C/EBPδ and STAT-1 Are Required for TLR8 Transcriptional Activity

Claudia Zannetti1, François Bonnay1, Fumihiko Takeshita2, Peggy Parroche1,5, Christine Ménétier-Caux2*, Massimo Tommasino3, and Uzma A. Hasan1

From the 1Infection and Cancer Biology Group, International Agency for Research on Cancer, Lyon 69008, France, 2Infection, Immunity, and Vaccination, INSERM U851, Faculty of Medicine, University of Claude Bernard, Pierre Benite 69310, France, 3UPR 9022, CNRS, Institute of Molecular and Cellular Biology, Strasbourg 67084, France, the 4Department of Molecular Biodefense Research, Yokohama City University Graduate School of Medicine, Yokohama 236-0004, Japan, and 5**Cytokines and Cancer, INSERM U590, Centre Léon Berard, Lyon 69008, France.

Toll-like receptor 8 (TLR8), which is expressed primarily in myeloid cells, plays a central role in initiating immune responses to viral single-stranded RNA. Despite the great interest in the field of TLR8 research, very little is known in terms of TLR8 biology and its transcriptional regulation. Here, we describe the isolation of the hTLR8 promoter and the characterization of the molecular mechanisms involved in its regulation. Reporter gene analysis and ChIP assays demonstrated that the hTLR8 regulation of the basal transcription is regulated via three C/EBP cis-acting elements that required C/EBPδ and C/EBPβ activity. In addition, we observed that R848 stimulation increases TLR8 transcriptional activity via an enhanced binding of C/EBPδ, and not C/EBPβ, to its responsive sites within the TLR8 promoter. Moreover, we showed that IFN-γ also increased TLR8 transcription activity via the binding of STAT1 transcription factor to IFN-γ activated sequence elements on the TLR8 promoter and enhanced TLR8 functionality. These results shed new light on the mechanisms involved during TLR8-mediated innate immune response.

Key players of the innate immune response are pattern-recognition receptors that sense microorganisms and activate antimicrobial defenses (1, 2). Pattern-recognition receptors also comprise Toll-like receptors (TLRs), a family of transmembrane proteins that recognize conserved microbial structures named pathogen-associated molecular patterns. TLR7, -8, and -9 are grouped together phylogenetically and are located in intracellular endolysosomal compartments to recognize viral infection in the form of foreign nucleic acids (3). Extensive research has been performed in the field of TLR7 and -9 with the assumption that TLR8 mobilizes in a similar manner (4); however, direct studies on TLR8 are in fact very limited. Restrictions in research are due to the existence of a nonfunctional TLR8 in mice, thus making in vivo studies difficult (5). The hTLR8 is predominantly expressed in lung and peripheral blood leukocytes and lies in close proximity to TLR7 on chromosome X (6). These receptors present overlapping specificity in sensing ssRNA viruses including HIV, sendai virus, coxsacki B virus, and parechovirus 1 (7–9) leading to a possible redundancy among these receptors. TLR8 is abundant in myeloid cells and induces more effectively TNF-α, IL-12, and IL-6, whereas TLR7 and TLR9 and not TLR8 (10, 11), are strongly detected in plasmacytoid dendritic cells and produce type I IFN in response to their respective ligands (5). Many studies have demonstrated that the mRNA levels of different TLRs change after activation of immune response (12, 13). These changes in TLR expression could have positive or negative consequences in regulation of an immune response. So far, the mechanisms responsible for the changes in TLR expression are poorly defined, and only few studies focused on the characterization of TLR transcriptional regulation (12–16).

C/EBP, CREB, Ets, and NF-κB transcription factors have been identified as important regulators of the transcription activity of the TLR9 promoter (13), whereas IRF-1 has been shown to play a role in inducing the TLR7 mRNA level after type I IFN stimulation (12). In terms of transcriptional regulation of TLR8, nothing is currently known. In this report, we describe the molecular mechanisms required for basic and inducible expression of the human TLR8 gene.

EXPERIMENTAL PROCEDURES

Stimuli Used—LPS (InvivoGen), R848 (InvivoGen), IFN-γ (catalog no. I3265, Sigma Aldrich), IL-6 (Peprotech), TNF-α (210-TA, R&D Systems), and uridine (Sigma Aldrich).

Cell Culture—THP-1 were kindly provided by Jurge Tschopp (University of Lausanne, Lausanne, Switzerland) and cultured as described previously (17). Peripheral blood mononuclear cells and monocyte-derived dendritic cells (moDCs) were generated as described previously (18). Monocytes were purified from peripheral blood mononuclear cells using CD14-positive selection (Miltenyi) and cultured as described previously (18). Huh-7 cells (kindly provided by Alex Weber, Deutsches Krebsforschungszentrum) and HEK293 were grown in the same conditions as described previously (19). Cloning of the hTLR8 Gene 5′-Flanking Region and Construction of hTLR8 Gene Promoter-dependent Luciferase Expression Vectors—Supplemental Fig. 1 shows the sequence of the cloned TLR8 promoter. The 5′-flanking region of the hTLR8 gene was...
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PCR-amplified from human genomic DNA with the Expand High FidelityPLUS PCR System (Roche Applied Science) using the primers as listed in supplemental Fig. 11 and cloned into the Nehl-HindIII site of the pGL3 basic vector (Promega).

Expression Vector Constructs—Expression vectors for Sp1 (PU.1), Sp1b, Sp1c, CREB1, C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε, c-Jun, c-Fos, p65, p50, c-Rel, IRF-3, and IRF-7 constitutively active have been described previously (13). Expression vectors for IRF-1, IRF-5, and IRF-7 have been kindly provided by Kate Fitzgerald (UMass Medical School).

Transient Transfection and Luciferase Assay—Transient transfections were performed in duplicate using FuGENE 6 (Roche Applied Science) as described previously (19).

Antibodies—Anti-C/EBPα (M-17), anti-C/EBPβ (C-19), and anti-Stat1 p84/p91 (E-23) antibodies were supplied by Santa Cruz Biotechnology. Antiphospho-Stat1 Tyr701 and anti-PARP antibodies were supplied by Santa Cruz Biotechnology. Antiphospho-Stat1 Tyr701 and anti-PARP antibodies were supplied by Santacruz Biotechnology. Anti-IgG control was supplied by Diagenode. Peroxidase-conjugated antibodies were supplied by Cell Signaling Technology. Anti-Stat1 p84/p91 (E-23) antibodies were supplied by Santa Cruz Biotechnology. Antiphospho-Stat1 Tyr701 and anti-PARP antibodies were supplied by Santa Cruz Biotechnology. Anti-IgG control was supplied by Diagenode. Peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies were from Promega.

Immunoblot Analysis—To obtain cytoplasmic and nuclear extracts 7 × 10⁶ cells were harvested, lysed as described previously (41). Twenty μg of protein extracts (determined by the Bradford assay, Bio-Rad) were used for immunoblotting. Proteins were detected using ECL (Amersham Biosciences).

Electrophoretic Mobility Shift Assay—EMSA and supershift assays were performed using the C/EBP (1) EMSA kit and Stat-1 EMSA kit (Panomics, Fremont, CA). For each binding reactions, 5 μg of nuclear extracts (see under “Experimental Procedures”) were used.

Chromatin Immunoprecipitation Assay—ChIP assay was performed using the Shearing Optimization kit and the One-Day ChIP kit (Diagenode, Philadelphia, PA). For THP-1 cells, the sonication cycle lasted 15 s with 5 sonatas at 20% of amplitude and was repeated six times. Immunoprecipitation was performed overnight on a rotating wheel at 4°C. 2.5 μl/reaction of DNA solution was used for qPCR. The primers used to amplify TLR8 promoter regions are listed in supplemental Fig. 11.

ELISA—THP-1 were seeded at 1 × 10⁵ cells/96 wells, pretreated for 3 h in triplicate with IFN-γ or left untreated, washed twice with PBS, and then stimulated with R848 and uridine. Supernatants were harvested 24 h after treatment for analysis of IL-8 or IL-6 (R&D Systems).

RT-qPCR—Total RNA was extracted from cells using the RNeasy mini kit (Qiagen, Hilden, Germany). cDNA was synthesized with the First Strand cDNA synthesis kit (Fermentas). M代码 has been used to perform qPCR with Mesa Green qPCR MasterMix Plus (Eurogentec), TLR8, TLR7, and αβ2-microglobulin-specific primers (supplemental Fig. 11). Each reaction was run in duplicate.

Statistical Analysis—GraphPad (version 5) was used to calculate unpaired and paired p values: *** extremely significant, p < 0.001; ** very significant, p = 0.001–0.01; *, significant, p = 0.01–0.05; and ns, p > 0.05.

RESULTS

Identification of the TLR8 Regulatory Region—The 16.5-kb TLR8 gene is located on chromosome X and is composed of three exons (20). Fig. 1A diagrammatically depicts the existence of two splice variants of TLR8 (isoform I and isoform II) with a sole transcriptional start site (6). We determined the expression of the two variants in cells known to express functional TLR8, i.e. monocytes (using the THP-1 cell line), peripheral blood mononuclear cells, and MoDCs. The presence of the TLR8 transcript levels were determined by RT-PCR using specific primers that would detect for the two spliced forms. We observed, however, that three bands were amplified in the three cell types tested (Fig. 1B). Sequencing of the amplified products led to the identification of isoform I (356 bp) and isoform II (220 bp) in the cell types tested (as verified using BLAST search). The isoform II was highly expressed in both THP-1 and primary cells. Because both isoforms contain the same transcriptional start site, we hypothesized that the upstream region of exon I is responsible for the transcriptional regulation of TLR8 gene. To consolidate this hypothesis, a comparative analysis of TLR8 genomic sequences derived from different mammals (dog, human and mouse) showed that the exon I upstream sequences were indeed highly conserved using FamilyRelations II software.

To determine whether this region exerts transcriptional regulatory functions, the genomic area spanning −3496 bp to +47 bp of the transcriptional start site was cloned into the luciferase reporter plasmid pGL3 and analyzed by transient transfection

FIGURE 1. The human TLR8 gene, the regulatory region, and splice variants. A, physical map of the human TLR8 gene. Exons 1–3 are indicated. Open and dark gray boxes represent the untranslated and coding regions, respectively. Black boxes mark the position of high homology sequences among mammals. The −3496-bp regulatory region is indicated above the TLR8 gene structure. Isoform I has an extended 19-amino acid (aa) N terminus encoded by exon 2. Isoform II uses an initiator methionine in exon 1 and lacks exon 2. The rest of the protein (1040 aa) is encoded by exon 3 shared by both spliced variants. B, determination of the TLR8 isoforms variants in THP-1, moDCs, and peripheral blood mononuclear cells. RT-PCR was performed using primers listed in supplemental Fig. 11. An asterisk indicates a nonspecific band. C, the −3496 TLR8 promoter is constitutively active in THP-1 cells. THP-1 cells were transfected with the −3496 TLR8 promoter cloned in front of the luciferase (Luc) reporter gene. Cells were harvested as described previously (19). The data are the mean of three independent experiments.
in THP-1 cells. The luciferase construct displayed a 10-fold increase in activity in comparison with the empty vector (Fig. 1C). We determined whether another 1.6-kb genomic fragment, which also is conserved among mammals and is located upstream exon II could display transcriptional activity when cloned in front of the luciferase reporter gene. Transient transfection experiments in THP-1 cells showed that this fragment was inactive (supplemental Fig. S2). The activity of the TLR8 promoter was myeloid cell-specific, as we did not observe luciferase expression in nonmyeloid cells such as HEK293 or Huh-7 cell lines (supplemental Fig. S3i and S3ii). Together, these results indicate that the regulatory regions responsible for the basal TLR8 transcription are comprised in a 3.5-kb-long region located upstream of exon I and their activity is myeloid cell-specific.

**C/EBPδ and C/EBPβ Regulate Basal Transcription Activity of the TLR8 Promoter**—Using MatInspector software (Genomatix), we performed a computational sequence analysis of the TLR8 promoter region and found no canonical TATA box. We identified the presence of several putative responsive elements such as CCAAT/enhancer binding proteins (C/EBP), STAT, IRF, Ets, and NF-κB family members (Fig. 2A). To determine their effect on TLR8 trans-activity, we co-transfected the TLR8 promoter with the constructs expressing different transcription factors including CRE-associating proteins (CREB1), Ets proteins (SpiB, SpiC, Spi1(PU.1)), C/EBPs (C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, and C/EBPε), interferon-regulated factors (IRF1, IRF3, IRF5, and IRF7), NF-κB proteins (p65, p50, and c-Rel) and AP-1 proteins (c-Fos and c-Jun).

**FIGURE 2.** The role of C/EBPδ and C/EBPβ in driving basal TLR8 expression. **A**, identification of the putative responsive elements on the TLR8 promoter. Motif scanning was performed (Genomatix), and putative transcription factor binding sites identified are indicated. **B** and **C**, characterization of the trans-activity of known transcription factors on the TLR8 promoter. THP-1 cells were co-transfected with the expression vectors for a panel of different transcription factors with the −3496 TLR8 promoter, and luciferase (Luc) activity was measured post-48 h. Values represent the mean of three independent experiments. **D**, THP-1 display a basal C/EBPδ DNA binding activity. A biotin-labeled C/EBP commercial probe was used in an EMSA using THP-1 nuclear extracts. For competition assays, an excess of the unlabeled probe was used. For supershift assays, antibodies against C/EBP family members are indicated above each line. E, ChIP assay determining the in vivo binding of C/EBPδ and C/EBPβ to the TLR8 promoter in THP-1 cells. Chromatin was immunoprecipitated with the indicated antibodies. A rabbit IgG isotype control was used as negative control. The sites A, B, C, D, and E on the TLR8 promoter were amplified by qPCR from DNA-transcription factor complexes, and the values obtained are referred as relative to the initial amount of DNA (input). F, deletion analysis of the TLR8 promoter. Progressively deleted TLR8 promoter fragments were transfected into THP-1. After 48 h, cells were processed as described previously (19). Values represent the mean of three independent experiments.
Overexpression of C/EBPδ strongly activated the −3496 TLR8 promoter (Fig. 2B), whereas a slight increase in luciferase activity (1.8-fold) was observed upon transfection of C/EBPβ. By contrast, the other transcription factors did not have any significant effect on the TLR8 promoter when expressed alone. We observed synergistic enhancements on the −3496 TLR8 promoter when co-expressing p50 with p65 or c-Rel proteins, suggesting a positive role played by NF-κB heterodimers (Fig. 2C). Conversely, the co-expression of additional transcription factors with C/EBPδ did not further increase TLR8 promoter activity versus C/EBPδ alone (Fig. 2C). To corroborate the role of C/EBPδ in the basal transcription of the TLR8, we performed an EMSA using nuclear extracts from the untreated THP-1 and C/EBP consensus probe. Two retarded bands were observed (Fig. 2D). Only the lower band was supershifted by an antibody against C/EBPδ but not by a C/EBPβ antibody (Fig. 2D).

We next determined whether C/EBPβ and/or C/EBPδ were able to bind to the TLR8 promoter. We identified five putative C/EBP responsive sites referred to as A, B, C, D, and E (Fig. 2A). We performed ChIP assays using THP-1 chromatin extracts. As shown in Fig. 2E, we observed a binding of both C/EBPβ and C/EBPδ at sites C (unpaired t test was applied compared with IgG control p < 0.006 and p < 0.0007 respectively, D, and E, which implies that these transcription factors play an important role in TLR8 basal transcription. No binding was observed at sites A and B (nonsignificant p values obtained for both C/EBPβ and C/EBPδ compared with IgG alone). Accordingly, in a deletion assay using 5′-flanking region luciferase gene hybrid constructs of the TLR8 gene promoter (Fig. 2F), the highest level of luciferase activity was observed with the −849/+47 construct, which excluded C/EBP sites A and B (p < 0.006, Student unpaired t test). These data support the crucial role played by sites C, D, and E to promote basal TLR8 expression. Additional deletions made from −849 to −396 and then from −396 to −172 regions, which excluded site C, then D and E, respectively, led to a further reduction of promoter basal activity.

TLR8 Transcription Regulation in Response to R848 and Proinflammatory Cytokines—Independent reports have showed previously that certain T helper 1 cell-induced cytokines, including IFN-γ, IL-6, TNF-α, and TLR agonists such as R848 and LPS up-regulated TLR8 expression in myeloid cells (21, 22). Using our model, we investigated the effect of the described cytokines on TLR8 transcription. As shown in Fig. 3A, IFN-γ strongly increased TLR8 mRNA by qPCR at 3 h. An increase in TLR8 mRNA also was observed after R848 treatment at 8 h. IL-6, TNF-α, and LPS also induced further TLR8 mRNA expression but only at later time points. These data indicate that IFN-γ and R848 may directly up-regulate TLR8 transcription, whereas IL-6, TNF-α, and LPS may induce secondary response genes that increase TLR8 mRNA levels. Accordingly, when we tested the effects of these cytokines on the −3496 TLR8 promoter in transient transfection experiments, we observed that IL-6 and TNF-α treatments induced marginal promoter activity compared with nonstimulated cells (supplemental Fig. 4A). IFN-γ significantly increased luciferase activity at 6 h (Fig. 3B, paired Student t test, p < 0.006) as well as at earlier time points (supplemental Fig. 4B). The induction of the promoter was also observed after 16 h with R848 (paired Student t test, p < 0.006). These data are coherent with the increase of TLR8 mRNA as we noted that R848 showed delayed kinetics in comparison to IFN-γ stimulation (Fig. 3A). To corroborate our data in a more physiological setting, we used moDC and human primary monocytes to examine TLR8 levels. Similarly to THP-1 cells, an induction of endogenous TLR8 gene was observed after treatments with IFN-γ for 3 h, and at later times with R848 (Fig. 3, C and D) or uridine (Fig. 3D). These results indicate that IFN-γ- and TLR8-mediated signaling pathways are involved in the induction of TLR8 gene. Because TLR7 and TLR8 present over-
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To verify whether C/EBPδ plays a role in the R848-mediated induction of TLR8 expression through the interaction with the cis-acting promoter elements, a ChIP assay was performed on C/EBPδ consensus sites A, B, C, D, and E in THP-1 stimulated with R848 for 8 h or left untreated (Fig. 4B). The qPCR analysis showed that in response to R848, THP-1 displayed an increased binding of C/EBPδ to sites C, D, and E, supporting the hypothesis that they are important for the R848-mediated TLR8 up-regulation (unpaired Student t test compared with C/EBPδ binding in nonstimulated cells; p < 0.008, p < 0.006, and p < 0.009, respectively). By contrast, C/EBPβ appeared not to be involved in this signaling pathway. In fact, C/EBPβ phosphorylated levels were not affected by TLR8 activation (supplemental Fig. 6), and no significant increase of binding of C/EBPβ to sites A, B, C, D, and E was observed after 8 h of R848 treatment in THP-1 in comparison to untreated cells (supplemental Fig. 7). Therefore, in response to R848, C/EBPδ, but not C/EBPβ, is recruited to the TLR8 promoter to further transactivate its expression, which suggests an auto-regulatory mechanism.

IFN-γ Triggers TLR8 Promoter Transactivation through STAT-1 Binding Sites—We had observed previously that IFN-γ increases the transcription of the TLR8 promoter (see Fig. 3, A and B). However, we did not note an increase in TLR8 promoter activity when we overexpressed IRF family members (Fig. 2B). Therefore, we looked at the status of phosphorylation of STAT1 in THP-1 stimulated with IFN-γ, IL-6, TNF-α, R848, and LPS versus untreated cells. We saw that phosphorylation and nuclear translocation of STAT-1 occurs at both 3.5 and 8 h after treatment with IFN-γ and R848 (Fig. 5A). We next performed gel shift experiments using nuclear extracts from THP-1 cells treated or not with IFN-γ and R848 for 3.5 h and a GAS probe. IFN-γ-stimulated cells displayed an enhanced DNA binding activity versus untreated cells (supplemental Fig. 8i). R848 had no effect on DNA binding activity. Incubation with STAT-1 antibody resulted in a decrease of IFN-γ-induced retarded band (supplemental Fig. 8ii).

We performed a computational analysis and identified four potential GAS cis-elements on the TLR8 promoter. These sites are referred to as GAS1, GAS2, GAS3, and GAS4 (Fig. 2A). To understand whether these sites are required for IFN-γ-mediated enhanced TLR8 transcription, a transient transfection assay was performed employing 5′ deletion mutants progressively lacking promoter regions with GAS-responsive elements. These data showed that IFN-γ-mediated TLR8 transactivation is affected partially when deleting the promoter region containing GAS2 and GAS3 elements (−1803 compared with −3496 unpaired Student t test, p < 0.05; Fig. 5B). A further decrease of promoter activity was observed when deleting the region containing the GAS4 site (−396 compared with −3496 unpaired Student t test, p < 0.05; Fig. 5B). To further demonstrate the in vivo interaction of STAT1 with the GAS sites on TLR8 promoter, a ChIP assay was performed using STAT-1 antibody. As shown in Fig. 5C, after 3.5 h of IFN-γ treatment, chromatin extracts from THP-1 cells contained STAT-1, which bound to GAS-1, -2, -3, and -4 sites. No binding was observed in untreated cells (Fig. 5C). These findings support a model in which TLR8 expression is induced upon IFN-γ treatment. Furthermore, priming THP-1 cells with IFN-γ for 3 h enhanced

lacking functions in sensing ssRNA motifs, we explored whether the two genes share similarities in terms of gene expression upon TLR8 alone (uridine) or TLR7/8 (R848) stimulation. We observed that TLR7 expression is induced by R848 at 3 h and by uridine at later times in THP-1 (supplemental Fig. 5A). No significant induction of TLR7 was detected in moDC (supplemental Fig. 5B). These data highlight the difference in gene expression among the two genes.

R848 Enhances C/EBPδ but Not C/EBPβ-mediated Induction of TLR8 Transcription—We have observed that C/EBPδ and C/EBPβ play an essential role in TLR8 basal transcription in monocytes. Moreover, previous studies have shown that CEBPδ was induced by proinflammatory signals, including TNF-α and IL-6, and that murine TLR4 stimulation leads to its activation in macrophages (23–25). We therefore determined whether activation of TLR8 signaling by R848 could lead to C/EBPδ induction. For this purpose, the levels of the activated CEBPδ were analyzed by immunoblotting from untreated THP-1 cells or treated with R848, LPS, IFN-γ, and cytokines produced by TLR8 activity such as IL-6 and TNF-α. Kinetics were performed at early time points indicative of a direct ligand effect. We observed that nontreated cells expressed C/EBPδ at a basal level (Fig. 4A). R848-TLR8 signaling triggered significant nuclear accumulation of C/EBPδ as did other proinflammatory stimuli starting at 8 h for IL-6, IFN-γ, and LPS and at 3.5 h with TNF-α. Notably, nontreated cells versus the R848-mediated accumulation of CEBPδ levels tightly correlated with the R848-mediated induction of TLR8 promoter activity (Fig. 3A). Furthermore, we confirmed that human TLR4 also induces C/EBPδ (24).

FIGURE 4. C/EBPδ nuclear accumulation and binding to TLR8 promoter specific sites is induced upon R848 treatment in THP-1. A, determination of C/EBPδ nuclear levels in THP-1 by immunoblotting. PARP immunodetection was performed as a loading control. B, ChIP determining the in vivo binding of C/EBPδ to the TLR8 promoter upon R848 treatment. Sheared chromatin from cells that have been stimulated ± R848 for 8 h was immunoprecipitated with C/EBPδ or IgG control antibodies. Sites A, B, C, D, and E on TLR8 promoter were amplified by qPCR, and specific binding was calculated relative to the initial amount of DNA (input). NT, not treated.
IL-8 and IL-6 production upon R848 or uridine treatment (Fig. 5D). Together, these results show that IFN-γ affects TLR8 transcriptional level through STAT-1 transcription factor and enhances TLR8-mediated functionality.

DISCUSSION

To date, it remains difficult to consolidate a specific role for TLR8 due to its overlapping function with TLR7. However, a direct role of TLR8 in innate immunity is emerging. Recent data reported HIV ssRNA sequences that are specific for TLR8 (26) and epidemiological studies showed the association of a functional TLR8 variant with HIV progression disease (27) and with pulmonary tuberculosis susceptibility (28). TLR8 activity is an important source of TNF-α in the sinovial membrane cultures in rheumatoid arthritis (29). Based on these findings, it is thus important to understand how this receptor is regulated prior to and during an immune response.

Here, we have characterized the mechanisms involved in basic TLR8 expression and identified cellular pathways responsible for TLR8 transcriptional activation upon exposure to R848 and/or IFN-γ. We found that members of the C/EBP family, δ and β, have a role in driving TLR8 basal expression. Although we did not observe binding of CEBP/β when performing the EMSA, ChIP experiments showed that both C/EBPs bind the three C/EBP sites (C, D, and E) within the TLR8 promoter. This apparent discrepancy is most likely due to the fact that EMSA was performed using a oligonucleotide containing a consensus C/EBP-binding sequence, whereas ChIP assays examined C/EBP binding to the cis-elements present on the endogenous TLR8 promoter. This apparent discrepancy is most likely due to the fact that EMSA was performed using a oligonucleotide containing a consensus C/EBP-binding sequence, whereas ChIP assays examined C/EBP binding to the cis-elements present on the endogenous TLR8 promoter. Several reports described the potential overlapping or synergistic functions of C/EBPβ and C/EBPδ because both transcription factors can bind the same cis-elements as homodimer or heterodimer complexes (30, 31).

Experiments in the mouse model showed that C/EBPβ and C/EBPδ are induced via TLR-MyD88-dependent signaling pathways, which lead to the induction of proinflammatory cytokines (31). Here, we show for the first time that in human cells TLR8 and TLR4 stimulation led to a nuclear accumulation of C/EBPδ but not C/EBPβ. This event correlated with an increase in TLR8 expression. The effect of TLR8 and/or TLR4
stimulation on C/EBPδ appears not to be direct since we and others (31) observed induction at eight hours post TLR8 and TLR4 stimulation. Many cytokines and chemokines are produced downstream of TLR signaling that lead to sequential activation of pathways that eventually accumulate on C/EBPδ gene transcriptional activation. Accordingly, the C/EBPδ gene has been shown to be positively regulated by multiple cytokines, including IL-6, growth factors and various transcription factors (25, 32), and a regulatory network involving initially c-Rel (24). The molecular events occurring after induction of C/EBPδ on the TLR8 promoter are still unknown. C/EBPδ is known to bind and recruit the histone acetylase CBP, which leads to chromatin opening (33) and nucleosome remodeling at the C/EBP-responsive site of the IL-12 p40 promoter (34). We can speculate the same mechanism for the TLR8 promoter. Further investigations in this direction will help to clarify this issue in terms of TLR8 transcription. We cannot exclude that other transcription factors are likely to be involved in the TLR8 transcription, which among them is the NF-κB family. This family comprises five different members p65 (RelA), RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52), which can form homo- or heterodimers and play a crucial role in the transcriptional regulation of many immunoregulated genes (35). Accordingly, we observed that NF-κB heterodimers p65/p50 and c-Rel/p50 transactivate the TLR8 promoter. Litvak and colleagues (24) showed that NF-κB activity is required for the transcriptional regulation of C/EBPδ. Thus, we cannot exclude that NF-κB may have a role in terms of regulating first C/EBPδ or simultaneously to activate the TLR8 promoter. Further studies must be performed to better characterize the role of NF-κB in TLR8 promoter transactivation.

We also describe the mechanisms through which IFN-γ induces the TLR8 transcript. IFN-γ-mediated TLR8 induction at the promoter and mRNA level occurs at early times after treatment, (starting from 2 h) and increases significantly post-12 h stimulation. We also observed that type I IFN transiently induces TLR8 at 2 h, yet this phenomenon was less evident than that induced by IFN-γ, as TLR8 levels returned to normal post-6 h stimulation (supplemental Fig. 9). The rapid TLR8 up-regulation upon IFN-γ treatment argues for a direct mechanism in terms of TLR8 transcription. The IFN-regulatory factor 1 (IRF1) is promptly induced after IFN-γ treatment (36) and is one of the major transcriptional activators of IFN-γ inducible genes. However, in our study, IRF1 did not affect TLR8 promoter activity in THP-1 cells, suggesting the involvement of another transcription factor. Sequence analysis of the TLR8 promoter identified four GAS sites. ChiP analysis revealed the binding of STAT-1 at the four GAS sites on the TLR8 promoter. The IFN-γ-mediated TLR8 induction has been observed in primary cells such as eosinophils (21) and freshly purified monocytes (supplemental Fig. 10).

Interestingly, a 3-h pretreatment with IFN-γ enhanced sensitivity of THP-1 cells to R848 and uridine (which is specific to TLR8) versus unprimed cells, supporting the possibility that IFN-γ could enhance TLR8 functionality. We also observed that TLR8 mRNA increased post-3 h treatment with IFN-γ. In monocytes, TLR8 has been shown to respond to ssRNA and induce IL-12 p70, which favors the differentiation of T helper 1 cells and the activation of natural killer cells (37, 38). An increase of IL-12 produced by monocytes in response to ssRNA is observed in presence of natural killer cells and appears to depend on IFN-γ production (39). Thus, we can hypothesize that the increased IL-12 induction by TLR8 may be due to the IFN-γ-mediated enhanced transcription and protein expression of TLR8. Although we have extensively examined TLR8 transcriptional regulation, protein expression is a technical obstacle. We tested a totality of four commercial available antibodies by Western blotting or by FACS analysis and found that for Western blotting, none of them worked in detecting either exogenous or endogenous TLR8 protein. For FACS, we were able to recognize only exogenously expressed TLR8 (data not shown).

TLR8 expression is mainly restricted to myeloid cells, (monocytes, macrophages, and moDCs) (4), which argues a cellular specific TLR8 transcription regulation. Coherently, we did not detect any TLR8 transcriptional activity in non-myeloid cells such as HUH-7 or HEK cell lines. Moreover, TLR8 promoter sequence appeared to share common features with other myeloid-specific promoters (16, 40) such as the lack of a TATA box, the presence of C/EBP-responsive elements, and purine-rich sequence motifs that are recognized by the Ets family. Regarding the role of Ets family members in TLR8 transcriptional regulation, we observed that overexpression of SpiC, SpiB, or Sp1 (PU.1) did not affect TLR8 promoter activity. However, the possibility that other members of the Ets family are involved in this event cannot be excluded.

In summary, our data describe and characterize the molecular events that trigger TLR8 transcriptional activation upon R848 stimulation and IFN-γ treatment and extend our understanding of tissue-specific TLR8 gene expression.

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