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Effect of Toll-like receptor 2 and 4 of corneal fibroblasts on cytokine expression with co-cultured antigen presenting cells

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ABSTRACT

Keratocytes are the first component to contact ocular pathogens when the epithelial barrier breaks down and the emerging evidences indicated keratocytes appeared to be one of the corneal cellular immune components. Little is known about the role of Toll-like receptors (TLRs) in keratocytes, although it has been well documented that keratocytes constitutively express various TLRs including TLR2 and TLR4. In this *in vitro* study, the authors focused on the role of keratocytes in corneal innate immune system and cross-talk of keratocytes with resident antigen presenting cells (APCs), especially through TLR2 and TLR4. Primary cultivated keratocytes (corneal fibroblasts) from C57BL/6 mice *per se* actively secreted pro-inflammatory cytokines, especially interleukin (IL)-6, with a dose-dependent manner in response to Pam3CSK4 or lipopolysaccharide (LPS) challenge. With co-culture of corneal fibroblasts with APCs *per se*, secretion of IL-6 and tumor necrosis factor (TNF)-α was markedly increased and it was counterbalanced by concurrent increase in IL-10 and tumor growth factor-β1. After Pam3CSK4 or LPS stimulation, this cytokine balance was completely broken down by overwhelming amplification of IL-6 and TNF-α secretion, especially in co-culture of corneal fibroblasts with macrophages, rather than with dendritic cells. Using corneal fibroblasts from TLR2 or TLR4 knockout mice, we could find the reversal of Pam3CSK4 or LPS-responsive dose-dependent increment in IL-6 and TNF-α. These results implied that corneal fibroblasts and their TLRs could be key components for the ocular homeostasis and pathogen-associated ocular innate immunity.

1. Introduction

Recognition of pathogen associated molecular patterns (PAMPs) by Toll-like receptors (TLRs) on antigen presenting cells (APCs) induces the expression of various pro-inflammatory cytokines and activates the effector functions of innate immune cells [1–3]. This downstream response has emerged as a key component of the innate immune system that detects microbial infection and triggers antimicrobial host defense [2–4].

While, TLR signaling in mucosal epithelial cells looked contribute to immunosilent environment in order to keep harmonious symbiosis, which depends on variable pathways [5]. Intracellular expression of TLR4 [6], lack of MD2 binding [7], or negative regulators including Toll-interacting protein (TOLLIP) and peroxisome proliferator-activated receptor-γ (PPARγ) seem to be the ways to elicit epithelial tolerance to lipopolysaccharide (LPS) [8–10]. Polarized apical activation of TLR9 by CpGODNs also displayed inhibition of inflammatory response, whereas basolateral stimulation of TLR9 led to NF-κB activation [11].

On the other hand, relatively little is known about the immunogenic versus immunomodulatory roles of the constitutive expression of TLRs in fibroblasts. Stimulation of corneal myofibroblasts with poly (I:C) or CpG-DNA was reported to enhance the production of interleukin (IL)-6, IL-8, and RANTES through TLR3 or TLR9 activation [12]. However, telomerase-immortalized human stroma fibroblasts (THSFs) which were pretreated with low-dose LPS and then challenged with *Aspergillus fumigatus*, resulted in diminished production of IL-6 and IL-8, suggesting inducible tolerance [13]. In addition, fibroblast-like synoviocytes from rheumatoid arthritis

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patients released heat shock protein (HSP) 70 and IL-10 through TLR4 signaling, indicating plausible immune modulatory role [14]. There has been still matter of controversy of the TLR's role depending on the various types of fibroblasts from various organs. Therefore, it needs to be further elucidated to understand immune-related roles in tissue specific fibroblasts.

Accordingly, in this study, we aimed to investigate the role of TLR2 and TLR4 on primary cultured keratocytes (corneal fibroblasts) to contribute to cross-talk with APCs, on assumption that corneal epithelial barrier is disrupted and keratocytes are directly stimulated by Gram positive or Gram negative pathogens. Simple application of TLR2 or TLR4 agonist could not directly stimulate keratocytes *in vivo*, because corneal epithelial cells have well organized TLRs' strategies to prevent unnecessary inflammatory responses to normal bacterial flora [4,6,15]. Therefore, artificial destruction of corneal epithelial barrier such as ethanol-assisted or mechanical debridement is necessary before application of TLR agonists. However, these procedures might induce nonspecific inflammation, which could stimulate TLRs other than TLR2/4 or could activate keratocytes and APCs via TLR-independent signaling. For these reasons, we prepared *in vitro* setting to simulate the condition that pathogens penetrate the epithelial barrier, directly contact with keratocytes, and selectively stimulate TLR2 or TLR4.

To investigate the responses of corneal fibroblasts *per se* to Gram positive or Gram negative bacteria, primary cultivated mouse keratocytes were stimulated by TLR2 or TLR4 agonist and then were evaluated for their cytokine secretion. To demonstrate the interaction between corneal fibroblasts and APCs, cytokine changes were assessed in co-culture condition of corneal fibroblasts with APCs under TLR2 or TLR4 agonist challenge. The reversal effect on cytokine secretion was checked out using corneal fibroblasts from TLR2 knockout (KO) or TLR4KO mice.

2. Materials and methods

All procedures used in this study conformed to the principles of the ARVO Statement regarding the Use of Animals in Ophthalmic and Vision Research and the study protocol was approved by the Research Ethics Committee at Seoul National University Hospital.

2.1. Culture of murine corneal resident cells

Specific-pathogen-free C57BL/6 (B6) mice (6- to 8-week old) were purchased from Orient Bio, Inc. (Seongnam, Korea). TLR2KO and TLR4KO mice (B6 background) were bred at a SPF animal facility, and were kindly provided by Dr. S.-Y Seong and Dr. S. Akira. TLR2 and 4 KO mice were confirmed for TLR2 and TLR4 gene KO by PCR genotyping.

Eight murine corneas were obtained from each species. The epithelium was removed using a surgical blade, and the Descemet's membrane was peeled off. The remaining stroma was treated with 1.2 U/ml dispase II (Roche, Basel, Switzerland) for 2 h at 37 °C, to which was added 5 ml (200 U) of type I collagenase (Worthington, Lakewood, NJ, USA) for 90 min at 37 °C. The tissues were shaken 3 times every 30 min. The harvested keratocytes were centrifuged at 1200 rpm for 5 min and the cell pellets inoculated into the culture dishes containing DMEM:F12 (1:1; Cambrex, East Rutherford, NJ, USA) with 10% fetal bovine serum (FBS; Cambrex) and 1% penicillin/streptomycin (PS). The primary keratocytes were cultured at 37 °C in a carbon dioxide incubator for 1–2 weeks. Passage 1 of corneal fibroblasts was used in this study.

Mouse dendritic cell line (DC 2.4) and mouse macrophage cell line (Raw 264.7), which were kindly provided by TJ Kim (Korea University Hospital, Seoul, Korea), were cultured in RPMI1640 with 10% FBS and 1% PS and in DMEM with 10% FBS and 1% PS, respectively.

2.2. TLR2 and TLR4 stimulation

First, basal secretion level and secretion change of pro- and anti-inflammatory cytokines in response to TLR2 or TLR4 agonist were checked out in corneal fibroblasts (1×10^4 cells) from B6 mice. To stimulate each TLRs, Pam3CSK4 (Invivogen, San Diego, CA, USA) was added to culture media as a TLR2 agonist, and LPS from *Escherichia coli* K12 strain (LPS-EK Ultrapure, Invivogen), as a TLR4 agonist, at various concentrations (1, 10, and 100 ng/mL for Pam3CSK4 and 1, 100, and 10,000 ng/mL for LPS).

Next, to evaluate production change of each cytokines in cross-talk between corneal fibroblasts and APCs, we co-cultured corneal fibroblasts with dendritic cells or macrophages (1:1, 5.0×10^3 cells each) for 24 h. Then, Pam3CSK4 or LPS was added to each media.

Finally, we investigated the direct involvement of TLRs on corneal fibroblasts in these changes. To evaluate whether TLR2 or TLR4 blocking could abrogate the change of cytokine secretion in response to challenge of each TLR agonist, we used corneal fibroblasts derived from TLR2KO or TLR4KO mice and then co-cultured them with APCs.

2.3. Cytokine quantification

At 24 h after TLR stimulation, secretion of pro-inflammatory (IL-6 and TNF-α) and anti-inflammatory (IL-10 and transforming growth factor (TGF)-β1) cytokines was determined in supernatants by commercial kits for enzyme-linked immunosorbent assay (ELISA) as per the manufacturer instructions (DuoSet ELISA; R&D Systems, Minneapolis, MN, USA). The concentration used for capturing and detector antibodies was 2000 and 200 ng/mL for IL-6; 800 and 200 ng/mL for TNF-α; 4000 and 400 ng/mL for IL-10; and 4000 and 200 ng/mL for TGF-β1. The standard curve was prepared by serial dilutions of a standard stock solution: a high standard of 1000 pg/mL for IL-6, and 2000 pg/mL for TNF-α, IL-10, and TGF-β1. All experiments were performed in triplicate, and repeated independently three times.

2.4. Statistical analysis

We used software SPSS 17.0 (Chicago, IL, USA). The differences of cytokine levels between individual groups were analyzed using the non-parametric Mann–Whitney *U* test. Statistical significance was accepted for P values of <0.05.

3. Results

3.1. Basal secretion level of cytokines from corneal fibroblasts under LPS or Pam3CSK4 challenge

Corneal fibroblasts from B6 mice secreted small amount of pro-inflammatory cytokines (IL-6 and TNF-α) and sparsely secreted anti-inflammatory cytokines (IL-10 and TGF-β1, data not shown) without TLR stimulation. After TLR4 or TLR2 stimulation, secretion of pro-inflammatory cytokines, especially IL-6, was significantly increased as a dose-dependent manner (9.2- or 1.9-fold increase in IL-6 secretion even at lowest concentration of each TLR agonist, respectively, Supplementary Fig. 1).

3.2. Interaction between keratocyte and APCs for the secretion of cytokines under TLR stimulation

Surprisingly, co-culture of corneal fibroblasts and APCs *per se* synergistically increased the secretion of all the pro-inflammatory and anti-inflammatory (especially IL-10) cytokines even without LPS or Pam3CSK4 challenge (Figs. 1–4, NS). The levels