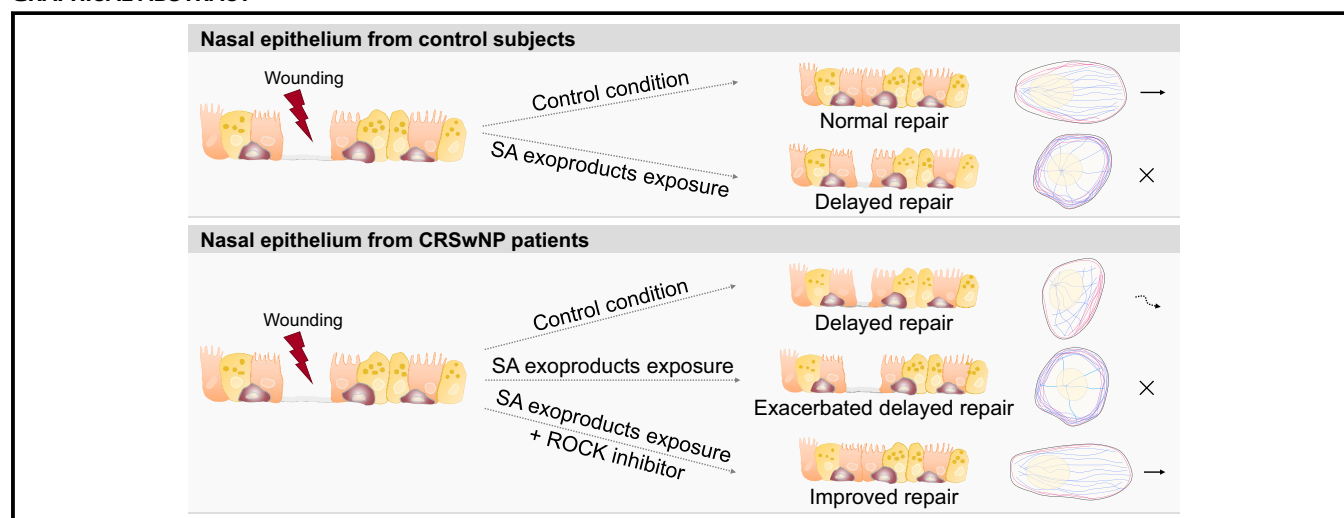


Staphylococcus aureus impairs sinonasal epithelial repair: Effects in patients with chronic rhinosinusitis with nasal polyps and control subjects



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GRAPHICAL ABSTRACT



Background: The effect of *Staphylococcus aureus* on nasal epithelial repair has never been assessed in patients with chronic rhinosinusitis with nasal polyps (CRSwNP). **Objective:** This study aimed to determine whether (1) nasal epithelial cell cultures from patients with CRSwNP and control

subjects repair differently; (2) *S aureus* exoproducts compromise nasal epithelial repair; (3) *S aureus* alters lamellipodial dynamics; and (4) deleterious effects could be counteracted by the Rho-associated coiled-coil kinase inhibitor Y-27632.

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Methods: Primary nasal epithelial cells (pNECs) collected during surgeries were cultured and injured under 3 conditions: (1) basal conditions, (2) exposed to *S aureus* exoproducts, and (3) exposed to *S aureus* exoproducts and Y-27632. Epithelial repair, lamellipodial dynamics, and cytoskeletal organization were assessed.

Results: Under basal conditions, pNEC cultures from patients with CRSwNP presented significantly lower repair rates and reduced lamellipodial protrusion length and velocity than those from control subjects. *S aureus* exoproducts significantly decreased repair rates and protrusion dynamics in both control subjects and patients with CRSwNP; however, the effect of *S aureus* on cell protrusions was more sustained over time in patients with CRSwNP. Under basal conditions, immunofluorescence assays showed significantly reduced percentages of cells with lamellipodia at the wound edge in patients with CRSwNP compared with control subjects. *S aureus* altered cell polarity and decreased the percentage of cells with lamellipodia in both groups. Finally, Y-27632 prevented the deleterious effects of *S aureus* exoproducts on CRSwNP repair rates, as well as on lamellipodial dynamics and formation.

Conclusions: *S aureus* exoproducts significantly alter epithelial repair and lamellipodial dynamics on pNECs, and this impairment was more pronounced in patients with CRSwNP. Importantly, Y-27632 restored epithelial repair and lamellipodial dynamics in the presence of *S aureus* exoproducts. (J Allergy Clin Immunol 2019;143:591-603.)

Key Words: *Staphylococcus aureus*, chronic rhinosinusitis, repair, nasal epithelial cells, lamellipodia, Rho-associated coiled-coil kinase inhibitor, Y-27632

Chronic rhinosinusitis (CRS) is defined as persistent inflammation of the sinonasal mucosa lasting for more than 12 weeks.¹ This disease affects 4.5% to 12% of the population in North American and European countries² and seriously compromises quality of life (QoL) indices in affected subjects.^{2,3} Patients with the chronic rhinosinusitis with nasal polyps (CRSwNP) phenotype have significantly greater impairment of disease-specific QoL than those with CRS without NPs.⁴ In addition, 40% of patients will present with endoscopic recurrence within 6 months after endoscopic sinus surgery (ESS),⁵ with 20% requiring revision surgery within 5 years.⁶

Despite the high personal, social, and economic effects of CRSwNP, its pathophysiology is still poorly understood. Increasing experimental evidence suggests that the epithelial barrier plays an important role in the disease process.^{7,8} In addition to its role as a physical barrier to pathogens and irritants, epithelial cells are also responsible for initiating and coordinating defensive responses. On stimulation, they secrete several cytokines, such as IL-8, IL-33, IL-25, and thymic stromal lymphopoietin, which serve to initiate multiple signaling pathways. In light of this, it is currently believed that reduced barrier function after damage to the epithelial lining is a potent contributor to type 2 immunity.⁹ Adequate epithelial repair after injury is essential to restore epithelial barrier integrity and to avoid subepithelial penetration of inhaled particles and deep tissue colonization with pathogenic bacteria,¹⁰ thereby perpetuating inflammation and facilitating subsequent infection.

Recent studies have suggested that epithelial repair and regeneration are altered in patients with CRSwNP. Yu et al¹¹ have shown impaired basal cell growth and proliferation, as

Abbreviations used

CRS:	Chronic rhinosinusitis
CRSwNP:	Chronic rhinosinusitis with nasal polyps
ESS:	Endoscopic sinus surgery
LB:	Luria Bertani
pNEC:	Primary nasal epithelial cell
ROCK:	Rho-associated coiled-coil kinase
SA _{inactive} :	Heat-inactivated 0.5% <i>S aureus</i> exoproducts

well as epithelial remodeling (hyperplasia and squamous metaplasia),¹² in patients with CRSwNP. Identification of specific factors influencing repair after injury might unravel innovative therapeutic targets for CRSwNP therapy.

Although it is believed that genetic factors can contribute to epithelial dysfunction in patients with CRSwNP, this nascent field remains in its infancy, and many early reports of candidate genes still require validation.¹³ In addition, the prevalence of CRS in the general population is too high to be determined only by genetic factors. Thus it is suspected that external environmental factors might play an important role in patients with this disease.

Staphylococcus aureus appears to be a good potential candidate. Indeed, *S aureus* is one of the most frequently recovered bacteria from patients with CRS¹⁴ and is more commonly present in patients with CRSwNP than in those with CRS without NPs.¹⁵ Intriguingly, it is often found deep under the epithelial surface at intracellular and intercellular locations.¹⁶⁻¹⁸ This appears to affect evolution of the disease: patients with intracellular *S aureus* have a greater risk of clinical and microbiological relapse after ESS.¹⁷ Effects by which this occur have been described, both relating to *S aureus* phenotypic changes after exposure to nasal epithelium¹⁹ and local modulation of the immune system.²⁰⁻²² *S aureus* might also have other pathogenic effects, possibly by contributing to the barrier dysfunction seen in patients with CRS.²³⁻²⁶ However, to date, the effect of *S aureus* on nasal epithelial repair has not been assessed formally.

In addition, mechanisms potentially implicated in epithelial dysfunction need to be identified. Several biological mechanisms are required for adequate epithelial repair, including cell migration, proliferation, and redifferentiation. Important components of these processes are the Rho family of small GTPases, including Rho, Rac, and Cdc42, which regulate actin cytoskeleton, cell migration, and proliferation in several types of epithelial cells.²⁷⁻²⁹ Rho-associated coiled-coil kinases (ROCKs) are serine/threonine kinases that are key regulators of cytoskeletal rearrangement. They mediate the formation of RhoA-induced stress fibers and focal adhesions and induce actomyosin contractility, cell-cell adhesion, and cell migration, differentiation, and proliferation.²⁷ Recently, eyedrops with ROCK inhibitors have shown *in vitro* and *in vivo* benefits on corneal wound healing.³⁰ This implies a role for the ROCK pathway in epithelial repair and suggest it as a potential therapeutic target.

The present study aims to (1) compare the epithelial repair characteristics of primary nasal epithelial cell (pNEC) cultures derived from control subjects and patients with CRSwNP, (2) investigate *S aureus* involvement in wound repair impairment, and (3) test whether Y-27632 (a ROCK inhibitor) could prevent negative *S aureus* effects.

TABLE I. Demographic data of patients whose samples were used at the present study

Data	Patients with CRSwNP	Control subjects
Age (y)	51.0 ± 9.5	50.3 ± 18.1
Sex	10 F/16 M	8 F/8 M
Previous ESS	10/26	—
Asthma	6/26	0/16
AERD	3/26	0/16
Smoking	6/26	1/16
IgE ≥100 UI/mL	5/26	—

Data are presented by frequency of events.

AERD, Aspirin-exacerbated respiratory disease; F, female; M, male.

METHODS

Surgical sample collection, cell isolation, and primary culture

This study was approved by the institutional review board of the Centre Hospitalier de l'Université de Montréal (protocol #HD04.025). All participants (aged ≥18 years) agreed to participate voluntarily and signed the informed consent form.

CRSwNP diagnosis was performed according to the 2004 American Academy of Otolaryngology–Head and Neck Surgery guidelines,³¹ and patients underwent ESS after failure of medical treatment. Control subjects underwent ESS for cerebral spinal fluid leak or nonsecreting pituitary adenomas. Exclusion criteria for both groups comprised immune deficiencies, neoplasia, ciliary dyskinesia, cystic fibrosis, and systemic immunosuppression. Tissues obtained during surgery at the level of the anterior ethmoidal bulla (control subjects) or the polyp itself (patients with CRSwNP) were collected immediately for subsequent cell isolation and culture.

After recovery, pNECs were isolated according to a standardized procedure.^{32–35} Briefly, collected samples were rinsed immediately and incubated overnight at 4°C in minimum essential medium (Life Technologies, Carlsbad, Calif) supplemented with 7.5% NaHCO₃ (Sigma-Aldrich, St Louis, Mo), 2 mmol/L L-glutamine (Life Technologies), 10 mmol/L HEPES (Thermo Fisher Scientific, Waltham, Mass), 0.05 mg/mL gentamicin (Sandoz, Bouchemville, Quebec, Canada), 50 U/mL penicillin-streptomycin, 0.25 µg/mL Fungizone (amphotericin B; Life Technologies), 0.1% protease, and 10 µg/mL DNase (Sigma-Aldrich).

After 18 hours, FBS was added to the solution to neutralize the protease-DNase activity, and pNECs were scraped gently off the remaining tissue. After removal of red blood cells by treatment with ACK lysis buffer (0.1 mmol/L NH₄Cl, 10 µmol/L KHCO₃, and 10 mmol/L EDTA), the cell suspension was characterized by means of immunodetection of specific epithelial markers, which indicated a mix of basal (CK13⁺), ciliated (β-tubulin⁺), and goblet (MUC5AC⁺) cells. This freshly isolated pNEC suspension was then seeded in T75 flasks coated with PureCol (Cedarlane Laboratory, Burlington, Ontario, Canada) and grown in CnT-17 to expand progenitor CK13⁺ basal cells until confluency (CELLnTEC Advanced Cell Systems, Bern, Switzerland) and supplemented with 100 U/mL penicillin and streptomycin. pNECs were then detached by using trypsin solution; 100,000 cells (second passage) were seeded into 24-well plastic plates (Corning, Corning, NY) and cultured in CnT-17. At 80% confluency CnT-17 medium was replaced by a mix of 1:1 volume of bronchial epithelial cell growth medium (CC4175; Lonza, Basel, Switzerland) and Dulbecco modified Eagle medium (Thermo Fisher Scientific) supplemented with 1.5 µg/mL BSA, 1 × 10^{−7} mol/L retinoic acid, and 100 U/mL penicillin-streptomycin.^{32–35}

S aureus strains

S aureus (Rosenbach 25923 strain; ATCC, Manassas, Va) was cultured in Luria Bertani (LB; Difco, Montreal, Quebec, Canada) agar under agitation at 250 rpm at 37°C. Planktonic bacterial cultures at the stationary phase after 24 hours of growth were centrifuged at 7200g for 10 minutes at room temperature. Supernatants were filtered with 0.22-µm cellulose acetate filters

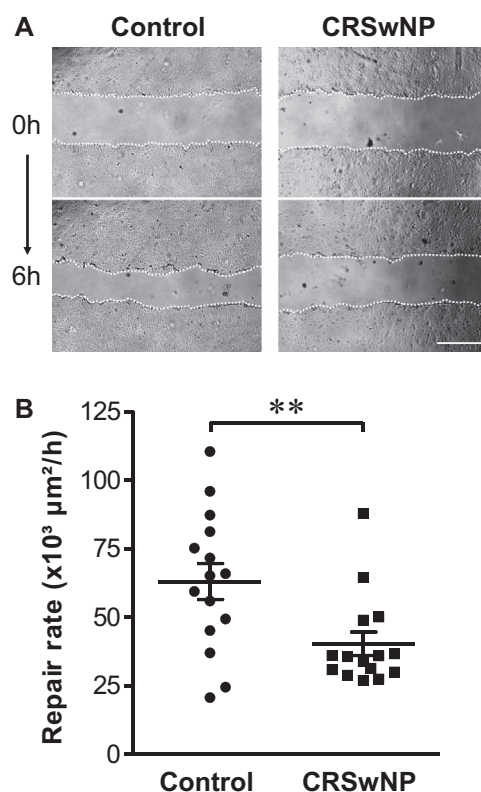


FIG 1. Nasal airway epithelial repair rate is poorest in epithelia of patients with CRSwNP than in that from control subjects under baseline conditions. Cell monolayers obtained from pNECs from control subjects or patients with CRSwNP were mechanically injured, and epithelial repair rates were measured over a 6-hour period. **A**, Representative pictures of injured epithelia from a control subject (left) and a patient with CRSwNP (right) were obtained from an inverted microscope (×4 magnification). Pictures were taken immediately after the injury (0h, top) and after a 6-hour period of repair (6h, bottom). Wounds have been brightened on the pictures, and dotted lines have been drawn along the wound edges for better visualization. **B**, Quantitative analysis of epithelial repair rate from 15 patients in each group. Samples were derived at the same time, and pNEC cultures were processed in parallel to compare repair rates in control subjects and patients with CRSwNP. Differences between rates in control subjects and patients with CRSwNP were assessed by using 1-way ANOVA, followed by Bonferroni *post hoc* tests. ***P* < .01.

(Corning) to remove bacteria, and filtrates containing *S aureus* exoproducts were placed in aliquots and stored at −80°C. One aliquot of this filtrate was heated at 95°C for 15 minutes to inactivate protease activity (for comparison to non-heat-inactivated aliquots). This aliquot was cooled after heating and stored for future assays.

Wound-healing assay

pNECs were injured mechanically (3 wounds per well and 3 wells per condition) by using a well-established and highly reproducible technique.^{32,34–37} After wounding, cultures were washed to remove detached cells and then treated randomly in one of the following conditions (performed in triplicates): (1) exposure to the negative control (0.5% [vol/vol] LB); (2) exposure to *S aureus* exoproducts (at both concentrations of 0.25% and 0.5% [vol/vol] *S aureus* filtrate); and (3) exposure to *S aureus* exoproducts (both concentrations of 0.25% and 0.5% [vol/vol] *S aureus* filtrate) and the ROCK inhibitor Y-27632 dihydrochloride (5 µmol/L; Sigma-Aldrich).

As a final assay, heat-inactivated *S aureus* exoproducts were used, and epithelial repair rates were reassessed at the following conditions: (1) negative control (0.5% [vol/vol] of LB); (2) exposure to *S aureus* exoproducts (at a concentration of 0.5% [vol/vol] *S aureus* filtrate); and (3) exposure to heat-

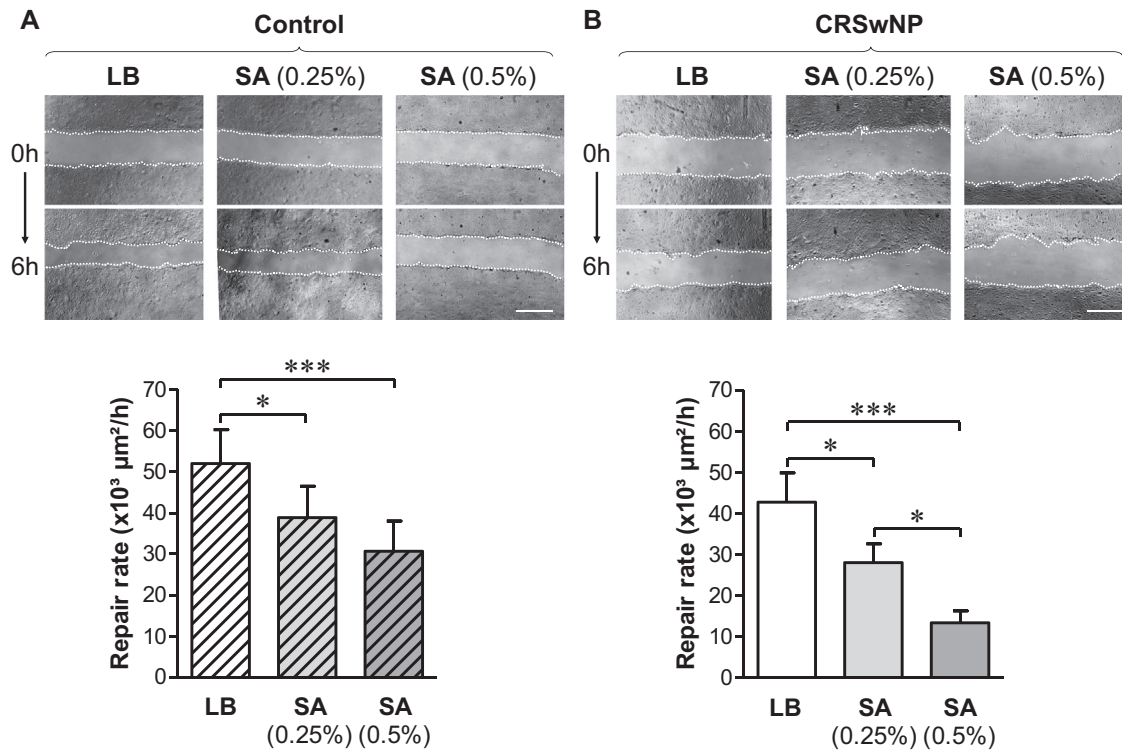


FIG 2. Exposure to *S aureus* exoproducts (SA) decreases nasal epithelial repair rates. pNEC monolayers obtained from control subjects (**A**) and patients with CRSwNP (**B**) were mechanically injured and treated with LB (left), 0.25% *S aureus* (middle), and 0.5% *S aureus* (right), and epithelial repair rates were measured over a 6-hour period. Upper panels, Representative pictures of injured epithelia from a control subject (Fig 2, A) and a patient with CRSwNP patient (Fig 2, B) were obtained with an inverted microscope ($\times 4$ magnification). Pictures were taken immediately after injury (0h, top) and after a 6-hour period of repair (6h, bottom). Wounds have been brightened on the pictures, and dotted lines have been drawn along the wound edges for better visualization. Quantitative analysis of epithelial repair rates from 11 control subjects (Fig 2, A) and 12 patients with CRSwNP (Fig 2, B) in control conditions (LB) and after exposure to *S aureus* exoproducts at 0.25% (vol/vol) and 0.5% (vol/vol). Values obtained in each condition were compared by using repeated-measures ANOVA, followed by Bonferroni *post hoc* tests. * $P < .05$ and *** $P < .001$.

inactivated *S aureus* exoproducts (concentration of 0.5% [vol/vol] *S aureus* filtrate).

A mark on wells allowed us to capture images of the wounds at exactly the same place at time 0 hours and time 6 hours by using an inverted microscope with a $\times 4$ objective (Nikon Eclipse TE 2000; Nikon, Tokyo, Japan). Images were registered with AxioVision 4.8 software (Carl Zeiss, Jena, Germany) and then analyzed with ImageJ software (National Institutes of Health, Bethesda, Md) to measure areas at time 0 hours and time 6 hours of wound healing and then to calculate repair rates (expressed in square micrometers per hour).

Analysis of lamellipodial dynamics by means of kymography

Brightfield time-lapse series were made on pNECs from patients with CRSwNP and control subjects plated in 2-well silicone culture inserts (#80209; Ibidi, Fitchburg, Wis) by using an inverted Axio ObserverZ1 microscope equipped with a CoolSNAP EZ CCD camera and a $\times 10/0.3$ NA air objective lens and then analyzed with AxioVision software.

Immediately before image acquisition, cells were treated with LB (control condition for *S aureus*) or LB plus water (control condition for *S aureus* plus Y-27632). Cells were then positioned into the incubation chamber (37°C in a 5% CO₂ atmosphere), and images were captured every minute for 2 hours to observe cell protrusions and retractions. Then cells were treated with either *S aureus* exoproducts (concentration of 0.5% [vol/vol]) or a mix of *S aureus* (concentration of 0.5% [vol/vol]) and Y-27632 (5 $\mu\text{mol/L}$), with recording pursued during the following 4 hours.

For each patient, on each time-lapse series, 4 to 6 cells exhibiting lamellipodia with consistent and dynamic protrusion/retraction cycles were chosen randomly for analysis. A 1-pixel-wide line crossing the cell membrane perpendicular to the lamellipodia was drawn, and this region was extracted from each image of the time-lapse series, as previously described by Choi et al.³⁸ Kymographs (time-space plots) were generated with the Multiple Kymograph plugin (J. Rietdorf, FMI, Basel, Switzerland, and A. Seitz, EMBL, Heidelberg, Germany) for ImageJ software. On kymographs, the x-axis represents time, and the y-axis represents distance, where each protrusion/retraction cycle appears as a peak (see Fig E1 in this article's Online Repository at www.jacionline.org). On each kymograph, segments following protrusions were manually retraced with the ImageJ segmented line tool. Data derived from these segmented lines were generated by using the read velocity tsp macro of ImageJ software and used to calculate the length and velocity of each protrusion. A total of at least 40 to 110 individual protrusion events per condition and at least 14 different cells were analyzed in 3 independent time-lapse series.

Immunofluorescence

pNECs were grown to confluency on coverslips (precision cover glasses thickness No. 1.5H; Marienfeld, Lauda-Königshofen, Germany) coated with PureCol. After injury, (3 mechanical wounds per coverslip), cell monolayers were washed and then treated with either (1) LB (concentration of 0.5% [vol/vol], negative control), (2) *S aureus* exoproducts (concentration of 0.5% [vol/vol] *S aureus* filtrate), or (3) *S aureus* exoproducts (concentration of

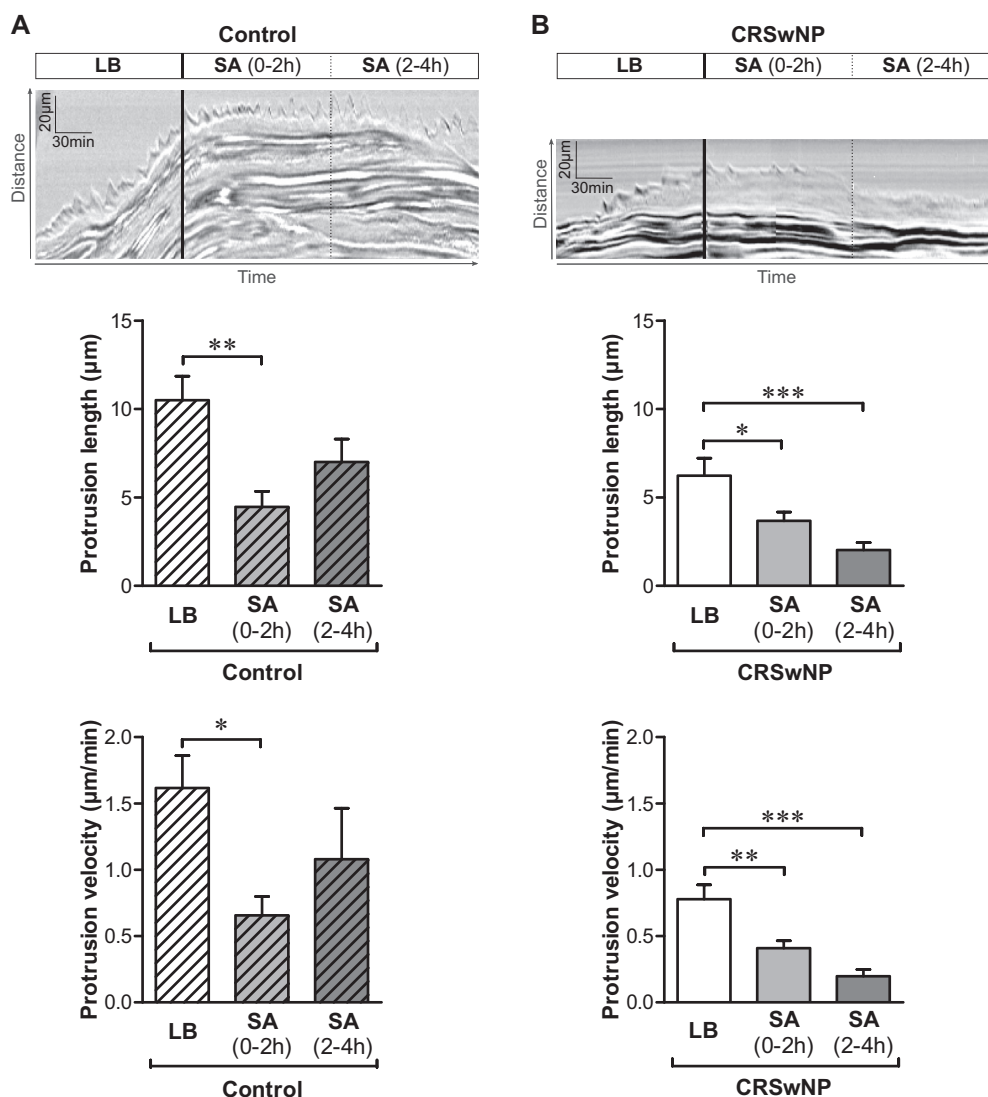


FIG 3. Lamellipodial dynamics of nasal epithelial cells from control subjects and patients with CRSwNP are affected by exposure to *S aureus* exoproducts (SA). pNECs from control subjects (**A**) or patients with CRSwNP (**B**) were treated with LB at the beginning of the experiment and imaged every minute for 2 hours. *S aureus* exoproducts were added, and recordings were pursued for the next 4 hours (SA [0-2h] and SA [2-4h]). *Upper panels*, Representative kymographs taken from time-lapse series of pNECs from a control subject (Fig 3, A) and a patient with CRSwNP (Fig 3, B). Kymographs depict variations in lamellipodial activity along a 1-pixel-wide line drawn perpendicularly to the cell membrane in sequential phase-contrast images. Ascending contours show lamellipodial protrusion, whereas descending contours show lamellipodial retraction. *Lower panels*, Quantitative analysis of lamellipodial dynamics in pNECs from control subjects (Fig 3, A) and patients with CRSwNP (Fig 3, B). Length and velocity of protrusions were calculated from slopes traced on the leading edge of cells on 14 to 15 kymographs (each kymograph was taken from 1 cell) from 3 control subjects (Fig 3, A) and 3 patients with CRSwNP (Fig 3, B). Means were calculated for 3 distinct periods: time 0 hours to 2 hours (LB, control condition), time 2 hours to 4 hours (SA [0-2h]), and time 4 hours to 6 hours (SA [2-4h]). Differences between groups (LB, SA [0-2h], and SA [2-4h]) were assessed by using repeated-measures ANOVA, followed by Bonferroni *post hoc* tests. * $P < .05$, ** $P < .01$, and *** $P < .001$.

0.5% [vol/vol] *S aureus* filtrate) and Y-27632 (5 $\mu\text{mol/L}$). After 4 hours of repair, cell monolayers were fixed with 4% paraformaldehyde in PBS for 5 minutes and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. After blocking (in 1% BSA in PBS for at least 1 hour at 4°C), fixed cells were incubated with the primary anti- β -tubulin antibody (1:1000 in PBS plus BSA 1%; Millipore, Etobicoke, Toronto, Ontario, Canada) for 1 hour at room temperature and then with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (1:2000 in PBS plus BSA 1% for 1 hour at room temperature; Thermo Fischer Scientific). F-actin was stained

with Phalloidin-Alexa Fluor 594 (1 unit; Thermo Fischer Scientific; in PBS + BSA 1% for 1 hour at room temperature), and nuclei were stained with 4'-6-diamidino-2-phenylindole dihydrochloride (1:1000 in PBS + BSA 1% for 5 minutes). Coverslips were mounted with ProLong Gold Antifade Reagent (Molecular Probes, Eugene, Ore) on SuperFrost glass slides (Thermo Fisher Scientific).

Images were acquired with an inverted confocal microscope (TCS SP5; Leica Microsystems, Wetzlar, Germany) by using an oil-immersion objective ($\times 63/1.4$). On immunofluorescence images, cells at the wound edge were

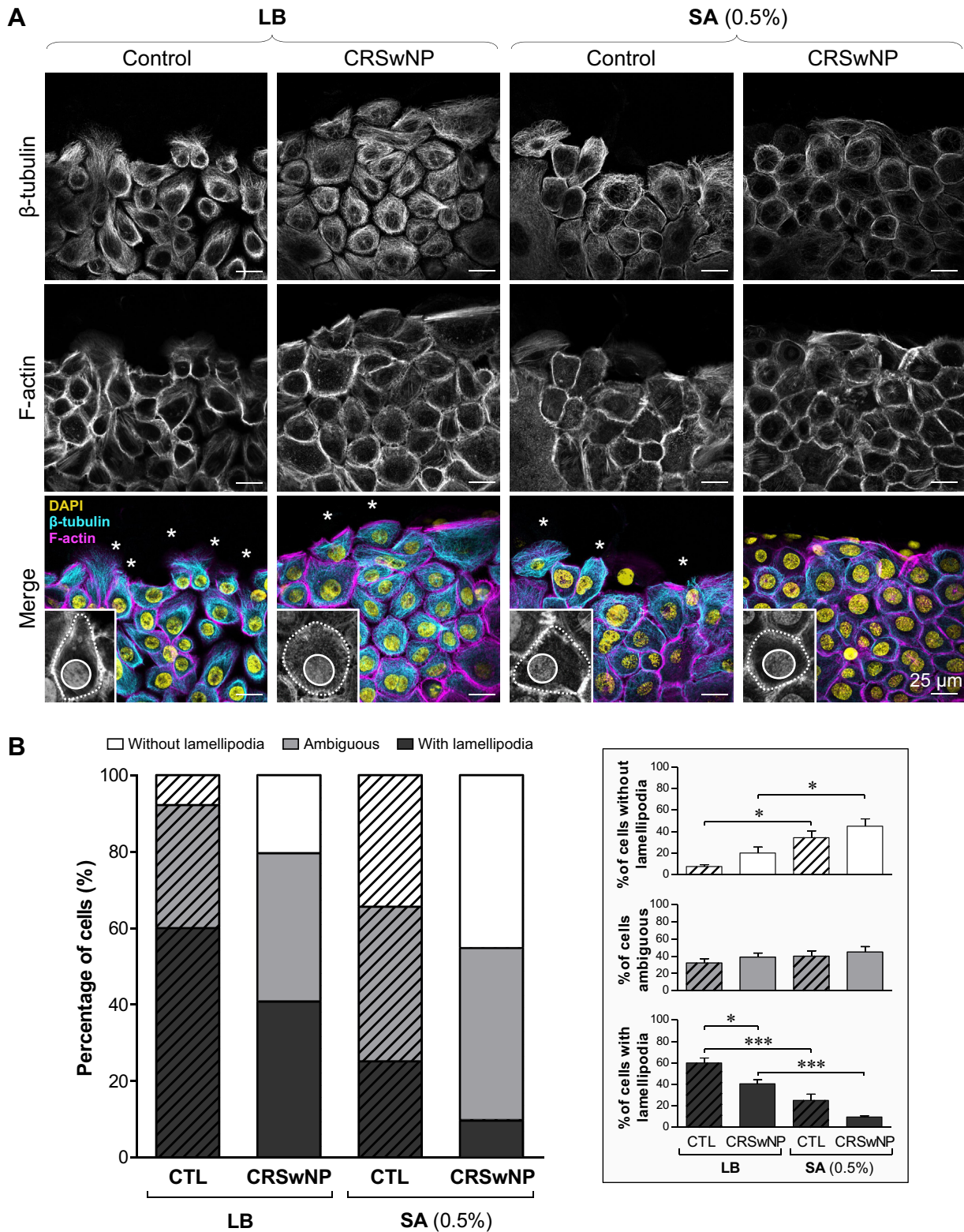


FIG 4. Exposure to *S aureus* exoproducts (SA) affects cytoskeletal organization of nasal epithelia from control subjects and patients with CRSwNP. **A**, Representative confocal microscopic images of nasal epithelia from control subjects and patients with CRSwNP mechanically injured and treated with either LB (control condition) or *S aureus* exoproducts (0.5%) for 4 hours. Cells were stained for microtubules (with anti- β -tubulin antibody, upper panels) and actin stress fibers (phalloidin staining, middle panels), and nuclei were labeled with 4'-6-diamidino-2-phenylindole dihydrochloride. Cells were visualized with a Leica TCS SP5 confocal microscope (magnification $\times 63$). Merged images are shown on lower panels. White asterisks

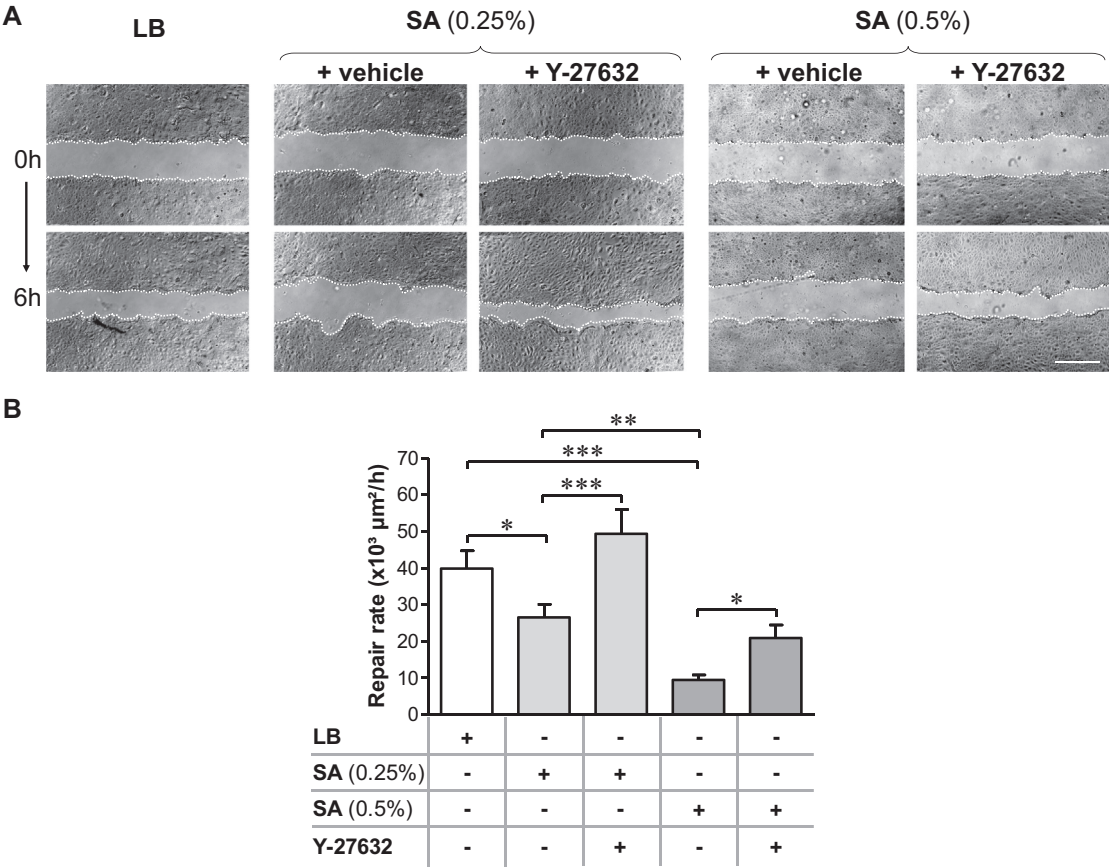


FIG 5. ROCK inhibitor Y-27632 prevented the deleterious effect of *S aureus* (SA) on epithelial repair rates of nasal epithelial cells from patients with CRSwNP. pNEC monolayers from patients with CRSwNP were injured and treated with LB plus vehicle (water), *S aureus* (0.25%) plus vehicle (water), and *S aureus* (0.25%) plus Y-27632, *S aureus* (0.5%) plus vehicle (water), or *S aureus* (0.5%) plus Y-27632, and then epithelial repair rates were calculated over a 6-hour period. **A**, Representative pictures of injured epithelia from a patient with CRSwNP were obtained under different conditions and taken at 0 hours (*top*) and 6 hours (*bottom*) after injury with an inverted microscope (×4 magnification). Wounds have been brightened on the pictures, and *dotted lines* have been drawn along the wound edges for better visualization. **B**, Quantitative analysis of epithelial repair rates from 15 patients with CRSwNP. Values obtained from each condition within the same group were compared by using repeated-measures ANOVA, followed by Bonferroni *post hoc* tests. **P* < .05, ***P* < .01, and ****P* < .001.

classified according to the categories “with” or “without” lamellipodia, as well as “ambiguous morphology” (see Fig E3 in this article’s Online Repository at www.jacionline.org).³⁹ Percentages of cells in each category were then calculated.

Statistical analysis

Data from all experiments are presented as means ± SEMs. ANOVA with Bonferroni *post hoc* tests were used to compare different conditions in the same group and to compare the same condition between groups. Comparisons

were considered statistically significant at a *P* value of less than .05. GraphPad Prism software (version 6.0; GraphPad Software, San Diego, Calif) was used to analyze all results.

RESULTS

A total of 35 samples from 19 patients with CRSwNP and 16 control subjects were included in this study. The demographic data for each group are presented in Table I.

indicate cell protrusions at the leading edge of the wounds. *Insets* are shown to emphasize cell shapes. *Scale bars* = 25 μm. If necessary, images were rotated to place wounds at the top of each photograph. **B**, Results of lamellipodial cell quantification. Percentages of cells with lamellipodia (dark gray), with ambiguous morphology (light gray), or without lamellipodia (white) at the wound edge were counted on immunofluorescence images of nasal epithelia from control subjects and patients with CRSwNP after mechanical injury and treatment with either LB or *S aureus* exoproducts (0.5%). *Left panel* shows repartition (in percentage) of cells according the 3 categories. Values are means ± SEMs from 4 independent experiments (≥80 cells per condition were classified). *Inset (right)* show results of statistical analysis. Differences between groups were assessed by using 1-way ANOVA, followed by Bonferroni *post hoc* tests. **P* < .05 and ****P* < .001.

Effect of *S aureus* on samples from control subjects and patients with CRSwNP

During the last years, the sinonasal epithelium has been intrinsically imbricated to CRSwNP physiopathology. Indeed, epithelial barrier defense is determinant to maintain mucosal homeostasis, because of either its physical (avoiding the entrance of pathogens) or functional (defense against pathogens through the release of inflammatory and antimicrobial substances) implications.⁴⁰⁻⁴³ To this end, the epithelium is a key inducer of both innate and adaptive inflammation.⁴⁰⁻⁴³ Very recently, Schleimer⁴³ proposed that mucosal injury and repair might be a key process during CRSwNP: a prolonged loss of epithelial barrier function of might facilitate the entrance of pathogens/inhalants and thus facilitate chronic inflammation. Thus we considered it essential to evaluate whether the repair ability of nasal epithelia differs between patients with CRSwNP and control subjects under baseline culture conditions.

For this purpose, pNECs from control subjects and patients with CRSwNP were cultured in parallel in the same conditions and then wounded to compare their repair rates. Our 2-dimensional cultures are composed predominantly of basal progenitor cells, which play a critical role in epithelial repair through their ability to migrate into the injured area and then proliferate.^{11,44} Repair rate analysis (Fig 1) in each pNEC culture from control subjects and patients with CRSwNP revealed variability among subjects within each group. However, the mean wound repair rates were significantly slower ($P < .01$) in pNEC monolayers from patients with CRSwNP than from control subjects under baseline conditions (LB; Fig 1, B).

We then assessed whether exposure to *S aureus* exoproducts could alter the wound-healing capacity of pNEC epithelia. We observed a significant decrease in the repair of control pNEC monolayers in the presence of 0.25% *S aureus* exoproducts (Fig 2, A). A stronger inhibitory effect ($P < .001$) was observed after exposure to *S aureus* exoproducts at 0.5% (41% decrease compared with repair rates in LB conditions). A dose-dependent inhibition of wound repair was observed in monolayers from patients with CRSwNP (34% and 69% decrease in the presence of 0.25% *S aureus* [$P < .05$ vs LB] and 0.5% *S aureus* [$P < .001$ vs LB], respectively; Fig 2, B).

To determine which component could be responsible for this deleterious response, we used heat-inactivated 0.5% *S aureus* exoproducts (SA_{inactive}) and reassessed their effect on the epithelial repair of pNEC monolayers from patients with CRSwNP (see Fig E2 in this article's Online Repository at www.jacionline.org). We observed that the epithelial repair was only weakly influenced by heat-inactivated 0.5% *S aureus* exoproducts (SA_{inactive} vs LB, 14% decrease [nonsignificant]), which was in contrast to active 0.5% *S aureus* exoproducts within the same pNEC cultures (LB vs *S aureus*, 88% decrease [$P < .001$] and *S aureus* vs SA_{inactive}, 86% increase [$P < .001$]).

Epithelial repair is dependent on several mechanisms, one of them being coordinated and directional cell migration.⁴⁵ Actin filaments organize themselves in a dynamic network and create a sheet-like membrane protrusion known as lamellipodia to optimize this lively process.⁴⁵⁻⁴⁷ Thus we further evaluated lamellipodia formation and orientation by using video kymograph analysis. We first confirmed that LB *per se* had no effect on cell movement (see Video E1 in this article's Online Repository at www.jacionline.org). Subsequently, we evaluated

kymographs of pNEC cultures from both control subjects and patients with CRSwNP before and after exposure to *S aureus* exoproducts.

Control cells depicted a mean protrusion length and velocity of $10.5 \pm 1.36 \mu\text{m}$ and $1.62 \pm 0.24 \mu\text{m}/\text{min}$, respectively, in LB conditions. Subsequent exposure to *S aureus* exoproducts induced a 57% ($P < .01$) and 60% ($P < .05$) decrease in protrusion length and velocity, respectively, followed by partial recovery of these parameters during the final 2 hours of the study observation (Fig 3 and see Video E2 in this article's Online Repository at www.jacionline.org). In cells from patients with CRSwNP, both protrusion length ($6.23 \pm 0.99 \mu\text{m}$) and velocity ($0.78 \pm 0.11 \mu\text{m}/\text{min}$) were altered at baseline (LB condition), and exposure to *S aureus* exoproducts significantly worsened the levels of protrusion length and velocity. Moreover, in contrast with control pNECs, the effect of *S aureus* was persistent throughout the entire period after exposure to SA exoproducts (Fig 3, SA 0-2h and SA 2-4h).

Finally, we evaluated the cellular cytoskeletal organization by using immunofluorescence experiments. Microtubules and actin microfilaments were stained with a specific β -tubulin antibody and fluorescent phalloidin, respectively. Under LB conditions, we observed that the leading cells at the wound edge of injured pNEC monolayers from control subjects exhibited more pronounced lamellipodial extensions (see cells marked with an asterisk, Fig 4, A, merge) than pNECs cultured from samples from patients with CRSwNP. However, pNECs from control subjects and patients with CRSwNP appeared to be polarized correctly and oriented toward the injured area (Fig 4, A). We did not observe a substantial difference in F-actin or β -tubulin distribution within pNECs from control subjects and patients with CRSwNP. However, further analysis and lamellipodial cell quantification at the wound edge showed distinct patterns for pNECs from control subjects and patients with CRSwNP (Fig 4, B). Indeed, the percentage of cells with lamellipodia was significantly lower in epithelia from patients with CRSwNP than in that from control subjects (control subjects: 60% vs patients with CRSwNP: 41%), whereas the percentage of cells without lamellipodia was greater (control subjects: 8% vs patients with CRSwNP: 20%; Fig 4, B).

pNECs from both control subjects and patients with CRSwNP were altered after exposure to *S aureus* exoproducts, exhibiting a more rounded shape, suggesting a loss in cell polarization and a reorganization of the microtubule cytoskeleton, with an increase in microtubules localized circularly under the plasma membrane (Fig 4, A). Additionally, lamellipodial cell quantification showed that *S aureus* exoproduct exposure induced significant decreases in the percentage of cells with lamellipodia at the wound edge (control subjects—LB: 60% vs *S aureus*: 25%; patients with CRSwNP—LB: 41% vs *S aureus*: 10%; Fig 4, B). Conversely, the percentage of cells without lamellipodia is significantly increased after *S aureus* exoproduct exposure in epithelia from control subjects (LB: 8% vs *S aureus*: 34%) and patients with CRSwNP (LB: 20% vs *S aureus*: 45%; Fig 4, B).

Effect of ROCK inhibitor Y-27632

Given the evidence that the Rho family of small GTPases is important for the wound-healing process in several cell types,^{27,30,45,48} we postulated that Y-27632, a potent ROCK inhibitor, could restore epithelial repair in pNECs despite the presence

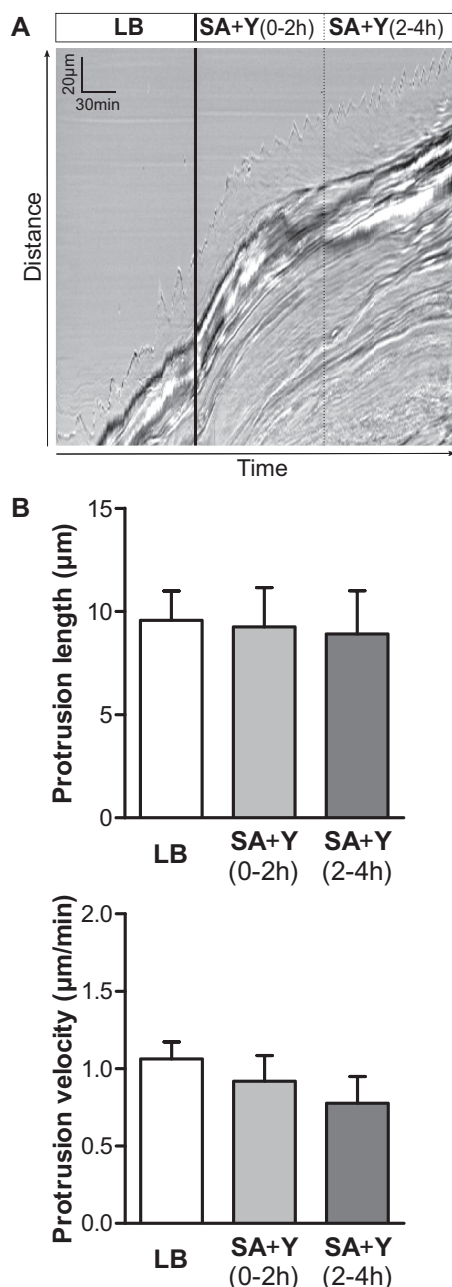


FIG 6. ROCK inhibition attenuates the negative effect of *S aureus* exoproducts (SA) on lamellipodial dynamics of nasal epithelial cells from patients with CRSwNP. pNEC monolayers from patients with CRSwNP were treated with LB plus vehicle (water) at the beginning of the experiment and imaged every minute for 2 hours. Then a combination of 0.5% of *S aureus* exoproducts and 5 μmol/L of the ROCK inhibitor Y-27632 (Y) was added, and recordings were made for the next 4 hours. **A**, Representative kymograph taken from phase-contrast time-lapse series of pNECs from a patient with CRSwNP (right). **B**, Quantitative analysis of lamellipodial dynamics. Length (upper panels) and velocity (lower panels) of protrusions (left panels) and retractions (right panels) were calculated from slopes traced on the leading edge of cells on 11 kymographs (each kymograph was taken from 1 cell) from 3 independent experiments (ie, 3 patients with CRSwNP; n = 3 patients and n = 11 cells). Means were calculated for 3 distinct periods: time 0 hours to 2 hours (LB, control condition), time 2 hours to 4 hours (SA+Y [0-2h]), and time 4 hours to 6 hours (SA+Y [2-4h]). Differences between groups were assessed by using repeated-measures ANOVA, followed by Bonferroni *post hoc* tests. All comparisons were established as nonsignificant after statistical analysis ($P > .05$).

of *S aureus* exoproducts. Because the effect of Y-27632 has been shown to be greatly influenced by drug concentration (lower concentrations have been associated with improved migration, proliferation, and wound healing,^{27,30,45,48} and higher doses have been reported to induce apoptosis in airway epithelial cells⁴⁹), we first determined in a pilot study that 5 μmol/L was the optimal dose for Y-27632 in pNECs. Indeed, treatment with 5 μmol/L Y-27632 in LB conditions led to an increase in epithelial repair rate of 23%, whereas increasing concentrations altered migration and induced pNEC apoptosis (data not shown).

pNEC monolayers were then exposed to *S aureus* exoproducts either in the presence or absence of Y-27632 (5 μmol/L; Fig 5). The drug inhibited the deleterious effect of *S aureus*, increasing the repair rate significantly by 1.86-fold when cocultured with 0.25% *S aureus* and by 2.22-fold with 0.5% *S aureus* compared with respective *S aureus* conditions in the absence of Y-27632.

We also explored whether the beneficial effect of Y-27632 on epithelial repair in the presence of *S aureus* exoproducts was related to an improvement in lamellipodial dynamics and migration. Although both protrusion length and velocity in pNECs from patients with CRSwNP were altered by exposure to *S aureus* exoproducts (Fig 3 and see Video E2), this deleterious effect was no longer observed (Fig 6 and see Video E3 in this article's Online Repository at www.jacionline.org) when pNECs from patients with CRSwNP were exposed simultaneously to 0.5% *S aureus* and Y-27632. Indeed, the length and velocity of protrusions measured after *S aureus* plus Y-27632 treatment were similar to those observed in the absence of *S aureus* (Fig 6, LB vs *S aureus* plus Y-27632).

Finally, we compared the cellular cytoskeletal organization in both the absence and presence of Y-27632 after exposure to *S aureus*. We first confirmed that, in the absence of Y-27632, *S aureus* exposure induced the cells to adopt a rounded shape, with lamellipodial extension substantially compromised at the leading edge of injury (Fig 7, A). Treatment with Y-27632 partially restored lamellipodial extensions: although the extension was not as prominent as under baseline conditions, most of the cells at the leading edge presented a more elongated shape and exhibited the lamellipodia pattern directed toward the injured area (Fig 7, A; see cells marked with an asterisk at the leading edge in LB and *S aureus* plus Y-27632 conditions). Interestingly, lamellipodial cell quantification showed that, after exposure to *S aureus* exoproducts combined with the Y-27632 compound, injured epithelia from patients with CRSwNP exhibited significantly more cells with lamellipodia (*S aureus*: 13% vs *S aureus* plus Y-27632: 65%) and significantly less cells without lamellipodia (*S aureus*: 43% vs *S aureus* plus Y-27632: 8%; Fig 7, B).

DISCUSSION

In the present study we document that there is an intrinsic defect in epithelial repair in patients with CRSwNP. In addition, we demonstrate that *S aureus* exoproduct exposure alters epithelial repair of normal cells and accentuates the abnormal repair in cells from patients with CRSwNP. Furthermore, we demonstrate that the effect of *S aureus* on cell protrusion is more sustained over time in pNEC cultures from patients with CRSwNP. This supports that barrier recovery dysfunction might represent an additional pathogenic mechanism by which *S aureus* contributes to CRSwNP development.

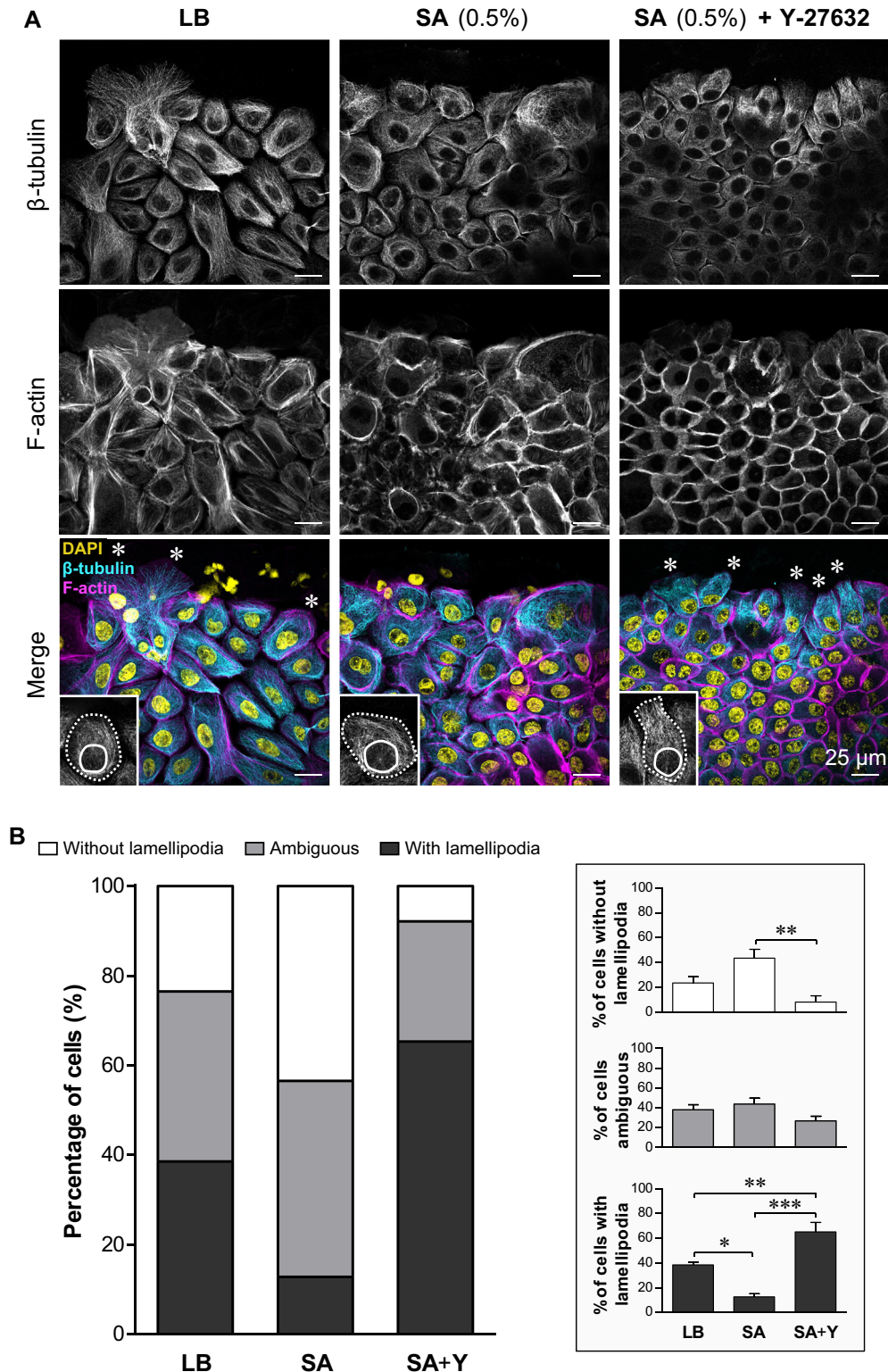


FIG 7. ROCK inhibition protects cytoskeletal disorganization of nasal epithelial cells from patients with CRSwNP caused by *S aureus* exoproduct (SA) exposure. **A**, Representative confocal microscopic images of nasal epithelia from patients with CRSwNP mechanically injured and treated with either LB plus vehicle (water, control condition), *S aureus* exoproducts (0.5%) plus vehicle (water), or *S aureus* (0.5%) exoproducts and Y-27632 (5 μ mol/L) for 4 hours. Cells were stained for microtubules (with an anti- β -tubulin antibody, upper panels) and actin stress fibers (phalloidin staining, middle panels), and nuclei were labeled with 4'-6-diamidino-2-phenylindole dihydrochloride. Cells were visualized with a Leica TCS SP5 confocal microscope (magnification $\times 63$). Merged images are shown on lower panels. White asterisks indicate protrusions at the leading edge of the wounds. Insets are shown to emphasize cell shapes. Scale bars = 25 μ m. If

Dysfunctional epithelial repair has been implicated in several diseases.^{29,50,51} *In vitro* studies have demonstrated the negative effect of inflammation and bacterial infection⁵²⁻⁵⁴ on this process and permitted assessment of potential therapies.^{55,56} For patients with CRSwNP, epithelial repair using pNEC models remains poorly studied, with only 2 articles addressing this topic^{32,54} and none comparing samples from patients with CRSwNP with those from control subjects. Wise et al⁵⁴ demonstrated a deleterious effect of T_H2 inflammation on epithelial repair by showing that IL-4 decreased the repair rate of pNECs obtained from healthy subjects. A role for bacterial influences was suggested by Ruffin et al,³² who demonstrated that exoproducts from *Pseudomonas aeruginosa* decreased epithelial repair significantly in pNECs obtained from patients with CRS with and without cystic fibrosis.

The difference between wound-healing rates after injury in epithelia from patients with CRSwNP and control subjects under basal conditions (ie, without inflammatory or bacterial products) suggests an intrinsic and transmissible defect in the repair processes of cells from patients with CRSwNP under genetic or epigenetic control. The genetic basis for a predisposition to impaired healing response to injury is not yet known but might be suggested by previous findings from population-based genetic association studies in patients with CRS. Indeed, a pooling-based genome-wide association scan has identified laminin A2 and B1 (*Lama2* and *Lamb1*) genes as top candidates related to CRS.⁵⁷ Laminin is associated with the extracellular matrix and integrin signaling and is essential for wound healing. Supporting a functional role for laminin, animal models showed that keratinocytes exposed to laminin peptides exhibited enhanced repair rate.⁵⁸

Polymorphisms in 2 potassium channels have also been postulated to be implicated in the pathogenesis of CRS in children.⁵⁹ This might also affect epithelial repair because several studies, including from our group,^{37,60,61} have demonstrated that potassium channels are essential for epithelial cell migration and proliferation and key processes for airway epithelial repair.

Finally, susceptibility to colonization with *S aureus* in patients with CRSwNP appears to have a genetic bias, which might also suggest additional mechanisms and novel potential therapeutic targets. Cormier et al⁶² showed that variation in the *Rac1* gene was associated with increased *S aureus* colonization in patients with CRS. As already described, RAC1 is a member of the Rho family of small GTPases, supporting that this pathway seems to be a reasonable target for therapy in patients with CRS. In support of this, Soong et al⁶³ showed that *S aureus* protein A activates the RhoA/ROCK signaling pathway to invade bronchial epithelial cells, leading to actomyosin contraction and disruption of tight junctions. Furthermore, Y-27632 was able to inhibit *S aureus* translocation in a dose-dependent manner. Although there are

no previous studies that have evaluated the effect of *S aureus* specifically on wound healing in nasal epithelial cells, this pathogen was related to worst epithelial repair in corneal epithelium⁵³ and keratinocytes.⁵²

Taken together, these findings suggest that *S aureus* has a major effect on nasal epithelial cells not by only inducing and directing the inflammatory pattern^{21,22} but also by facilitating its access to the epithelium and its persistence as an intracellular agent.^{16,17,19} In this study we observed that *S aureus* exoproducts significantly decreased epithelial repair rates in pNECs from control subjects and patients with CRSwNP, whereas heat-inactivated exoproducts did not elicit any harmful effect. This suggests that heat-sensitive proteins, such as proteases, secreted by *S aureus* could be responsible for the deleterious effect of *S aureus*. In agreement with our data, Marano et al⁵² reported that compounds secreted by *S aureus* biofilms inhibited keratinocyte migration and proliferation and that this effect was abolished after heat inactivation. Moreover, proteomic analysis revealed that several secreted proteins, among them α -hemolysin, alcohol dehydrogenase, fructose-bisphosphate aldolase, lactate dehydrogenase, and epidermal cell differentiation inhibitor, can contribute to the observed delay in wound healing. Moreover, another study²⁶ points out that α -hemolysin exposure induced strong actin cytoskeleton remodeling and altered the expression profile of proteins involved in lamellipodial structures. Actually, many different types of virulence factors are secreted by *S aureus*, including metallo-, serine- and papain-like cysteine proteases. Bacterial proteases, such as *P aeruginosa* elastase,^{32,46,64} have been shown to play a critical role in airway epithelial repair impairment. Although *S aureus* proteases might also be involved in the repair delay of the nasal airway epithelia induced by *S aureus*, further study will be required to identify the nature of the exoproducts responsible for this harmful effect of *S aureus*.

S aureus exoproducts can influence the contraction of the cell cytoskeleton and finally alter lamellipodia. Two previous studies have shown that hemolysin A, a specific *S aureus* exotoxin, changed actin cytoskeletal reorganization^{25,26} and reduced lamellipodia and focal contacts²⁶ in airway epithelial cells. Indeed, this was exactly what we observed: the pool of *S aureus* exoproducts changed cell polarity, decreased lamellipodial extension and the percentage of cells at the wound edge exhibiting lamellipodia, and inhibited lamellipodial dynamics. Moreover, cells from patients with CRSwNP were less predisposed to recover after exposure to *S aureus* exoproducts.

Along with the increased epithelial permeability,²³ dysfunctional epithelial repair might be an important initial step in which *S aureus* could induce epithelial dysfunction and initiate and perpetuate the chronic inflammatory response present in patients with CRSwNP. Tan et al¹⁷ observed that the presence of intracellular *S aureus*, which is facilitated by all the processes reported

necessary, images were rotated to place the wounds at the top of each photograph. **B**, Results of lamellipodial cell quantification. Percentages of cells with lamellipodia (dark gray), with ambiguous morphology (light gray), or without lamellipodia (white) at the wound edge were counted on immunofluorescence images of nasal epithelia from control subjects and patients with CRSwNP after mechanical injury and treatment with either LB plus vehicle (LB), *S aureus* exoproducts (0.5%) plus vehicle, or *S aureus* exoproducts (0.5%) plus Y-27632 (5 μ mol/L; SA+Y). *Left panel* shows repartition (in percentages) of cells according to the 3 categories. Values are means $\pm$ SEMs from 4 independent experiments (≥ 110 cells per condition were classified). *Inset (right)* shows results of statistical analysis. Differences between groups were assessed by using 1-way ANOVA, followed by Bonferroni *post hoc* tests. Statistical differences are indicated as follows: * $P < .05$, ** $P < .01$, and *** $P < .001$.

above, was related to a greater chance of clinical and microbiological relapse after ESS.

In addition to showing a new mechanism by which *S aureus* can alter epithelial function, this study explored a new strategy to counteract the negative effect of *S aureus* by targeting the Rho/ROCK signaling pathway. The use of this drug was justified by the fact that *S aureus*, to promote invasion and proliferation within eukaryotic cells, can alter actin polarization by inhibiting Rho GTPase signaling.^{63,65-67} Also, Cormier et al⁶² reported that a polymorphism in the *Rac1* gene (a member of Rho GTPase signaling) could be clinically related to greater predisposition to *S aureus* colonization among patients with CRS.

Our study shows that the deleterious effect of *S aureus* on repair rate and lamellipodia of pNEC cultures from patients with CRSwNP was prevented by ROCK inhibition with Y-27632. To the best of our knowledge, this is the first time that Y-27632 has been used on this purpose on nasal epithelial cells, but its positive effect on wound healing has been demonstrated already on corneal^{27,30} and pulmonary⁴⁵ epithelial cells. Furthermore, Y-27632 has been used to repress epithelial barrier disruption^{63,68} and *S aureus* translocation into epithelial cells.⁶³ Thus this pathway might be a reasonable target for therapy, especially in patients with CRSwNP colonized by *S aureus*.

Altogether, our results show that epithelial repair and lamellipodia are already impaired in cells from patients with CRSwNP under baseline conditions. Furthermore, in infectious conditions nasal epithelial cells show a delayed epithelial healing process and defective lamellipodia formation, which might contribute to altered epithelial integrity and function, thus facilitating access of bacteria and inhaled particles to deeper unprotected areas. Nevertheless, the different response in epithelial repair and lamellipodial dynamics in both a baseline situation and after exposure to *S aureus* exoproducts observed between cells from patients with CRSwNP and control cells might depict an additional susceptibility of patients with CRSwNP to nasal mucosa injuries.

Finally, the present study explores a relatively new aspect of the physiopathology of patients with CRS in a controlled and reproducible system. However, the positive response to a Rho-ROCK inhibitor observed in this study should be confirmed in animal and clinical studies to determine whether this beneficial effect on the recovery of epithelial integrity would be reproduced in *in vivo* assays, as well as to verify the safety of such an epithelium-modifying strategy.

Clinical implications: Sinonasal epithelia from patients with CRSwNP exhibits a defect in wound healing compared with epithelia from control subjects, which is worsened by exposure to *S aureus*. This phenomenon might be prevented by ROCK-targeted therapies.

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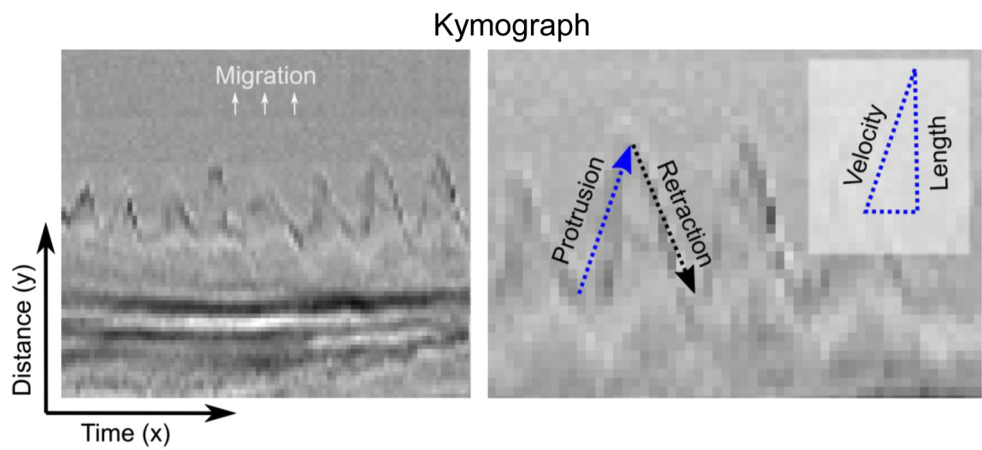


FIG E1. Kymographic analysis. Kymograph taken from a phase-contrast time-lapse series showing local protrusion–retraction cycles. *Blue and black dotted arrows* indicate protrusion and retraction phases, respectively. Protrusion and retraction length and velocity were calculated by using the multiple kymograph and tsp050706 plugin for ImageJ software by tracing a segmented line along the edge of lamellipodia on kymographs.

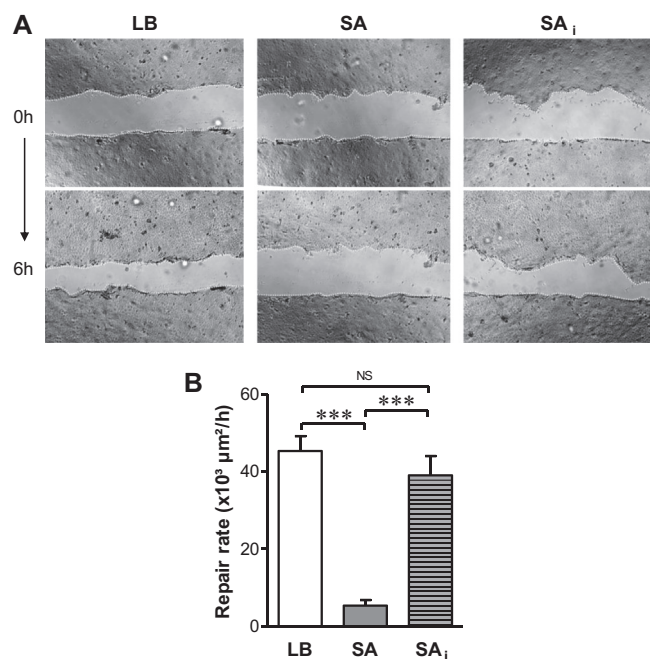


FIG E2. Heat inactivation of *S aureus* exoproducts (SA) prevents the negative effect of *S aureus* exoproducts on repair rates of nasal epithelia from patients with CRSwNP. pNEC monolayers obtained from pNECs from patients with CRSwNP were mechanically injured and treated with LB (left), *S aureus* exoproducts (0.5%; SA – medium), or heat-inactivated SA exoproducts (0.5%; SA_{inactive} – right), and epithelial repair rates were measured over a 6-hour period. Heat inactivation was performed by heating *S aureus* exoproducts at 95°C during 15 minutes. **A**, Representative pictures were taken immediately after injury (0h, above) and after 6 hours of repair (6h, below) with an inverted microscope ($\times 4$ magnification). Wounds have been brightened on the pictures for a better visualization. **B**, Quantitative analysis of epithelial repair rates from 3 patients. Values obtained from each situation within the same group were compared by using repeated-measures ANOVA, followed by Bonferroni *post hoc* tests. *** $P < .001$. NS, Nonsignificant.

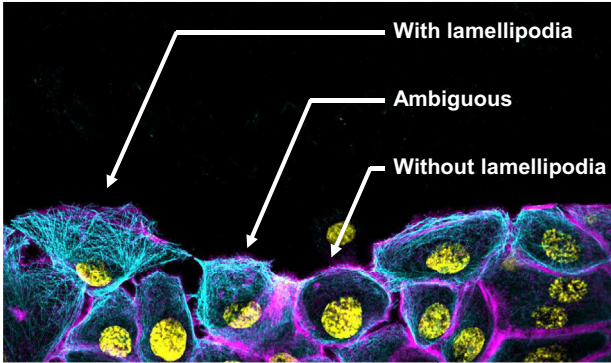


FIG E3. Lamellipodia quantification method. Example of cells classified according to the categories with or without lamellipodia as well as with ambiguous morphology. Percentages of cells in each category were then calculated.