Biochemical and Biophysical Research Communications 404 (2011) 419–423

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Contribution of IPS-1 to polyI:C-induced cytokine production in conjunctival epithelial cells

Mayumi Ueta [a,b,*], Taro Kawai [c], Norihiko Yokoi [a], Shizuo Akira [c], Shigeru Kinoshita [a]

[a] Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan
[b] Research Center for Inflammation and Regenerative Medicine, Faculty of Life and Medical Sciences, Doshisha University, Kyoto, Japan
[c] Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

ARTICLE INFO

Article history:
Received 23 November 2010
Available online 3 December 2010

Keywords:
RIG-I
MDA5
IPS-1
Conjunctival epithelium
TLR3
PolyI:C

ABSTRACT

We previously demonstrated that ocular surface epithelium expressed TLR3 and that its ligand, polyI:C, stimulation induced the secretion of inflammatory cytokines and type I IFN. It was recently reported that RIG-I and MDA5 also recognize viral dsRNA mimicking polyI:C. In this study, we investigated whether RIG-I and/or MDA5 contribute to polyI:C-inducible responses in conjunctival epithelium. The expression of RIG-I, MDA5, and TLR3 in human conjunctival epithelium was examined by RT-PCR and their up-regulation after polyI:C stimulation by quantitative RT-PCR and immunoblot analysis. Human conjunctival epithelial cells also expressed RIG-I, MDA5 and TLR3 mRNA and protein. The expression of RIG-I and MDA5, but not of TLR3, was markedly up-regulated upon polyI:C stimulation. We also examined the function of IPS-1 (an adaptor molecule common to RIG-I and/or MDA5) and TLR3 in conjunctival epithelium using IPS-1 KO and TLR3 KO mice. To analyze *in vivo* murine conjunctival epithelial cells, 10 μl of a 100 μg/ml polyI:C solution were delivered subconjunctivally and as eye drops, then conjunctival epithelial cells were subjected to gene expression analysis. We focused on 10 transcripts up-regulated in murine conjunctival epithelium upon polyI:C stimulation. *Cxcl10, Mx1, Ifi44, Ifi203, Iigp2* and *Rtp4* were dominantly regulated by IPS-1, *Ccl5* by TLR3, and *Rsad2*, *Mx2* and *Cmpk2* were regulated by TLR3 and IPS-1. Our results showed that conjunctival epithelial cells express RIG-I and MDA5, and IPS-1, an adaptor molecule common to RIG-I and MDA5, contributes to polyI:C-inducible cytokine production in conjunctival epithelial cells.

1. Introduction

Innate immunity, the early host defense against microbes, is primarily studied in host immune-competent cells such as macrophages. The ability of cells to recognize pathogen-associated molecular patterns (PAMPs) depends on the expression of a family of Toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [1]. Although TLRs detect PAMPs either on the cell surface or the lumen of intracellular vesicles such as endosomes or lysosomes, RLRs and NLRs are cytosolic detection systems for intracellular PAMPs. RLRs belong to the RNA helicase family that specifically detects RNA species derived from viruses in the cytoplasm; they coordinate anti-viral programs via the induction of type I interferon (IFN) [1].

Elsewhere we reported that human ocular surface epithelium expresses TLR3, which recognizes double-stranded (ds) RNA mimicking polyinosine–polycytidylic acid (polyI:C) (a synthetic dsRNA), and that polyI:C stimulation induces the secretion of inflammatory cytokines such as IL (interleukin)-6, IL-8, and type I IFN such as IFN-β [2,3]. Moreover, our gene expression analysis of primary human conjunctival epithelial cells (PHCjECs) using oligonucleotide microarrays to examine the comprehensive effects of stimulation by polyI:C determined that transcripts including *CXCL11, RIG-I, IL28A, CXCL10, CCL5, CCL4, MDA5, IL7R, TSLP, CCL20*, and *ICAM-1*, were up-regulated more than 10-fold by polyI:C stimulation.

Abbreviations: IFN, interferon; IPS-1, IFN-β promoter stimulator-1; PAMPs, pathogen-associated molecular patterns; TLRs, Toll-like receptors; RIG-I, retinoic acid-inducible gene-I; RLRs, retinoic acid-inducible gene-I-like receptors; NOD, nucleotide-binding oligomerization domain; NLRs, nucleotide-binding oligomerization domain-like receptors; polyI:C, polyinosinic:polycytidylic acid; PHCjECs, primary human conjunctival epithelial cells; CXCL, chemokine (C–X–C motif) ligand; CCL, chemokine (C–C motif) ligand; MDA5, melanoma-differentiation-associated gene 5; TSLP, thymic stromal lymphopoietin; ICAM-1, intercellular adhesion molecule-1; Rsad2, radical *S*-adenosyl methionine domain containing 2; Cmpk2, cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial; Mx, myxovirus (influenza virus) resistance; Ifi44, interferon-induced protein 44; Ifi203, interferon-activated gene 203; ligp2, interferon-inducible GTPase 2; Rtp4, receptor transporter protein.

* Corresponding author at: Department of Ophthalmology, Kyoto Prefectural University of Medicine, 465 Kajiicho, Hirokoji, Kawaramachi, Kamigyoku, Kyoto 602-0841, Japan. Fax: +81 75 251 5663.
E-mail address: mueta@koto.kpu-m.ac.jp (M. Ueta).

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.11.136

This finding suggests that polyI:C stimulation could not only induce anti-viral innate immune responses, but that it could also regulate allergic reactions in PHCjECs [4,5].

The cytoplasmic helicase proteins, RIG-I (retinoic-acid-inducible protein I; also known as Ddx58) and MDA5 (melanoma-differentiation-associated gene 5; also known as Ifih1) have been reported to be implicated in the recognition of viral dsRNA. Our results show that new receptors that recognize dsRNA and polyI:C, RIG-I, and MDA5, are also expressed in PHCjECs [4,5].

Because, not only TLR3, but also RIG-I and MDA5 detect viral dsRNA, we examined the expression of RIG-I and MDA5 in human conjunctival epithelium, and investigated the function of IPS-1, an adaptor molecule common to RIG-I and/or MDA5, using IPS-1 KO mice to examine whether RIG-I and/or MDA5 contribute to polyI:C-inducible responses in conjunctival epithelium.

2. Materials and methods

2.1. Human conjunctival epithelial cells and PHCjECs

Our study was approved by the institutional review board of Kyoto Prefectural University of Medicine, Kyoto, Japan; all experiments were conducted in accordance with the tenets of the Declaration of Helsinki. Written informed consent was obtained from all patients after they were given a detailed explanation of the purpose of the research and the experimental protocols.

For RT-PCR we obtained conjunctival epithelial cells from healthy volunteers by brush cytology using previously described methods [6]. For immunoblot analysis and quantitative RT-PCR, PHCjECs harvested from conjunctival tissue obtained at conjunctivochalasis surgery were cultured as described elsewhere [7]. Briefly, conjunctival tissues were washed and immersed for 1 h at 37 °C in 1.2 U ml^{-1} purified dispase (Roche Diagnostic Ltd., Basel, Switzerland), and epithelial cells were detached, collected, and cultured in low-calcium defined keratinocyte-SFM medium containing defined growth-promoting additives (Invitrogen, Carlsbad, CA) including insulin, epidermal growth factor, fibroblast growth factor, and 1% antibiotic–antimycotic solution. Using this method, cell colonies usually became visible within 3–4 days. After they reached 80% confluence in 7–10 days, the cultured PHCjECs were used in subsequent procedures.

2.2. RT-PCR assay

Total RNA was isolated from human conjunctival epithelial cells with TRIzol (Invitrogen) according to the manufacturer's instructions. For RT-PCR we employed the SuperScript™ preamplification system (Invitrogen). Amplification was with DNA polymerase (Takara, Shiga, Japan) for 35 cycles at 94 °C for 1 min, 64 °C for 1 min, and 72 °C for 1 min for human RIG-I, MDA5, and TLR3 (GeneAmp®; PE Applied Biosystems, Foster City, CA, USA). The primers were: (forward) 5′-CCTATGCAGCTCCGCCTCGC-3′ and (reverse) 5′-GCCACGGAACCAGCCTTCCT-3′ for human RIG-I; (forward) 5′-GCAAGAGCATCCCCGGAGCC-3′ and (reverse) 5′-TCGTGGCCCC TCCAACACCA-3′ for human MDA5; (forward) 5′-CGCCAACTTCA-CAAGGTA-3′ and (reverse) 5′-GGAAGCCAAGCAAAGGAA-3′ for human TLR3; and (forward) 5′-CCATCACCATCTTCCAGGAG-3′ and (reverse) 5′-CCTGCTTCACCACCTTCTTG-3 for human GAPDH. The integrity of RNA was electrophoretically confirmed on ethidium bromide-stained 1.5% agarose gels.

2.3. Immunoblot analysis of the protein expression of RIG-I, MDA5, and TLR3

Protein expression in PHCjECs was examined by immunoblot analysis using a previously described method [8]. The cells were incubated for various periods with 25 μg/ml polyI:C, washed twice, and then lysed by immersion in 0.2 ml ice-cold CelLytic M Cell Lysis reagent (Sigma–Aldrich Corp., St. Louis, MO). SDS–PAGE was on a NuPAGE electrophoresis system (Invitrogen) according to the manufacturer's instructions. Separated proteins were transferred to a polyvinylidene difluoride membrane using the iBlot Gel Transfer device (Invitrogen) according to the manufacturer's instructions. The membrane was incubated in 5% skim milk with 0.1% Tween 20/TBS buffer for 1 h at room temperature for blocking. Immunoblot analysis was with rabbit anti-RIG-I or rabbit anti-MDA5 (Cell Signaling Technology, Inc., Danvers, MA) or rabbit anti-TLR3 antibodies (Abcam, Cambridge, MA) for first antibodies and with HRP-conjugated donkey anti-rabbit IgG antibodies (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for secondary antibodies. Protein detection was with the ECL Plus Western blotting Detection System (GE Healthcare).

2.4. Mice and reagents

C57BL/6 and Balb/c mice were purchased from CLEA (Tokyo, Japan); C57BL/6 background *IPS-1*$^{-/-}$ mice [9] and Balb/c background *TLR3*$^{-/-}$ mice [10] were a gift from Professor Akira. Wild-type C57BL/6 and Balb/c mice were the respective controls. All mice were used at 8–12 weeks of age; they were maintained on a 12-h light/dark cycle under specific pathogen-free conditions. All experimental procedures were approved by the Committee on Animal Research of Kyoto Prefectural University of Medicine, Kyoto, Japan and all studies were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

PolyI:C was purchased from Invivogen (San Diego, CA). For the *in vivo* analysis of murine conjunctival epithelial cells we prepared a 100 μg/ml polyI:C solution in 50% VISCOAT® (Alcon Laboratories Ltd., Fort Worth, TX)/PBS to assure the *in vivo* stimulation by polyI:C of murine conjunctival epithelium. VISCOAT® is an ophthalmic viscosurgical product used in cataract surgery; it is a sterile, non-pyrogenic, viscoelastic solution of highly purified, non-inflammatory medium molecular-weight sodium chondroitin sulfate and sodium hyaluronate. PolyI:C solution (10 μl) was injected subcon junctivally and dropped into the eyes, respectively.

2.5. Quantitative reverse transcription polymerase chain reaction (RT-PCR) assay

Total RNA was isolated from murine conjunctival epithelium and PHCjECs using the RNeasy Mini kit (QIAGEN, Valencia, CA)

Table 1
The primers for quantitative RT-PCR assay of murine samples.

Gene	Primer for quantitative RT-PCR: forward and reverse
Cxcl10	5′-TGAATCCGGAATCTAAGACCATCAA-3′
	5′-AGGACTAGCCATCCACTGGGTAAAG-3′
Mx1	5′-CTGAGATGACCCAGCACCTGAA-3′
	5′-CTCCAGGAACCAGCTGCACTTAC-3′
Rsad2	5′-TGGGGATGCTGGTGCCCACT-3′
	5′-ACCCCGGACCTGTGGCTGTT-3′
Ifi44	5′-TCGGTGCCAGCCTCGGTACA-3′
	5′-TGCAGGCACTTTTCTTGCTGCCA-3′
Iigp2	5′-TTCCCCACAGGACCCTGCCC-3′
	5′-AGCAACGGGGCAACCTGCTC-3′
Cmpk2	5′-CCTTGCGACCACCCGGACAC-3′
	5′-ATGCGCAGTCCACCTGCACC-3′
Ccl5	5′-CACCACTCCCTGCTGCTT-3′
	5′-ACACTTGGCGGTTCCTTC-3′
Rtp4	5′-GGGATGGAGGCAGCACCAGC-3′
	5′-CGTGCCTGGCCCTGCGATTT-3′
Ifi203	5′-TGTGAGAGAATTAAGGCACCAAGGAGA-3′
	5′-ACTTTCAACACCATCACTTGTTTGGGA-3′
Mx2	5′-GTGCGGCCCTGCATTGACCT-3′
	5′-GGCCACTCCAGACAGTGCTTCTAGT-3′

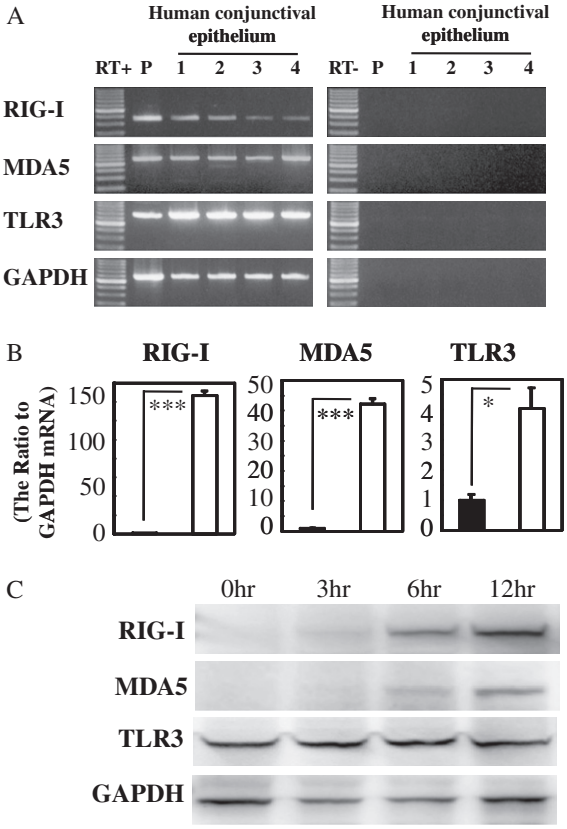

Fig. 1. Up-regulation of RIG-I, MDA5, and TLR3 expression in human conjunctival epithelial cells stimulated with polyI:C. (A) We examined the expression of RIG-I, MDA5, and TLR3 in human conjunctival epithelial cells by RT-PCR assay. The positive control was mRNA isolated from human mononuclear cells. The expected length of PCR products for RIG-I (360 bp), MDA5 (601 bp), and TLR3 (689 bp) was obtained from the positive control and the human conjunctival epithelial cells. (B) We used quantitative RT-PCR assay to examine changes in the mRNA expression of the *RIG-I*, *MDA5*, and *TLR3* genes after the stimulation of PHCjECs with polyI:C. The quantification data were normalized to the expression of the housekeeping gene GAPDH. The *Y* axis shows the increase in specific mRNA over unstimulated samples. Data are representative of 3 separate experiments and show the mean ± SEM from one experiment carried out in 3 wells per group. (C) Up-regulation by polyI:C stimulation of the protein expression of RIG-I and MDA5 in PHCjECs. Data are representative of 3 independent experiments.

according to the manufacturer's instructions. For the RT reaction we used the SuperScript™ Preamplification kit (Invitrogen). Quantitative RT-PCR was on an ABI-prism 7700 instrument (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The primers for the murine samples are shown in Table 1. The primers and probes for human RIG-I, MDA5, and TLR3 were purchased from Applied Biosystems. The results were analyzed with sequence detection software (Applied Biosystems). Quantification data were normalized to the expression of the housekeeping gene GAPDH.

3. Results

3.1. RT-PCR assay of the expression of RIG-I, MDA5, and TLR3 in human conjunctival epithelial cells

The expected length of the PCR products for RIG-I (360 bp), MDA5 (601 bp), and TLR3 (689 bp) was obtained from the positive controls (mRNA isolated from human mononuclear cells) and from conjunctival epithelial cells, suggesting that human conjunctival epithelial cells express RIG-I, MDA5, and TLR3 mRNA (Fig. 1A). To confirm the specificity for the detection of RIG-I, MDA5, and TLR3 mRNA we isolated and sequenced the PCR products. The obtained sequences were identical to the human RIG-I, MDA5, and TLR3 mRNA sequence.

3.2. Up-regulation of the expression of RIG-I, MDA5 and TLR3 in polyI:C-stimulated human conjunctival epithelial cells

The expression of mRNA in *RIG-I, MDA5,* and *TLR3* genes was up-regulated by polyI:C stimulation (Fig. 1B). Up-regulation was particularly pronounced in *RIG-I* and *MDA5*. Immunoblot analysis of polyI:C-stimulated PHCjECs to examine changes in the protein expression of RIG-I, MDA5, and TLR3 showed marked up-regulation in RIG-I and MDA5 but not TLR3 (Fig. 1C).

3.3. Up-regulated transcripts in polyI:C-stimulated murine conjunctival epithelium

For gene expression analysis of polyI:C-stimulated murine conjunctival epithelium, polyI:C was delivered by eye drops and subconjunctival injection.

We selected the top 15 genes of the ratio of polyI:C-administered sample signal: vehicle-administered sample signal (data not shown) and focused on the 10 genes whose expression was confirmed to be up-regulated more than 5-fold in polyI:C-stimulated samples compared to unstimulated samples using quantitative RT-PCR (Fig. 2). They were *Rsad2* (radical *S*-adenosyl methionine domain containing 2), *Cxcl10* (chemokine (C–X–C motif) ligand 10), *Cmpk2* (cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial), *Mx1* (myxovirus (influenza virus) resistance 1), *Ifi44* (interferon-induced protein 44), *Mx2* (myxovirus (influenza virus) resistance 2), *Ifi203* (interferon-activated gene 203), *Iigp2* (interferon-inducible GTPase 2), *Rtp4* (receptor transporter protein 4), and *Ccl5* (chemokine (C–C motif) ligand 5).

3.4. Change in the expression of transcripts in murine polyI:C-stimulated conjunctival epithelium

Quantitative RT-PCR to compare the gene expression of the above 10 transcripts upon polyI:C stimulation showed that all but *Ccl5* were significantly down-regulated in the conjunctival epithelium of IPS-1 KO compared with wild-type mice (Fig. 3A).

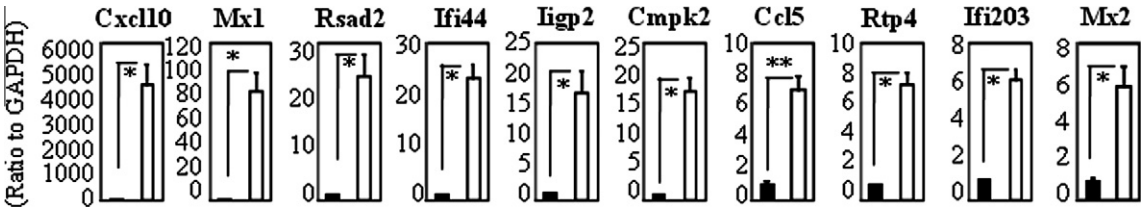

Fig. 2. Transcripts up-regulated in murine polyI:C-stimulated conjunctival epithelium. In murine conjunctival epithelium, the expression of mRNA in 10 transcripts up-regulated by 6-h stimulation with polyI:C was 5-fold higher than in unstimulated samples. The quantification data were normalized to the expression of the housekeeping gene GAPDH. The *Y* axis shows the increase in specific mRNA over unstimulated samples. Data are representative of 3 separate experiments and show the mean ± SEM from one experiment carried out in 3 mice per group (*$p < 0.05$, **$p < 0.005$).

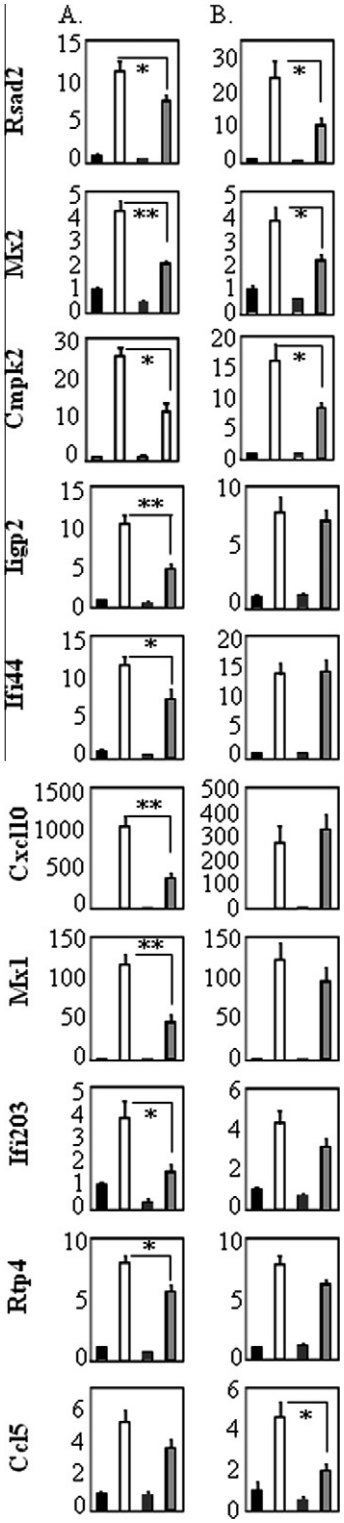

Fig. 3. Comparison of changes in the expression of transcripts induced by the stimulation of conjunctival epithelium with polyI:C in wild-type (C57BL/6)- and IPS-1 KO mice and in wild-type (Balb/c)- and TLR3 KO mice. Expression of mRNA in 10 transcripts up-regulated by 6-h stimulation of conjunctival epithelium with polyI:C in wild-type (C57BL/6)- and IPS-1 KO mice (A), and in wild-type (Balb/c)- and TLR3 KO mice (B). The quantification data were normalized to the expression of the housekeeping gene GAPDH. The *Y* axis shows the increase in specific mRNA over unstimulated samples. Data are representative of 3 separate experiments and show the mean ± SEM from one experiment carried out in 6 mice per group ($^{*}p < 0.05$, $^{**}p < 0.005$).

PolyI:C could be recognized not only by RIG-I and MDA5, whose common adaptor molecule is IPS-1, but also by TLR3 whose adaptor molecule is TRIF. To compare the gene expression of the above 10 polyI:C-induced transcripts in wild-(Balb/c) and TLR3 KO mice, we performed quantitative RT-PCR. We found that *Rsad2, Mx2, Cmpk2*, and *Ccl5*, but not *Cxcl10, Mx1, Ifi44, Ifi203, Iigp2,* and *Rtp4* were significantly down-regulated in the conjunctival epithelium of TLR3 KO compared to wild-type mice (Fig. 3B).

Our results suggest that *Cxcl10*, *Mx1*, *Ifi44*, *Ifi203*,*Iigp2*, and *Rtp4* were dominantly regulated by RIG-I or/and MDA5, that *Ccl5* were dominantly regulated by TLR3, and that *Rsad2*, *Mx2*, and *Cmpk2* were regulated by both TLR3 and RIG-I or/and MDA5.

4. Discussion

Our results demonstrate that conjunctival epithelial cells express TLR3, RIG-I and MDA5, and that not only TLR3 but also RIG-I and/or MDA5 contribute to polyI:C-inducible immune responses because *Rsad2, Mx2, Cmpk2*, *Cxcl10, Mx1, Ifi44, Ifi203, Iigp2*, and *Rtp4* were significantly down-regulated in conjunctival epithelial cells of IPS-1 knock-out compared to control mice. Moreover, *Rsad2, Mx2, Cmpk2*, and *Ccl5*, but not*Cxcl10, Mx1, Ifi44, Ifi203, Iigp2,* and *Rtp4* were also significantly down-regulated in the conjunctival epithelium of TLR3 KO compared to wild-type mice.

We posit that *Cxcl10, Mx1, Ifi44, Ifi203, Iigp2*, and *Rtp4* are dominantly regulated by IPS-1 (RIG-I or/and MDA5), *Ccl5* by TLR3, and that *Rsad2*, *Mx2*, and *Cmpk2* are regulated by both TLR3 and IPS-1 (RIG-I or/and MDA5). Our studies indicate that IPS-1 signaling plays an important role in eliciting innate immune responses in conjunctival epithelial cells.

Although the TLR family detects PAMPs either on the cell surface or in the lumen of intracellular vesicles such as endosomes or lysosomes, recent studies have shown the existence of a cytosolic detection system for intracellular PAMPs [1]. These cytosolic PRRs include RLRs and NLRs. RLRs, which include RIG-I and MDA5, belong to the RNA helicase family that specifically detects RNA species derived from viruses in the cytoplasm; they coordinate anti-viral programs via the induction of type I IFN [1].

According to Lai et al. [11] following injury, TLR3 is required for part of the normal inflammatory response in the skin epithelium; TLR3-deficient mice produced significantly less IL-6 and TNF-α at the wound edge than wild-type controls. Torres et al. [12] reported that TLR3/TRIF-dependent pathway activation was required for the development of the dsRNA-induced exacerbation of allergic reactions; they were reduced in TRIF (−/−) mice. They also found that *in vivo* and *in vitro* treatment with dsRNA amplified airway epithelial cytokine production. Our findings document that *Ccl5* were dominantly regulated by TLR3, and that *Rsad2*, *Mx2*, and *Cmpk2* were regulated by TLR3 and IPS-1 (RIG-I or/and MDA5); *Rsad2*, *Mx2*, *Cmpk2*, and *Ccl5* were significantly down-regulated in conjunctival epithelium of TLR3 KO compared with wild-type mice. These observations document that TLR3 contributes to the production of cytokine and to inflammation.

In intestinal epithelial cells, the production and expression of polyI:C-inducible type I IFN were dependent on the activation of RIG-I and IPS-1 [13]. We found that *Cxcl10, Mx1, Ifi44, Ifi203, Iigp2*, and *Rtp4* were dominantly regulated by IPS-1 (RIG-I or/and MDA5), and that *Rsad2*, *Mx2*, and *Cmpk2* were regulated by both TLR3 and IPS-1 (RIG-I or/and MDA5); *Rsad2*, *Cxcl10, Mx1, Ifi44, Mx2, Cmpk2, Ifi203, Iigp2*, and *Rtp4* were significantly down-regulated in conjunctival epithelium of IPS-1 KO compared to wild-type mice.

Moreover, polyI:C incorporated by dendritic cells is accessible to both TLR3 and MDA5; they activate respective signaling pathways for the robust induction of cytokines [14]. Similarly, in conjunctival

epithelial cells, polyI:C-inducible cytokine production requires signaling through both TLR3 and IPS-1 (RIG-I or/and MDA5).

Rsad2 is an interferon-inducible protein that inhibits many DNA and RNA viruses [15]. *Mx2* is also an interferon-regulated gene that selectively inhibits hanta virus replication [16]. *Cmpk2*, a pyrimidine nucleoside monophosphate kinase, is thought to be involved in macrophage activation and inflammatory responses [17].

In our study, *Rsad2,* and *Mx2*, which exert anti-viral actions, and *Cmpk2* involving in inflammatory responses, were regulated by TLR3 and IPS-1 (RIG-I or/and MDA5).

Iigp2 plays a cell-autonomous role in IFN-γ-mediated chlamydia inhibition [18] and *Ifi44* is associated with hepatitis C virus infection although its function is unknown [19]. *Cxcl10* is expressed primarily in response to a wide range of DNA and RNA viruses; it plays a role in the recruitment of leukocytes during inflammation [20]. Its expression is also increased in allergic diseases; it was elevated in the epidermis of patients with atopic dermatitis [21] and in the bronchoalveolar lavage fluid of patients with severe asthma [22]. *Mx1* is an interferon-regulated gene that selectively interferes with the multiplication of influenza viruses [23]. *Ifi203*, a member of the *Ifi-200* gene family, is induced by type I and type II interferons; it has been reported as a regulator of cell proliferation and differentiation, and plays a role in apoptotic and inflammatory processes [24]. *Rtp4* is a member of the receptor transport protein (RTP) family; it participates in the export of odorant and taste receptors [25]. We document that these 6 transcripts (*Cxcl10, Mx1, Ifi44, Ifi203, Iigp2*, and *Rtp4*) are dominantly regulated by IPS-1 (RIG-I or/and MDA5).

Like *Cxcl10*, *Ccl5* is up-regulated in the presence of viral infection [26]; it is also up-regulated in the skin lesions of chronic atopic dermatitis [27]. Our study shows that *Ccl5*, which play a role in inflammation and allergy, were dominantly regulated by TLR3. Elsewhere we reported that TLR3 regulated the late-phase reaction of experimental allergic conjunctivitis in a mouse model; eosinophilic conjunctival inflammation was reduced in TLR3 KO mice and exacerbated in TLR3 transgenic mice [10]. These findings suggest that TLR3 can induce anti-viral innate immune responses and that it exerts other functions such as the regulation of allergic reactions. Others [28] reported that in the absence of viral infection TLR3 amplified immune responses during acute inflammatory processes, a phenomenon that may involve TLR3 stimulation by endogenous RNA from necrotic cells. Innate immunity can respond to endogenous molecules released by host cells as a result of necrosis, pathogen infection, damage, injury, and certain pathological conditions that are directly or indirectly recognized by TLRs, NLRs, RLRs, and by yet to be identified sensors [1]. It is possible that endogenous RNA from tissues or cells may stimulate not only TLR3 but also RIG-I or/and MDA5.

In summary, our results showed that conjunctival epithelial cells express RIG-I and MDA5, and IPS-1, an adaptor molecule common to RIG-I and MDA5, contributes to polyI:C-inducible cytokine production in conjunctival epithelial cells.

Acknowledgments

This work was supported in part by grants-in-aid for scientific research from the Japanese Ministry of Health, Labour and Welfare, the Japanese Ministry of Education, Culture, Sports, Science and Technology, the Kyoto Foundation for the Promotion of Medical Science, the National Institute of Biomedical Innovation of Japan, and the Intramural Research Fund of Kyoto Prefectural University of Medicine, and the Shimizu Foundation for Immunological Research Grant.

References

[1] T. Kawai, S. Akira, The roles of TLRs, RLRs and NLRs in pathogen recognition, Int. Immunol. 21 (2009) 317–337.
[2] M. Ueta, J. Hamuro, H. Kiyono, S. Kinoshita, Triggering of TLR3 by polyI:C in human corneal epithelial cells to induce inflammatory cytokines, Biochem. Biophys. Res. Commun. 331 (2005) 285–294.
[3] M. Ueta, S. Kinoshita, Innate immunity of the ocular surface, Brain Res. Bull. 81 (2010) 219–228.
[4] M. Ueta, S. Kinoshita, Ocular surface inflammation mediated by innate immunity, Eye Contact Lens 36 (2010) 269–281.
[5] M. Ueta, K. Mizushima, N. Yokoi, Y. Naito, S. Kinoshita, Gene-expression analysis of polyI:C-stimulated primary human conjunctival epithelial cells, Br. J. Ophthalmol. (2010).
[6] K. Kojima, M. Ueta, J. Hamuro, Y. Hozono, S. Kawasaki, N. Yokoi, et al., Human conjunctival epithelial cells express functional Toll-like receptor 5, Br. J. Ophthalmol. 92 (2008) 411–416.
[7] M. Ueta, K. Mizushima, N. Yokoi, Y. Naito, S. Kinoshita, Expression of the interleukin-4 receptor alpha in human conjunctival epithelial cells, Br. J. Ophthalmol. 94 (2010) 1239–1243.
[8] M. Ueta, K. Mizushima, N. Yokoi, Y. Naito, S. Kinoshita, Gene-expression analysis of polyI:C-stimulated primary human conjunctival epithelial cells, Br J Ophthalmol (2010).
[9] T. Kawai, K. Takahashi, S. Sato, C. Coban, H. Kumar, H. Kato, et al., IPS-1, an adaptor triggering RIG-I- and MDA5-mediated type I interferon induction, Nat. Immunol. 6 (2005) 981–988.
[10] M. Ueta, S. Uematsu, S. Akira, S. Kinoshita, Toll-like receptor 3 enhances late-phase reaction of experimental allergic conjunctivitis, J. Allergy Clin. Immunol. 123 (2009) 1187–1189.
[11] Y. Lai, A. Di Nardo, T. Nakatsuji, A. Leichtle, Y. Yang, A.L. Cogen, et al., Commensal bacteria regulate Toll-like receptor 3-dependent inflammation after skin injury, Nat. Med. 15 (2009) 1377–1382.
[12] D. Torres, A. Dieudonne, B. Ryffel, E. Vilain, M. Si-Tahar, M. Pichavant, et al., Double-stranded RNA exacerbates pulmonary allergic reaction through TLR3: implication of airway epithelium and dendritic cells, J. Immunol. 185 (2010) 451–459.
[13] Y. Hirata, A.H. Broquet, L. Menchen, M.F. Kagnoff, Activation of innate immune defense mechanisms by signaling through RIG-I/IPS-1 in intestinal epithelial cells, J. Immunol. 179 (2007) 5425–5432.
[14] H. Kumar, S. Koyama, K.J. Ishii, T. Kawai, S. Akira, Cutting edge: cooperation of IPS-1- and TRIF-dependent pathways in poly IC-enhanced antibody production and cytotoxic T cell responses, J. Immunol. 180 (2008) 683–687.
[15] G. Shaveta, J. Shi, V.T. Chow, J. Song, Structural characterization reveals that viperin is a radical *S*-adenosyl-L-methionine (SAM) enzyme, Biochem. Biophys. Res. Commun. 391 (2010) 1390–1395.
[16] H.K. Jin, K. Yoshimatsu, A. Takada, M. Ogino, A. Asano, J. Arikawa, et al., Mouse Mx2 protein inhibits hantavirus but not influenza virus replication, Arch. Virol. 146 (2001) 41–49.
[17] Y. Xu, M. Johansson, A. Karlsson, Human UMP-CMP kinase 2, a novel nucleoside monophosphate kinase localized in mitochondria, J. Biol. Chem. 283 (2008) 1563–1571.
[18] I. Miyairi, V.R. Tatireddigari, O.S. Mahdi, L.A. Rose, R.J. Belland, L. Lu, et al., The p47 GTPases Iigp2 and Irgb10 regulate innate immunity and inflammation to murine *Chlamydia psittaci* infection, J. Immunol. 179 (2007) 1814–1824.
[19] L.C. Hallen, Y. Burki, M. Ebeling, C. Broger, F. Siegrist, K. Oroszlan-Szovik, et al., Antiproliferative activity of the human IFN-alpha-inducible protein IFI44, J. Interferon Cytokine Res. 27 (2007) 675–680.
[20] J.M. Farber, Mig and IP-10: CXC chemokines that target lymphocytes, J. Leukoc. Biol. 61 (1997) 246–257.
[21] S. Klunker, A. Trautmann, M. Akdis, J. Verhagen, P. Schmid-Grendelmeier, K. Blaser, et al., A second step of chemotaxis after transendothelial migration: keratinocytes undergoing apoptosis release IFN-gamma-inducible protein 10, monokine induced by IFN-gamma, and IFN-gamma-inducible alpha-chemoattractant for T cell chemotaxis toward epidermis in atopic dermatitis, J. Immunol. 171 (2003) 1078–1084.
[22] S. Ying, B. O'Connor, J. Ratoff, Q. Meng, C. Fang, D. Cousins, et al., Expression and cellular provenance of thymic stromal lymphopoietin and chemokines in patients with severe asthma and chronic obstructive pulmonary disease, J. Immunol. 181 (2008) 2790–2798.
[23] M.A. Horisberger, Interferons, Mx genes, and resistance to influenza virus, Am. J. Respir. Crit. Care Med. 152 (1995) S67–S71.
[24] M. Mondini, S. Costa, S. Sponza, F. Gugliesi, M. Gariglio, S. Landolfo, The interferon-inducible HIN-200 gene family in apoptosis and inflammation: implication for autoimmunity, Autoimmunity 43 (2010) 226–231.
[25] H. Saito, M. Kubota, R.W. Roberts, Q. Chi, H. Matsunami, RTP family members induce functional expression of mammalian odorant receptors, Cell 119 (2004) 679–691.
[26] C. Prehaud, F. Megret, M. Lafage, M. Lafon, Virus infection switches TLR-3-positive human neurons to become strong producers of beta interferon, J. Virol. 79 (2005) 12893–12904.
[27] E. Gros, C. Bussmann, T. Bieber, I. Forster, N. Novak, Expression of chemokines and chemokine receptors in lesional and nonlesional upper skin of patients with atopic dermatitis, J. Allergy Clin. Immunol. (2009).
[28] K.A. Cavassani, M. Ishii, H. Wen, M.A. Schaller, P.M. Lincoln, N.W. Lukacs, et al., TLR3 is an endogenous sensor of tissue necrosis during acute inflammatory events, J. Exp. Med. 205 (2008) 2609–2621.