Brief Definitive Report

The Human Toll Signaling Pathway: Divergence of Nuclear Factor κB 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 κB (NF-κB)–inducing kinase (NIK) are both involved in subsequent steps of NF-κB activation. Conversely, a dominant negative version of TRAF6 failed to block hToll–induced activation of stress-activated protein kinase/c-Jun NH₂-terminal kinases, thus suggesting an early divergence of the two pathways.

Key words: Toll • interleukin 1 receptor • nuclear factor κB • c-Jun NH₂-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-1R I) and the IL-1R accessory protein (IL-1R AcP) (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-κB 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-κB 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-κB–inducing kinase (NIK) (8, 11, 12). Finally, NIK activates the IκB kinase complex (including IKKα and IKKβ) that directly phosphorylates IκBα (13–17).

In this study, we identified and molecularly ordered the mediators of the hToll–induced NF-κB and stress-activated protein kinase (SAPK)/c-Jun NH₂-terminal kinase (JNK) activation cascade.

Materials and Methods

Expression Vectors and Transfection. TRAF6-Flag, ΔTRAF6-Flag (or TRAF6 298–522), ΔTRAF2-Flag (or TRAF2 87–501), MyD88-AU1, ΔMyD88-AU1 (or MyD88 152–296), IRAK, NIK (KK-AA), and HA-p46SAPKγ–pCDNA3 expression vectors have been described (8, 18). Expression vectors for NH₂-terminal Flag-tagged hToll and ΔhToll (amino acids 1–666) were constructed by inserting PCR-generated cDNA fragments lacking...
the coding sequence for the signal peptide, into the mammalian expression vector pFlag-CMV-1 (Eastman Kodak Co., Rochester, N.Y.). CD4/Toll has been described previously (2).

Human embryonic 293 or 293T cells were transiently transfected by the calcium phosphate method with the indicated plasmids. The total amount of DNA was kept constant by supplementation with an empty vector (pCDNA3; Invitrogen Corp., Carlsbad, CA). CD4/Toll has been described previously (2).

Monocytes were separated from fresh blood of healthy donors as described previously (19). Total RNA was isolated and analyzed as described previously (19).

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Northern Blotting Analysis. Human embryonic 293 or 293T cells were transiently transfected by the calcium phosphate method with the indicated plasmids. The total amount of DNA was kept constant by supplementation with an empty vector (pCDNA3; Invitrogen Corp., Carlsbad, CA). CD4/Toll has been described previously (2).

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Figure 1. hToll expression and induction of NF-κB.

(A) Human monocytes were treated with LPS (100 ng/ml) for different periods of time and analyzed for their hToll mRNA content by Northern blotting. Two distinct transcripts specific for hToll are detected and induced by LPS stimulation. (B) Ectopic expression of hToll-Flag and CD4/Toll but not the mutant version ΔhToll-Flag activate NF-κB in 293T cells in a dose-dependent manner, as measured by NF-κB reporter gene activity. (C) Equal amounts of hToll-Flag and ΔhToll-Flag are produced upon ectopic expression in 293T cells. 3.2 μg of each expression construct were used for this experiment. IP, Immunoprecipitation. BLOT 1, Immunoblotting analysis.

Figure 2. Functional and structural evidence of MyD88 recruitment to the hToll signaling complex. (A) Sequence alignment of human MvD88 (amino acids 152–296), hToll (657–840), hIL-1R ACP (391–570), and hIL-1R I (381–569). Alignment was performed with ClustalW software. Shading, Identical amino acids with a score <3. Boxes, Identical amino acids with a score = 0. Dots, Conserved amino acids that are essential for IL-1RI to signal (reference 20). (B) MvD88 associates with hToll but not with a truncated version of hToll (ΔhToll) lacking the cytoplasmic region sharing sequence similarity with MvD88. 293T cells were transfected with hToll-Flag, ΔhToll-Flag, or IL-1R ACP-Flag as a positive control together with AU1-tagged MvD88. The presence of MvD88 that coprecipitated with the receptors was detected by immunoblotting with a rabbit polyclonal antiserum to MvD88. (C) ΔMvD88 inhibits hToll-induced but not the unrelated TNFR-2–induced NF-κB activity. 1 μg of receptors and 1.5 μg of ΔMvD88 were transfected. Data are expressed as the percentage of receptor-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. 1A, 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 extracellular 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 activity in human embryonic 293T cells at levels similar to the CD4/hToll-induced NF-κB activity.

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-1R AcP served as a positive control. Cells were transfected and analyzed as in Fig. 2B. (B) IRAK-2 binds very weakly to hToll. IL-1R AcP served as a positive control. Cells were transfected and analyzed as in A. (C) ΔTRAF6 but not the unrelated ΔTRAF2 attenuates CD4/hToll-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.

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.

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. 2A) (20). Since we have shown recently a homophilic interaction to occur between the IL-1RαCp 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-κB activation, failed to bind MyD88 (Fig. 2B).

A mutant version of MyD88 (∆M MyD88), encoding only for the COOH-terminal IL-1R-like domain, abrogates IL-1RI/IL-1RαCp-induced NF-κB activation (8); given this, we analyzed whether ∆M MyD88 could act as a dominant negative inhibitor of hToll-induced NF-κB activation. As predicted, ∆M MyD88 specifically inhibited hToll-induced but not TNFR2-induced NF-κB activity, lending functional credence to the interaction occurring between hToll and MyD88 (Fig. 2C). 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-1RαCp, 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. 3A and B), thus suggesting that it may not represent a relevant hToll signal transducer.

NF-κB 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-κB activation mediated by TNFR1 and TNFR2 (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-κB activity. ΔTRAF6 but not ΔTRAF2 significantly impaired hToll-induced NF-κB activity, suggesting that TRAF6 may act as an additional downstream mediator of the hToll-induced NF-κB activation cascade (Fig. 3C).

The protein kinase NIK has been shown to act as a general mediator of TRAF-induced NF-κB activation (12); once activated, NIK directly phosphorylates and activates the Iκκα/β complex, which is responsible for Iκκα phosphorilation and subsequent NF-κB 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-κB activation induced by Fas, TNF, and IL-1 (12). Given this, we asked whether NIK(KK-AA) could inhibit hToll-induced NF-κB activation. NIK(KK-AA) abrogated NF-κB activity triggered by hToll ectopic expression (Fig. 3C) as well as by TRAF6 overexpression (not shown).

In addition to inducing activation of NF-κB, 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 TNFR1 occurs through a TRAF2-dependent pathway, as a dominant negative version of TRAF2 acts to inhibit both NF-κB and c-Jun activation induced by TNF (18, 26, 27). In contrast, a dominant negative version of NIK, which abrogates TNF-induced NF-κB activation, fails to inhibit c-Jun phosphorylation, supporting a model wherein TNF-mediated NF-κB 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 ΔToll-Flag resulted in activation of SAPK-JNK as indicated by specific phosphorylation at Thr 183 and Tyr 185 (Fig. 4A). 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, ΔMyD88 but not ΔTRAF6 acted to inhibit hToll-triggered JNK phosphorylation (Fig. 4B). Importantly, under the same experimental conditions, both ΔMyD88 and ΔTRAF6 abrogated hToll-induced NF-κB activation (96 and 90% inhibition, respectively). Additionally, ΔTRAF6 alone, as well as ΔMyD88, 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-κB 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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References

1. Medzhitov, R., and A. Janeway, Jr. 1997. Innate immunity: the virtues of a nonclonal system of recognition. C. R. 324: 295–298.

2. Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 388:394–397.

3. Rock, F.L., G. Hardiman, J.C. Timans, R. Kastelein, and F.J. Dinarello. 1996. Biologic basis for interleukin-1 in disease. Blood 87:2095–2147.

4. Bazan, J.G., and G. Ju. 1997. A family of human receptors structurally related to the dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 86:973–983.

5. Greenfeder, S.A., M. Labow, R.A. Melli, and N.L. Malinin. 1997. Amino acids conserved in interleukin-1 receptors (IL-1Rαs) and the Drosophila Toll protein are essential for IL-1R signal transduction. J. Biol. Chem. 272:13757–13765.

6. Gay, N.J., and F.J. Keith. 1991. Drosophila Toll and IL-1 receptor. Nature 351:355–356.

7. Dinarello, C.A. 1996. Biologic basis for interleukin-1 in disease. Blood 87:2095–2147.

8. Muzio, M., F. Re, M. Sironi, A. Minty, D. Caput, P. Ferrara, A. Mantovani, and F. Colotta. 1994. Interleukin-13 induces the production of interleukin-1 receptor antagonist (IL-1ra) and the expression of the mRNAs for the intracellular (kinase) form of IL-1ra in human myelomonocytic cells. Blood 83:1738–1743.

9. Hegov-Caric, A., T. Baldari, G. Macchia, J.L. Telford, and M. Melli. 1992. Amino acids conserved in interleukin-1 receptors (IL-1Rs) and the Drosophila Toll protein are essential for IL-1R signal transduction. J. Biol. Chem. 267:2605–2609.

10. Rothe, M., S.C. Wong, W.J. Henzel, and D.V. Goeddel. 1994. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75kDa tumor necrosis factor receptor. Cell 78:681–692.

11. Rothe, M., V. Sarma, V.M. Dixit, and D.V. Goeddel. 1995. TRAF2-mediated activation of NF-κB by TNF receptor 2 and CD40. Science 269:1424–1427.

12. Derijard, B., M. Hibi, I.-H. Wu, T. Baret, B. Su, T. Deng, M. Karin, and R. Davis. 1994. JNK1: a protein kinase stimulated by TRAF2-dependent activation of NF-κB. Cell 78:548–554.

13. Muzio, M., W. Henzel, and X. Gao. 1996. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75kDa tumor necrosis factor receptor. Cell 78:681–692.

14. Rothe, M., S.C. Wong, W.J. Henzel, and D.V. Goeddel. 1994. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75kDa tumor necrosis factor receptor. Cell 78:681–692.

15. Adler, V., A. Polotskaya, F. Wagner, and A.S. Kraft. 1992. Affinity-purified c-Jun amino-terminal protein kinase requires serine/threonine phosphorylation for activity. J. Biol. Chem. 267:1092–1098.

16. Natoli, G., A. Costanzo, F. Moretti, M. Fulco, C. Balsano, and M. Levrero. 1997. Tumor necrosis factor (TNF)-mediated activation of IκB kinase (IKK)-α and -β requires serine/threonine phosphorylation for activity. J. Biol. Chem. 272:9444–9451.

17. Zandi, E., M. Delhase, M. Hayakawa, and M. Karin. 1997. The IκB kinase complex contains two kinase subunits, IκKα and IκKβ, necessary for IκB phosphorylation and NF-κB activation. Cell 91:243–252.

18. Natoli, G., A. Costanzo, A. Ianni, D.J. Templeton, J.R. Woodgett, C. Balsano, and M. Levrero. 1997. Activation of SAPK/JNK by TNF receptor-1 through a noncytotoxic TRAF2-dependent pathway. Science 275:200–203.

19. Muzio, M., F. Re, M. Sironi, N. Polentarutti, A. Minty, D. Caput, P. Ferrara, A. Mantovani, and F. Colotta. 1994. Interleukin-13 induces the production of interleukin-1 receptor antagonist (IL-1ra) and the expression of the mRNA for the intracellular (kinase) form of IL-1ra in human myelomonocytic cells. Blood 83:1738–1743.

20. Hegov-Caric, A., T. Baldari, G. Macchia, J.L. Telford, and M. Melli. 1992. Amino acids conserved in interleukin-1 receptors (IL-1Rs) and the Drosophila Toll protein are essential for IL-1R signal transduction. J. Biol. Chem. 267:2605–2609.

21. Muzio, M., W. Henzel, and D.V. Goeddel. 1994. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75kDa tumor necrosis factor receptor. Cell 78:681–692.