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The role of toll-like receptor 3 in chronic contact hypersensitivity induced by repeated elicitation

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ABSTRACT

Background: Accumulating evidence suggests that Toll-like receptor (TLR)-3 signaling is involved in non-infectious immune and inflammatory reactions as well as in viral infections. The skin of patients with atopic dermatitis (AD) is often infected with virus and bacteria, leading to the aggravation of atopic symptoms. These findings suggest TLR3 signaling may be involved in the pathogenesis of AD, but the exact role of TLR3 in AD remains to be defined.
Objective: The purpose of this study was to investigate the role of TLR3 in chronic contact hypersensitivity reactions induced by repeated elicitation, resembling the features of AD.
Methods: Wild-type (WT) and Toll-like receptor 3 knockout (Tlr3 KO) mice were sensitized, and chronic contact hypersensitivity reactions were elicited in their ear skin via repeated application of a hapten, 2,4,6-trinitro-1-chlorobenzene (TNCB) or oxazolone.
Results: The Tlr3 KO mice exhibited less ear swelling, less leukocyte infiltration into the skin, and lower serum total IgE levels than WT mice after hapten challenge. The Tlr3 KO mice also displayed lower expression levels of inflammatory cytokines (interleukin (IL)-33, IL-4, IL-10, and interferon-ɤ in their TNCB-treated ear skin than WT mice.
Conclusion: These results showed that TLR3 deficiency suppressed the development of chronic contact hypersensitivity reactions, suggesting that TLR3 signaling may participate in the pathogenesis of AD.

1. Introduction

Toll-like receptors (TLRs) are germline-encoded pattern recognition receptors that play a central role in host cell recognition and the response to microbial pathogens. There are at least 11 mammalian TLR, which are expressed on immune cells, such as macrophages, dendritic cells, granulocytes, natural killer cells, and T cells, as well as non-immune cells, such as fibroblasts and epithelial cells [1–3]. TLR3 recognizes double-stranded RNA, a component of the lifecycle of most viruses, and induces immune responses that are characterized by the production of type I interferons and proinflammatory cytokines [1–3].

There is increasing recognition of the role of TLR3 signaling in non-infectious immune and inflammatory reactions as well as in viral infections. Recently, we have found that TLR3 signaling is closely associated with the pathogenesis of allergic and irritant contact dermatitis [4]. Our findings also imply that endogenous danger signals, that are associated with inflammatory tissue damage, may activate TLR3 signaling in keratinocytes and dermal fibroblasts of the inflamed skin [4]. In addition to its role in skin

Abbreviation: TLR, Toll-like receptor; AD, atopic dermatitis; WT, wild-type; *Tlr3* KO, *Toll-like receptor 3* knockout; TNCB, 2,4,6-trinitro-1-chlorobenzene; IL, interleukin; TSLP, thymic stromal lymphopoietin; IFN-ɤ, interferon-ɤ; ELISA, enzyme-linked immunosorbent assay; HEKa, Human Epidermal Karatinocytes, adult; KCMH-1, mouse keratinocyte-derived squamous cell carcinoma cell line.

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inflammation, we have found that TLR3 enhances experimental allergic conjunctivitis by inducing the production of thymic stromal lymphopoietin (TSLP) in conjunctival epithelial cells [5]. In addition, Nuolivirta et al. demonstrated that the exacerbation of virus-induced asthma is associated with TLR expression, especially TLR3 expression [6]. Furthermore, infants with allergic diseases exhibit increased perinatal TLR3 responses [7]. Upregulated expression of TLR 2, 3, and 4 has also been detected in allergic rhinitis [8]. Taken together, these findings suggest that TLR3 signaling is deeply involved in the pathogenesis of allergic disorders.

Atopic dermatitis (AD) is a chronic inflammatory skin disorder characterized by increased levels of type 2 cytokines, such as interleukin (IL)-4 and IL-10; high serum IgE levels; and eosinophilic tissue inflammation [9]. The skin of patients with AD is often infected with virus such as herpes simplex virus or molluscum contagiosum virus and bacteria such as *Staphylococcus aureus*, leading to the aggravation of atopic symptoms. It has been reported that monocytes of patients with intrinsic AD express TLR2 and TLR4 more strongly than those of healthy controls [10]. In addition, Novak et al. has demonstrated that the *TLR9* promoter polymorphism may influence patients' susceptibility to atopic eczema, particularly in patients with the intrinsic variant of atopic eczema [11]. Kubo et al. reported that a TLR3-mediated mechanism is involved in TSLP production in AD [12]. Thus, it is likely that signaling of TLRs including TLR3 is involved in the pathogenesis of AD, but the exact role of TLR3 in AD remains to be defined.

Several animal models of AD including a hapten-induced mouse model have been reported [13]. Chronic elicitation via repeated epicutaneous application of the hapten leads to elevation of serum IgE levels and a shift in the time course of chronic hypersensitivity reactions from the typical delayed-type to the early-type response [14]. In addition, chronic exposure induces type 1 cytokine production during the primary response, leading to a shift in the pattern of antigen-induced cytokine expression toward the induction of type 2 cytokine production [15]. The inflammatory responses observed in chronic lesions share many of the histopathological, immunological, and clinical features of human AD [13].

In this study, we examined whether TLR3 signaling affects the pathological mechanisms of AD using *Toll-like receptor 3* knockout (*Tlr3* KO) and wild-type (WT) mice. The mice were sensitized to a hapten, 2,4,6-trinitro-1-chlorobenzene (TNCB) or oxazolone, and then chronic contact hypersensitivity reactions were elicited in their ear skin via the repeated application of the hapten. We measured the ear thickness of the mice at various time points; performed histological examinations of their ear skin; measured their serum total IgE levels; and examined the release of type 1 cytokines (interferon (IFN)-ɤ and IL-2), type 2 cytokines (IL-4, IL-10, IL-13, IL-33 and TSLP).

2. Materials and method

2.1. Mice

Male BALB/c mice were obtained from Shimizu Laboratory Supplies (Kyoto, Japan), and were used at the age of 6 to 8 weeks. Male *Tlr3* KO mice were generated as described previously [16], back-crossed more than 7 generations with BALB/c mice [5], and used at the age of 6 to 8 weeks. All of the mice were housed in a specific pathogen-free barrier facility and screened regularly for pathogens. This experimental procedure was approved by the Committee for Animal Research of Kyoto Prefectural University of Medicine.

2.2. Sensitization and elicitation procedure

The mice were sensitized via the epicutaneous application of 25 μl 1% TNCB (Tokyo Kasei, Tokyo, Japan) and 1% oxazolone (Sigma-Aldrich, St. Louis, MO) in acetone/olive oil (4:1) to the shaved abdominal skin of the mice at 7 days before the first elicitation procedure (day 0). Then, 20 μl (10 μl on the dorsal side and 10 μl on the ventral side) of 1% TNCB were repeatedly applied to the right ear from day 0 to day 30 at 2-day intervals. The same amount of acetone/olive oil (4:1) was administered to the left ear as a control. Ear thickness was measured at a predetermined site with a dial thickness gauge (Shinwa Rules, Niigata, Japan). Ear thickness was measured before each elicitation procedure. In the detailed time course analysis of ear swelling, ear thickness was measured before and 0.5, 1, 3, 6, 9, 12, 24, 36, and 48 h after the elicitation procedure on days 0, 8, 14, 20, and 28.

2.3. Histological assessment

The ears of some of WT mice and *Tlr3* KO mice were excised at 6 and 9 h after the challenge on day 8, respectively. The ears of the remaining mice were excised at 6 h after the challenge on day 30. After being excised, the ears were fixed with 10% formalin and embedded in paraffin. Sections (5 μm) were cut using a microtome and stained with hematoxylin and eosin. Immunostaining included anti-mouse CD4 monoclonal antibody (clone 4SM95: eBioscience, San Diego, CA), anti-mouse CD8a monoclonal antibody (clone 4SM16; eBioscience), macrophage-specific anti-mouse F4/80 rabbit monoclonal antibody (clone SP115: Spring Bioscience, Pleasanton, CA). The presence of mast cells was confirmed by the characteristic toluidine blue metachromasia. Dermal leukocyte infiltration was evaluated by averaging the number of leukocytes present in at least 5 fields for each lobe (magnification: ×400).

2.4. Measurement of serum total IgE levels

Blood was collected from the lateral caudal vein on days 0, 8, and 30, and serum levels of total IgE were analyzed using an enzyme-linked immunosorbent assay (ELISA) kit for IgE (BD Biosciences-Pharmingen, San Diego, CA).

2.5. Measurement of cytokine levels in the skin tissue by PCR

Quantitative real-time PCR was performed on an ABI Prism 7100 (Applied Biosystems, Foster City, CA), as described previously [17]. To detect IFN-ɤ, IL-2, IL-4, IL-10, IL-13, IL-33 and TSLP mRNA, whole ears were cut into pieces on day 8, put into 0.5 ml of RNAlater (Ambion, Austin, TX), snap-frozen in liquid nitrogen, and stored at −80 °C until use. Total RNA was isolated from the frozen whole ears by homogenizing them in TRI reagent. For the reverse transcription, we used an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The primers and probes for mouse IFN-ɤ, IL-2, IL-4, IL-10, IL-13, IL-33, TSLP and glyceraldehyde-3-phosphate dehydrogenase were obtained from Takara Bio (Otsu, Japan). The results were analyzed with sequence detection software (Applied Biosystems). The expression levels of the target molecules are shown relative to that of glyceraldehyde-3-phosphate dehydrogenase.

2.6. Measurement of IL-33 levels in the skin tissue by ELISA

The skin tissue was excised from WT and *Tlr3* KO mice at 0, 6, and 24 h after TNCB challenge on day 8, and then homogenized using gentle MACS dissociator (Milteny Biotec, Bergisch Gladbach, Germany) and a Bio-Plex Cell Lysis Kit (Bio-Rad, Hercules, CA) according to the manufacture's instructions. The concentrations of

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