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SPECIES-SPECIFIC REGULATION OF TOLL-LIKE RECEPTOR 3 GENES IN MEN AND MICE

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SUMMARY

Toll-like receptor 3 (TLR3) belongs to a family of evolutionary conserved innate immune recognition molecules and recognizes double-stranded (ds)RNA, a molecular pattern associated with viral infections. Earlier studies suggested a differential expression pattern in men and mice, the molecular basis for this observation, however, was unknown. Here we demonstrate that species-specific differences in tissue expression and responses to lipopolysaccaride (LPS) coincide with the presence of different, evolutionary non-conserved promoter sequences in both species. Despite the overall unrelatedness of TLR3 promoter sequences, mRNA expression of both TLR3 orthologues was induced by interferons, particularly by IFN-β. The basal and IFN-β-induced activation of promoters from both species largely depended on similar interferon regulatory factor (IRF) elements which constitutively bound IRF-2 and recruited IRF-1 after stimulation. In murine macrophages, IFN-β-induced TLR3 up-regulation required IFNAR1, STAT1, and in part IRF-1, but not the Janus kinase (Jak) family member Tyk2. We also show that LPS specifically upregulates TLR3 expression in murine cells through the induction of autocrine/paracrine IFN-β. In humans, however, IFN-β induced upregulation of TLR3 was blocked by pretreatment with LPS, despite the efficient induction of IRF-1. Our findings reveal a mechanistic basis for the observed differences as well as similarities in TLR3 expression in men and mice. The IFN-β -TLR3 link further suggests a role of TLR3 in innate and adaptive immune responses to viral infections. It will be interesting and important to clarify whether the observed differences in the transcriptional regulation of TLR3 influence innate immune responses in a species-specific manner.

INTRODUCTION

Vertebrate Toll-like receptors (TLRs[1]) recognize conserved microbial structures (pathogen-associated molecular patterns, PAMPs). Upon ligation they activate conserved intracellular signaling pathways, leading to the up-regulation of co-stimulatory molecules or the secretion of cytokines – responses that are necessary to combat infection in vertebrates (as reviewed in (1;2)).

Two recent studies have implicated Toll-like receptor 3 (TLR3) in the recognition of double-stranded RNA, a molecular pattern associated with viral infections (3;4). Activation of the receptor with a chemical analog of double-stranded RNA, polyriboinosine-polyribocytidylic acid (poly(I:C)), was shown to induce the activation of NF-kB and the production of type I interferons (IFNs) in human and murine TLR3 expressing cell types (3;4). TLR3-deficient (TLR3-/-) mice exhibited reduced responses to poly(I:C), and reduced production of the inflammatory cytokines IL-6, IL-12 and TNF (3). A contribution of TLR3 to antiviral immunity, however, remains to be demonstrated.

Interestingly, different TLR3 expression patterns have been reported in mice and humans, a phenomenon also observed for at least three other TLR family members: TLR2, TLR4 and TLR9 (for a review see Ref (5)). Within the human hematopoietic compartment, TLR3 mRNA expression has been shown to be restricted to dendritic cells (6), as was the cytokine response to poly(I:C) stimulation (7). In mice, TLR3 is also strongly expressed in macrophages and its expression is markedly induced upon LPS stimulation – a feature that has not been observed in human cells (3).

Since the differential expression pattern of TLR3 could have a significant impact on TLR3 function in humans and mice, we have further characterized TLR3 expression and analyzed gene regulatory mechanisms acting in both species. We found that the non-coding regions, including 5'-exons and proximal promoter regions of *TLR3* genes are different in both species, as is the

cell type-specificity and the regulated expression upon stimulation with LPS. However, despite the overall unrelatedness of promoter sequences, both species share the up-regulation of TLR3 by IFN-β. Evolutionary conservation of TLR3 induction by interferons indicate that this regulatory feature may be important during viral infections in both species.

EXPERIMENTAL PROCEDURES:

Chemicals. All chemical reagents used were purchased from Sigma-Aldrich (Berlin, Germany) unless otherwise noted. Protease inhibitors are from Roche Biochemicals. Purified lipopolysaccharide (LPS) from *Salmonella abortus equi* (LPS_{SAE}) was a gift from C. Galanos (Freiburg, Germany). LPS from *Salmonella minnesota* (LPS_{SM}) and *Escherichia coli* (serotype 055:B5; LPS_{EC}) were purchased from Sigma-Aldrich. Oligonucleotides were synthesized by TIB Molbiol (Berlin, Germany). Antisera for supershift analyses were purchased from Santa Cruz.

Mice. The mutant mouse strain deficient in Tyk2 was generated by gene targeting, as described previously (8). To obtain a pure C57BL6 background Tyk2 deficient mice were backcrossed with C57BL6 mice for 8 generations. IFN-β–, IFNAR1-, STAT1- and IRF-1-deficient mice (C57BL6 background) have been described previously (9-12). Wildtype C57BL6 and Balb/c mice were obtained from Charles River.

Cells. Monocytes were isolated and cultured to generate macrophages or immature dendritic cells as described earlier (13). The human monocytic cell line THP-1 and the murine macrophage cell line RAW264.7 were maintained in RPMI 1640 medium plus 10 % FCS and supplements. THP-1 cells were differentiated for 48-72 h by adding PMA (10^{-8} M) to the culture medium. To prepare peritoneal macrophages, mice (12 weeks of age) were injected intraperitoneally (i.p.) with 2 ml of 4% thioglycollate medium (DIFCO). Three days later, peritoneal exudate cells were isolated from the peritoneal cavity by washing with PBS solution (GIBCO). Cells were cultured in endotoxin-free DMEM/5%FCS (GIBCO) and after 1 hour medium was changed to remove non-adherent cells. Adherent monolayer cells were used as peritoneal macrophages. Peritoneal macrophages were cultured in DMEM medium (GIBCO) supplemented with 5% FCS (GIBCO). Murine bone marrow derived macrophages (BMMs) were cultured as described previously (14).

RNA-preparation, Real-time-PCR. Total RNA was isolated from different cell types by the guanidine thiocyanate/acid phenol method (15). RNA (1 µg) was reverse transcribed using Superscript II MMLV-RT (Invitrogen). Real-time PCR was performed on a Lightcycler (Roche) using the Quantitect kit (Qiagen) according to the manufacturer's instructions. Primers used were: TLR3 (specific for human and mouse): sense 5`-TCA CTT GCT CAT TCT CCC TT-3`, antisense 5`-GAC CTC TCC ATT CCT GGC-3`. TLR2 (mouse): sense 5`-TTC TGA GTG TAG GGG CTT C-3`, antisense 5`-CCC AGA AGC ATC ACA TGA C-3`. ß-Actin: sense 5`-TGA CGG GGT TCA CCC ACA CTG TGC CCA TCT A-3`, antisense 5`-CTA GAA GCA TTT GTG GTG GAC GAT GGA GGG-3`. Cycling parameters were: denaturation 95°C, 15 min, amplification 95°C, 15 s, 56°C, 20 s, 72°C, 25 s, for 45 cycles. The product size was initially controlled by agarose gel electrophoresis and melting curves were analysed to control for specificity of the PCR reactions. TLR data were normalized for expression of the housekeeping gene β-actin. The relative units were calculated from a standard curve plotting 3 different concentrations of log dilutions against the PCR cycle number (CP) at which the measured fluorescence intensity reaches a fixed value. The amplification efficiency E was calculated from the slope of the standard curve by the formula: $E = 10^{-1/slope}$. E_{TLR3} was in the range of 1,78 to 2,14, E_{TLR2} ranged from 1,76 to 1,84. For each sample data of 3 independent analyses were averaged.

RNA ligase-mediated RACE-PCR.. Ten µg of total RNA from monocyte-derived DC or LPS-stimulated murine BMMs were used for cDNA synthesis with the FirstChoice™ RLM-RACE Kit (Ambion). The following TLR3-specific primers were used to amplify full-length 5'-cDNA fragments of human or murine TLR3, respectively: hTLR3-OUT (5'- TGT GAA GTT GGC GGC TGG -3') and hTLR3-IN (5'- CAG GTG GCT GCA GTC AGC AAC-3'); mTLR3-OUT (5'- GTC AGC TAC GTT GTA TCT CAC AGT G -3') and mTLR3-IN1 (5'- ACA CCC TTT CAT GAT TCA GCC -3') or mTLR3-IN2 (5'- ACA CCA GAA TCC ATA GGG AC -3'). PCR-

products from both species were cloned into pCR2.1-TOPO (TOPO Cloning Kit, Invitrogen) and inserts from at least 10 individual plasmid-containing bacterial colonies derived from each cell type were re-amplified by PCR and directly sequenced (performed by GENEART, Regensburg, Germany).

Plasmid construction and purification. A 588 bp genomic fragment of the human TLR3-promoter was amplified from human genomic DNA using the Expand High Fidelity PCR system (Roche Biochemicals) and the primers hTLR3p_S (5'- GAT CAG ATC TCA GCT TTG CCA TGT TTG G -3') and hTLR3p_AS (5'- ACG TGA ATT CTG TTG GAT GAC TGC TAG CCT TTC C -3'). Primer sequences were derived from a BAC clone containing the TLR3 sequence deposited in the Genbank database (Genbank accession no AC104070). The obtained PCR fragment was subcloned into pGL3-B (Promega) and sequenced. Deletions of the hTLR3(-588) construct were generated by PCR using primers hTLR3(-400)_S (5'- GTC AAG ATC TTC GCA TGA GTC TAG CAG -3') or hTLR3(-200)_S (5'- GAC TAG ATC TGG TTT GAA ACG CCT CTC TG -3') together with the vector-specific primer GL2 (Gibco). Two fragments of the proximal murine TLR3 promoter (including intron 1 and exon2) were similarly amplified from mouse genomic DNA using the primers mtlr3-F1_S (5'- TGC AAG ATC TGA GTG TAG CCA TGA GCC AGG -3') and mtlr3-F2_AS (5'- CAT CAA GCT TCT ATC TTC TTT TGG TGC GCG -3'). Deletions of the resulting mtlr3(-1368) construct were generated using the internal *Sac* I (-966) or *EcoR* I (-429) restriction sites. Mutations of putative transcription factor binding sites were carried out by PCR-mediated mutagenesis using the following primers: human ISRE/IRF element: htlr3IRF-M_S (5'-TTT TCA AGC TTT ACA CGC ACT TTC GAG AGT G-3') and htlr3IRF-M_AS (5'-CAC TCT CGA AAG TGC GTG TAA AGC TTG AAA A-3'); human STAT element: htlr3STAT-M_S (5'-CCT TTG CCC TTC TTA TGA TGC ACC AAA CAT AA-3') and htlr3STAT-M_AS (5'-TTA TGT TTG GTG CAT CAT AAG AAG GGC AAA GG-3'); murine exon 2 ISRE/IRF element: mtlr3IRFi-M_S (5'-CTC TCT CAA CTT AAG ACG CAC

TTT CAG GCT GA-3') and mtlr3IRFi-M_AS (5'-TCA GCC TGA AAG TGC GTC TTA AGT TGA GAG AG-3'); murine intron 1 ISRE/IRF element: mtlr3IRFo-M_S (5'-GGT AAG TGA ATG GCA CGC ACT TTG TTT AGA CA-3') and mtlr3IRFo-M_AS (5'-TGT CTA AAC AAA GTG CGT GCC ATT CAC TTA CC-3'). DNA sequence analysis was performed by GENEART (Regensburg, Germany). For transfections, plasmids were isolated and purified using the Endofree Plasmid Kit from Qiagen.

Transient and stable DNA transfections. THP-1 cells were transfected in duplicates using DEAE-dextran as described previously (16). Undifferentiated THP-1 cells were cultivated for 48 h before harvesting. In stimulation experiments, THP-1 cells were treated with PMA the day after transfection and harvested after 72 h. Cell lysates were assayed for firefly and renilla luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) on a Sirius luminometer (Berthold). Firefly luciferase activity of individual transfections was normalized against renilla luciferase activity. RAW264.7 cells were transfected using SuperFect reagent (Qiagen) according to the manufacturer's instructions as described (17). Duplicate transfections were harvested after 24h and cell lysates assayed for firefly luciferase activity using the Luciferase Reporter Assay System (Promega). Firefly luciferase activity of individual transfections was normalized against protein concentration measured using a BCA assay (Sigma). For stimulation experiments, RAW264.7 cells were transfected in 10 cm tissue culture dishes as above using linearized reporter constructs (10 µg) as well as a plasmid (pCDNA3) carrying the neomycin resistance gene (5 µg). Cells were selected for stable integration of plasmid DNA by culturing cells in RPMI 1640 medium supplemented with 350 µg G418 for two to three weeks. Stably transfected cells were pooled, expanded and 7.5 x 10^5 cells/ml were seeded into six-well plates in duplicates the day before stimulation. Cells were harvested at the indicated timepoints and cell lysates were assayed as above.

Nuclear extracts and electrophoretic mobility shift assay. Nuclear extracts were prepared as

described previously (16). Double-stranded oligonucleotides corresponding to the STAT- or IRF- elements were labeled with α-[^{32}P]dGTP using Klenow DNA polymerase. Sequences of IRF-motifs are indicated in the figures, sequences of other oligonucleotides were: human STAT/GAS element: human STAT-motif 5'-CTT TGC CCT TCT TGG AAT GCA CCA-3'; mutated human STAT-motif (5'-CTT TGC CCT TCT T***at g***AT GCA CCA -3'); consensus GAS-element: 5'-CTT TGC ATT TCC CCG AAA TCA CCA-3'. The binding reaction contained 2.5 µg of nuclear extract protein, 0.5 µg of poly d(I/C), 20 mM HEPES pH 7.9, 20 mM KCl, 1mM DTT, 1mM EDTA, pH 8.0, 5% glycerol and 20 nmol of probe DNA in a final volume of 10µl. Antisera used in supershift analyses were added after 15 min and samples were loaded onto polyacrylamide gels after incubating at room temperature for a total of 30 min. Buffers and running conditions used have been described (16). Gels were fixed in 5% acetic acid, dried and autoradiographed.

RESULTS

TLR3 mRNA expression in murine and human mononuclear phagocytes. Recently published data suggests a differential expression pattern of TLR3 in human and murine mononuclear cells (5). To directly compare the mRNA expression of TLR3 in human and murine cells, we designed PCR primers complementary to TLR3 sequences which are identical in both species. Real-time PCR using various mononuclear cell types from both species confirmed the previously observed predominant expression of human TLR3 in 'immature' myeloid dendritic cells (s. Figure 1). Highest TLR3 expression levels in mice, however, were detectable in macrophages, which expressed the highest TLR3 mRNA levels of all tested cell types. These results indicated that cell type-specific regulation of TLR3 might be different between the two species.

Species-specific TLR3 regulation in response to LPS or IFNs. Previously published data indicated a differential, species-specific response in LPS-stimulated cells regarding TLR3 expression (3;6;18). Additionally, TLR3 expression in human macrophages was shown to be markedly induced upon stimulation with IFN-α (19). To systematically compare the inducible expression patterns of TLR3 in both species, TLR3-expressing monocytic cells from both species were analyzed for TLR3 expression after stimulation with LPS, as well as type I or II interferons (IFN-β and IFN-γ, respectively), the latter being important mediators during viral infections. As shown in Figure 2, all three stimuli induced TLR3 mRNA in murine bone marrow-derived macrophages after 4h, with TLR3 being down-regulated to basal levels after 24 h. In the murine RAW264.7 macrophage cell line, again, all three stimuli up-regulated TLR3 mRNA at 4h. In contrast to bone marrow-derived macrophages, IFN-γ led to a further increase in TLR3 expression after 24 h in RAW264.7 cells. In human monocyte-derived cell types, a consistent up-regulation of TLR3 expression was only observed in the presence of IFN-β. Shown are two representative examples (out of four) for monocyte-derived dendritic cells from different donors. While expressing different basal levels of TLR3 mRNA, primary blood monocytes and

monocyte-derived macrophages up-regulated TLR3 in a comparable fashion in response to IFN-β (data not shown). The response to IFN-γ in human cells was variable depending on the donor. While the human monocytic cell lines MonoMac6 and THP-1 proved unresponsive under normal culture conditions, TLR3 mRNA was IFN-β-inducible in PMA-differentiated macrophage-like THP-1 cells (data not shown). In contrast to murine TLR3, we never observed a significant up-regulation of human TLR3 in response to LPS.

Determination of transcriptional start sites and proximal promoters. To further analyse the regulatory mechanisms of TLR3 expression in both species, we determined TLR3 transcriptional start sites by RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) PCR using total RNA derived from LPS-stimulated murine RAW264.7 macrophages or human monocyte-derived dendritic cells as described in Experimental Procedures. Comparison of the obtained 5'-cDNA sequences and publicly available genomic sequences revealed the complete structures of the human and murine TLR3 genes (see Figure 3). The translation start codon of human TLR3 is located in exon II whereas the murine coding sequence begins in exon IV. Murine TLR3 mRNA can be initiated from two alternative promoter regions preceding exon I or exon II, respectively, the latter being predominantly utilized in LPS-stimulated RAW264.7 macrophages. While the nucleotide sequences around coding regions of human and murine TLR3 share a high degree of homology (75% identity), sequence comparison using a ClustalW algorithm did not reveal a significant level of homology (3-16 % identical nucleotides) between the proximal promoter regions and 5'-UTRs of murine and human TLR3 genes. Computational analysis revealed a differential organization of the proximal promoter sequences from both species. Whereas the human gene contains a TATA-like element, the murine promoters are TATA-less and instead contain several putative binding sites for the myeloid and B-cell specific transcription factor PU.1 (sequences and structures of the proximal promoter regions are shown in Figure 3). To facilitate further analysis of mechanisms regulating the differential expression pattern of TLR3

in humans and mice, we cloned fragments of the 5'-proximal promoter regions of both TLR3 genes into a luciferase reporter plasmid. Transient transfection analysis was performed in the human monocytic cell line THP-1 and the murine macrophage cell line RAW 264.7 to determine the basal activity of proximal TLR3 promoter regions. As shown in Supporting Figure 8, human TLR3 promoter constructs were weakly active in both cell lines. Constructs containing either both alternative murine promoters or the downstream promoter alone were strongly active in murine macrophages, but only weakly in the human monocytic cell line, suggesting that the proximal promoters display different activities in human and murine cell lines.

TLR3 promoter elements and signaling molecules involved in IFN signalling. Initial transfection experiments indicated that the proximal TLR3 promoter regions from both species are responsive to IFN-β treatment in both human and murine cells. To determine *cis*-elements required for IFN-β-mediated induction of promoter activity, we mutated putative binding sites that could be involved in interferon-regulated gene responses, including two putative ISRE/IRF elements in the mouse promoter and an ISRE/IRF as well as a STAT element in the human promoter (see Figure 4A). In human PMA-differentiated THP-1 cells, wildtype TLR3 constructs of both species significantly responded to IFN-β after 24h, while only a marginal induction of promoter activity was observed after 4h. Mutation of the putative ISRE/IRF element in the human promoter abolished both basal and induced activity. Mutation of the nearby human STAT-site reduced basal activity to approximately 50 % and also abrogated the IFN-β-induced activation (Figure 4B). In murine RAW264.7 cells, promoters from both species were activated by IFN-β after 4h. Induction of both promoters by IFN-γ was delayed as compared to IFN-β. Promoters were only weakly induced by LPS in transfection experiments performed in RAW264.7 cells (Figure 4C). These results were similar to those obtained for endogenous TLR3 expression in RAW264.7 cells upon stimulation (see Figure 2B). Mutation of the inner ISRE/IRF element drastically reduced the basal activity of the murine TLR3 promoter in

RAW264.7 macrophages (Figure 4D) and resulted in complete loss of IFN-β-induced upregulation of promoter activity (Figure 4E). Mutation of an upstream ISRE/IRF element had no effect on the basal and the IFN-β induction of the TLR3 promoter (Figure 3D and 3E). Above results suggest that despite the overall unrelatedness of TLR3 promoter structures in mouse and man, promoters of both species contain functionally important ISRE/IRF elements which are similar in sequence and located close to the transcription start sites. To identify the nuclear factors binding these elements under basal and induced conditions, gelshift experiments were performed using nuclear extracts of IFN-β-treated and untreated human dendritic cells and murine RAW264.7 macrophages. As demonstrated by competition and supershift assays (shown in Figure 5), IRF-2 constitutively bound the IRF motif of human and murine origin, whereas IRF-1 was recruited after stimulation in both human and murine cells. Antibodies against other IRF family members (IRF-3, -4, -7, -8 and -9) or STAT-proteins (STAT1-6) did not change the observed band pattern in gelshift assays (data not shown), indicating that IRF-1 and IRF-2 are the major factors binding IRF-sites in both species. In initial cotransfection studies, both IRF-1 and IRF-2 were able to transactivate the human promoter in HT-29 cells (data not shown). We also performed gelshift assays to identify the nuclear proteins binding to the human STAT-motif. The STAT-motif specifically competed with the binding of STAT1 to a known STAT1 (GAS) binding site, indicating that STAT1 may be able to bind this site. However, we were unable to detect binding of STAT1 directly to the human STAT-motif in gelshift assays (data not shown). For the murine gene, the role of several interferon signalling components in IFN-β induced TLR3 up-regulation including the IFN-α/β receptor (IFNAR1), STAT1, Tyk2 as well as IRF-1 was analyzed in either peritoneal macrophages or bone marrow-derived macrophages from knock-out mice lacking the respective genes. As shown in Figure 6, up-regulation of TLR3 by IFN-β in peritoneal macrophages depends on IFNARI, but not on the Janus kinase (Jak) family member Tyk2. The induction of TLR3 in bone marrow-derived macrophages also depended on

STAT1 as well as IRF-1, albeit to a lesser degree, indicating that other family members may compensate for IRF-1 deficiency.

Mechanism of LPS-induced TLR3 regulation in mice and men. Since initial attempts failed to identify a role of NF-κB in LPS-induced TLR3 upregulation, and LPS is known to induce type I interferons, we analyzed the putative role of type I interferons in LPS-induced up-regulation of TLR3 in mice. As shown in Fig. 7A, a blocking antiserum against IFN-β partially inhibited the LPS-induced upregulation of TLR3, whereas an anti-IFN-α antiserum had no effect. Since LPS-induced autocrine IFN-β could not be completely blocked by anti-IFN-β antibody pretreatment, probably due to high local IFN-β concentrations and short intervention path length between secretion and autocrine action, we analyzed LPS-induced TLR3 expression in peritoneal macrophages derived from mice deficient in IFN-β. As shown in Figure 7B, the LPS response was almost completely blocked in IFN-β-deficient macrophages. Again, downstream signaling of IFN-β did not require the Janus kinase (Jak) family member Tyk2 since Tyk2-deficient mice induced TLR3 normally upon stimulation. In comparison, the induction of TLR2 expression by LPS was analyzed. In line with previous studies demonstrating the activation of its proximal promoter through NF-κB sites, induction of TLR2 occurred in the absence of IFN-β.

Since IFN-β also increases TLR3 mRNA expression in humans, two possible mechanism could lead to the lack of TLR induction by LPS in humans. Firstly, in contrast to the murine counterparts, human monocytic cell types might not produce sufficient amounts of type I interferon to activate the autocrine feedback loop in human cells and to up-regulate TLR3 expression. However, in gelshift assays LPS treatment induced IRF-1 binding to the IRF element of the human TLR3 promoter comparable to IFN-β-induced IRF-1 binding (s. Supporting Figure 9), which is in line with earlier studies demonstrating the production of type I interferons in response to LPS stimulation by human dendritic cells. As a second possibility, LPS-induced

regulatory factors might interact with the human, but not the murine promoter, to prevent up-regulation of TLR3 by autocrine IFN-β. To test the latter possibility, we assayed TLR3 induction in monocyte-derived dendritic cells pretreated with LPS for 2h before IFN-β stimulation. As shown in Figure 7C, LPS pre-treatment of human monocyte-derived dendritic cells completely prevented the up-regulation of TLR3 by subsequently added IFN-β, supporting the second hypothesis.

DISCUSSION

Toll-like receptors (TLRs) are a family of evolutionary conserved pattern recognition molecules which play an essential role in mammalian innate immune defense (1;2). Recent observations suggest that the expression of several TLR orthologues, namely TLR2, TLR3, TLR4 and TLR9, markedly varies between mice and humans - differences among species include the expression of TLR transcripts in different cell types and dissimilar transcription regulation upon cellular activation (for a review see Ref. (5)). The present study investigates the molecular mechanisms underlying the observed species-specific variations in TLR3 expression. We show that TLR3 genes in mice and men are controlled by non-conserved, distinct promoter regions, which likely explains the observed cell-type specific expression patterns and different responses to LPS stimulation.

Using real-time PCR with species-overlapping primers, we could extend previously published observations, showing that among the human and murine cell types tested, the basal TLR3 expression levels are by far the highest in murine macrophages. In contrast, human monocytes and macrophages express relatively low basal levels of TLR3 and as observed before, human monocyte-derived dendritic cells express the highest TLR3 levels within the assayed human cells. The murine proximal TLR3 promoter was highly active in the murine macrophage cell line RAW264.7, whereas the human promoter was weakly active in these cells, indicating that the murine promoter regions contain elements required for a strong expression in macrophages. In line with these observations, the murine promoter (but not the human) contains a number of putative binding sites for the macrophage- and B-cell-specific transcription factor PU.1, which has been implicated in the regulation of a number of macrophage-specific genes (20;21). It is likely that the human promoter contains elements favoring TLR3 expression in human monocyte-derived dendritic cells. However, further investigations will be necessary to identify the exact elements regulating the basal cell type-specific expression in both species. In addition

to the species variations in basal TLR3 expression, TLR3 has previously been shown to be induced by LPS in murine macrophages, but not in human cells. We have confirmed these observations for different human and murine cell types. Using blocking antibodies as well as knock-out mice, we were able to demonstrate that in murine macrophages, TLR3 mRNA is up-regulated by LPS-induced autocrine IFN-β. A similar mechanism of IFN-β-mediated, LPS-induced transcriptional upregulation was recently demonstrated for iNOS, MCP-5 and IP-10 genes (22-24). In human cells, however, LPS prevented TLR3 upregulation in response to subsequent IFN-β treatment. This may indicate the action of inhibitory factors induced by LPS that either are missing in murine cells or interact with human regulatory sites which are not present in the murine gene. Initial transfection experiments indicate that the inhibitory effect of LPS on TLR3 upregulation is not mediated through the proximal promoter alone. Further experiments are necessary to identify the regulatory mechanisms controlling the observed effect of LPS on TLR3 expression in human cells.

Although the biological consequence of species-specific variations in TLR expression remains to be analyzed, changes in cellular expression patterns - in particular the cell type specificity of TLRs and a cell's ability to induce their expression upon cellular activation - are likely to have a significant impact on TLR function in the immune defense. The recent establishment and analysis of transgenic mouse lines expressing the complete murine *Tlr4* gene supports the hypothesis that TLR regulation on the transcriptional level represents an important aspect of TLR immunologic function. In these mice, IL-6 secretion upon challenge with lipopolysaccharide (LPS) is directly proportional to the number of *Tlr4* transgene copies (25). In the future it will be important to clarify whether the observed differences in the transcriptional regulation of TLR3 (or other TLRs) are also seen on protein level and whether they influence innate immune responses in a species-specific manner.

Interestingly, despite the evolutionary unrelatedness of the proximal TLR3 promoter regions,

TLR3 transcripts in both species are markedly up-regulated after stimulation with IFN-β. IFN-β is one of the first cytokines released by a virus-infected cell and induces an antiviral protection program in neighboring cells (26). This includes the up-regulation of TLR3, sensitizing nearby epithelial cells and APCs for activation by the viral PAMP double-stranded RNA. This effect appears to be mediated - at least in part - by similar IRF-binding sites in promoters of both species. We showed that in mice (and likely in humans) interferon-induced up-regulation of TLR3 transcripts proceeds through an IFN α/β receptor, STAT1 and IRF-1-dependent but Tyk2-independent pathway. Tyk2 deficiency leads to a partially defective IFN-β response in macrophages (8), however, the IFN-β induced upregulation of TLR3 seems to be independent of Tyk2. Both TLR3 promoters may also be targets for IRF-2 activation since mutation of the proximal IRF binding site abolishes basal activity in transient transfections and IRF-2 was able to transactivate the human TLR3 promoter in co-transfection assays (data not shown). In the case of many IFN-inducible genes, IRF-2 appears to repress IRF-1 mediated gene activation (27), however, TLR3 may be one of the few cases that include VCAM-1 (28) and histone H4 (29) where IRF-2 acts as a transcriptional activator rather than a repressor.

In conclusion, our observations suggest that the human and murine *TLR3* genes are regulated through different promoter regions that likely mediate the observed species- specific variations in TLR3 expression. Further investigations are necessary to clarify whether the observed differences in basal cell type-specific and induced expression of TLR3 influence immune responses in both species. The marked up-regulation of TLR3 in response to type I interferons that was found to be mediated through the Jak-STAT-IRF signaling pathway, however, appears to be conserved in mice and men, indicating that this regulatory feature may be an important aspect of TLR3 biology.

Footnotes

* These authors contributed equally to this work

[1] Abbreviations used in this paper: dsRNA, double-stranded RNA; IFN, interferon; IRF, interferon regulatory factor; ISRE, IFN-stimulated response element; LPS, lipopolysaccharide; PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptor.

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FIGURE LEGENDS

Fig. 1 **Differential basal expression of TLR3 in human and murine myeloid cell types**. Real-time quantitative PCR (Lightcycler) was performed on cDNAs prepared from the indicated human (open bars) and murine (solid bars) cell types as described in Experimental Procedures (BM, bone marrow; MO, monocyte; DC, dendritic cell; MAC, macrophage). Lightcycler analysis was performed in triplicate, and results were normalized to β-actin expression. Data represent mean values ± SD of at least two independent RNA preparations.

Fig. 2 **Real-time quantitative PCR (Lightcycler) analysis of inducible TLR3 expression.**

Real-time quantitative PCR (Lightcycler) was performed on cDNAs prepared from *A*. murine bone-marrow derived macrophages (Balb/c), *B*. the murine macrophage cell line RAW264.7, and *C*. and *D*. human monocyte-derived dendritic cells from two representative donors (out of four) either untreated (open bars) or treated with recombinant IFN-β (100 U/ml; dark gray bars), IFN-γ (100 U/ml; light gray bars) or LPS (human cells: 100 ng/ml LPS_{SAE}; murine cells: 1 µg/ml LPS_{EC}; black bars). Lightcycler results were normalized to β-actin expression. Data represent mean values ± SD of three independent Lightcycler analyses.

Fig. 3 **Structure and sequence of the human and murine TLR3 promoters.** ***A***. Physical maps of human and murine TLR3 genes. Proximal promoter regions (light grey) and exons (non-coding sequences: dark grey, coding sequences: black) are shown as boxes. Indicated sequence regions were compared using the *ClustalW* algorithm. Sequence similarity is given as percent identity between species. Note that TLR3 transcripts are initiated in exon 1 and exon 2, indicating the presence of two alternative promoters. ***B*** and ***C***. Promoter sequences of human and murine TLR3 genes, respectively. Potential binding sites for transcription factors are in bold italics and boxed in gray. Nucleotide sequences of exons are in capital lettering and transcription start sites determined by RLM-RACE-PCR are marked with arrows. The size of arrows indicates

the number of fragments obtained for each start site. Start sites of the published full-length sequences are indicated with their respective Genbank accession number. The limits of deletion constructs used in this study are also indicated.

Fig. 4 **Mutational analysis of inducible TLR3 promoter activity.** *A*. Schematic representation of reporter constructs. Putative binding sites for IFN regulated factors are indicated. Crosses mark the presence of a mutation at a particular site. *B*. Human THP-1 cells were transiently transfected with the indicated plasmids and treated with PMA as described in Experimental Procedures. 24 h and 4 h prior to harvesting, cells were treated with IFN-β (100 U/ml) or left untreated. Luciferase activities are shown relative to untreated cells transfected with the empty pGL3-B vector. *C*. Murine RAW264.7 cells were stably (*C*. and *E*.) or transiently (*D*.) transfected with the indicated plasmids as described in Experimental Procedures. 24 h (*C*.) and 4 h (*C*., *E*.) prior to harvesting, cells were treated as indicated (IFN-β: 100 U/ml; IFN-γ: 100 U/ml or LPS_{EC}: 1 μg/ml). Induction of luciferase activity is shown either relative to activities for untreated cells (fold induction; *C*. and *E*.) or relative to the activity of the wild-type construct (*D*.). Values are the mean ± SD obtained from 3 independent experiments (*B*. - *E*.).

Fig. 5 **Binding of nuclear proteins to the proximal human and murine IRF sites.** *A*. Sequence alignment of consensus ISRE and IRF sites and murine and human TLR3 promoter sequences. *B., C.* Labeled hIRF or mIRF oligonucleotide was used in EMSA with nuclear proteins from human DC (*B*.) or RAW264.7 macrophages (*C*.) untreated (0h) or treated with IFN-β for 2h. Addition of unlabeled oligonucleotides for competition analysis (lanes 2-5 and 7-10 in *C*.) or antisera against IRF-family transcription factors (lanes 3-5 and 7-10 in *B*. and lanes 13-15 and 17-19 in *C*.) are indicated above each lane. IRF-1- and IRF-2-containing complexes are marked with arrows, antibody supershifts with 'SS' and unspecific complexes with an asterisks.

Fig. 6 **Analysis of signaling molecules involved in TLR3 mRNA induction by IFN-β.** *A*. Peritoneal macrophages from two wildtype, IFNAR1- or Tyk2-deficient mice were left untreated or treated with IFN-β (100 U/ml) for 2 h. Real-time quantitative PCR (Lightcycler) was performed on cDNAs prepared from above cell types and results were normalized to β-actin expression and to unstimulated controls. Data represent mean values ± SD of three independent Lightcycler analyses. Bone-marrow derived macrophages from STAT-1 deficient mice (-/-) as well as wildtype littermates (*B*.) and bone-marrow derived macrophages from IRF-1 deficient mice (-/-) as well as heterozygous littermates (+/-) (*C*.) were left untreated or treated with IFN-β (100 U/ml) for 2 h. Real-time quantitative PCR (Lightcycler) results were normalized to β-actin expression and to unstimulated controls. Data represent mean values ± SD of three independent RNA preparations.

Fig. 7 **Regulation of TLR3 expression by LPS.** *A*. Murine RAW264.7 cells were treated with blocking antisera against IFN-α or –β before stimulation with either recombinant IFN-β (20 U/ml) to control for the specificity of both antisera, or LPS_{EC} (1 μg/ml) for 4 h. *B*. Peritoneal macrophages from two wildtype, IFN-β- or Tyk2-deficient mice were left untreated or treated with LPS_{SM} (100 ng/ml) for 4 h. *C*. Monocyte-derived dendritic cells (one representative experiment out of two is shown) were pretreated (or left untreated) with LPS_{SAE} (100 ng/ml) for 2 h before stimulation with or without IFN-β for 4h and 24 h. Real-time quantitative PCR (Lightcycler) was performed on cDNAs prepared from above cell types and results were normalized to β-actin expression (*A*. and *C*.) and to unstimulated controls (*B*.). Data represents the mean of three independent Lightcycler analyses ± SD.

Figure 1

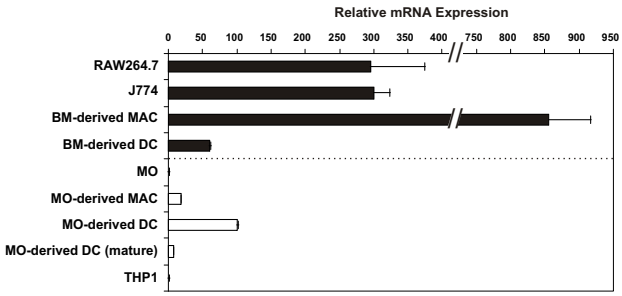


Figure 2

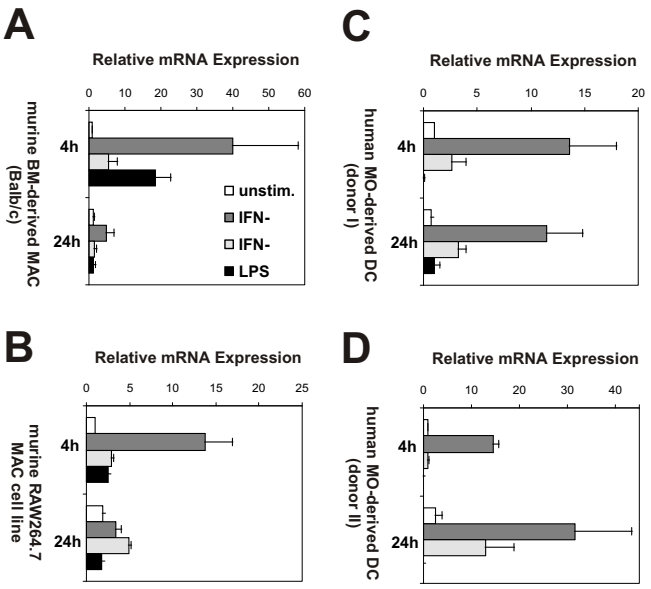
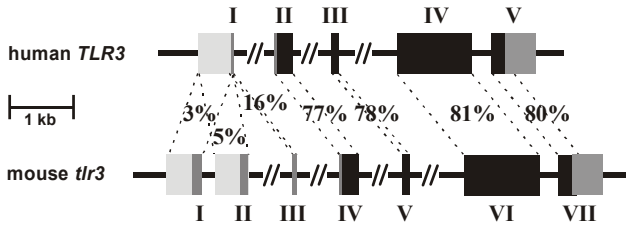


Figure 3

A



B

```
                TLR3-588                                                Ets
-588  tgccatgttt ggctctttct ctgctgtgaa actcagttttc ttcctggatc aagaggttaa
                                          GATA                  SMAD
-528  tttctgccgg tgttatttta cagtgttgca aagagataaa agccagacga aagaagggaa
-468  agtacttcag ctttgattag ggatttttt ttctgtccct gtgaccatac actactcaat
                TLR3-400
-408  tctgacagct tcgcatgagt ctagcagaaa attgagggat tcactgttat tccttaactt
                SMAD      Sp1
-348  gcagaggtcc agacccgccc acatcaaatg gtgaggtccc cactttcaac tttagctgtt
      C/EBP
-288  gctaaatctg tgacccttc aatgaaatcc aaggatctca gcttaaaaaa agttaaaata
                                TLR3-200
-228  aaataaaaac taggtgtttt tcagaggcgg tttgaaacgc ctctctgagg ttgtcagaga
      Sox                       Ets/STAT(GAS)                 TATA-like Box
-168  ttgtttttgt tttctccctt tgccctttctt ggaatgcacc aaacataaaa gcatttttt
                (BC017954, BG401802)      ISRE/IRF      (BI489939)
-108  tcaagcttta cttttCACTTT CGAGAGTGCC GTCTATTTGC CACACACTTC CCTGATGAAA
                (HSU88879)                                    Intron II
-48   TGTCTGGATT TGGACTAAAG AAAAAAGGAA AGGCTAGCAG TCATCCAAgt aagtgaaggt
```

C

```
                tlr3-1368                 Ets/PU.1
-1368 gagtgtagcc atgagccagg taaccacagg aagtaacctc ctggggccag ggaagagcaa
-1308 tttgcaccag gatacaaagt tacttcttgc tttcacttct gtggccattc acctttcca
                                    AP-1                      NF-κB
-1248 ctttcacctc tgccaaacaa aggaactatt gactgagagc tcaggcactc ccctcttctg
-1188 gcttcaagac aagaagatca ggtcaggtgc tctcccattt cttgtatgct ttattacgtt
-1128 taacttttt tttaaaacta aataacaacc tggctgaata ctatgtcatt gctcatctgt
-1068 aatgaaaaga aaactgactt cattcccttg caactccgtt tctttaacca gttggagtcc
                                          tlr3-966
-1008 tcatagctaa ttgaggaaga aggagttaat cccgagctct tctctgacca aacagatgcc
-948  tactgatgcc taatttgtgg gttttgtgga aaactgggcc tgcttgagta agaaaggctc
                                                PU.1
-888  tttgtgacac ccagtgctgg accaaacttc tgccttcctt cctccctccc tgctcccttc
           (BB647755)                     NF-κB
-828  ccccaTTGTT ATTTCCCGGC CTAGTTGTTT CTGGGAGTTT CCTGCTTTTG AGGCAGGACC
          (BB664065)          (BB657013)      Ets/PU.1
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                                          GATA                  Intron I
-708  CCTAAGAGAT TAAGTTCGAC TTTACTCATT TTGCAAAGAA GATAAAGCGA GGTAAgtgaa
          ISRE/IRF
-648  tggctttcac tttgtttaga cacatcccat ttccccacac ggccggtccc tgttggcccc
-588  tcccactatg cgcgattccc gaccagcact ctgggtctct gggcggcagt cccaacctca
-528  gaccatgcac tgcgcatatc acaggtttgc aaggatctga aacccactgc agagggcttg
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          IRF
-348  ccctttaaaaa gaaactaaaa aagggtaaca gtttaataac aatttaactg tttcgctgaa
                                    Sp1
-288  aatatcctct ctctctccct ctccctctcc cccctcccc ccctcctct ctctctctct
                Intron I (alternative)   ISRE/IRF   Intron I          Ets
-228  ctctctctct ctctctctct ctctcaactt aagTTTCACT TTCAGGCTGA AAGGAAATGT
-168  GTGATTTAAA AAAAAAAGTC TGAATGAAAA CTAAGGGGAT GCAGGACCTC AGGCTGAGTA
          (BB666360, BB666361)
-108  AAATCAGAAT GTACTTGGCT TTTGAGGTTG ACGCACCTGT TCTCTATCTG GGAAGTTAAA
          (BB554514)                                          Intron II
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Figure 4

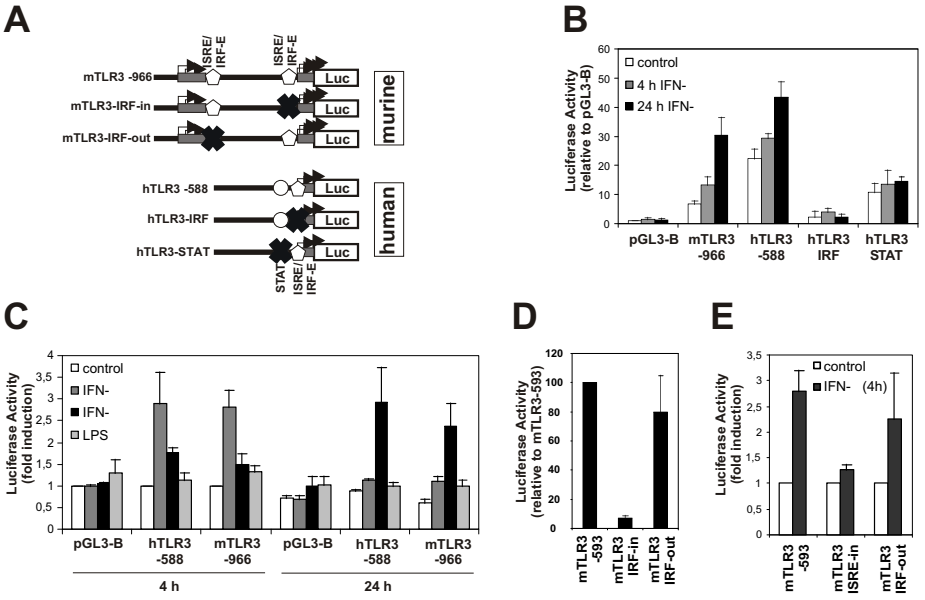


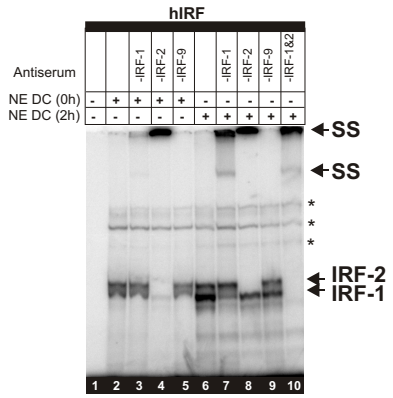
Figure 5

A

cons. ISRE	$^{A}_{G}$NGAAANNGAAACT
hISRE/IRF	TCTCGAAAGTGAAAGTAAA
mISRE/IRF-in	GCCTGAAAGTGAAACTTAA
cons. IRF	GAAA$^{G/T}_{C}$GAAA$^{G/T}_{C}$

B

hIRF 5'-AGCTTTAC**TTTCACTTTC**GAGAGTGC-3'



C

mIRF-in 5'-CTCAACTTAAG**TTTCACTTTC**AGGCTGA-3'
mIRF-in*M* 5'-CTCAACTTAAG***acg*CACTTTC**AGGCTGA-3'
hIRF 5'-AGCTTTAC**TTTCACTTTC**GAGAGTGC-3'
hIRF*M* 5'-AGCTTTAC***acg*CACTTTC**GAGAGTGC-3'

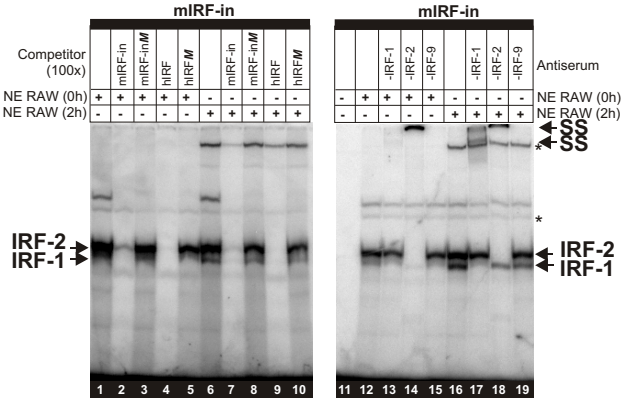


Figure 6

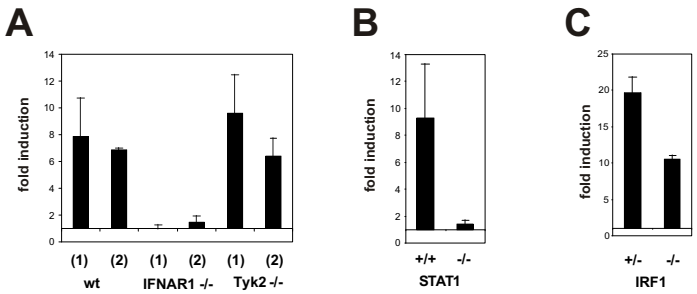
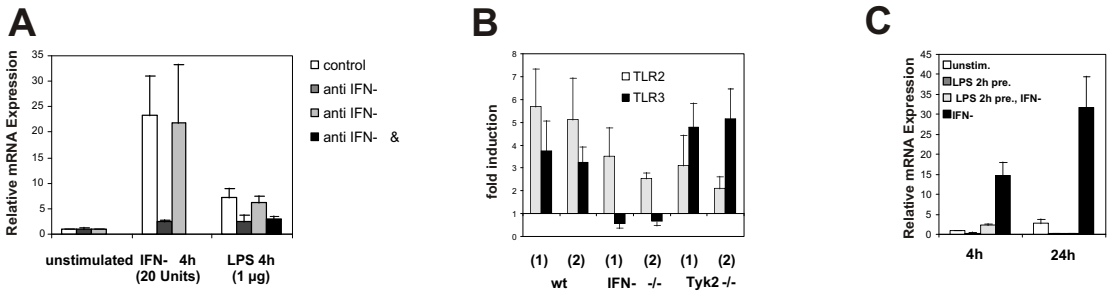


Figure 7

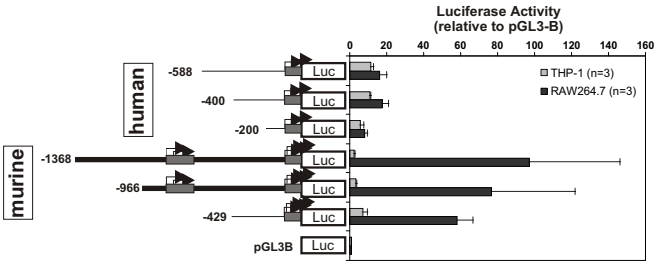


Supporting Figure Legends

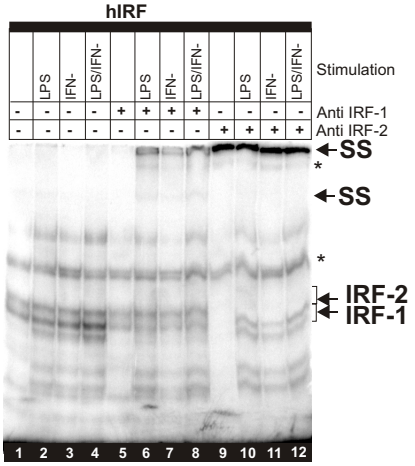
Supporting Fig. 8 **Deletion analysis of the human and murine TLR3 promoters.** Each deletion mutant was transiently transfected into human monocytic THP-1 cells and murine RAW264.7 macrophage cells as described in Materials and Methods. Luciferase activity is relative to the empty pGL3-B vector and values are the mean + SD obtained from 3 independent experiments.

Supporting Fig. 9 **IRF1/2 binding to the proximal human IRF site after stimulation with LPS.** Labeled hIRF oligonucleotide was used in EMSA with nuclear proteins from human DC untreated (0h) or treated with 100 ng/ml LPS_{SAE} for 4h (lanes 2, 6 and 10), IFN-β (100 U/ml) for 2h (lanes 3, 7 and 11), and IFN-β for 2h after 2h pretreatment with LPS (lanes 4, 8 and 12). Addition of antisera against IRF-1 and IRF-2 transcription factors are indicated above each lane. IRF-1- and IRF-2-containing bands appearing as doublets are marked with arrows, antibody supershifts with 'SS' and unspecific complexes with an asterisks.

Supporting Figure 8



Supporting Figure 9



Species-specific regulation of toll-like receptor 3 genes in men and mice

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