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TLR10 Is a Negative Regulator of Both MyD88-Dependent and -Independent TLR Signaling

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TLRs are central components of the innate immune system which, upon recognition of bacterial, fungal or viral components, activate intracellular signals that lead to protective inflammatory responses. Among the 10-member human TLR family, TLR10 is the only remaining orphan receptor without a known ligand or signaling function. Murine TLR10 is a disrupted pseudogene, which precludes investigation using classic gene knockout approaches. We report here that TLR10 suppressed the production of an array of cytokines in stably transfected human myelomonocytic U937 cells in response to other TLR agonists. This broad TLR suppressive activity affects both MyD88- and TRIF-inducing IFN-β–mediated signaling pathways upstream of IκB and MAPK activation. Compared with nontransgenic littermate controls, monocytes of TLR10 transgenic mice exhibited blunted IL-6 production following ex vivo blood stimulation with other TLR agonists. After i.p. injection of LPS, lower levels of TNFα, IL-6, and type 1 IFN were measured in the serum of TLR10 transgenic mice compared to nontransgenic mice, but did not affect mouse survival in an LPS-induced septic shock model. Finally, treatment of human mononuclear cells with a monoclonal anti-TLR10 Ab suppressed proinflammatory cytokines released by LPS stimulation. These results demonstrate that TLR10 functions as a broad negative regulator of TLR signaling and suggests that TLR10 has a role in controlling immune responses in vivo. ***The Journal of Immunology*****, 2016, 196: 3834–3841.**

Toll-like receptors are type 1 transmembrane receptors that are part of a broad class of innate immune receptors known as pattern-recognition receptors. TLRs serve as the first line of defense against infectious pathogens by initiating protective inflammatory responses following the direct sensing of bacterial, fungal or viral components. Recognition of a cognate microbial ligand by the extracellular leucine-rich repeat domain of each TLR leads to receptor dimerization (1). This event dimerizes two C-terminal Toll/IL-1 homology domains (TIR) that provide a scaffold for the recruitment of other cytosolic TIR domain–containing adaptor molecules that propagate intracellular signaling (2, 3).

Humans possess 10 TLR family members, numbered 1 through 10, subsets of which are expressed in leukocytes and various tissue cells (4, 5). TLRs 1, 2, 4, 5, and 6 traffic to the plasma membrane, sense microbial and fungal cell wall components and stimulate the production of classic proinflammatory molecules. TLRs 3, 7, 8, and 9 are located in endosomal compartments, sense viral and bacterial nucleic acids and are best known for their ability to stimulate the production of Type 1 IFNs (3). Almost all TLRs use the TIR domain–containing adaptor MyD88, which upon recruitment to dimerized TLRs at the plasma membrane induces the activation of NF-κB and other transcription factors that promote expression of classic proinflammatory cytokines (6). The TIR domain–containing adaptor inducing IFN-β (TRIF) is used by both TLR3 and endosomal TLR4 and, in a MyD88-independent manner, drives the expression of type 1 IFN production through activation of the transcription factor IFN regulatory factor 3 (3).

To date, we have a clear understanding of the ligand recognition, signaling, and biological functions of human TLRs 1 through 9. In contrast, TLR10 is the only remaining orphan human TLR without a confirmed ligand, signaling pathway, or biological function. The TLR10 gene was first cloned in 2001 and various studies have revealed strong transcriptional expression in the lymphoid tissues including the spleen, lymph node, thymus, and tonsils. TLR10 is expressed among a number of leukocyte subtypes and perhaps most prominently by B cells (4, 5). A major obstacle toward defining a function for TLR10 is that several retroviral insertion elements have rendered TLR10 a pseudogene in mice thus precluding a classic knockout mouse model (7). Although TLR10 is disrupted in mice, every other mammal sequenced to date contains an undisrupted TLR10 gene, including numerous primate species, domestic animals, and a variety of rodents (8, 9).

TLR10 is most homologous to TLR1 and TLR6, which both cooperate with TLR2 to mediate inflammatory responses to a variety of microbial lipids including bacterial lipoproteins (8, 9). Previously, we have shown that TLR10 is capable of binding the TLR2/1 ligand PAM_3CSK_4 in cooperation with TLR2. The resulting dimer is able to recruit MyD88 although no TLR-associated events including the transcriptional activation of NF-κB, IL-8, and IFN-β promoters has been detected (10). Sequence analysis has revealed that the TIR domain of TLR10 is less conserved and has a calculated rate of evolutionary change that is higher than that of any other TLR (8). This finding suggests that TLR10 may have a unique function among the TLR family.

To assess the function of human TLR10, we have generated stably transfected monocytic cells lines, a transgenic mouse model, and biologically active mAbs to the TLR10 receptor. Collectively,

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Abbreviations used in this article: pI:C, polyinosinic:polycytidylic acid; TIR, Toll/IL-1 homology domain; TRIF, TIR domain–containing adaptor inducing IFN-β.

our findings support the idea that TLR10 is an anti-inflammatory receptor. Importantly, we also show that the TLR10-mediated suppression broadly affects both MyD88- and TRIF-dependent signaling pathways, making TLR10 a potential global suppressor of TLR signaling.

Materials and Methods

Reagents

Abs specific for phospho-p38 (clone D3F9), phospho-JNK (clone 81E11), phospho-ERK (clone D13.14.4E), IκBα (clone 44D4), and β-actin (clone 13E5) were obtained from Cell Signaling Technologies (Beverly, MA). Anti-FLAG Ab (clone M2) were obtained from Sigma-Aldrich (St. Louis, MO). FITC conjugated CD11b (clone 3A33) was obtained from Abcam (Cambridge, MA). Directly conjugated Abs to Ly6G (clone 1A8) and IL-6 (clone MP5-20F3) were obtained from BioLegend (San Diego, CA). The synthetic triacylated lipopeptide PAM_3CSK_4 was purchased from Alexis Biochemicals (San Diego, CA); LPS from *Escherichia coli* strain K235, O111:B4 and polyinosinic:polycytidylic acid (pI:C) were from Sigma-Aldrich. Monoclonal Abs against the extracellular domain of recombinant human TLR10 were produced in-house. One clone, 3C10C5, is commercially available. The Ab clone used in this study is 5C2C5. The isotype control used was MOPC-21, a murine IgG1 with no known specificity.

Plasmid constructs

Expression constructs for TLR10, TLR1, TLR1-10, TLR10-1, CD4-TLR10, CD4-TLR1, MyD88, TRIF, IL-8 promoter driven luciferase, IFN-β promoter driven luciferase, and Renilla-luciferase transfection control are previously described (10). The pMX-IRES-Puro expression vector, used for the production of retrovirus, was purchased from Cell Biolabs (San Diego, CA). This vector was then modified to include a preprotrypsin leader sequence and a FLAG linker region into which the coding region of TLR10 was subcloned to generate pMX-FLAG-TLR10. The CMV-FLAG-TLR10 plasmid, used in the generation of TLR10 transgenic mice, was constructed by inserting the TLR10 coding sequence lacking the endogenous signal peptide into pFLAG-CMV vector (Sigma-Aldrich).

Development of stable cell lines

The NIH3T3 amphoteric packaging line was plated in 10-cm dishes and transfected with either pMX-FLAG-TLR10 or the empty pMX-FLAG vector using FuGene 6 according to the manufacturer's instructions (Roche, Indianapolis, IN). Forty-eight hours after transfection, viral supernatants were cleared of cell debris and applied to parental U937 cells during log phase growth. Twenty-four hours after viral transduction, virus was removed and stably transduced cells were selected in media containing 2 μg/ml Puromycin (Sigma-Aldrich). Batch-derived cells were expanded and surface expression of the FLAG epitope was verified.

Cell culture and TLR stimulation

Human cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-Glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Stably transduced U937 cells were kept under selection in the presence of 2 μg/ml puromycin (Sigma-Aldrich). To investigate cytokine production, cells were plated at 5×10^4 cells per well in 96-well plates and differentiated with PMA at 100 ng/ml for 48 h. Cells were then washed and allowed to rest in fresh media for 24 h. Media was replaced before cells were stimulated with 50 ng/ml PAM_3CSK_4, 50 μg/ml pI:C, or 50 ng/ml LPS. Cells were stimulated overnight with the indicated agonist, and cell-free supernatants were collected for cytokine analysis.

Transient transfection assays

HEK293T cells were transfected in 12-well plates using FuGene 6 with vectors encoding either MyD88 or TRIF constructs (20 ng/ml), TLR vectors (100 ng/ml), either IL-8 or IFN-β luciferase reporter (150 ng/ml), and *Renilla* luciferase as a transfection control (50 ng/ml). Luciferase readings were obtained using the Dual-Luciferase Assay Kit (Promega, Madison, WI) according to the manufacturer's instructions.

Real-time PCR analysis

U937 cell lines were plated at 2×10^6 cells/ml and differentiated for 48 h in 100 ng/ml PMA. Media was changed and cells were allowed to rest for 24 h before stimulation with 100 ng/ml PAM_3CSK_4 for 4 h. After stimulation, cells were harvested and RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany). RNA quality was assessed by spectrophotometry and agarose gel electrophoresis. Specific mRNA transcripts were quantified using the SABiosciences TLR pathway qPCR Array (Qiagen), according to the manufacturer's instructions. The array includes analysis of 84 genes involved in TLR signaling plus five housekeeping genes for normalization and controls for genomic DNA contamination, RNA quality, and general PCR performance.

Analysis of MAPK signaling and NF-κB activation

Vector control and TLR10-expressing U937 cells were stimulated with either 50 ng/ml PAM_3CSK_4, 50 μg/ml pI:C or 50 ng/ml LPS for the indicated time periods. Cell lysates were prepared from 1×10^7 cells in RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1% Nonidet P-40, 1 mM EDTA) supplemented with the Halt protease and phosphatase inhibitor mixture (Thermo Scientific, Rockford, IL). Equal amounts of protein lysate were loaded on SDS-PAGE gels, transferred to PVDF membranes and blotted using specific Abs (Cell Signaling Technologies, Beverly, MA).

Development of TLR10 transgenic mice

CMV-FLAG-TLR10 plasmid (10) was digested with Spe1 and Stu1, and 2 μg of purified linearized CMV-FLAG-TLR10 dsDNA was injected into C57BL/6 (Harlan Laboratories) fertilized oocytes. Early embryos were then transferred into Swiss Webster pseudopregnant fosters to generate transgenic founders. Pups were weaned and genotyped using a primer set specific to the 5′ end of the transgene: forward 5′-ACA AAG ACG ATG ACG ACA AGC-3′ and reverse 5′-AAT AGA ACC GAT GTC TTA GC-3′. A second primer set was specific to the 3′ end of the transgene: forward 5′-ACT TTG TCC AGA ATG AGT GG-3′ and reverse 5′-TAT TAG GAC AAG GCT GGT GG-3′. All transgenic mice were generated by the Transgenic Mouse Core Facility at University of Illinois at Urbana–Champaign under an approved Institutional Animal Care and Use Committee protocol.

Southern blot analysis

Mouse tail genomic DNA was isolated through proteinase K treatment and phenol:chloroform:isoamylalcohol (25:24:1) purification. Five micrograms of genomic DNA was digested with Sac1 and separated on a 1% agarose gel. DNA was blotted onto Immobilon-Ny^+ membrane (Millipore), washed with 6X SSC Buffer and then cross-linked using a ultraviolet Stratalinker (Agilent Technologies). Hybridization was carried out at 65°C with a biotin-labeled 3′-transgene fragment containing the hGH polyA sequence of the CMV-FLAG-TLR10 vector as described by the manufacturer (South2North; Thermo Scientific). Transgene copy number was estimated from copy number standards containing varying amounts of unlabeled probe DNA.

Tissue RNA extraction and RT-PCR

Approximately 100 mg of mouse tissue was homogenized in 1 ml Trizol (Ambion) reagent using a Dounce homogenizer (Wilmad laboratory glass). RNA was then purified with PureLinkTM RNA purification kit (Ambion) and used to generate first-strand cDNA with SuperScript III reverse transcriptase (Invitrogen). PCR was carried out using the forward primer 5′-ACA AAG ACG ATG ACG ACA AGC-3′ and the reverse primer 5′-AAT AGA ACC GAT GTC TTA GC-3′.

Western blotting of TLR10

Mouse tissue was ground with a Dounce homogenizer in 1 ml RIPA lysis buffer containing protease and phosphatase inhibitors. Cell homogenate was then incubated on ice for 2 h followed by centrifugation at top speed for 15 min at 4°C. Total protein was separated on a 7% SDS-PAGE gel, blotted onto PVDF membrane (GE Healthcare) and probed with HRP conjugated anti-FLAG Ab (clone M2).

Mouse whole blood stimulation and intracellular IL-6 staining

Mouse whole blood was collected from the lateral tail vein of age- and gender-matched, nontransgenic and transgenic mice. Fifty microliters of whole blood was mixed with an equal volume of RPMI 1640 medium containing 500 ng/ml PAM_3CSK_4, 100 μg/ml pI:C, or 200 ng/ml LPS and incubated at 37°C with gentle agitation overnight. Centrifuged supernatants were assessed for cytokines by standard ELISA. For intracellular IL-6 staining, 100 μl mouse whole blood was stimulated with 500 ng/ml LPS in the presence of Brefeldin A for 6 h. RBCs were lysed with ACK buffer and peripheral blood leukocytes were stained with anti-mouse CD11b and Ly6G Abs. Permeabilized cells were stained with directly conjugated anti-mouse IL-6 Ab or isotype control Ab.

Intraperitoneal injection and shock assay

Age- and gender-matched nontransgenic and transgenic mice (8–10 wk old) were injected i.p. with a high dose of LPS from *E. coli* O111:B4 (20–25mg/kg). Tail blood was collected at 1 and 4 h postinjection, and serum was prepared and assayed for cytokines as described above. In the shock assay, injected mice were monitored for survival for up to 7 d.

Cytokine assays

Human IL-6 and IL-8 and mouse IL-6 and TNF-α were analyzed by paired-Ab ELISA according to manufacturer's instructions (Invitrogen, Grand Island, NY). Type 1 IFN was detected using an ISRE-L929 reporter cell line bioassay (a gift from Dr. Bruce Beutler, University of Texas Southwestern Medical Center). ISRE-L929 cells were plated at 2×10^4 cells per well in a 96-well format. Cell culture media was replaced 24 h later with cell-free supernatant from either stimulated U937 cells or mouse serum. ISRE-L929 cells were incubated for an additional 6 h, and luciferase activity was detected in cell lysates using a luciferase reporter assay system (Promega).

Human mononuclear cell stimulation

Primary human mononuclear cells were obtained from venous blood of consenting healthy adult volunteers under an approved institutional review board protocol. Blood was mixed 1:1 in leukocyte isolation buffer (1× PBS, 2 mM EDTA, 2% FBS) before centrifuging over a Ficoll gradient (1.077 g/L) at $1100 \times g$ for 15 min with no brake. The resulting buffy coat was washed twice in cell culture media before plating 100 μl of ~$2–5 \times 10^4$ cells in a 96-well plate. Cells were allowed to preincubate in the presence of either an isotype control (clone MOPC-21) or anti-TLR10 Ab (clone 5C2C5) before stimulation with LPS (10 ng/ml). After 24 h, cell-free supernatants were collected and assayed by ELISA.

Statistical analysis

All data were analyzed with the two-tailed Student *t* test unless otherwise indicated.

Results

TLR10 is a suppressor of TLR2/1-induced responses

To examine the biologic function of TLR10, we stably expressed the receptor in U937 cells, a human myelomonocytic line that lacks detectable endogenous TLR10 expression (Fig. 1A). Using MMLV retrovirus, an N-terminally tagged FLAG-TLR10 construct was stably transduced into U937 cells, and TLR10 expression was confirmed with both RT-PCR and flow cytometry using an anti-FLAG Ab (Fig. 1A, 1B). The stably transduced cell line exhibited no differences in cell growth or viability compared with the parental or empty vector (MMLV) control lines (S. Jiang, unpublished observations). To explore potential signaling outputs of TLR10, we compared the MMLV and TLR10 cell lines for the expression of 84 genes known to be targets of TLR signaling following stimulation with the TLR2/1 lipopeptide agonist PAM_3CSK_4. Compared with MMLV-U937 cells, the RNA message for 11 genes had lower PAM_3CSK_4 induction levels in TLR10-U937 cells. These genes included those encoding the proinflammatory cytokines TNF-α, IL-1α, IL-6, and IFN-β. These genes also included the anti-inflammatory cytokine IL-10, which was also suppressed in TLR10-U937 cells compared with control MMLV-U937 cells (Supplemental Table I). Supernatants from cells stimulated with varying concentrations of PAM_3CSK_4 confirmed that TLR10-U937 cells secreted significantly lower levels of both IL-6 and TNF-α (data not shown). These data suggest that TLR10 mediates an anti-inflammatory function in U937 cells stimulated with TLR2/1 lipopeptide agonist.

TLR10 inhibits TLR-induced inflammatory responses

To examine the specificity of TLR10 suppression, we stimulated the U937 cell lines with TLR agonists in addition to those for TLR2/1 (PAM_3CSK_4), including TLR2/6 (MALP-2), TLR3 (pI:C) and TLR4 (LPS). Compared with MMLV-U937 cells, TLR10-U937 cells secreted significantly less IL-6 in response to all the TLR agonists tested (Fig. 1C). The secretion of type 1 IFNs following stimulation with the TLR3 agonist pI:C (Fig. 1D) was also marked by a decreased in TLR10-U937 cells. The cell lines had indiscernible differences in IL-6 production in response to IFN-β and indiscernible differences in type 1 IFN production in response to TNF-α. Taken together, these results support the idea that TLR10 broadly inhibits production of both IL-6 and IFN-β induced by a variety of TLRs, but not the production of these cytokines induced through other signaling pathways.

TLR10 suppresses IκBα degradation and the phosphorylation of MAPKs

To gain insight into the mechanism of TLR10-mediated suppression, we analyzed the effect of TLR10 on various canonical TLR signaling pathways, including the activation of NF-κB and various MAP kinases. As shown in Fig. 2, PAM_3CSK_4, pI:C, and LPS each triggered IκBα degradation within 30 min of TLR stimulation in vector control cells. However, degradation of IκBα was consistently inhibited in TLR10-expressing cells, suggesting that TLR10 suppresses NF-κB signaling upstream of IκBα degradation. TLR10-U937 cells also exhibited reduced ERK, JNK, and p38 phosphorylation levels compared with vector control cells following stimulation with TLR agonists PAM_3CSK_4, pI:C, or LPS. Taken together, these results indicate that TLR10 acts as a broad suppressor of TLR-induced proinflammatory signaling including NF-κB and MAP kinase signaling pathways.

TLR10 suppresses MyD88 and TRIF signaling

The ability of TLR10 to suppress responses broadly from a variety of TLRs suggests that this receptor inhibits MyD88-dependent signaling. To assess this, we examined the effect of TLR10 on HEK293T cells expressing MyD88. As expected, the overexpression of MyD88 in HEK293T cells resulted in strong constitutive induction of an IL-8 promoter-driven luciferase reporter (Fig. 3A). Consistent with its suppressive role, coexpression of TLR10 resulted in an 8-fold reduction of MyD88-induced IL-8 luciferase activity. In contrast, coexpression of TLR1, the closest homolog of TLR10, had no measureable effect.

The inhibitory effect on TLR3-induced IFN-β production strongly suggests that TLR10 also suppresses MyD88-independent signaling mediated by the TLR adaptor TRIF. To assess this, we examined the effect of TLR10 on HEK293T cells expressing TRIF, which induces constitutive activation of IFN regulatory factor 3, thereby driving type 1 IFN production. The overexpression of TLR10 resulted in a marked reduction of type 1 IFN production, as measured by a bioassay, whereas coexpression of TLR1 had no measureable effect (Fig. 3B). Importantly, TLR10 suppression of both MyD88- and TRIF-mediated pathways is dose dependent (Supplemental Fig. 1). The data together suggest that TLR10 acts as a negative regulator of proinflammatory TLR signaling, targeting both MyD88- and TRIF-dependent TLR signaling.

To assess the roles of the extracellular LRR domain and the intracellular TIR domain of TLR10 in suppression, we generated chimeric receptors between TLR1 and TLR10. Interestingly, neither the construct containing the extracellular domain of TLR1 and the intracellular domain of TLR10 (TLR1-10) nor the reverse chimeric receptor (TLR10-1) were able to suppress MyD88- or TRIF-dependent activation, demonstrating that both domains of TLR10 are required for its suppressive function (Fig. 3A, 3B). To assess the role of receptor dimerization, we replaced the extracellular domain of TLR10 with that of CD4—an approach that has been shown to cause constitutive TIR domain-mediated signaling because CD4 naturally forms dimers. Similar to full-length TLR10, CD4-TLR10 inhibited both MyD88- and TRIF-dependent signaling. In contrast, CD4-TLR1 had no effect on either MyD88 or TRIF signaling (Fig. 3C, 3D). Cellular expression of all the chimeric receptors was confirmed by flow cytometry (Supplemental Fig. 2). Taken together,

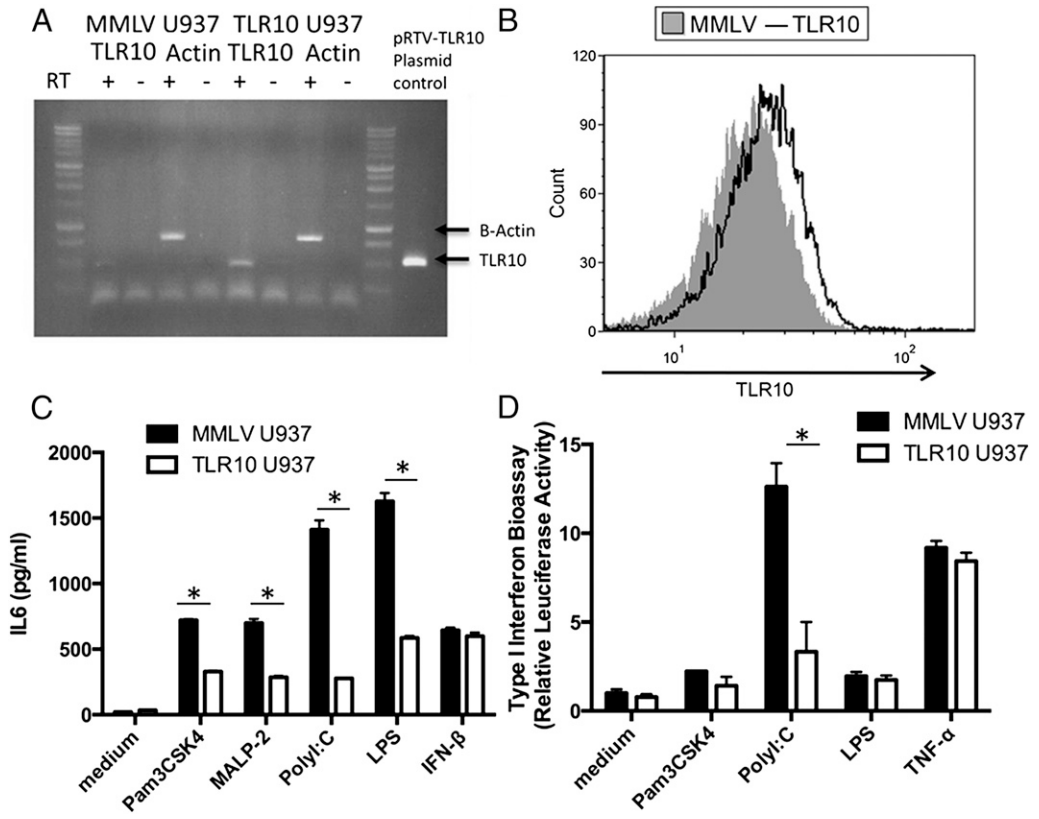

FIGURE 1. Stable TLR10 expression in U937 cells suppresses TLR-induced cytokine production. Parental U937 cells were transduced with either MMLV encoding FLAG-tagged TLR10 or an empty control MMLV. Stably transduced cells were isolated after 2 wk of selection under 2 μg/ml puromycin. (**A**) RT-PCR analysis of TLR10 expression in MMLV-U937 versus TLR10-U937 cell lines. (**B**) Surface staining of TLR10 transfected and control cells using an anti-FLAG Ab. Cells were gated on forward and side scatter characteristics. (**C**) TLR10-U937 and MMLV-U937 control cells were differentiated for 48 h in PMA. Cells were stimulated with the indicated agonist overnight, after which IL-6 was measured in cell-free supernatants using a paired-Ab ELISA. (**D**) Type 1 IFN was measured using an ISRE-L929 luciferase reporter assay. Luciferase activity was normalized to that of unstimulated MMLV control cells. Error bars represent the SD of three independent samples, and statistical analysis was performed using two-tailed paired Student *t* test. $*p < 0.01$

these data suggest that TLR10 functions as a homodimer to suppress both MyD88- and TRIF-dependent TLR signaling.

Development of TLR10 transgenic mice

To explore the potential function of TLR10 in vivo, we developed a transgenic mouse in which an N-terminally FLAG-tagged human TLR10 is constitutively expressed behind a strong viral promoter. Thirty-two pups were derived from oocyte injection of the vector and then screened for the transgene with 14 PCR-positive founders further analyzed by Southern blot (Fig. 4A). A founder with an intermediate copy number of the transgene and broad tissue expression was selected from among the 14 candidates for further analysis (Fig. 4B). TLR10 expression levels were highest in the spleen with confirmed expression in blood leukocytes (Fig. 4C).

Founder mice showed no overt developmental abnormalities and reproduced at expected Mendelian ratios. However, two founders

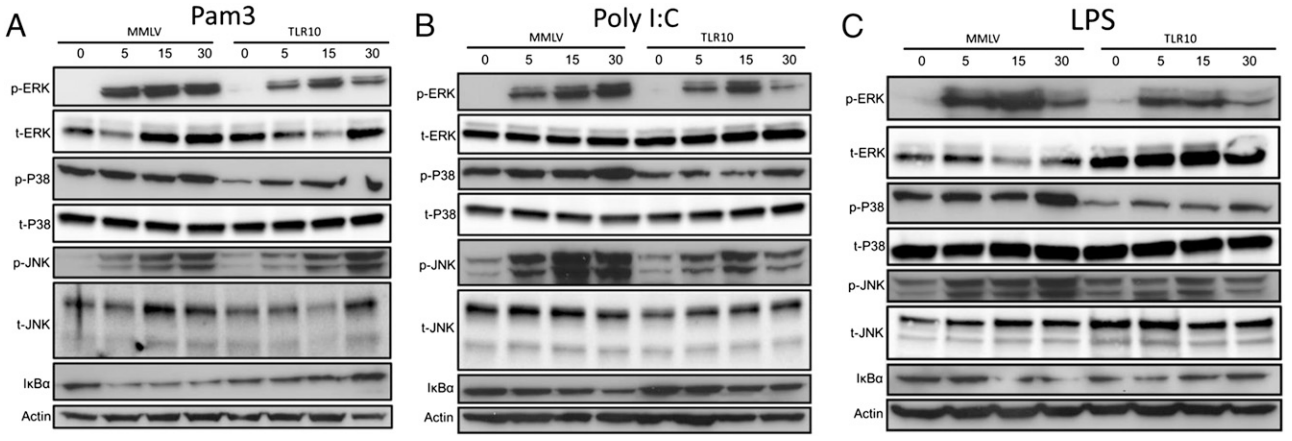

FIGURE 2. TLR10 suppresses phosphorylation of MAPKs and degradation of IκB. TLR10-U937 and MMLV-U937 control cells were stimulated with (**A**) 50 ng/ml PAM_3CSK_4, (**B**) 50 μg/ml pI:C, or (**C**) 50 ng/ml LPS. Cell lysates were analyzed by immunoblotting for the indicated signaling targets. Data are representative of at least three independent experiments.

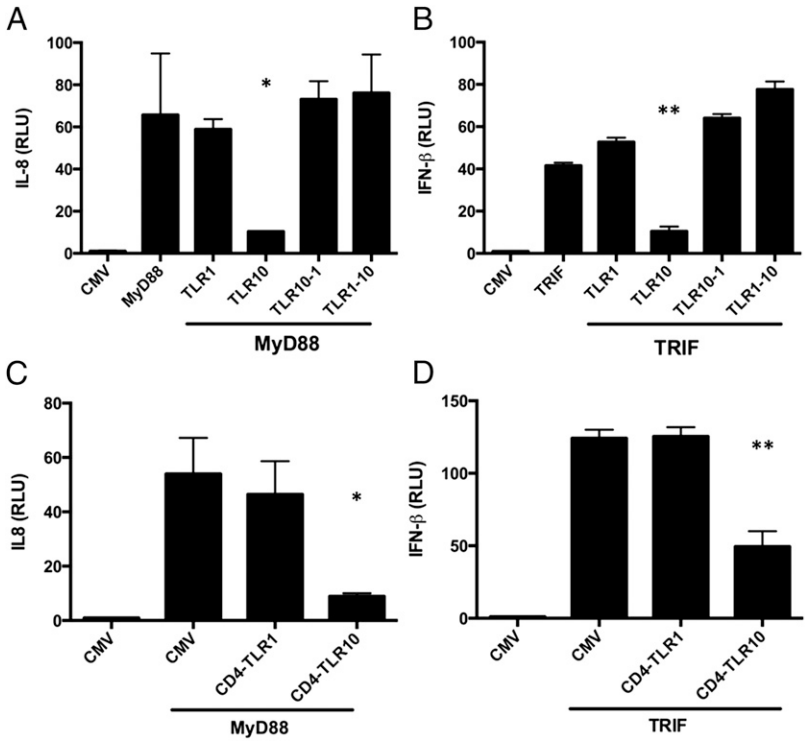

FIGURE 3. TLR10 suppresses both MyD88 and TRIF signaling. HEK293T cells were cotransfected with the indicated TLR construct and either (**A** and **C**) MyD88 and an IL-8 promoter-driven luciferase construct or (**B** and **D**) TRIF and an IFN-β–driven luciferase reporter construct. Results indicate fold induction of luciferase over empty vector after normalizing each sample for transfection efficiency using *Renilla* luciferase. Error bars represent the SD of three independent samples, and statistical analysis was performed using two-tailed paired Student *t* test. $*p < 0.05$, $**p < 0.005$

that exhibited the highest level of TLR10 expression across a variety of tissues developed lethal urethral tract infections in the first 2 mo of life. Necroscopy revealed a high concentration of Gram-positive bacteria, including *Staphylococcus aureus* and species of *Enterococcus*, in the urine (X. Li, unpublished observations). These observations suggest that high levels of TLR10 expression in mice may suppress the immune system, allowing for the opportunistic overgrowth of commensal bacteria.

TLR10 transgenic mice exhibit reduced TLR-induced responses

To determine the effect of TLR10 expression on murine blood leukocytes, we examined IL-6 production following ex vivo stimulation of whole blood with different TLR agonists. Production of IL-6 in whole blood of TLR10 transgenic mice was significantly lower than that of nontransgenic control mice in response to PAM_3CSK_4, pI:C, and LPS (Fig. 5A). The reduced cytokine production is not due to any significant differences in the numbers of

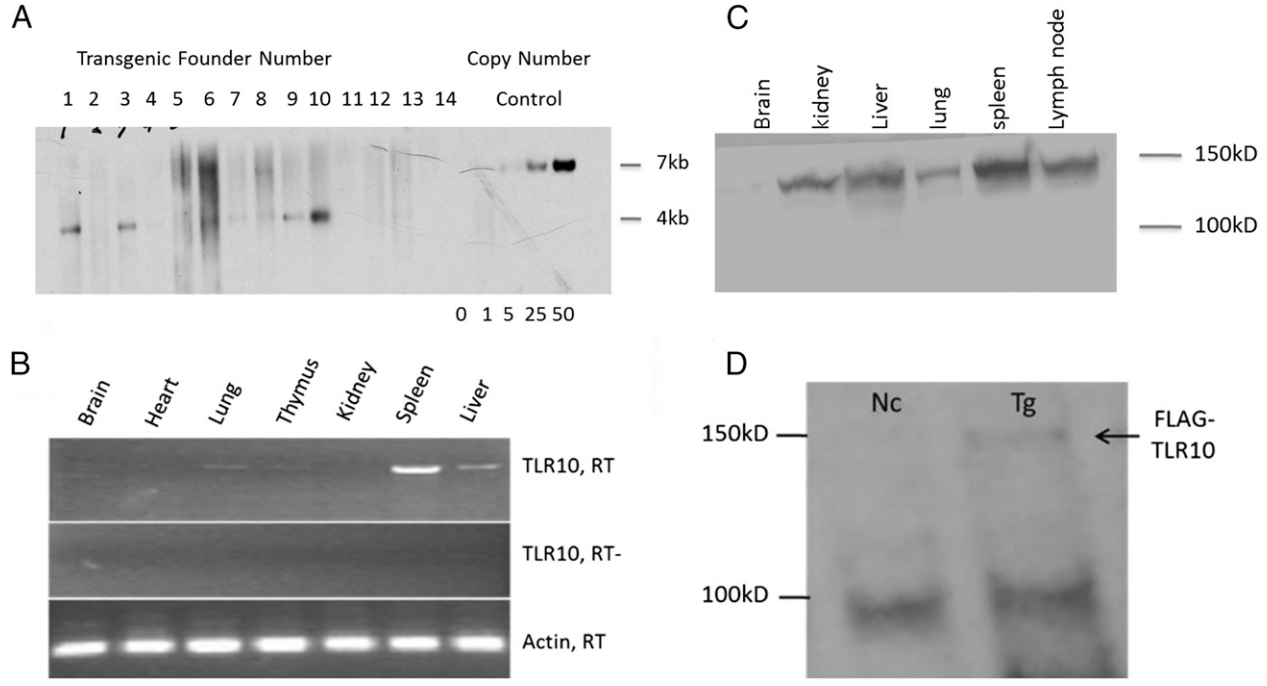

FIGURE 4. Generation of a TLR10 transgenic mouse under a constitutive CMV promoter. (**A**) Southern blot analysis of genomic DNA from 14 TLR10-positive founder mice with varying copy number insertions. (**B**) Reverse transcriptase dependent detection of FLAG-TLR10 in various tissues of a transgenic mouse. Western blots of (**C**) various tissues or (**D**) peripheral blood leukocytes from transgenic (Tg) and nontransgenic (Nc) mice using the anti-FLAG Ab to detect TLR10. FLAG-TLR10 migrates as an ~150-kDa band, whereas a nonspecific band appears at ~100 kDa.

peripheral blood monocytes or neutrophils (Supplemental Fig. 3). To identify the cell types targeted by TLR10-mediated suppression, we stimulated mouse peripheral blood ex vivo with LPS and measured intracellular IL-6 by flow cytometry. Monocytes ($CD11b^+$, $Ly6G^-$), but not neutrophils ($CD11b^+$, $Ly6G^+$), were shown to have suppressed IL-6 production in response to LPS stimulation compared with equivalent cells from nontransgenic control mice (Fig. 5B, 5C). These data confirm our previous findings in human myelomonocytic cell lines and suggest that, even in a murine background, human TLR10 maintains its function as an inhibitor of proinflammatory cytokine production.

TLR10 suppresses in vivo response to LPS

To assess the role of TLR10 in vivo, we measured blood cytokine levels in mice following i.p. injection with a sublethal dose of LPS. The induction of IL-6, TNF-α, and type 1 IFNs were all reduced in the TLR10 transgenic mice compared with the nontransgenic littermate control mice (Fig. 6A). To determine whether TLR10 can protect mice from LPS-induced septic shock, we monitored survival following i.p. injection of mice with a high, almost uniformly fatal, dose of LPS. However, no significant difference in either mortality or time-to-death was observed between TLR10 transgenic and nontransgenic mice (Fig. 6B). These data show that TLR10 is able to suppress a broad array of TLR responses in both MyD88- and TRIF-dependent manners, but it is not capable of protecting mice from LPS-induced septic shock in vivo.

Endogenous TLR10 suppresses human mononuclear cell activation

To assess the effect of TLR10 engagement on proinflammatory responses, we incubated human mononuclear cells isolated from the peripheral blood of healthy donors with either an anti-TLR10 Ab or a nonspecific isotype-matched control Ab. These cells were then stimulated overnight with the TLR4 agonist LPS. Compared with an isotype control Ab, the anti-TLR10 Ab suppressed the secretion of both IL-6 and TNF-α from the mononuclear cells of two independent donors (Fig. 7). These data show that Ab engagement of endogenous human TLR10 in primary human cells suppresses inflammatory responses mediated by MyD88.

Discussion

Previously we have shown that TLR10, either alone or with TLR2, fails to activate proinflammatory responses typically associated with this family of receptors (10). In this study, results stemming from a variety of experimental approaches support the idea that TLR10 functions as a broad suppressor of other TLRs with inhibitory activity toward both MyD88- and TRIF-dependent signaling. We believe that, as a suppressor, TLR10 functions as a homodimer, as evidenced by the fact that replacement of the extracellular domain of TLR10 with CD4, but not the extracellular domain of TLR1, results in a receptor that retains full suppressive function. Consistent with this notion is the observation that the crystal structure of the TLR10 TIR domain was solved as a symmetric homodimer (11). In addition, using gel filtration chromatography, we have found that the TLR10 extracellular domain purifies as a homodimer (data not shown). Together, these observations suggest that the extracellular and TIR domains of TLR10 contribute to homodimerization and that this event can be driven by either overexpression, in cell lines or mice, or through engagement of the endogenous receptor with a divalent mAb.

Among members of the TLR family, TLR10 is most homologous to TLRs 1 and 6, with the highest homology observed within the signaling TIR domain (9). In the crystal structure of the TLR10 TIR domain, a portion of the BB loop forms part of the dimer interface, but much of the loop is exposed and available to interact with TIR domain adaptor molecules such as MyD88 and TRIF (11). In this context, there are notable differences in key residues of the BB loop that mediate intracellular signaling; these include a 2-aa insertion just before the BB loop and amino acid changes within residues of the BB loop itself. Whether these difference are responsible for the suppressive activity of TLR10 remains to be investigated.

In support of our studies, another report published last year revealed TLR10 as an anti-inflammatory receptor that suppresses TLR2-mediated inflammatory responses in human mononuclear cells and a transgenic mouse model (12). In that study, TLR10 was shown to suppress the release of a number of proinflammatory cytokines in response to the TLR2 agonists PAM_3CSK_4 and whole *Borrelia burgdorferi*. In support, genetic polymorphisms in TLR10 have been shown to associate with variability of responses to bacterial lipopeptide (13). A number of possible mechanisms for suppression are proposed, including possible competition for ligand or coreceptors and the induction of anti-inflammatory cytokine expression. This study extends the suppressive function of TLR10 to that of other TLR family members and to both MyD88- and TRIF-dependent signaling pathways. Our analysis of signaling pathways indicate that TLR10 acts proximally in the signaling cascade through a currently undefined mechanism.

Two other studies have proposed an opposite, proinflammatory function for TLR10. One study showed that siRNA-mediated

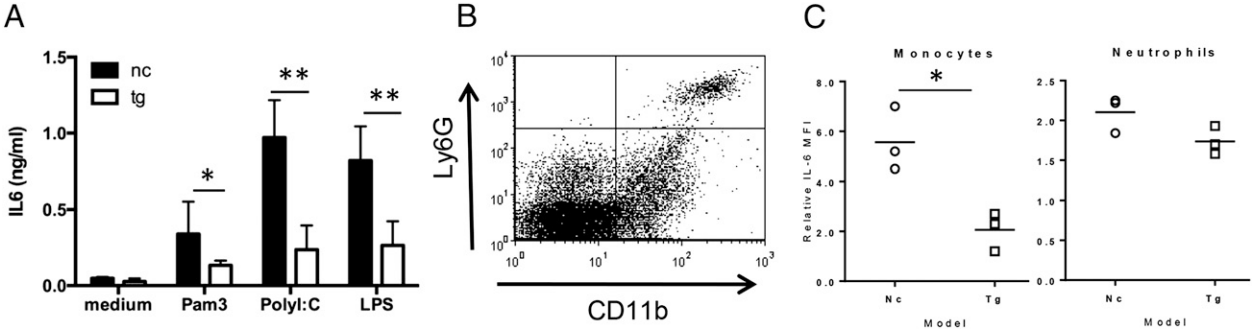

FIGURE 5. Blood monocytes of TLR10 transgenic mice exhibit suppressed cytokine production in response to TLR agonists. (**A**) Whole blood from either TLR10-transgenic (tg) or nontransgenic (nc) littermate controls was stimulated with the indicated agonists, and IL-6 release was measured in cell-free supernatants by ELISA. (**B**) Whole blood was stimulated with LPS, and IL-6 production was measured by intracellular staining in monocyte ($CD11b^+$, $Ly6G^-$) and neutrophil ($CD11b^+$, $Ly6G^+$) populations. Dot plot represents ungated cell populations. (**C**) Median fluorescent intensity (MFI) of IL-6 is shown relative to unstimulated cells. Error bars represent the SD of three independent samples, and statistical analyses were performed using two-tailed paired Student *t* test. $*p < 0.05$, $**p < 0.01$

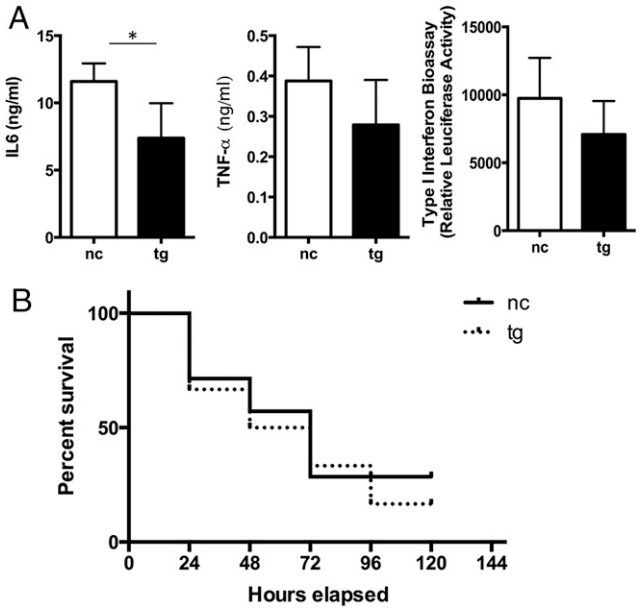

FIGURE 6. TLR10 transgenic mice exhibit suppressed responses to injected LPS, but are not protected in a model of septic shock. Four TLR10-transgenic (tg) or four nontransgenic littermate controls (nc) were injected i.p. with 25 mg/kg LPS. (**A**) Blood was collected at 1 and 4 h postinjection, and serum TNF-α and IL-6 levels were measured using a paired Ab ELISA. Serum type 1 IFN levels were measured using the ISRE-L929 reporter line. Error bars represent the SD of four mice, and statistical analysis was performed using one-tailed Student *t* test. (**B**) Six TLR10-transgenic (tg) and seven nontransgenic (nc) mice were injected i.p. with 25 mg/kg LPS and monitored for survival, with results plotted in Kaplan-Meier format. $*p < 0.05$.

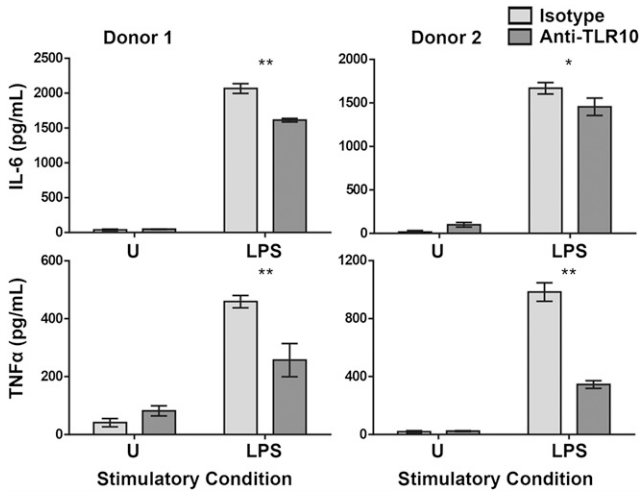

FIGURE 7. Anti-TLR10 Ab inhibits TLR-induced activation of PBMCs. Human PBMCs from two separate donors were preincubated with either an anti-TLR10 Ab or isotype matched control Ab prior to stimulation with the indicated agonist for 24 h. Cell-free supernatants were collected, and IL-6 and TNF-α production was assayed using an Ab-paired ELISA. Error bars represent SEM of three intradonor replicates, and statistical analysis was performed using the Holm–Sidak *t* test assuming equal population scatter. $*p < 0.05$, $**p < 0.01$.

knock down of endogenous TLR10 in HT-29 colonic epithelial cells blunted inflammatory responses to *Listeria monocytogenes* (14). Another study, showed that knockdown of TLR10 in the THP-1 myelomonocytic cell line inhibited cellular responses to the H1N1 and H5N1 flu virus strains (15). Interestingly, both studies revealed broad effects on proinflammatory cytokine and type 1 IFN production that were dependent on infection of cells with live pathogens, but not with heat-killed pathogens. Because both of these organisms replicate in the cytoplasm, intracellular forms of TLR10 may have a proinflammatory function following recognition of a PAMP associated with virulence (16). In the study of flu virus, transfection studies suggested that TLR10 responds to viral ribonuclear protein complexes (15). Although these findings contrast our own and others (12), it is noteworthy that we have engaged endogenous TLR10 at the cell surface, where it may possess an opposing signaling function.

The finding that TLR10 is a negative regulator among members of the TLR family is perhaps not surprising (17). Even within the closely related IL-1R family, whose members have TIR domains and use MyD88 to propagate signaling, are two inhibitory receptors known as ST2 (18) and SIGIRR (19). The transmembrane receptor ST2 has been shown to suppress NF-κB activation mediated by TLR2, TLR4, and TLR9, but not TLR3 by sequestering the adaptor proteins MyD88 and MAL. SIGIRR has been shown to inhibit MyD88-dependent signaling through its interactions with TRAF6 (TNF receptor-associated factor 6) and IRAK (IL-1R–associated kinase 1) (19). Thus, there is precedence for regulatory or inhibitory function among members of the TLR/IL-1R superfamily.

The biologic function of TLR10 might not be truly understood until a natural ligand has been discovered. Nevertheless, because sustained TLR signaling is an underlying feature of a wide variety of chronic inflammatory conditions, including many autoimmune diseases and cancers (13), the characterization of TLR10 as a broadly acting suppressor of TLR activation has far-reaching therapeutic implications.

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Disclosures

The authors have no financial conflicts of interest.

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