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# *Toxoplasma gondii*-Derived Profilin Triggers Human Toll-Like Receptor 5-Dependent Cytokine Production

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**Key Words**
*Toxoplasma gondii* · Monocytes · Humans · Pathogen-associated molecular patterns · Pattern recognition receptors · Toll-like receptor

**Abstract**
Up to a third of the world's population is infected with *Toxoplasma gondii*. Natural infection in humans can be life threatening during pregnancy and in immunocompromised individuals. Toll-like receptor (TLR) 11 is the mouse innate sensor that recognizes *T. gondii* profilin; however, in humans the *TLR11* gene leads to transcription of no functional protein. Herein, by using a multiple sequence alignment phylogenetic analysis program between human and mouse species, we found that human *TLR5* seems to be the evolutionarily closest member of the TLR gene family to mouse *tlr11*. We therefore asked whether human TLR5 could mediate IL-6, IL-8 and IL-12p70 production in response to the *T. gondii* profilin. We found that this was the case both in human cell lines as well as peripheral blood monocytes. Moreover, TLR5 neutralization and gene silencing mediated specific ablation of cytokine production after profilin exposure. Finally, peripheral blood monocytes carrying the TLR5 R392X mutation failed to produce cytokines in response to stimulation with profilin. Taken together, the results presented herein reveal a previously unappreciated cross-recognition of a relevant human pathogen-derived pathogen-associated molecular pattern.



## Introduction

Microbial recognition by the innate immune system is mediated by a multitude of cellular and endosomal membrane-bound as well as intracellular receptors. *Toxoplasma gondii*-derived pathogen-associated molecular patterns (PAMPs), namely cyclophilin-18 and profilin, have been shown to be recognized by receptors present in macrophages and dendritic cells, triggering cell activation and production of proinflammatory cytokines, including IL-1β, IL-6 and IL-12. While cyclophilin-18 is recognized by both mouse and human CCR5 [1, 2], profilin has been shown to mediate powerful cytokine production from mouse dendritic cells via activation of Toll-like receptor (TLR) 11 [3]. In fact, TLR11, which was previously found to mediate recognition of uropathogenic bacteria, has been identified as a major component and is essential for the development of the protective immune response in infected mice through the induction of massive IL-12

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production by dendritic cells. IL-12-mediated induction of type 1 immunity is crucial for containing parasite replication and mediating long-term immunity to infection. However, due to the presence of several stop codons, transcription of the human *TLR11* gene does not produce a functional protein [4]. Yet, as we show here, human cells are responsive to *T. gondii* profilin. Therefore, we asked whether there could be a functional ortholog for mouse TLR11 that is responsible for recognition of *T. gondii* profilin in humans. To do so, we performed evolutionary genetic taxa comparisons. We found that TLR11 is, perhaps, the most ancient TLR family member and that the subsequent members of this family of genes were derived from successive gene duplications. Both human and mouse TLR5 seemed to be evolutionarily the oldest relatives of mouse TLR11. This result led us to hypothesize that human TLR5 could have conserved (or rescued) mouse TLR11 biological function and mediate *T. gondii* profilin recognition. To test this hypothesis, we systematically examined whether human cell lines as well as peripheral blood monocytes expressed functional TLR5, followed by examining their cytokine response to *T. gondii* profilin in the absence of TLR5 through loss-of-function approaches [antibody (Ab)-mediated neutralization and siRNA gene silencing]. Our results show conclusively that *T. gondii* profilin induces a TLR5-dependent proinflammatory response by human monocytes.

## Materials and Methods

*Reagents and Cells*

IgA anti-human (hu)TLR5, recombinant flagellin and recombinant *T. gondii* profilin were purchased from Invivogen, and proteinase K was purchased from Roche. Human embryonic kidney (HEK) 293 cells were purchased from ATCC (CRL-1573.3) and grown in 10% FCS RPMI medium. Peripheral CD14+ blood monocytes were purified from whole blood from healthy donors using Ficoll density gradient and a highly specific monocyte isolation kit (CD14+ antibody magnetic labeled beads, Miltenyi). Proteinase K digestion of flagellin and profilin was performed as described previously [5, 6]. Briefly, proteinase K-agarose was reconstituted in endotoxin-free water to 10 mg/ml, incubated at 4°C for 2 h and washed 5 times with endotoxin-free water. Digestion buffer was prepared by supplementing PBS with 2.7 mM KCl, 1.5 mM $K_2PO_4$, 137 mM NaCl and 8.1 mM $Na_2PO_4$. Subsequently, 100 μg of flagellin or profilin were incubated in digestion buffer with proteinase K-agarose slurry on a shaking platform for 3 h at 37°C, followed by centrifugation and harvesting of supernatants. Both the cell lines and human peripheral blood monocytes were cultured overnight with native or proteinase K-predigested PAMPs, with or without anti-huTLR5 Ab. Culture supernatants were harvested and stored at –40°C until assayed for cytokine production.

*Evolutionary Relationships of Taxa*

The evolutionary history was inferred using the neighbor-joining method [7]. The evolutionary distances were computed using the Poisson correction method [8] and are in the units of the number of amino acid substitutions per site. The analysis involved 20 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 102 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [9, 10] and with ClustalW2-Phylogeny [11].

*Human Cytokine Measurements*

Human IL-6, IL-8, IL-12p40 and IL-12p70 levels were evaluated in culture supernatants using ELISA Duo-Set kits from R&D.

*TLR5 Flow Cytometry Analysis*

HEK293 cells and human peripheral blood monocytes were incubated with mouse R-phycoerythrin (PE)-labeled anti-huTLR5 mAb (clone 85B152.5, Enzo Life Sciences) or isotype mouse IgG2a-PE control Ab in FACS buffer (surface staining) or Perm-Wash solution (surface and intracellular staining; BD) for 30 min. Cells were then washed in FACS buffer, resuspended and acquired for flow cytometry analysis. Data were analyzed using FlowJo software.

*siRNA TLR5 Gene Silencing*

Control (sc-37007) and TLR5-specific (sc-40253) siRNA oligos were obtained from Santa Cruz Biotechnology. Gene silencing was performed using a transfection kit from Amaxa, following their specific instructions. Briefly, highly enriched peripheral blood CD14+ monocytes were transfected with control and TLR5-specific siRNAs using a nucleofector device and transfection reagent (Amaxa) in media. Afterwards, cells were placed in a 24-well plate with prewarmed transfection media and incubated for 24 h. Green fluorescent protein-labeled empty vector control was used to determine the transfection efficiency by flow cytometry. To verify the *TLR5* gene silencing, we analyzed TLR5 expression in transfected monocytes by flow cytometry using mouse R-PE-labeled anti-huTLR5 (Enzo Life Sciences). In order to test the functional ablation of TLR5 expression, transfected monocytes that showed decreased TLR5 protein levels were stimulated with flagellin and/or profilin (1 μg/ml) for 24 h, and supernatants were harvested and assayed for cytokine production by ELISA.

*TLR5 (R392X) Genotyping*

Genomic DNA samples (25 ng) from 35 peripheral blood monocytes were isolated and screened for TLR5 (R392X, rs5744168). Genotyping was carried out by allelic discrimination real-time PCR using the following primers: WT TLR5 T5–10, forward, 5′-ATGGGAGACCACCTGGACCTTCTCC-3′; T5–30, reverse, 5′-GGAGATGGTTGCTACAGTTTGCAACGG-3′. PCR primers for TLR5 (R392X) were T5–10, forward, and T5–31, reverse, 5′-GAGATCCAAGGTCTGTAATTTTTCCAGG-3′ [12]. End-point analysis was performed by high-resolution melting curve analysis using LightCycler 480 software (Roche).

*In vitro huTLR5 Ectodomain Binding Assay*

An in vitro huTLR5 ectodomain binding assay was performed as indicated by the manufacturer's instructions, as follows. Flagellin and profilin (Invivogen; 100 ng/ml in PBS) were incubated overnight in 96-well ELISA plates. Wells were washed 3 times with PBS

and incubated with titration curves of huTLR5-Fc (Invivogen; 100–3.125 ng/ml) with PBS alone or with flagellin or profilin (100 ng/ml). After a 2-hour incubation, wells were washed 5 times with PBS and incubated with anti-human IgG1-horseradish peroxidase conjugates for 1 h. Wells were developed with TMB substrate, and optical density was measured at 405 nm. Nonlinear regression curves were plotted, normalized and analyzed using Prism software.

*Statistical Analysis*

Student's t test was performed to determine statistical significance of differences ($p < 0.05$) between control and treated groups using the GraphPad software.

## Results

*Human TLR5 and Mouse tlr11 and tlr12 Are Part of an Ancient Cluster within the TLR Phylogenetic Tree*

Human innate immune system cells can recognize the presence of *T. gondii* parasites and produce proinflammatory cytokines, including IL-12 [13–15]. *T. gondii* profilin was shown to be a major component of innate recognition by mouse innate cells via activation of TLR11 [3]. The human *TLR11* gene is not translated due to the presence of a stop codon within its coding region [4], and to date, there are no homologs of mouse *tlr12*. We hypothesized that *T. gondii* profilin activates human cells through interaction with another TLR present in human cells. The approach to select which candidates to test was to examine the comparative evolutionary background of the TLR gene family between humans and mice. Figure 1 shows a phylogenetic tree comparing the amino acid sequences for TLRs 1–13 using the neighbor-joining method. Interestingly, the data indicate that mouse TLR11 is the most ancient member of this family, with all subsequent clusters derived from gene duplications and amino acid substitutions. In this regard, the oldest event gave origin to a cluster with mouse TLR12 and with human and mouse TLR5. Later, clusters containing TLRs 1, 2, 3, 4, 6 and 10 and, more recently, another cluster containing TLRs 7, 8 and 9 were derived. Based on these observations, we hypothesized that human TLR5 could potentially perform the microbial recognition executed by mouse TLR11. Although this method is limited with regards to interpretations that indicate complete evolutionary estimation, for the question posed in this article, we consider that it fulfilled its potential as a general sequence comparison analysis of gene family evolution between the two species based on amino acid sequences. We therefore raised the hypothesis that human TLR5 is involved in innate recognition and induction of cytokine production by *T. gondii*-derived profilin.

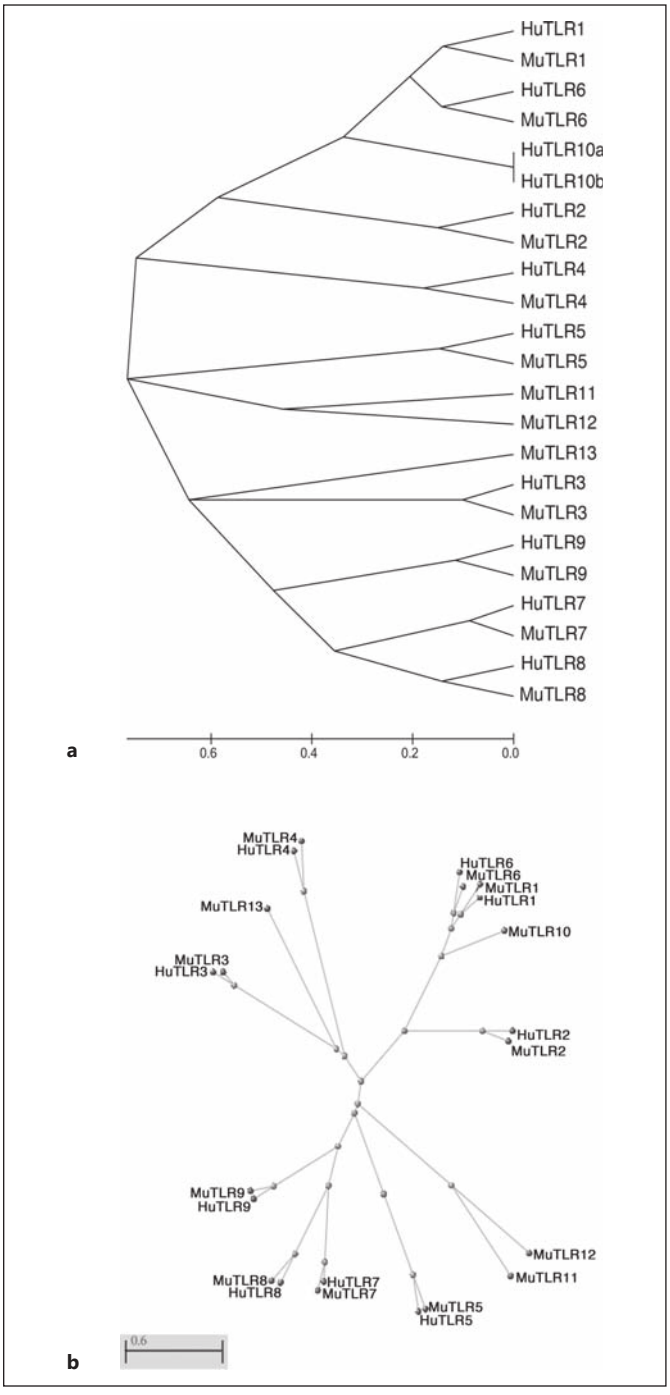


**Fig. 1.** Evolutionary relationship comparison of the TLR gene family between human and mouse. The evolutionary history was inferred by the neighbor-joining method using a MEGA5 cladogram tree (**a**) or a ClustalW2-Phylogeny radial tree (**b**). The optimal tree with the sum of the branch length equal to 7.94970641 is shown. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 20 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 102 positions in the final dataset.

*HEK293 Cells Are TLR5+ and Respond to Both Flagellin and Profilin in a TLR5-Dependent Manner*

Next, we focused on investigating the potential involvement of human TLR5 in the recognition of *T. gondii* profilin. We adopted a widely known approach using the HEK293 cell line transfected with the respective TLRs. However, to our surprise, we noticed that in the presence of both *T. gondii* profilin and the prototypical TLR5 ligand, flagellin, there was significant IL-8 production from nontransfected cells, independent of the presence of *TLR5*-containing plasmid. At this point, we followed up on testing whether HEK293 cells expressed detectable amounts of human TLR5. As shown in figure 2a, we found significant levels of TLR5 in HEK293 cells. On the other hand, THP-1 cells did not express detectable levels of TLR5 above isotype control Ab staining. These results suggest that the profilin-triggered IL-8 response in HEK293 cells could be derived from activation of this receptor.

In fact, figure 2b shows that both flagellin and profilin triggered a dose-dependent IL-8 production from HEK293 cells but not THP-1 cells (fig. 2b). Upon transfection with human but not mouse TLR5, HEK293 cells produced extremely high levels of IL-8 in response to flagellin (fig. 2c) and profilin (fig. 2d). Such a potent yet nonphysiological response overshadows the endogenous TLR5-triggered cytokine production. Moreover, mAb-mediated neutralization of human TLR5 inhibited IL-8 production by HEK293 cells in response to flagellin and profilin but not lipopolysaccharide (LPS) stimulation (fig. 2e–g). Therefore, these data clearly indicate that TLR5 expressed in HEK293 cells triggers IL-8 production in response to both flagellin and *T. gondii*-derived profilin.

*Human Peripheral Blood-Derived CD14+ Monocytes Produce Proinflammatory Cytokines in Response to Flagellin and Profilin in a TLR5-Dependent Manner*

To establish a role for human TLR5 in the recognition of *T. gondii* profilin in a more physiological context, we next aimed to evaluate the production of proinflammatory cytokines by peripheral blood monocytes in response to flagellin, profilin and LPS. The TLR5 surface expression profile was established by flow cytometry. Freshly isolated human peripheral blood monocytes (CD14+ cells) displayed membrane as well as intracellular TLR5 above background staining (fig. 3a). Upon exposure to flagellin, profilin and LPS, we observed significant induction of IL-6 and IL-12p70 by peripheral CD14+ monocyte cultures, while cells incubated with medium alone showed almost undetectable production (fig. 3b–g). Moreover, preincubation with a neutralizing anti-TLR5 mAb abolished cytokine induction by flagellin and profilin but not by LPS, thus confirming the specific TLR5 activation by both flagellin and profilin (fig. 3b–g). Notably, predigestion of both flagellin and profilin using proteinase K also prevented cytokine induction in these cultures but not in LPS-treated ones (fig. 3b–g), therefore ruling out the potential effect of nonpeptide contaminants in the induction of cytokine production. Taken together, these results provide solid evidence that human peripheral blood monocytes are activated by *T. gondii* profilin in a TLR5-sensitive manner.

*TLR5 Gene Silencing Inhibits the Response of Human Monocytes to Flagellin and Profilin*

To further establish the role of TLR5 in mediating cytokine induction by human monocytes, we inhibited *TLR5* gene expression by transfection with siRNA-coding plasmids. Figure 4a shows the effect of *TLR5* siRNA transfection versus control siRNA transfection on the cell membrane TLR5 expression levels as determined by flow cytometry. Figure 4b and c show that while control siRNA-transfected cells presented production of IL-6 and IL-12p70 in response to all microbial stimulants, there was a significant reduction in cytokine production by cells transfected with *TLR5* siRNA after stimulation with both flagellin and profilin. Taken together, these results indicate that TLR5 is a required component of the human monocyte response to *T. gondii*-derived profilin.

*TLR5 (R392X) Peripheral Blood Monocytes Are Unresponsive to T. gondii Profilin Stimulation and Hyporesponsive to Tachyzoite Exposure in vitro*

Human polymorphisms of the TLR5 gene had been described previously to be relevant in several infectious diseases and chronic inflammatory diseases, including Legionnaires' disease [12], Crohn's disease [16], cystic fibrosis [17] and obesity [18]. In particular, the mutation R392X, which leads to the insertion of a stop codon at the position 392, leads to complete loss of TLR5 protein expression. R392X is a highly frequent (up to 10%) mutation among Caucasians of European background [12]. Notably, *TLR5* (R392X) cells were shown be unresponsive to flagellin stimulation [12]. Here, we aimed to establish a more physiological model to further dissect the function of TLR5 in mediating monocyte cytokine responses to *T. gondii* profilin. To do so, we determined TLR5 expression in purified CD14+ monocytes. Figure 5a shows a histogram overlay profile from monocytes

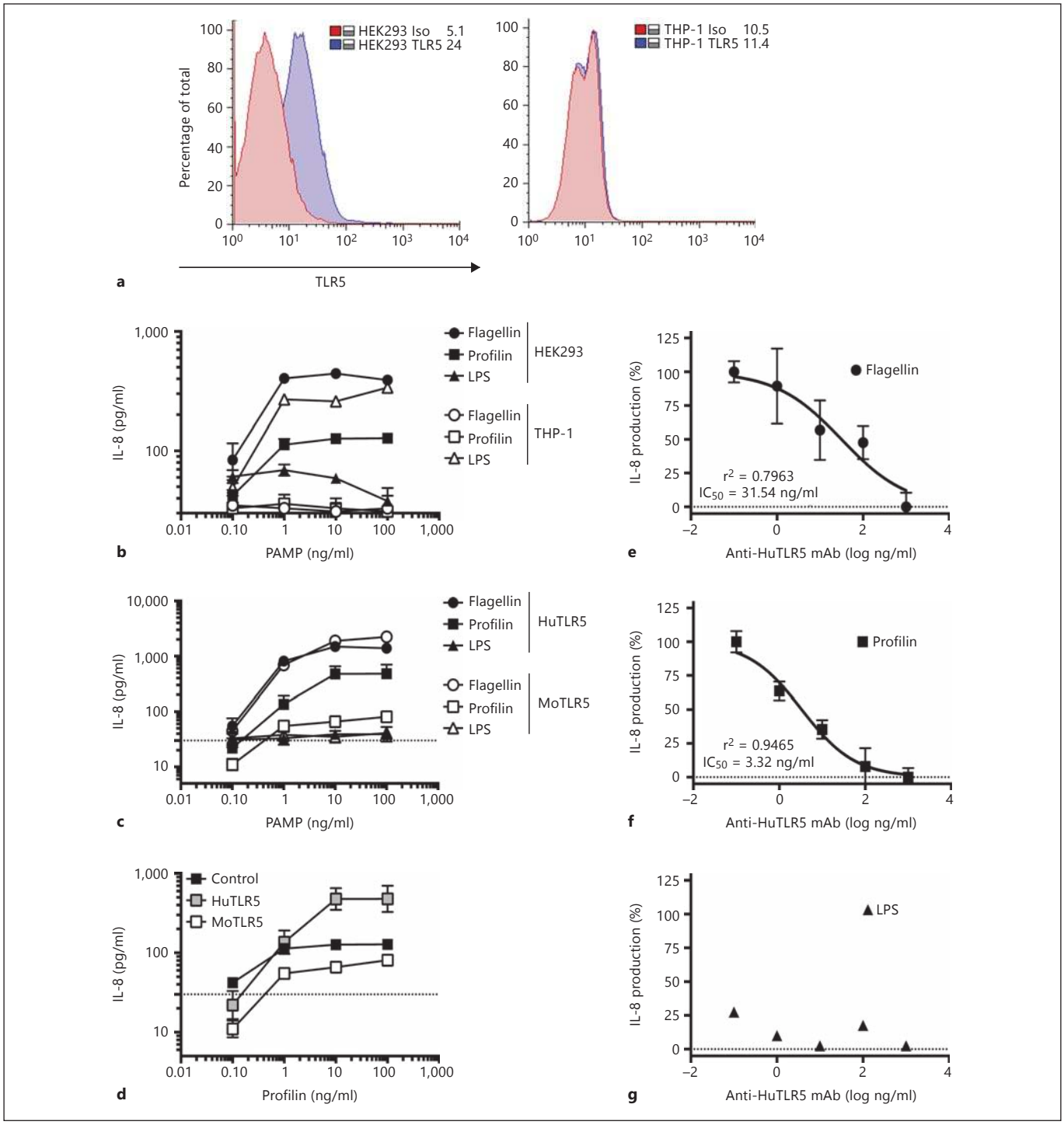


**Fig. 2.** Endogenously expressed human TLR5 in HEK293 cells mediates IL-8 responses to flagellin and *T. gondii*-derived profilin. **a** HEK293 or THP-1 cells were suspended in FACS buffer with PE-labeled anti-huTLR5 antibody. Cells were then washed and acquired for flow cytometry. Data shown are histogram overlays of samples stained with isotype antibody control (IgG2a-PE) and anti-TLR5. **b–d** Subsequently, cells were stimulated with several concentrations of recombinant flagellin C (**b**), recombinant *T. gondii* profilin (**c**) or LPS (**d**) for 24 h. **e–g** HEK293 cells were plated and incubated in the presence of medium alone or with anti-TLR5 mAb, as indicated, followed by stimulation with flagellin (**e**), profilin (**f**) or LPS (**g**) for 24 h. Supernatants were harvested and assayed for the presence of IL-8 by ELISA. Regression curves and $r^2$ and median inhibitory concentration ($IC_{50}$) values for anti-TLR5 mAb-mediated inhibition of IL-8 responses to flagellin, profilin or LPS are shown. Data shown are representative of at least 3 independent experiments.

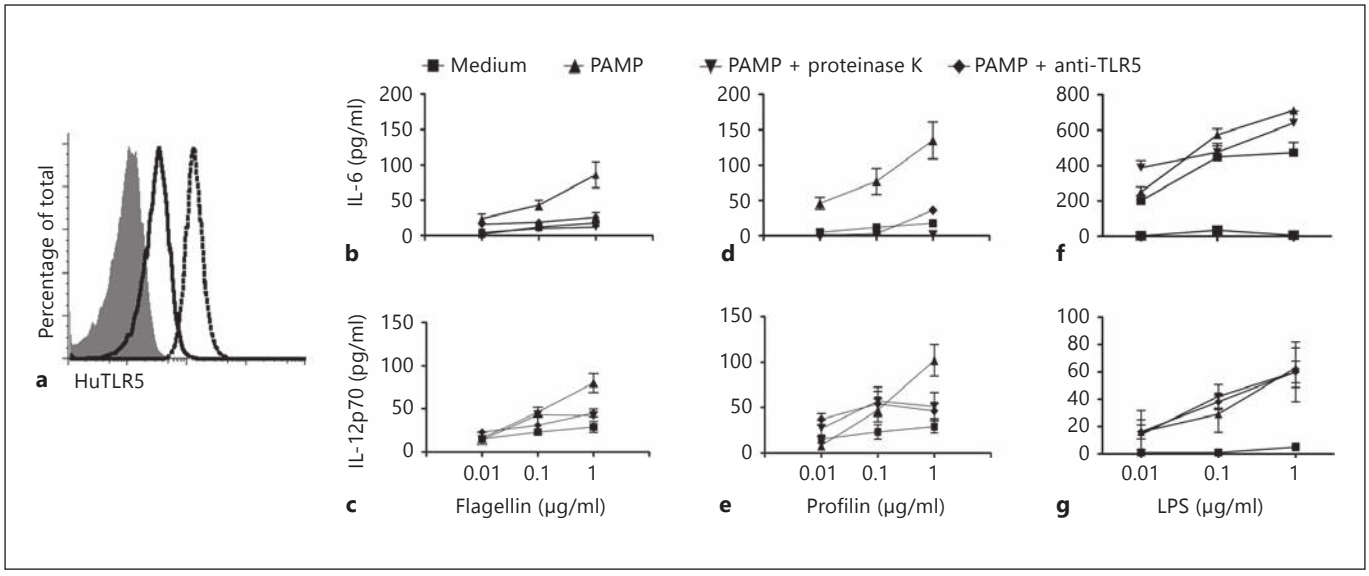


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**Fig. 3.** *T. gondii*-derived profilin triggers a TLR5-sensitive human peripheral blood-derived monocyte proinflammatory cytokine production. **a** Peripheral blood monocytes were purified and stained for intact (solid line) and permeabilized cells (dotted line). CD14-MACS bead-purified peripheral blood monocytes were suspended in FACS buffer with or without PermWash buffer followed by incubation with PE-conjugated isotype control IgG2a (gray histogram) or anti-TLR5 mAb. **b–g** Cells were plated and incubated with medium alone, flagellin (**b**, **c**), profilin (**d**, **e**) or LPS (**f**, **g**) in PBS (PAMP), PAMP predigested with proteinase K (PAMP + proteinase K) or anti-huTLR5 mAb (PAMP + anti-TLR5; 1–0.1 μg/ml). After incubation for 24 h, supernatants were harvested and assayed for IL-6 (**b**, **d**, **f**) or IL-12p70 (**c**, **e**, **g**) by ELISA. Data shown are representative of 3 independently performed experiments.

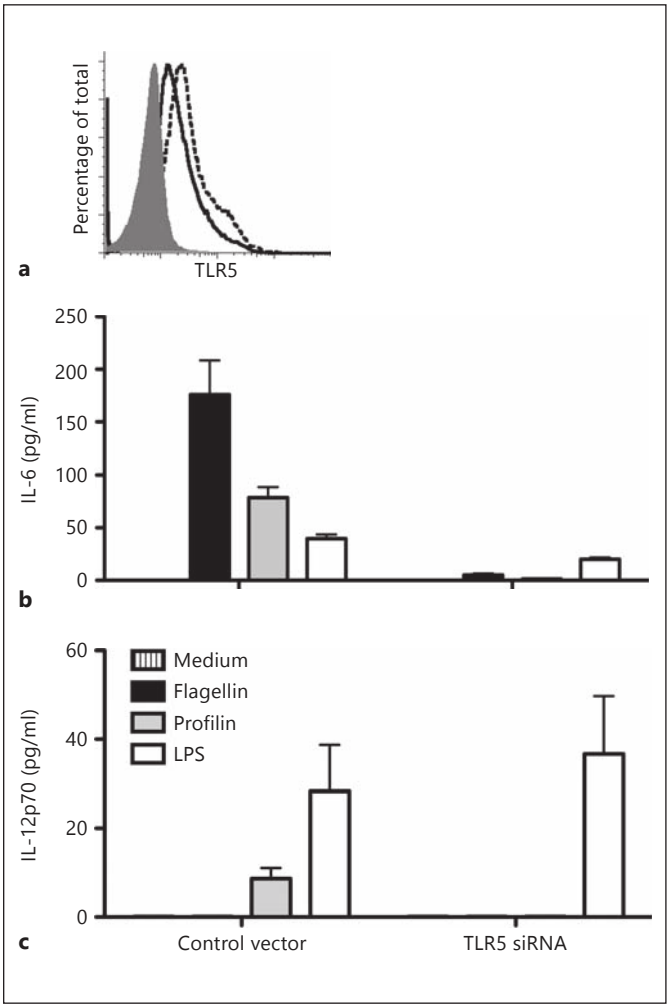


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**Fig. 4.** siRNA-mediated silencing of human *TLR5* inhibits profilin- and flagellin-mediated proinflammatory cytokine production by human peripheral blood-derived monocytes. Peripheral blood-derived monocytes were isolated and electroporated in the presence of medium alone, control siRNA or human TLR5 siRNA oligos. Cells were then stained for TLR5 as described in figure 3. **a** Transfected cells were gated and TLR5 expression was analyzed. A histogram overlay of isotype control-stained cells (gray histogram), control siRNA-transfected cells (dotted line) or TLR5 siRNA-transfected cells (solid line) is shown. **b**, **c** Cells were plated and incubated in the presence of medium alone, flagellin, profilin or LPS for 24 h. Culture supernatants were then harvested and assayed for IL-6 (**b**) and IL-12p70 (**c**) by ELISA. Data shown are representative of 3 independently performed experiments.

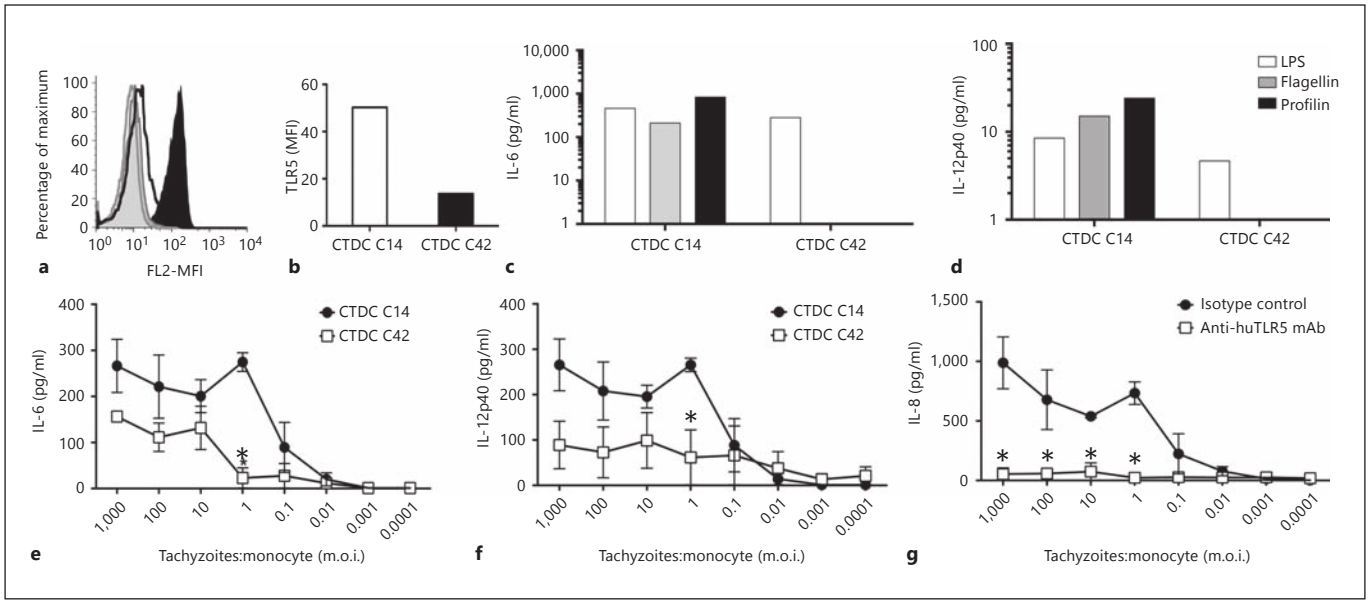


**Fig. 5.** *TLR5 (R392X)* mutation abolishes monocyte cytokine production after *T. gondii* profilin stimulation. Peripheral blood CD14+ monocytes were purified and stained as described in figure 3. **a** Histogram overlays of isotype control versus TLR5 staining of CD14+ monocytes obtained from two donors (CTDC C42, gray histogram; CTDC C14, black histogram). Empty histograms represent isotype control Ab staining (black line for CTDC C14 and gray line for CTDC C42). **b** Mean fluorescence intensity (MFI) of the samples. **c**, **d** Cell suspensions from the same donors were then cultured in the presence of medium alone, LPS, flagellin or profilin (all at 1 μg/ml) for 24 h. Supernatants were harvested and assayed for IL-6 (**c**) and IL-12p40 (**d**) by ELISA. **e**, **f** Peripheral blood monocytes from donors CTDC C14 and CTDC C42 were incubated in the presence of live *T. gondii* Rh strain tachyzoites (m.o.i. ranging from 1,000 to 0.0001); 24 h later supernatants were harvested and assayed for IL-6 (**e**) and IL-12p40 (**f**). **g** HEK293 cells were plated and incubated in the presence of isotype control Ab or anti-huTLR5 mAb and live tachyzoites as indicated in e and f. Culture supernatants were harvested and assayed for IL-8 by ELISA. Data shown are representative of triplicate samples from 2 independent experiments. * $p < 0.05$ between donors or between isotype control and anti-huTLR5 mAb treatment as determined by t test.

that expressed low and high levels of TLR5. Figure 5b shows the mean fluorescence intensity of such samples and the low/high profiles of TLR5 expression within CD14+ cells. We then confirmed by real-time genotyping that the cells that showed low levels of TLR5 staining also showed high detection using primers containing the R392X mutation (online suppl. fig. 1, www.karger.com/doi/10.1159/000362367). We then examined their cytokine profile in response to LPS, flagellin and profilin. Figure 5c and d show IL-6 and IL-12p40 levels induced above background (unstimulated control) values. LPS stimulation triggered increased production of all cytokines tested in cells from both donors. On the other hand, flagellin and profilin triggered IL-6 and IL-12p40 production from $TLR5^{high}$ but not from TLR5 R392X cells (fig. 5c, d), thus providing evidence that a fully functional TLR5 is required for a monocyte response to *T. gondii* profilin. To further establish the biological relevance of TLR5-mediated recognition of *T. gondii* profilin, we exposed $TLR5^{WT}$ and $TLR5^{R392X}$ peripheral blood monocytes to live *T. gondii* Rh strain tachyzoites at several multiplicities of infection (m.o.i.'s) and assayed for IL-6 and IL-12p40 by ELISA. Figure 5e (IL-6) and figure 5f (IL-12p40) show that $TLR5^{WT}$ and $TLR5^{R392X}$ peripheral blood monocytes presented m.o.i.-dependent cytokine production in response to tachyzoite exposure; however, $TLR5^{R392X}$ monocytes showed significant reduction of cytokine production at 1 m.o.i. (fig. 5e, f), thus suggesting a minor but nonetheless relevant role for the TLR5-mediated cytokine response to live parasite in monocytes. In light of these results, we exposed HEK293 cells to live *T. gondii* Rh strain tachyzoites (same m.o.i. range as in fig. 5e, f) in the presence of isotype control Ab or neutralizing anti-TLR5 mAb and assayed for IL-8 production, as described in figure 2. Figure 5g shows that HEK293 cells produced IL-8 in response to tachyzoite exposure in an m.o.i.-dependent manner while in the presence of isotype control Ab. However, human TLR5 neutralization completely abolished the HEK293 IL-8 response to live tachyzoites in vitro. This suggests that ep-

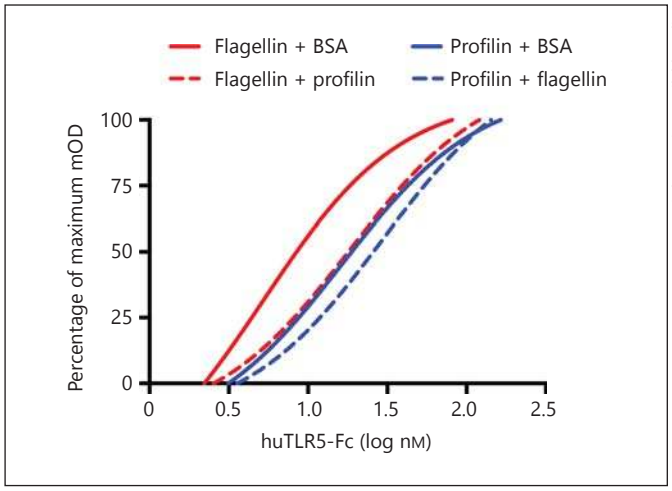


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**Fig. 6.** Flagellin and profilin bind to the ectodomain of human TLR5 in vitro. Flagellin or profilin (1 μg/ml) were immobilized on ELISA plates. Wells were then incubated with increasing concentrations of huTLR5-Fc fusion protein (ranging from 1.5 to 200 μg/ml) in the presence of 1 μg/ml BSA, profilin or flagellin for 2 h. Wells were washed 3 times with PBS-Tween 0.5%, followed by incubation with anti-human IgG-horseradish peroxidase conjugates. HuTLR5-Fc binding was determined colorimetrically using TMB substrate in an ELISA plate reader. Data were then normalized to a percentage of maximum values and nonlinear regression curve fit using Prism. Data shown are means of triplicate samples from 1 of 2 independent experiments. mOD = Milli-optical density.

ithelial cells (such as HEK293), which have a more limited range of PAMP recognition machinery, use mostly TLR5/profilin interaction for inducing cytokine production. On the other hand, monocytes, which express a wider spectrum of pattern recognition receptors, are capable of responding to live tachyzoites through TLR5-independent pathways.

*Flagellin and Profilin Share Common Binding Sites within the Ectodomain of Human TLR5*

Our results consistently show a human TLR5-dependent cytokine response to *T. gondii* profilin within both myeloid and nonmyeloid compartments. The relative contribution of this pathway remains to be established in human toxoplasmosis; however, it suggests profilin as a novel ligand for human TLR5. To document such interaction, we took advantage of binding assays using human TLR5 ectodomain/human IgG Fc (huTLR5-Fc) fusion protein. Figure 6 shows binding curves of huTLR5-Fc preincubated with BSA to both flagellin and profilin. In order to investigate whether flagellin could compete for profilin TLR5 binding sites (and vice versa), we pre-exposed huTLR5-Fc to the competitor prior to incubating with the plate-bound ligand. Interestingly, we found minor cross-competition between flagellin and profilin (fig. 6), thus suggesting distinct binding sites among the two ligands with minor overlap within TLR5.

## Discussion

Some studies have shown an overlap of TLR5 and TLR11 in the mouse system, with TLR5-dependent responses to previously assigned TLR11 ligands [19–21] and vice versa [22]. This set of overlapping activity might be rooted in the selective pressure for recognition of PAMPs from pathogens well adapted to their hosts. Our functional clustering of the TLR gene family from humans and mice suggests an older relationship between TLR11 (supposedly the oldest TLR in both species) and TLR5 – the first gene theoretically product of an ancient *tlr11* gene duplication event. Despite the evolutionary distance, our results suggest that function and microbial ligand affinity is conserved between human TLR5 and mouse TLR11. Interestingly, overlap with regard to mouse TLR5 and TLR11 ligand specificity has been reported previously; however, a thorough comparative study of TLR5 and TLR11 ligands in mouse cells has not yet been conducted to date.

Previous literature relied vastly on the HEK293 transfection system to test ligand specificity with a great degree of reproducibility. Nevertheless, our study raises a central issue for the correct interpretation of these results. We have shown here strong evidence supporting the endogenous expression of TLR5 in nontransfected HEK293 cells. Past analysis of TLR/PAMP interaction in transfected HEK293 cells was certainly made under very high gene expression levels (more than 1,000 times higher than baseline) by comparing untreated versus stimulated cells (usually represented as fold increase over control). However, this method introduces a bias in the interpretation of the results due to the fact that TLR activation by PAMP in nontransfected or mock-transfected cells is proportionally increased. However, the magnitude of the TLR activation signal is too high in transfected cells as for the signal levels observed in nontransfected cells to be appreciated after ligand exposure. Another potential complicating factor with this method of analysis is that the signals coming from subtle affinity changes between receptor and PAMPs are minimized by the extremely high activation threshold over baseline. In fact, the commercial source for the use of HEK293 cells in a TLR/NOD

reporter assay alerts to the endogenous baseline levels of TLR3, TLR5 and NOD1 in these cells (Invivogen, catalogue No. 293-LacZ). Moreover, several previous reports indicated increased endogenous TLR5 expression in HEK293 cells [23–25]. Therefore, our results are consistent with several lines of published data.

Human cells show an obvious response to *T. gondii* profilin that is independent of any cognate signal (i.e. CD40L, IFN-γ), an observation that highlights the innate character of this interaction. However, it is not clear that profilin is the only PAMP from this protozoan to trigger a human innate cytokine response in vivo. The mouse model suggests a very complex scenario, where several receptor/ligand pairs play a relevant role early after infection in vivo. As such, TLR11 is required for profilin-triggered cytokine production [3], while TLR9 has been shown to mediate some response [26]. However, both TLR11- and TLR9-deficient mice show resistance to acute infection, while MyD88-deficient mice quickly succumb to infection [27]. Moreover, we and others have shown the activation of CCR5-dependent cytokine dendritic cell responses by exposure to cyclophilin-18 from *T. gondii* [1, 28]. CCR5-deficient mice also showed high mortality upon infection concomitant with lower type 1 cytokine production [1].

More recently, a series of studies have shown that the TLR11-mediated response to *T. gondii* is compounded by coactivation of TLR12, as well as TLR7/TLR9 triggering by parasite RNA/DNA [29]. In the absence of all these pathways combined, mice show a susceptibility phenotype that resembles *T. gondii*-infected MyD88-deficient hosts [29]. Such a complex response can be further supported by the observations using UNC93B1-deficient mice, in which the activation of TLRs 3, 7 and 9 by RNA/DNA is abolished [30]. Taking all these observations together with the fact that humans have a truncated nonfunctional *TLR11* gene and no homolog for mouse *tlr12*, we propose here that TLR5 'fills in' for the absent human TLR11. Further interactions resulting from recognition of parasite RNA and DNA in the context of profilin-initiated responses remain to be further characterized. Our experiments were performed using recombinant profilin to focus on a specific ligand/receptor interaction, although crude parasite lysates (soluble tachyzoite antigen) can trigger monocyte cytokine production (J.A., personal observations). Furthermore, proteinase K digestion of recombinant profilin completely abolished cytokine induction by this molecule, thus suggesting that potential nucleotide, polysaccharide or other nonpeptide contamination is unlikely.

The relative contribution of TLR5 to the protection against toxoplasmosis in humans, especially within populations in which there is high frequency of the TLR5 R392X mutant, remains to be fully investigated. Finally, the biological implications of the studies presented here open a new venue for PAMP-based vaccine adjuvants. Vaccine research using the mouse system has not accounted for the potential role of TLR5/profilin interaction seen in human cells, as we showed here. The use of profilins as vaccine adjuvants has been proposed previously [31]. Our results clearly identify that the receptor/ligand interaction involved in profilin recognition in humans is therefore highly relevant for the future development of PAMP-based vaccine adjuvants as well as other clinical applications.


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## Disclosure Statement

The authors declare no conflict of interest.

## References


1 Aliberti J, Valenzuela JG, Carruthers VB, Hieny S, Andersen J, Charest H, Reis e Sousa C, Fairlamb A, Ribeiro JM, Sher A: Molecular mimicry of a CCR5 binding-domain in the microbial activation of dendritic cells. Nat Immunol 2003;4:485–490.

2 Yarovinsky F, Andersen JF, King LR, Caspar P, Aliberti J, Golding H, Sher A: Structural determinants of the anti-HIV activity of a CCR5 antagonist derived from Toxoplasma gondii. J Biol Chem 2004;279:53635–53642.

3 Yarovinsky F, Zhang D, Andersen JF, Bannenberg GL, Serhan CN, Hayden MS, Hieny S, Sutterwala FS, Flavell RA, Ghosh S, Sher A: TLR11 activation of dendritic cells by a protozoan profilin-like protein. Science 2005;308:1626–1629.

4 Ishii KJ, Uematsu S, Akira S: 'Toll' gates for future immunotherapy. Curr Pharm Des 2006;12:4135–4142.

5 Zhang D, Zhang G, Hayden MS, Greenblatt MB, Bussey C, Flavell RA, Ghosh S: A toll-like receptor that prevents infection by uropathogenic bacteria. Science 2004;303:1522–1526.

6 Luong M, Zhang Y, Chamberlain T, Zhou T, Wright JF, Dower K, Hall JP: Stimulation of TLR4 by recombinant HSP70 requires structural integrity of the HSP70 protein itself. J Inflamm (Lond) 2012;9:11.

7 Saitou N, Nei M: The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4:406–425.

8 Zuckerkandl E, Pauling L: Molecules as documents of evolutionary history. J Theor Biol 1965;8:357–366.

9 Kumar S, Nei M, Dudley J, Tamura K: MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 2008;9:299–306.
10 Tamura K, Dudley J, Nei M, Kumar S: MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 2007;24:1596–1599.
11 Goujon M, McWilliam H, Li W, Valentin F, Squizzato S, Paern J, Lopez R: A new bioinformatics analysis tools framework at EMBL-EBI. Nucleic Acids Res 2010;38:W695–W699.
12 Hawn TR, Verbon A, Lettinga KD, Zhao LP, Li SS, Laws RJ, Skerrett SJ, Beutler B, Schroeder L, Nachman A, Ozinsky A, Smith KD, Aderem A: A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to Legionnaires' disease. J Exp Med 2003;198:1563–1572.
13 Subauste CS, Wessendarp M: Human dendritic cells discriminate between viable and killed Toxoplasma gondii tachyzoites: dendritic cell activation after infection with viable parasites results in CD28 and CD40 ligand signaling that controls IL-12-dependent and -independent T cell production of IFN-gamma. J Immunol 2000;165:1498–1505.
14 Seguin R, Kasper LH: Sensitized lymphocytes and CD40 ligation augment interleukin-12 production by human dendritic cells in response to Toxoplasma gondii. J Infect Dis 1999;179:467–474.
15 Aldebert D, Durand F, Mercier C, Brenier-Pinchart MP, Cesbron-Delauw MF, Pelloux H: Toxoplasma gondii triggers secretion of interleukin-12 but low level of interleukin-10 from the THP-1 human monocytic cell line. Cytokine 2007;37:206–211.
16 Gewirtz AT, Vijay-Kumar M, Brant SR, Duerr RH, Nicolae DL, Cho JH: Dominant-negative TLR5 polymorphism reduces adaptive immune response to flagellin and negatively associates with Crohn's disease. Am J Physiol Gastrointest Liver Physiol 2006;290:G1157–G1163.
17 Blohmke CJ, Park J, Hirschfeld AF, Victor RE, Schneiderman J, Stefanowicz D, Chilvers MA, Durie PR, Corey M, Zielenski J, Dorfman R, Sandford AJ, Daley D, Turvey SE: TLR5 as an anti-inflammatory target and modifier gene in cystic fibrosis. J Immunol 2010;185:7731–7738.
18 Al-Daghri NM, Clerici M, Al-Attas O, Forni D, Alokail MS, Alkharfy KM, Sabico S, Mohammed AK, Cagliani R, Sironi M: A nonsense polymorphism (R392X) in TLR5 protects from obesity but predisposes to diabetes. J Immunol 2013;190:3716–3720.
19 Chassin C, Tourneur E, Bens M, Vandewalle A: A role for collecting duct epithelial cells in renal antibacterial defences. Cell Microbiol 2011;13:1107–1113.
20 Arikawa K, Nishikawa Y: Interleukin-8 induction due to diffusely adherent Escherichia coli possessing Afa/Dr genes depends on flagella and epithelial Toll-like receptor 5. Microbiol Immunol 2010;54:491–501.
21 Andersen-Nissen E, Hawn TR, Smith KD, Nachman A, Lampano AE, Uematsu S, Akira S, Aderem A: Cutting edge: Tlr5-/- mice are more susceptible to Escherichia coli urinary tract infection. J Immunol 2007;178:4717–4720.
22 Mathur R, Oh H, Zhang D, Park SG, Seo J, Koblansky A, Hayden MS, Ghosh S: A mouse model of Salmonella typhi infection. Cell 2012;151:590–602.
23 Yang D, Chen Q, Su SB, Zhang P, Kurosaka K, Caspi RR, Michalek SM, Rosenberg HF, Zhang N, Oppenheim JJ: Eosinophil-derived neurotoxin acts as an alarmin to activate the TLR2-MyD88 signal pathway in dendritic cells and enhances Th2 immune responses. J Exp Med 2008;205:79–90.
24 Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdorfer B, Giese T, Endres S, Hartmann G: Quantitative expression of toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol 2002;168:4531–4537.
25 Matsumoto M, Kikkawa S, Kohase M, Miyake K, Seya T: Establishment of a monoclonal antibody against human toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. Biochem Biophys Res Commun 2002;293:1364–1369.
26 Minns LA, Menard LC, Foureau DM, Darche S, Ronet C, Mielcarz DW, Buzoni-Gatel D, Kasper LH: TLR9 is required for the gut-associated lymphoid tissue response following oral infection of Toxoplasma gondii. J Immunol 2006;176:7589–7597.
27 Scanga CA, Aliberti J, Jankovic D, Tilloy F, Bennouna S, Denkers EY, Medzhitov R, Sher A: Cutting edge: MyD88 is required for resistance to Toxoplasma gondii infection and regulates parasite-induced IL-12 production by dendritic cells. J Immunol 2002;168:5997–6001.
28 Aliberti J, Reis e Sousa C, Schito M, Hieny S, Wells T, Huffnagle GB, Sher A: CCR5 provides a signal for microbial induced production of IL-12 by CD8 alpha+ dendritic cells. Nat Immunol 2000;1:83–87.
29 Andrade WA, Souza Mdo C, Ramos-Martinez E, Nagpal K, Dutra MS, Melo MB, Bartholomeu DC, Ghosh S, Golenbock DT, Gazzinelli RT: Combined action of nucleic acid-sensing toll-like receptors and TLR11/TLR12 heterodimers imparts resistance to Toxoplasma gondii in mice. Cell Host Microbe 2013;13:42–53.
30 Melo MB, Kasperkovitz P, Cerny A, Konen-Waisman S, Kurt-Jones EA, Lien E, Beutler B, Howard JC, Golenbock DT, Gazzinelli RT: UNC93B1 mediates host resistance to infection with Toxoplasma gondii. PLoS Pathog 2010;6:e1001071.
31 Yarovinsky F, Kanzler H, Hieny S, Coffman RL, Sher A: Toll-like receptor recognition regulates immunodominance in an antimicrobial CD4+ T cell response. Immunity 2006;25:655–664.