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Toll-like receptor 4 plays an important role to enhance bacterial clearance from the nose in synergy with triggering receptor expressed on myeloid cells (TREM)-1 expression on polymorphonuclear neutrophils

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ABSTRACT

Objective: Acute rhinosinusitis (ARS) is among the most common infectious diseases. Neutrophils play a major role in innate host defenses against pathogenic microorganisms such as fungi and bacteria. Recently, in neutrophils, ligation of the triggering receptor expressed on myeloid cells (TREM)-1 was found to activate the full spectrum of neutrophil effector mechanisms, including the release of inflammatory mediators, degranulation, phagocytosis, and oxidative burst in synergy with Toll-like receptors (TLRs). In this study, we investigated the effect of TREM-1 on the functions of neutrophils in relation to TLR4 in a nasal and nasopharyngeal inflammation mouse model via nontypeable *Haemophilus influenzae* (NTHi) intranasal inoculation.

Methods: We used C3H/HeJ (TLR4-deficient) mice, which arose spontaneously and have non-functional TLR4 protein, and normal wild-type (WT) C3H/HeN mice. Mice were inoculated intranasally with NTHi (10^7 cfu/mouse) to investigate the effects of TLR4 on the function of Neutrophils. We examined the kinetics of bacterial clearance and inflammatory cell infiltration in nasal washes at 6, 12, 24, and 72 h after inoculation. The expression of TREM-1 on neutrophils, and TREM-1 mRNA expression in neutrophils in the nasal washes were examined by flow cytometric analysis and RT-PCR.

Results: Bacterial counts of NTHi from nasal washes were significantly lower in WT mice than in TLR4-mutant mice after inoculation. The numbers of inflammatory cells in nasal washes were significantly higher in WT mice at 6 h, 12 h, and 24 h after inoculation than in TLR4-deficient mice. The expression of TREM-1 protein on neutrophils and the mRNA levels were greater in WT mice than in TLR4-mutant mice. The concentrations of soluble TREM-1 in WT nasal washes were also significantly higher than in those of TLR4-deficient mice.

Conclusion: TREM-1 may play an important role together with TLR4 in the nasopharyngeal clearance of NTHi by neutrophils. Further studies will need to clarify the innate immune responses of neutrophils via TLR4 to prevent NTHi infection.

1. Introduction

Acute rhinosinusitis (ARS) is among the most common infectious diseases in children and adults, and is usually caused by a viral infection (rhinovirus, adenovirus, influenza, or parainfluenza), which generally persists up to 10 days [1] before a secondary bacterial infection emerges. Nontypeable *Haemophilus influenzae* (NTHi) is considered one of the major pathogens in otitis media (OM) and ARS [2,3]. Current treatment options depend mainly on the use of antibiotics but are facing serious challenges from emerging antibiotic-resistant strains [4]. Thus, the development of a new strategy to prevent these diseases is an urgent goal for public health.

In the last decade, Toll-like receptors (TLRs) have emerged as key regulators of mammalian innate immune responses to infection and are critical players in both innate and adaptive immune responses [4]. Within the TLR family, TLR4 mediates lipopolysaccharide (LPS) or lipooligosaccharide (LOS) responsiveness and recognizes Gram-negative bacteria, including NTHi, via the LPS/LOS moiety present on the surface of these microorganisms [5]. Specific polymorphisms in human TLR4 genes are present in individuals and associated with increased susceptibility to OM in children [6]. In chronic rhinosinusitis, TLR2 and TLR4 share a close relationship with TGF-β1 and the histological features of remodeling [7]; however, little is known about the role of TLR4 in relation to ARS. Recently, it was found that the triggering receptor

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expressed on myeloid cells 1 (TREM-1) is an activating receptor expressed on neutrophils and monocytes, and the engagement of TREM-1 on the surface of the cell membrane leads to activation of a cascade of intracellular events that result in inflammatory effects, such as cytokine production, degranulation of neutrophils, and phagocytosis. The ligands of TREM-1 have not been clarified, although several putative ligands have been proposed, for example, platelets, HMGB1, HSP70, peptidoglycans, and endotoxins [8]. Moreover, some of the biological effects of TREM-1 are attributed to its interaction with the TLR4/LPS-receptor complex [9].

Clarification of the immune response mediated by TLR4 in relation to TREM-1 in the upper respiratory tract may help to establish a strategy for the treatment of ARS caused by NTHi infection. In this study, we investigated the kinetics of humoral and related cellular responses in C3H/HeJ (TLR4-deficient) mice, which have a spontaneous nonfunctional TLR4 protein, and normal wild-type (WT) C3H/HeN mice after intranasal challenge with NTHi to examine the dynamics between TREM-1 expression and TLR4. We examined the kinetics of mucosal clearance of NTHi from the nostrils, the migration of neutrophils to the nasal cavities, and the role of the TLR4/TREM-1 interactions during NTHi intranasal challenge.

2. Materials and methods

2.1. Animals

C3H/HeJ and C3H/HeN mice were purchased from Charles River Laboratories (Atsugi, Japan). All mice were maintained in a pathogen-free facility until they were 6 weeks old, at which time they were used for the experiments. All experiments were approved by the Committee on Animal Experiments of Oita University (Oita, Japan:P028001).

2.2. Nasal challenge with live NTHi

NTHi strain 76, which was isolated from the nasopharynx of a patient with OM with effusion at Oita University, was used for the nasal challenge.

An aliquot of stored bacteria was cultured on chocolate or blood agar plates overnight at 37 °C with 5% CO_2 for 16 h. The concentration of bacterial cells was determined from the optimal density at a wavelength of 600 nm. Thereafter, a bacterial suspension of 10^9 cfu/mL in phosphate-buffered saline (PBS) was prepared and stored on ice. We confirmed the bacterial concentration by counting the colonies after overnight incubation. The bacterial suspension was injected into the nasal cavities following a method described previously [10]. A micropipette was inserted into both nostrils, and 10 μL (10^7 cfu) of live NTHi suspension was slowly injected. At 6, 12, 24, and 72 h after inoculation, 6 mice from each group were euthanized by intraperitoneal administration of pentobarbital solution. Nasal cavities were washed with 200 μL physiological saline at the time of decapitation to obtain the nasal washes. Untreated mice were used as controls and evaluated using the same procedures.

2.3. Bacterial and inflammatory cell counts

Nasal washes were diluted serially with PBS and 10 μL from each diluted sample was plated on chocolate agar. Bacterial colonies were counted after overnight incubation to measure the rates of NTHi clearance from the nasal cavities. Inflammatory cells in the nasal wash samples were also counted by flow cytometry. Granulocytes were gated, and the number of cells in each nasal wash was counted.

2.4. Histological evaluation

The heads of the decapitated mice were immersed in 10% neutral-buffered formalin fixative for 6 h and decalcified in 0.12 M ethylenediaminetetraacetic acid (EDTA, pH 7.5) for 2 weeks at 4 °C. The tissues were dehydrated through graded series of ethanol, cleared in xylene, and embedded in paraffin. Serial paraffin sections (6 μm) containing the nasal cavity were prepared for hematoxylin-eosin (H&E) staining. Untreated mice were used as controls and evaluated using the same histological procedures. Horizontal sections of nose containing turbinates were observed by light microscopy and histological images (magnification x200) were captured by Leica MC120HD (Leica Microsystems, Wetzlar, Germany). The number of inflammatory cells in the nasal mucosae of inferior turbinate was counted and analyzed at three sites of the turbinate. Three mice were used for this experiment.

2.5. Concentration of soluble TREM-1 in nasal washes

We investigated the concentration of soluble TREM-1 in the nasal washes in another set of mice. Nasal washes were collected from 5 to 8 mice from each group, as described before, centrifuged at 120 × *g* for 10 min, and the supernatants were collected and stored at −80 °C until the assays were performed. The levels of soluble TREM-1 in the nasal washes from WT mice and TLR4 mutant mice were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN). Standard curves were generated from the known concentrations of the cytokines provided by the manufacturer. Standard curves were used to quantify the amount of each cytokine based on the level of spectrophotometric absorbance in each sample by regression analysis.

2.6. Expression of TREM-1 on neutrophils in nasal cavities

The expression of TREM-1 on neutrophils in the nasal washes was examined by flow cytometry. After the washes were centrifuged, the precipitates were collected, and fluorescein isothiocyanate (FITC)-conjugated anti-Ly-6G monoclonal antibodies (mAb: Beckman Coulter, Brea, CA), and phycoerythrin-conjugated anti-TREM1 mAb (R&D Systems) were used. The immunofluorescent intensities of neutrophils were analyzed using CellQuest software (Becton Dickinson, San Jose, CA). Five independent experiments were performed, each with a pool of 3 mice.

2.7. TREM-1 mRNA expression in inflammatory cells in nasal washes

The nasal washes were centrifuged, supernatants were discarded, and RNA was extracted from the precipitate. The samples contained uniform concentrations of RNA and were measured by RT-PCR. The expression of TREM-1 mRNA in inflammatory cells in the nasal washes was also examined by RT-PCR. The extraction of total cellular RNA and the synthesis of first-strand cDNA were performed with a commercially available RNA extraction and reverse transcription kit (Qiagen, Tokyo, Japan). The total volume of RNA used in the assays for each group was adjusted to 0.25 μg. Each cDNA sample was then used as a template for a PCR amplification mixture containing forward and reverse primers for TREM-1, forward and reverse primers for 18S rRNA (internal control), and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). PCR amplifications of the target cytokine and internal control 18S rRNA were performed in a single well of a capped 96-well optical plate. RT-PCR data were analyzed using a StepOnePlus Real-Time PCR system (Applied Biosystems). Final quantification was derived using the comparative CT (threshold cycle) method, and was reported as the fold difference relative to calibrator cDNA (cells in nasal washes from each mouse without NTHi challenge as controls) prepared in parallel with the experimental cDNA. Data are expressed as the arithmetic means of the CT values from 3 independent experiments (± standard deviation [SD]), each from a pool of 3 mice.