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TLR4-mediated induction of TLR2 signaling is critical in the pathogenesis and resolution of otitis media

Anke Leichtle[1,2], **Michelle Hernandez**[3], **Kwang Pak**[1], **Kenshi Yamasaki**[4], **Chun-Fang Cheng**[1], **Nicholas J. Webster**[5], **Allen F. Ryan**[1], and **Stephen I. Wasserman**[6]

[1]Department of Surgery/Otolaryngology, University of California San Diego, La Jolla, California, USA

[2]Department of Otolaryngology, University of Bonn, Bonn, Germany

[3]Department of Pediatrics/Allergy, Immunology, Rheumatology, and Infectious Diseases, University of North Carolina at Chapel Hill School of Medicine, North Carolina, USA

[4]Department of Medicine/Dermatology, University of California San Diego, La Jolla, California, USA

[5]Department of Medicine/Endocrinology, University of California San Diego, La Jolla, California, USA

[6]Department of Medicine/Rheumatology, Allergy and Immunology, University of California San Diego, La Jolla, California, USA

Abstract

Otitis media is the most prevalent childhood disease in developed countries. The involvement of Toll-like receptors (TLRs) in otitis media pathophysiology has been implicated by studies in cell lines and association studies of TLR gene polymorphisms. However, precise functions of TLRs in the etiology of otitis media *in vivo* have not been examined. We investigated the inflammatory response to nontypeable *Haemophilus influenzae* using a model of otitis media in wild-type, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice by gene microarray, qPCR, immunohistochemistry, Western blot analysis and histopathology. Toll-like receptor-2$^{-/-}$ and TLR4$^{-/-}$ mice exhibited a more profound, persistent inflammation with impaired bacterial clearance compared to controls. While wild-type mice induced tumor necrosis factor-α (TNF) after non-typeable *H. influenzae* challenge, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice lack TNF induction in the early phase of otitis media. Moreover, lack of TLR2 resulted in a late increase in IL-10 expression and prolonged failure to clear bacteria. Toll-like receptor-4$^{-/-}$ mice showed impaired early bacterial clearance and loss of TLR2 induction in early otitis media. Our results demonstrate that both TLR2 and TLR4 signalling are critical to the regulation of infection in non-typeable *H. influenzae*-induced otitis media. Toll-like receptor-4 signalling appears to induce TLR2 expression, and TLR2 activation is critical for bacterial clearance and timely resolution of otitis media.

Keywords

Haemophilus influenzae; innate immunity; otitis media; TLR; TNF

Correspondence to: Allen F. Ryan, PhD, Department of Surgery/Otolaryngology, University of California San Diego, 9500 Gilman Drive #0666, La Jolla, CA 92093, USA. Tel: +1 858 534 4594; Fax: +1 858 534 5319; afryan@ucsd.edu.
A. Leichtle and M. Hernandez contributed equally to this work. A. Ryan and S. Wasserman contributed equally to the supervision of this work.

Introduction

Otitis media is the most prevalent childhood disease in developed countries, accounting for more office visits and drug purchases than any other disease from ages 6 months to 6 years.[1,2] Predisposing factors to otitis media include Eustachian tube dysfunction, prior viral infection and allergy.[3–5] However, bacterial infection is the dominant etiology of acute and/or recurrent otitis media, leading to hyperplasia of the middle ear mucosa, middle ear effusion and leukocyte infiltration of the middle ear cavity.[6,7] A common otitis media pathogen is non-typeable *Haemophilus influenzae*, which since the advent of pneumococcal vaccines accounts for a growing percentage of otitis media.[8]

Non-typeable *H. influenzae* activates Toll-like receptor (TLR) signaling pathways. Toll-like receptors are a class of pattern recognition receptors recognizing molecules associated with microbial pathogens and playing a key role in innate immunity.[9,10] They are considered the first line of host defense in response to infection. Activation of TLR induces pro-inflammatory cytokines including tumor necrosis factor-α (TNF) and antiinflammatory cytokines such as interleukin-10 (IL-10) via signaling cascades including the myeloid differentiation primary response gene 88 (MyD88)–NF-κB, TRIF–IFR3 and/or MAP kinase-dependent pathways.[11] Nontypeable *H. influenzae* contains molecules that serve as ligands for TLRs. Peptidoglycans and petidoglycanassociated proteins, such as outer membrane protein P6, are ligands for TLR2.[12] Lipooligosaccharide (LOS), with a molecular structure closely related to lipopolysaccharide (LPS), activates cellular signals via both TLR2 and TLR4.[11,13] Injection of peptidoglycan or LPS into the middle ear can mimic pathological changes of otitis media: mucosal inflammation, leukocytosis, edema, middle ear pressure abnormalities and an infiltrate of macrophages into the subepithelial space.[14,15] While middle ear responses to TLR2 and TLR4 ligands are well documented, the role played by these receptors in otitis media pathogenesis has received less attention. In middle ear epithelial cell lines, nontypeable *H. influenzae* induces TLR2 expression[12,16] and TLR2 activation regulates the expression of pro-inflammatory cytokines and mucin genes.[17,18] Moreover, polymorphisms in the TLR4 gene are associated with increased susceptibility to otitis media in children,[19] as are polymorphisms in the gene for TNF, a major proinflammatory target of TLR signaling.[19,20] Also polymorphisms in IL-10, which often opposes TLR signaling, are associated with otitis media.[19] Experimentally, reduced short-term responses to non-typeable *H. influenzae* from 6 h to 3 days were reported in the middle ears of C3H/HeJ mice, which express a non-functional TLR4.[21] Since TLR deficiencies have been shown to induce significant abnormalities in the recovery from bacterial infection at other sites,[22,23] we sought longer-term studies of non-typeable *H. influenzae*-induced otitis media to understand the pathology of chronic otitis media, which shows persistent infection and inflammation.

Our study evaluated the roles of TLR2 and TLR4, for up to 21 days, in a well-established experimental model of otitis media induced by non-typeable *H. influenzae*.[24] We demonstrate that both TLR2 and TLR4 signaling are critical for efficient host defense of the middle ear. Genetic loss of TLR2 or TLR4 is associated with prolonged and persistent otitis media. Our results further suggest that early TLR4 signaling is required to up-regulate expression of TLR2 and influence the downstream molecular targets TNF and IL-10. These findings support a predominant role for TLR2 in nontypeable *H. influenzae*-induced otitis media, primed by non-typeable *H. influenzae* interaction with TLR4.

Materials and Methods

Animals

Experiments were performed according to NIH guidelines and approved by the IRB of the San Diego VA Medical Center. Toll-like receptor-2$^{-/-}$ and TLR4$^{-/-}$ mice on a C57BL/6 background (6 × crossed) were originally generated and generously supplied by Akira and colleagues.[25,26] Age-matched C57BL/6 control mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and rats from Harlan Sprague Dawley (Indianapolis, IN, USA).

Bacteria

Haemophilus influenzae strain 3655 (non-typeable, biotype II) was used at a concentration of 10^5–10^6 bacteria/ml to induce an inflammatory response in the middle ear. The inocula were prepared as described elsewhere.[27]

Surgery

A total of 72 mice per strain (TLR2$^{-/-}$, TLR4$^{-/-}$ and C57BL/6) were divided into groups of 9 mice for each experimental time point (3 each for histopathology, bacterial culture and qPCR). For DNA microarrays, 40 C57BL/6 mice per time point were used. For Western blotting, 2–8 rats per time point were used. As described previously, 24 animals were anesthetized and an incision made along the midline of the ventral neck. The bullae were exposed by soft tissue dissection and a hole was carefully made with a 20-gauge needle to avoid underlying middle ear structures. Using a 27-gauge needle, each middle ear was injected with 5 μl of non-typeable *H. influenzae* inoculum. Any excess inoculum was wicked from the surgical field, carefully avoiding the opening in the bulla. The opening was then sealed with soft tissue and the skin incision closed. Uninoculated animals (time 0) served as controls.

DNA microarray

Forty middle ear mucosae were harvested at 0, 6 and 48 h after non-typeable *H. influenzae* inoculation. Tissue was homogenized in TRIzol™ (Invitrogen, Carlsbad, CA, USA) and total RNA extracted. RNA was labeled and hybridized to two Affymetrix MU430 2.0 microarrays per time point. Each time point was repeated, to obtain two independent samples. Expression of TLR transcript levels were evaluated using variance-modeled posterior inference (VAMPIRE)[28] and expressed as fold change relative to time 0. Significance was determined, after Bonferroni correction at a level of 0.05.

Histology

Middle ears were harvested at 0, 6, 12 h and 1, 2, 3, 5, 10, 14, 21 days post inoculation, processed and sectioned. Sections from standardized locations were digitally recorded and mucosal thickness and percent area of the middle ear lumen occupied by inflammatory cells was computer-averaged as described previously.[24] The numbers of neutrophils and macrophages comprising middle ear infiltrates were counted in five randomly selected clusters of cells for each middle ear (×400) by two independent observers and computer-averaged.

Immunohistochemistry

Paraffin-embedded, formalin-fixed tissue sections were deparaffinized and re-hydrated in xylene, ethanol and PBST. Endogenous peroxidases (20 min incubation in 0.3% H_2O_2) and endogenous biotin (Avidin/Biotin Blocking Kit, Vector Laboratories, Burlingame, CA, USA) were removed. For antigen retrieval, sections were incubated in proteinase K (DAKO,

Carpinteria, CA, USA) for 7 min, blocked with 1% BSA in PBS and incubated with anti-TLR4 (Abcam, Cambridge, MA, USA) or anti-TLR2 (Abcam) primary antibody in PBS, 0.1% BSA, washed in PBS and detected with horse radish peroxidase (HRP) anti-rabbit secondary antibodies (DAKO) and AEC peroxidase substrate kit (Vector Laboratories) per the manufacturers' instructions.

Bacterial clearance

Bacterial presence was evaluated and analyzed from at least three wild-type, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice per time point by obtaining a 1 μl loop sample from each middle ear for non-typeable *H. influenzae* culture as described previously.[29] Classification of the degree of colonization per plate: 0 indicates no colony-forming units (CFUs), 1 indicates CFUs in one quadrant, 2 indicates CFUs in two quadrants, 3 indicates CFUs in three quadrants and 4 indicates CFUS in all four quadrants.

mRNA quantification by qPCR

Expression of selected genes in the middle ear during non-typeable *H. influenzae*-induced otitis media was assessed by qPCR in wild-type, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice. At least 6 middle ear mucosae per time point and genotype were isolated. mRNA was extracted, reverse transcribed, and 1 μg/μl of cDNA was amplified using pre-developed TaqMan qPCR primers (Applied Biosystems, Foster City, CA, USA) for TLR2 (Mm00442346_m1), TLR4 (Mm00445273_m1), TNF (Mm00443258_m1) and IL10 (Mm00439616_m1) in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Fold induction was calculated using the comparative threshold cycle (Ct) method.[30] Relative expression of each target gene was normalized to levels of GAPDH (Mm03302249_g1) and compared to uninfected mucosa.

Western blot analysis

For Western blot, preliminary data showed protein levels in mice that were insufficient for detection. Since non-typeable *H. influenzae*-induced otitis media is quite similar in wild-type mice and rats, 31 rats were used for protein level determination. Mucosae from 2–8 middle ears of non-typeable *H. influenzae*-injected rats were pooled per time point. Rats uninoculated (0 h) and at 6, 12 h and 1, 2, 3, 5 and 10 days after non-typeable *H. influenzae* inoculation were sacrificed under general anesthesia for three independent Western blot replications as described elsewhere.[32] Rabbit polyclonal anti-TLR2 (Abcam) and anti-TLR4 (IMGENEX, Cambridge, MA, USA) antibodies were used at 0.2–0.4 μg/ml; with a mouse anti-rabbit, horseradish peroxidase-conjugated secondary antibody (1:15,000; Jackson ImmunoResearch, West Grove, PA, USA). According to the antibody source data sheets, TLR2 is expected to run at 86 kDa, while TLR4 runs at 90 kDa in Western blots, slightly below the molecular weights predicted from their amino acid structures (RefSeq). β-Actin served as control.

Statistical analysis

Data were analyzed with ANOVA using StatView statistics calculation software as described elsewhere.[32] Differences between groups were considered significant at $P < 0.05$. Descriptive statistics for bacterial load from middle ear cultures was performed according to Hernandez *et al.*[29]

Results

Non-typeable *H. influenzae* infection triggers expression of TLRs in the middle ear

To assess the influence of non-typeable *H. influenzae* on the expression pattern of individual TLRs in the middle ear mucosa, gene array analysis was performed in C57BL/6 mice. Among the expressed TLRs in the middle ear mucosa, we found a nearly 10-fold up-regulation of TLR2 mRNA at 6 h after non-typeable *H. influenzae* challenge (Fig. 1A). More modest, but still significant, up-regulation was detected for several other TLR genes at 6 h and 48 h. However, TLR4 mRNA showed no change at 6 h or 48 h. By Western blot, TLR2 protein was increased from 12 h to 5 days (Fig. 1B). Toll-like receptor-4 protein was barely detectable at 6 h and 2 days, and weakly induced 3–10 days after non-typeable *H. influenzae* inoculation. Immunohistochemistry confirmed that middle ear epithelial cells expressed TLR2 and much lesser amounts of TLR4 (Fig. 1C).

Toll-like receptor-2$^{-/-}$ and TLR4$^{-/-}$ mice show altered otitis media in response to non-typeable *H. influenzae*

We next assessed the middle ear response to nontypeable *H. influenzae* in TLR2$^{-/-}$ and TLR4$^{-/-}$ mice. Wild-type mice displayed significant thickening of the middle ear mucosa and cellular infiltration in the middle ear lumen at 2 days (Fig. 2a, 3 and 4). Then the mucosal thickness dramatically recovered and the cellular infiltrate had almost cleared by 5 days in wild-type mice (Fig. 2b, 3 and 4), and the middle ear was histologically normal by day 10 (Fig. 2c, 3 and 4). While, less cellular infiltration in the middle ear lumen was observed in TLR2$^{-/-}$ mice at 2 days, mucosal hyperplasia was similar to wild-type middle ear (Fig. 2d and 4). Mucosal thickening of TLR2$^{-/-}$ mice persisted at 5 days and 10 days, accompanied by a massive cellular infiltrate (Fig. 2e, f, 3 and 4). TLR4$^{-/-}$ mice showed both mucosal hyperplasia and cellular infiltration similar to wild-type middle ear at 2 days (Fig. 2g, 3 and 4) and thickness persisted at 5 days (Fig. 2h and 3). Partial recovery of mucosal hyperplasia and clearance of the cellular infiltrate was seen on day 10 (Fig. 3 and 4). These results suggested that both TLR2 and TLR4 are necessary to appropriately clear the middle ear inflammation induced by non-typeable *H. influenzae*.

Toll-like receptors influence leukocyte types in the middle ear during otitis media

The most prominent cells in inflammatory infiltrates after bacterial infection are neutrophils and macrophages, and their recruitment can be mediated by cytokines and chemokines through TLR signaling. As expected, neutrophils appeared rapidly in the wildtype mice middle ear, peaking at 1 day and declining until essentially none were observed at 5 days (Fig. 5A). In the absence of TLR2, neutrophil recruitment showed a remarkable resurgence at 5 days and 10 days, whereas TLR4$^{-/-}$ mice exhibited a faster decline in these cells than wild-type mice.

Macrophages predominated from 2–5 days in wildtype middle ears, peaking at 3 days and declining to baseline at 10 days (Fig. 5B). Macrophages in TLR2$^{-/-}$ mice paralleled wild-type middle ears, but persisted in the middle ear lumen through day 14. Middle ears from TLR4$^{-/-}$ mice also showedmacrophage recruitment similar to the wild-type, although fewer were present at 5 days.

Bacterial clearance is reduced in TLR2$^{-/-}$ and TLR4$^{-/-}$ mice

We further examined middle ear bacterial clearance in TLR-deficient mice (Table 1). In wild-type mice, the proportion of culture-positive middle ears increased from 4/6 with a colonization score of 4 on day 1, to 6/6 (score of 3) on day 2, and dropped to 3/6 (score of 1) on day 3. Thereafter, no bacteria were recovered from wild-type middle ears. Middle ears from TLR2$^{-/-}$ mice were consistently culture-positive throughout the observed period, with

3/6 positive middle ears from 1–21 days after inoculation (except for 2/6 on day 5). The bacterial colonization score was 4 on day 1, followed by 1–2 thereafter. Middle ears from TLR4$^{-/-}$ mice displayed robust culture positivity, with 4/6 or 3/6 from day 1 to day 10 after inoculation, and colonization scores were between 1.3–3.5. However, in contrast to TLR2$^{-/-}$ middle ears, only 1/6 TLR4$^{-/-}$ middle ears (score of 1) was culture positive at 14 days, and non-typeable *H. influenzae* was cleared by day 21.

Toll-like receptor-2 and TLR4 interact in the middle ear

To determine if TLR redundancy plays a role in otitis media persistence, we evaluated TLR2 and TLR4 expression in wild-type and TLR-deficient mice by qPCR. Confirming the microarray data (Fig. 1A), TLR2 mRNA was robustly induced at 6 h in the wild-type middle ear mucosa, followed by rapid down--regulation (Fig. 6A). At day 5, TLR2 gene expression showed a modest second increase.

Interestingly, TLR4$^{-/-}$ mice showed dysregulation of TLR2 expression with lack of early TLR2 induction at 6 h and delayed induction at 5 days and 10 days compared to wild-type mice (Fig. 6A). Immunohistochemistry confirmed little TLR2 expression at 1 day after non-typeable *H. influenzae* inoculation in TLR4$^{-/-}$ middle ear mucosa, however TLR2 was detectable at 5 days (Fig. 6B). Consistent with the microarray data (Fig. 1A), TLR4 was not significantly induced by nontypeable *H. influenzae* in wild-type or TLR2$^{-/-}$ mice, neither mRNA (data not shown), nor protein (data not shown). These results suggest that the rapid TLR2 increase in early-phase, non-typeable *H. influenzae*-induced otitis media in wild-type mice requires TLR4. They further suggest that the lack of TLR2 induction and signaling in early-phase otitis media results in delayed bacterial clearance in TLR4-deficient mice, while resolution of otitis media is accompanied by TLR2 induction on days 5 and 10.

Lack of TLR4 and TLR2 alters expression of TNF and IL-10

Both TNF and IL-10 are inducible by TLR signaling. Recent reports implicating genetic polymorphisms of TNF and IL-10 in risk for otitis media[19,20] prompted us to determine whether expression of these cytokines is altered during non-typeable *H. influenzae* otitis media in TLR2$^{-/-}$ and TLR4$^{-/-}$ mice. Indeed, in TLR2$^{-/-}$ mice, TNF induction was significantly reduced at 6 h compared to wild-type, which demonstrate rapid and robust TNF expression (Fig. 6C). A decrease of TNF mRNA upon non-typeable *H. influenzae* inoculation was even more evident in TLR4$^{-/-}$ mice (although expression at baseline was higher than in wild-type or TLR2$^{-/-}$ mice).

Expression of the anti-inflammatory cytokine IL-10, was undetectable in wild-type mice throughout the entire period of observation (Fig. 6D). However, up-regulation of IL-10 was very strong in TLR2$^{-/-}$ mice at 3 days and 5 days after non-typeable *H. influenzae* exposure. In TLR4$^{-/-}$ mice, IL-10 was significantly induced only at day 1.

Discussion

In this investigation, we found that both TLR2 and TLR4 contribute to the pathogenesis of non-typeable *H. influenzae*-induced otitis media in mice. After non-typeable *H. influenzae* infection of wild-type mice, TLR2 mRNA expression is tightly up-regulated in the middle ear mucosa, peaking at 6 h after non-typeable *H. influenzae* exposure and declining rapidly, leading to increased protein expression for the next few days. The consequences of this regulation are seen in the production of pro-inflammatory mediators, as peak induction of TNF mRNA coincides with the peak in TLR2 expression, followed by little detectable TNF message thereafter.

The importance of interactions between TLR2 and TLR4 on downstream signaling was further highlighted in our study. Induction of TNF was strongly reduced in TLR2$^{-/-}$ middle ear. Moreover, the early TLR2 and TNF expression seen in wild-type animals was absent in TLR4$^{-/-}$ animals, rendering TLR4$^{-/-}$ mice functionally deficient in TLR2 as well, unable to mount a pro-inflammatory response during the early stages of infection. This lack of key pro-inflammatory signals, during the period of time that innate immune mechanisms normally predominate, would be expected to alter the middle ear response to bacterial challenge.

Indeed, mice lacking either TLR2 or TLR4 exhibit aberrant otitis media, with impaired bacterial clearance and more persistent inflammatory changes. Toll-like receptor-2$^{-/-}$ mice showed a reduced initial response to non-typeable *H. influenzae* within the first days after bacterial inoculation, followed by an exuberant late mucosal hyperplasia and neutrophil infiltration into the middle ear that occurred primarily after otitis media had resolved in the wild-type counterparts and failure to clear non-typeable *H. influenzae* even at 21 days. In contrast, the middle ears of TLR4$^{-/-}$ mice showed a small early enhancement of mucosal response, with a modestly prolonged mucosal hyperplasia late in otitis media, but eventual resolution of bacterial infection. These results suggest that both TLR2 and TLR4 are essential to a timely recovery from non-typeable *H. influenzae*-induced otitis media. Toll-like receptor-2 appears most critical for the later clearance of neutrophils from the middle ear and for complete elimination of bacteria, and TLR4 appears to be more important for priming and mounting the initial host response through contact with bacterial pathogen associated molecular patterns and perhaps through exposure to various potential endogenous ligands for the TLR family[33] that are present in the middle ear, such as hyaluronic acid,[34] fibronectin[35,36] surfactant,[37] heat shock protein 70,[34] heparan sulfate,[38] and defensins 1 and 2.[39] Indeed, we found slightly higher basal levels of TNF gene induction in TLR4$^{-/-}$ mice than in TLR2$^{-/-}$ or wild-type mice. TLR4-deficient C3H/HeJ mice have been reported to develop spontaneous chronic otitis media.[40]

The ability of TLR4 to respond to ligands appears to be important early in otitis media, as demonstrated by higher bacterial titers in the middle ear for the first several days of otitis media in TLR4$^{-/-}$ mice. Our results are consistent with other models,[41,42] in which TLR4 signaling is most important early in a response to nontypeable *H. influenzae*. However, TLR4$^{-/-}$ mice showed eventual resolution of cellular infiltration, mucosal thickness and bacterial clearance. It is interesting that the late clearance of bacteria in TLR4-deficient mice was temporally associated with TLR2 induction.

In contrast to the responses of TLR4$^{-/-}$ mice, TLR2$^{-/-}$ mice showed lower initial levels of bacteria, perhaps mediated by TLR4, but were never completely clear of non-typeable *H. influenzae* during the 21-day observation period. This underscores the importance of TLR2 for late responses to non-typeable *H. influenzae* and for bacterial clearance. It can even be speculated that the eventual clearance of bacteria from TLR4$^{-/-}$ middle ears is mediated by the late expression of TLR2 seen in this model. When clearance of non-typeable *H. influenzae* is compared between TLR2$^{-/-}$ and wild-type mice, our results are inconsistent with the idea that TLR2 deficiency enhances initial host responses to bacteria, as has been suggested.[42]

As noted, redundancy may occur between TLRs. At relatively low infection titers, as used here, TLR-deficient mice may exhibit a normal or near-normal response to infection. However, the temporal dependence of early TLR2 expression upon TLR4 signaling indicates that there is a more complex relationship between TLR2 and TLR4 in the middle ear response to non-typeable *H. influenzae*. Other investigators have noted that TLR4 responses to pathogens can occur earlier than those of TLR2.[41] One reason for this may be

the ability of TLR4 to signal via TRIF, and to induce the expression of interferons (IFNs),[43] as well as via MyD88. This IFN pathway has been suggested to mediate the most immediate TLR responses to infection, followed by MyD88 signaling inducing expression of pro-inflammatory cytokines like TNF.[13] While mutation of the Toll receptor renders *Drosophila* highly susceptible to fungal and other infections,[44] mutation of individual TLRs in mammals has not been found to be as deleterious, suggesting that there is a degree of functional redundancy amongst mammalian TLRs.[45] Studies have demonstrated an interaction between TLRs such that induction of TLR2 or TLR4 can enhance expression of TLR4 or TLR2, respectively.[42,46] Our data suggest that early TLR4 signaling is required for the initial expression of TLR2 in response to non-typeable *H. influenzae*, although other signaling mechanisms eventually up-regulate TLR2 even in the absence of TLR4. Li and colleagues[12] have reported that responses to non-typeable *H. influenzae* are strongly mediated via TLR2-dependent pathways in middle ear epithelial cells. Our data similarly indicate that TLR2, which signals exclusively via MyD88, is more important for otitis media resolution than TRL4. However, although they were unable to clear non-typeable *H. influenzae* completely, TLR2$^{-/-}$ mice did partially reduce bacterial titers, mucosal hyperplasia and middle ear leukocyte infiltration by day 21. Thus TLR4, other TLRs or other innate immune receptors can mediate a response to nontypeable *H. influenzae*, although with some delay and reduced efficiency. Supporting this conclusion is our finding that MyD88$^{-/-}$ mice, which are deficient in signaling via most TLRs, have an otitis media phenotype more severe than that seen in TLR2$^{-/-}$ mice.[29]

Since both TLR2 and TLR4 can signal via MyD88 and NF-κB to induce pro-inflammatory cytokines, our results suggest that early TLR2- and TLR4-dependent production of such cytokines, as exemplified by TNF, is critical to the response to non-typeable *H. influenzae*.

The response of the wild-type mouse middle ear to non-typeable *H. influenzae* is similar to that seen in other models of infectious disease. O'Grady *et al*.[47] noted strong increases in TNF and other pro-inflammatory cytokines within hours of endotoxin instillation into the lung, but no change in IL-10, as also reported for bacterial pneumonia by Manderscheid *et al*.[48] These observations reflect the multifunctional role of TNF in bacterial clearance and inflammation. Using a bacterial pneumonia model, Fujita *et al*.[49] found that TNF receptor 1 deficiency compromises bacterial killing by macrophages and dysregulates inflammatory cytokine production, while TNF receptor 2 deficiency lead to a marked accumulation of neutrophils, enhanced inflammation and death. Lack of anti-inflammatory cytokines such as IL-10[50] in the wild-type middle ear is also presumably related to recovery from infection. Sun *et al*.[51] found that induced over-expression of IL-10 significantly decreased bacterial clearance from the lung.

The pattern of cytokine production observed in wildtype mice was severely disrupted in TLR-deficient animals. The TNF production was greatly reduced in TLR2 and TLR4 knock-out mice, which presumably contributed to the decreased bacterial clearance observed during otitis media. However, in TLR2-deficient mice, a remarkable increase in middle ear IL-10 expression was observed, at 3–5 days after nontypeable *H. influenzae* inoculation. This may help to explain why bacteria persisted much longer in these animals than in TLR4 knock-out mice, when TNF production was decreased in both. This result also suggests the possibility that late IL-10 expression in wild-type mice is actively suppressed by TLR2, and lack of this receptor may unmask IL-10 production. Alternatively, TLR2 deficiency may increase the ingress of IL-10-producing cells. These seem unlikely to be the neutrophils that were greatly increased in the TLR2-deficient middle ear late in otitis media, but could be a subset of the macrophages[52] that we observed in the middle ear at higher numbers in TLR2-deficient animals than in controls beginning on day 3. Obonyo *et al*.[53] found that macrophages produce IL-10 in response to TLR4 activation by *Helicobacter pylori*.

Toll-like receptors and cytokines may play similar roles in clinical otitis media. In humans the IL-10-1082 A/A genotype, associated with a low IL-10 production, was reported to be associated with protection from otitis media after vaccination.[19] In fact, it is well known that susceptibility to otitis media has a strong genetic component[54,55] and gene polymorphisms in TLR4, TLR co-receptor CD14 and the TLR target TNF have been reported to be significantly more common in otitisprone children than in control children.[19,20,56]

The present study underscores the different and interactive roles played by TLR2 and TLR4 signaling for an appropriate host response to non-typeable *H. influenzae* in the middle ear. The persistence of otitis media noted and the relevance of TLRs in the response of humans to infection[19,20,56] suggest the need for additional investigation into TLR signaling pathways and their effector molecules in human otitis media. Knowledge of these innate immune pathways may suggest interesting targets for pharmacological manipulation, particularly in treating therapy-resistant, chronic, slow-to-resolve, or persistent otitis media.

Conclusions

Induction of TLR2 via TLR4 signaling is important for the timely resolution of non-typeable *H. influenzae*-induced otitis media. Failure otitis media recovery in TLR2$^{-/-}$ and TLR4$^{-/-}$ mice is associated with decreased TNF and enhanced IL-10 expression, suggesting that abnormalities of innate immunity may contribute to otitis media in humans and that innate immune pathways could be targets of otitis media therapy.

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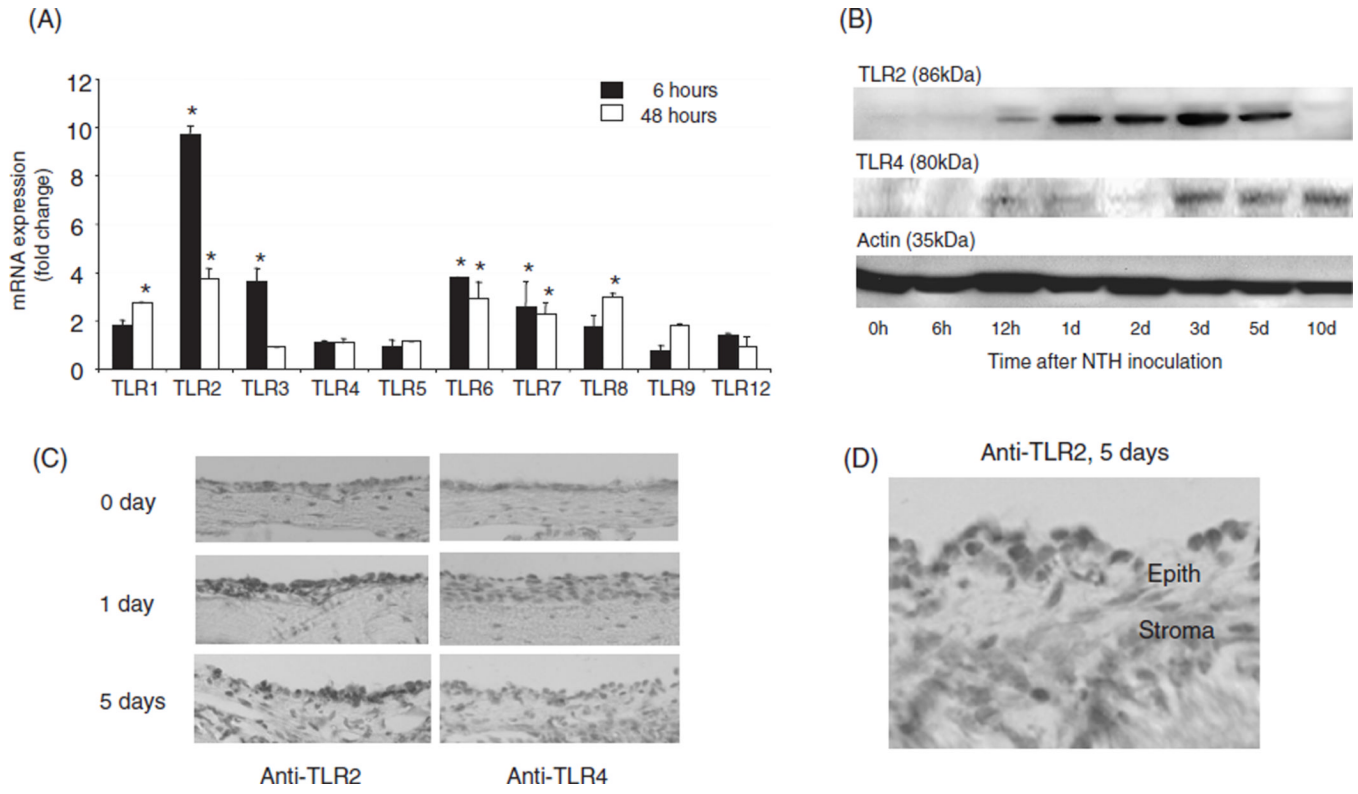

Fig. 1.

Toll-like receptor-2 is strongly induced by non-typeable *H. influenzae* in the middle ear mucosa of wild-type mice. (A) Gene array data at 6 h and 48 h after non-typeable *H. influenzae* inoculation, expressed as fold change from 0 h ($n = 40$ middle ears/time; $*P < 0.05$). (B) Western blot (rat middle ear) with β-actin control ($n = 2–8$ middle ears per group). (C) Representative protein expression of TLR2 and TLR4 in the middle ear mucosa at 0, 1 and 5 days after non-typeable *H. influenzae*. (D) Detail of TLR2 expression in the middle ear mucosa 5 days after non-typeable *H. influenzae*, showing TLR2 in cells of the mucosal epithelium (Epith).

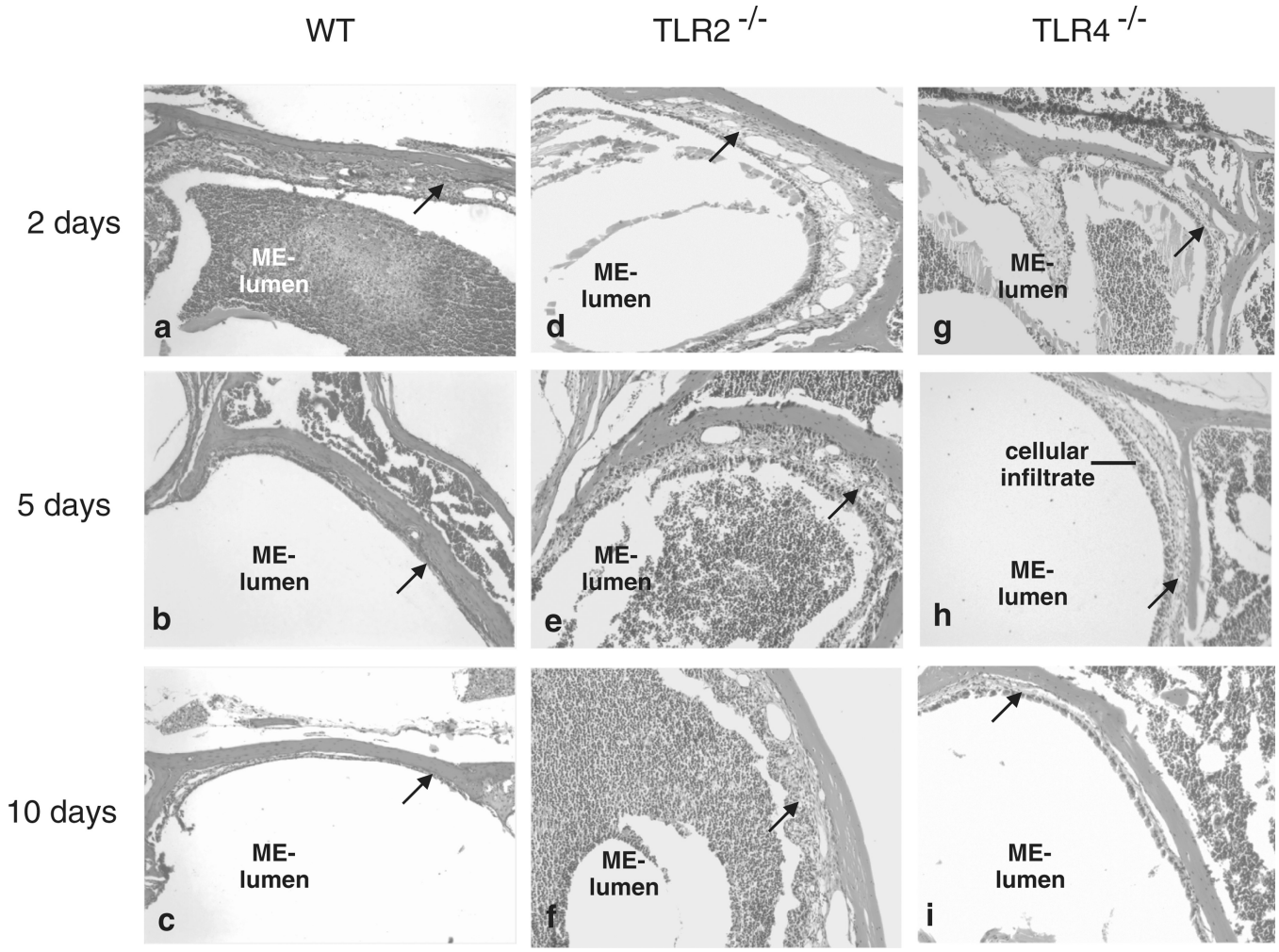

Fig. 2.

Representative histopathology of middle ears at 2, 5 and 10 days after non-typeable *H. influenzae* challenge. Middle ear response to non-typeable *H. influenzae* in wild-type middle ears (a–c) differs from that noted in TLR2$^{-/-}$ (d–f) and TLR4$^{-/-}$ (g–i) middle ears. Hematoxylin and eosin staining at 2, 5 and 10 days after non-typeable *H. influenzae* challenge (×40 magnification). Arrows indicate the middle ear mucosa (MEM).

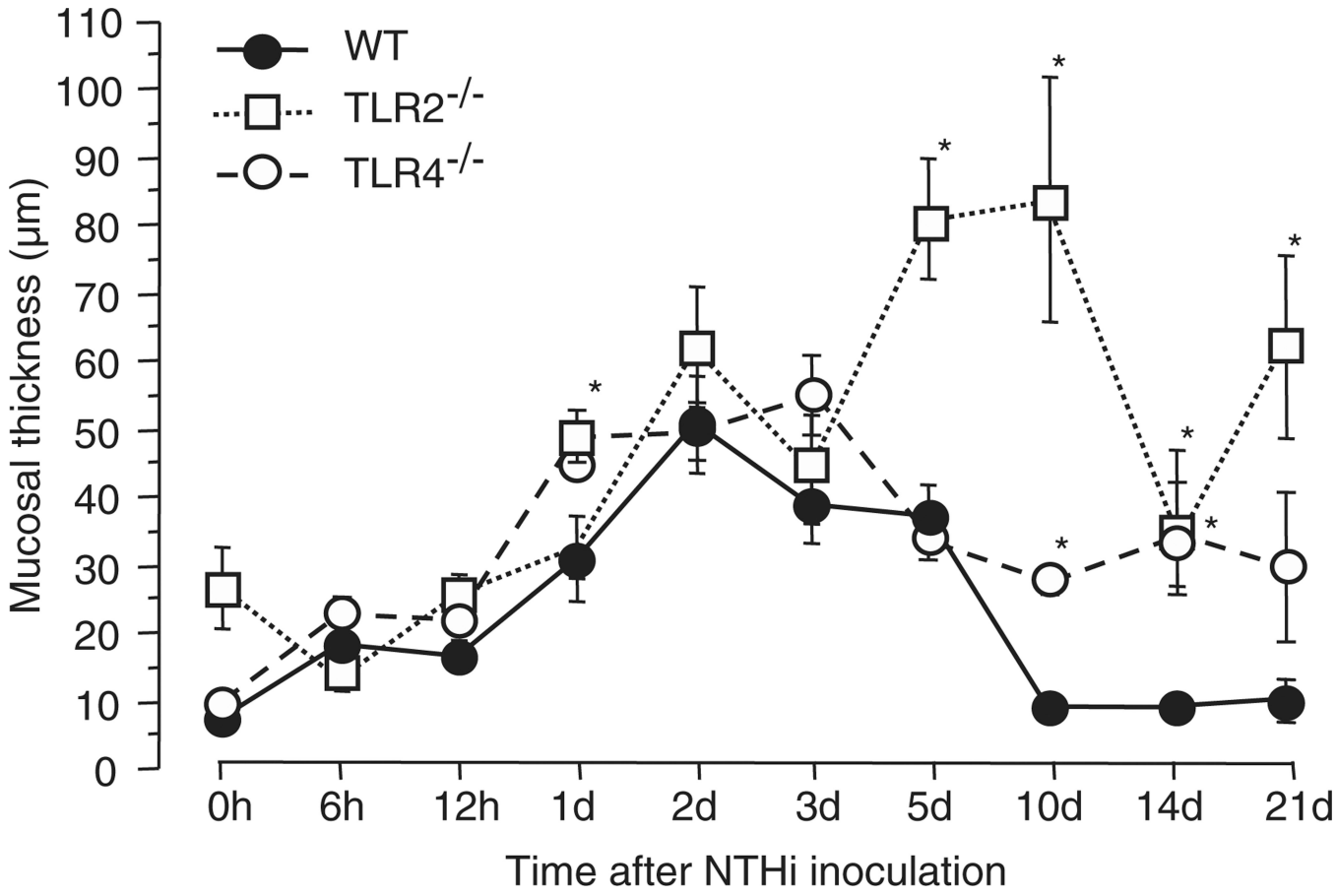

Fig. 3.

A quantitative evaluation of mucosal thickness and cellular infiltration of the middle ear cavity throughout the course of otitis media. Mucosal thickness is greater in TLR2$^{-/-}$ and TLR4$^{-/-}$ than in wild-type mice (n = 6–8 middle ears per time point; bars represent the mean ± SEM; *P <0.05).

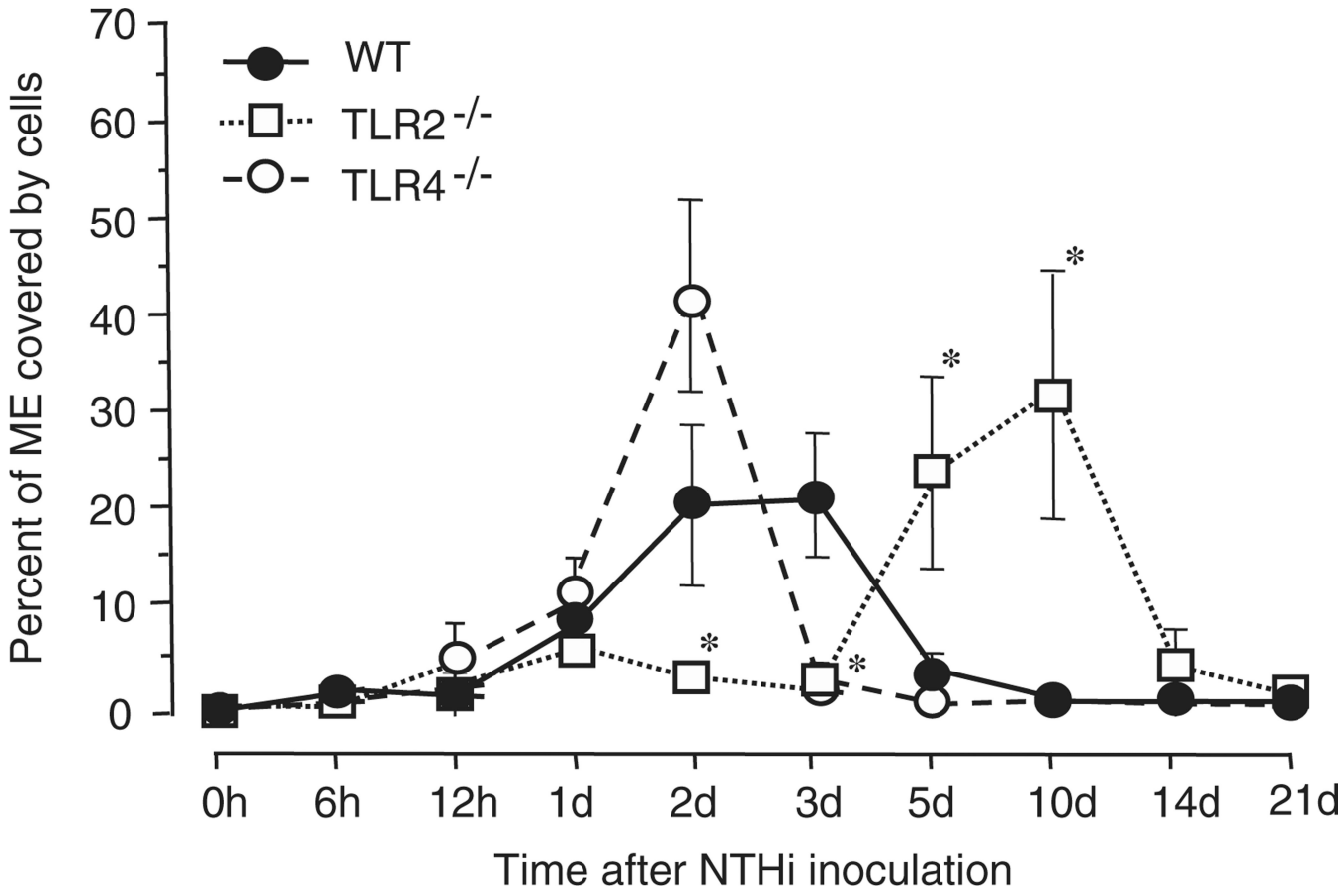

Fig. 4.

Infiltration of the middle ear cavity by leukocytes after nontypeable *H. influenzae* inoculation. Leukocyte infiltration is substantially delayed in TLR2$^{-/-}$ mice. Toll-like receptor-4$^{-/-}$ mice exhibit more leukocytes than wild-type mice 2 days after inoculation, but an earlier recovery (n = 6–8 middle ears per time point; bars represent the mean ±SEM; *P <0.05).

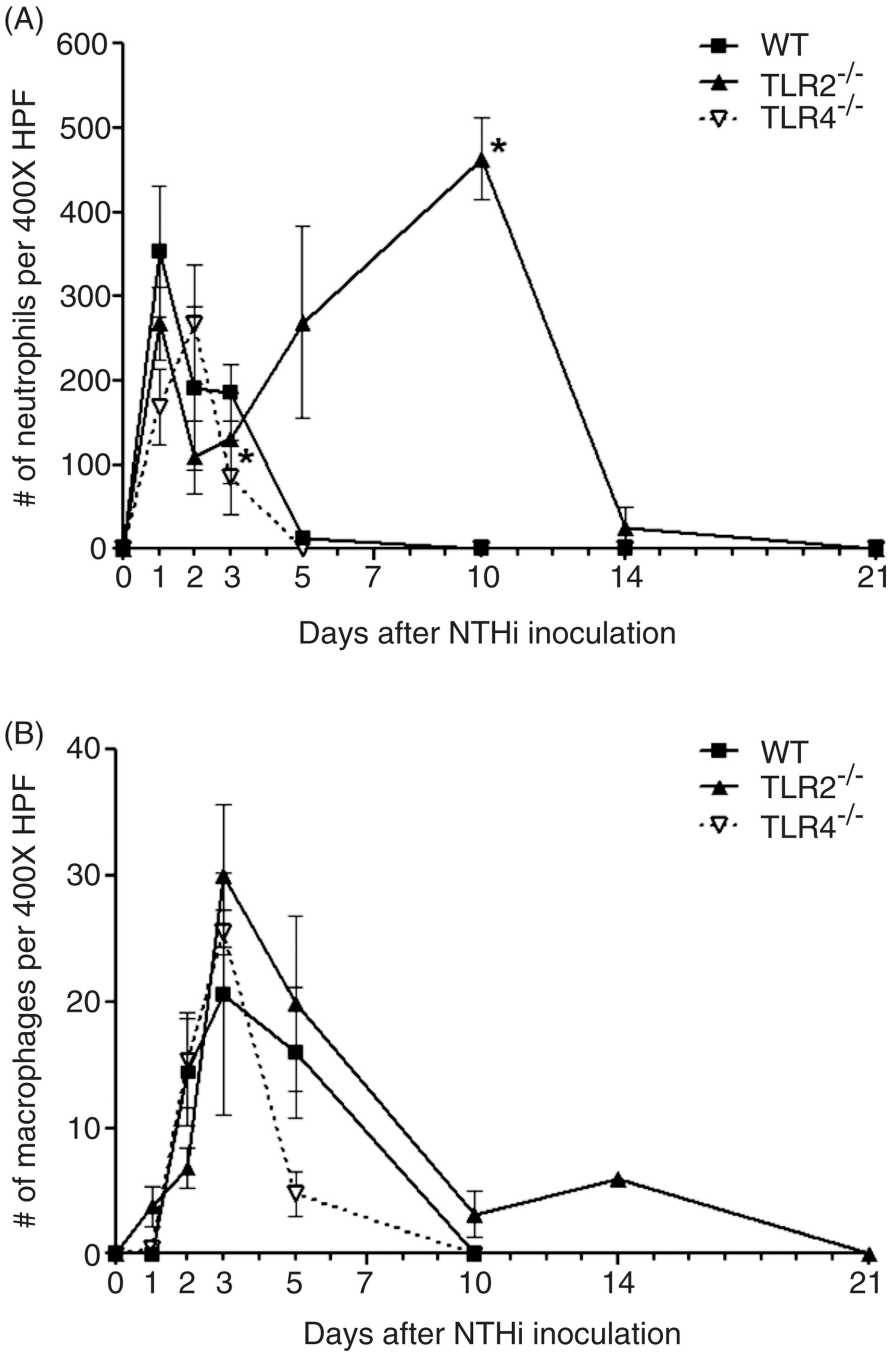

Fig. 5.

Leukocyte numbers measured in middle ear infiltrates in wildtype and TLR$^{-/-}$ mice. (A) Neutrophils showed a striking, late influx at 5 and 10 days in TLR2$^{-/-}$ mice and were slightly but significantly reduced in TLR4$^{-/-}$ mice at 3 days. (B) Toll-like receptor-2$^{-/-}$ mice displayed prolonged macrophage presence in the middle ear, until 14 days after non-typeable *H. influenzae* inoculation (n = 6 middle ears per time point; bars represent ± SEM; *P<0.05).

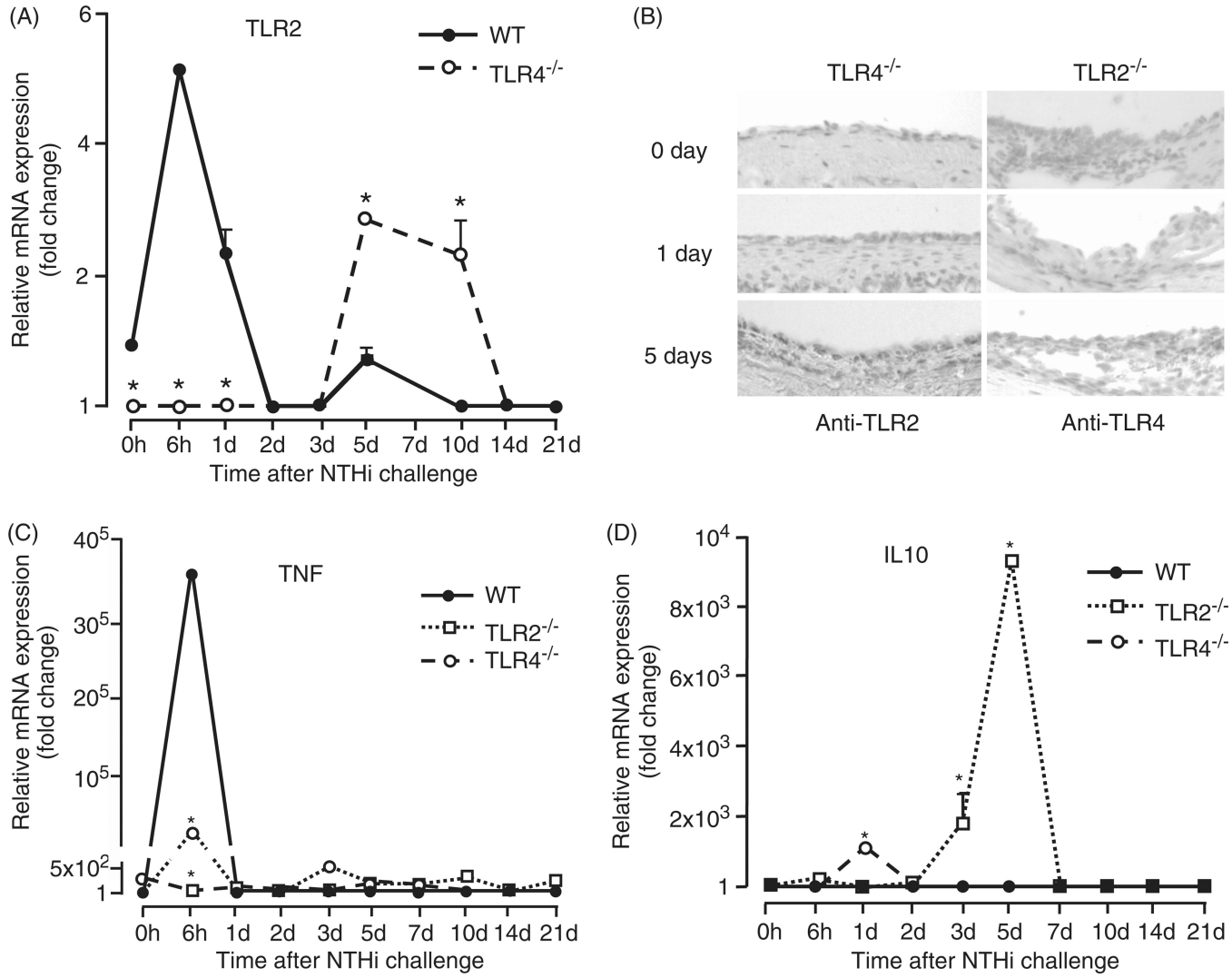

Fig. 6.

Reduced early TLR-signaling in the middle ear mucosa of $TLR^{-/-}$ mice. (A) Toll-like receptor-2 mRNA expression in wild-type and $TLR^{-/-}$ middle ears during non-typeable *H. influenzae*-induced otitis media by qPCR. (B) Reduced early TLR2 and TLR4 protein expression in $TLR^{-/-}$ mucosa, compared to wild-type by immunohistochemistry (Fig. 1B). (C) Tumor necrosis factor-α and (D) IL-10 gene expression measured by qPCR, normalized to GAPDH and compared to uninfected wild-type mucosa (n = >6 middle ears per/point; mean±SEM; *P <0.05).

Table 1

Impaired bacterial clearance of TLR2$^{-/-}$ and TLR4$^{-/-}$ middle ears.

	C57BL/6		TLR2$^{-/-}$		TLR4$^{-/-}$	
Time after non-typeable *H. influenzae* instillation	**Culture positive plates**	**Mean bacterial colonization**	**Culture positive plates**	**Mean bacterial colonization**	**Culture positive plates**	**Mean bacterial colonization**
Day 1	4/6	4	3/6	4	4/6	2.3
Day 2	6/6	3	3/6	1	4/6	1.3
Day 3	3/6	1	3/6	2	3/6	3.3
Day 5	0/6	0	2/6	1	4/6	3.5
Day 10	0/6	0	3/6	1.3	3/6	2
Day 14	0/6	0	3/6	2	1/6	1
Day 21	0/6	0	3/6	1	0/6	0

Mean bacterial colonization of the culture positive plates was evaluated using semi-quantitative analysis of bacterial colonization: 0 indicates no CFUs, 1 indicates one quadrant with CFUs, 2 indicates two quadrants with CFUs, 3 indicates three quadrants with CFUs and 4 indicates four quadrants with CFUs. Data represent positive culture plates out of six.

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