Human TLR8 senses UR/URR motifs in bacterial and mitochondrial RNA

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Abstract

Toll-like receptor (TLR) 13 and TLR2 are the major sensors of Gram-positive bacteria in mice. TLR13 recognizes Sa19, a specific 23S ribosomal (r) RNA-derived fragment and bacterial modification of Sa19 ablates binding to TLR13, and to antibiotics such as erythromycin. Similarly, RNase A-treated Staphylococcus aureus activate human peripheral blood mononuclear cells (PBMCs) only via TLR2, implying single-stranded (ss) RNA as major stimulant. Here, we identify human TLR8 as functional TLR13 equivalent that promiscuously senses ssRNA. Accordingly, Sa19 and mitochondrial (mt) 16S rRNA sequence-derived oligoribonucleotides (ORNs) stimulate PBMCs in a MyD88-dependent manner. These ORNs, as well as S. aureus-, Escherichia coli-, and mt-RNA, also activate differentially expressed human monocytoid THP-1 cells, provided they express TLR8. Moreover, Unc93b1−/− and Tlr8−/−THP-1 cells are refractory, while endogenous and ectopically expressed TLR8 confers responsiveness in a UR/URR RNA ligand consensus motif-dependent manner. If TLR8 function is inhibited by suppression of lysosomal function, antibiotic treatment efficiently blocks bacteria-driven inflammatory responses in infected human whole blood cultures. Sepsis therapy might thus benefit from interfering with TLR8 function.

Keywords bacteria; human TLR8; mitochondrial; ribosomal; RNA

Subject Categories Immunology; Microbiology; Virology & Host Pathogen Interaction

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and human/mouse cross-reactive monoclonal antibody (mAb, named T2.5) was ineffective unless the hiSa solution was incubated with single-stranded (ss) RNA-specific RNase A (Fig 1A) [5,15]. We thus concluded that both Sa19 “like” ssRNA segments as well as TLR2 ligands are candidates of S. aureus-derived immune stimulatory PAMPs.

Also, self-RNA–antimicrobial peptide complexes stimulate human dendritic cells (DCs) via TLRs and the endosymbiotic theory implies that self-mitochondria originate from bacteria [16,17]. Since 23S bacterial rRNA comprises Sa19, we analyzed the sequence of its 16S mtrRNA human, cattle, mouse and rat orthologs and designed specific 19-mer Sa19 “like” oligoribonucleotides (ORNs, Figs 1B and 18). We also used two Sa19 adenine (A) 7 variants that lack “GGAAAGA” motifs are contained in other regions of 16S mtrRNAs [18]. None of the Sa19 “like” RNA segments applied activated mTLR13, and only *mtPTL carrying a TLR7-prone “UAU” motif

### Results and Discussion

**Specific bacterial and mitochondrial RNAs activate human immune cells dependent on MyD88 and Unc93B1 but largely independent on TLR7**

In quest for a human equivalent of TLR13, we analyzed whether the 23S rRNA segment Sa19—with 19 bases of appropriate length [14]—or heat-inactivated S. aureus (hiSa) stimulate PBMCs. Both compounds were active. Thus, blockade of TLR2 with a neutralizing antibody implicate a novel therapeutic interference in acute inflammation pathology.

#### Figure 1. Specific bacterial and mitochondrial ribosomal RNA segments and fractions induce proinflammatory but not type I IFN cytokine production through an endosomal TLR.

A. Cytokine release by PBMCs challenged with heat-inactivated S. aureus (hiSa), and/or RNAs A (RA)-treated solution, or Sa19 (TLR13 activating ORN) upon TLR2 blockade (T2.5; n.p., not performed; **P ≤ 0.01, n = 3, unpaired t-test).

B. Alignment of Sa19 and mitochondrial 16S rRNA Sa19 “like” segment (mt, mitochondrial; PTL, peptidyl transferase loop; D, domain; _t, transition region) sequence with common core motif (blue) and uracils (red U); *conserved in human (Hs), cattle (Bt), mouse and rat; G/A underlined, mutated core motif; "a, 6N methylation of adenosine 7.

C. Cytokine release of PBMCs transfected with ORNs including TLR7/8 ligand RNA40 (n = 3).

D. E. Activity of PBMCs upon bacterial RNA challenge, pretreatment with chloroquine in (D) only (S. Svedberg, r, ribosomal; tot, total; n = 3).

F. Activity of PBMCs upon mitochondrial (mt) RNA challenge (Hs, human; triangle, increasing doses; LFA, Lipofectamine 2000; n = 3).

G. Activity of PBMCs challenged with ORNs and total S. aureus (Sa) RNA (MyD88**), mutant MyD88 expression not impairing LPS-driven IL-8 production; n = 2.

H. Responsiveness of undifferentiated (undiff) and 3- or 8-day PMA-differentiated (ddi) THP-1 cells to Sa19 challenge (n = 3).

I. Activity of parental and Unc93B1**−−/−−ddiTHP-1 cells challenged with ORNs (n = 3).

Data information: Graphs show mean ± SD; *, unchallenged; n.d., not detected; P3C, Pam3CSK4, Loxo, loxoribine.
activated the murine immune system at all and through TLR7 (Fig EV1C–H) [14,19]. Total mRNA was refractory (Fig EV1D), as if *mtPTL* was rendered inactive in the context of total mtRNA. This finding was reminiscent of TLR13 “muteness” toward the *E. coli* Sa19 within total RNA [5].

We next analyzed human PBMCs for their responsiveness to different bacteria and self-derived RNAs. While all Sa19 “like” ORNs applied including TLR13-silent, point-mutated, and methylated ones induced robust TNF production in PBMCs, surprisingly none triggered substantial type I interferon (IFN) release (Fig 1C). This result indicated a lack of TLR7 involvement because TLR7 ligands such as RNA40 and bacterial RNA induce PBMC type I interferon (IFN) production by activating plasmacytoid DC TLR7 [14,19]. Next, we analyzed bacterial rRNA activities (Fig EV2A) [5,9,10]. 23S, 16S, and 5S rRNA of both Gram-positive and Gram-negative bacteria induced substantial TNF release from PBMCs, provided their lysosomes were functional (Fig 1D). While total bacterial RNA encompassing tRNA triggered substantial IFNγ release, neither bacterial rRNAs, nor total mtRNA hardly induced IFNγ release, while the latter RNA elicited strong IL-6 production (Figs 1E and F, and EV2B).

PBMCs of a human individual expressing a nonfunctional Glu53ΔMyD88 mutant [20] failed to respond to *S. aureus* RNA and to Sa19 “like” ORNs (Fig 1G). Given that 3-day differentiated (3ddi) THP-1 cells turned out to be responsive toward Sa19 (Fig 1H), we applied THP-1 cells lacking expression of Unc93B1 (a chaperon translocating TLRs from the endoplasmic reticulum to endosomes) [21,22]. Like undifferentiated and 8ddiTHP-1 cells, *Unc93b1*−/− 3ddiTHP-1 counterparts failed to respond toward Sa19 “like” ORNs (Fig 1I).

**Upregulation or overexpression of TLR8 confers uridine-dependent specific bacterial and mitochondrial RNA responsiveness while TLR8 knockout abrogates it**

*Unc93b1*−/−3ddiTHP-1 cells were also insensitive to large bacterial rRNAs (Fig 2A) [12]. However, they responded well to hiSa and *E. coli* (Ec) via TLR2 (Fig 2B and C). Next, we comparatively profiled transcriptions of undifferentiated, 3ddi-, and 8ddiTHP-1 cells. Selection of non-interleukin receptors that were at least twofold upregulated on the mRNA level in 3ddiTHP-1 cells, yielding a candidate molecule criteria out of which we considered mTLR13-like TLR8 as most promising candidate (Figs 2D and EV2C). In contrast to mTLR13, hTLR8 carries a z-loop (Fig EV2D), which is deleted in mTLR8 [13]. In order to substantiate our selection of hTLR8, we analyzed its “gain and loss of” function. All Sa19 “like” ORNs that contained sparse U (such as Sa19 and Sa19 point mutants) substantially became enhanced by the RNA specificity of TLR8 might be guided by the U and G content of ssRNA [14]. We thus transfected Sa19 “like” ORNs differing in their U content (Fig 1B) and admixed U nucleosides into TLR8+ cells. The stimulatory power of ORNs that contained sparse U (such as Sa19 and Sa19 point mutants) substantially became enhanced by co-application of U nucleoside. Notably, ORN HsmtD1 containing like Sa19 just one U strongly activated cells. U-less Sa12 containing a CGG motif failed to activate TLR8, while its mutant Sa12s6U carrying an A6U mutation resulting in containment of a UAA motif, included in duplicate in BtmtD3_4, while HsmtD1 carries a UGA motif, strongly activated cells (Fig 3E–H). Our data imply UR/URR rather than mere UG/UGG as RNA ligand consensus motif (Figs 3F and H, and EV3B) [13].

**Bacterial infection-driven cell activation is TLR8 dependent and inhibited by chloroquine in whole blood**

Next, we explored the impact of hTLR8 on protective interventions during infection. To test this, THP-1 cells pretreated with TLR2 neutralizing mAb were seeded with viable *S. aureus*, or with *E. coli*. In TLR8−/−3ddiTHP-1 cells, inhibition of activation by TLR2 blockade was substantial (Fig 4A). Furthermore, a combination of chloroquine—a broadly established lysosomal function inhibitor—and T2.5 pretreatment significantly impacted PBMCs. Specifically, a contribution of TLR2 to both, TNF and IFNγ production upon Gram-positive bacteria-driven activation, was barely detectable since chloroquine alone was efficient, which was true also for Gram-negative bacterial infection in respect to IFNγ but not TNF release (Figs 4B and EV3C). The failure of the control stimulus (and TLR8 ligand BtmtD3_4 to induce substantial IFNγ production while triggering that of TNF to similar degrees as compared to both infections implicated involvement of further endosomal pattern recognition in bacterial infections such as of tRNA through TLR7 (Figs 4B and EV3C). Also within whole blood, chloroquine inhibited TNF release more strongly upon infection with Gram-positive bacteria as compared to infection with Gram-negative bacteria, while its impact on IL-6 production (strongly induced by lipopeptide/TLR2) was the opposite (Figs 4C and EV3D). Altogether, these data imply TLR8 as one major bacteria- and self-mitochondria sensor and hint at an anti-inflammatory potential of TLR8 blockade in sepsis. Whether this potential extends toward other clinical syndromes, such as trauma-induced sterile inflammation, or autoimmunity, remains to be evaluated.

**Perspective and concluding remarks**

Upon submission of this study, four reports on Gram-positive bacterial RNA sensing by hTLR8 or on mTLR13 structure have been published and largely summarized [26–30]. Thus, *S. aureus* as well as *Streptococcus pyogenes* and *Streptococcus agalactiae* and *Listeria*...
monocytogenes total RNAs activate hTLR8 but not TLR7 [26,28]. Total RNAs of further Gram-positive and probiotic bacteria, as well as Enterococcus faecalis (EC-12)-derived 23S and 16S rRNA drive TLR8-dependent, yet TLR7-independent IL-12 production [27]. Our results extended these and earlier findings by implicating SS beyond 23S and 16S rRNA of S. aureus and also Gram-negative E. coli as well as mtrRNA as immune stimulatory P-/DAMPs that activate hTLR8 with their UR/URR motif segments [11,13].

Function restricting TLR8 mutation in mice might underlie their TLR13 expression and thus the A7 methylation mediated Sa19 camouflage, which is inoperative in other tetrapodes including human, macaque, and hog (Figs 1C, EV3E and EV4). Evolution of

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Figure 2. Transcriptome as well as gain- and loss-of-function analyses implicate TLR8 as bacteria and mitochondria ribosomal RNA sensor.

A Cytochrome release of 3-day differentiated (ddi) THP-1 cells toward stimulation with TLR ligands and bacterial RNAs (S. Svedberg; r, ribosomal; tot, total; n = 3).
B, C Responsiveness of Unc93b1−/−3ddiTHP-1 cells treated with T2.5 and challenged with heat-inactivated (hi) bacteria (triangle, decreasing doses; n = 3).
D Ratios of constitutive mRNA amounts in 3- versus 8ddi (gray bar) and of each of both versus undifferentiated TLR3 cells (black or white bar, respectively) according to a transcriptome profiling result (n = 1).
E, F NF-kB-driven relative luciferase activity (Rel. luc. act.) of hTLR8-overexpressing HEK293 and murine RAW264.7 cells, respectively, upon challenges (vector, empty plasmid; n = 3).
G Transfection of TLR3 siRNA impairs 3ddiTHP-1 cell responsiveness (n = 3).

Data information: Graphs show mean ± SD; *P ≤ 0.05, **P ≤ 0.01, unpaired t-test; −, unchallenged; P3C, Pam3CSK4, n.d., not detected; S. aureus; E. coli.

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Figure 3. TLR8 recognizes UR/URR motif containing RNA.

A, B Cytokine release of 3-day differentiated (ddi) THP-1 cells challenged with (A) ORNs or (B) bacterial RNA fractions (tot, total; S. Svedberg; r, ribosomal).
C Responsiveness of Tlr8−/−3ddiTHP-1 cells challenged with heat-inactivated (hi) bacteria (triangle, decreasing doses; *P ≤ 0.05, **P ≤ 0.01, unpaired t-test) upon TLR2 blockade (T2.5).
D Responsiveness of 3ddiTHP-1 cells upon challenge with human (hs) mitochondrial (mt) RNA (triangle, increasing doses).
E–H NF-kB-driven relative luciferase activity (Rel. luc. act.) of hTLR8+ HEK293 cells or cytokine release by PBMCs all transfected with the RNAs indicated and additional uridine (U) if indicated (n.p., not performed; vector, empty plasmid).

Data information: Graphs show mean ± SD; n = 3; −, unchallenged; P3C, Pam3CSK4; S. aureus; E. coli; n.d., not detected.
TLR8 as a bacteria sensor might have resulted from perpetuating confrontation with either soil bacteria producing an MLS antibiotic, or bacteria expressing resistance conferring methyltransferase only, or bacteria carrying a respective 23S rRNA mutation upon fish–tetrapod evolutionary transition [31]. Since immune escape of such bacteria could have handicapped host survival, promiscuous ssRNA sensing via TLR8 is of advantage. The price to be paid, however, is reactivity toward endogenous ssRNA with which the immune system of hTLR8 transgenic mice can hardly cope [32,33].

Materials and Methods

Materials

RNase A, phorbol 12-myristate 13-acetate (PMA), U nucleosides, chloroquine, and gentamicin were applied at 20 μg/ml, 200 nM, 5 mM, 5 or 20 μg/ml (PBMCs or whole blood, respectively), and 10 μg/ml respectively (all Sigma). Cells were pre-incubated for 30 min for TLR2 blockade with 20 μg/ml T2.5 [3] (Hycult Biotech). Pam3CSK4 was applied at 0.1 μg/ml (EMC Microcollections). LPS (0111:B4), CL075, RNA40, loxoribine, and R848 were applied to cells at 0.1 or 1 μg/ml (human or murine cells, respectively), 2.5 μg/ml, 0.5 μg/ml, 600 μM, and 10 or 5 μg/ml (human or murine cells, respectively), respectively (all Invivogen). ODN 1668 was applied at a concentration of 10 μM (MWG).

Bacterial challenges

HiSa and hiEc were applied at concentrations of 1 × 10^8, 10^7, and 10^6 cfu/ml or a concentration of 1 × 10^7 cfu/ml only. The hiSa solution was incubated with RNase A for 1 h at 37°C wherein the 37°C incubation alone did not abrogate the stimulation capacity [5]. THP-1 cells were infected with 1 × 10^8, 10^7, and 10^6 cfu/ml of S. aureus and 1 × 10^7, 10^6, and 10^5 cfu/ml of E. coli. PBMCs with 1 × 10^6, 5 × 10^5, 1 × 10^5, and 1 × 10^4 cfu/ml of S. aureus and 5 × 10^4,
1 × 10^4, 5 × 10^3, and 1 × 10^3 cfu/ml of *E. coli*, and whole blood with 1 × 10^7, 10^6, and 10^5 cfu/ml of *S. aureus* and 1 × 10^6, 10^5, and 10^4 cfu/ml of *E. coli*. After 1 h, gentamicin was added to stop bacterial growth [3].

**Cell culture**

BM or splenocytes from mice and PBMCs generated from humans of wt genotype if not indicated otherwise as well as RAW264.7 (murine macrophagoid), HepG2 (human hepatocytoid), THP-1 (human monocytoid), and HEK293 (human fibroblastoid) cell line cells were grown under regular cell culture conditions [3]. THP-1 cells were differentiated with PMA (1 × 10^7 cells/96-well plate) for either 24 h followed by 3 days culture in PMA-free medium (3ddi), or 72 h followed by 5 days culture in PMA-free medium (8ddi). Whole blood was drawn into monovettes (Braun, 8 ml) prefilled with Bivalirudin (5.3 mg) and aliquoted in 200 µl portions [34]. Blood was drawn and PBMCs generated from buffy coats (provided by P. Horn) upon approval by the local ethics committee from healthy donors (14-5804-B0, University Hospital Essen) and from a patient expressing a nonfunctional Glu53A/157,159AGAG MyD88 mutant, approved through ethics vote of the local committee (282/11, University of Freiburg, Germany).

**Bacteria**

*S. aureus* (DSMZ 20231) and a clinical isolate clone of *E. coli* [3] were used for infections and challenges as heat-inactivated bacteria *in vitro or in vivo*, or as sources of RNA preparations used for cell challenges by their transfection. Bacteria were inoculated with 16-h-grown preparatory cultures (agitation, 37°C, *E. coli* in LB, *S. aureus* in BHI medium). In the logarithmic growth phase, bacteria were pelleted and collected in PBS for RNA preparation, heat inactivation 100°C, 15 min), or infection. Bacterial samples (taken immediately prior to inactivation) were titrated by plating.

**Cytokine measurement and immunoblot analysis**

Enzyme-linked immunosorbent assay (ELISA, human IL-8, IL-6, and TNF, used also for macaque samples, mouse IL-6, TNF, and KC all R&D, capture and detection antiseria *swine IL-6, Bethyl Laborato- ries, recombinant swine IL-6, Kingfisher Biotech Inc.*) and luminex or ELISAs (ebioscience) for human IFNα were performed according to the manufacturer’s instructions. Supernatants from murine BMs and splenocytes or THP-1 cells (human) were sampled for ELISA 16 h after challenge start, while those of human PBMCs and whole blood were sampled after a 24-h time period.

**Mitochondria and subsequent mtRNA preparation**

Liver was harvested from Wistar rats. Tissue as well as harvested cultured HepG2 cells was homogenized in ice-cold isolation buffer (2 mM Hepes, 220 mM mannitol, and 70 mM sucrose, pH 7.4). Homogenized suspension was spun at 494 g (Sorvall ST16 cell centrifuge) for 3 min at 4°C. Supernatant was transferred to a fresh tube and spun. This washing step was repeated twice. Clear supernatant was spun at 26,000 g for 10 min at 4°C. Pellet was resuspended in ice-cold isolation buffer, and the centrifugation step was repeated twice which resulted in a pure mitochondria preparation. Pellets were resuspended in TRI reagent (Sigma) for RNA isolation (as described before [5]). A total of 0.25, 0.5, 1, or 2 µg per 200 µl mtRNA was applied to PBMCs and 0.5, 1, or 2 µg per 200 µl to THP-1 cells.

**Transfection of ORNs, and bacterial and mtRNA preparations**

RNA40 was transfected principally using LyoVec (Innoven) which was also used for ORNs, bacterial and mitochondrial RNAs challenge of BMs. RAW264.7 cells were transfected with Sa19 by LyoVec and with BtmtD3_4 by pLA (Sigma). HEK293 cells were transfected with ORNs with Lipofectamine 2000 (Life Technologies). All bacterial and mitochondrial RNA preparations were transfected with Lipofectamine 2000 in THP-1 cells and PBMCs except for BtmtD3_4 and *mtPTL* for the transfection of which pLA was used. ORNs (IBA) were transfected at a concentration of 100 pmol per 200 µl. Bacterial RNAs were transfected as follows: 1 µg (total RNA), 600 ng (23S and 16S rRNA), and 400 ng (5S rRNA) bacterial RNAs per 200 µl to challenge cells.

**TLR8 mRNA knockdown**

Forty-eight hours upon differentiation, THP-1 cells were transfected with siRNA (Qiagen) toward human TLR8 (ID S102642458) as well as scrambled variant (ID 1027280, negative control). Lipofectamine RNAiMAX Transfection Reagent (Life Technologies) was used according to the manufacturer’s instructions. Twenty-four hours post-transfection cells were challenged with stimulants in fresh medium. Supernatants were analyzed by ELISA. RNA was isolated and analyzed via RT-PCR as described before [5]. The primers used for PCR were (MWG eurofins) as follows: 18S rRNA, (sense) 5'-GAGCATC and 5'-GAGGATC AACCAGGAAG-3', (antisense) 5'-TGCCGTAGCTACATTG-3'; human Tlr8, (sense) 5'-GAGGATC AACCAGGAAG-3', (antisense) 5'-TGCCGTAGCTACATTG-3'.

**TLR8 knockout**

*Tlr8<sup>−/−</sup>-THP-1 cells were generated using the CRISPR/Cas9 technology as previously described [22]. In brief, cells were transfected with two plasmids encoding a CMV-mCherry-Cas9 expression cassette and a U6 promoter-driven sgRNA, respectively, by electroporation. The CRISPR target site used for TLR8 was AGTCCAGC ATGGAGCACTGAGG. THP-1 cells expressing mCherry-Cas9 upon electroporation were enriched by FACs sorting and cloned by limiting dilution. Two weeks later, single-cell clones were picked and expanded. Of these clones, genomic DNA was extracted and subjected to a first-level PCR using the following primer pair: 5'-ACACCTTCTGATACAGGCAGGTCCTGAGTGAGG AA TGCTGCACT-3' and 5'-TACACCTTTGATACAGGCAGGTCCTGAGTGAGG AA TGCTGCACT-3'. In second-level PCRs, barcode primers unique for individual clones were used and PCR products were subjected to deep sequencing using the MiSeq (Illumina) benchtop sequencing system. Raw sequencing data were analyzed by applying the online tool OutKnocker.org and cell clones harboring out-of-frame mutations within the TLR8 gene were identified.
Gene array

For gene expression analysis of undifferentiated versus 3ddi versus 8ddiTHP-1 cells, RNA was prepared using TRI reagent (as described before [5]) from cells analyzed in parallel for their Sa19 responsiveness (Fig 1H). Total RNA concentration and purity were measured with a NanoDrop 1000 spectrophotometer (NanoDrop Technologies/Thermo Scientific). RNA integrity was assessed using an Agilent Bioanalyzer nano chip. Biotinylated cRNA was prepared according to the Affymetrix ExpressKit protocol starting from 200 ng total RNA. Following fragmentation, 10 μg of cRNA was hybridized for 16 h at 45°C on GeneChip HG-U133Plus_2. GeneChips were washed and stained in the Affymetrix Fluidics Station 450 using the Affymetrix hybridization, wash, and staining Kit. Arrays were scanned in a GeneChip 3000 scanner with G7 update. The data were analyzed with MAS5 using Affymetrix default analysis settings and global scaling to a target intensity of 1,000 as normalization method. The microarray data from this publication have been submitted to the NCBI GEO database http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67264.

Reporter gene assay

Murine TLR13 and human TLR8 expression plasmids were transfected together with luciferase reporter plasmids into HEK293 cells for analysis of NF-κB-driven firefly and constitutive renilla luciferase activities upon calcium phosphate precipitation, as previously described [5]. RAW264.7 cells were transfected with the same plasmids using TurboFect (Life Technologies) following the manufacturer’s instructions. 16 h after challenge, cell lysate was analyzed for luciferase activity (96-well plate Berthold luminometer).

Statistics

Results were analyzed for statistical significance using the Student’s t-test for unpaired samples. Each illustrated column represents mean ± SD (error bars) of triplicate data points except for whole blood experiments results of which are represented by duplicate data points.

Further Methods are described in the Appendix Supplementary Methods.

Expanded View for this article is available online.

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Author contributions

AK, MO, and CC designed and performed most experiments, analyzed data, interpreted results, and participated in manuscript writing; DB and SR performed protein sequence alignment and in silico expression analyses; JK and PH performed comparative analysis of MyD88-defective human blood; AMS, JS, and HH prepared PBMCs and performed analyses; SS and JB designed experiments; HW designed experiments and wrote the manuscript; VH generated Thrb1−/−-THP-1 cells; and CJK overlooked the project, designed most experiments, analyzed and interpreted results, and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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