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Secretory IgA accumulated in the airspaces of idiopathic pulmonary fibrosis and promoted VEGF, TGF-β and IL-8 production by A549 cells

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Summary

Secretory IgA (SIgA) is a well-known mucosal-surface molecule in first-line defense against extrinsic pathogens and antigens. Its immunomodulatory and pathological roles have also been emphasized, but it is unclear whether it plays a pathological role in lung diseases. In the present study, we aimed to determine the distribution of IgA in idiopathic pulmonary fibrosis (IPF) lungs and whether IgA affects the functions of airway epithelial cells. We performed immunohistochemical analysis of lung sections from patients with IPF and found that mucus accumulated in the airspaces adjacent to the hyperplastic epithelia contained abundant SIgA. This was not true in the lungs of non-IPF subjects. An *in-vitro* assay revealed that SIgA bound to the surface of A549 cells and significantly promoted production of vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β and interleukin (IL)-8, important cytokines in the pathogenesis of IPF. Among the known receptors for IgA, A549 cells expressed high levels of transferrin receptor (TfR)/CD71. Transfection experiments with siRNA targeted against TfR/CD71 followed by stimulation with SIgA suggested that TfR/CD71 may be at least partially involved in the SIgA-induced cytokine production by A549 cells. These phenomena were specific for SIgA, distinct from IgG. SIgA may modulate the progression of IPF by enhancing synthesis of VEGF, TGF-β and IL-8.

Keywords: fibrosis, human, lung, secretory IgA

Introduction

Immunoglobulin A (IgA) is composed of two heavy chains (α chain) and two light chains (κ/λ chain), and is the most abundant immunoglobulin on mucosal surfaces [1]. In secretions, IgA is present mainly in dimeric form, i.e. two monomers are linked by a chain, the 'J chain'. The dimer binds to polymeric immunoglobulin receptors (pIgR) expressed on the basal surface of epithelial cells, is transported to the apical side and is released with the cleaved pIgR extracellular domain, a secretory component (SC) [2], called secretory IgA (SIgA). SIgA's basic function is as a first-line defense by neutralizing extrinsic pathogens and antigens on mucosal surfaces, thereby protecting mucosal tissues from infections and allergic sensitization [2–4]. In more recent years, attention has been focused on other immunomodulatory and pathological roles of SIgA.

SIgA on the intestinal mucosa contributes to intestinal homeostasis by controlling commensal bacteria and inducing immunological tolerance via cross-talk between the host and its intestinal contents [5]. SIgA binds to gastrointestinal M cells of Peyer's patches, is transported to the subepithelial dome region and may influence immunological tolerance [5]. Interestingly, SIgA exerts its effects on tissue cells not only under physiological conditions, but also in pathological states. In celiac disease, gliadin-specific SIgA binds to transferrin receptor (TfR)/CD71 that is over-expressed on intestinal epithelial cells and is transported to subepithelial areas, leading to accumulation of gliadin [6]. IgA is associated with the pathogenesis of IgA nephropathy. It is thought that mainly high-molecular-weight serum IgA is deposited in the kidney, but SIgA can also play a pathogenic role by promoting proinflammatory cytokine production by mesangial cells via an unknown receptor [7]. Regarding hematocytes, cell surface

binding and cross-linking of SIgA induces degranulation of eosinophils [8] and basophils [9], which can exacerbate allergic reactions. Despite these findings showing binding of SIgA to myriad cells, evoking multiple physiological and pathological effects, there has been no investigation of whether or how SIgA influences the cells comprising respiratory mucosal tissue.

Idiopathic pulmonary fibrosis (IPF) is the most common disease among idiopathic interstitial pneumonia, and has a poor prognosis [10]. It is thought that repeated injury to alveolar epithelial cells and aberrant repair due to excess fibrotic signaling lead to progressive and irreversible fibrosis [11,12]. The pathological findings include accumulation of fibroblasts and myofibroblasts, excess extracellular matrix deposition, formation of so-called fibroblastic foci [13] and abnormal epithelial cells, some of which have an alveolar type 2 (AT2) cell-like identity [14]. Moreover, it is notable that mucus, having an altered composition [15,16], accumulates in IPF lungs [16,17]. The amount of mucus accumulation in IPF lungs is associated with the degree of lung inflammation and FVC decline [17]. These novel findings led to a hypothesis that drainage impairment caused by aberrant mucus production affects the disease progression in IPF lungs, but the precise mechanism has not been elucidated.

In this study, we show that epithelia of remodeled tissue in IPF lungs was exposed to SIgA-rich mucus that had accumulated in the airspaces. An *in-vitro* assay revealed that SIgA bound to the surface of A549 cells, a cell line with AT2 cell phenotype, and promoted production of vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β and interleukin (IL)-8, cytokines reported to be involved in the pathogenesis of IPF [12,18–22]. We also found that TfR/CD71 was involved in the cytokine production evoked by SIgA stimulation.

Materials and methods

Reagents

The following reagents were purchased as indicated: purified human secretory IgA (MP Biomedicals, Santa Ana, CA, USA); phosphate-buffered saline (PBS) and fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA); citrate buffer (Muto Pure Chemicals Co., Tokyo, Japan); histofine antigen retrieval solution pH 9 (Nichirei, Tokyo, Japan); Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium, hydrogen peroxide, 0·5 M ethylenediamine tetraacetic acid (EDTA) and methanol (Wako Pure Chemical Industries, Osaka, Japan); 0·25% trypsin (Life Technologies Corporation, Grand Island, NY, USA); carbonate–bicarbonate buffer (Sigma Aldrich, St Louis, MO, USA); and BioFX TMB One Component HRP Microwell Substrate (Surmodics IVD, Inc., Eden Prairie, MN, USA).

The following antibodies were purchased as indicated: fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD71 monoclonal antibody (mAb) (IgG2aκ, clone CY1G4), phycoerythrin (PE)-conjugated mouse anti-human CD89 mAb (IgG1κ, clone A59), allophycocyanin (APC)-conjugated mouse anti-human CD206 mAb (IgG1, clone 15-2), APC-conjugated mouse anti-human CD209 mAb (IgG2a, clone 9E9A8), PE-conjugated mouse anti-human CD351 mAb (IgG1κ, clone TX61), FITC-conjugated mouse IgG2aκ isotype control (clone MOPC-173), PE-conjugated mouse IgG1κ isotype control (clone MOPC-21) and mouse IgG1κ isotype control (clone MOPC-21) (BioLegend, San Diego, CA, USA); PE-conjugated mouse anti-asialoglycoprotein receptor (ASGP-R) mAb (IgG1κ, clone 8D7) and PE-conjugated mouse IgG1κ isotype control (clone MOPC-21) (BD Biosciences, San Jose, CA, USA); PE-conjugated goat $F(ab')_2$ anti-mouse IgG pAb, APC-conjugated mouse IgG1 isotype control (clone P3.6.2.8.1) and APC-conjugated mouse IgG2a isotype control (clone eBM2a) (eBioscience, San Diego, CA, USA); APC-conjugated mouse anti-human IgA mAb (IgG1, clone IS11-8E10) (Miltenyi Biotec, Bergisch Gladbach, Germany); goat horseradish peroxidase (HRP)-conjugated anti-IgA antibody (polyclonal, whole IgG) (Bethyl Laboratories, Inc., Montgomery, TX, USA); mouse anti-human transferrin receptor/CD71 mAb (IgG1, clone H68.4) and mouse anti-human secretory component mAb [IgG1, clone COMPO2 (SC-05)] (Thermo Fisher Scientific); and mouse anti-β 1, 4-galactosyltransferase 1 mAb (IgG1) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

Immunohistochemistry

Formalin-fixed, paraffin-embedded lung tissue samples from three non-IPF patients and samples from three IPF patients who underwent lobectomy for lung cancer were used. Non-diseased parts of the resected lungs from the non-IPF patients were used as controls. The diagnosis of IPF was based on the official American Thoracic Society/European Respiratory Society/Japanese Respiratory Society/Latin American Thoracic Society (ATS/ERS/JRS/ALAT) statement, and diagnosis of usual interstitial pneumonia was confirmed by an independent pathologist. The study was approved by the Ethics Committee of Tokyo Hospital. Written informed consent was obtained from each of the patients before surgery.

After deparaffinization and blocking of endogenous peroxide with 0·9% hydrogen peroxide in methanol, heat-induced antigen retrieval was performed in citrate buffer pH 6 at 98°C for 40 min for secretory component staining and in histofine antigen retrieval solution pH 9 at 98°C for 40 min for IgA and TfR/CD71 staining. Immunohistochemistry was performed using a Vectastain ABC kit (Funakoshi Co., Ltd., Tokyo, Japan) in combination

with an avidin/biotin blocking kit (Vector Laboratories, Inc., Burlingame, CA, USA). Mouse anti-human secretory component mAb, goat anti-human IgA mAb and mouse anti-human transferrin receptor/CD71 mAb were used at 40 μg/ml, 0·2 μg/ml and 0·5 μg/ml, respectively, and comparison was made to sections stained with isotype-matched control antibodies at the same concentrations. The color was developed by the diaminobenzidine (DAB) reaction using a peroxidase substrate kit DAB (Vector Laboratories, Inc.). Photographs were taken with a Keyence fluorescence microscope BZ-X710 (Keyence Japan, Osaka, Japan).

Cells and culture

An adenocarcinomic human alveolar basal epithelial cell line, A549, was maintained in DMEM/Ham's F-12 medium containing 5% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C, 5% CO_2 until the experiments.

Flow cytometric analysis

SIgA binding to the A549 cell surface was analyzed by flow cytometry. After detaching from the plates with 5 mM EDTA in PBS, 2×10^5 cells were washed with fluorescence activated cell sorter (FACS) buffer (PBS containing 3% FBS), blocked with human polyclonal IgG at 20 μg/ml, and incubated with or without SIgA at 300 μg/ml for 30 min at 4°C. For binding inhibition assay with IgG, human polyclonal IgG was added as well as SIgA at a final concentration of 300 μg/ml. After washing with FACS buffer, the cells were incubated with APC-conjugated mouse anti-human IgA mAb for 30 min at 4°C. Then the cells were washed again with FACS buffer, resuspended in FACS buffer and analyzed by FACSVerse (BD Biosciences). For each sample, at least 10 000 events were collected, and dot-plots and histograms were generated using FlowJo (Tree Star Inc., Ashland, OR, USA).

For binding inhibition assay with EDTA, the cells were preincubated with 10 mM EDTA in FACS buffer for 15 min at 4°C. Then human polyclonal IgG at 20 μg/ml was added for blocking, and finally SIgA was added. For binding inhibition assay with trypsin treatment, the cells were preincubated with 0·25% trypsin for 5 min at room temperature, washed with FACS buffer, blocked with human polyclonal IgG at 20 μg/ml, and finally SIgA was added. After incubation for 30 min at 4°C, the cells were washed with FACS buffer and incubated with APC-conjugated mouse anti-human IgA mAb for 30 min at 4°C. The cells were washed with FACS buffer and analyzed by flow cytometry in the same manner as described above. To investigate the cell surface expression of each of TfR/CD71, FcαRI/CD89, mannose receptor/CD206, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN)/CD209, Fcα/μR/CD351, asialoglycoprotein receptor (ASGP-R) and β 1, 4-galactosyltransferase 1, after blocking with human polyclonal IgG at 20 μg/ml, 2×10^5 cells were stained with specific antibody, or with isotype control antibody for 30 min on ice. Cells stained with non-conjugated antibody were incubated with PE-conjugated anti-mouse IgG antibody for 30 min on ice. Finally, the cells were analyzed by flow cytometry as described above.

Quantitation of cell-derived cytokines

A549 cells were seeded at 1×10^5/well in 24-well plates. After 24 h, the cells were incubated with SIgA or human polyclonal IgG at 300 μg/ml in FBS-free DMEM/Ham's F-12 medium for 24 or 48 h. For concentration analysis, the cells were incubated with SIgA at 10, 30, 100 and 300 μg/ml or human polyclonal IgG at 300 μg/ml for 24 h. The levels of VEGF-A and IL-8 in the culture supernatants were measured by cytometric bead array (CBA; BD Biosciences), according to the manufacturer's instructions. The levels of TGF-β1 in the culture supernatants were measured by human/mouse TGF-β1 ELISA Ready-SET-Go (Thermo Fisher Scientific), according to the manufacturer's instructions. The supernatants were stored at −80°C until assay.

Real-time quantitative polymerase chain reaction (RT–PCR) analysis

A549 cells were seeded at 1×10^5/well in 24-well plates. After 24 h, the cells were incubated with or without SIgA at 300 μg/ml in FBS-free DMEM/Ham's F-12 medium, and the total RNA was then extracted from the cultured cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The extracted mRNA was reverse-transcribed to cDNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). RT–PCR was performed using primers and Taqman probes designed by Thermo Fisher Scientific, Inc. Data were calculated by the $^{\Delta\Delta}$Ct method using the cDNA and, as a reference, β-actin cDNA. The relative quantitation (RQ) values were calculated using the following equation: $RQ = 2^{-\Delta\Delta Ct}$.

Down-regulation of TfR/CD71 by siRNA transfection

A549 cells were transfected with small interference RNA (siRNA) against TfR/CD71 (Sigma Genosys siRNA Service, Hs_TFRC_9216_s: #1590001; 5′rGrGUUrArCUrGrGrGrCrArAUUUrCUrATT3′, Hs_TFRC_9216_as: #1590002; 5′UrArGrArArAUUrGrCrCrCrArGUrArArCrCTT3′) (Sigma Aldrich) or control RNA (Sigma Genosys siRNA Service, Mission_SIC-001_s, Mission_SIC-001_as) at 1000 nM by the electroporation method using 4D-Nucleofector and a SF cell line 4D-Nucleofector X kit L (Lonza, Basel, Switzerland), according to the manufacturer's instructions. The transfected cells

were cultured for 24 h and then used for the experiments.

Statistics

Statistical analyses were performed using an unpaired, two-tailed *t*-test for pairwise comparisons, a one-sample *t*-test for comparisons of relative values and analysis of variance (ANOVA) with Tukey's test for multiple comparisons. Statistical significance was defined as a *P*-value of less than 0·05. All data are expressed as the mean ± standard error of the mean (s.e.m.) of three to 12 independent experiments.

Results

Accumulated mucus containing SIgA was in direct contact with epithelia in IPF lungs

We first performed immunohistochemical analysis of lung sections from the control subjects and IPF patients to investigate the morphological relationships between SIgA and epithelial cells. The details of the control subjects and IPF patients are summarized in Table 1. We found that, in IPF lungs, accumulated mucus was highly positive for both IgA and SC and was in direct contact with the epithelia of remodeled tissue (Fig. 1e–g,i–k). In contrast,

Table 1. Characteristics of the IPF patients and control subjects

	IPF patients			Control subjects		
Age (years)	73	78	69	72	63	80
Gender	Male	Male	Female	Female	Female	Female
Smoking history	Yes	Yes	Yes	Never	Never	Never
Brinkman index	1000	1600	800	–	–	–
Pulmonary function tests						
FVC% predicted	80·1	79	83·4	98·2	133·9	127·5
DLco% predicted	29·9	Not performed	40·8	Not performed	Not performed	Not performed
Medication for IPF	No	No	Pirfenidone	–	–	–

DLco = diffusing capacity for carbon monoxide; FVC = forced vital capacity; IPF = idiopathic pulmonary fibrosis.

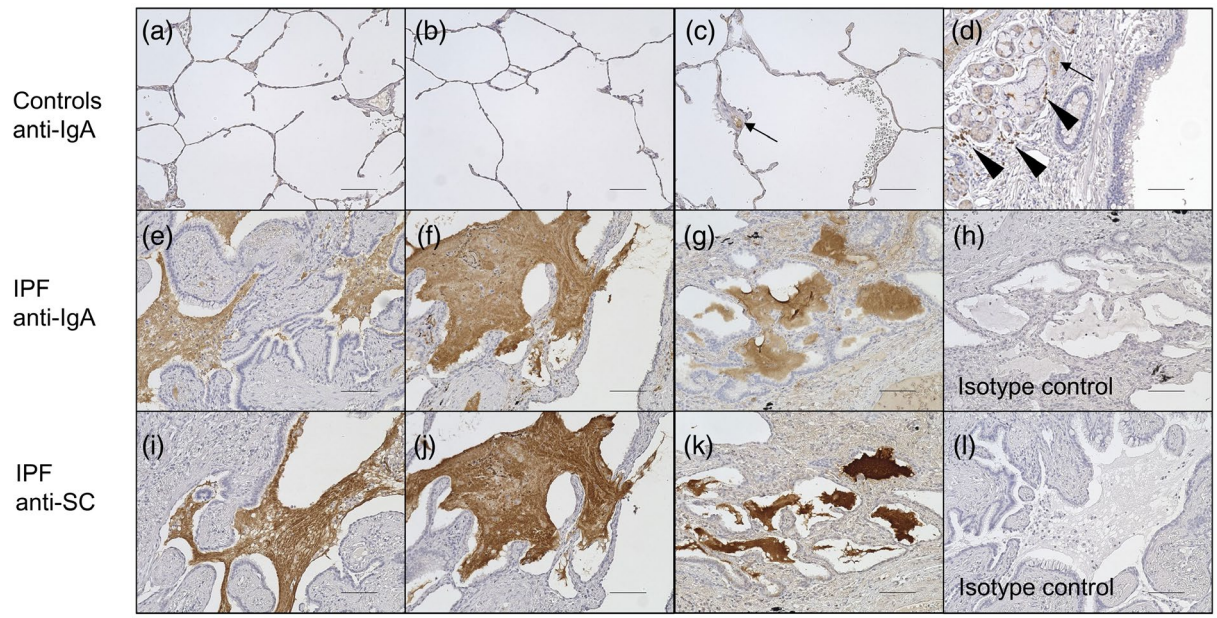

Fig. 1. Immunohistochemistry of lung sections of control subjects (n = 3) and idiopathic pulmonary fibrosis (IPF) patients (n = 3). Lung sections were stained with anti-immunoglobulin (Ig)A antibody (controls, a–d; IPF, e–g) or anti-SC antibody (IPF, i–k). Representative images from all patients' lung sections are shown. Accumulated mucus was highly positive for IgA and was adjacent to the epithelia of remodeled tissue in IPF lungs (e–g). These findings were not observed in control lungs (a–c), while bronchial glands, mononuclear cells around bronchial glands (d, arrowhead) and serum in vessels (c,d, arrow) were stained by anti-IgA antibody. The mucus in IPF lungs was also positive for secretory component (SC) (i–k). Non-specific staining was not observed with the isotype control antibodies (isotype control for anti-IgA antibody, h; isotype control for anti-SC antibody, l). The bars represent 100 μm.

mucus stained with anti-IgA antibody was not observed in the alveoli of control lungs, whereas their bronchial glands, cells infiltrated around the glands [23] and sera in vessels were clearly stained for IgA, as expected in their normal state (Fig. 1a–d).

These results indicate that (1) aberrantly accumulated mucus in IPF lungs were rich in SIgA, the production and distribution of which are restricted to bronchi in normal states [23] and (2) the epithelia of remodeled tissue in IPF lungs were exposed to that mucus.

SIgA bound to the surface of A549 cells

Next, using flow cytometry, we found that SIgA clearly bound to the surface of A549 cells (Fig. 2a). The binding was not inhibited by polyclonal human IgG (Fig. 2b), indicating that SIgA does not share its receptor with IgG. Because some IgA receptors bind to IgA cation-dependently [24,25], we also examined whether the SIgA binding to A549 cells is cation-dependent or -independent. As shown in Fig. 2c, the SIgA binding was not affected by chelation with EDTA. Conversely, trypsin treatment significantly reduced the SIgA binding to A549 cells (Fig. 2d), showing that a trypsin-sensitive protein expressed on the surface of A549 cells is involved in SIgA binding.

These results indicate that SIgA binds to A549 cells in a cation-independent and trypsin-sensitive manner.

SIgA-induced VEGF-A, TGF-β1 and IL-8 production by A549 cells

Following the binding assay, we investigated for VEGF, TGF-β and IL-8 production by A549 cells stimulated with SIgA. We measured VEGF-A and TGF-β1 because they are well-studied subtypes known to be involved in the pathogenesis of IPF [21,22]. The levels of VEGF-A and TGF-β1 in the culture supernatant were significantly increased by high-dose SIgA stimulation for both 24 and 48 h (Fig. 3a). IL-8 was also up-regulated in a dose-dependent manner and increased significantly in response to high-dose SIgA (Fig. 3a). Conversely, human polyclonal IgG was unable to similarly induce cytokine production

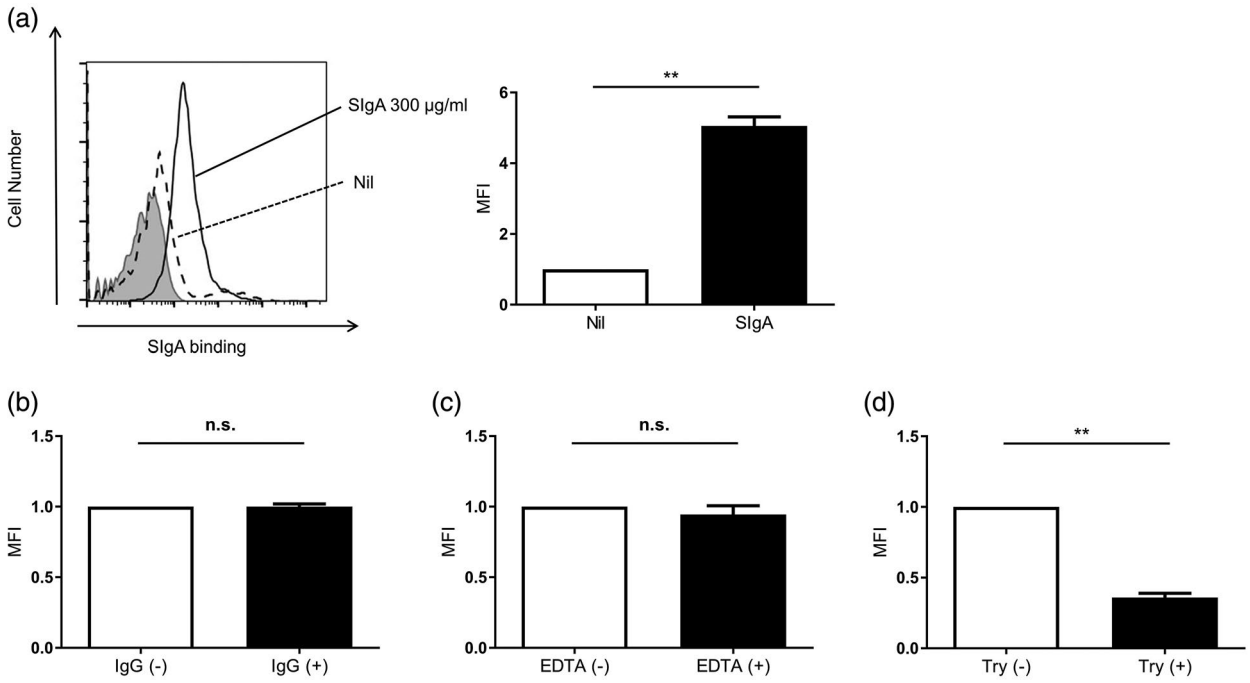

Fig. 2. Secretory immunoglobulin A (SIg)A bound to the surface of A549 cells. A549 cells were incubated with SIgA at 300 μg/ml (solid line) or without SIgA dashed line), followed by staining with allophycocyanin (APC)-conjugated anti-IgA antibody. The shaded area shows cells without staining. Representative histograms from three independent experiments are shown. Mean fluorescence intensity (MFI) of the histograms from three independent experiments are shown as relative values to those of cells incubated without SIgA (□). Bars represent the standard error of the mean (s.e.m.). $^{**}P < 0{\cdot}01$. (b) A549 cells were incubated with SIgA at 300 μg/ml and with (■) or without (□) IgG at 300 μg/ml. The cells were stained and analyzed as described above. MFI of the histograms from three independent experiments are shown as relative values to those of cells incubated without (□) IgG. Bars represent the s.e.m.; n.s. = not significant. (c) A549 cells were incubated with SIgA at 300 μg/ml and with (■) or without (□) ethylenediamine tetraacetic acid (EDTA). The cells were stained and analyzed as described above. MFI of the histograms from three independent experiments are shown as relative values to those of cells incubated without (□) EDTA. Bars represent the s.e.m.; n.s. = not significant. (d) After the treatment with (■) or without (□) trypsin, A549 cells were incubated with SIgA at 300 μg/ml. The cells were stained and analyzed as described above. MFI of the histograms from three independent experiments are shown as relative values to those of cells without trypsin treatment (□). Bars represent the standard error of the mean (s.e.m.). $^{**}P < 0{\cdot}01$.

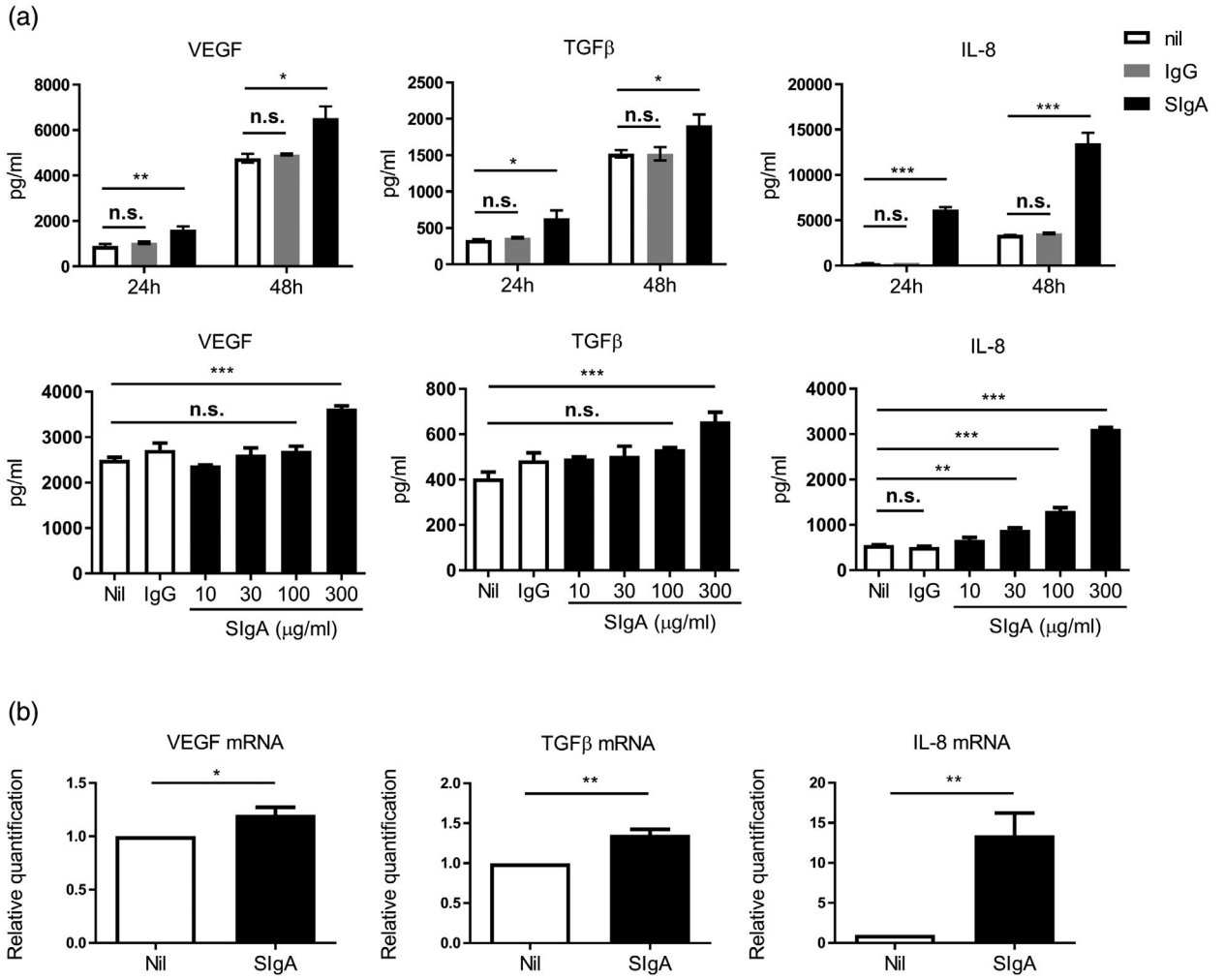

Fig. 3. Production of each of vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β and interleukin (IL)-8 was enhanced by secretory immunoglobulin A (SIg)A stimulation. (a) Upper panels show the levels of VEGF, TGF-β and IL-8 in the culture supernatant after stimulation with SIgA or IgG at 300 μg/ml for 24 and 48 h. Lower panels show the levels of VEGF, TGF-β and IL-8 in the culture supernatant after stimulation with SIgA at the indicated concentrations or IgG at 300 μg/ml for 24 h. After stimulation with SIgA under each condition, the levels of VEGF, TGF-β and IL-8 in the culture supernatant were measured. Bars represent the standard error of the mean (s.e.m.) (n = 3–5); n.s. = not significant. *P < 0·05, **P < 0·01, *** P < 0·001. (b) mRNA expressions of VEGF and IL-8 at 6 h after stimulation with (■) or without (□) SIgA at 300 μg/ml and those of TGF-β 24 h after stimulation. Bars represent the s.e.m. (n = 5–6). *P < 0·05, **P < 0·01.

(Fig. 3a). Moreover, SIgA up-regulated mRNA expression for both VEGF-A and IL-8 at 6 h and for TGF-β1 at 24 h (Fig. 3b).

Collectively, SIgA up-regulated A549 cells production of VEGF-A, TGF-β1 and IL-8 at both the protein and mRNA levels. These phenomena were specific for SIgA, and not seen with human polyclonal IgG.

A549 cells and hyperplastic epithelial cells in IPF lungs expressed TfR/CD71

We next investigated the cell-surface expression of seven well-established IgA receptors: TfR/CD71 [26], FcαRI/CD89 [27], mannose receptor/CD206 [28], DC-SIGN/CD209 [24], Fcα/μR/CD351 [29], ASGP-R [30] and β 1, 4-galactosyl-transferase 1 [31]. Flow cytometry found that only TfR/CD71 was expressed on the surface of A549 cells (Fig. 4a). Because TfR/CD71 is trypsin-sensitive [32], these findings are compatible with our finding that the SIgA binding to A549 cells was trypsin-sensitive (Fig. 2d).

We also investigated expression of TfR/CD71 by epithelial cells of IPF lungs. Immunohistochemical analysis revealed that hyperplastic epithelial cells in IPF lungs highly expressed TfR/CD71 compared to alveolar epithelial cells in the control lungs (Fig. 4b).

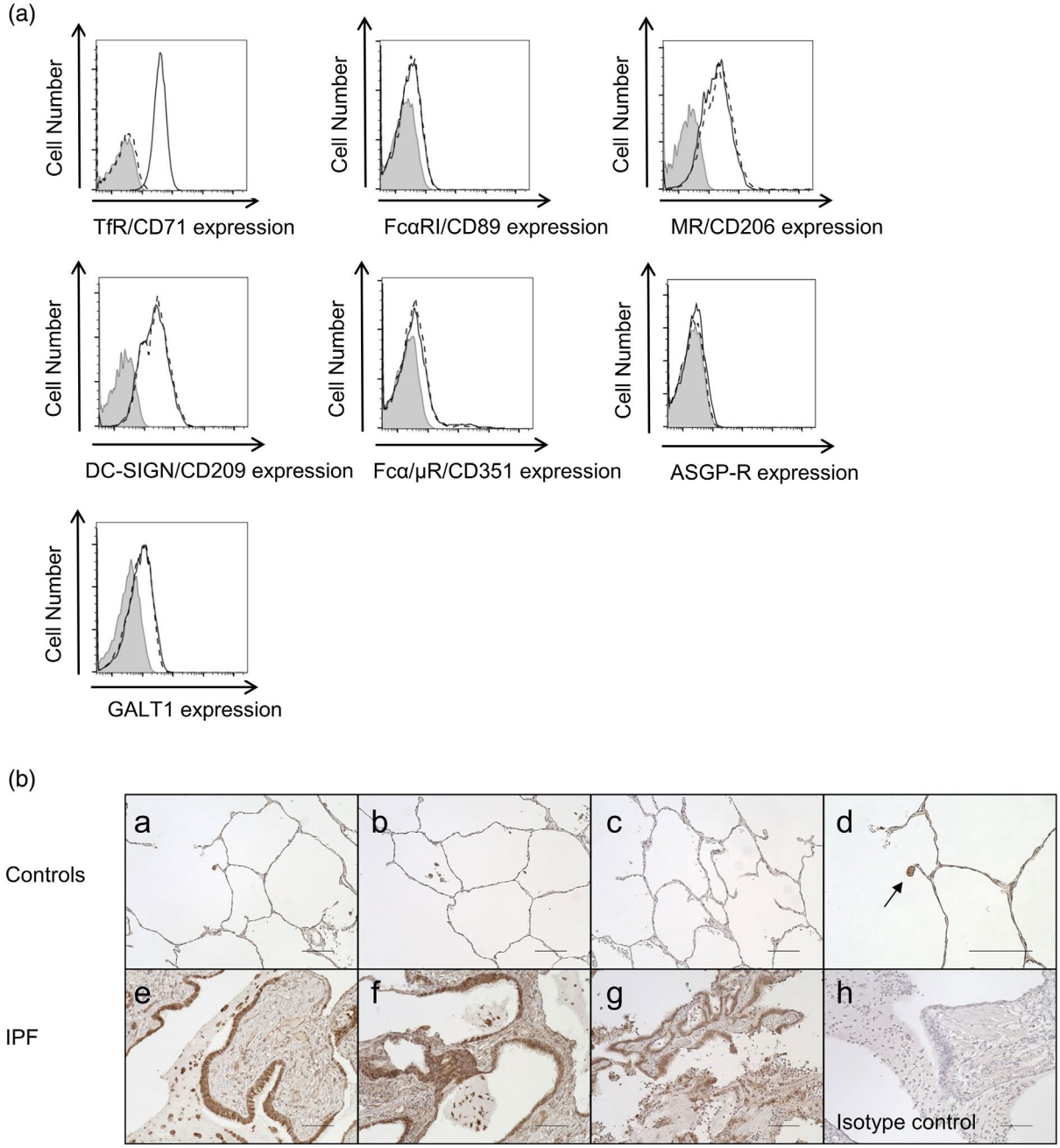

Fig. 4. A549 cells and epithelial cells in idiopathic pulmonary fibrosis (IPF) lungs expressed transferrin receptor (TfR)/CD71. (a) Expressions of TfR/CD71, FcαRI/CD89, mannose receptor (MR)/CD206, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN)/CD209, Fcα/μR/CD351, asialoglycoprotein receptor (ASGP-R) and β 1, 4-galactosyltransferase 1 (GALT1) on the surface of A549 cells were examined. A549 cells were stained with specific antibodies for the indicated receptors (solid line) or with isotype control antibody (dashed line). Expression of each receptor was analyzed by flow cytometry. Representative histograms from three independent experiments are shown. The shaded area shows cells without staining. (b) Immunohistochemistry of lung sections from control subjects (n = 3) and IPF patients (n = 3). Lung sections were stained with anti-TfR/CD71 antibody (controls, a–d; IPF, e–g). Representative images from all patients' lung sections are shown. The epithelial cells of remodeled tissue in IPF lungs were clearly positive for TfR/CD71 (e–g). These findings were not observed in normal control lungs (a–c), while alveolar macrophages were positive for TfR/CD71 (d, magnified figure of image; a, arrow). Non-specific staining was not observed with the isotype control antibodies (h). The bars represent 100 μm.

TfR/CD71 was involved in the VEGF, TGF-β and IL-8 production evoked by SIgA

Finally, we examined the effect of siRNA targeting TfR/CD71 on cytokine production in response to SIgA. A549 cells were transfected with TfR/CD71 siRNA to knock down its expression. Flow cytometric analysis revealed significant suppression of expression of cell-surface TfR/CD71 at 24 h after transfection (Fig. 5a), and that knock-down persisted for up to 48 h after transfection. After transfection with siRNA or control RNA, A549 cells were stimulated with SIgA at 300 μg/ml. As shown in Fig. 5b, VEGF-A, TGF-β1 and IL-8 production by TfR/CD71 siRNA-treated cells was significantly decreased compared to the control RNA-treated cells.

These results suggest that TfR/CD71 is at least partially involved in the cytokine production induced by SIgA stimulation.

Discussion

In this study, we show that accumulated mucus in the airspaces of IPF lungs contained abundant SIgA and was in direct contact with the epithelia of remodeled tissue (Fig. 1). We also found that SIgA bound to the surface of A549 cells (Fig. 2a), leading to production of profibrotic and inflammatory cytokines; namely, VEGF, TGF-β and IL-8 (Fig. 3). Among multiple cytokines involved in the pathogenesis of IPF, VEGF and TGF-β play pivotal roles [12,18–22] and are the targets of anti-fibrotic agents used currently in clinical practice [33–35]. Importantly, one of the main sources of these cytokines is from hyperplastic AT2 cells [21,36]. IL-8 is an inflammatory cytokine known to be a potent chemotactic factor for neutrophils [37], which are often observed to be recruited to IPF lungs [38,39]. The level of serum IL-8 and the bronchoalveolar lavage fluid neutrophil count are associated with the

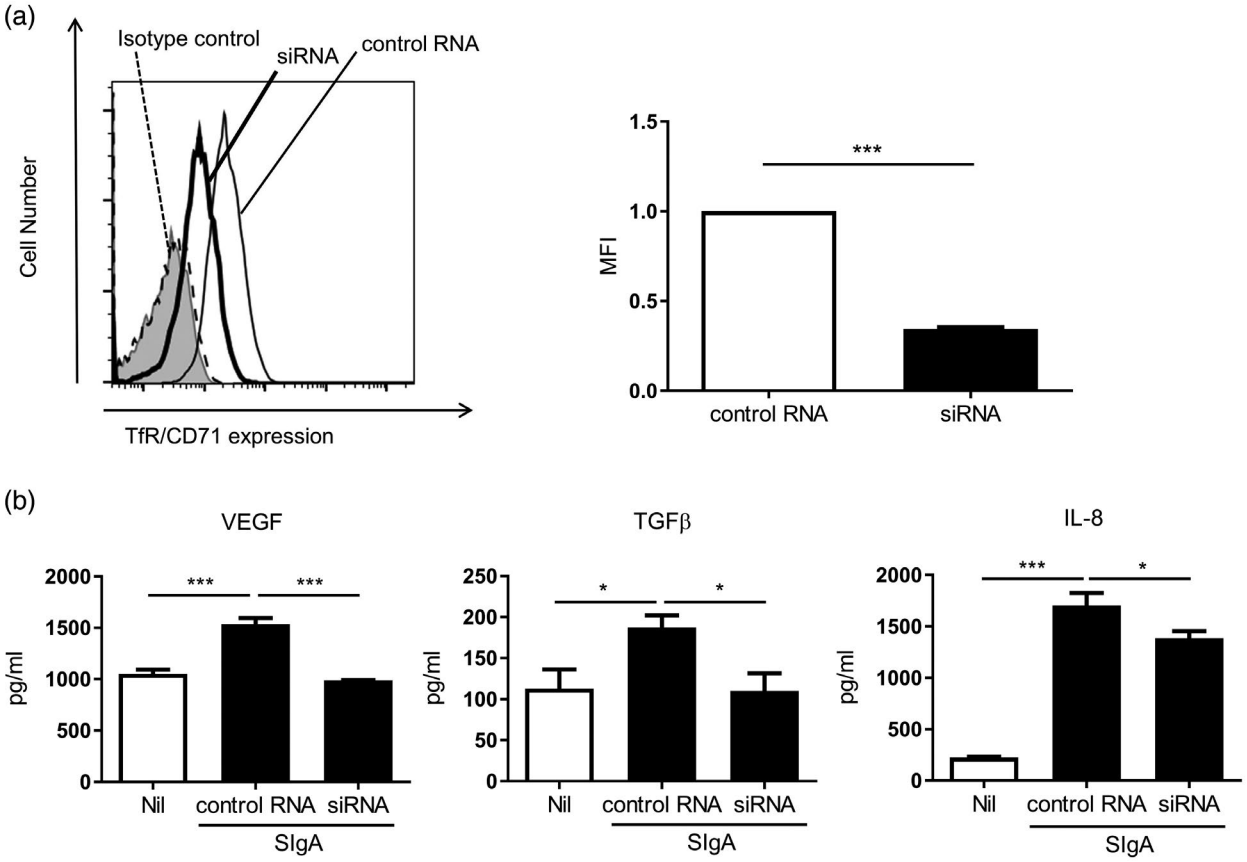

Fig. 5. Knock-down experiments on transferrin receptor (TfR)/CD71. (a) A549 cells were transfected with TfR/CD71 siRNA (bold line) or negative control RNA (solid line). The cells were stained with specific antibody for TfR/CD71 or with isotype control antibody (dashed line) and analyzed by flow cytometry. Representative histograms from three independent experiments are shown. The shaded area shows cells without staining. Mean fluorescence intensity (MFI) of the histograms from three independent experiments are shown as relative values. Bars represent the standard error of the mean (s.e.m.). ***$P < 0{\cdot}001$. (b) After transfection with TfR/CD71 siRNA or control RNA, A549 cells were stimulated with secretory immunoglobulin A (SIg)A at 300 μg/ml for 24 h. The levels of vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β and interleukin (IL)-8 in the culture supernatant were measured. Bars represent the s.e.m. ($n = 4$–12); n.s. = not significant. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$.

prognosis of IPF [20,39]. Taken together, SIgA may be involved in the progression of IPF by promoting production of these mediators by epithelial cells in remodeled tissue in IPF lungs.

The serum IgA concentration is reported to reflect the severity of liver fibrosis in non-alcoholic fatty liver disease [40] and also the prognosis of IPF [41]. While IgA can be a biomarker of fibrosis based on these findings, it is not clear whether IgA itself up-regulates profibrotic cytokine synthesis. TGF-β can be a confounding factor, because it induces IgA class-switch [42] and promotes fibrosis in the liver and lungs [12,22,43]. Interestingly, serum IgA1 from patients with IgA nephropathy was reported to induce TGF-β synthesis via the renin–angiotensin system in human mesangial cells [44]. In line with this, our previous study showed that SIgA induces inflammatory cytokine production and collagen synthesis by human lung fibroblasts [45]. In our present study, using alveolar epithelial cells, we show for the first time that high-dose SIgA can induce production of two profibrotic cytokines, VEGF and TGF-β.

TfR/CD71 was first reported as a serum IgA receptor expressed on mesangial cells in IgA nephropathy [26]. It was also shown to function as an SIgA receptor [6], although it is still controversial whether TfR/CD71 binds SIgA [6] or not [46]. Our preliminary experiments, using recombinant TfR/CD71 and an ELISA technique, confirmed that SIgA bound to TfR/CD71 to the same extent as serum IgA (Supporting information, Fig. S1). Moreover, among well-established IgA receptors – namely, TfR/CD71, FcαRI/CD89, mannose receptor/CD206, DC-SIGN/CD209, Fcα/μR/CD351, ASGP-R and β 1, 4-galactosyltransferase 1 – only TfR/CD71 was expressed on A549 cells (Fig. 4a). In general, compared to slowly proliferating cells, highly proliferating cells express high levels of TfR/CD71 to meet their iron demands, and this was also the case with A549 cells [47–49]. Hyperplastic epithelial cells in IPF lungs also expressed a high level of TfR/CD71 (Fig. 4b), which is compatible with the finding that serial gene expressions related to cell proliferation are up-regulated in abnormal epithelial cells with an AT2 cell-like identity in IPF lungs [14]. In addition, our knock-down experiment using siRNA suggests that TfR/CD71 may be at least partially involved in the cytokine production induced by SIgA stimulation (Fig. 5b). To further confirm the importance of TfR/CD71 on airway epithelial cells, the expression levels of TfR/CD71 and cytokine synthesis evoked by SIgA were also investigated using BEAS-2B, a bronchial epithelial cell line that expresses TfR/CD71 on its surface. BEAS-2B cells expressed TfR/CD71, but no other established SIgA receptors were detectable on its surface. SIgA induced cytokine production, including IL-8, by BEAS-2B (data not shown).

Despite these findings, we must note that our study has some important limitations. First, our knock-down study using siRNA targeted TfR/CD71, but we cannot conclude that it is the only functional receptor for SIgA, because IL-8 production evoked by SIgA stimulation was not suppressed to the control level (Fig. 5b). There may be other unknown IgA receptor(s) [7], or TfR/CD71 might work only as a molecule that uptakes SIgA intracellularly, while there are other intracellular receptors or signal transducers. Secondly, our *in-vitro* assay did not use epithelial cells obtained from IPF lungs. Although there is an emerging novel method for maintaining iPS cell-derived AT2 cells *in vitro* [50], it is still impossible to culture disease-specific epithelial cells from IPF lungs without their phenotype being changed. A549 is one of the ideal alternative materials available, because it shares some important traits with abnormal epithelial cells in IPF lungs. That is, A549 cells have a AT2 phenotype, and cell proliferation signals are up-regulated with high TfR/CD71 expression, as noted previously [49]. Finally, structural differences among individual SIgA samples obtained from different sources should be taken into account. Although the basic structure of IgA is preserved, IgA is variously modified by glycosylation [1] and, importantly, the bioactivity of IgA is likely to be altered by glycosylation. Aberrant glycosylation is an important factor in the pathogenesis of IgA nephropathy [46], so we should note that the response of A549 cells to SIgA could vary depending on the differences in glycosylation among the SIgA samples.

In conclusion, we show that SIgA bound to A549 cells and evoked production of profibrotic and inflammatory cytokines involved in the pathogenesis of IPF. Further studies are needed to investigate whether SIgA contributes to and modifies the progression of IPF *in vivo*, and to confirm the involvement of TfR/CD71. Our study sheds new light on the role of SIgA in respiratory diseases from a novel viewpoint.

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Disclosure

The authors have no conflicts of interest to declare.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. TfR/CD71 - SIgA ELISA.