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SIGIRR inhibits toll-like receptor 4, 5, 9-mediated immune responses in human airway epithelial cells

Chun Zhang · Xueling Wu · Yunfeng Zhao · Zhaoxia Deng · Guisheng Qian

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Abstract Human airway epithelial cells (HAEC) may contribute to acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) through toll-like receptors (TLRs)-mediated molecular mechanisms. TLRs exist on the surface of HAEC where binding to their cognate ligands initiates airway inflammation. Single immunoglobulin interleukin-1 receptor-related protein (SIGIRR) is a member of the toll-interleukin-1 receptor (TIR) family that can negatively modulate the immune response. We carried out studies to characterize SIGIRR modulation of TLR-mediated immune response in HAEC and to define its mechanisms of action. Following treatment with various concentrations of LPS, flagellin and CpG DNA, the levels of cognate TLRs 4, 5, and 9 were measured in the supernatants of HAEC over-expressing the SIGIRR molecule. Moreover, the interaction of the TLR adaptor myeloid differentiation factor 88 (MyD88) with SIGIRR in response to LPS-, flagellin- and CpG DNA-stimulation was examined by co-immunoprecipitation. The findings from this study revealed that overexpression of SIGIRR in HAEC stimulated by LPS, flagellin or CpG DNA resulted in attenuated production of the inflammatory mediators IL-6 and TNF-α. This attenuation was not the result of decreased expression of TLR4, 5 or 9, but rather a sequestration of MyD88 to the TLRs. In conclusion, SIGIRR can inhibit TLR4, 5, and 9-mediated immune responses in HAEC and may be a valuable therapeutic target for the prevention of ALI/ARDS.

Keywords SIGIRR · Toll-like receptor · Human airway epithelial cell · MyD88 · Proinflammatory cytokines · Inflammation

Introduction

Human airway epithelial cells (HAEC) comprise the first line of lung defence against local and primary immune challenges; as such, HAEC play an important role in the development of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) [1]. Excessive activation of toll-like receptors (TLRs) located on the surface of HAEC can lead to overstimulation of the host inflammatory process, whereby normal immune response to an infection become detrimental. ALI and ARDS develop as a consequence of an uncontrolled hyperinflammatory response which is predominantly characterized by excessive cytokine release from HAEC.

Microbe-derived pathogen-associated molecular pattern (PAMP) molecules have been shown to induce innate immune responses in human bronchus via TLR recognition [2]. Binding of microbial antigens to their cognate TLRs

Chun Zhang and Xueling Wu contributed equally to this work.

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C. Zhang · X. Wu · G. Qian (✉)
Institute of Respiratory Medicine, Xinqiao Hospital, Third Military Medical University, Chongqing 400037, People's Republic of China
e-mail: qiangs001@yahoo.com.cn

Y. Zhao
Department of Respiratory Medicine, The Affiliated Zhongda Hospital of Southeast University, Nanjing 210009, People's Republic of China

Z. Deng
Department of Intensive Care Unit, SouthWest Hospital, Third Military Medical University, Chongqing 400039, People's Republic of China

leads to gene expression of nuclear factor κB (NF-κB) and activator protein-1(AP-1) transcription factors and activation of the interferon regulatory factor (IRF)3/7 defence signalling pathways [3]. Ultimately, transcription of proinflammatory genes, whose protein products are implicated in the development of airway inflammation (IL-6, TNF-α, MCP-1, IFN-γ), is initiated and leukocytes are recruited and activated at the inflammatory site. The local cytokine milieu, therefore, is likely to play an important role in the modulation of bronchial inflammation and in the appearance of allergic symptoms [4].

TLRs induce inflammatory reactions by activating signalling pathways mediated by TIR domain-containing adaptor proteins, such as myeloid differentiation factor 88 (MyD88), toll-interleukin-1 receptor (TIR) domain containing adaptor protein (TIRAP), toll/IL-1-receptor domain-containing adaptor inducing IFN (TRIF) and TRIF-related adaptor protein (TRAM) [5–7]. Among these adaptors, MyD88 is the primary protein for signal transduction to gene transcription pathways involving the IL-1 receptor family and all TLRs, except TLR3 [8]. It has been suggested that MyD88 particularly mediates innate immune-related functions, whereas TRIF-mediated adaptive function appears to be involved in innate and adaptive immunity [9, 10]. Genetic ablation of MyD88 signalling pathways prevented hyper-inflammation and attenuated the pathogenic consequences of sepsis [11]. The evolutionary relationship of toll-like cytoplasmic domains, identified by in silico homology search using MyD88 as a database query sequence, has indicated that SIGIRR is a close relative of MyD88 and both share a proximal common ancestor with the IL-1R/IL-18R/ST2 family of TIR-bearing receptors [12, 13]. SIGIRR has a highly conserved TIR domain, but it deviates from complete homology to IL-1R by two amino acids (Ser447 and Tyr536) that have been shown to be essential for signalling [14]. Overexpression of SIGIRR by dendritic cells inhibits IL-1- and IL-18-mediated NF-κB activation [15]. In vivo, SIGIRR-deficient mice generated on the BALB/c background presented with increased susceptibility to endotoxin shock [16]. Moreover, SIGIRR promotes resistance against *Pseudomonas aeruginosa* corneal infection by down-regulating type 1 immunity [17]. Histological analysis of colon sections from gut-epithelial-specific SIGIRR-transgenic mice revealed significantly reduced inflammation and less tissue damage as compared to SIGIRR$^{-/-}$ mice [18]. Taken together, the results of these studies demonstrate that SIGIRR is a key molecule in the control of TLR-mediated inflammation; however, little is known about the role of SIGIRR in HAEC. More importantly, the detailed mechanism by which SIGIRR suppresses TLR function remains unclear. Our study was designed to determine whether and how SIGIRR inhibits TLRs-mediated innate immune response in HAEC.

We investigated the expression of inflammatory cytokines in SIGIRR over-expressing H292 cells, a cell line similar to HAEC after stimulation with LPS, flagellin and CpG DNA. Subsequently, the potential link between SIGIRR and MyD88 was examined in H292 cells by co-immunoprecipitation assay.

Materials and methods

Reagents

Fetal bovine serum (FBS) and all other cell culture reagents were obtained from HyClone (Logan, UT, USA). Flagellin and lipopolysaccharide (LPS) from *Escherichia coli* serotype O55:B5 was purchased from ALEXIS Biochemicals (San Diego, CA) and Sigma (St. Louis, MO), respectively. The CpG DNA 2006 oligonucleotide (5′-TCGTCGTTT TGTCGTTTTGTCGTT-3′) was synthesized in a phosphorothioate-modified form by SBS Genetech Co., Ltd (Beijing, China). Antibodies to SIGIRR (AF990; R&D Systems, Minneapolis, MN), MyD88 (CSA-510; Stressgen, San Diego, CA), TLR4 (MAB1478; R&D Systems), TLR5 (19d759.2; Abcam, Cambridge, UK), TLR9 (14-9099; eBioscience, San Diego, CA) and β-ACTIN (sc-1616; Santa Cruz Biotech Inc, Santa Cruz, CA) were used for Western-blot assay. Secondary antibodies of peroxidase-conjugated Affinipure rabbit anti-goat IgG (H + L), goat anti-rat or mouse IgG (H + L) and goat anti-mouse IgG (H + L) were purchased from Beijing Golden Bridge Biotech (Beijing, China). SYBR PrimeScript™ reverse transcription polymerase chain reaction (RT-PCR) kit was purchased from TaKaRa Bio, Inc. (Dalian, China). Phenylmethylsulfonyl fluoride (PMSF), Tween 20, Triton X-100, Nonidet 40 (NP-40) were each purchased from Sigma.

Cell culture

The human lung mucoepidermoid carcinoma cell line (NCI-H292) (CRL-1848; ATCC, Manassas, VA) was cultured in RPMI 1640 (Invitrogen, Paisley, UK) supplemented with 10% FBS in the presence of penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified chamber with 5% CO_2. For serum deprivation, confluent cells were washed twice with phosphate-buffered saline (PBS) and re-cultured in RPMI 1640 with 0.2% FBS. The recombinant stable cell lines were cultured with 400 μg/ml G418 sulfate (Amersco Inc, Solon, OH) in the medium.

Plasmid construction and transfection

The SIGIRR gene was cloned into the pEGFP-N1 expression plasmid (Clontech Europe, St. Germain-en-Laye, France) by placing the SIGIRR in-frame with the N-terminus of the EGFP protein after a six-amino acid linker sequence of LGCRRW. SIGIRR DNA fragments were generated by PCR from the plasmid pReceiver-Lv19 (EX-V0554; GeneCopoeia, Inc., Guangzhou, China) using the following pair of primers: 5′-TAG CTA GAA TTC TGA TGC CAG GTG TCT GTG AT-3′ (forward) and 5′-AGT CAG GAT CCC GCA TAT CAT CC T TGG ACA CC-3′ (reverse). The PCR product was digested with *EcoRI*/*BamH*I restriction enzymes and inserted into the pEGFP-N1 vector to produce the recombinant plasmid SIGIRR-EGFP. Cells were transfected with SIGIRR-EGFP, or the empty vector control pEGFP-N1, with Lipofectamine 2000^{TM} transfection reagent (Invitrogen) according to the manufacturer's instructions; stably transfected cell lines were selected with 800 μg/ml G418 (Amersco). Cell lines were designated as: H292, wildtype; EH292, transfected with empty vector pEGFP-N1; RH292, transfected with recombinant plasmid SIGIRR-EGFP.

RNA extraction and real-time quantitative RT-PCR

Total RNA was isolated using the TRIZOL reagent (Invitrogen), according to the manufacturer's protocol. The expression of TLR4, 5, and 9 and SIGIRR mRNAs in H292 cells were assessed by real-time quantitative RT-PCR as previously described [19], including both a negative control and a positive (housekeeping) gene control. Oligonucleotide primers were obtained from TaKaRa Bio, Inc. (supplemental Table 1). SIGIRR mRNA was compared between two stably over-expressing H292 cell lines by using a double standard curves relative quantitation, and the results were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control. Real-time reverse transcription was performed on a Rotor-Gene 3000 (Corbett Research, Australia). The PCR parameters were set as 95°C for 2 min, followed by 40 cycles of 95°C for 5 s and 60°C for 20 s. All reactions were performed in triplicate, and reports were generated by Rotor-Gene Real-Time Analysis Software 6.0.

Immunocytochemistry

RH292, EH292, and H292 cell monolayers were processed for analysis by confocal microscopy. At 48 h post-transfection, cell monolayers were fixed with 4% paraformaldehyde in PBS, incubated with 5 μg/ml DAPI (Beyotime Institute of Biotechnology, Jiangsu, China) and mounted on slides. Furthermore, RH292 cells were fixed with 4% paraformaldehyde for 20 min, and permeabilized with 0.1% Triton X-100 for 30 min. Cells were incubated with or without primary antibody (1:250) (anti-SIGIRR) overnight at 4°C, rinsed in PBS, then incubated with secondary antibody conjugated to Rhodamine (TRITC) at 1:1000 dilution (Beijing Golden Bridge Biotech) for 90 min at 37°C. After several rinses, RH292 cells were counterstained with DAPI for 2 min and mounted on glass slides. Samples were observed using a confocal Leica TCS SP5 (Leica Microsystems, Wetzlar, Germany).

Inflammatory cytokines assay

RH292, EH292 and H292 cells were plated at 1×10^6 cells/well in 12-well flat bottom tissue culture plates. When growth reached 70–80% confluence, the cells were cultured for a further 24 h in serum deprivation conditions, as described above. Finally, the cells were treated for 6 h with different stimuli at various concentrations, including flagellin (1, 0.2 and 0.05 μg/ml), LPS (1, 0.2 and 0.05 μg/ml) and CpG DNA 2006 (1, 0.2 and 0.05 μM). Subsequently, concentrations of IL-6 and TNF-α in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA; Jingmei Biotech Co. Ltd, Shenzhen, China). At least three sets of replicates were examined for each experimental group.

Western-blot analysis

RH292, EH292 and H292 cells were treated with different stimuli including LPS (1 μg/ml), flagellin (0.1 μg/ml) and CpG DNA 2006 (0.5 μM) for 24 h. At 0, 1, 6, or 24 h post-stimulation, the expression of TLR4, 5, and 9 and SIGIRR were detected. Briefly, cells were harvested, washed in cold PBS buffer, pelleted by centrifugation, and lysed in ice-cold lysis buffer (30 mM Tris-HCl, pH7.5, 150 mM NaCl, 1% NP-40 and 1 mM PMSF) for 30 min. Cell debris was pelleted by centrifugation for 10 min at $13{,}000 \times g$. Supernatants were separated on 10% SDS-PAGE, transferred to nitrocellulose membrane (Millipore, Bedford, MA) and blocked in a 5% solution of nonfat dry milk prepared in 1 × PBS with 0.05% Tween 20. Blots were incubated with primary antibody diluted in PBS for overnight at 4°C, after which they were washed three times for 10 min each with PBS, detected with horseradish peroxidase-conjugated secondary antibody diluted 1:5000 in PBS + 5% nonfat milk, and developed using the enhanced chemiluminescence method (ECL Plus; Amersham Biosciences, Little Chalfont, UK) following the manufacturer's protocol.

Co-immunoprecipitation

RH292, EH292 and H292 cells were stimulated for 6 h with LPS (1 μg/ml), or flagellin (0.1 μg/ml), or CpG DNA 2006 (0.5 μM), followed by incubation with lysis buffer as described [20]. Cell extracts were incubated with 1 μg SIGIRR antibody overnight at 4°C with 20 μl protein A-Sepharose beads (Pharmacia Biotech, Inc., Piscataway, NJ). After incubation, the beads were washed four times with lysis buffer, separated by SDS-PAGE, transferred to Immobilon-P membranes (Millipore) and analyzed by immunoblotting.

Statistical analysis

All results have been presented as means ± SD. Student *t* tests were used to compare two groups; ANOVA was used with Tukey's multiple comparison for multiple groups. Values of $P < 0.05$ were regarded as statistically significant. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software for Windows, version 11.0 (SPSS Inc, Chicago, IL).

Results

Localization of SIGIRR in HAEC

Cellular localization of the fusion protein SIGIRR-EGFP was analyzed in H292 cells by confocal microscopy. The fluorescence signal of the EGFP-fused SIGIRR protein was observed at 48 h post-transfection (Fig. 1). SIGIRR-EGFP fluorescent signals were superimposed at the plasma and nuclear membrane (Fig. 1Ad–f). In contrast, the EGFP fluorescence detected in cells transfected with empty vector pEGFP-N1 was diffusely localized throughout both the cytoplasmic and nuclear compartments, but not at the plasma membrane (Fig. 1Aa–c). As a control, the H292 (wild type) cells did not emit any detectable fluorescence (data not shown).

The cellular localization of SIGIRR was further analyzed in RH292 cells by immunostaining with anti-SIGIRR antibody as the primary antibody. Positive staining signals appeared to only correspond with outer membranes of RH292 cells (Fig. 1Ba) and SIGIRR-EGFP fusion protein was located mainly on the plasma membranes of RH292 cells (Fig. 1Bb). SIGIRR labelled with EGFP or TRITC were colocalized (Fig. 1Bc). Positive staining signals were not observed in the negative controls without the addition of primary antibody (Fig. 1Bd). These results indicated that SIGIRR was expressed on the plasma membrane of the H292 HAEC cell line.

SIGIRR suppressed proinflammatory cytokines production in HAEC in response to LPS, flagellin and CpG DNA 2006 exposure

To determine whether overexpression of SIGIRR affected TLR4, 5, or 9-mediated innate immune responses, we selected the stably transfected cell line RH292-2 as the representative RH292 cells for further investigation. RH292-2 overexpressed the target gene SIGIRR substantially more than other stably transfected and control cells as evidenced by real-time RT–PCR (Fig. 2). Subsequently, levels of proinflammatory cytokines IL-6 and TNF-α were assayed by ELISA from culture supernatants of the RH292 cells that were treated with the indicated concentrations of LPS, flagellin or CpG DNA 2006 for 6 h. H292 cells and EH292 stimulated with the same factors were used as controls. Results showed that the protein expression of IL-6 and TNF-α in RH292 cells decreased significantly as compared to the controls, and no significant difference was found between the controls, H292 cells and the EH292 cells (Fig. 3a and b). Results indicated that SIGIRR was a common suppressor in TLR4, 5, and 9-mediated signalling pathways that worked by negatively regulating IL-6 and TNF-α gene expression.

SIGIRR did not modulate TLR4, 5, 9 expression in HAEC

Since overexpression of SIGIRR can attenuate TLR4, 5, and 9-mediated proinflammatory signals, we investigated whether TLR4, 5, or 9 mRNA expression was also down-regulated in SIGIRR overexpressed HAEC. The mRNA and protein levels of TLR4, 5, and 9 did not exhibit significant differences between SIGIRR over-expressing H292 cells and control cells (Fig. 4). Furthermore, the protein levels of TLR4, 5, and 9 in different cells treated with LPS (1 μg/ml), flagellin (0.1 μg/ml) or CpG DNA 2006 (0.5 μM) for 24 h were measured. The results showed that the protein levels of TLR4, 5, and 9 decreased obviously in response to stimulation with cognate ligands over 1 h, but the protein levels increased gradually in a time-dependent manner for up to 24 h (Fig. 5), indicating overexpression of SIGIRR did not affect the TLR4, 5, or 9 expression in HAEC. Moreover, the protein expression pattern of SIGIRR in each experimental group of cells was similar to TLR4, 5, and 9 following ligand stimulation (Fig. 6).

SIGIRR interacted with MyD88 following TLR4, 5, and 9 activation by their cognate ligands in HAEC

As MyD88 is believed to be the key adaptor protein of most TLR-mediated signalling pathways, we next examined

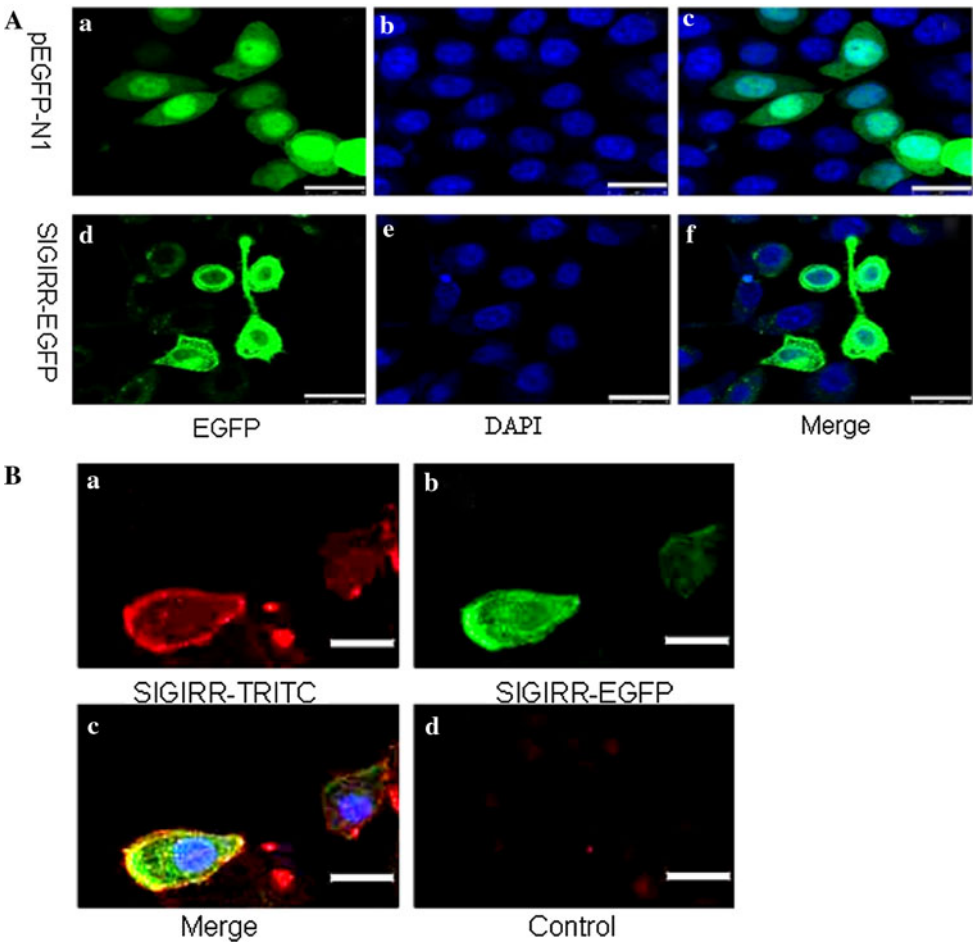

Fig. 1 Localization of SIGIRR protein. Panel **A**, Confocal fluorescence microscopic images of H292 cells after transfection with pEGFP-N1 (*a–c*) or SIGIRR-EGFP (*d–f*). EGFP and SIGIRR-EGFP fusion protein presence were detected by the EGFP signal (*a*, *d*). The nuclei of the cells were counterstained with DAPI signal (*b*, *e*), and the merged images illustrate co-localization of EGFP and SIGIRR signals (*c*, *f*). Panel **B**, Immunofluorescence of SIGIRR overexpressed in H292 cells. SIGIRR immunostaining with TRITC-labelled secondary antibody confirmed SIGIRR expression on the cellular surface (*a*). SIGIRR-EGFP fusion protein was detected by EGFP signal (*b*). The merged images illustrate co-localization of TRITC and SIGIRR-EGFP signals (*c*), the nuclei of the cells were counterstained with DAPI signal (*c*), and the negative control was incubated without primary antibody (*d*). *Bars* represent 25 μm

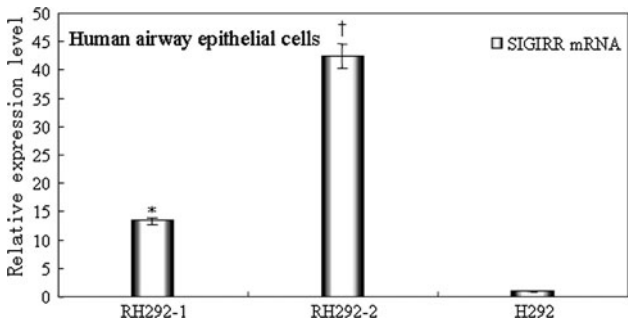

Fig. 2 The mRNA levels of SIGIRR in HAEC transfected with SIGIRR-EGFP. RH292-1 and RH292-2 were two clones derived from H292 cells that were stably transfected with recombinant SIGIRR-expressing plasmid. SIGIRR mRNA in RH292-2 and RH292-1 increased by 44-fold and 14-fold, respectively, as compared to untransfected H292. Data represents the mean ± SD from three independent experiments; * $P < 0.05$ vs H292; † $P < 0.05$ vs H292

whether MyD88 was involved in the SIGIRR impaired TLR4, 5, and 9-mediated inflammation signals. Cell extracts from the H292 untransfected and H292 transfectants stimulated with LPS (1 μg/ml), flagellin (0.1 μg/ml) and CpG DNA 2006 (0.5 μM) for 1 h were co-immunoprecipitated with anti-SIGIRR antibody, followed by Western-blot analysis with antibody against MyD88 or SIGIRR. As shown in Fig. 7a, minimal MyD88 interacted with SIGIRR in H292 cells in the absence of stimulation and the similar result was obtained in EH292 or RH292 (data not shown). However, obvious amounts of MyD88 complexed with SIGIRR could be detected upon TLR ligand stimulation (Fig. 7a). Moreover, MyD88 combined with SIGIRR in RH292 cells was detected at a much higher level than in H292 or EH292 (Fig. 7a and b). The results demonstrated that SIGIRR attenuated the TLR4, 5, and 9-mediated inflammation signals through increased interaction of MyD88 with SIGIRR after ligand stimulations in HAEC.

Discussion

In the present study, localization of SIGIRR in HAEC was described for the first time using fusion protein SIGIRR-EGFP and confocal fluorescence microscopy to demonstrate that SIGIRR-EGFP was expressed at the plasma membrane in a similar manner as endogenous SIGIRR. The membrane exclusive localization of the SIGIRR-EGFP was compatible with a protein that functions as a transmembrane

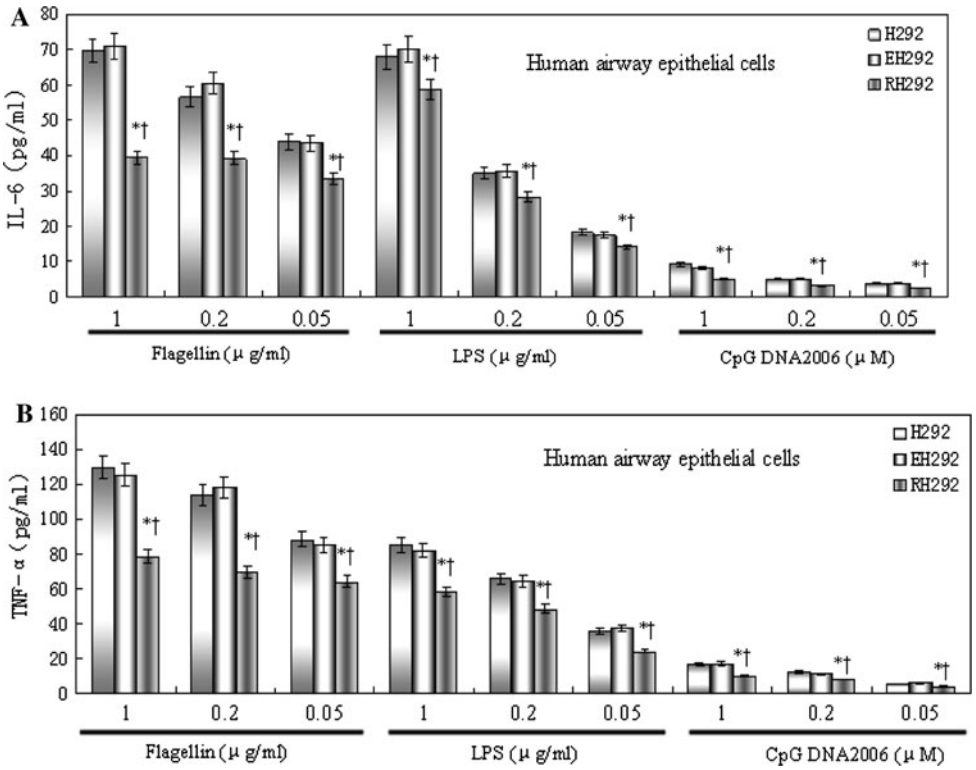

Fig. 3 Overexpression of SIGIRR impact on the induction of proinflammatory cytokines from HAEC activated by TLR4, 5, or 9 ligands. Release of IL-6 (**a**) and TNF-α (**b**) from airway epithelial cells (1×10^6/well) activated by the specific ligands for 6 h in a 12-well plate was determined by ELISA. Protein expression of IL-6 and TNF-α in the supernatants of RH292 cells decreased significantly as compared to H292 and EH292 cells. Data represents the mean ± SD from three independent experiments. H292, wildtype; EH292, transfected with empty vector; RH292, transfected with recombinant plasmid SIGIRR-EGFP. † $P < 0.05$, RH292 vs EH292; * $P < 0.05$, RH292 vs H292

protein receptor. Furthermore, proinflammatory cytokines IL-6 and TNF-α were found to be decreased significantly in SIGIRR overexpressed HAEC as compared to controls after stimulation with LPS, flagellin and CpG DNA. TLR 4, 5, and 9 protein expression levels were not down-regulated by SIGIRR in over-expressing HAEC, and the protein expression patterns of SIGIRR and TLR 4, 5, and 9 exhibited similar time-dependent patterns after stimulation. The similar expression patterns indicated that the parallel change of positive regulators (TLRs) and negative regulator (SIGIRR) for inflammation signals ensured the necessary protective response while avoiding an excessive reaction. Finally, increased amounts of adaptor MyD88 binding to SIGIRR in over-expressing HAEC following stimulation was confirmed by co-immunoprecipitation.

A previous report showed that SIGIRR interfered with recruitment of the receptor-proximal signalling components through its intracellular TIR domain [20]; however, the EGFP protein was fused to the C-terminus of SIGIRR in our study, likely blocking this domain. In fact, negative modulation of SIGIRR remained in HAEC because the EGFP-fused SIGIRR protein carried a six-amino acid flexible linker which contained no bulky or peptide chain-bending amino acid residues [21]. The eukaryotic expression vector pEGFP-N1 has also been previously applied successfully in studies involving airway inflammation [22].

Cell type-specific regulation of SIGIRR has been evidenced in recent studies by others. Increased production of IL-1 and TNF-α by *Mycobacterium tuberculosis*-infected dendritic cells of $Tir8^{-/-}$ mice was observed in vitro [23]. Moreover, the expression of SIGIRR mRNA was found to be up-regulated and to inhibit NF-κB activation in monocytes from septic patients [24]. The same negative regulation also was observed in gut epithelial cells [25, 26]; additionally, resident myeloid cells were found to contribute to TLR-mediated antimicrobial immunity in the kidney, and this function was controlled by SIGIRR [27]. The underlying molecular mechanism(s) responsible for these differences and the exact cell type playing a regulatory role with SIGIRR remain to be elucidated. Possibly, polymorphisms in the SIGIRR gene and/or post-translational modifications are associated with the development of inflammation. For instance, a single nucleotide polymorphism in the TLR 8 and 9 genes has been associated, respectively, with susceptibility to coronary artery disease (CAD) and systemic lupus erythematosus (SLE) in a Chinese population [28, 29]. Monocytes and renal tubular epithelial cells have been observed to express the same splice variant of SIGIRR mRNA, but the resultant SIGIRR protein was differentially N- and O-glycosylated in these cell types [27]. As discussed above, expression and regulation of SIGIRR is circumstantial and dependent upon different modifications (such as phosphorylation, alkylation, and sulfation, but not glycosylation) that may vary between cell types and functions.

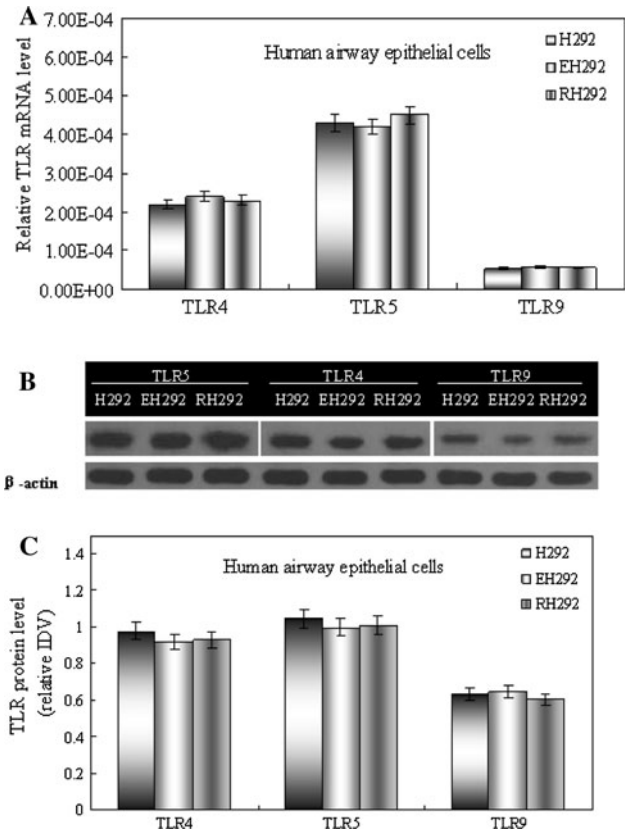

Fig. 4 TLR4, 5, and 9 expression in HAEC. Panel **a**, TLR4, 5, and 9 mRNA levels were quantitated and normalized to the β-actin control. Panel **b**, Western blot analysis of TLR4, 5, and 9 protein expression in H292 cells. The mRNA and protein levels of TLR4, 5, and 9 did not exhibit significant differences between RH292 cells and control cells. The data shown are representative of three independent experiments. H292, wildtype; EH292, transfected with empty vector; RH292, transfected with recombinant plasmid SIGIRR-EGFP. Panel **c**, TLR4, 5, and 9 protein levels were quantitated and normalized to the β-actin control. Data has been expressed as the mean ± SD ($n = 3$) for integrated density values (IDV)

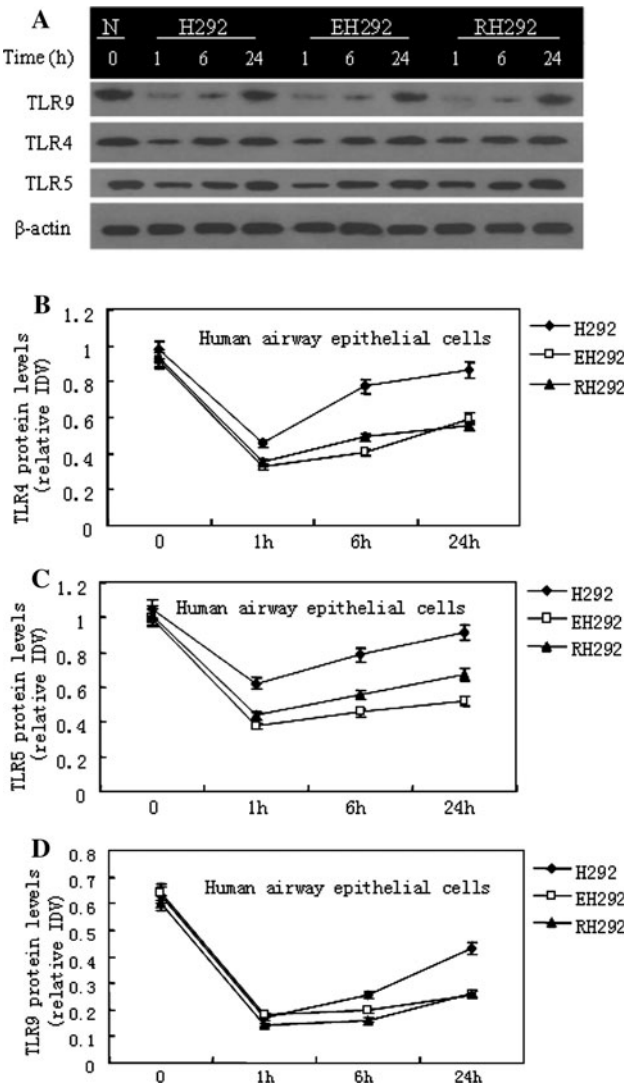

Fig. 5 TLR4, 5, and 9 expression in HAEC exposed to LPS (1 μg/ml), flagellin (0.1 μg/ml) and CpG DNA 2006 (0.5 μM) for 24 h. Panel **a**, Western blot analysis of TLR4, 5, and 9 protein levels in H292, EH292 and RH292 at 1, 6, and 24 h, respectively. N, H292 cells; EH292, transfected with empty vector; RH292, transfected with recombinant plasmid SIGIRR-EGFP. Panel **b**, **c** and **d**, The intensity of bands was quantitated and normalized to the β-actin control. The protein levels of TLR4, 5, and 9 decreased obviously in response to stimulation with cognate ligands for 1 h, but the protein levels increased gradually in a time-dependent manner for up to 24 h. Data represents the mean ± SD ($n = 3$) for IDV at each time point

To date, although the SIGIRR membrane receptor has no defined ligand, both the extracellular Ig domain and the intracellular TIR domains are known to be important for SIGIRR to inhibit IL-1 signalling, because SIGIRR inhibits heterodimerization between IL-1RI and IL-1RAcP through its extracellular Ig domain. In contrast, only the TIR domain is necessary for SIGIRR to inhibit TLR-mediated signalling [20]. However, SIGIRR interacts minimally with adaptor MyD88 in untreated HAEC, and was found to combine with MyD88 in response to stimulation in the present study. This observed interaction might be due to a higher binding activity of either SIGIRR or MyD88. Meanwhile, overexpression of SIGIRR was able to overcome the TLR-induced down-regulation of SIGIRR and may, therefore, have suppressed the cytokine production so that proinflammatory cytokines decreased significantly from RH292 as compared to H292 or EH292. Figure 7b described the amount of precipitated MyD88 as being higher in RH292 cells as compared to controls; this might be due to a higher amount of SIGIRR in the cells or a higher binding activity of either SIGIRR or MyD88. It can only be addressed by precipitation of endogenous SIGIRR for further analysis. Thus, one more important question that remains to gain a more comprehensive understanding of this mechanism concerns the pathways and molecules that lead to SIGIRR binding to MyD88. Biochemical analysis

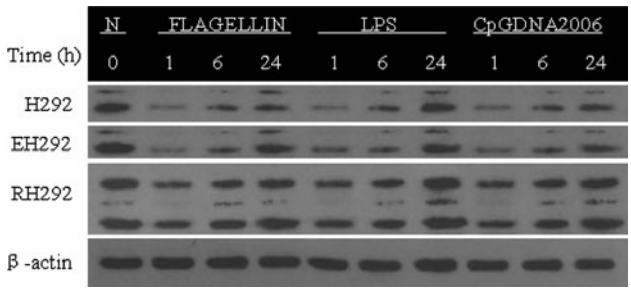

Fig. 6 SIGIRR expression in HAEC exposed to LPS (1 μg/ml), flagellin (0.1 μg/ml) and CpG DNA 2006 (0.5 μM). The protein level was measured by Western blot at 0, 1, 6, and 24 h. N, H292 cells; EH292, transfected with empty vector; RH292, transfected with recombinant plasmid SIGIRR-EGFP. The protein levels of SIGIRR decreased obviously in response to stimulation for 1 h, but the protein levels increased gradually in a time-dependent manner for up to 24 h. Representative experiments are shown

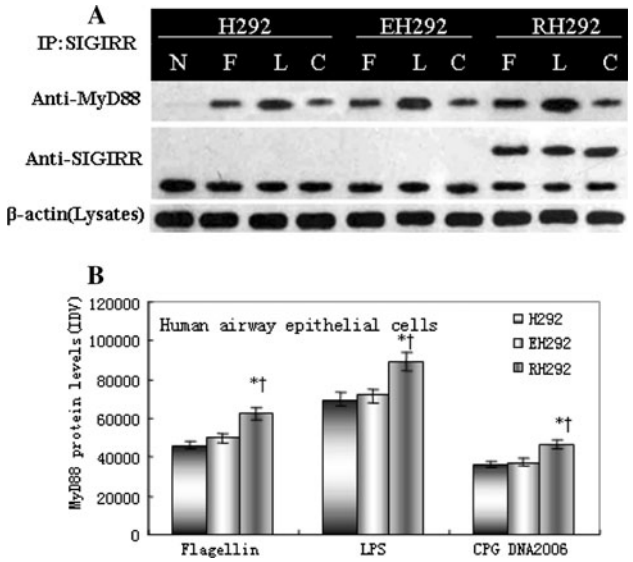

Fig. 7 Detection of MyD88 protein complexed with SIGIRR in HAEC. Panel **a**, cell extracts prepared from wtH292 cells (N) and transfectants stimulated with LPS (1 μg/ml), flagellin (0.1 μg/ml) or CpG DNA 2006 (0.5 μM) for 1 h were co-immunoprecipitated with antibody against SIGIRR, followed by Western blot analysis with anti-MyD88 antibodies. β-actin expression was used as an internal control. H292, wildtype; EH292, transfected with empty vector; RH292, transfected with recombinant plasmid SIGIRR-EGFP. F, flagellin; L, LPS; C, CpG DNA2006. Panel **b**, MyD88 protein levels were quantitated as IDV. Data represents the mean ± SD ($n = 3$). † $P < 0.05$, RH292 vs EH292; * $P < 0.05$, RH292 vs H292

has already revealed that the bipartite nature of MyD88 allows it to form homodimers through TIR-TIR(upstream) and DD-DD(downstream) domain interactions, and that it exists as a dimer when recruited to the receptor complex [30, 31]. Mechanistic studies have indicated that SIGIRR works by being recruited through its TIR domain to the TIR domain of target receptors, where it may then sequester the key signalling proteins IRAK and TRAF6 and prevent signal propagation [32]. Likewise, the interaction of SIGIRR and MyD88 may prevent the dissociation of the IRAK-IRAK4 from MyD88, thereby inhibiting the formation of the IRAK-TRAF6 complex. Gong et al. have provided yet another example of how SIGIRR may exert its inhibitory effect, namely through blocking the molecular interface of TLR4, TLR7 and the MyD88 adaptor mainly via its BB-loop region [33]. A low molecular weight MyD88 mimic, modelled on a tripeptide sequence of the BB-loop in the TIR domain, has been suggested as an intracellular target site for anti-inflammatory drug action [34]. Moreover, evidence has been presented to support the idea that SIGIRR may be acting as a negative regulator of MyD88-dependent TLR signalling. More significantly, SIGIRR is most prominently expressed in organs with an epithelial component. This fact may indicate an important role for tolerance to microbes in the airway and other epithelial tissues. Ragnarsdóttir et al. showed that reduced levels of the SIGIRR were present in patients with asymptomatic bacteriuria [35].

Taken together, negative regulation of TLR signalling may be required to avoid inappropriate (and detrimental) inflammatory responses. The results obtained from the study presented herein suggest that SIGIRR will be a promising therapeutic target towards the reduction of ALI/ARDS development.

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