

Claudin-1 expression in airway smooth muscle exacerbates airway remodeling in asthmatic subjects

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Background: Increased airway smooth muscle (ASM) mass is an essential component of airway remodeling and asthma development, and there is no medication specifically against it. Tight junction (TJ) proteins, which are expressed in endothelial and epithelial cells and affect tissue integrity, might exist in other types of cells and display additional functions in the asthmatic lung.

Objective: The aim of this study was to investigate the existence, regulation, and function of TJ proteins in ASM in asthmatic patients.

Methods: The expression and function of TJ proteins in primary ASM cell lines, human bronchial biopsy specimens, and a murine model of asthma were analyzed by means of RT-PCR, multispectral imaging flow cytometry, immunohistochemistry, Western blotting, 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester staining, tritiated thymidine incorporation, wound-healing assay, and luminometric bead array.

Results: Increased claudin-1 expression was observed in ASM of asthmatic patients, as well as in a murine model of asthma-like airway inflammation. Whereas IL-1 β and TNF- α upregulated claudin-1 expression, it was downregulated by the T_H2 cytokines IL-4 and IL-13 in primary human ASM cells. Claudin-1 was localized to the nucleus and cytoplasm but not to the cell surface in ASM cells. Claudin-1 played a central role in ASM cell proliferation, as demonstrated by increased ASM cell proliferation seen with overexpression and decreased proliferation seen with small interfering RNA knockdown of claudin-1. Overexpression of claudin-1 induced vascular endothelial growth factor and downregulated IL-6, IL-8, and IFN- γ -induced protein 10 production by ASM cells. Claudin-1 upregulation by IL-1 β or TNF- α was suppressed

by dexamethasone but not by rapamycin, FK506, or salbutamol.

Conclusion: These results demonstrate that claudin-1 might play a role in airway remodeling in asthmatic patients by means of regulation of ASM cell proliferation, angiogenesis, and inflammation. (J Allergy Clin Immunol 2011;127:1612-21.)

Key words: Airway remodeling, airway smooth muscle, asthma, claudin-1, cytokine, IFN- γ -induced protein 10, proliferation, tight junction, treatment, vascular endothelial growth factor

It has been estimated that 300 million persons have asthma.¹ Asthma is characterized by airway inflammation, airway hyper-responsiveness (AHR), and airway obstruction, which are usually reversible in patients with mild asthma.² However, when it is not adequately treated, permanent irreversible structural changes in the airways result in airway remodeling.³ An excessive repair process takes place with increased airway smooth muscle (ASM) mass, inflammatory cell infiltration, subepithelial fibrosis, mucous metaplasia, and bronchial angiogenesis.^{4,5} Increased ASM mass, which consists of hyperplasia, hypertrophy, and migration of ASM, is the most important component of airway remodeling in asthmatic patients.^{6,7} Treatment strategies are being developed that are focused on 3 mechanisms, including decreased ASM proliferation, augmented ASM apoptosis, and reduced ASM migration into the smooth muscle layer.⁸ However, it seems difficult to treat increased ASM mass because no specific medication has been demonstrated thus far, and numerous studies are being performed to find novel molecular targets and modes of treatment.⁹⁻¹¹

Tight junctions (TJs) normally exist in endothelial and epithelial cells. They mainly play a role in barrier function by tightly connecting neighboring cells and restricting the passage of substances through the paracellular pathway.¹² The TJ network consists of transmembrane proteins and cytoplasmic peripheral proteins. Transmembrane proteins include at least 4 types termed claudins, occludins, junctional adhesion molecules, and crumbs.¹³ The zonula occludens (ZO) family proteins link transmembrane proteins to the underlying cytoskeleton. To date, 24 members of the claudin family have been identified in mice and human subjects.¹⁴ Claudin-1, which is a main structural component of TJs, is a 23-kd membrane protein and has 4 transmembrane domains with 2 extracellular loops in epithelial cells.¹² Claudin-1 expression has been reported in the heart, brain, lung, liver, kidney, and testis.¹² Recent reports have revealed some additional roles for TJ proteins. Some of the claudin families expressed in tumor cells are involved in their growth and metastatic behavior, although the effect depends on cell types and is still under investigation.¹⁵ Claudin-1 is expressed in melanoma, which is a malignant neoplasm of nonepithelial origin, and contributes to the motility of

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Abbreviations used

AHR:	Airway hyperresponsiveness
ASM:	Airway smooth muscle
CFSE:	5-(and-6)-Carboxyfluorescein diacetate succinimidyl ester
IP-10:	IFN- γ -induced protein 10
NF- κ B:	Nuclear factor κ B
PMA:	Phorbol 12-myristate 13-acetate
siRNA:	Small interfering RNA
TJ:	Tight junction
VEGF:	Vascular endothelial growth factor
ZO:	Zonula occludens

the cells.¹⁶ Furthermore, claudin-1 expressed in Langerhans cells and lymph node dendritic cells promotes their adhesion and migration.¹⁷

As a part of our studies that investigate novel ways to fight severe remodeling in asthmatic patients, we demonstrate here that a TJ protein, claudin-1, is highly expressed in ASM in asthmatic patients. The present study is focused on its regulation and functions and suggests a novel mechanism in airway remodeling.

METHODS

Subjects

Bronchial biopsy specimens were obtained from 3 nonasthmatic subjects (1 male and 2 female subjects) and 5 patients with severe persistent asthma (1 male and 4 female patients). The study was approved by the responsible regional and cantonal ethics commissions. Human primary ASM cells from bronchi were purchased from Promocell (Heidelberg, Germany) and Lonza (Visp, Switzerland). Two different cell lines were expanded in smooth muscle growth medium (SmGM-2, Lonza) in a humidified atmosphere containing 5% CO₂ at 37°C and frozen into liquid nitrogen for the further experiments (passage 3).¹⁸

Overexpression of claudin-1

Full-length claudin-1 cDNA was amplified from human primary ASM cells after RNA extraction and reverse transcription, as described below, with the exception of using oligo-dT priming instead of random hexamers. For amplification of claudin-1, the following primers were used. The forward primer sequence with a *Nco*I restriction site is 5'-TTG GCC ATG GCC AAC GCG GGG CTG CAG CTG TTG GGC -3', and the reverse primer sequence with a *Nhe*I restriction site is 5'-TAG GCT AGC CTA CAC GTA GTA GTC TTT CCC GCT GGA AGG TGC -3'. The resulting 570-bp fragment was subcloned into the *Nco*I/*Nhe*I site of the expression vector pORF (InvivoGen, San Diego, Calif) and transformed into *Escherichia coli* NovaBlue (DE3) strains (Novagen, San Diego, Calif). After sequence verification by means of sequencing of the DNA insert, as previously described,¹⁹ a clone harboring a correct sequence was grown overnight at 37°C in a shaking incubator in 200 mL of 2*YT broth (Bio101) supplemented with 100 μ g/mL ampicillin, and the plasmid DNA was isolated the following day with the QIAfilter Plasmid Midi Kit (Qiagen, Hombrechtikon, Switzerland), according to the manufacturer's protocol. pORF without any insert was used as a control plasmid.

One day before transfection, 9×10^4 ASM cells were seeded into a 12-well plate. For transfection, the complexes of 1.6 μ g of plasmid DNA and 4 μ L of Lipofectamine 2000 (Invitrogen, Basel, Switzerland) were prepared, according to the manufacturer's instructions, and added with 1 mL of medium in total. After 4 hours of incubation, the medium was replaced. Twenty-four hours after transfection, cells were used for further investigations, as previously described.²⁰ Optimal conditions were determined by preliminary experiments.

Knockdown of claudin-1

Transfection reagents and small interfering RNA (siRNA) were purchased from Ambion (Austin, Tex). For transfection, a proprietary blend of polyamines (siPORT *Amine*) that delivers siRNA into mammalian cells was used. Three different Silencer Select Pre-designed siRNA of claudin-1 were tested, and the best one was selected for further experiments. The forward primer sequence is 5'-CAA UAG AAU CGU UCA AGA Att -3', and the reverse primer sequence is 5'-UUC UUG AAC GAU UCU AUU Gcc -3'. Silencer Select Negative Control #1 siRNA was used as a negative control.

One day before transfection, 6×10^4 ASM cells were seeded into a 12-well plate. The complexes of siRNA (20 nmol/L final concentration) and 4 μ L of transfection agent were prepared, according to the manufacturer's instructions, and added together with 1 mL of medium in total to transfection wells. Twenty-four hours after transfection, the medium was replaced, and the cells were used for the further experiments, as previously described.²¹ Optimal conditions were determined by preliminary experiments.

Proliferation analysis

ASM cell proliferation was analyzed by using 3 different methods, including a 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) proliferation assay, a tritiated thymidine incorporation assay, and a wound-healing assay. For the CFSE proliferation assay, after transfection, ASM cells were harvested and labeled with 5 μ mol/L CFSE (Invitrogen) in 1 mL of medium for 6 minutes at 4°C in the dark. After washing twice, cells were seeded in a 6-well plate. ASM cells were irradiated with 100 kVp by means of radiography for 15 minutes as a nonproliferative control. On the fifth day, CFSE was detected by using flow cytometry (Beckman Coulter, Brea, Calif).

For the tritiated thymidine incorporation assay, cells were incubated for a certain period, and 1 μ Ci of tritiated thymidine (Hartmann Analytic GmbH, Braunschweig, Germany) was added to each well (corresponds to a final concentration of 5 μ Ci/mL) during the last 24 hours. After the plate was frozen and thawed, incorporation of labeled nucleotide was determined by using a LKB β plate reader (GE Healthcare, Zurich, Switzerland), as previously described.²²

For the wound-healing assay, after transfection, the ASM cell monolayer was scratched with a 10- μ L pipette tip, and cellular debris was removed by means of washing. Microscopic pictures were taken at certain time points after scratching at $\times 50$ magnification with an AxioCam (Carl Zeiss AG, Feldbach, Switzerland).

Statistical analysis

Statistical analysis was performed by using the Wilcoxon signed-rank test in paired conditions and the unpaired *t* test. Differences in *P* values of .05 or less were considered significant.

For more information on cell cultures, reagents, isolation of RNA and cDNA synthesis, quantitative real-time PCR, immunofluorescence staining, staining for multispectral imaging flow cytometry, Western blotting, determination of cytokine concentrations, quantification of the wound-healing model, and the murine model of airway inflammation see [Table E1](#) and the [Methods](#) section in this article's Online Repository at www.jacionline.org.

RESULTS

Claudin-1 was upregulated by IL-1 β or TNF- α and downregulated by IL-4 or IL-13 in human primary ASM cells

Human primary ASM cells were investigated for the expression of TJ proteins. Cells were cultured together with several cytokines, and relative mRNA expressions of major TJ proteins were analyzed. Although claudin-1, claudin-4, occludin, ZO-1, and ZO-2 were all detectable, claudin-1 showed significant

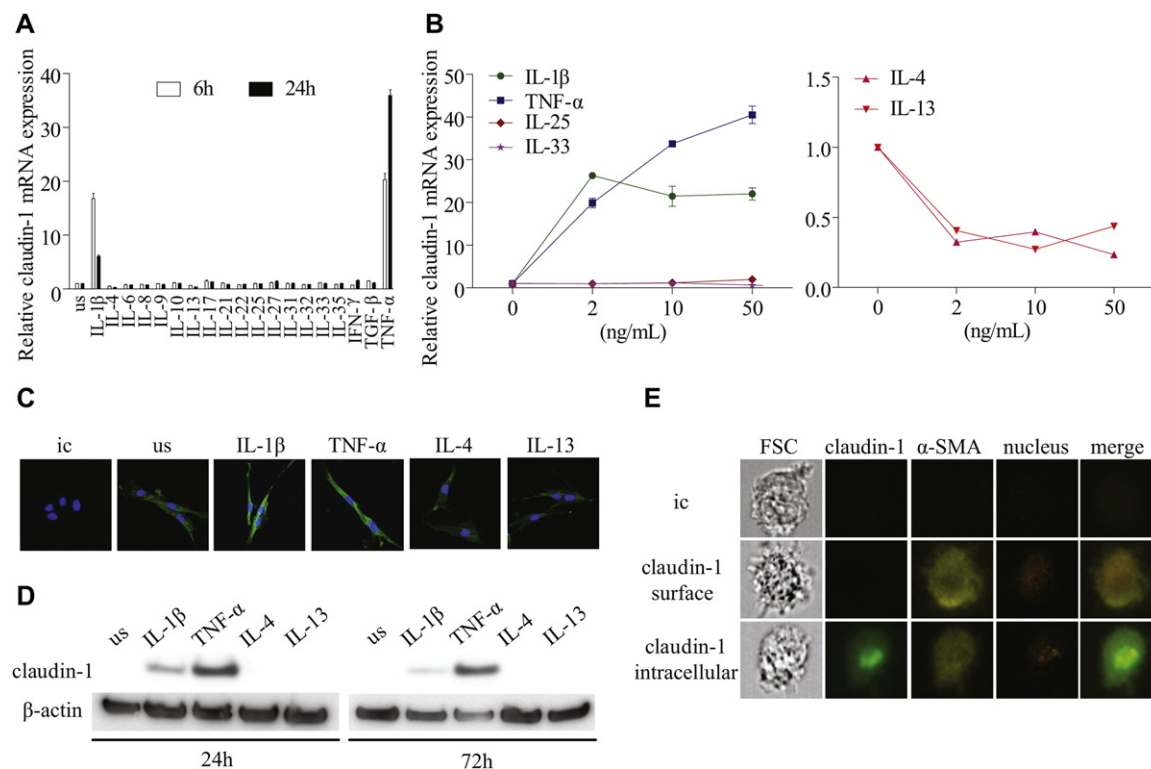


FIG 1. Claudin-1 is upregulated by IL-1β and TNF-α and downregulated by IL-4 and IL-13 in human primary ASM cells. Human primary ASM cells were incubated with 50 ng/mL (A), different concentrations (B), or 10 ng/mL (C and D) of cytokines. Relative claudin-1 mRNA expression (Fig 1, A and B) and claudin-1 protein levels were detected by means of immunofluorescence staining at 24 hours (Fig 1, C), Western blotting (Fig 1, D), and multispectral imaging flow cytometry (E). Results are representative of 2 (Fig 1, D and E) or 3 independent experiments with 2 different ASM cell lines. Fig 1, A and B, shows means ± SEMs. FSC, Forward scatter; ic, isotype control; us, unstimulated.

regulation among TJ proteins by several proinflammatory and T_H2 cytokines, and accordingly, it was further investigated in detail (Fig 1, A, and see Fig E1 in this article's Online Repository at www.jacionline.org). The mRNA levels of claudin-4, occludin, ZO-1, and ZO-2 showed changes in response to several cytokines on which we did not focus in the present study. Claudin-4 was downregulated by IL-1β, IL-4, IL-13, and TNF-α. Occludin and ZO-2 were downregulated by IL-1β at 24 hours (see Fig E1).

The proinflammatory cytokines IL-1β and TNF-α highly upregulated claudin-1 expression in ASM cells at 24 hours (Fig 1, B). On the other hand, the T_H2 cytokines IL-4 and IL-13 significantly downregulated claudin-1 expression. The 2 new cytokines with both proinflammatory and T_H2 functions, namely IL-25 and IL-33, did not show any effect on claudin-1 expression. The suppressive effects of IL-4 and IL-13 were also observed on claudin-1 mRNA expression upregulated by IL-1β and TNF-α (see Fig E2 in this article's Online Repository at www.jacionline.org). These findings were confirmed with the expression of claudin-1 protein by means of immunofluorescence staining and Western blotting (Fig 1, C and D). Almost no claudin-1 expression was observed in unstimulated cells in Western blots. TNF-α highly and continuously upregulated claudin-1, whereas IL-1β had a relatively low effect (Fig 1, D). These changes in claudin-1 protein expression were quite parallel to changes in mRNA expression

(see Fig E3 in this article's Online Repository at www.jacionline.org).

Localization of claudin-1 in ASM cells was also investigated by means of multispectral imaging flow cytometry. Although surface staining of claudin-1 showed no expression on the cell surface, intracellular staining demonstrated that claudin-1 was expressed both in the nucleus and the cytoplasm (Fig 1, E).

Among the above-mentioned cytokines, only TNF-α induced ASM cell proliferation, whereas IL-1β, IL-4, IL-13, IL-25, and IL-33 did not show any effect (see Fig E4 in this article's Online Repository at www.jacionline.org).

Claudin-1 overexpression highly stimulated ASM cell proliferation

Because ASM cell proliferation is an essential feature of airway remodeling,⁶ we investigated whether claudin-1 might regulate their proliferation in experiments in which claudin-1 was overexpressed in primary human ASM cells. Transfection of claudin-1-pORF highly and specifically upregulated its mRNA expression (Fig 2, A) and protein levels (Fig 2, B and C). ASM cell proliferation was analyzed in claudin-1-overexpressing and control ASM cells by using 3 different methods, namely a CFSE staining assay, a tritiated thymidine incorporation assay, and a wound-healing assay. The CFSE dilution and tritiated thymidine incorporation assays both showed more proliferation in

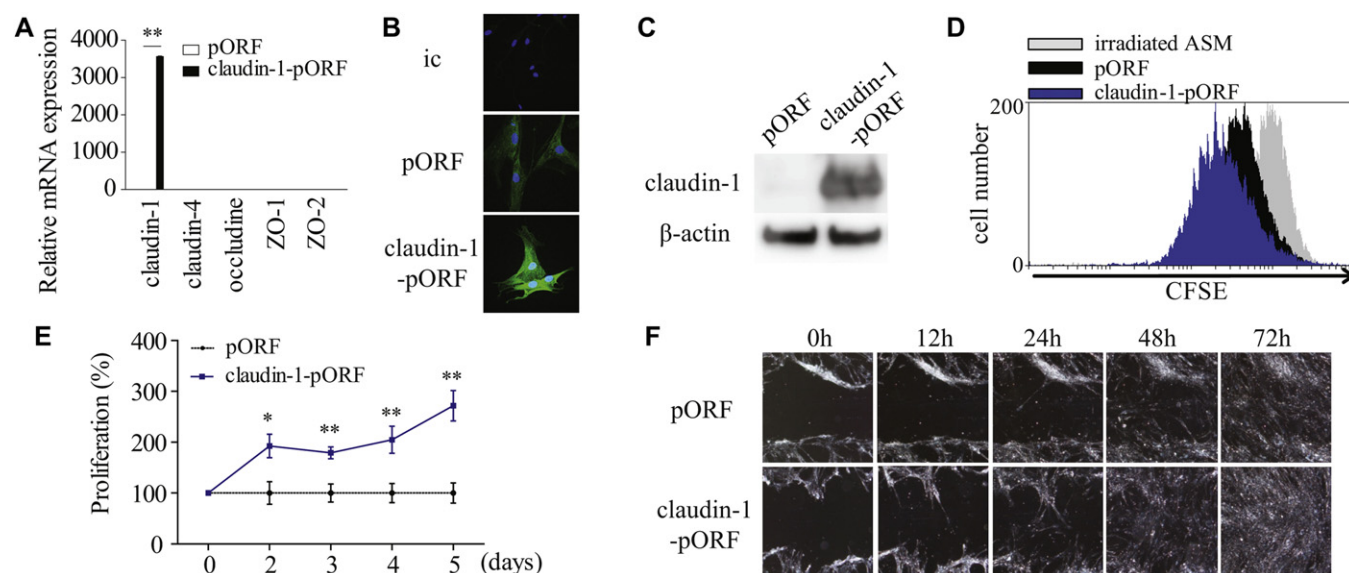


FIG 2. Claudin-1 overexpression stimulates ASM cell proliferation. An empty vector (*pORF*) or a vector containing claudin-1 (*claudin-1-pORF*) was transfected into primary ASM cells. Relative mRNA expression of TJ molecules was analyzed (**A**), and claudin-1 protein levels were analyzed by means of immunofluorescence (**B**) and Western blotting (**C**). Cell proliferation was determined by using a CFSE proliferation assay (**D**), a tritiated thymidine incorporation assay (**E**), and a wound-healing assay (**F**). Fig 2, A, B, D, and F are representative of at least 3 independent experiments. Fig 2, E, is from 4 independent experiments. Fig 2, A and E, show means $\pm$ SEMs. * $P < .05$ and ** $P < .01$.

the claudin-1-transfected ASM cells compared with that seen in the control ASM cells (Fig 2, D and E). These findings were visually confirmed by the wound-healing assay (Fig 2, F, and see Fig E5, A, in this article's Online Repository at www.jacionline.org). ASM cells overexpressing claudin-1 recovered quickly and closed the scratch-induced wound significantly faster than control cells.

Claudin-1 knockdown decreased ASM cell proliferation

Claudin-1 was knocked down by means of transfection with siRNA to ensure the function of claudin-1 in ASM cell proliferation. Transfection of claudin-1 siRNA specifically diminished its mRNA expression at 24 hours (Fig 3, A), and claudin-1 protein was clearly suppressed after 48 hours (Fig 3, B). In contrast to the findings in overexpression experiments, claudin-1 siRNA knockdown showed slightly decreased ASM cell proliferation. This could be because of a small amount of claudin-1 expression in ASM cells under resting conditions (Fig 3, C-E, and see Fig E5, B). For this reason, claudin-1 or control siRNA-transfected ASM cells were stimulated with TNF- α , which induced both claudin-1 expression and ASM cell proliferation. TNF- α upregulated claudin-1 expression, which was significantly suppressed by claudin-1 siRNA (Fig 3, F). Claudin-1 knockdown showed a significant decrease of proliferation in these ASM cells (Fig 3, G and H, and see Fig E5, C). The intensities of wound healing were calculated and are demonstrated in Fig E5. TNF- α induced almost full wound healing after 36 hours, and the effect of siRNA knockdown of claudin-1 was demonstrated after 24, 36, and 48 hours.

Together, these overexpression and siRNA suppression data demonstrate that claudin-1 plays an important role in ASM cell proliferation.

Claudin-1 expression was suppressed by dexamethasone

ASM cells were incubated with and without IL-1 β or TNF- α in the presence or absence of each medical compound, and relative claudin-1 mRNA expression was analyzed after 24 hours to determine the effect of drugs on claudin-1 expression. Three immunosuppressive compounds, including dexamethasone, rapamycin, and FK506, and salbutamol, which is a short-acting β_2 -adrenergic receptor agonist, were studied. No significant difference of claudin-1 expression was observed by using these medical compounds in unstimulated ASM cells. The effect of IL-1 β on the upregulation of claudin-1 expression was highly suppressed only by dexamethasone but not by the other compounds (Fig 4, A). The same results were observed in ASM cells stimulated with TNF- α . Because salbutamol is a short-acting agent, the kinetics of claudin-1 expression with salbutamol was also investigated, showing no effects on claudin-1 expression (see Fig E6 in this article's Online Repository at www.jacionline.org). These results were confirmed by the analysis of claudin-1 protein expression. ASM cells were incubated with and without IL-1 β or TNF- α in the presence of 0.1 μ mol/L dexamethasone, and claudin-1 protein was analyzed after 24 hours by means of immunofluorescence staining. Again, dexamethasone suppressed the upregulation of claudin-1 induced by IL-1 β or TNF- α (Fig 4, B).

Claudin-1 regulates IL-6, IL-8, vascular endothelial growth factor, and IFN- γ -induced protein 10

Supernatants of claudin-1-overexpressing ASM cell cultures were analyzed to find which cytokines, chemokines, or both are regulated by claudin-1. Twenty-four hours after the overexpression of claudin-1, ASM cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin or left unstimulated

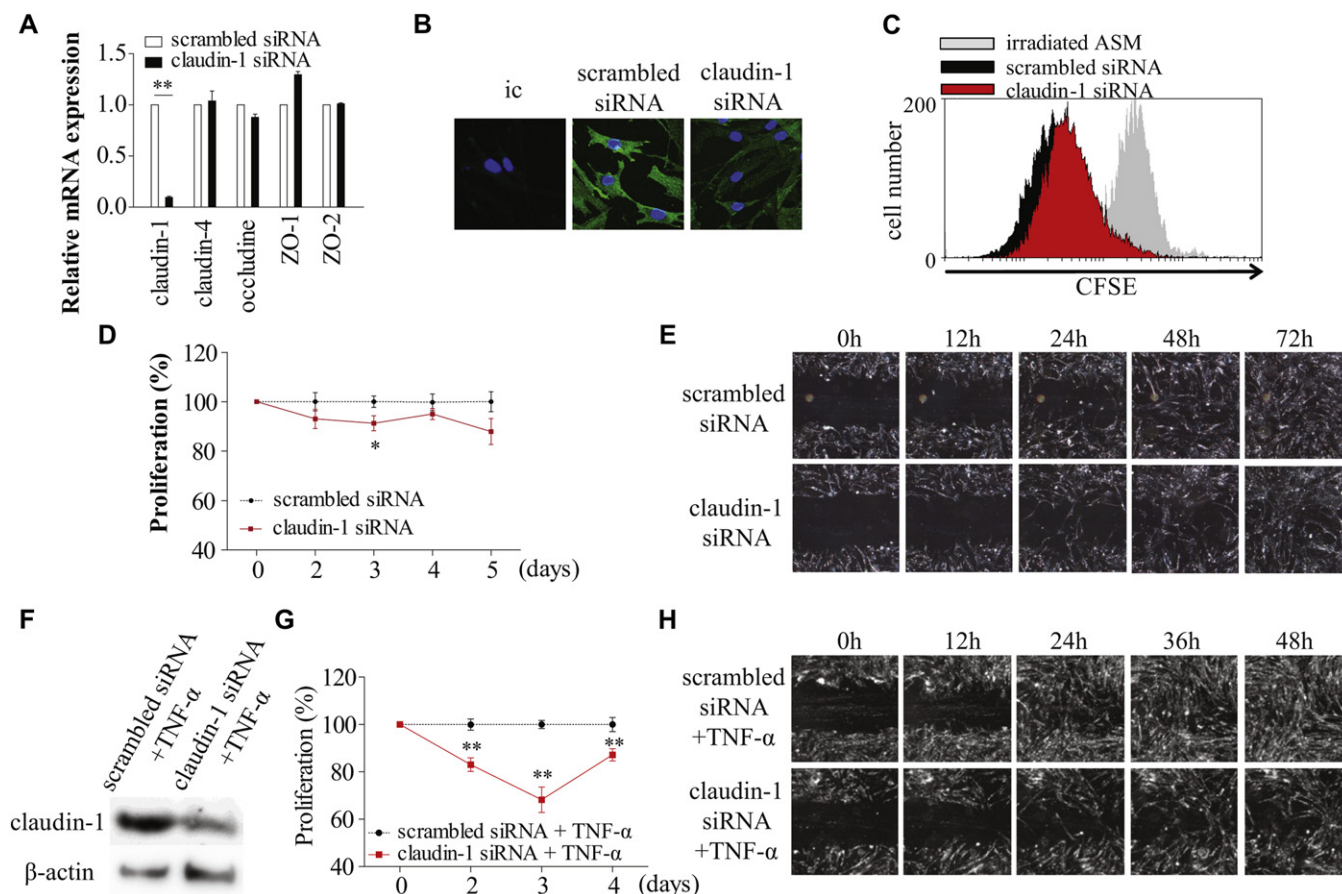


FIG 3. siRNA knockdown of claudin-1 decreased ASM cell proliferation. Scrambled siRNA or claudin-1 siRNA was transfected. Claudin-1 expression (**A** and **B**) and cell proliferation (**C–E**) were analyzed as in Fig 2. siRNA-transfected ASM cells were stimulated with 10 ng/mL TNF- α 24 hours after transfection, and claudin-1 protein (**F**) and cell proliferation (**G** and **H**) were analyzed under TNF- α -stimulated conditions. Fig 3, **A**, **B**, **C**, **E**, **F**, and **H**, are representative of at least 2 independent experiments. Fig 3, **D** and **G**, are from at least 4 independent experiments. Fig 3, **A**, **D**, and **G**, show means $\pm$ SEMs. * $P < .05$ and ** $P < .01$. ic, Isotype control.

for 24 hours. Protein concentrations of 27 cytokines and chemokines in supernatants were determined by using the luminometric bead array. In these measurements IL-1 β , IL-1 receptor α , IL-2, IL-4, IL-5, IL-7, IL-10, IL-15, IL-17, eotaxin, GM-CSF, platelet-derived growth factor, and TNF- α were undetectable. IL-9, IL-12, IL-13, fibroblast growth factor, granulocyte colony-stimulating factor, IFN- γ , monocyte chemoattractant protein 1, macrophage inflammatory protein 1 α and 1 β , and RANTES were detectable in small amounts without showing any difference with overexpression of claudin-1. Four of these, IL-6, IL-8, IFN- γ -induced protein 10 (IP-10), and vascular endothelial growth factor (VEGF), were detectable in high levels (in nanograms per milliliter). Overexpression of claudin-1 significantly upregulated VEGF and downregulated the proinflammatory cytokines IL-6, IL-8, and IP-10 in resting conditions (Fig 5, **A**). PMA/ionomycin stimulation significantly upregulated VEGF and downregulated IP-10 in claudin-1-overexpressing cells, whereas IL-6 and IL-8 expression was not altered (Fig 5, **B**). Taken together, claudin-1 in ASM cells plays a further important role in other aspects of airway remodeling and inflammation because VEGF as an angiogenic factor efficiently contributes to essential airway remodeling features of asthma, such as angiogenesis of the chronic asthmatic lung when proinflammatory factors were downregulated.

Claudin-1 expression was increased in asthmatic lung

The expression of claudin-1 in asthmatic patients and asthma-like inflammation in lungs of mice was analyzed to investigate the role of claudin-1 in asthmatic patients *in vivo*. In human subjects claudin-1 was highly expressed in ASM sections from patients with severe persistent asthma compared with that seen in nonasthmatic subjects (Fig 6, **A**). Similarly, claudin-1 expression, which was detected in epithelial cells as expected, was significantly increased in ASM in ovalbumin-treated murine lungs (Fig 6, **B**). These results suggest that claudin-1 might play a critical role in airway remodeling in asthmatic patients because nonasthmatic human subjects and noninflamed lungs of control mice both showed very little claudin-1 expression in ASM.

DISCUSSION

In the present study we demonstrate that TJ proteins, particularly claudin-1, are expressed and regulated in ASM cells in human asthmatic patients and in a murine model of asthmatic lung inflammation. Thus far, there has been no report on the role and description of TJ protein expression and function on ASM cells. Only 1 study describes the relationship between TJs and ZO-2 in vascular smooth muscle cells, which promotes its

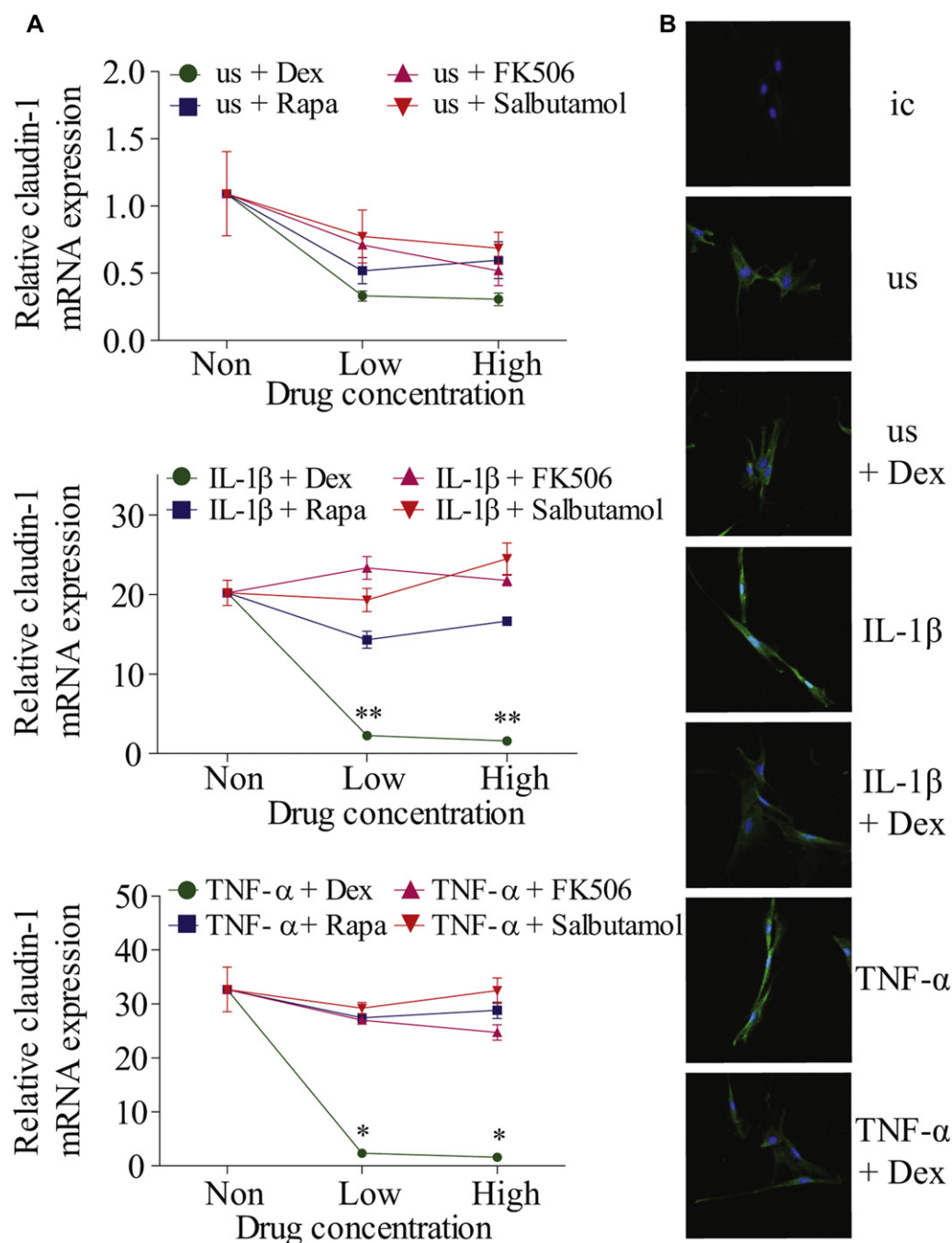


FIG 4. Claudin-1 expression is suppressed by dexamethasone (Dex). ASM cells were incubated with and without 50 ng/mL of cytokines in the presence or absence of each compound, and relative claudin-1 mRNA expression (**A**) and protein levels (**B**) were analyzed after 24 hours. Data are representative of 3 independent experiments. Fig 4, A, shows means $\pm$ SEMs. * $P < .05$ and ** $P < .01$. ic, Isotype control; Rapa, rapamycin; us, unstimulated.

proliferation through regulation of signal transducer and activator of transcription 1, and this suggests that ZO-2 can be a molecular target in the pathological state of vascular remodeling in patients with cardiovascular diseases.²³

Proinflammatory cytokines, such as IL-1 β or TNF- α , which are important for the pathogenesis of asthma, upregulate claudin-1 expression in ASM, whereas classical T_H2 cytokines, such as IL-4 or IL-13, which have been linked to allergic responses,

downregulate it. Although both IL-1 β and TNF- α upregulated claudin-1 expression, only TNF- α promoted ASM cell proliferation. TNF- α highly and continuously upregulated claudin-1 expression, whereas the peak of claudin-1 expression by IL-1 β was at 12 hours at the mRNA level, and it gradually decreased. This difference in intensity and kinetics might partially play a role in this effect. Furthermore, ZO-2 expression, which has been known to promote vascular smooth muscle cell proliferation,

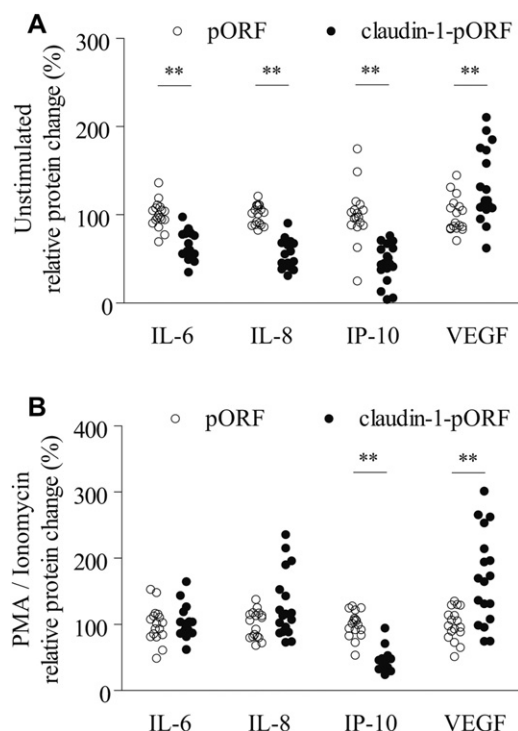


FIG 5. Claudin-1 overexpression upregulates VEGF and downregulates IL-6, IL-8, and IP-10 in ASM cells. Twenty-four hours after the transfection, ASM cells were left unstimulated (**A**) or stimulated with PMA and ionomycin (**B**) for 24 hours. Cytokine and chemokine concentrations in supernatants were determined by using the luminometric bead array, and relative percentage change against the negative control was shown. The ranges of secreted cytokines and chemokines were as follows: IL-6, 296 to 4,448 pg/mL; IL-8, 257 to 3,435 pg/mL; IP-10, 662 to 81,462 pg/mL; and VEGF, 263 to 2,500 pg/mL. Data are from 6 independent experiments in triplicate of 18 samples. $^{**}P < .01$.

was upregulated by TNF- α but not by IL-1 β . In addition, other factors that were influenced by these 2 cytokines could affect proliferation. Obviously there are many factors that influence cell proliferation. ASM cells proliferate in response to at least 2 major groups of mitogens. One comprises polypeptide growth factors that activate receptors with intrinsic receptor tyrosine kinase activity, and the other comprises contractile agonists that ligate receptors linked to heterotrimeric guanosine triphosphate-binding proteins.⁶

It seemed that claudin-1 was localized to both the nucleus and cytoplasm but not to the cell surface, suggesting that it is not a typical component of TJ structure in ASM cells. As observed in the present study, in human colon cancers claudin-1 was expressed not only in the cell membrane but also in the nucleus and cytoplasm.²⁴

Overexpression and siRNA knockdown experiments were performed to investigate the pure effect of claudin-1 on ASM proliferation. Both overexpression and siRNA knockdown were claudin-1 specific, enabled live cells for further experiments, and did not affect other TJ proteins. Overexpression of claudin-1 clearly showed the increase of ASM cell proliferation. However, siRNA knockdown of claudin-1 slightly decreased it, most probably because of a small amount of claudin-1 expression in resting cells. To overcome this, claudin-1 siRNA knockdown together with TNF- α stimulation significantly showed the role of

claudin-1 in ASM cell proliferation. Although the role of claudin-1 in cell proliferation seems to depend on cell types and a difference has been observed between transformed cell types of tumors and primary cells,¹⁵ several studies have provided evidence that claudin-1 expression is related to cell proliferation. Claudin-1 expression is increased in human colon cancers, particularly in metastatic lesions.²⁴ Claudin-1 is critical to induce invasive behavior in human liver cells, and the activation of the c-Abl-protein kinase δ signaling pathway is required for the claudin-1-induced acquisition of the malignant phenotype.²⁵ In patients with inflammatory bowel disease, claudin-1 expression is increased and correlates positively with inflammatory activity, suggesting that increased claudin-1 expression might be involved at early stages of transformation in inflammatory bowel disease-associated neoplasia.²⁶

The upregulation of claudin-1 by proinflammatory cytokines is suppressed by dexamethasone. It has been known that epithelial TJ proteins, including claudin-1, are internalized through a nuclear factor κ B (NF- κ B)-dependent pathway, and NF- κ B activation induced by mucosal T cells is required for the opening of TJs.²⁷ NF- κ B activation also seems to be required for claudin-1 upregulation in ASM cells because dexamethasone strongly inhibits NF- κ B.²⁸ In contrast, neither the immunosuppressive compounds rapamycin, which inhibits IL-2 response, nor FK506, which inhibits IL-2 production by T cells, suppressed claudin-1 upregulation, and neither did salbutamol, a β_2 -adrenergic receptor agonist frequently used for asthma attacks.

We further investigated whether some essential proinflammatory cytokines and chemokines are regulated by claudin-1. Overexpression of claudin-1 demonstrated a positive association with VEGF and a negative association with IP-10, irrespective of whether ASM cells were stimulated. IL-6 and IL-8, the 2 proinflammatory cytokines decreased in ASM cells, are similar to the proinflammatory chemokine IP-10. It is very important to stress here that all 3 essential proinflammatory molecules were downregulated by claudin-1 overexpression. It has been well known that VEGF expression is increased in tissues from asthmatic patients^{7,29,30} and its levels directly correlate with disease activity.³¹ Moreover, VEGF induces asthma-like phenotypes, such as inflammation, vascular remodeling, edema, mucous metaplasia, and AHR, and there is more smooth muscle hyperplasia in lung-targeted VEGF-transgenic mice.³² Meanwhile, downregulation of proinflammatory cytokines/chemokines might play an important role in inflammation and the tissue-remodeling process. IP-10 plays a role in the generation and delivery of an effector T-cell response³³ and chemotaxis of CXCR3-expressing T_H0 and T_H1 effector cells.³⁴ It is also well known as a potent inhibitor of angiogenesis both *in vitro* and *in vivo*.^{35,36} In addition, expression of IP-10, which is decreased in lung tissue treated with bleomycin, significantly reduces bleomycin-induced pulmonary fibrosis, the number of lung endothelial cells, and angiogenesis.³⁷ A recent study has reported that myocardial infarction in IP-10^{-/-} mice shows a significantly high number of infiltrated α -smooth muscle actin-positive myofibroblasts compared with wild-type infarcts but exhibits reduced recruitment of α -smooth muscle actin-positive cells that expressed CXCR3.³⁸ Furthermore, there is evidence that oral corticosteroids cause a significant increase in IP-10 expression in asthmatic airway mucosa,³⁹ which concurs with our findings that dexamethasone suppresses the upregulation of claudin-1 expression, which indirectly results in increased IP-10 levels. On the

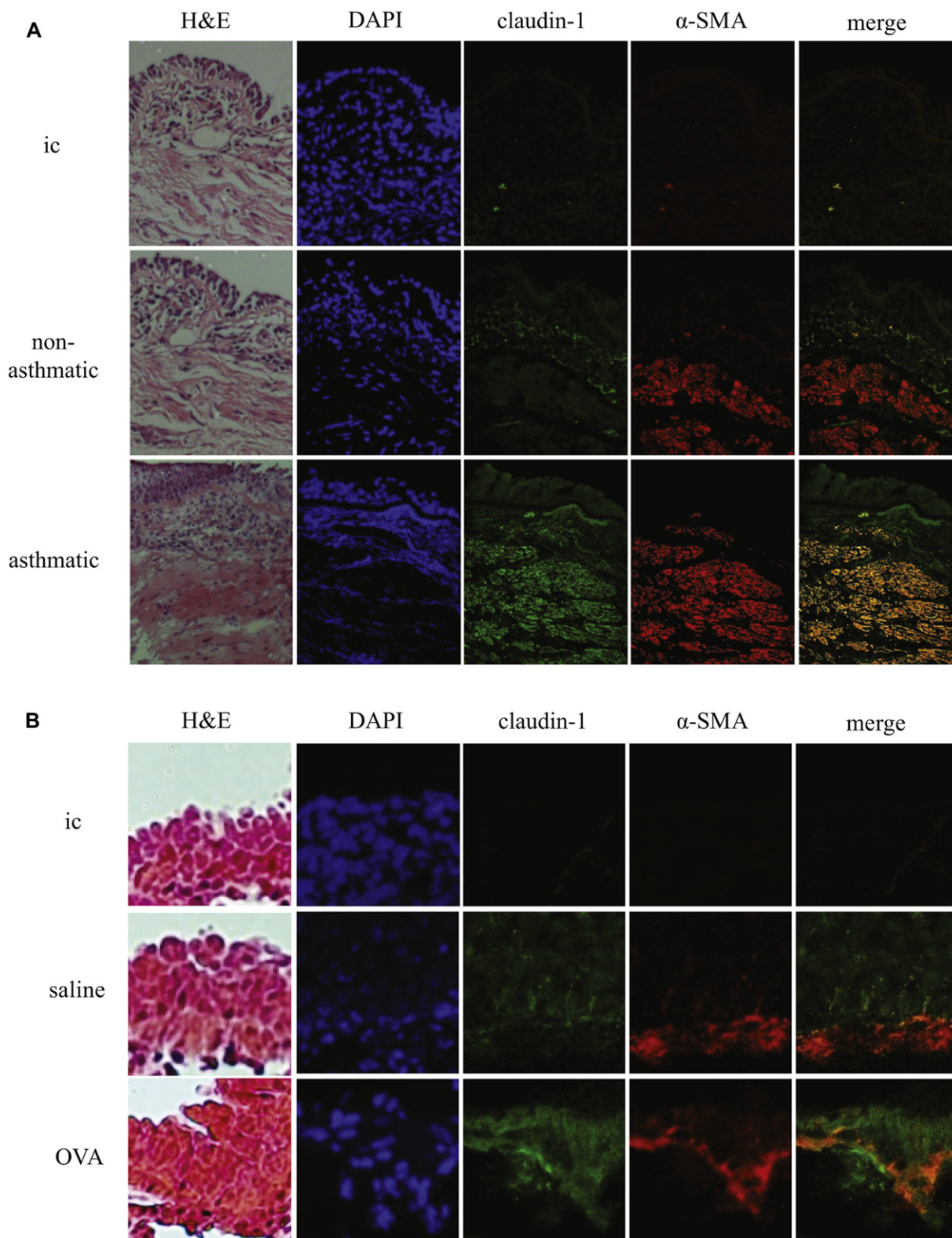


FIG 6. Claudin-1 expression is increased in asthmatic lungs. **A**, Human bronchial biopsy specimens from patients with severe persistent asthma and nonasthmatic subjects were analyzed. **B**, An ovalbumin (OVA)-induced murine model of airway inflammation was analyzed. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue), claudin-1 was stained with Alexa Fluor 488 (green), and α -smooth muscle actin (α -SMA) was stained with Cy3 (red). Data shown are from 1 representative staining. H&E, Hematoxylin and eosin; ic, isotype control.

other hand, IP-10 also shows a proasthmatic function, which contributes to AHR and T_H2 -type inflammation in a murine model of asthma.⁴⁰

In addition to these findings with primary ASM cell lines, immunofluorescence staining shows claudin-1 expression in ASM is increased in asthmatic lungs both from humans and mice. Although T_H2 -mediated allergic inflammation is predominant in asthmatic airways, proinflammatory cytokines also play a key role in a chronic, established inflammatory phase of asthma.⁴¹ The balance of IL-1 β and TNF- α levels versus IL-4 and IL-13 levels might be important for claudin-1 expression. It seems that the concentration of T_H2 cytokines *in vivo* might not be sufficient to suppress claudin-1 expression in the presence of IL-1 β and TNF- α . It should be stressed here that T_H2 cytokines alone did not show any direct effect on ASM cell proliferation.

Taken together, upregulation of claudin-1 can be a target of inflammation leading to airway remodeling in asthmatic patients. In normal tissue repair there is a focus on 3 key mechanisms: (1) the inflammatory cascade should be downregulated; (2) the injured cells should receive proliferative capacity and try to increase in number and mass to close the wound; and (3) the tissue should show increased angiogenesis capacity to feed and regenerate remodeling areas. All of these events are taking place in lungs from asthmatic patients, and claudin-1 expression in ASM cells plays an important role in all of the key aspects of airway remodeling.

We thank Dr Kyoko Fujita for critical review and discussions and the AO Research Institute, Davos, Switzerland, for housing and maintaining the mice.

Clinical implications: The regulation of claudin-1 by proinflammatory and T_H2 cytokines in ASM suggests a role in and represents a possible therapeutic target in airway remodeling in asthma.

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METHODS

Cell cultures

In all experiments ASM cells, passage between 4 and 6, were used in RPMI 1640 medium supplemented with 1 mmol/L sodium pyruvate, 1% minimal essential medium, nonessential amino acids and vitamins, and 2 mmol/L L-glutamine, all from Invitrogen, and 10% heat-inactivated FCS from Sera-Lab (Sussex, United Kingdom). One day before the stimulation, cells were harvested by means of trypsinization (trypsin/EDTA solution, neutralized by trypsin-neutralizing solution; Clonetics-BioWhittaker, Walkersville, Md) and seeded into 6-, 12-, or 24-well culture plates at a density of approximately 20,000/cm², which forms 80% to 90% confluency. Afterward, different stimulants were added to ASM cell cultures in the growth phase of the cell cycle and incubated for the indicated time points.

Reagents

Recombinant human IL-1 β , IL-6, IL-8, IL-9, IL-10, IL-13, IL-17A, and IFN- γ were purchased from PeproTech (London, United Kingdom); IL-4, IL-25, IL-27, IL-31, IL-32, IL-33, TNF- α , and TGF- β were from R&D systems (Abingdon, United Kingdom); IL-21 was from eBioscience (San Diego, Calif); and IL-35 was from Alexis (Lausen, Switzerland). Dexamethasone, rapamycin, and FK506 were purchased from Sigma-Aldrich (St Louis, Mo), and salbutamol was from GlaxoSmithKline (Brentford, United Kingdom). The concentrations of dexamethasone were 0.01 and 0.1 μ mol/L, concentrations of rapamycin were 0.1 and 1 μ mol/L, concentrations of FK506 were 0.01 and 0.1 μ mol/L, and concentrations of salbutamol were 1 and 10 μ mol/L.

Isolation of RNA and cDNA synthesis

Total RNA was isolated with the RNeasy mini kit (Qiagen), according to the manufacturer's protocol. Reverse transcription was performed with TaqMan reverse transcription reagents (Fermentas, Nunningen, Switzerland) using random hexamer primers, according to the manufacturer's protocol.

Quantitative real-time PCR

The PCR primers and probes were designed based on the sequences reported in GenBank with the Primer Express software version 1.2 (Applied Biosystems, Foster City, Calif). All primers were purchased from Microsynth (Balgach, Switzerland) and were verified for efficacy over a 4-log concentration range. Primer sequences are listed in Table E1. The prepared cDNAs are amplified by using iTaq SYBR Green Supermix with ROX (Bio-Rad, Basel, Switzerland), according to the manufacturer's recommendations, in an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Relative quantification and calculation of the range of confidence was performed by using the comparative $\Delta\Delta$ CT method. Elongation factor 1 α was used as an endogenous control in human subjects, and glyceraldehyde-3-phosphate dehydrogenase was used in mice. All amplifications were conducted in triplicate.

Immunofluorescence staining

Cells were fixed with 70% ethanol for 10 minutes and were blocked with goat serum (Dako, Glostrup, Denmark) diluted 10-fold with 1% BSA (Fluka, Buchs, Switzerland) in PBS (Invitrogen) for 15 minutes. Next, cells were stained with 2.5 μ g/mL monoclonal mouse anti-claudin-1 antibody labeled with Alexa Fluor 488 (Invitrogen) or the same amount of murine IgG1- κ labeled with Alexa Fluor 488 (Invitrogen) as an isotype control for 1 hour in a humidity chamber. For immunohistochemistry of murine lung tissue, frozen sections were consecutively stained with 3.5 μ g/mL monoclonal anti- α -smooth muscle actin Cy3 conjugate (Sigma-Aldrich) for 1 hour. For immunohistochemistry of human lung tissue embedded in paraffin sections, antigen retrieval was made by boiling sodium citrate buffer (pH 6.0) for 4 minutes in a pressure cooker before staining. Then biopsy specimens were fixed for 8 minutes with 4% formaldehyde (Fluka), followed by permeabilization with detergent (0.1% Triton [Fluka] and 0.02% SDS [Roth, Karlsruhe, Germany] in PBS) for 5 minutes. They were blocked with goat serum diluted 10-fold with 1% in PBS for 15 minutes and stained with 5 μ g/mL rabbit anti-claudin-1 (Invitrogen) or the same amount of rabbit immunoglobulin as an isotype control for 1 hour in a humidity chamber. Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) was used as a secondary

antibody and consecutively stained with Cy3-labeled anti- α -smooth muscle actin for 1 hour. After mounting with VectaShield mounting medium containing 4', 6-diamidino-2-phenylindol (DAPI; Vector Laboratories, Burlingame, Calif), cells were analyzed with a Leica TCS SPE confocal microscope (Leica Microsystems AG, Glattbrugg, Switzerland), and images were acquired with a resolution of 1024 $\times$ 1024 pixels.

Staining for multispectral imaging flow cytometry

Anti-claudin-1 mAb and murine IgG1- κ isotype control were used as described above. Anti-human α -smooth muscle actin labeled with phycoerythrin and murine IgG2a isotype control were purchased from R&D Systems. For surface staining of claudin-1, cells were stained with 2.5 μ g/mL anti-claudin-1 antibody for 30 minutes at 4°C ahead of intracellular staining. For fixation and permeabilization, the BD Cytofix/Cytoperm kit was used according to the manufacturer's instructions (BD biosciences, San Diego, Calif). Intracellular α -smooth muscle actin and claudin-1 were stained with 2.5 μ g/mL anti- α -smooth muscle actin antibody with or without anti-claudin-1 antibody for 30 minutes at 4°C. Subsequently, nuclei were stained with 7-AAD (Beckman-Coulter, Fullerton, Calif) for 15 minutes at 4°C. Cells were analyzed by using ImageStreamX (Amnis, Seattle, Wash).

Western blot analysis

Cells were lysed in lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mmol/L NaCl, and 20 mmol/L Tris HCL [pH 8.0] containing protease inhibitors) for 30 minutes on ice. Cell debris was separated by means of centrifugation. Protein concentration was determined with the BCA-Kit (Pierce, Cheshire, United Kingdom), according to the manufacturer's instruction. Equal amounts of total protein were denaturated for 10 minutes at 80°C in 4 $\times$ NuPage LDS Sample buffer (Invitrogen) containing dithiothreitol, separated on 4% to 12% BisTris NuPage gradient gels (Invitrogen), and transferred onto a Hybond-P polyvinylidene difluoride membrane (GE Healthcare). Membranes were blocked with 3% milk in TBS-T (50 mmol/L Tris [pH 7.6], 150 mmol/L NaCl, and 0.1% Tween-20) and incubated overnight with primary antibody (rabbit anti-claudin-1, Invitrogen; mouse anti- β -actin, Cell Signaling, Danvers, Mass) in 1.5% milk in TBS-T at 4°C. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit, anti-mouse, Cell Signaling) for 1 hour at room temperature. Proteins were visualized with a chemiluminescence reagent (ECL plus, GE Healthcare) with Image Reader LAS-1000 Pro version 2.5 software and analyzed with an Aida Image Analyzer.

Determination of cytokine concentrations

ASM cells were stimulated with or without 0.5 μ g/mL PMA and 10 μ g/mL ionomycin (Sigma-Aldrich) for 24 hours. The supernatant was harvested, and cytokine/chemokine concentrations were determined by using 27-plex with the cytometric bead array (Bio-Rad, Hercules, Calif), according to the manufacturer's protocol.

Quantification of wound-healing model

The intensity of cell growth inside the scratch fields was calculated to quantify the wound-healing model, as follows. The wound area of each photo was divided into 6 areas, and an average intensity of cellularity of each area was calculated by using Medical Image Processing Analysis & Visualization (MIPAV) software. An average intensity of each time point was compared with that of the starting point (0 hours).

Murine model of airway inflammation

An ovalbumin-induced murine model of airway inflammation was performed, as follows. Female BALB/c mice were sensitized with 20 μ g of ovalbumin (Sigma-Aldrich, Grade IV) mixed with 500 μ g of alum (Pierce) by means of intraperitoneal injection on days 0, 14, and 21. Consecutively, 1% ovalbumin was inhaled in nebulized form for 20 minutes on days 26, 27, and 28. Lungs were resected from all mice on day 29. Mice were housed at the AO Research Institute, Davos, Switzerland, in individually ventilated cages for the duration of the study, and all experimental procedures were carried out in accordance with Swiss law.

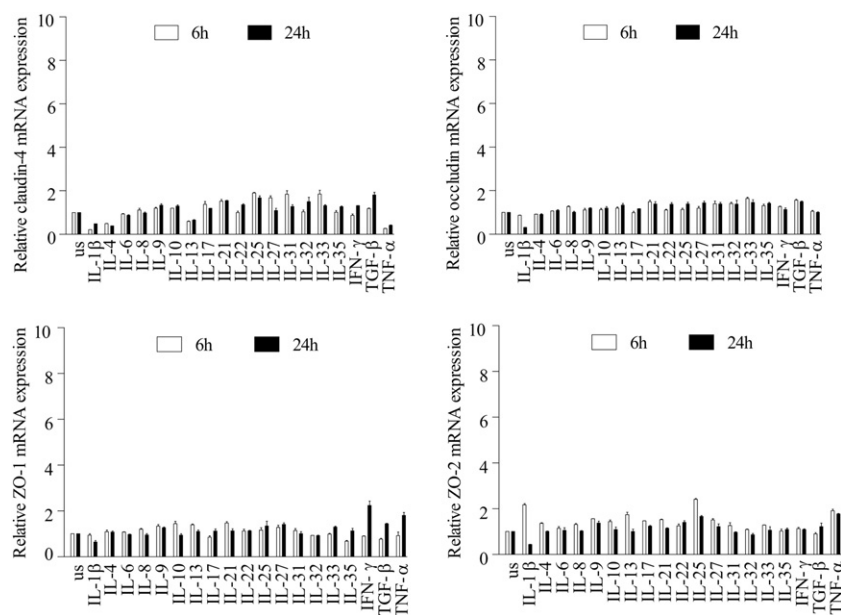


FIG E1. Expression of claudin-4, occludin, ZO-1, and ZO-2 in human primary ASM cells. Human primary ASM cells were incubated with 50 ng/mL of each cytokine, and relative claudin-4, occludin, ZO-1, and ZO-2 mRNA expressions were analyzed at 6 and 24 hours. Results are representative of 3 independent experiments. Means $\pm$ SEMs are shown.

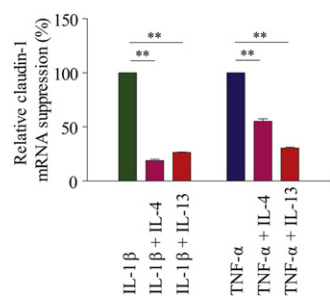


FIG E2. Effect of T_H2 cytokines on claudin-1 expression. Human primary ASM cells were stimulated with 10 ng/mL of each cytokine, and the percentage of claudin-1 mRNA suppression at 24 hours was shown. Results are representative of 3 independent experiments. Means $\pm$ SEMs are shown. ***P* < .01.

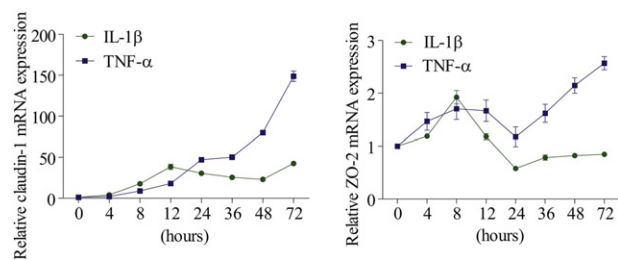


FIG E3. Kinetic change of claudin-1 and ZO-2 expression. Human primary ASM cells were stimulated with 10 ng/mL of each cytokine, and relative claudin-1 and ZO-2 mRNA expressions were analyzed at each time point. Results are representative of 3 independent experiments. Means $\pm$ SEMs are shown.

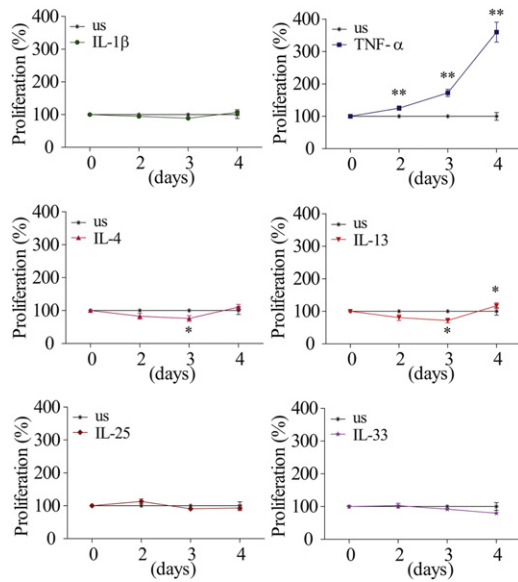


FIG E4. Effect of cytokines on ASM cell proliferation. ASM cells were incubated and stimulated with 10 ng/mL of each cytokine in a 96-well flat-bottom plate for a certain period, and tritiated thymidine was added to each well in the last 24 hours. The proliferation of stimulated ASM cells was compared with that of unstimulated (*us*) ASM cells, and the ratio was shown. Results are means $\pm$ SEMs from 3 independent experiments. * $P < .05$ and ** $P < .01$.

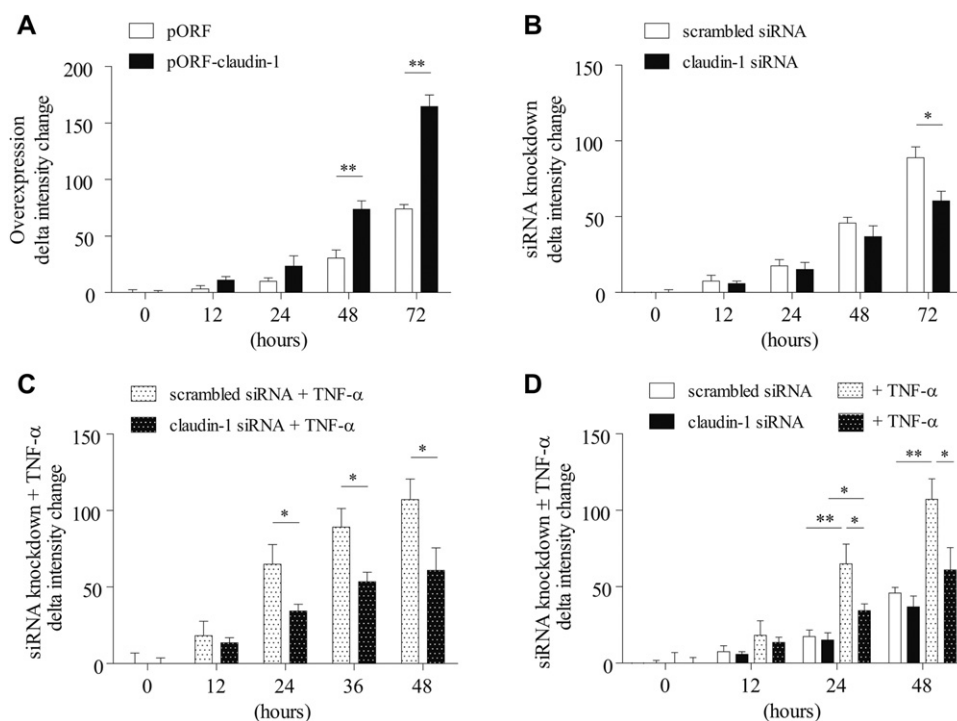


FIG E5. Quantification of the wound-healing model. The wound-healing models shown in Figs 2, F, 3, E, and 3, H, were quantified as A, B, and C, respectively. D, Comparison of the role of claudin-1 in wound healing in the presence or absence of TNF- α . The intensity of cell growth inside the scratch fields was calculated, and that of each time point was compared with that of the starting point. * $P < .05$ and ** $P < .01$.

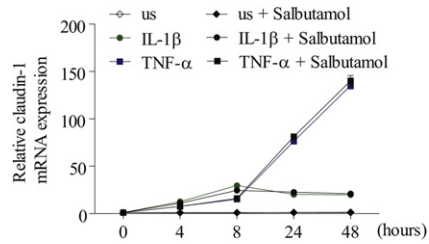


FIG E6. Time course of claudin-1 expression by salbutamol. Human primary ASM cells were stimulated with 50 ng/mL of each cytokine in the presence or absence of 10 μ mol/L salbutamol, and claudin-1 mRNA expression was analyzed at each time point. Results are representative of 2 independent experiments. Means $\pm$ SEMs are shown. *us*, Unstimulated.

TABLE E1. Claudin-1 expression in ASM exacerbates airway remodeling in asthmatic patients

Human elongation factor 1 α (EF-1 α)	
Forward	5'- CTG AAC CAT CCA GGC CAA AT -3'
Reverse	5'- GCC GTG TGG CAA TCC AAT -3'
Human claudin-1	
Forward	5'- CAG TCA ATG CCA GGT ACG AAT TT -3'
Reverse	5'- AAG TAG GGC ACC TCC CAG AAG -3'
Human claudin-4	
Forward	5'- TGT ACC AAC TGC CTG GAG GAT -3'
Reverse	5'- GAC ACC GGC ACT ATC ACC ATA A -3'
Human occludin	
Forward	5'- GAT GAG CAG CCC CCC AAT -3'
Reverse	5'- GGT GAA GGC ACG TCC TGT GT -3'
Human ZO-1	
Forward	5'- ACA GTG CCT AAA GCT ATT CCT GTG A -3'
Reverse	5'- TCG GGA ATG GCT CCT TGA G -3'
Human ZO-2	
Forward	5'- CGG TTA AAT ACC GTG AGG CAA A -3'
Reverse	5'- GGG AAC CAC TGG GTG TAA TTC A -3'