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# Differential induction of gene promoter constructs by constitutively active human TLRs

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**Abstract**

Antigen presenting cells can sense microorganisms through activation of members of the Toll like receptor family (TLRs), which initiate signals leading to transcription of many inflammation-associated genes. TLRs and IL-1R, through their TIR domains, activate NFκB and mitogen-activated protein kinase pathways and upregulate a set of specific target genes. Recent evidence points to several differences in signaling pathways activated by individual TLRs. To evaluate the basic signaling potential of individual TIR signaling domains, we generated constitutively active versions of all known human TLRs by fusing mouse CD4 extracellular portion with the TLR transmembrane and TIR domains. A panel of promoters from genes known to be activated by TLRs as well as artificial promoter constructs with transcription factor binding sites were selected to measure their response in the presence of constitutively active CD4TLR fusion molecules. These studies show for the first time that a unique panel of promoters appears to be highly induced by CD4TLR1, 6 (TLRs that usually function through heterodimerisation with TLR2), and CD4TLR10. We also observed that CD4TLR4 is the most potent gene activator compared to all other ten human TLRs. Preliminary analyses of several promoter deletions showed that TLRs use different sequence elements to activate these reporters. In addition, since different ligands for a single TLR (e.g., TLR9) can induce different pathways, the CD4TLR fusions seem to activate all the pathways and therefore can be used to assess the overall signaling capacity of a given TLR. Finally, analysis of promoter constructs induced by the only orphan TLR, TLR10, allowed the identification of the ENA78 promoter as a tool for screening its ligands.


The Toll-like receptor family are germ-line encoded receptors playing an essential role in initiating the immune response against pathogens. The 10 human TLRs that have been identified so far recognize a wide variety of pathogen-associated molecular patterns (PAMPs) from bacteria, viruses, and fungi, as well as certain host-derived molecules [1]. TLRs are type I transmembrane glycoproteins with extracellular domain composed of numerous leucine-rich repeats and an intracellular region containing a TIR homology domain. The extracellular domain features a remarkable plasticity in terms of ligand recognition, since one TLR can recognize structurally diverse ligands from unrelated sources [2]. The TIR domains are responsible for signal transmission from the receptor to the intracellular signaling cascades which result in induction of transcription factors controlling expression of a variety of genes involved in host defense. The TIR domains of TLRs interact with several TIR domain-containing adaptor molecules (MyD88, TIRAP, TRIF, and TRAM) which in turn activate a cascade of events resulting in transcription factor induction [3]. With the exception of TLR3, all TLRs seem to recruit MyD88, and some TLRs (i.e., TLR4, TLR2) can interact with several adaptors and generate diverse effects on gene transcription [4]. Although some TLRs signal through MyD88 only, the pattern of genes induced by individual TLRs seems to vary, and it is not clear what determines the functional outcome of signaling for each TLR. In addition, various factors may effect the quality of TLR signaling, i.e., TLR expression level, cell type, ligand concentration, presence of other TLRs, etc.

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In the present study we attempted to analyze the effect of constitutively active forms of all 10 TLRs on the activity of several reporter constructs in one cell line to provide information concerning the basic signaling capacity of individual TLRs. In this system, all TLRs are activated in the same way through association of extracellular CD4 domains, which avoids issues emanating from differential effects of ligands for a single TLR, requirements for co-receptors, absence of a known ligand, etc. We have chosen promoters from several genes which have been reported to be activated by TLRs, as well as artificial promoters containing specific transcription binding sites. These promoters were cloned in front of the luciferase reporter gene and the level of activation by individual TLRs was measured after co-transfection with CD4-TLR fusions in recipient cells.

We have found that each CD4TLR fusion activates the promoter constructs with a different profile and that certain TLRs can activate promoters through different mechanisms as judged by the analysis of promoter deletions. We were also able to determine which promoters were activated by TLR10, the only orphan TLR, and which can be used for screening for TLR10 ligands.

## Experimental procedures

*Constitutively active TLRs*. Constitutively active TLRs (CD4TLRs) were constructed by fusing cDNAs encoding the extracellular domain of murine CD4 to the transmembrane and cytoplasmic domains of human TLRs 1–10. All constructs were cloned into the pcDNAAmp vector. See Appendix for further details.

*Full length TLRs and MD-2*. TLR7 (GenBank Accession No. AF240467 and AY035889), TLR9 (GenBank Accession No. AF245704) were amplified from a human cDNA library and cloned into a CMV expression vector. The human TLR5, TLR6, TLR10, and p.DisplayMD2 flagged constructs were from Dr. Elisabeth Bates (Schering–Plough, France). pUNOTLR1 and pUNOTLR3 were purchased from Invivogen, France. The human TLR2 and TLR4-Flag constructs were kind gifts from Dr. Mantovani, Universita degli Studi di Milano, Italy. The pMetTLR8 plasmid was obtained from Vincent Flacher (Schering–Plough, France) and subcloned into pQCXNneo vector (Clontech, France).

*Reporter constructs*. Promoters IL-2 (GenBank Accession No. AJ006884), IL-4 (GenBank Accession No. Y18933), IL-6 (GenBank Accession No. AF048692), IL-8 [5], IL-18 [6], TNFα (GenBank Accession No. AB048818), STAT-6 (GenBank Accession No. AF067572), IFNβ [7], IP-10 [5], and ENA-78 [5] were amplified from genomic DNA and cloned into the pGL3 basic luciferase vector (Promega, France). NFκB and AP-1 constructs were obtained from Clontech, France. All sequences were human with the exception of the mouse IFNβ promoter [8].

*Deletions*. Specific promoter deletions were made in promoters IL-8, IP-10, ENA78, and STAT 6 at every 200 bps and cloned into the pGL3 vector (see Appendix for primer sequences and Fig. 4).

*Cell culture conditions*. HEK293T cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 2 mM L-glutamine. Cells were cultured at 37 °C with 5% $CO_2$.

*Transfections*. 293T cells were transiently transfected with 100 ng of reporter construct together with 25 ng CD4TLR expression vector in 24-well tissue culture plates with cells at 50% confluency. In addition, 1 ng of a construct directing expression of *Renilla* luciferase (under the control of the constitutively active CMV promoter) was used to normalize the transfection efficiency. Cells were harvested and analyzed for luciferase activity 24 and 48 h post-transfection.

*Luciferase assay*. Cells were harvested and analyzed simultaneously for firefly and *Renilla* luciferase activity using the Perkin–Elmer Firelite reporter assay reagents. Each experiment was repeated three times; results generally deviated by less than 10% of the mean value.

*Western blot analysis*. 293T cells were seeded into 6-well plates and the following day 300 ng of each CD4TLR construct was transfected. With each transfection 50 ng of reporter vector GFP was added to normalize expression levels of the CD4TLR constructs. At 24 h post-transfection cells were then lysed in a lysis buffer containing 1.0% Nonidet-P40, 150 mM NaCl, 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, and protease inhibitor cocktail (Calbiochem, France). The cell lysates were separated by SDS–PAGE and transferred onto a PVDF membrane (Millipore, France). The membrane was then probed with a mouse CD4 antibody (Pharmingen, France) and with an anti GFP antibody (Roche, France).

*ELISA*. Biological validation of our reporter studies was confirmed with IL-8, RANTES, and ENA-78 ELISAs. Medium was collected from 293 or 293T cells 24 and 48 h post-transfection with the CD4-TLR constructs. Secretion of IL-8, RANTES, and ENA-78 was measured using kits provided from R&D systems. Each experiment was repeated three times; results generally deviated by less than 10% of the mean value.

## Results and discussion

### *Expression of CD4TLR constructs*

To evaluate the basic signaling potential of individual TIR signaling domains, we generated constitutively active constructs of all known human TLRs by fusing mouse CD4 extracellular portion with the TLR transmembrane and TIR domains. Expression of the fusion proteins was evaluated by Western blot analysis (Fig. 1). We demonstrated that CD4TLR1–4 and 7–9 were expressed at similar levels whereas 5, 6, and 10 were expressed at a lower level.

Our selection of promoters was based on the experiments performed by Granucci et al. [9,10], who identified several cytokine and transcription factor genes which were upregulated in a mouse dendritic cell line stimulated with LPS. Fig. 2 lists promoters activated by the CD4TLR constructs in 293Ts, the majority of which encode cytokines that are regulated by TLR signaling. ENA78 was selected as it belongs to the IL-8 chemokine family and is heavily secreted in 293T cells when

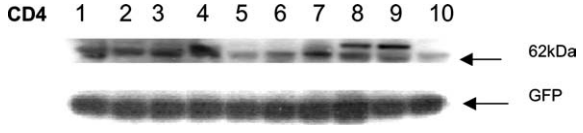


Fig. 1. Expression of the CD4TLR constructs. 293T cells were co-transfected with CD4TLR plasmids and GFP-expressing plasmid to normalize transfection efficiency. Cells were harvested 24 h post-transfection and Western blot analysis was performed using an anti-CD4 mouse antibody (BD, France) and anti-GFP antibody.

**A**

24h

| | NEG | | CD4 T1 | | CD4 T2 | | CD4 T3 | | CD4 T4 | | CD4 T5 | | CD4 T6 | | CD4 T7 | | CD4 T8 | | CD4 T9 | | CD4 T10 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *fold* | *sd* | *fold* | *sd* | *fold* | *sd* | *fold* | *sd* | *fold* | *sd* | *fold* | *sd* | *fold* | *sd* | *fold* | *sd* | *fold* | *sd* | *fold* | *sd* | *fold* | *sd* |
| **IFNbm** | 1,0 | 0,0 | 1,0 | 0,0 | 1,7 | 0,6 | 5,3 | 0,6 | 4,0 | 0,0 | 4,0 | 0,0 | 1,0 | 0,0 | 3,7 | 1,2 | 12,3 | 1,5 | 9,0 | 0,0 | 1,0 | 0,0 |
| **IL2** | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 |
| **IL4** | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 5,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 5,3 | 1,5 | 2,2 | 0,3 | 5,3 | 0,6 | 6,0 | 0,0 |
| **IL6** | 1,0 | 0,0 | 5,0 | 0,8 | 4,7 | 0,6 | 5,7 | 1,2 | 5,3 | 0,6 | 5,0 | 1,0 | 7,7 | 0,6 | 8,0 | 1,0 | 7,3 | 0,6 | 4,7 | 0,6 | 5,0 | 0,0 |
| **IL8** | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 63,3 | 4,2 | 120,0 | 10,0 | 62,3 | 7,4 | 1,0 | 0,0 | 21,7 | 2,9 | 1,0 | 0,0 | 5,0 | 0,0 | 1,0 | 0,0 |
| **IL18** | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 11,0 | 0,0 | 8,0 | 1,0 | 4,0 | 1,0 | 5,7 | 0,6 | 1,0 | 0,0 | 1,0 | 0,0 | 3,7 | 0,6 | 1,0 | 0,0 |
| **ENA78** | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 50,7 | 0,6 | 41,3 | 2,3 | 41,0 | 1,0 | 52,0 | 1,0 | 43,3 | 4,2 | 60,0 | 5,0 | 120,0 | 1,0 |
| **TNFA** | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 5,0 | 1,0 | 5,0 | 0,0 | 5,0 | 3,0 | 5,3 | 0,6 | 4,3 | 0,6 | 2,7 | 0,6 | 8,0 | 1,0 | 8,0 | 0,0 |
| **STAT6** | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 11,7 | 0,6 | 120,3 | 0,6 | 210,0 | 10,0 | 1,0 | 0,0 | 3,3 | 0,6 | 4,3 | 5,8 | 1,0 | 0,0 | 1,0 | 0,0 |
| **NFKB** | 1,0 | 0,0 | 1,0 | 0,0 | 12,3 | 0,6 | 4,3 | 0,6 | 564,7 | 12,0 | 43,3 | 4,6 | 7,3 | 0,6 | 17,2 | 0,3 | 12,3 | 1,5 | 14,3 | 0,6 | 1,0 | 0,0 |
| **IP10** | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 120,7 | 1,2 | 201,7 | 7,6 | 1,0 | 0,0 | 2,7 | 0,6 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 |
| **AP1** | 1,0 | 0,0 | 1,0 | 0,0 | 7,3 | 0,6 | 3,3 | 0,6 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 4,7 | 0,6 |

**B**

48h

| | NEG | | CD4 T1 | | CD4 T2 | | CD4 T3 | | CD4 T4 | | CD4 T5 | | CD4 T6 | | CD4 T7 | | CD4 T8 | | CD4 T9 | | CD4 T10 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *fold* | *sd* | *fold* | *sd* | *fold* | *sd* | *fold* | *sd* | *fold* | *sd* | *fold* | *sd* | *fold* | *sd* | *fold* | *sd* | *fold* | *sd* | *fold* | *sd* | *fold* | *sd* |
| **IFNbm** | 1,0 | 0,0 | 1,0 | 0,0 | 5,3 | 1,5 | 12,0 | 2,0 | 14,7 | 1,2 | 5,0 | 0,0 | 1,0 | 0,0 | 6,3 | 0,6 | 13,3 | 1,5 | 12,7 | 2,3 | 1,0 | 0,0 |
| **IL2** | 1,0 | 0,0 | 1,3 | 0,6 | 1,0 | 0,0 | 1,0 | 0,0 | 2,3 | 0,6 | 1,0 | 0,0 | 1,0 | 0,0 | 5,3 | 0,6 | 3,0 | 1,0 | 1,0 | 0,0 | 10,0 | 1,0 |
| **IL4** | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 2,3 | 1,5 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 5,7 | 2,1 | 76,7 | 35,1 |
| **IL6** | 1,0 | 0,0 | 1,0 | 0,0 | 2,0 | 0,8 | 1,2 | 0,3 | 2,0 | 1,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,7 | 1,2 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 |
| **IL8** | 1,0 | 0,0 | 1,0 | 0,0 | 1,3 | 0,6 | 5,7 | 0,6 | 66,7 | 11,5 | 51,7 | 12,6 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 |
| **IL18** | 1,0 | 0,0 | 1,0 | 0,0 | 55,0 | 5,0 | 66,7 | 26,0 | 1,0 | 0,0 | 1,3 | 0,6 | 5,3 | 1,5 | 1,3 | 0,6 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 |
| **ENA78** | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 110,7 | 10,0 | 120,0 | 26,5 | 127 | 11,5 | 111,7 | 10,4 | 107,3 | 6,8 | 630,0 | 17,3 | 120,0 | 26,5 |
| **TNFA** | 1,0 | 0,0 | 23,3 | 2,9 | 24,3 | 4,0 | 42,3 | 2,5 | 85,3 | 4,7 | 5,0 | 1,0 | 5,0 | 1,0 | 4,0 | 1,0 | 7,7 | 0,6 | 6,3 | 2,1 | 8,0 | 1,0 |
| **STAT6** | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 |
| **NFKB** | 1,0 | 0,0 | 1,0 | 0,0 | 463,3 | 33,3 | 156,7 | 22,0 | 12909 | 1525 | 332,3 | 11,0 | 139 | 13,3 | 383 | 12,9 | 220 | 6,0 | 112,7 | 16,9 | 36,0 | 2,0 |
| **IP10** | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 124,0 | 2,6 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 |
| **AP1** | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 1,0 | 0,0 | 3,0 | 1,7 | 1,0 | 0,0 | 1,0 | 0,0 | 5,0 | 1,0 | 4,0 | 1,0 | 1,0 | 0,0 | 7,0 | 2,6 |

Fig. 2. Activation of promoter constructs by CD4TLRs. Two hundred and ninety-three cells were transfected with indicated CD4TLR expression plasmids and different promoter constructs and cells were harvested (A) 24 h or (B) 48 h post-transfection for measurement of luciferase activity. Three independent experiments were performed (mean result of promoter activation), showing the potential of each CD4TLR construct to activate the panel of different promoters in HEK 293T cells.

activated in the presence of PMA [5]. All regulatory regions down to the transcription start site of the selected promoters were cloned into the pGL3 luciferase expression vector. Artificial promoters contained several binding copies for NFκB and AP-1, respectively. We initially included in our study BCL-X, IFNα, IL-12p40, and ISRE promoters, however, these constructs did not give reproducible results in 293T cells following transfection with the CD4TLR constructs (data not shown).

*Activation of promoter constructs by CD4TLRs*

CD4TLRs were co-transfected with the promoter constructs into 293T cells, harvested, and analyzed for luciferase activity 24 and 48 h post-transfection. Overall, the majority of CD4TLRs were able to induce NFκB, including CD4TLR2 and 6. Furthermore, CD4TLR1 and 6 activated the IL-6 promoter independent of heterodimerization with TLR2 [11]. We also observed that CD4TLR4 is the most potent gene activator compared to all other 10 human TLRs, possibly due to the recruitment of all four TIR adapters in the TLR4 signaling pathway.

The data summarized in Fig. 2 then allowed us to examine more specifically the differences emerging from TLR signaling. Here we discuss the induction of IFNβ, IL-18, IL-4, and ENA-78 as these genes are not usually associated with TLR signaling.

*IFNβ*

Prior to the generation of the Th1 pathway is the induction of IFN-I (Type I interferons), cytokines that link innate and adaptive immunity by inducing the secretion of IFNγ. Dendritic cells make high levels of IFN-I in response to viral infection and activation of TLRs 3, 4, 7, and 9 [12]. Our results show that CD4TLR3, 4, 5, 7, 8, and 9 have the potential to induce the IFNβ promoter. Gautier et al. (unpublished) in our laboratory observed that monocyte-derived DCs can produce certain levels of IFNβ following stimulation of TLR8 with R848. Stimulation of TLR5 signaling has not been shown to stimulate IFN production so far, and the reason why CD4TLR5 induces IFNβ promoter is not clear.

*IL-18*

IL-18 is a cytokine that plays an important role in the Th1 response, primarily by its ability to induce IFN-γ production by T cells and natural killer (NK) cells. The IL-18

reporter was activated by CD4TLR2, 3, 5, 6, and 9. Evidence that these TLRs are capable of inducing IL-18 is supported by the observation that treatment of DCs with purified listerial LTA (TLR2 ligand) yielded high levels of IL-18 release, but only minimal IL-12 production. However, stimulation of DCs with LPS (TLR4 ligand) conversely induced significant amounts of IL-12 production, but no IL-18 [10]. Here we observed and furthermore validated, a response that complements the findings of Kolb-Maurer et al. [10], where at 48 h post-transfection a significant induction of the IL-18 promoter by CD4TLR2 was observed but not by CD4TLR4.

*IL-4*

An essential component of the Th2 response is IL-4, a cytokine which has pleiotropic effects on the immune system. IL-4 is primarily produced by T cells, mast cells, and basophiles and drives T cells to differentiate into Th2 IL-4 producing cells. Fig. 2 reveals that the IL-4 promoter is induced by CD4TLR4, 7, 8, 9, and 10 (76-fold). Our data show a direct induction of IL-4 through TLR activation; these results may imply the possible involvement of TLRs in regulating a direct Th2 shift in cytokine production.

*ENA78*

This chemokine is upregulated during an inflammatory response by eosinophils [13] and LPS-stimulated intestinal epithelial cells [14]. ENA-78 has been classified as a neutrophil chemoattractant belonging to the IL-8 chemokine family [15] and there is currently no evidence supporting the role of TLRs in recruiting neutrophils during an innate response to pathogens. Fig. 2 reveals that the majority of Toll like receptors activate this promoter (CD4TLR4–10), although regulation of this gene amongst the TLRs appears to differ (examined below). Therefore, these observations clearly indicate the role of TLRs in inducing genes which are not typically associated with in TLR signaling.

*TLR9*

From these data we also show the convenience of using the CD4 constructs to understand the signaling mechanisms potentially mediated by TLR9. The TLR9 ligands consist of two distinct types of CpG ODN which differ in their capacity to stimulate antigen presenting cells: CpG-A induce high amounts of type-I IFN in plasmacytoid dendritic cells (PDCs), while CpG-B induce PDC maturation and are potent activators of B cells, but stimulate only small amounts of IFNα/β [15]. It seems that CD4TLR9 can mimic the signaling induced by both types of CpGs by inducing NFκB, IFNβ, IL-4, IL-6, IL-8, and IL-18.

*TLR10*

Finally, we identify for the first time genes that may be induced by TLR10. TLR10 appears to be organized in the same phylogenetic tree with TLR1, 2, and 6 and therefore may act as co-receptor for TLR2. TLR10 is mainly expressed by B cells [16] as well as eosinophils: [17]. CD4TLR10 activates NFκB, TNFα, IL-6, and ENA-78 promoters and showed the strongest induction of AP-1 and IL-4 as compared to other CD4TLRs. There are reports describing ENA-78 secretion by eosinophils [13] but not by B cells. CD4TLR10 induces the IL-6 promoter, which is a cytokine secreted mainly by activated B cells. Since its discovery no group has been able to determine the ligand for this receptor and gene induction of ENA-78 (based on our findings) may be used as a read-out in attempts to understand how TLR10 becomes activated.

We next addressed the question, whether the reporter activity seen at 48 h was due to direct TLR signaling and not due to a secondary effect. Supernatants from CD4TLR transfected cells were removed 24 h post-transfection and placed onto cells transfected with NFκB reporter only. Presence of the supernatants did not have any effect on the activity of the reporter (data not shown), indicating that the response at 48 h was not due to secondary effects caused by the initial round of CD4TLR signaling.

*Biological validation*

To verify our reporter studies we analyzed the production of IL-8 in supernatants transfected with the CD4 fusion molecules vs IL-8 luciferase activity and RANTES secretion vs IFNβ luciferase expression. Medium was harvested 24 and 48 h post-transfection. Figs. 3A and C compare IL-8 reporter with IL-8 secretion. Observations show that the reporter activity reflects biological induction of IL-8 in the cases of CD4TLRs 4, 5, 7, and 9. No IL-8 secretion was induced by CD4TLR3 and conversely no reporter IL-8 activity was seen in CD4TLR6, however, IL-8 secretion was observed. This may be due to the higher sensitivity of an ELISA compared to the luciferase assay. To validate activity seen with IFNβ reporter a RANTES, ELISA was performed as IFNβ was undetectable in 293T cells. A common feature in the genes encoding IFNβ and RANTES is that the same multiple regulatory elements are required for their activation in response to viral infection [18]. Therefore, the activity of IFNβ was confirmed via RANTES secretion as induction of both of these genes requires similar transcription factors to initiate gene expression. Validation for CD4TLR3, 4, 7, and 8 but not for 5 or 9 (Figs. 3B and D) was observed. Hence, for some CD4TLRs we were unable to confirm activity of the IFNβ promoter based on RANTES secretion. This may be due to the presence of response elements induced by CD4TLR5 and 9 which

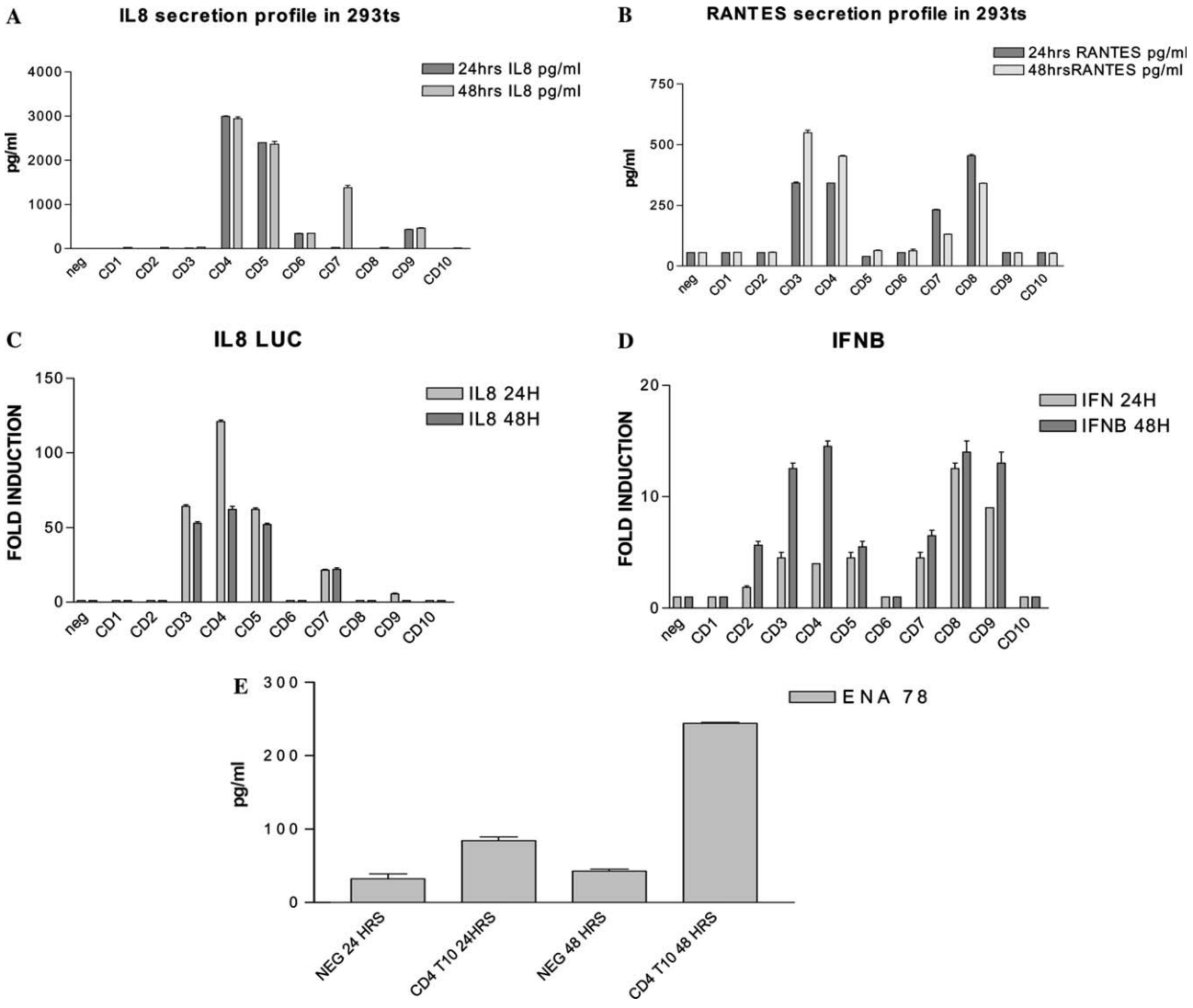


Fig. 3. CD4TLRs induce IL-8, RANTES, and ENA-78 secretion. For IL-8 and RANTES measurements by ELISA, medium was collected 24 and 48 h post-transfection with the CD4TLR constructs. Neat and 1:10 dilutions of the supernatant were used to measure IL-8 and RANTES using Quantikine ELISA kits from R&D systems. Graphs (A,B) compare IL-8 secretion and reporter activity, respectively. Graphs (C,D) compare RANTES secretion and IFNB reporter activity, respectively. (E) *CD4TLR10 induces ENA 78 secretion.* Medium was collected 24 and 48 h post-transfection with the CD4TLR10 construct. Neat and 1:10 dilutions of supernatant were used to measure ENA 78 using Quantikine ELISA kits from R&D systems. Representative experiment out of three experiments is shown.

were adequate to activate the IFNβ promoter but not to induce RANTES secretion in this experimental assay. Interestingly, CD4TLR8 induces the IFNβ reporter and the secretion of RANTES which further suggests that TLR8 may be involved in the IFN-I response. To confirm reporter data with CD4TLR10 construction, ENA-78 was measured in 293T supernatants from cells transfected with CD4TLR10 (Fig. 3E). ENA-78 was secreted at 24 and 48 h post-transfection, here reporting for the first time biological evidence of TLR10 signaling. Therefore, these findings support our reporter studies (in most experimental cases) and also provide new information in respect to TLR10 activation where the natural ligand is still not known.

*Promoter deletions*

Since many of the CD4TLRs activated the same reporter construct, we decided to generate 5′-terminal deletions of Stat6, IL-8, IP-10, and ENA78 promoters in order to analyze whether all CD4TLRs use the same promoter region to activate transcription of a given gene. These deletions were co-transfected with various CD4TLRs and the effect of deletions was assessed by comparison with the activation of the full-length promoter construct. Fig. 4 shows in a schematic way the position of various potential transcription factor binding sites in selected promoters and the position of deletions in respect to the transcription factor binding sites. Fig. 5A shows the

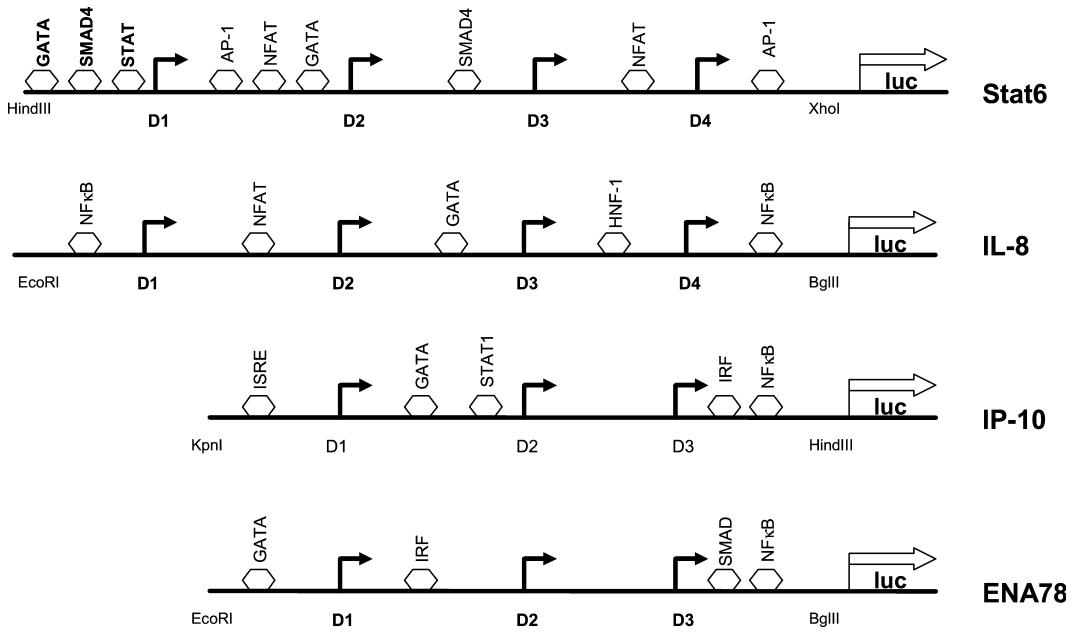


Fig. 4. Schematic drawing indicating position of various transcription factor binding sites in Stat6, IL-8, IP-10, and ENA78 promoters. Positions of deletions are marked D. The transcription factor binding sites were analyzed using the MatInspector software (Genomatix) with core similarity 1.000.

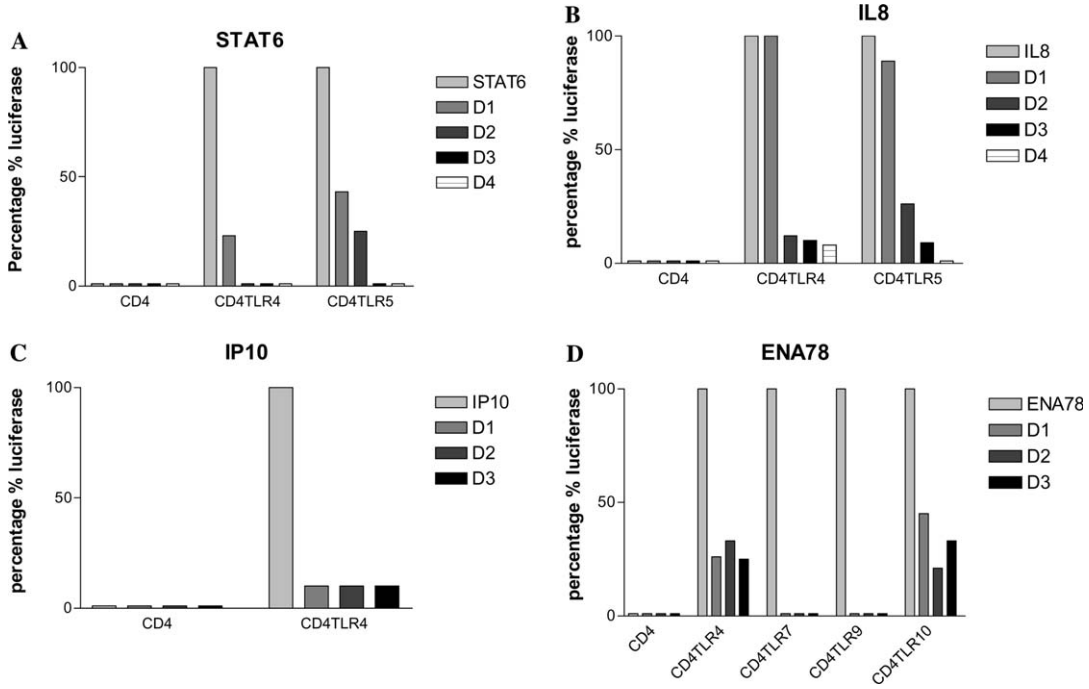


Fig. 5. Activity of promoter deletions in the presence of CD4TLRs. Deletions in Stat6, IL-8, IP-10, and ENA78 promoters were generated and transfected with various CD4TLRs and cells were harvested 24 h post-transfection. (A) CD4TLR4 or 5 vs STAT6 and deletions, (B) CD4TLR4 or 5 vs IL-8 and deletions, (C) CD4TLR4 vs IP-10 and deletions, and (D) CD4TLR4, 7, 9, and 10 vs ENA 78 and deletions. Results are plotted as percentage of activity of a given deletion against the activity of the full-length promoter. One experiment out of three independent transfections is shown.

effect of CD4TLR4 and 5 on Stat6 promoter deletions. Deleting the first ~400 bp (D1) results in a significant loss of activation by both TLRs compared to the full-length promoter while additional deletion (D2) rendered the promoter unresponsive to CD4TLR4 but not to CD4TLR5. It seems therefore that CD4TLR4 and CD4TLR5 can activate Stat6 promoter at least in part by stimulating transcription factors present in the deletion D1 (AP-1, NFAT, and GATA) and that CD4TLR5 acts through additional sites present in deletion D2. Deletion of the first ~200 bp in IL-8 promoter did not cause a major change in responsiveness to CD4TLR4 and 5,

although this construct lost a potential NFκB site. Further deletions D2 and D3 respond to a lesser extent and deletion D4 (which still contains NFκB site) is no longer activated by CD4TLR5 while it conserved some inducibility in the presence of CD4TLR4. Deleting the first part of IP-10 promoter with ISRE binding site (Fig. 5C) results in a loss of responsiveness to CD4TLR4 and further deletions did not affect the response. Analysis of ENA78 promoter deletions showed that CD4TLR7 and 9 require the 5′ part of the promoter while CD4TLR4 and 10 lose responsiveness after deleting the first part of the promoter but continue to activate this promoter by elements present in the deletion D3 (SMAD, NFκB) [19]. Further analyses will be required to show whether indicated transcription factors indeed participate in regulation of these promoters by TLRs.

These experiments show that although individual CD4TLRs activate a common set of genes, each CD4TLR activates the tested promoter constructs through induction of different transcription factors. This implies that the mechanisms underlying the regulation of these promoters by TLR signaling may differ and might affect the kinetics and the scale of gene expression.

In this study, we have compared the signaling potential of all 10 human TLRs by co-transfection of their constitutively active versions with a panel of promoter constructs in a single cell line. Our results show that CD4TLRs can be used as a fairly accurate tool for analyzing promoter responsiveness to TLR ligands and that some promoters identified in this study (e.g., ENA78) can be used for screening for ligands activating TLR10. We have identified promoters IL-18, IFNβ, IL-4, and ENA78 as potentially useful tools for analysis of TLR signaling, since these genes were not previously described to be activated through TLRs. We have also observed that TLRs can potentially activate a Th2 response. In addition, our results evaluated the signaling potential of TLRs which tend to heterodimerize with others and have shown to have an intrinsic signaling capacity in their homodimeric form. Finally, we found that different TLRs can activate promoters through distinct sequence elements which may have an effect on the scale and tissue specific expression of target genes.

## Appendix

| CD4-TLRS | |
|---|---|
| CD4 FW: | 5′ GGA AGC TTA CCA CCA TGT GCC GAG CCA TC |
| CD4 REV: | 5′ AAC TCG AGC ACT GTC TGG TTC ACC CCT C |
| T2 FW: | 5′ ATT CTC GAG CTC TCG GTG TCG GAA TGT CAC A |
| T2 REV: | 5′ GCG TCT AGA CTA GGA CTT TAT CGC AGC TCT C |
| T3 FW: | 5′ AGC CTC GAG TCA TCT TGC AAA GAC AGT GCC |
| T3 REV: | 5′ GCC TCT AGA TTA ATG TAC AGA GTT T |
| T5 FW: | 5′ CTC TCG AGA AGT TCT CCC TTT TCA TTG TAT GCA C |
| T5 REV: | 5′ ATT CTA GAT TAG GAG ATG GTT GCT ACA GTT TGC |
| T6 FW: | 5′ GC CTC GAG GTC ACT ACC CAG AAA GTT ATA GAA |
| T6 REV: | 5′ CGG TCT AGA CGA CTG TAC TAT TAT CCA TCA TCC |
| T7 FW Sal: | 5′ ACC GTC GAC GAT CTG ACT AAC CTG ATT CTG |
| T7 REV Xba: | 5′ GCA TCT AGA CTA GAC CGT TTC CTT GAA CAC C |
| T9 FW: | 5′ AAC TCG AGT GCC TGG ATG AGG CCC TCT C |
| T9 REV: | 5′ AGT CTA GAC TAT TCG GCC GTG GGT CCC |
| T10 FW Sal: | 5′ AAC GTC GAC GAT TCA TAC ACC TGT GAA TAC C |
| T10 REV Sal: | 5′ GTT GTC GAC TTA TAG ACA ATC TGT TCT CAT CAG |

CD4-T1, T4, and T8 were kind gifts from Vincent Flacher, Schering–Plough, France. All three sequences were subcloned into pCDNA.AMP. PCR was performed on TLR plasmids as templates.

| Promoter deletions | |
|---|---|
| *STAT-6* | |
| REV: | 5′ CCT TAT GCA GTT GCT CTC CAG |
| FW: | d1: 5′ AGG CTC GAG CTA ATC TCA AGT ACT TCA GGA C |
| | d2: 5′ AGG CTC GAG ACG GAG TCT TGT TCT GTC AC |
| | d3: 5′ AGG CTC GAG TCT CAT TTG AGG GAT TGA CAC |
| | d4: 5′ AGG CTC GAG GGA CAA GCC AAT GGA CAG AG |

(*continued on next page*)

**Appendix** (*continued*)

| | |
|---|---|
| *ENA-78* | |
| REV: | 5′ AAC GGT ACC TAC TCC CTT CTA GCT G |
| FW: | d1: 5′ AGA GGT ACC GAA GTC CAG AGA TTC ATA AAG TC |
| | d2: 5′ AGA GGT ACC GCT CCT GTT ACT TTG GTT CC |
| | d3: 5′ AGA GGT ACC AGA TAC TCC CTT CTA GCT G |
| *IL-8* | |
| REV: | 5′ AAC AGA TCT CGA GGA AGC TTG TG |
| FW: | d1: 5′ AGA GGT ACC ATA AGA ACC CTT CCT TCC |
| | d2: 5′ AGA GGT ACC GCA CCA CTT TCT GGA GC |
| | d3: 5′ AGA GGT ACC ACA TTA CTC AGA AAG TTA CTC |
| | d4′: 5′ AGA GGT ACC ATG ATC TTG TTC TAA CAC CTG |
| *IP-10* | |
| REV: | 5′ CCT TAT GCA GTT GCT CTC CAG |
| FW: | d1: 5′ AGA GGT ACC GAC TGC TAT AAG ACG TGA AAC |
| | d2: 5′ AGA GGT ACC GTC CAG GTA AAT CAC TGT TC |
| | d3: 5′ AGA GGT ACC TAG AAT GGA TTG CAA CTT TTG |

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