

## **The Human Toll Signaling Pathway: Divergence of Nuclear Factor $\kappa$ B and JNK/SAPK Activation Upstream of Tumor Necrosis Factor Receptor-associated Factor 6 (TRAF6)**

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### **Summary**

The human homologue of *Drosophila* Toll (hToll) is a recently cloned receptor of the interleukin 1 receptor (IL-1R) superfamily, and has been implicated in the activation of adaptive immunity. Signaling by hToll is shown to occur through sequential recruitment of the adapter molecule MyD88 and the IL-1R-associated kinase. Tumor necrosis factor receptor-activated factor 6 (TRAF6) and the nuclear factor  $\kappa$ B (NF- $\kappa$ B)-inducing kinase (NIK) are both involved in subsequent steps of NF- $\kappa$ B activation. Conversely, a dominant negative version of TRAF6 failed to block hToll-induced activation of stress-activated protein kinase/c-Jun NH<sub>2</sub>-terminal kinases, thus suggesting an early divergence of the two pathways.

**Key words:** Toll • interleukin 1 receptor • nuclear factor  $\kappa$ B • c-Jun NH<sub>2</sub>-terminal kinase/stress-activated protein kinase

Immune response to infection requires the production of cytokines and costimulatory molecules by antigen-presenting cells. Distinct cell-associated receptors on myelomonocytic cells, such as CD14, allow the recognition of pathogen-associated molecules and trigger natural immune response by inducing the production of inflammatory cytokines that subsequently signal to activate adaptive immunity (1).

The molecular mechanisms that control the initial induction of these signals upon infection have been explored recently. In particular, a novel transmembrane receptor homologous to the *Drosophila* Toll, human Toll (hToll, also called TLR4), has been cloned recently (2, 3). The *Drosophila* Toll protein controls the potent antifungal response in *Drosophila* adults (4). Analogously, hToll has been shown to signal activation of adaptive immunity in humans by inducing the expression of B7.1, IL-6, and IL-8; thus, it represents a key molecule for the switching from natural to acquired immunity. However, the biochemical transduction pathway triggered by hToll was ill-defined (2).

hToll is a type I orphan receptor with an extracellular portion containing leucine-rich repeats, and a cytoplasmic domain significantly similar to the intracellular portion of the IL-1R type I (IL-1RI) and the IL-1R accessory protein (IL-1RAcP) (2, 5, 6); these observations suggest that they may use an analogous molecular framework for signaling.

IL-1 triggers the activation of distinct transcription factors, including NF- $\kappa$ B and c-Jun/activator protein 1, that

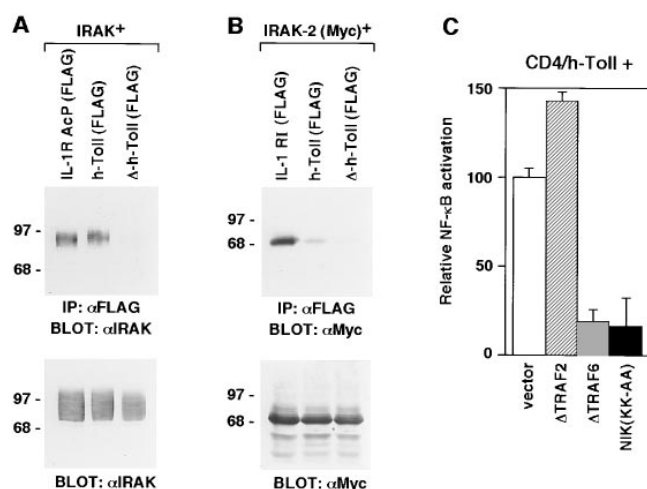
subsequently drive the transcriptional induction of several cytokine genes (7). The molecular events occurring from the IL-1R signaling complex to the induction of NF- $\kappa$ B activity have been characterized recently; in particular, the adapter protein MyD88 recruits two distinct putative Ser/Thr kinases, namely IL-1R-associated kinase (IRAK) and IRAK-2, to the receptor complex (8–10). IRAK and IRAK-2 interact subsequently with the adapter molecule TNFR-activated factor (TRAF) 6, which bridges them to the protein kinase NF- $\kappa$ B-inducing kinase (NIK) (8, 11, 12). Finally, NIK activates the I- $\kappa$ B kinase complex (including IKK $\alpha$  and IKK $\beta$ ) that directly phosphorylates I- $\kappa$ B $\alpha$  (13–17).

In this study, we identified and molecularly ordered the mediators of the hToll-induced NF- $\kappa$ B and stress-activated protein kinase (SAPK)/c-Jun NH<sub>2</sub>-terminal kinase (JNK) activation cascade.

### **Materials and Methods**

**Expression Vectors and Transfection.** TRAF6-Flag,  $\Delta$ TRAF6-Flag (or TRAF6 298–522),  $\Delta$ TRAF2-Flag (or TRAF2 87–501), MyD88-AU1,  $\Delta$ MyD88-AU1 (or MyD88 152–296), IRAK, NIK(KK-AA), and HA-p46SAPK $\gamma$ -pCDNA3 expression vectors have been described (8, 18). Expression vectors for NH<sub>2</sub>-terminal Flag-tagged hToll and  $\Delta$ hToll (amino acids 1–666) were constructed by inserting PCR-generated cDNA fragments lacking





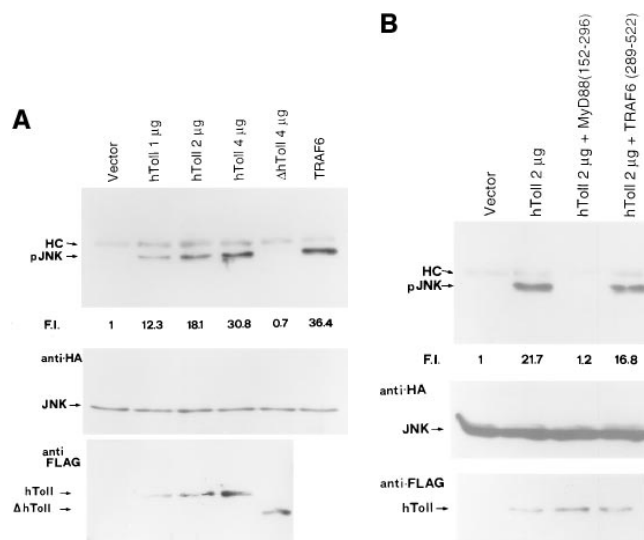
**Figure 3.** NF-κB activation by hToll occurs through IRAK, TRAF6, and NIK. (A) IRAK is recruited to hToll but not to the inactive truncated version of hToll (ΔhToll). IL-1RAcP served as a positive control. Cells were transfected and analyzed as in Fig. 2 B. (B) IRAK-2 binds very weakly to hToll. IL-1RI served as a positive control. Cells were transfected and analyzed as in A. (C) ΔTRAF6 but not the unrelated ΔTRAF2 attenuates CD4/Toll-induced NF-κB activity. A dominant negative mutant version of the downstream molecule NIK [NIK(KK-AA)] also inhibits CD4/hToll-induced NF-κB activity.

Santa Cruz Biotechnology, Inc.). An aliquot of the cell lysate was also analyzed for NF-κB activation as above.

## Results and Discussion

The expression and eventual regulation of specific transcripts encoding for hToll were analyzed in distinct cell types that play a critical role in the natural immune response. In particular, human monocytes were separated from healthy donors and treated with the bacterial product LPS for different periods of time. As shown in Fig. 1 A, specific transcripts for hToll were present in these cells and were induced significantly after treatment with LPS. These observations suggest that modulation of a nonclonal receptor, namely hToll, after exposure to infectious agents may play a regulatory role in the natural immune response. Of note, PMN and dendritic cells also transcribed hToll mRNA at different levels (our unpublished observations).

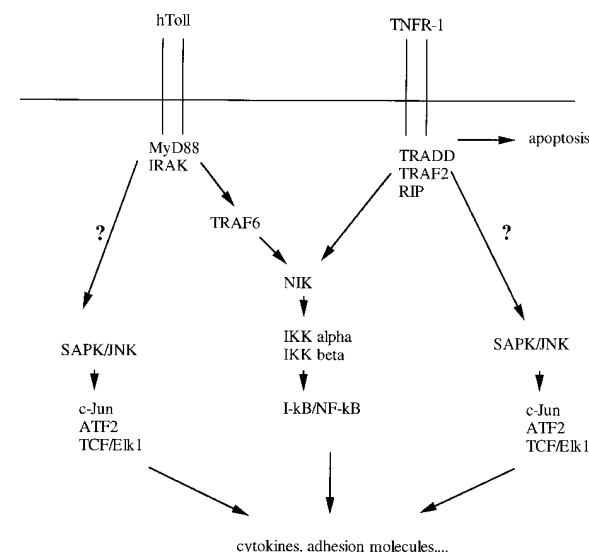
We next analyzed the hToll signaling pathway at the molecular level. A chimeric version of hToll in which the extra cellular portion was substituted with the corresponding region of CD4 (CD4/hToll) has been shown previously to induce NF-κB activation in Jurkat cells (2). We engineered distinct expression constructs encoding for Flag epitope-tagged hToll (hToll-Flag) or for a truncated version of hToll that lacks most of the cytoplasmic portion (ΔhToll-Flag). Ectopic expression of hToll-Flag but not ΔhToll-Flag induced NF-κB activation in human embryonic 293T cells at levels similar to the CD4/Toll chimeric protein that served as a positive control (Fig. 1, B and C). From these observations, it is apparent that either CD4-driven or ectopic expression-forced aggregation of the cytoplasmic portions of distinct hToll receptors is sufficient to trigger a signaling cas-



**Figure 4.** hToll-induced SAPK activation requires MyD88 but not TRAF6. (A) hToll activates SAPK/JNK in a dose-dependent manner, as determined by the presence of the active phosphorylated form of JNK (pJNK). F.I., Fold induction calculated by normalizing pJNK with JNK. HC, IgG heavy chain. (B) MyD88 (152-296) (ΔMyD88) but not TRAF6 (289-522) (ΔTRAF6) abolishes hToll-induced SAPK/JNK phosphorylation. F.I., Fold induction calculated by normalizing pJNK with JNK. HC, IgG heavy chain.

cade that leads ultimately to activation of the transcription factor NF-κB. Given this, one could speculate that an as yet unidentified hToll ligand binds to hToll and induces its oligomerization and subsequent signaling cascade, in a manner similar to IL-1 and TNF with their cognate receptors.

hToll shares significant sequence similarity with distinct members of the IL-1R family, including IL-1RI, IL-1RAcP, and MyD88; of note, Phe 513 and Trp 514 in IL-1RI, which



**Figure 5.** Overview of hToll and TNFR-1 signaling pathways. The diagram shows that the bifurcation of NF-κB and JNK/SAPK activation by hToll or TNFR-1 occurs at different levels with respect to the specific TRAFs. TRADD, TNFR-1-associated death domain. RIP, Receptor-interacting protein.

are conserved in all of these proteins, have been shown to be essential for IL-1RI to signal (Fig. 2 A) (20). Since we have shown recently a homophilic interaction to occur between the IL-1RAcP and MyD88 throughout their homologous domains (8), we asked whether hToll and the adapter protein MyD88 could interact. Upon coexpression, MyD88 and hToll formed an immunoprecipitable complex; in contrast, a mutant version of hToll, which lacks the region of homology to MyD88 and which was unable to induce NF- $\kappa$ B activation, failed to bind MyD88 (Fig. 2 B).

A mutant version of MyD88 ( $\Delta$ MyD88), encoding only for the COOH-terminal IL-1R-like domain, abrogates IL-1RI/IL-1RAcP-induced NF- $\kappa$ B activation (8); given this, we analyzed whether  $\Delta$ MyD88 could act as a dominant negative inhibitor of hToll-induced NF- $\kappa$ B activation. As predicted,  $\Delta$ MyD88 specifically inhibited hToll-induced but not TNFR-2-induced NF- $\kappa$ B activity, lending functional credence to the interaction occurring between hToll and MyD88 (Fig. 2 C). From these observations, it is apparent that both IL-1R and hToll recruit the adapter protein MyD88 to their respective signaling complex.

IRAK and IRAK-2 are two additional proximal mediators of the IL-1R signaling complex; IRAK is recruited to the IL-1RAcP, whereas IRAK-2 preferentially binds IL-1RI (8, 10). Given this, we asked whether IRAK or IRAK-2 could interact with hToll. Upon ectopic expression, IRAK and hToll formed an immunoprecipitable complex. In contrast, IRAK-2 bound only weakly to hToll compared with IL-1RI (Fig. 3, A and B), thus suggesting that it may not represent a relevant hToll signal transducer.

NF- $\kappa$ B activation induced by a number of cytokine receptors is mediated by members of the TRAF adapter family. TRAF2, for example, plays a critical role in NF- $\kappa$ B activation mediated by TNFR-1 and TNFR-2 (21, 22). TRAF6 has been implicated in the IL-1 signaling pathway and has been shown to complex with IRAK and IRAK-2 downstream to the receptor signaling complex (8, 11). Therefore, we determined whether dominant negative versions of either could act to inhibit hToll-induced NF- $\kappa$ B activity.  $\Delta$ TRAF6 but not  $\Delta$ TRAF2 significantly impaired hToll-induced NF- $\kappa$ B activity, suggesting that TRAF6 may act as an additional downstream mediator of the hToll-induced NF- $\kappa$ B activation cascade (Fig. 3 C).

The protein kinase NIK has been shown to act as a general mediator of TRAF-induced NF- $\kappa$ B activation (12); once activated, NIK directly phosphorylates and activates the IKK $\alpha$ / $\beta$  complex, which is responsible for I- $\kappa$ B $\alpha$  phosphorylation and subsequent NF- $\kappa$ B activation (13–17). Dominant negative versions of NIK, in which the critical

lysine has been mutated to alanine [NIK 429–430 (KK-AA)], act to inhibit NF- $\kappa$ B activation induced by Fas, TNF, and IL-1 (12). Given this, we asked whether NIK(KK-AA) could inhibit hToll-induced NF- $\kappa$ B activation. NIK(KK-AA) abrogated NF- $\kappa$ B activity triggered by hToll ectopic expression (Fig. 3 C) as well as by TRAF6 overexpression (not shown).

In addition to inducing activation of NF- $\kappa$ B, distinct inflammatory cytokines also induce SAPK, also known as JNK. The active phosphorylated form of SAPK binds to and phosphorylates the transcription factors c-Jun, activating transcription factor 2 (ATF2), and ternary complex factor (TCF)/Elk1 (23–25). In particular, activation of SAPK/JNK by the TNFR-1 occurs through a TRAF2-dependent pathway, as a dominant negative version of TRAF2 acts to inhibit both NF- $\kappa$ B and c-Jun activation induced by TNF (18, 26, 27). In contrast, a dominant negative version of NIK, which abrogates TNF-induced NF- $\kappa$ B activation, fails to inhibit c-Jun phosphorylation, supporting a model wherein TNF-mediated NF- $\kappa$ B and c-Jun pathways bifurcate at TRAF2 (28, 29). Therefore, we analyzed whether hToll induced SAPK/JNK. Ectopic expression of increasing amounts of hToll-Flag but not  $\Delta$ hToll-Flag resulted in activation of SAPK $\gamma$  as indicated by specific phosphorylation at Thr 183 and Tyr 185 (Fig. 4 A). Overexpression of TRAF6 also activated SAPK as described previously (28). To identify mediators of hToll-mediated JNK activation, we cotransfected 293 cells with hToll and dominant negative versions of either MyD88 or TRAF6. Surprisingly,  $\Delta$ MyD88 but not  $\Delta$ TRAF6 acted to inhibit hToll-triggered JNK phosphorylation (Fig. 4 B). Importantly, under the same experimental conditions, both  $\Delta$ MyD88 and  $\Delta$ TRAF6 abrogated hToll-induced NF- $\kappa$ B activation (96 and 90% inhibition, respectively). Additionally,  $\Delta$ TRAF6 alone, as well as  $\Delta$ MyD88, failed to activate JNK (data not shown). Collectively, these observations indicate that although ectopic expression of TRAF6 induced SAPK, a dominant negative version of TRAF6 failed to inhibit hToll-induced JNK phosphorylation. Given this, one could speculate that although TRAF6 overexpression is sufficient to activate JNK/SAPK, endogenous TRAF6 does not provide a significant contribution to JNK/SAPK activation by hToll (Fig. 5).

Regardless, MyD88 appears to represent the most upstream mediator of the hToll-mediated signaling cascade, which ultimately activates NF- $\kappa$ B and c-Jun, thus driving transcriptional activation of several cytokines and costimulatory molecules. Therefore, it may represent a potentially useful therapeutic target for controlling the molecular switch from the innate to the adaptive immune response.

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