphorylation following IRM stimulation. While discrete tyrosine residues appear essential to TLR8 activation by IRMs, the bulk of tyrosine phosphorylation paradoxically occurs within a timeframe that would not be anticipated for the initiation of receptor signalling.

In addition to the wt-HA–TLR8, we next examined tyrosine phosphorylation of HA–TLR8-bearing mutations in the putative PI3K p85-binding motif, YXXM (Figure 6A). The double mutant, FXXL (Y1048F/M1051L), had substantial increase in neither tyrosine phosphorylation nor TLR8 protein. This observation also corresponded well to a decreased NF-κB response and IL-8 secretion seen with these mutants (results not shown).

It should be noted that tyrosine phosphorylation, although greatly diminished, is still observed in the FXXL double mutant. Since direct p85 binding to this motif is dependent on phosphorylation of its resident tyrosine, this suggests that other TLR8 tyrosine(s), in addition to that in the p85-binding motif, are subject to phosphorylation. Other tyrosine to phenylalanine mutations in the cytosolic domain of TLR8 also result in a loss of its signalling and overall levels of its tyrosine phosphorylation. Compared with the wt Box 1 sequence, Y898XXYXY904, the double mutant, F898XXYXXF904, fails to signal, while the single mutant, Y898XXF904, signals normally (Figures 2 and 6). As shown in Figure 6(B), the phosphorylation occurring at the latter time points in the wt is largely abated in the F898XXYXXF904 double mutant. Although phosphorylation is diminished in the FXXYXXF904 mutant, total TLR8 protein species increase, unlike that seen in the FXXL mutant. As such, mutation of tyrosine residues in either the Box 1 segment or in the C-terminal YXXM motif results in diminished late phosphorylation.

Clearly, loss of general tyrosine phosphorylation upon phenylalanine substitution of a particular tyrosine cannot be strictly attributed to its being the site of phosphate addition. Substitution of critical tyrosine residues may have significant effects on function through less direct effects on TLR8 conformation and downstream interactions with adaptor molecules.

In order to determine if the YXXM sequence in TLR8 is indeed a p85-subunit-binding site, we sought to detect the association of the regulatory subunit p85 of PI3K with wt-HA–TLR8 and motif mutants following IRM treatment by immune co-precipitation.
IRM-induced modification and regulation of TLR8

As shown in Figure 6, the p85 subunit was co-precipitated with wt-HA–TLR8. The p85-binding-domain double mutant, FXXL, showed no p85 association. The same is seen with the FXXM single mutant (results not shown). Association of p85 with wt-HA–TLR8 was observable within 1 h and was increasing up to 4 h with the levels remaining similar up to 8 h. The 2 h point coincides temporally with the increase in tyrosine phosphorylation.

Also shown in Figure 6(B) is the effect of the Box 1 mutations on p85 binding. The F898XXYXXF904 double mutant is neither heavily phosphorylated nor does it bind p85 to the degree seen in the wt. This underscores the fact that mutation of tyrosine in one locale can affect functions at another, perhaps through changes in protein conformation or intracellular trafficking. Alternatively, p85 association with TLR8 need not be direct but via association of the latter with a p85-binding adaptor molecule. In this case, loss of p85 association with TLR8 would be a failure to recruit such an adaptor.

In follow-up to the observation of p85 binding, a role for PI3K was investigated by determining the effects of two PI3K inhibitors, wortmannin and LY294002, on TLR8-mediated NF-κB activation. Also examined was change in the phosphorylation state of Akt (thymoma viral proto-oncogene; also called protein kinase B), an obligate downstream effector of PI3K [18,19]. The conduct of numerous experiments in the HEK-293 system consistently failed to reveal either inhibition of TLR8-mediated NF-κB activation by PI3K inhibitors or Akt phosphorylation following TLR8 activation. Immunoblotting with antibody to

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PI3K p110 isoforms failed to reveal the presence of the catalytic subunit in these immune complexes. As is the case with p85 in other cellular systems, the precise role of the association of the PI3K p85 regulatory subunit with TLR8, direct or indirect, remains to be determined [20–22].

MAPK activation is an early event following TLR8 activation

Since the major modifications seen so far in TLR8 following IRM treatment occur at relatively late times, we sought to examine early events to determine if the late tyrosine phosphorylation could be a secondary response to an IRM-induced secreted cytokine. We confirmed the early activation of ERK1/2, p38 and JNK MAPKs. As shown for ERK1/2 and JNK in Figure 7, 3M-003 activation of these MAPKs is TLR8-dependent and occurs within the first 15 min of compound exposure. TLR8 is thought to be localized intracellularly in the endoplasmic reticulum [23,24]. As such, it might be reasoned that the apparent delay in TLR8 phosphorylation, and its coincidence with the responses monitored in Figure 2, is due to inefficient access of the IRM to this intracellular compartment in the HEK-293 model system as compared with human PBMCs. However, the rapid TLR8-dependent activation of the MAPKs would not support this suggestion.

The activation of NF-κB is both a consequence of TLR8 activation and a prerequisite for TLR8 induction of cytokines. To determine if such cytokines functioning in an autocrine fashion stimulate late TLR8 phosphorylation, their synthesis was inhibited by pretreatment of the cells with the proteasome inhibitor MG-132 (carbobenzoxy-L-leucyl-L-leucyl-leucinal) [25,26]. Whereas TLR8 induction of cytokines is not dependent on proteosomal degradation of IκBα [6], processing of both the p50 and p52 subunits of NF-κB is [27]. MG-132 was shown to completely inhibit 3M-003-stimulated IL-8 formation (results not shown) and the NF-κB luciferase reporter at a concentration of 2 μM (Figure 7A). In addition, HA–TLR8-transfected HEK-293 cells were pretreated with 2 μM MG-132 and then left without 3M-003 stimulation (zero time) or with 3M-003 for the times indicated. HA–TLR8 was immunoprecipitated from the cell lysates, prepared for Western blotting and then probed with anti-HA antibody and, subsequently, with anti-phosphotyrosine antibody. The lysates were also analysed directly by Western blotting to determine the status of JNK and ERK.

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Figure 6 Tyrosine phosphorylation of wt-TLR8 and TLR8 tyrosine mutants in response to IRM

(A) Tyrosine phosphorylation of wt-TLR8 and TLR8-PI3K motif mutant. (B) Tyrosine phosphorylation of wt-TLR8 and TLR8-Box 1 mutant. Stable NF-κB luciferase HEK-293 cells expressing wt-HA–TLR8 or mutant-HA–TLR8 were treated with 20 μM of TLR7/8 (mixed) IRM, 3M-003, for various time points. Cell lysates were immunoprecipitated with anti-HA antibody and Western blotted with anti-HA antibody, anti-phosphotyrosine antibody and anti-p85 antibody. These results are typical of three independent experiments.
IRM-induced modification and regulation of TLR8

Figure 7  Effect of proteasome inhibitor MG-132 on the activation of NF-κB, MAPK and TLR8 induced by IRM

Stable NF-κB luciferase HEK-293 cells were transfected with wt-TLR8, pretreated with MG-132 at the concentrations indicated and subsequently challenged with 10 μM of TLR7/8 (mixed) IRM, 3M-003, for 4 h. MG-132 inhibition of NF-κB activation was monitored by determination of luciferase activity (A). Also, stable NF-κB luciferase HEK-293 cells were transfected with wt-TLR8 and challenged with 10 μM 3M-003, or pretreated with 2 μM MG-132 and then challenged with 10 μM 3M-003 for the times indicated. Cell lysates were immunoprecipitated with anti-HA antibody and Western blotted with anti-HA antibody and anti-phosphotyrosine antibody. Activation of JNK and ERK was determined by Western blotting of the whole cell lysates using phospho-specific antibodies as described in the Experimental section (B). These results are typical of three independent experiments.

MAPKs. As shown in Figure 7(B), inhibition of the activation of NF-κB and its downstream cytokines has minimal or no effect on increased TLR8 protein expression or TLR8 tyrosine phosphorylation. This demonstrates that the TLR8 protein expression and phosphorylation effects noted are independent of the activation of NF-κB.

Modification of TLR8

Close examination of Western blot images of anti-HA-immunoprecipitated HA–TLR8 reveals the appearance of additional anti-HA reactive species following cell activation with 3M-003. The increase in HA–TLR8 expression at later time points is composed of an apparent faster migrating species and an anti-HA reactive band at higher molecular mass (Figure 8). To examine the relationship of these new species to that of HA–TLR8 observed prior to treatment with 3M-003, samples were immunoprecipitated with anti-HA antibody, separated by using SDS/PAGE, identified in the stained gel, carefully excised from the gel and prepared for sequencing by LC/MS/MS as described in the Experimental section. The information in Table 2 summarizes the results from these studies relative to bands 1, 2 and 3 from the gel-resolved immunoprecipitates (Figure 8).

The major or highest scoring protein, immunoprecipitated with anti-HA antibody, was identified in each of the bands as isoform 1 of hTLR8. This indicates that significant modification of this protein occurs, in addition to tyrosine phosphorylation, leading to both slower and faster migrating species.

As shown, ubiquitin was the second major protein identified in bands 1 and 2. In separate experiments, anti-HA immunoprecipitates derived from HA–TLR8-transfected cells were
Figure 8 Tyrosine phosphorylation of wt-TLR8 induced by IRM results in modification of TLR8

Stable NF-κB luciferase HEK-293 cells expressing wt-HA–TLR8 were treated with 20 µM of TLR7/8 (mixed) IRM, 3M-003, for various time points. Cell lysates were immunoprecipitated with anti-HA antibody and Western blotted with anti-HA antibody. Band 1 is a higher molecular mass species and band 3 is a faster migrating species.

separated by using SDS/PAGE, blotted and probed with either FL-76 rabbit polyclonal anti-ubiquitin antibody or FK-2 mouse monoclonal anti-ubiquitin antibody. Both of these antibodies recognize ubiquitin associated with the two higher molecular mass proteins co-immunoprecipitated with TLR8 using anti-HA antibody (Figure 9). As is particularly evident in Figure 9(B), using the mouse monoclonal FK-2 antibody, ubiquitin is not seen to be associated with faster migrating proteins, which would be expected for potential degradation products of TLR8. The DEAH box protein, nDHII, was identified with TLR8 as a protein in the faster migrating band 3. Any role it may have relative to TLR8 is unknown.

DISCUSSION

TLR8 is one of four TLRs that respond to oligonucleotide (RNA and DNA)-like structures. Each of these resides intracellularly in the endosomal compartment and includes TLR3, TLR7 and TLR9 [1,23,24]. In addition, TLR7 and TLR8 are known to respond to small purine-like molecules [28,29]. 8-Oxo-guanine-like molecules such as loxoribine are reported to be TLR7 agonists [30] and 3M Pharmaceuticals imidazooquinoline IRMs are potent agonists of TLR7 and/or TLR8 [5,31]. ssRNA has been shown to be the biological agonist of TLR7 and TLR8 [32]. While some evidence has been reported for direct or indirect ligand binding of cognate agonists to the other TLRs [33–36], no such evidence exists for either TLR7 or TLR8. Our own work has included exploration with biologically active 125I-labelled IRM photoaffinity probes, immobilization of IRM on supports for affinity isolation, and similarly immobilized IRMs for surface plasmon resonance detection of binding, and panning of phage displayed human PBMC cDNA libraries. As such, little is known about the regulation of TLR8 signalling and less still of how these small molecule agonists activate the TLR8 pathway in the absence of detectable TLR binding.

Recent reports have suggested that properties of TLR8 differ from those of other TLRs. While it is known that the pro-inflammatory cytokines induced by TLR8 are regulated by NF-κB, the pathway from TLR8 to NF-κB activation appears not to include the degradation of IRAK1, although the pathway is IRAK1-dependent. TLR8-stimulated activation of NF-κB is dependent on MEKK3 rather than TAK1 and results in IκBα phosphorylation but not its degradation [6]. Small molecule stimulation of TLR8 activity is also subject to modulation with DNA oligonucleotides. Although the same oligonucleotides have no apparent stimulatory activity on their own, they dramatically enhance the TLR8 response to IRMs [31]. TLR8 also engages in functional interactions with TLR7 and TLR9. In HEK-293 cells, co-expression of TLR8 with either TLR7 or TLR9 inhibits the capacity of the latter two to respond to their agonists [8]. To better understand

Table 2 Identification of proteins immunoprecipitated with anti-HA antibody from 3M-003-treated HEK-293 cells transfected with HA–TLR8

<table>
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<th>Rank</th>
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<th>Mass</th>
<th>Accession number</th>
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the activities and regulation of TLR8, we have undertaken the present studies utilizing HEK-293 cells as a model system.

TLR8 is endowed with 13 tyrosine residues in its cytoplasmic domain. Three of these are found in the Box 1 portion (Y109, XXYXYX114) of the TIR domain and conservative phenylalanine mutation of either Tyr109 or Tyr114 results in a loss of TLR8 signalling in response to either IRMs or ssRNA. It is of interest to note that TLR8 is the only TLR with this configuration of tyrosine residues in Box 1 (Figure 1). With regard to its Box 1 sequence, TLR8 has greater similarity to the IL-1R-related family of receptors [1]. This family includes orphan receptors such as TIGIRR1 (three immunoglobulin-domain-containing IL-1R-related protein 1), IL-1RAP1 (IL-1R accessory protein 1), SIGIRR (single immunoglobulin-domain-containing IL-1R-related protein) and T1/ST2 (suppressor of tumorigenicity 2). It is of interest to note that neither SIGIRR nor ST2 appears to have signalling activity, but they do act as inhibitors of the signalling of IL-1R and various TLRs [37,38]. hTLR8 exhibits both properties of the signalling TLR family members and the inhibitory IL-1R family members. Both human and murine TLR8, when co-transfected in HEK-293 cells with either TLR7 or TLR9, inhibit their ability to signal in response to TLR7 agonists or TLR9 agonists respectively [39]. It is widely accepted that murine TLR8 is inactive; however, its main function in murine cells may be that of a negative modulator of other endosomal TLRs.

We have demonstrated that Y898F mutation, the N-terminal tyrosine in Box 1, inhibits TLR8 signalling. All signalling TLRs have tyrosine in this position, while non-signalling TLR1, TLR6 and TLR10 have phenylalanine. We have observed that mutation of this N-terminal Box 1 tyrosine in TLR4, TLR7, TLR8 and TLR9 inhibits the signalling of these TLRs to their respective agonists (R. Rajagopal, A. S. Waller, J. D. Mendoza and P. D. Wightman, unpublished work). Previous reports establish similar effects on TLR3 signalling by Tyr → Phe mutation within Box 1 of this TLR, although its sequence pattern is YXXXHXXXY [40,41]. It may be deduced that tyrosine phosphorylation in Box 1 plays a critical role in TIR adaptor binding. Similarly, it has been demonstrated that mutation of the N-terminal Phe → Tyr in TLR1 and TLR6 Box 1 imparts to these TLR the ability to bind MyD88 which was absent from the wt. However, no effect is seen with analogous mutation within the Box 1 of TLR10 [14]. While the Box 2 region of the TIR is thought to represent a major point of contact with MyD88 [42], tyrosine phosphorylation in Box 1 may exert a conformational effect on the nearby BB loop, which is conducive to TIR–TIR homotypic interactions either in TLR dimerization or TLR/MyD88 binding. Homology modelling comparing the TIR of TLR8 with the crystal structures of the TIR of TLR1, TLR2 and IL-1RAP1 is being pursued to address this question.

The YXXM putative P13K p85-binding motif also appears critical to TLR8 function. Conservative mutation within this motif abrogates TLR8 signalling in HEK-293 cells as determined from inhibition of IL-8 synthesis and activation of the NF-κB reporter assay. For this to actually function in p85 binding, it is essential that Tyr108 be phosphorylated. Indeed we have demonstrated that tyrosine residues in TLR8 are phosphorylated and that TLR8 function is inhibited by inhibitors of tyrosine kinases. We also show p85 to be co-immunoprecipitated with TLR8 following its stimulation with 3M-003, suggesting that this may be a functional p85-binding motif. We show that inactivation of TLR8 signalling by mutation in Box 1 also inhibits p85 binding. As such, it is not possible to identify specific sites of TLR8 tyrosine phosphorylation, from among the 13 available, by scanning mutation of tyrosine residues and should be accomplished by physical/chemical methods.

Although p85 is associated with TLR8 following IRM treatment, direct or not, we are not able to show any effect of wortmannin or LY294002 on TLR8 signalling in HEK-293 cells. As noted above, we also do not observe IRM-induced activation or phosphorylation of Akt. Previous studies claim P13K to exert either stimulatory or inhibitory regulation on TLR signalling and, accordingly, show inhibition or enhancement of TLR function in the presence of these P13K inhibitors [40,43–45]. Depending on the inhibitor and the cytokine being measured, our results for the PBMC in Table 1 exhibit both. In the case of TLR9 function, P13K appears to be instrumental in shutting CpG DNA to vesicular TLR9 and P13K inhibitors block the overall TLR9-mediated cytokine response [46]. Flagellin activation of TLR5 in T84 cells results in stimulation of IL-6 and IL-8 formation. Pretreatment with wortmannin relieves the negative modulation exerted by P13K and enhances production of these cytokines [43]. In marked contrast, LY294002 has been shown to inhibit flagellin-induced IL-8 production in NCM460 cells [44]. This study also shows indirect association of p85 with TLR5 mediated through binding to the YXXM motif in MyD88. Others have reported p85 association with TLR2 and TLR3 following agonist stimulation and the signalling of these TLRs is wortmannin-sensitive [40]. Most recently, Hazeki et al. [47] have provided evidence that P13K is a general negative modulator to TLR signalling and cites evidence from TLR2-, TLR4- and TLR9-based systems for their conclusions. Suggestions regarding the role of P13K in TLR signalling can be influenced by the system(s) used in individual studies. Some reports are based on primary cells and native components, while others use recombinant model cell systems using transfected components. Other conclusions are based on the use of wortmannin and/or LY294002 P13K inhibitors. It is instructive to note that the data for these compounds within a given system do not necessarily agree. Where one inhibits, the other may not [47]. Some of them use recombinant dominant-negative P13K constructs, while others use siRNA (small interfering RNA) or other knock-down methodologies. At present, it is not prudent to make generalized statements about the role(s) of P13K in TLR signalling. This remains an open area of investigation.

It is not without precedent that p85 association with a receptor plays a role independent of p110, phosphatidylinositol phosphorylation or Akt activation. The p85 regulatory subunit has several motifs that would support interaction with other signalling elements. These motifs include three SH2 domains (Src homology 2 domains), a single SH3 domain and a RhoGAP (Rho GTPase-activating protein) domain [48].

The small GTP-binding protein Rac1 has been shown to bind to p85 through the RhoGAP domain. p85 has been shown to serve an adaptor function in T-cells leading to the activation of NFAT (nuclear factor of activated T-cells) transcription in T-cell hybridomas as well as normal splenocytes and that this is dependent on its interaction with Rac1. Using chimaera constructs of CD28 and p85, they have been shown to function in concert with TCR (T-cell receptor)/CD3 to stimulate IL-2 production [48]. Rac1 has been shown previously to regulate IL-1-induced NF-κB activation in an IκB-independent manner [49]. Perhaps of further relevance has been the finding that the recruitment of Rac1 and P13K p85 to the cytosolic domain of TLR2 is essential for the activation of NF-κB in response to heat-killed Staphylococcus aureus in HEK-293 cells [50]. That this pathway is reported to be IκB-independent may be relevant to the finding that TLR8 activation is independent of IκBα degradation [6].

In addition, the p110-independent association of p85 with IRS-1 (insulin receptor substrate-1) has been reported to mediate the joining of these components in a sequestration complex in the cytosol, resulting in the down-regulation of IGF (insulin-like
growth factor) receptor signalling [51]. As such, p110-independent p85 binding has been associated with both activation and down-regulation of receptor signalling.

Our finding that ubiquitin and nDHI are immunoprecipitated with IRM-activated TLR8 was neither sought nor anticipated. We would not have probed the TLR8 immunoprecipitates in Western blots with antibodies to either of these proteins. These proteins were identified by HPLC/MS/MS in an effort to confirm that the three forms of TLR8 observed in Western blots were, indeed, TLR8 (see Figure 8).

Others have demonstrated ubiquitination of TLRs and have suggested that this is a signal leading to their down-regulation and degradation by the proteasome [52]. It has also been demonstrated that TLR4 ubiquitination leads to association with a Vps (vacuolar protein sorting), Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate), via its UIM (ubiquitin interaction motif), leading to its degradation in the lysosome [53]. Based on the observations with TLR4, which are dynamin- and clathrin-dependent, this is suggested as a general model for all TLRs, independent of their transmembrane disposition at the cell surface or at the endosome [54].

Recent reports demonstrate, however, that sorting proteins with UIMs including epsin, Eps15 (epidermal-growth-factor-receptor pathway substrate 15), the transcription factor Sp5 (specificity protein 5) and Hrs have much higher affinity for polyubiquitin chains than monoubiquitin. As such, these sorting proteins are unable to bind monoubiquitinated proteins unless the protein is decorated with multiple monoubiquitinations [55].

In the case of TLR8, sequencing of the tryptic peptides suggests that these are monoubiquitin chains bound to TLR8. The ubiquitin peptide sequences show that all of the major lysine residues are points of cleavage by trypsin. The polyubiquitination of a given lysine residue would prevent trypsin cleavage [56,57]. In addition, examination of Figure 9 shows that ubiquitin recognition by antibody is limited to the 150 kDa and higher molecular mass species.

Like TLR9, TLR8 is localized in the endoplasmic reticulum. Like CpG oligonucleotides, IRMs have been demonstrated to rapidly localize in endosomes (R. Rajagopal, A. S. Walker, J. D. Mendoza and P. D. Wightman, unpublished work). Previous reports have demonstrated that the shuttling of TLR9 and its CpG agonist from one vesicular compartment to another is a critical part of its regulation. CpG oligonucleotides accumulate initially in early endosomes and subsequently migrate to tubular lysosomes. TLR9 resides in the endoplasmic reticulum at rest but moves to the CpG-containing compartment following cell exposure to this agonist [58]. Monoubiquitin attached to membrane proteins serves as a signal for internalization into the endocytic pathway, the endosome itself being a site of active signalling. Similarly, monoubiquitin attached to endocytic membrane proteins is a signal for sorting of cargo into vesicles that bud into the late endosome lumen for delivery into the lysosome [59]. As such, monoubiquitination of TLR8 may be essential to its trafficking towards (activation) or from (down-regulation) its IRM agonist.

We have shown that TLR8 expressed in HEK-293 cells is subject to covalent modification following IRM treatment and engages in protein–protein interactions beyond those anticipated for a TLR, e.g. MyD88 and IRAKs. TLR8 has numerous tyrosine residues in its cytosolic domain and some of these appear essential to normal TLR8 signalling. These include Tyr<sup>890</sup> and Tyr<sup>890</sup> in Box 1 and Tyr<sup>1048</sup> in the p85-binding motif. We show further that TLR8 is tyrosine-phosphorylated with late hyperphosphorylation being commonly observed. TLR8 covalent modifications include apparent monoubiquitination, which may play a critical role in intracellular trafficking of TLR8 in response to agonist treatment.

TLR8 is also shown to associate with the p85 regulatory subunit of PI3K. We see neither activation of downstream Akt nor modulation of TLR8 responses by PI3K inhibitors wortmannin or LY294002 and suggest that p85 may function in the HEK-293 system as an adaptor similar to that role suggested for in T-cells and the activation of the NFAT transcription. The observation of TLR8 association with nDHI is novel and unanticipated. Its precise role in TLR8 regulation can only be speculated at present. The fact that these modifications are occurring at late times following IRM-stimulated MAPK activation suggests at least two alternative explanations: (i) they are part of a complex mechanism for the downstream regulation of the receptor, or (ii) the TLR8/HEK-293 model system is an anomaly at least as would be expected for receptor stimulus–response coupling. These observations pose many questions regarding the regulation of TLR8 in primary human cells and to address them fully requires the development of validated reagents, such as highly selective antibodies to endogenous TLR8 (and TLR7) to support similar studies in purified primary human cell populations.

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