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Ontogeny of Toll-Like Receptors *Tlr2* and *Tlr4* in Mice

KIRSI HARJU, VIRPI GLUMOFF, AND MIKKO HALLMAN

Department of Pediatrics and Biocenter Oulu, FIN-90014 University of Oulu, Oulu, Finland

ABSTRACT

Toll-like receptors (*Tlr*) have recently been linked to the immunostimulatory function of microbial toxins in human and mice. *Tlr* signals activation of nuclear factor κB that leads to the production of a number of proinflammatory mediators. *Tlr4* mediates the endotoxin-induced inflammatory response, whereas *Tlr2* may be involved in the response to yeast and Gram-positive bacterial products. To better understand age-related changes in acute inflammatory response, we studied the ontogeny of *Tlr2* and *Tlr4* mRNA in murine fetal lung, liver, and placenta by quantitative reverse transcriptase-PCR. Different expression patterns were seen between the tissues and between the *Tlr*. This is in accordance with the evidence that there are differences in the receptors for different microbial toxins and that the response is organ specific. We additionally show that the expression of *Tlr* was dependent on the stage of differentiation. In the liver, the levels of *Tlr2* and *Tlr4* were high regardless of the age. In the lung, *Tlr2* and *Tlr4* expression levels were barely detectable in immature fetus (d 14–15). *Tlr2* and *Tlr4* were increased several-fold during prenatal development and further increased after birth. The present results support the finding of a deficient inflammatory response of the immature lung to microbial toxins. **(*Pediatr Res* 49: 81–83, 2001)**

Abbreviations

LPS, lipopolysaccharide
***Tlr*,** Toll-like receptor

Bacterial LPS (also known as endotoxin) as a constituent of the cell wall of Gram-negative bacteria is a major causative agent of septic shock. LPS starts a complex cascade of events in responsive cells, particularly in monocytes and macrophages, that leads to the production of endogenous mediators such as proinflammatory cytokines IL-1, tumor necrosis factor-α, IL-6, IL-8, and a number of other mediators. In Gram-positive bacteria, the major immunostimulatory components of the cell wall include peptidoglycan and lipoteichoic acid (1, 2).

A cell membrane component required for LPS-induced immunostimulation was recently identified to be a *Tlr* (3–5). According to the latest studies, *Tlr2* and *Tlr4* recognize different bacterial cell wall components. *Tlr4* has been shown to mediate LPS-induced signal transduction (6, 7), whereas *Tlr2* may mediate the response to yeast and Gram-positive bacteria (2, 8). Macrophages contain a surface protein called CD14, which binds ligands such as LPS (5, 7). However, CD14 does not participate directly in signaling. Rather, *Tlr* are essential for the innate immune response. Whereas the extracellular domain of *Tlr*, compatible with CD14, discriminates between pathogens, the cytoplasmic tail of *Tlr* triggers the cascade of intracellular mediators, leading to the activation of the nuclear factor κB and of the inflammatory response. *Tlr* are the mammalian homologues of the *Drosophila* Toll family that controls the dorsoventral patterning in the developing embryo and the antimicrobial response in the adult fly (9). So far, at least six of *Tlr Drosophila* have been identified in humans and mice (10). It has been proposed that *Tlr* control the switch from the innate to adaptive immune response (11). *Tlr2* and *Tlr4* initiate the transmembrane signaling that leads to activation of nuclear factor κB and induction of a number of inflammatory mediators (11, 12).

At present, there is little evidence of factors controlling the expression of *Tlr* in mammalian tissues (9, 10). The present study was undertaken to find out whether the expressions of *Tlr2* and *Tlr4* reveal trends during perinatal development and whether they are organ specific. The result is consistent with the possibility that the expression of *Tlr* controls the primary immune response to microbes. We propose that deficiencies in *Tlr* contribute to susceptibility to pulmonary infections during the perinatal period.

METHODS

The present study was approved by the Animal Experimentation Board of the University of Oulu.

Extraction of RNA. Tissues were collected from mouse fetuses of different ages, known within ± 12 h. The age of the

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Correspondence: Mikko Hallman, M.D., Ph.D., Department of Pediatrics, P.O. Box 5000, FIN-90014 University of Oulu, Oulu, Finland.
Reprint requests: Kirsi Harju, M.D., Department of Pediatrics, P.O. Box 5000, FIN-90014 University of Oulu, Oulu, Finland.
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adults was 3 mo. The first day after conception was called d 0. The animals were killed by decapitation. The placenta, fetal lungs, and liver were frozen in liquid nitrogen. The tissues were ground in liquid nitrogen, and total RNA was isolated by Trizol reagent (GIBCO BRL, Life Technologies, Inc., Grand Island, NY, U.S.A.).

Quantitative reverse transcriptase-PCR analysis. The absolute amounts of RNA were too small to be analyzed by Northern analysis. Half a microgram of the total RNA was used in each reverse transcriptase reaction. The quantitative PCR were driven by ABI PRISM 7700 Sequence Detection System (Perkin Elmer, Norwalk, CT, U.S.A.). The validation and reliability of this quantitative reverse transcriptase-PCR method has been reported (13). The primer sequences used for *Tlr2* were 5′-GCCACCATTTCCACGGACT-3′ and 5′-GGCTTCCTCTTGGCCTGG-3′, and the TaqMan probe sequence was 5′(FAM)-TGGTACCTGAGAATGATGT-GGGCGTG-(TAMRA)3′. The primer sequences used for *Tlr4* were 5′-CCTCTGCCTTCACTACAGAGACTTT-3′ and 5′-TGTGGAAGCCTTCCTGGATG-3′, and the TaqMan probe sequence was 5′(FAM)-CCTGGTGTAGCCATTGCTGC-CAACA-(TAMRA)3′. All results were normalized to 18S rRNA. The expression levels of *Tlr2* and *Tlr4* in each organ and in the whole body were related to each other. The whole body expression levels of *Tlr2* and *Tlr4* mRNA in 12-d fetus were very similar and valued as 1.0.

RESULTS

To study the ontogeny of *Tlr2* and *Tlr4* in mouse, the lungs, liver, and placenta were collected, and total RNA was isolated. The relative *Tlr* levels were quantified by reverse transcriptase-PCR. All results, normalized to 18S rRNA, were expressed as arbitrary units relative to the value 1.0 assigned to the whole fetus aged 12 d. The results showed very different expression patterns between tissues and between *Tlr2* and *Tlr4*. In the lung, there was a several-fold increase in the *Tlr2* and *Tlr4* mRNA expression levels from a fetal age of 14–15 d to the term. In the adult lung, expression levels of *Tlr2* and *Tlr4* were higher than the expression levels in the newborn (Fig. 1). The regression lines for *Tlr2* and *Tlr4* were different from zero ($p < 0.0001$).

In the liver, both *Tlr2* and *Tlr4* were prominent, and no developmental trends were detected (Fig. 2). In the placenta, on the other hand, the expression of *Tlr4* was higher by 1 order of magnitude than that of *Tlr2* (Fig. 3). The placental *Tlr4* mRNA decreased during the second half of pregnancy ($p < 0.01$), whereas no significant trends in *Tlr2* were evident.

DISCUSSION

In the present study, we have demonstrated for the first time that *Tlr2* and *Tlr4* mRNA expression is both tissue specific and dependent on the age. In the lung, the *Tlr* expression levels in immature fetuses were very low. *Tlr* increased 8-fold during the last trimester of murine pregnancy (from the late pseudoglandular to terminal sac stage) and further increased 2.5-fold after birth. The expression levels of *Tlr2* and *Tlr4* were similar. In contrast, the fetal liver showed 1 to 2 orders of magnitude higher *Tlr* mRNA expression levels than did the lung, and there were no remarkable changes in the expression during the fetal or postnatal life. In the placenta, the mRNA expression levels of *Tlr4* during midpregnancy were higher by 1 order of magnitude than those of *Tlr2*.

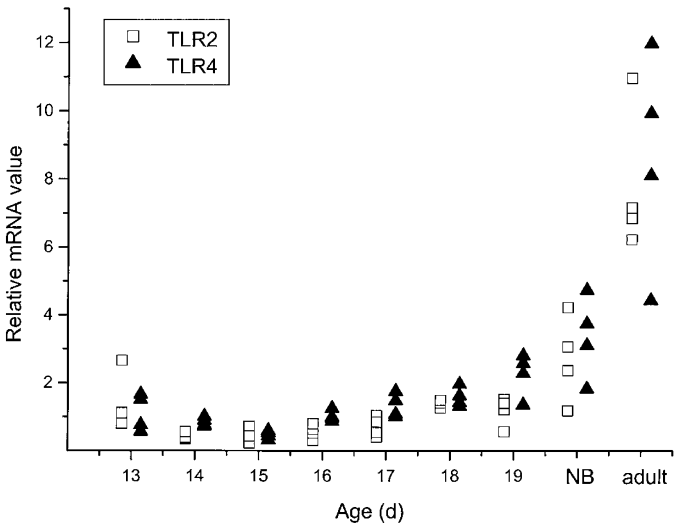

Figure 1. Pulmonary expression levels of *Tlr2* and *Tlr4* in fetal, newborn, and adult mice. Four independent analyses were performed for each age group. *Tlr* mRNA levels are expressed as relative values; the whole body expression level of *Tlr* in 12-d-old fetus was valued as 1.0. *NB* indicates newborn; age < 20 h.

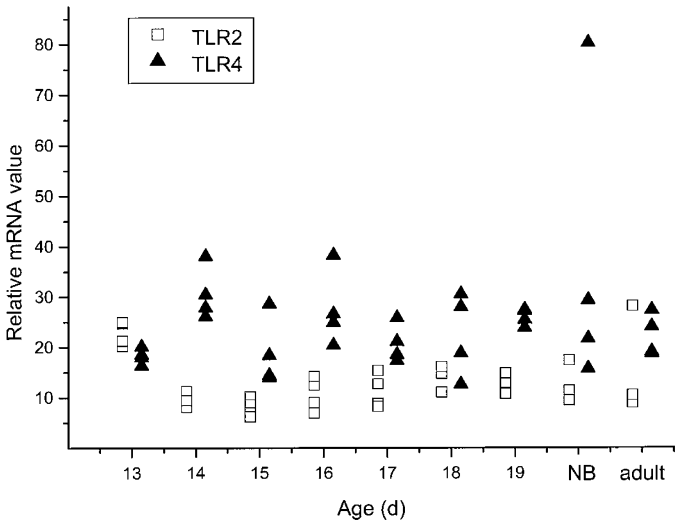

Figure 2. Hepatic expression levels of *Tlr2* and *Tlr4* in fetal, NB, and adult mice. Four or three (NB and adult) independent analyses were performed for each age group. *Tlr* mRNA levels are expressed as relative values; the whole body expression level of *Tlr* in 12-d-old fetus was valued as 1.0.

Tlr2 and *Tlr4* are expressed in macrophages (7, 14). Their expression levels influence the intensity of the inflammatory response to microbial toxins (6). The low levels of *Tlr4* (1000 or fewer *Tlr* molecules per nonstimulated macrophage) (15) complicate the detection of *Tlr* in normal tissues by use of immunohistochemistry or *in situ* hybridization. *Tlr* expression levels were increased after the exposure to the LPS or the cytokines (16). At present, it is unknown whether the observed increase in *Tlr* in the lung tissue was due to the increase in the

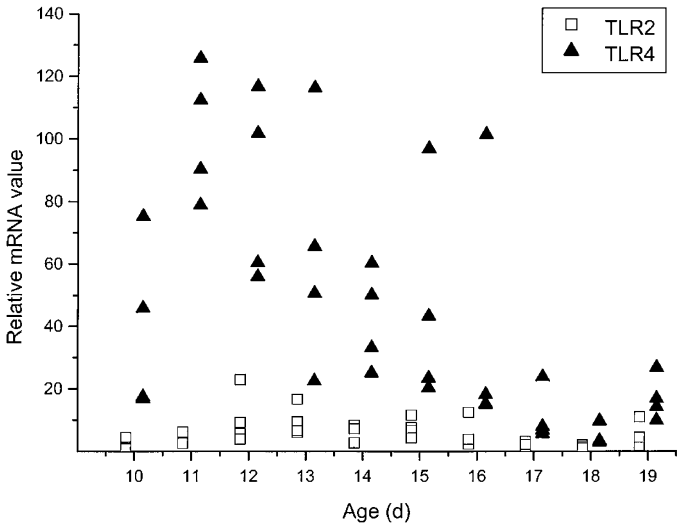

Figure 3. Expression levels of *Tlr2* and *Tlr4* in murine placenta. Four independent analyses were performed for each age group. *Tlr* mRNA levels are expressed as relative values; the whole body expression level of *Tlr* in 12-d-old fetus was valued as 1.0.

pulmonary content of macrophages, to the expression level of *Tlr* in the pulmonary macrophages, or to both. In the lung, the tissue concentration of macrophages tends to increase during prenatal development, whereas the number of alveolar macrophages increases rapidly after birth (17).

Whether the *Tlr* mRNA expression levels control the responsiveness of the innate immune system remains to be proven. It is of interest to note that some premature newborn infants are affected by hyperacute fulminant pneumonia due to Gram-positive (Group B *Streptococcus*, in particular) or Gram-negative (*Escherichia coli* and others) bacteria. In these cases, the initial acute phase response was deficient (18, 19). These data are supported by deficient responsiveness of the immature lung to LPS *in vitro* and an increase in the pulmonary LPS responsiveness toward term (20). Investigation of the genes and gene products responsible for deficient primary inflammatory response would help in the design of new strategies for prevention of life-threatening infections and inflammatory diseases.

REFERENCES

1. Scletter J, Heine H, Ulmer AJ, Rietschel ET 1995 Molecular mechanism of endotoxin activity. Arch Microbiol 164:383–389
2. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S 1999 Differential roles of *Tlr2* and *Tlr4* in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 11:443–451
3. Medzhitov R, Preston-Hurlburt P, Janeway CA 1997 A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 388:394–397
4. Kirschning CJ, Wesche H, Ayres TM, Rothe M 1998 Human Toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. J Exp Med 188:2091–2097
5. Yang R-B, Mark MR, Gray A, Huang A, Xie MH, Zhang M, Goddard A, Wood WI, Gurney AL, Godowski PJ 1998 Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. Nature 395:284–288
6. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogava T, Takeda K, Akira S 1999 Toll-like receptor 4 (TLR4)- deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as *Lps* gene product. J Immunol 162:3749–3752
7. Chow JC, Young DW, Golenbock DT, Christ WJ, Gusovsky F 1999 Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. J Biol Chem 274:10689–10692
8. Lien E, Sellati TJ, Yoshimura A, Flo TH, Rawadi G, Finberg RW, Carroll JD, Espevik T, Ingalls RR, Radolf JD, Golenbock DT 1999 Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. J Biol Chem 274:33419–33425
9. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF 1998 A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci USA 95:588–593
10. Takeuchi O, Kawai T, Sanjo H, Copeland NG, Gilbert DJ, Jenkins NA, Takeda K, Akira S 1999 TLR6: a novel member of an expanding Toll-like receptor family. Gene 231:59–65
11. Muzio M, Natoli G, Saccani S, Levrero M, Mantovani A 1998 The human Toll signalling pathway: divergence of nuclear factor κB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6). J Exp Med 187:2097–2101
12. Kopp EB, Medzhitov R 1999 The Toll-receptor family and control of innate immunity. Curr Opin Immunol 11:13–18
13. Winer J, Kwang C, Jung S, Shackel I, Williams PM 1999 Development and validation of real-time quantitative reverse transcriptase polymerase chain reaction for monitoring gene expression in cardiac myocytes *in vitro*. Anal Biochem 270:41–49
14. Underhill DM, Ozinsky A, Hajjar AM, Stevens A, Wilson CB, Bassetti M, Aderem A 1999 The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature 401:811–814
15. Beutler B 2000 Tlr4: central component of the sole mammalian LPS sensor. Curr Opin Immunol 12:20–26
16. Frantz S, Kobzik L, Kim Y-D, Fukazawa R, Medzhitov R, Lee RT, Kelly RA 1999 J Clin Invest 104:271–280
17. Sherman MP, Ganz T 1992 Host defense in pulmonary alveoli. Ann Rev Physiol 54:331–350
18. Fantuzzi G, Badolato R, Oppenheim JJ, O'Grady NP 1999 New insights into the biology of the acute phase response. J Clin Immunol 19:203–214
19. Aikio O, Vuopala K, Pokela M-L, Hallman M 2000 Diminished inducible nitric oxide synthase expression in fulminant early-onset neonatal pneumonia. Pediatrics 105:1013–1019
20. Väyrynen O, Glumoff V, Kangas T, Hallman M 1999 Endotoxin-induced changes in expression of surfactant proteins are dependent on the degree of lung maturity. Pediatr Res 45:896A(abstr)