Published in final edited form as:
Biochem J. ; 421(2): 301–310. doi:10.1042/BJ20090013.

NF-κB activation primes cells to a pro-inflammatory polarized response to a TLR7 agonist

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Abstract

Toll-like receptor 7 (TLR7) mediates anti-viral immunity by recognizing ssRNA viruses. Small molecular weight TLR7 agonists have been approved, or are being evaluated, for treatment of cancers or infectious diseases. Although TLR7 is predominantly expressed in a restricted set of immune cell types including plasmacytoid dendritic cells (pDCs), it is also expressed in non-native expressing cells (e.g., hepatocytes) under certain circumstances. To elucidate the molecular basis of TLR7 induction by pro-inflammatory stimulation and the subsequent cellular responses in these non-native TLR7-expressing cell types, we firstly cloned and characterized the 5′-promoter region of *TLR7*. The proximal region of this promoter drives the transcription of the *TLR7* gene. Pro-inflammatory stimuli activated *TLR7* transcription via a NF-κB binding motif in this region, and this activation could be blocked by mutation of the NF-κB binding site or addition of NF-κB inhibitors. Further studies showed that pretreatment of the Hep3B hepatocytes with TNF-α or IL-1 rendered them responsive to TLR7 activation by a TLR7 agonist. However, distinct from TLR7 activation in pDCs, which respond to stimulation with Th1 polarized cytokine production, TLR7 induction by pro-inflammatory signals in hepatocytes reconstitutes the NF-κB-dependent cascade but not the IRF7-dependent cascade, resulting in a pro-inflammatory polarized response rather than a Th1 polarized response. These results indicate that inflammatory stimulation is capable of priming cells to respond to TLR7 agonist with an immune response that differs from that in native TLR7-expressing cells.

Keywords

TLR receptor; cell activation; inflammation; gene regulation; nuclear factor-κB; interferon regulatory factor

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INTRODUCTION

Toll-like receptors (TLRs) are the key sensors of microbes in innate immune cells that play an important role in initiation of host defense [1,2]. Ten TLRs (TLR1 to TLR10) have been identified in humans. TLR7 belongs to a subfamily of the TLRs, comprising TLR3, TLR7, TLR8, and TLR9, which recognizes nucleic acids [3,4]. This subfamily of TLRs is generally referred to as endosomal TLRs because they are located in intracellular endosomes unlike the other TLRs, which are expressed on the cell surface. In addition, this subfamily of TLRs is also referred to as antiviral TLRs because of their potent ability to induce type I interferon (IFN) [5–7].

TLR7 and TLR8 have high sequence homology, and phylogenetically are closest to each other [4]. As a result, the two receptors have some overlap in their ligand recognition. TLR7/8 recognize synthetic guanosine- or uridine-rich single-stranded RNA (ssRNA), and various ssRNA viruses such as vesicular stomatitis virus, sendai virus, coxsackie B virus, parechovirus, dengue virus, influenza virus, and human immunodeficiency virus (HIV). Synthetic agonists for TLR7/8 with molecular weights ranging from 200–400 Da have been identified and are structurally related to nucleic acids. Whereas imiquimod, loxoribine, and isatoribine selectively activate TLR7, 3M-002 selectively activates TLR8. Others such as 3M-011 and R848 (resiquimod) activate both receptors [8,9].

Similar to the other TLRs, the TLR7/8 contain an ectodomain consisting of multiple leucine-rich repeats and a cysteine-rich domain, a transmembrane region, and a cytoplasmic Toll/IL-1 receptor (TIR) domain. Upon stimulation, TLR7/8 recruits the TIR-domain-containing adapter protein, MyD88, to its cytoplasmic TIR domain. This homotypical interaction initiates both a pro-inflammatory signaling cascade and an anti-viral signaling cascade. In the pro-inflammatory signaling pathway, formation of a complex of MyD88, IRAK1, IRAK4, and TRAF6 leads via activation of TAK1 to the activation of NF-κB and production of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-12. In the anti-viral signaling pathway, this MyD88/IRAK1/IRAK4/TRAF6 complex activates IRF7, leading to the production of type I IFNs [10–13]. The production of type I IFNs is particularly important in TLR-elicited antiviral and antitumor responses because these cytokines activate transcription of many IFN-inducible genes that influence protein synthesis, growth arrest and cell death, as well as enhance the maturation, differentiation, and proliferation of dendritic cells, natural killer cells, and cytotoxic T cells [14–16]. Because this Th1 immune response promotes killing of virus-infected cells and tumor cells, TLR7/8 agonists are being evaluated in clinical investigations for their therapeutic efficacy in treatment of various infectious diseases and cancers [8,9].

TLR7 and TLR8 are preferentially expressed in immune cells with distinct patterns of expression. TLR7 is preferentially expressed in plasmacytoid dendritic cells (pDCs), B cells, and likely some monocytes [17,18]; whereas TLR8 is expressed in myeloid dendritic cells (mDCs), macrophages and neutrophils but not in pDCs [18–20]. These distinct expression patterns result in different cytokine production profiles following *in vivo* administration of TLR7 and TLR8 agonists. TLR7 stimulation of B cells induces differentiation and proliferation but only a low level of cytokines, whereas stimulation of pDCs induces IFN-α since they are a major IFN-α–producing cell. Thus, the expression pattern results in Th1 polarized cytokine production in response to TLR7 agonists, whereas TLR7/8 agonists tend to induce production of higher levels of inflammatory cytokines, chemokines, and cell adhesion molecules [5,6].

Accumulating evidence indicates that viral infections and chronic inflammation are frequently associated with elevated TLR7 expression in a variety of non-native expressing cell types. For example, TLR7 expression is enhanced in PBMCs in HIV- or HCV-infected patients and in systemic lupus erythematosus patients [21–23]. Enhanced TLR7 expression was detected in

the synovium of rheumatoid arthritis patients, and in bacteria- or virus-infected cells, such as in nontypeable haemophilus influenza and herpes simplex virus 1 infected epithelial cells, and in HCV-infected hepatocytes [24–27]. Although expression of TLR7 in non-native expressing cells may generate a response that differs from that in native TLR7 expressing cell types, the molecular mechanism by which induction of TLR7 is controlled, and the biological consequences of up-regulation of this receptor in these cells have not been elucidated.

Here we cloned and characterized the TLR7 promoter to define the factor(s) that govern TLR7 expression in response to inflammatory stimulation in human Hep3B hepatocytes, and compared the induction and activation of TLR7 in these cells with that in native TLR7-expressing pDCs. These non-TLR7-expressing cells were selected for these studies because they are responsive to the pro-inflammatory stimuli, TNF-α and IL-1. Thus, TLR7 may be inducible by pro-inflammatory stimuli in these cells as has been seen in the other non-native TLR7-expressing cell types [21–27]. In addition, they are hepatocyte-derived cells and a number of TLR7 agonists are under investigation as therapeutics for HCV and HBV infections [8,9]. Our results indicate that inflammatory stimulation induces TLR7 expression in the hepatocytes through NF-κB activation, and distinct from TLR7 activation in pDCs, subsequent cell activation by the TLR7 agonist activates only the pro-inflammatory cascade but not the anti-viral cascade.

MATERIALS AND METHODS

Reagents and Antibodies

LPS (Re595) from *Salmonella minnesota* R595, PMA, Poly(I:C), and CpG-ODN (TCGTCGTTTTGTCGTTTTGTTT) were purchased from Sigma (St. Louis, MO). R848 was purchased from GLS Synthesis (Worcester, MA). Pam_3CSK4, Ro106-9920, IKK inhibitor III, U0126, JNK inhibitor II, SB203585, LY294002 and JAK inhibitor I were purchased from Calbiochem (San Diego, CA). TNF-α, IL-1, IFN-α, IFN-γ, and EGF were purchased from PeproTech Inc. (Rocky Hill, NJ). Anti-human TLR7 antibody was purchased from Imgenex (San Diego, CA).

Cell Culture, RNA isolation and RT-PCR analysis

Human pDCs were purchased from MatTek Corp. (Ashland, MA), and grown in DCP-MM medium. Human Hep 3B hepatocytes were cultured in DMEM supplemented with 10% fetal bovine serum and non-essential amino acids. These cells were treated with various stimuli and inhibitors for different period of times as indicated in each experiment. Total RNAs were isolated from cells using a RNA isolation kit (Qiagen, Valencia, CA). First strand cDNA was then prepared from total RNA samples using a SuperScript™ preamplification kit (Invitrogen, Carlsbad, CA). PCR amplifications were performed using an Expand HI Fid PCR kit (Roche, Indianapolis, IN). The sequence of the gene-specific primers used are as follows: TLR7 (5-CCAACTGACCACTGTCCCTGAGAG-3′, 5′-GGTAACCAGTCCCTTTCCTCGAGAC-3′), TLR8 (5′-GAAGTCAGTAGTCTGAAGCACCTCG-3′ 5′-CTCATCCATTAGCCTCTGCAAAGCC-3′), TNF-α (5′-CATGATCCGGGACGTGGAGCTGG-3′, 5′-TCACAGGGCAATGATCCCAAAGTAG-3′), ELAM-1 (5′–CTGGAGAGTTCACCTTCAAATCATCC-3′, 5′-GGATGTAAGAAGGCTTTTGGTAGCTTC-3′), IFN-α (5′-ATGGCCTTGACCTTTGCTTTACTG-3′, 5′-CTGCCTTGACAACCTCCCAGGCAC-3′), NF-κB, p65 (5′-ACTGTGTGACAAGGTGCAGAAAGAG-3′, 5′ CTGCTGAAACTCGGAGTTGTCGAC-3′), IRF7 (5′-CAACCAAGGCTCCTGGAGAGGGAC-3, 5′-CTCCAGCTCCATAAGGAAGCACTC-3′),

GAPDH (5′-TGAAGGTCGGAGTCAACGGATTTGGTCG-3′, 5′-CATGTGGGCCATGAGGTCCACCACCAC-3′). PCR products were visualized by electrophoresis on a 1% agarose gel after staining with ethidium bromide.

Cloning and Characterization of TLR7 Promoter

Genomic DNA sequence for *TLR7* containing exon 1 and 5′-upstream region was retrieved from the human genome database at NCBI (accession number: NT_011757). Based on this sequence, PCR primers were designed. A DNA fragment containing the 5′-flanking region spanning from −1001 to +100 was PCR amplified using Expand High Fidelity PCR System (Roche, Indianapolis, IN) with human genomic DNA as template (Clonetech, Palo Alto, CA). This DNA fragment was subcloned into the pGL3-basic luciferase reporter vector (Promega, Madison, WI) for DNA sequencing to confirm the sequence. The putative transcription factor binding sites were analyzed by an AliBaba2.1 computer program (http://www.gene-regulation.com/pub/programs.html) [28], and a MatInspector computer program (http://www.genomatix.de/products/MatInspector/) [29].

Plasmid Constructs

DNA fragments containing truncated *TLR7* promoter regions were generated by PCR amplification using primers listed in Table S1. The forward primers contain a Kpn 1 site, and the reverse primers contain a Hind III site. DNA fragments containing point mutants in the TLR7 promoter region were generated by two-step PCR amplification procedures. A forward primer containing mutations introduced into the putative transcription factor binding site as shown in Table S1 was used with a reverse primer to generate the first DNA fragment. This DNA fragment was used as a reverse primer with a forward primer to generate a full length DNA fragment. These amplified DNA fragments were subcloned into a pGL3 basic vector at the Kpn1 and Hind III sites to generate luciferase reporter constructs with deletion or point mutant in the TLR7 promoter.

Nuclear Run-on Assay

Nuclear run-on assays were performed as previously described [30], with minor modification. Briefly, 9×10^6 Hep3B cells were washed twice with PBS before lysis in buffer containing10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl, 150 mM sucrose, and 0.5% NP-40. The nuclear run-on reactions were performed by incubating the isolated nuclei for 1 h at 30°C in a transcription buffer containing 10 mM Tris-HCl, pH 8, 2.5 mM $MgCl_2$, 100 mM KCl, 2 mM each of ATP, GTP, CTP, biotin-16-UTP (Roche, Indianapolis, IN), and 1 unit/μl RNAse inhibitor (Invitrogen, Carlsbad, CA). The reactions were stopped by adding RNeasy lysis buffer followed by total RNA purification using RNeasy mini kit (Qiagen, Valencia, CA). Biotin-labeled RNA transcripts were isolated using a μMACS streptavidin kit (Miltenyi Biotech, Aubum, CA) according to the manufacturers' protocol. RT-PCR was performed as described above to analyze the transcriptional activities of TLR7 and GAPDH.

Transient Tansfection, and Luciferase Reporter Assay

Hep3B cells were plated in 12-well plates and transfected on the following day using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). To investigate the TLR7 promoter region, the cells were co-transfected with pGL3 basic vector or various reporter constructs with deletion or point mutant of TLR7 promoter, and β-galactosidase plasmid as indicated. Sixteen hours later, the cells were lysed and luciferase activity was analyzed using reagents from Promega Corp. (Madison, WI). To determine the induction of the TLR7 promoter by various stimuli, the cells were co-transfected with/out TLRs expression vectors, TLR7 promoter constructs and β-galactosidase plasmid as indicated. Twelve hours later, the cells were treated with various stimuli as indicated for 6 h and analyzed for the luciferase activity. The relative luciferase

activities were calculated as fold of induction compared with the pGL3 basic vector control, or an unstimulated vector control.

Flow Cytometry Analysis

Cells were fixed and permeablized with a Cytofix/Cytoperm kit (BD Biocisence, San Diego, CA) according to the manufacturers' procedures. Induction of TLR7 was analyzed by FACSCalibur (Becton Dickinson, San Jose, CA) using the antibody as described above.

Measurement of Cytokine Production

The concentration of TNF-α and IFN-α in culture medium were measured by ELISA using reagents from eBioscience (San Diego, CA), and PBL InterferonSource (Piscataway, NJ).

Statistical Analysis

Statistical significance between two groups was determined by Student's *t*-test. All groups were from three or more independent experiments. Statistical significance was determined as $p< 0.05$.

RESULTS

Transcriptional activation of TLR7 expression in Hep3B cells by TNF-α and IL-1

To study the regulation of TLR7 expression in non-native TLR7-expressing cells in response to various stimuli, Hep3B cells were left untreated or treated with cytokines (10 ng/ml of TNF-α, IL-1, IFN-α, and IFN-γ), TLR ligands (100 ng/ml of Pam_3Cys, LPS and 1 μM of R848), or mitogens (10 ng/ml of EGF and 10 nM of PMA) for 4 h before measuring TLR mRNA by RT-PCR with gene-specific primers. TNF-α and IL-1, two pro-inflammatory cytokines, but no other stimuli, induced increased expression of the TLR7 transcript. Distinct from TLR7, induction of TLR8 was not observed in any of the stimulated cells (Figure 1A). By comparison, TLR7 mRNA was more abundant in pDCs, native TLR7-expressing cells, and was not increased further by treatment with TNF-α or IL-1 (Figure 1B). The regulation of TLR7 in Hep3B cells by TNF-α and IL-1 was further investigated by a nuclear run-on assay. In this assay, the transcription rate of the *TLR7* gene in cells was reflected in the abundance of TLR7 transcript in the run-on RNAs. The result indicated that consistent with the steady state levels of TLR7 mRNA in the total nuclear RNA (Figure 1C, left panel), the levels of run-on transcript for TLR7 in TNF-α– or IL-1–treated samples were increased (Figure 1C, right panel). Together, these results suggest that TNF-α and IL-1 treatment increases the transcription rate of *TLR7* in hepatocytes.

Cloning and characterization of the 5′ flanking region of the TLR7 gene

To investigate further the molecular mechanism by which *TLR7* is transcriptionally controlled, we cloned the 5′ flanking region of the *TLR7* gene. Previously, we have used rapid amplification of cDNA ends (RACE) to determine the 5′-end sequence, and the genomic structure of the *TLR7* gene. *TLR 7* is located on chromosome Xp22, with the transcriptional region distributed in three exons, and the putative transcription start site located in exon 1 (Figure 2A and Ref 4). To further refine the transcription start site, we searched the EST database at NCBI for the most upstream 5′-end of TLR7 cDNA sequences and found 6 EST sequences for TLR7 with the same 5′-end start site. This site is located in exon 1, and is 17 bp upstream to the previously determined transcription start site. This site was designated as the putative transcription start site (Figure 2A and 2B). Having determined the 5′-end of the TLR7 transcriptional region, we next cloned the 5′-flanking region of the human *TLR7* gene by PCR amplification using human genomic DNA as template. The sequence of this 5′-flanking region, ranging from −547 to +92 relative to the putative transcription start site, is shown in Figure 2B, and putative transcription

factor binding sites were predicted using an AliBaba2.1 program and a MatInspector program [28,29].

Analysis of TLR7 promoter activities

To investigate whether this 5′ flanking region contains any promoter activity to drive *TLR7* transcription, a set of serial deletion constructs, as shown in Figure 3, were generated by fusing truncated forms of the 5′ flanking region to the 5′-end of a luciferase reporter gene in pGL-3 basic vector. We first transiently transfected Hep3B cells for 16 h with these constructs to determine their basal promoter activities using a luciferase reporter assay. In cells transfected with the construct pGL(−496/+53) containing a fragment spanning from −496 to +53, a 2–3 fold increase in luciferase activity was generated compared to cells transfected with control pGL-3 basic vector. This basal promoter activity was not significantly reduced when the 3′-end of this fragment was deleted to +14, but completely lost when deleted to −13, suggesting that the promoter region for *TLR7* is located upstream to position +15 in the 5′-flanking region. We further dissected this promoter region using a set of constructs generated by progressive deletion from the 5′-end of a DNA fragment spanning from −1001 to +15. The constructs containing −205 to +15, −135 to +15, and −110 to +15 fragments generated the highest promoter activity in cells. The promoter activity was reduced to basal levels when the 5′ region was extended upstream to −496 or deleted downstream to −34 (Figure 3). These results suggested that the 5′ flanking region from −34 to −110 may contain positive regulatory elements, and the region from −205 to −496 may contain negative regulatory elements for control of basal *TLR7* transcription.

TLR7 promoter construct is responsive to pro-inflammatory stimulation

Because TNF-α and IL-1 induced expression of *TLR7* (Figure 1), we focused on identifying the elements within the 5′ flanking region responsible for the TNF-α– and IL-1–dependent induction of *TLR7* expression. We first utilized the pGL(−496/+15) construct to determine whether this 5′-flanking region is sufficient for responsiveness of *TLR7* to TNF-α and IL-1 stimulation. Hep3B cells were transiently transfected with this reporter construct and then treated with TNF-α, IL-1, different TLR ligands, or mitogens. As expected, its activity was enhanced in TNF-α– or IL-1–treated cells but not in cells treated with other stimuli (Figure 4A). Since TLR ligands are potent in provoking pro-inflammatory responses, we reasoned that the unresponsiveness of this promoter to TLR ligands may be due to insufficient expression of various TLRs in these cells. To address this question, Hep3B cells were co-transfected with the pGL(−496/+15) construct and different TLR expression vectors, including TLR3, TLR5, TLR7 and TLR9, and then treated with 20 μg/ml of poly(I:C), 0.1 μg/ml of flagellin, 1 μM of R848 or CpG-ODN, respectively. The TLR7 promoter was activated by TLR3, TLR5, TLR7 and TLR9 activation (Figure 4B). Together, these results indicate that pro-inflammatory stimuli activate TLR7 transcription, and the cis-responsive element is located within this 5′-flanking region.

NF-κB activation is required for induction of TLR7 transcription

In order to define the location of the cis-responsive element within the 5′ flanking region that is required for pro-inflammatory stimuli induced TLR7 expression, Hep3B cells were transiently transfected with a set of reporter constructs generated by progressive deletion from 5′-end of the fragment spanning −496 to +15, and then treated with TNF-α or IL-1. The luciferase activity in the cells transfected with a pGL(−85/+15) construct was similar to that achieved with other constructs containing longer upstream promoter regions (up to −496), whereas induction was completely abolished in cells transfected with the pGL(−34/+15) construct (Figure 5A). This suggested that the region from −85 to −34 contains binding sites for cis-responsive elements required for induction of *TLR7* by pro-inflammatory stimuli.

Computer analysis using an AliBaba2.1 program and a MatInspector program predicted putative binding sites in this region for three transcription factors, namely, SP1, RxR-β and NF-κB (Figure 2B). To investigate the functional significance of these elements in driving *TLR7* transcription, we used site-directed mutagenesis to abolish each of these sites within the promoter region in a DNA fragment spanning from −205 to +15 to generate reporter constructs shown in Figure 5B. Mutation of the SP1 site decreased the basal promoter activity, and mutation of RxR-β only partially decreased the basal activity; however, induction by TNF-α or IL-1 was unaffected by either mutation. In contrast, mutation of the NF-κB binding site did not affect basal promoter activity, but the responsiveness to TNF-α or IL-1 was completed abolished (Figure 5B).

To further investigate this finding, Hep3B cells were transiently transfected with the pGL (−205/+15) construct and treated with inhibitors of various signaling pathways including Ro106-9920 (NF-κB inhibitor), IKK inhibitor III, U0126 (MEK1 and MEK2 inhibitor), JNK inhibitor II, SB203580 (p38 MAP kinase inhibitor), LY294002 (PI3K inhibitor), and JAK inhibitor I. Responsiveness of the TLR7 promoter to TNF-α or IL-1 stimulation was attenuated in Ro106-9920– and IKK inhibitor III–treated cells, but was unaffected by the other inhibitors (Figure S2A). Consistent with this, the levels of TLR7 transcript induced by TNF-α and IL-1 were attenuated in Ro106-9920– or IKK inhibitor III–treated cells (Figure S2B). Thus, analysis of the *TLR7* promoter suggests that NF-κB activation is required for induction of *TLR7* transcription.

Induction of TLR7 protein expression in Hep3B cells by pro-inflammatory cytokines

Although expression of TLR7 in Hep3B cells was detectable by RT-PCR, the expression was not at a functional level because stimulation with a potent TLR7 agonist, R848, failed to induce cytokine production in these cells, but transfection with a TLR7 expression vector restored the cellular response (Figure 4B). To investigate whether treatment with pro-inflammatory stimuli would enable these cells to respond to TLR7 agonists, we first determined the kinetics of induction of TLR7 transcript after TNF-α or IL-1 stimulation, and then examined expression of TLR7 protein. RT-PCR analysis indicated that the TLR7 transcript was increased in cells 4 h after TNF-α or IL-1 stimulation and remained elevated for 20 h following stimulation (Figure 6A). Flow cytometry analysis showed an increased level of TLR7 protein 12 h after stimulation (Figure 6B). These results indicated that in parallel to the increased level of TLR7 transcript, the protein level increased following stimulation, although whether this expression level was sufficient to support cellular responses to TLR7 agonist remained to be seen.

Pro-inflammatory cytokines prime Hep3B cells to a polarized response to TLR7 agonist

Ligation of TLR7 activates both a NF-κB–dependent signaling pathway, leading to production of pro-inflammatory cytokines, and an IRF-7–dependent signaling pathway, leading to the production of IFN-α [10–13]. Looking first at activation of the NF-κB signaling pathway, Hep3B cells pretreated with TNF-α or IL-1 were transfected with an endothelial-leukocyte adhesion molecule 1 (ELAM-1) promoter controlled luciferase reporter gene, and then stimulated with vehicle control or 1 μM R848 for 6 h. The result indicated that the ELAM-1 promoter was activated in response to TLR7 activation (Figure S3A). This result was confirmed by analyzing the induction of endogenous ELAM-1 transcript using RT-PCR. As expected, R848 increased the level of ELAM-1 transcript in the pretreated cells (Figure S3B). Because the ELAM-1 promoter is driven by NF-κB activation [31], these results indicated that the NF-κB signaling pathway was activated in response to TLR7 activation in these TNF-α– or IL-1–pretreated cells. We next compared the induction of TNF-α by TLR7 activation in the pretreated Hep3B cells and pDCs because TNF-α is representative of the pro-inflammatory cytokines induced in response to NF-κB activation following ligation of TLR7 agonist [5,6]. Production of endogenous TNF-α at both the transcript and the secreted protein levels was undetectable

in Hep3B cells that were not pretreated but was induced by R848 in the pretreated Hep3B cells, consistent with induced NF-κB activation (Figure 7A, 7B and S3A, S3B). By comparison, TNF-α expression was readily induced by TLR7 agonists in untreated pDCs and was not further induced by pretreatment of the pDCs with TNF-α or IL-1 (Figure 7C).

We next investigated the induction of IRF7 signaling pathway dependent cytokine production by TLR7 activation in the pretreated Hep3B cells and pDCs. In contrast to TNF-α production, neither the IFN-α transcript nor the secreted protein in cell culture medium were induced in the pretreated Hep3B cells by R848 stimulation (Figure 8A and B). This is in distinct contrast to pDCs where IFN-α was induced by TLR7 agonists in untreated cells and its expression was not further enhanced by pretreatment with TNF-α or IL-1 (Figure 8C). We reasoned that the lack of IFN-α production in response to TLR7 activation in pretreated Hep3B cells may be due to the absence of a major component in the IRF7 signaling pathway in these cells, in particular, IRF7 itself which is largely restricted to leukocytes [11,32]. To investigate this, we compared the expression of IRF7 and NF-κB p65 in unstimulated and TNF-α– or IL-1–stimulated Hep3B cells and pDCs. The results indicated that while NF-κB p65 was detected in all the cells, IRF7 was only detected in pDCs (Figure 8D), thus confirming that the lack of induction of IFN-α in pretreated Hep3B cells may be due to insufficient IRF7.

Overall, these results demonstrate that the pretreatment of Hep3B cells with TNF-α or IL-1 primes them to respond to TLR7 agonist with a cytokine production profile polarized to the pro-inflammatory cascade. This is distinct from the immune response induced by TLR7 agonists in the native TLR7 expressing pDCs.

DISCUSSION

TLR7 is preferentially expressed in a limited set of immune cells including plasmacytoid dendritic cells (pDCs), B cells, and likely some monocytes [17,18]. Nevertheless, substantial evidence has shown induction of TLR7 expression by viral infections and chronic inflammation in a variety of non-native TLR7-expressing cell types [21–27]. In this study, we investigated the regulation of *TLR7* expression by pro-inflammatory cytokines in Hep3B hepatocytes in an attempt to elucidate the molecular basis for induction of TLR7, and the subsequent cellular response to TLR7 agonists in a non-native TLR7-expressing cell type as compared to that in a native TLR7-expressing cell type. The Hep3B cell line was selected firstly, because TLR7 is not expressed to a functional level in these cells as evidenced by their unresponsiveness to TLR7 agonist, and that exogenous TLR7 restores the NF-κB mediated response (Figure 4B); and secondly, they are responsive to the pro-inflammatory stimuli, TNF-α and IL-1 (Figure S1). Thus, this cell type might be representative of the other non-native TLR7-expressing cell types in which TLR7 is inducible in response to pro-inflammatory stimulation [21–27].

We cloned and characterized the putative promoter region of the *TLR7* gene. The low basal promoter activity observed with a construct spanning from −1001 to +15 is consistent with the observation that TLR7 was not expressed to a functional level in these cells. Deletion analysis suggested that the distal region may contain negative regulatory elements to suppress basal transcription. In addition, the 5′-proximal region lacks TATA box consensus initiator sequences, or the GC-rich region regions found in housekeeping genes. These findings may explain the low basal expression level of TLR7 in Hep3B cells, as well as the cell-type-restricted expression patterns of TLR7. Similarly, the 5′-proximal region of human *TLR4* does not contain a TATA box, or GC-rich region, and its expression is primarily detected in myeloid cells (monocytes, macrophages and dendritic cells) and some B lymphocytes [33]. Other TLR genes, such as human *TLR2* and mouse *TLR9*, do not contain a TATA box in their 5′-proximal region either, and their expression is also cell type restricted [34–37].

The nuclear run-on assay indicated that induction of TLR7 by TNF-α and IL-1 was due to increased transcription and therefore, we focused our study on determining the factors that govern pro-inflammatory cytokine-induced expression of the *TLR7* gene. Analysis of TLR7 promoter spanning from −496 to +15, which included the cis elements responsive to TNF and IL-1 stimulation, showed a single putative binding site for IRF-1, SRF, NF-κB, and two sites for SP1. Further analyses localized the responsive elements within the −85 to −34 region which contains the SP1, RxR-β, and NF-κB binding sites but not the IRF-1 and SRF sites. While the IRF-1 binding site was suggested to be involved in IFN-β induced TLR7 expression in dendritic cells [38], it did not appear to be required for the response of *TLR7* to pro-inflammatory stimulation in these hepatocytes, suggesting that different regulatory elements may be required for TLR7 induction by different stimuli in different cell types.

Subsequent studies using site-directed mutagenesis and inhibitors of NF-κB signaling indicated that the NF-κB binding site was essential for induction of *TLR7*. The SP1 site was involved in control of basal TLR7 expression, but neither the SP1 nor the RxR-β site were involved in the control of promoter activation induced by TNF-α and IL-1. SP1 is a ubiquitously expressed transcription factor. SP1 binding sites are frequently found in the promoter of NF-κB regulated genes, including TLR2, HIV, ICAM-1, GM-CSF, CD40, and OX40 [34,35,39–43]. Other studies have suggested a role for the SP1 family transcription factors in the regulation of several myeloid-specific genes, including human TLR2. Since SP1 transcription factor is ubiquitiously expressed, it was suggested that these factors might regulate myeloid-specific genes through collaboration with other more tissue-restricted transcription factors [33–35,44–46].

NF-κB and IRF7 activation are the two key downstream signaling events following TLR7 activation and lead to the production of pro-inflammatory cytokines and IFNs, respectively [10–13]. The balance of production between these two classes of cytokines could determine the outcome of therapies with TLR7 agonists, with production of IFNs favoring a beneficial Th1 immune response and production of pro-inflammatory cytokines favoring an inflammatory response [5,6]. Since different cells respond to TLR7/8 activation with production of a unique set of cytokines [5,6], the cellular response to TLR7 agonist may be governed by the content of signaling molecules in the TLR7-expressing cells. Thus, by comparing native and non-native TLR7-expressing cells, we asked whether the induction and activation of TLR7 in response to inflammatory stimulation differs in the two cell types and how this difference affects the outcome of TLR7 activation.

It is known that activation of TLR7 *in vivo* generates a Th1 immune response, hence the beneficial therapeutic effects, and a major reason for this is that pDCs, a major native TLR7-expressing cell type, are also the major producers of type I IFNs. pDCs contain more abundant TLR7 than the untreated or TNF-α– or IL-1–treated Hep3B cells (Figure 1B). Treatment of pDCs with TNF-α or IL-1 does not further enhance the abundance of TLR7 or the response to TLR7 activation (Figures 1B, 7 and 8). The reason for these observations is still unclear. One possibility is that *TLR7* transcription is fully activated at the basal level in these cells and, thus, is no longer able to be further enhanced by NF-κB activation.

In contrast, TNF-α or IL-1 treatment of Hep3B cells increases TLR7 expression and primes cellular response to R848 stimulation. Although R848 is a TLR7/8 agonist, its activity is solely mediated by TLR7 in these pretreated cells because only TLR7, and not TLR8, is up-regulated by TNF-α or IL-1 treatment (Figure 1). We also chose to use R848 in these experiments because its effective concentration for cell stimulation is much lower (~ 100 fold) than imiquimod or loxoribine, which are specific to TLR7 [47,48]. In the pre-treated Hep3B cells, R848 activated production of NF-kB dependent gene, ELAM-1 and TNF-α. Thus, the NF-κB signaling cascade is intact in these cells and is activated by TLR7 agonists following induction of TLR7 by pro-inflammatory stimulation, suggesting that TLR7 is the only missing component in the NF-κB

pathway in the Hep3B cells. Indeed, the results in Figure 4B show that expression of exogenous TLR7 activates the NF-κB–driven TLR7 promoter in these hepatocytes.

In contrast to the NF-κB signaling cascade, the IRF7 cascade is not functional in pretreated Hep3B cells since production of IFN-α is not induced by TLR7 activation in these cells. This result suggested the deficiency of another component(s) in addition to the TLR7 in the IRF7 signaling cascade in the Hep3B cells. IRF7 is a critical component required for the induction of type I IFNs by TLR7 activation. pDCs derived from IRF7-deficient mice are incapable of producing type I IFNs in responding to TLR7 stimulation [49]. IRF3 is structurally the most similar to IRF7, and is critical to TLR3/4-mediated activation of production of IFN-β, but is not able to replace the function of IRF7 in TLR7-activated type I IFN production [49]. Basal expression of IRF7 is restricted to pDCs and some other leukocytes, and is only very weakly expressed in most other cell types, although its expression is induced in response to type I IFNs signaling [11,32]. Accordingly, the expression of IRF7 was not detected in the untreated, and pre-treated Hep3B cells (Figure 8D), indicating that the IRF7 could be the missing component, or at least is one of the missing components in the IRF7 cascade. Consistent with these results, a previous study has shown that in cord blood pDCs expression of type I interferon induced in response to TLR7 activation is largely reduced due to a deficiency of IRF7, whereas NF-κB–mediated cytokine production is only partially affected [50].

In conclusion, this study has revealed an essential role of NF-κB activation in TLR7 induction by pro-inflammatory stimuli, and uncovered a molecular mechanism of how inflammatory stimulation can prime cells to respond to TLR7 activation in a manner that differs from that in native TLR7-expressing cells. Imiquimod, a small molecular weight TLR7 agonist, has been approved for the topical treatment of external genital warts infection, actinic keratosis and superficial basal cell carcinoma. Other TLR7 agonists are being evaluated in preclinical or clinical investigations for treatment of cancers, or infectious diseases. Of these, the clinical trials with ANA975, for treatment of HCV infection, have been suspended at phase Ib because of unacceptable adverse effects due to the intense immune response provoked following daily administration of this drug in an animal toxicology study [5,6]. Cancer cell death, viral infection or therapeutic treatment with TLR7 agonists frequently result in differing extents of inflammatory assaults. Our results suggest that the inflammatory response generated via these mechanisms might increase the complexity of therapy with TLR7 agonists.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was support in part by grants from the National Institutes of Health (GM069652 to T.H.C., AI054128 to W.M.C.), California Tobacco-Related Disease Research Program (16RT-0103 to T.H.C.), National Natural Science Foundation of China (30572116, 30672389, 2007AA021010, 2007CB914804 to R.X.)

Abbreviation used

TLR
Toll-like receptor

TNF-α
tumor necrosis factor-α

IL-1
interleukin-1

IFN interferon

EGF epidermal growth factor

LPS lipopolysaccharide

Poly(I C), polyinosinic acid-polycytidylic acid

PMA Phorbol 12-myristate 13-acetate

SP1 stimulating factor1

SRF serum response factor

NF-κB nuclear factor kB

IRF interferon regulatory factor

MyD88 myeloid differentiation factor 88

IRAK IL-1 R associated-kinase

TRAF6 TNFR-activated factor 6

TAK1 TGF-β-activating kinase

HIV human immunodeficiency virus

HCV hepatitis C virus

PBMC peripheral blood mononuclear cell

pDC plasmacytoid dendritic cell

mDC myeloid dendritic cell

ELISA enzyme-linked immunosorbent assay

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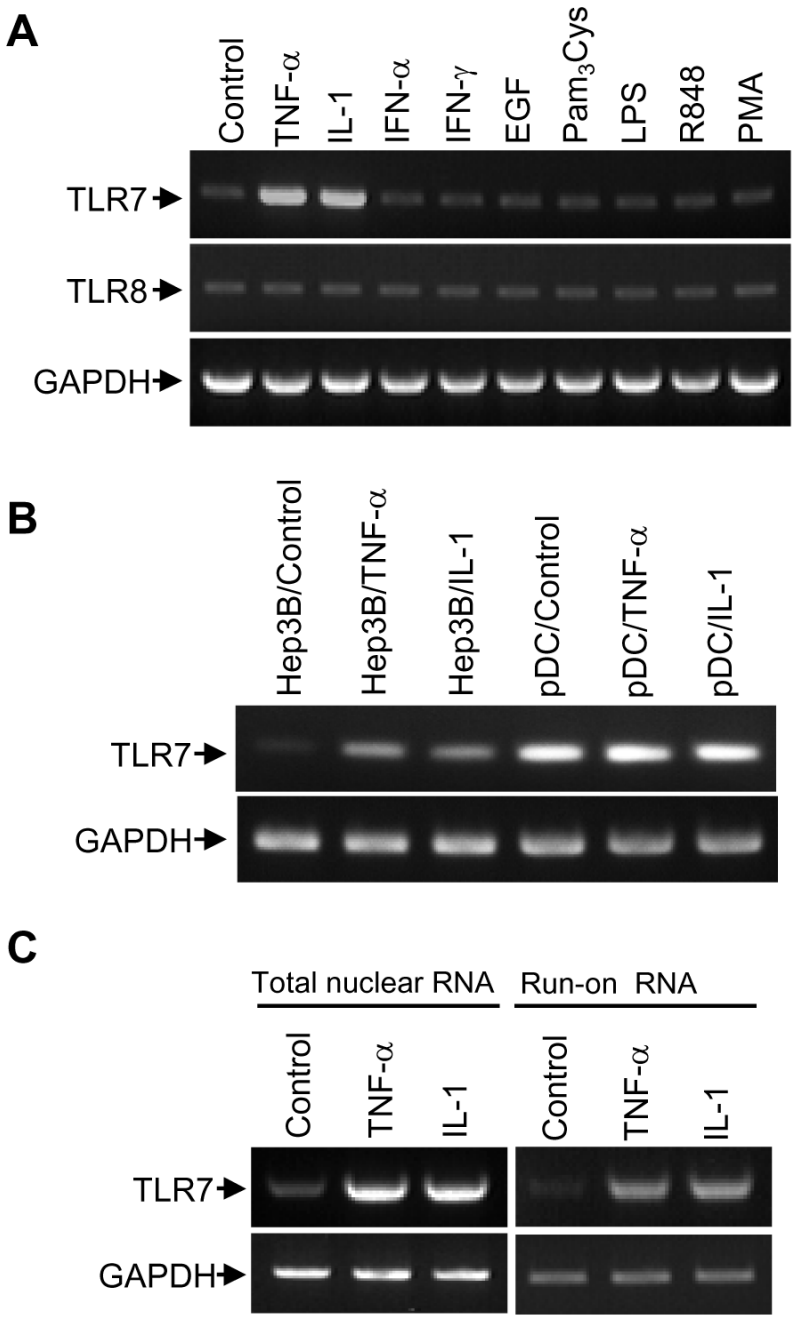

Figure 1. Transcriptional regulation of TLR7 expression

(**A**)Hep3B cells were treated with various stimuli for 4 h. (**B**)Hep3B cells and pDCs were treated with TNF- α or IL-1 for 4 h. Induction of TLR7 expressions in these cells were analyzed by RT-PCR. (**C**) Nuclear run-on reaction was preformed to determine the transcription rate of *TLR7* following TNF-α or IL-1 stimulation. TLR7 transcripts in the total nuclear RNA pool, and the run-on RNA pool were determined by RT-PCR. In these PCR reactions, amplification of GAPDH was performed to control for equivalent amounts of cDNA used as template.

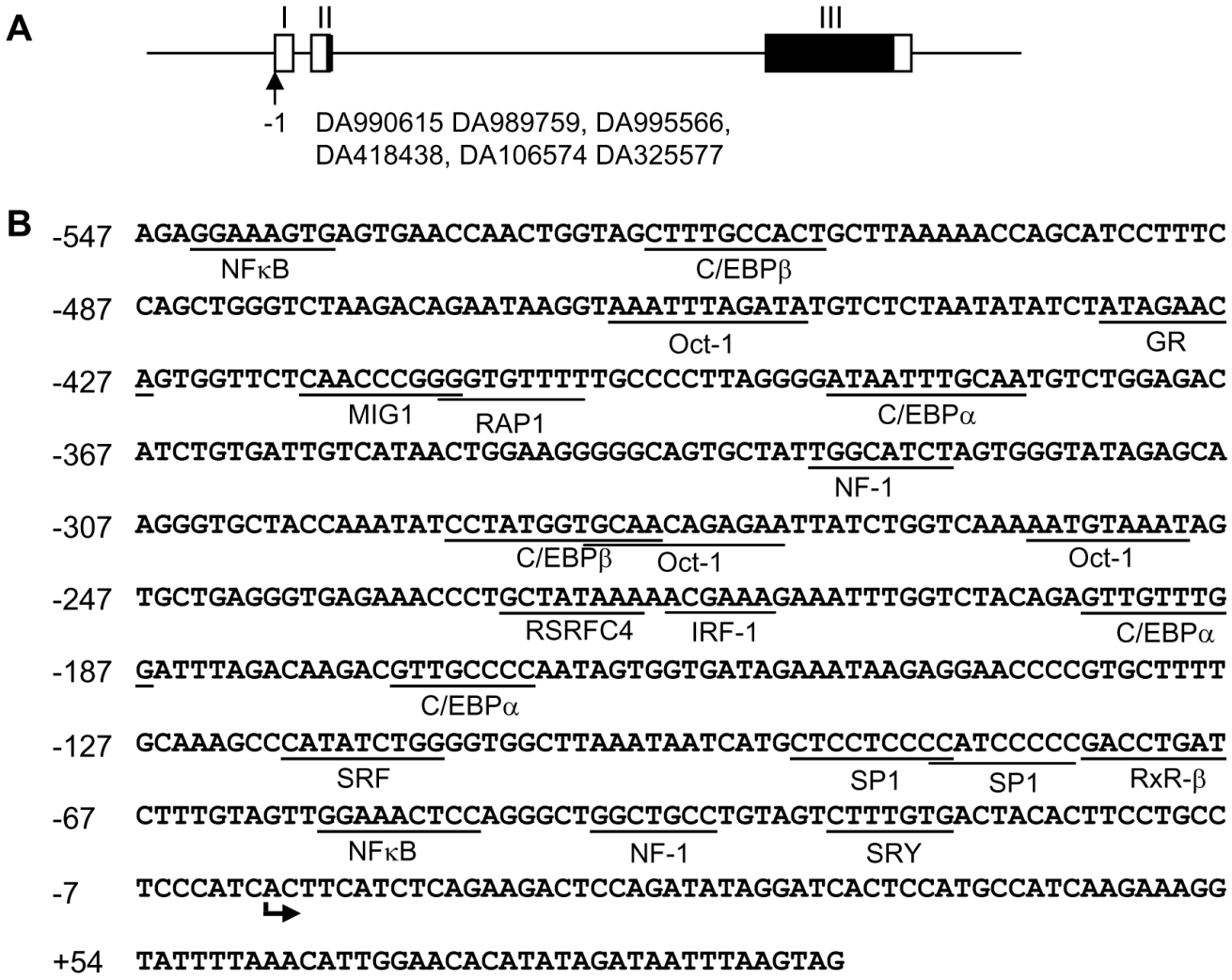

Figure 2. Structural analysis of the *TLR7* 5′-flanking region

(**A**) Schematic representation of the genomic structure of the *TLR7* gene. Exons are numbered by Roman numerals. The black bars show the coding regions, open bars show the non-coding regions. The arrow under exon 1 shows the putative transcription start site which was determined by the most distal TLR7 EST sequences identified from DNA sequence databases. The accession numbers of these EST sequences are shown below the figure. (**B**) Characterization of the putative promoter of *TLR7*. Putative transcription factor binding sites are analyzed by computer programs and underlined. The putative transcription start site (+1) is indicated by the bent arrow. The numbering at left side is relative to the transcription start site.

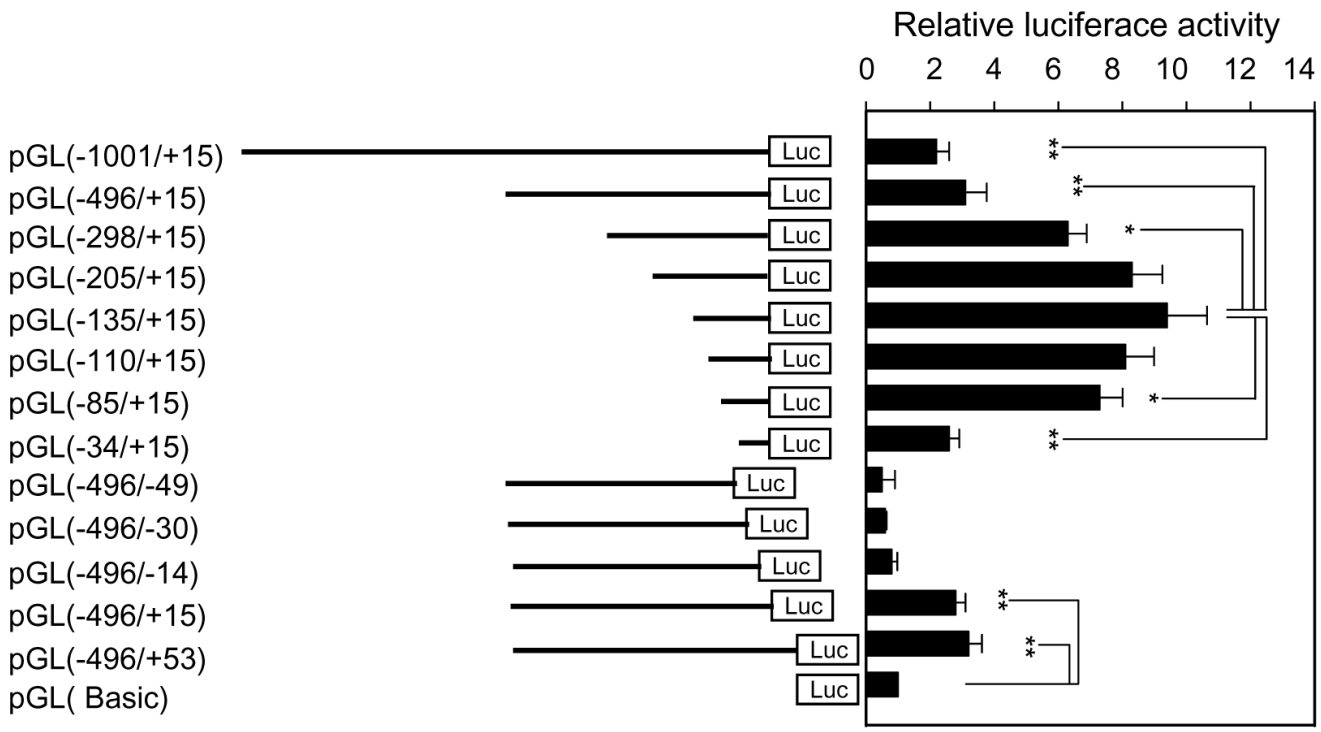

Figure 3. Deletion analysis of *TLR7* promoter activity

Serial deletion constructs containing different lengths of TLR7 promoter coupled to the luciferase reporter gene in the pGL3-basic reporter plasmid were generated. The 5′- and 3′-end positions of the promoter region are shown by the numbers in the parentheses. These reporter constructs were transfected into Hep3B cells and the cells were harvested for luciferase assays to analyze the promoter activity 16 h after transfection. The results were presented as mean ± SD (n=4). *, $p<0.05$. **, $p<0.01$.

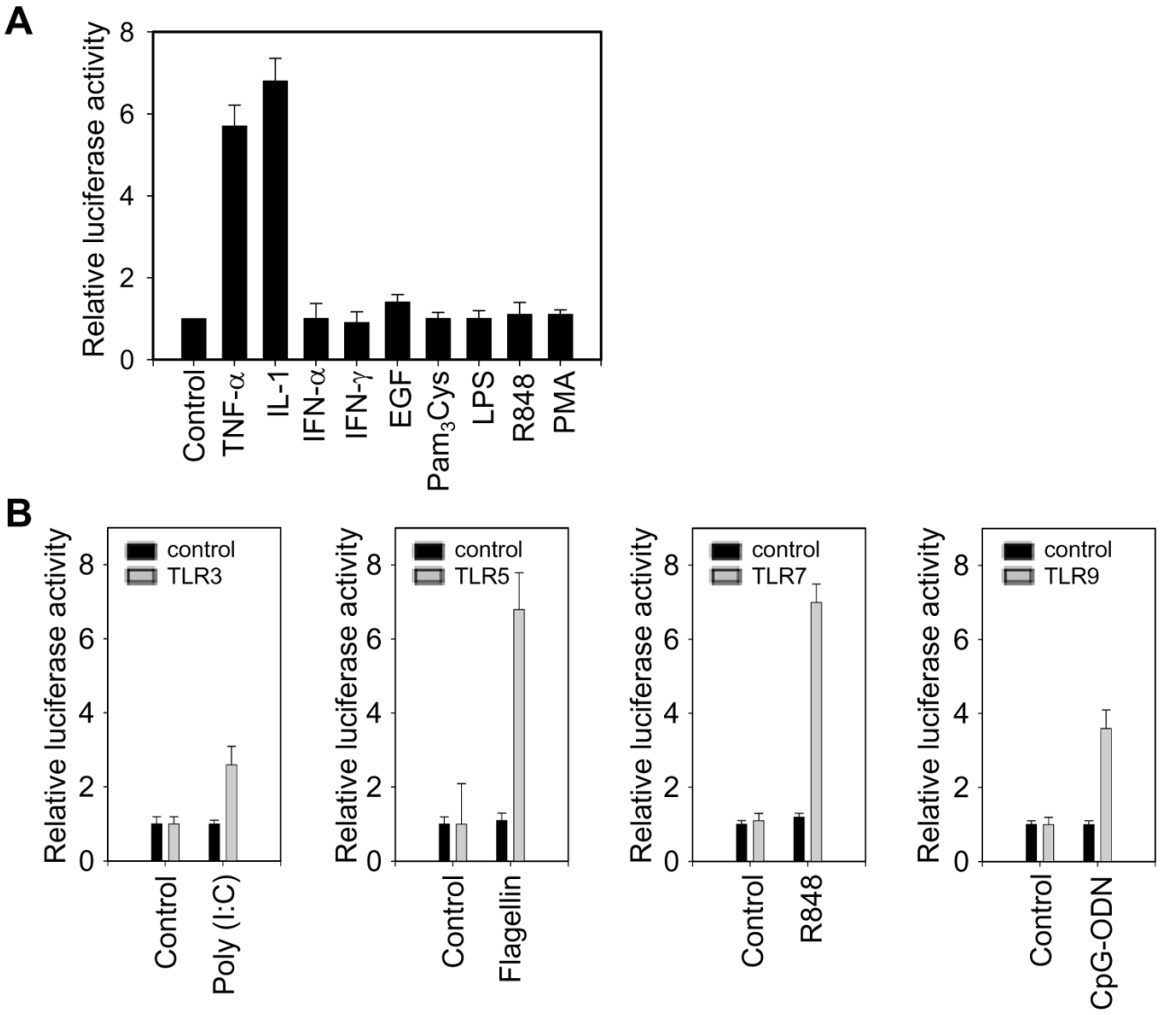

Figure 4. Inflammatory stimuli activate the *TLR7* promoter

(**A**) Hep3B cells were transfected with the pGL(−496/+15) luciferase reporter construct for 12 h. (**B**) Hep3B cells were co-transfected with the pGL(−496/+15) luciferase reporter construct and expression vector for different TLRs as indicated for 12h. These cells were treated with various stimuli for 6 h, and then harvested for luciferase assays to analyze the induced *TLR7* promoter activity. The results were presented as mean ± SD (n= 3).

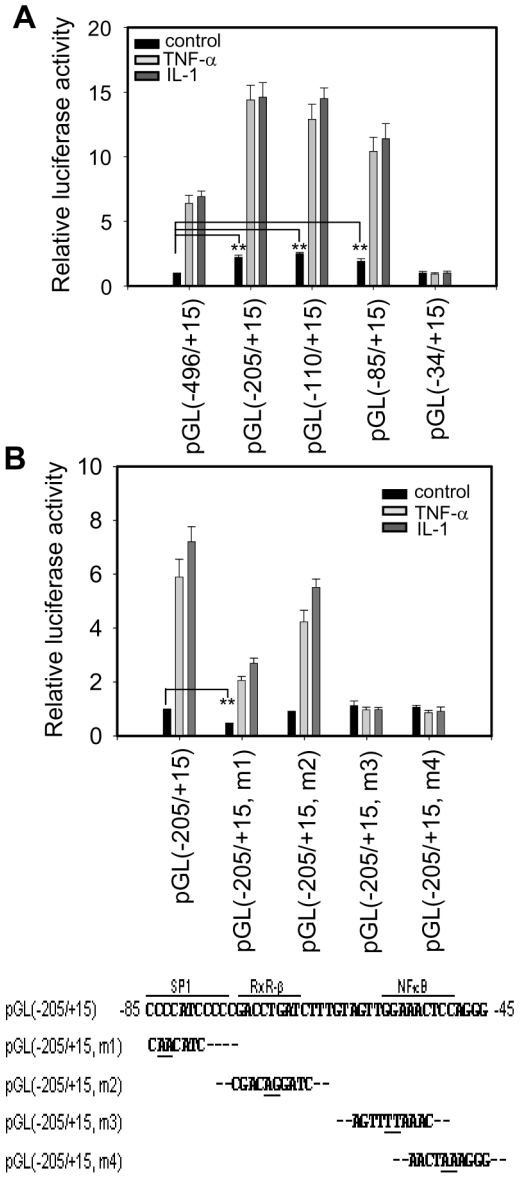

Figure 5. Localization of the TNF-α and IL-1 responsive element in the TLR7 promoter

(**A**) Hep3B cells were transfected with a set of luciferase reporter constructs containing progressively deleted TLR7 promoter regions. (**B**) Hep3B cells were transfected with luciferase reporter constructs containing the *TLR7* promoter with point mutations in the SP1, RxR-β or NF-κB binding site. At 12 h after transfection, these cells were treated with TNF-α and IL-1 for 6 h before being harvested for luciferase assays to analyze promoter activity. The mutated nucleotides in each construct are shown below the figure. The results were presented as mean ± SD (n=3). **, $p<0.01$.

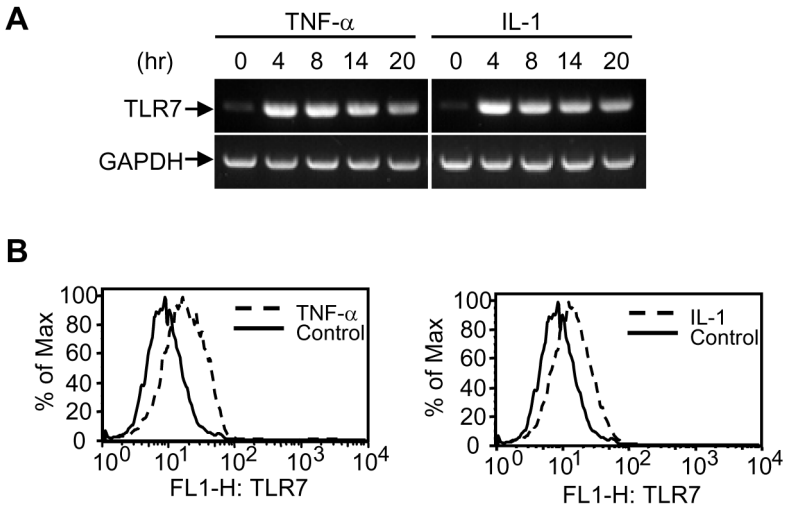

Figure 6. Time course of TNF-α and IL-1 induced TLR7 expression

Hep3B cells were treated with TNF-α and IL-1. (**A**) The levels of TLR7 and GAPDH transcript at different times after stimulation were determined by RT-PCR. (**B**) The expression of TLR7 protein 12 h after stimulation was assessed by antibody staining and FACS analysis.

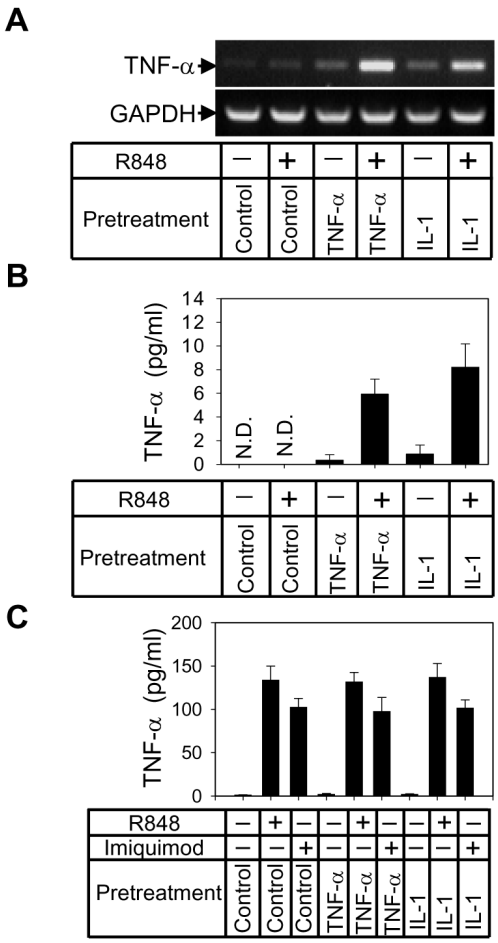

Figure 7. Activation of TNF-α production in TNF-α and IL-1 pretreated cells

(**A**)Hep3B cells were pretreated with TNF-α or IL-1 for 12 h, washed, and left for 5 h. The pretreated cells were treated with R848 for 4h and analyzed by RT-PCR for the levels of TNF-α transcript. (**B**) The Hep3B cells, pretreated as in (**A**), were treated with R848 for 16h, and the production of TNF-α protein analyzed by ELISA. (**C**) pDCs were pretreated with TNF-α or IL-1 for 12 h, washed, and left for 5 h. The pretreated cells were treated with 1 μM of R848 or 5 μM of imiquimod for 16h, and TNF-α production analyzed by ELISA.

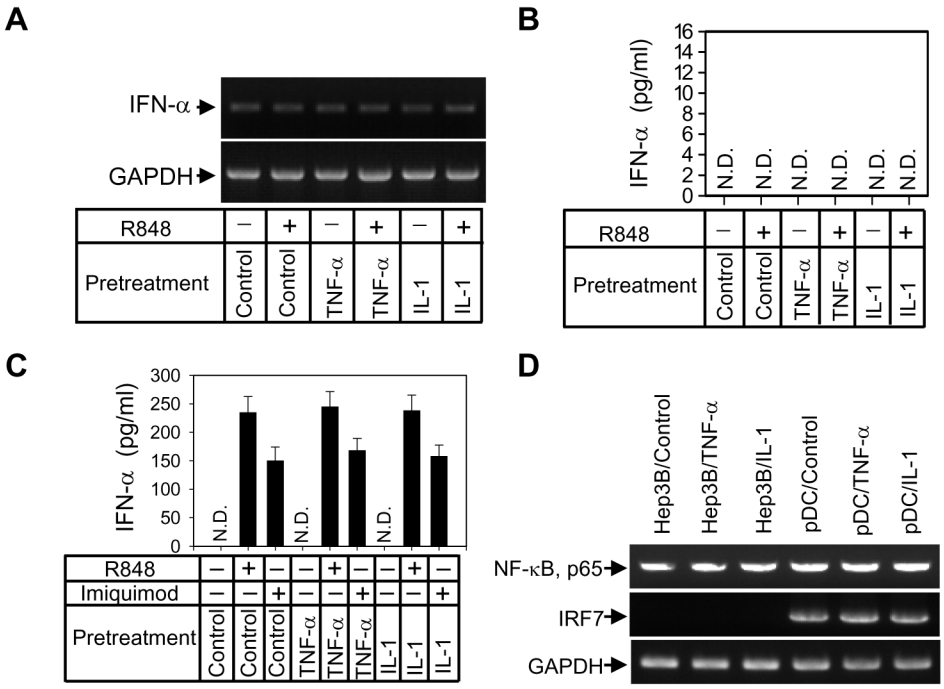

Figure 8. Activation of IFN-α production in TNF-α and IL-1 pretreated cells

(**A** and **B**)Hep3B cells, pretreated as in Figure 7 were treated with R848 and analyzed for IFN-α transcript (**A**) and protein (**B**). (**C**) pDCs, pretreated as in Figure 7 were treated with R848 or imiquimod, and IFN-α production analyzed by ELISA. (**D**) Hep3B cells and pDCs were treatedwith TNF-α or IL-1 for 4 hr, total RNA was isolated, and the expression levels of NF-κB p65 and IRF7 mRNA were determined by RT-PCR.