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ORIGINAL ARTICLE

Dupilumab prevents nasal epithelial function alteration by IL-4 in vitro: Evidence for its efficacy

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Abstract

Background: Chronic rhinosinusitis with nasal polyp (CRSwNP) is a typical type 2 inflammation involving interleukin (IL)-4 and IL-13. Dupilumab is a fully human monoclonal antibody targeting IL-4 receptor α subunit, thereby blocking signaling by both cytokines. Our hypothesis was that IL-4 and IL-13, by inducing a severe epithelial dysregulation, are involved in CRSwNP pathogenesis. This study aimed to evaluate the in vitro direct effect of IL-4, IL-13, and dupilumab on nasal epithelial functions.

Methods: Nasal polyps and control mucosa from 28 patients, as well as human nasal epithelial cells (HNEC) from 35 patients with CRSwNP were used. Three major epithelial functions were investigated: the epithelial barrier function (characterized by transepithelial electrical resistance measurements and tight junction protein expression), the ciliary motion (characterized by the ciliary beating efficiency index), and wound healing (characterized by the wound repair

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rate) under various stimulations (IL-4, IL-13, and dupilumab). The main outcome was a significant change in epithelial functions following exposure to IL-4, IL-13, and dupilumab for 48 h in the basal media.

Results: IL-4 (1, 10, and 100 ng/mL) but not IL-13 induced a significant decrease in occludin and zonula-occludens protein expression, ciliary beating efficiency, and wound repair rate in HNEC. Dupilumab (0.04 mg/mL) had no effect on HNEC and specifically restored all epithelial functions altered when cells were exposed to a 48-h IL-4 stimulation.

Conclusion: Dupilumab, in vitro, restored epithelial integrity by counteracting the effect of IL-4 on the epithelial barrier (increased epithelial permeability, decreased ciliary beating efficiency, and decreased wound repair rate).

KEYWORDS

chronic rhinosinusitis, ciliary motility, epithelial cell, paranasal sinus diseases

1 | INTRODUCTION

In western Europe, up to 11% of the population is affected by chronic rhinosinusitis with nasal polyps (CRSwNP),¹ an inflammatory disease of the airways² with a type 2 (T2) inflammatory pattern in 85% of CRSwNP cases.³ CRSwNP is characterized by epithelial damage including goblet cell metaplasia and decreased ciliated cell differentiation.² The nasal epithelium is an important mechanical barrier and responds to environmental insults by inducing the production of T2 promoting cytokines.⁴ To ensure their barrier function, neighboring human nasal epithelial cells (HNEC) are connected to one another via a tight junction (TJ) protein complex. In CRSwNP patients, loss of TJ function and/or expression has been observed⁵; the disruption of the epithelial barrier leads to a chronic inflammatory response that fails to resolve.^{6–9} T2 inflammation, epithelial dysfunction, and numerous cytokines appear to be key factors in the pathogenesis of CRSwNP and the formation of nasal polyps.^{2,10,11}

IL-4 and IL-13 are key T2 cytokines sharing a common receptor subunit (IL-4 receptor α subunit, IL-4R α ¹²). High levels of IL-4 and IL-13 have already been reported in the mucus of patients with CRSwNP,^{13,14} and both IL-4 and IL-13 are deeply involved in airway remodeling.^{15–21} IL-4 has been shown to reduce the expression of the TJ proteins zonula-occludens (ZO-1) and occludin,²² but these results remain debated as other authors did not find any impact of IL-4 on TJ.²³ IL-4 but not IL-13 was found to decrease wound repair rate (WRR) in vitro²⁴ and increase epithelial permeability.²³ Conversely, IL-4 and IL-13 were both shown to induce epithelial migration.²² Dupilumab, a fully human monoclonal antibody (mAb) that binds IL-4R α , thereby blocking signaling by both cytokines,

has been shown to be effective in multiple T2 diseases (asthma, atopic dermatitis, and CRSwNP).^{25,26} IL-4 blockade potently prevents B-cell activation, immunoglobulin E (IgE) production, and pathogenic T-cell lung infiltration, while inhibition of IL-13 is required to prevent goblet cell metaplasia.²⁶ All these results support our main hypothesis that IL-4 and IL-13 are responsible for severe epithelial dysregulation, which can be measured by testing nasal epithelial functions. Moreover, by targeting IL-4R α , dupilumab should have a positive impact on these same functions, explaining why it became the optimum treatment for T2 diseases. Nevertheless, the direct effect of dupilumab on the nasal epithelial barrier in vitro has never been evaluated.

The main aim of the present study was to evaluate the direct effect of IL-4, IL-13, and dupilumab on nasal epithelial functions in CRSwNP.

2 | MATERIALS AND METHODS

2.1 | Tissue and cell collection

First, the mRNA expression of IL-4, IL-13, as well as the protein expression of occludin were analyzed in nasal polyps and control mucosa ($n = 28$). For control mucosa, small-sized septal mucosa fragments were obtained from patients operated on for non-inflammatory and non-tumoral diseases ($n = 14$). Nasal polyps were taken from patients suffering from CRSwNP with or without comorbidities such as aspirin-exacerbated respiratory diseases (AERD; $n = 14$); no allergic fungal rhinosinusitis (AFRS) or CRS sin NP (CRSsNP) patients were included in this study. Tissues were frozen in liquid nitrogen

before protein and nucleic acid extraction. The study was approved by an institutional review board (agreement number DC-20193746, December 2019).

2.2 | Primary culture of HNEC

Using primary cultures of HNEC at the air–liquid interface (ALI), the mRNA expression pattern of IL-4 receptors and the effect of IL-4, IL-13, and dupilumab on transepithelial electrical resistance (TEER), TJ protein expression, ciliary beating efficiency, and wound repair mechanisms were evaluated. To analyze TJ protein expression, two proteins of this complex, occludin and ZO-1, were chosen in this study.^{27,28}

The nasal polyps from 35 patients operated on for CRSwNP were obtained and immediately placed in culture medium and transported to the laboratory for HNEC isolation and culture, as previously described by Coste et al.⁶ (Supporting Information). The approach used to establish primary cultures of HNEC at ALI was the same as that developed more than 20 years ago by our team.^{7,29–31} As described previously, nasal polyps were taken from patients suffering from CRSwNP with or without comorbidities such as AERD (no AFRS or CRSsNP patients). All patients abstained from oral corticosteroids during the month before the surgery and had given informed consent (agreement by the local ethics committee, CPP IDF X 2016-01-01). The epithelial nature of ALI-cultured HNEC was confirmed by flow cytometry and cytokeratin 14 immunofluorescence analysis. After intense proliferation during the first week, the HNEC reach their differentiated stage during the third week of culture and were used herein from day 21 onwards.^{6,31}

In order to test the effect of IL-4, IL-13, and dupilumab on epithelial function, the same various conditions were tested for each function (barrier, ciliary beating, repair): IL-4 alone, IL-13 alone, dupilumab alone, IL-4 with dupilumab, anti-IL-6 alone, and IL-4 with anti-IL-6. All were placed in the basal media for 48 h (referred as 48 h of cytokine exposure in Section 3); basal medium alone was used as control. IL-4 and IL-13 were tested at 1, 10, and 100 ng/mL concentrations, according to previous studies on epithelial cells.^{11,23,24,32} Dupilumab was used at a concentration of 0.04 mg/mL, which is the equivalent concentration of that available in the tissue after a subcutaneous injection (Supporting Information). IL-6 is a multifunctional cytokine that plays an important role in inflammation and CRSwNP pathogenesis¹¹; the latter however does not target IL-4R α . In order to assess whether the effect of dupilumab (an IgG4 of 147 kDa) was specific, a neutralizing monoclonal isotype antibody targeting human IL-6 was used (anti-IL-6, 145 kDa, Invivogen). This

mAb, called anti-IL-6, was used at the same concentration than dupilumab (0.04 mg/mL).

2.3 | IL-4 and IL-13 mRNA expression and occludin protein expression in nasal polyps and control mucosa

To evaluate IL-4 and IL-13 mRNA expression, total RNA from the nasal polyps and control mucosa was isolated using a NucleoSpin RNA II kit (Macherey-Nagel). Occludin protein expression was evaluated using Western blot. Details appear in [Supporting Information](#).

2.4 | IL-4 receptor α expression in HNEC (mRNA)

To evaluate IL-4R α mRNA expression in HNEC, total RNA from HNEC, after 21 days of culture, was isolated using a NucleoSpin RNA II kit (Macherey-Nagel; [Supporting Information](#)).

2.5 | Occludin protein expression in HNEC (Western blot)

To evaluate the effects of IL-4, IL-13, and dupilumab on epithelial barrier function, the in vitro expression of occludin was quantified using Western blot in HNEC ([Supporting Information](#)).

2.6 | ZO-1 protein expression in HNEC (immunolabeling)

To evaluate further the effects of IL-4, IL-13, and dupilumab on epithelial barrier function, after TEER measurements were performed (see below), labeling of actin and immunolabeling of ZO-1 were performed ([Supporting Information](#)). To focus on the TJ belt, we chose to acquire all images at the apical pole of the cell. Immunolabeling and labeling were performed three times, but only representative images are shown. In all conditions, the same parameters (labeling intensity and magnifications) for ZO-1 and actin expression were used to ensure that a qualitative analysis could be performed.

2.7 | TEER in HNEC (electrical resistance)

TEER provides a physical measure of the electrical resistance across the epithelial cell barrier and is widely used as

an indicator of in vitro permeability.³³ TEER was measured using an epithelial volt ohm meter with an ENDOHM-12 electrode (World Precision Instruments) as described previously.³⁴ Only HNEC with a TEER > 200 Ω/cm^2 were used (Supporting Information).

2.8 | Ciliary beating efficiency in HNEC (high-speed video microscopy)

The effect of IL-4 and IL-13 on the ciliary beat frequency (CBF) of respiratory epithelium has already been described and has been shown to contribute to the impaired mucociliary clearance in vitro.³⁵ Although, CBF is not the best tool to evaluate ciliary beating efficiency, another one described by our team, the efficiency index of ciliary clearance was chosen for the present study.^{36,37} In order to evaluate further the effects of IL-4, IL-13, and dupilumab on the ciliary beating efficiency using the latter, all analyses were performed at controlled room temperature (20°C–25°C) in vitro in the same primary culture of HNEC with the same conditions as previously described in the methods. Therefore, microbead velocity was used as a marker of the flow generated by the ciliary beating to evaluate the shear stress induced by cilia on the fluid (mean ciliary efficiency index [in mPa]), as previously reported^{34,36,37} (Supporting Information).

2.9 | Wound repair rate in HNEC (time-lapse images)

The in vitro wound repair assay was carried out using a model of mechanical injury adapted from a previously described method performed on fully differentiated cultures of HNEC.³⁸ The repair mechanism was evaluated under the same conditions as previously described in the methods. Supporting Information are also available.

2.10 | Statistical analysis

Data are expressed as mean \pm standard error of the mean (SEM). SEM was used instead of standard deviation as an estimate of variability among observations because of the size of the samples. Comparisons on tissue samples were made using the Mann–Whitney test. Comparisons of TJ expression, TEER, ciliary beating efficiency, and WRR between cytokine stimulated conditions and basal medium as controls on HNEC were performed using the Wilcoxon test. The latter allows to compare two measurements of a quantitative variable for unpaired data. It is an alternative to the Student's *t*-test when its hypotheses are not valid. A *p*-value ≤ 0.05 was considered as significant. The statistical

analyses were performed using the R software (v. 4.1.2, R Foundation for Statistical Computing, www.r-project.org).

3 | RESULTS

3.1 | IL-4 and IL-13 were overexpressed and occludin protein expression was decreased in nasal polyps

IL-4 and IL-13 mRNA were overexpressed in nasal polyps ($n = 9$) when compared to control mucosa ($n = 9$; $p = 0.0006$ and 0.0002 , respectively; Figure S1A,B). The protein expression of occludin was decreased in nasal polyps ($n = 5$) when compared to control mucosa ($n = 5$; $p = 0.0159$; Figure S1C).

3.2 | IL-4 decreased occludin protein expression in HNEC and dupilumab counteracted this effect

First, we confirmed the mRNA expression of the IL-4R α in HNEC when compared to negative control (leukemia inhibitory factor receptor, Figure S2). In IL-4 stimulated HNEC, occludin expression was significantly decreased for the 100 ng/mL concentration of IL-4 when compared to control ($p = 0.0079$; Figure 1). No significant changes were observed with IL-13. Dupilumab counteracted the decreased expression of occludin in IL-4 stimulated HNEC whereas anti-IL-6 had no effect (Figure 1). ALI cultures of HNEC stimulated on day 21 with IL-4 1, 10, and 100 ng/mL for 48 h showed a decrease in ZO-1 labeling (Figure 2A–D), whereas no changes were observed with IL-13 (Figure S3). The effects of IL-4 were specifically counteracted by dupilumab (Figure 2F–H).

3.3 | IL-4 but not IL-13 decreased TEER in HNEC

The mean \pm SEM baseline TEER measurement across all culture wells used for the analysis was $411 \pm 144 \Omega/\text{cm}^2$ ($n = 8$, 64 wells; Figure 3A) prior to cytokine exposure (16 wells corresponding to $n = 2$ patients were discarded due to TEER < 200 Ω/cm^2). The control well experiments showed a mild decrease in TEER over the 48 h of cytokine exposure (92.7% of baseline values, $381 \pm 112 \Omega/\text{cm}^2$ at 48 h; Figure 3A). After 48 h of cytokine exposure, only IL-4 (1 ng/mL) exposure revealed a significant decrease in TEER (73.4% of baseline values, $343 \pm 89 \Omega/\text{cm}^2$ at 48 h vs. $467 \pm 130 \Omega/\text{cm}^2$ at baseline, $p = 0.05$; Figure 3A). No significant difference was observed at 48 h for IL-13 at all concentrations tested when compared with unstimulated

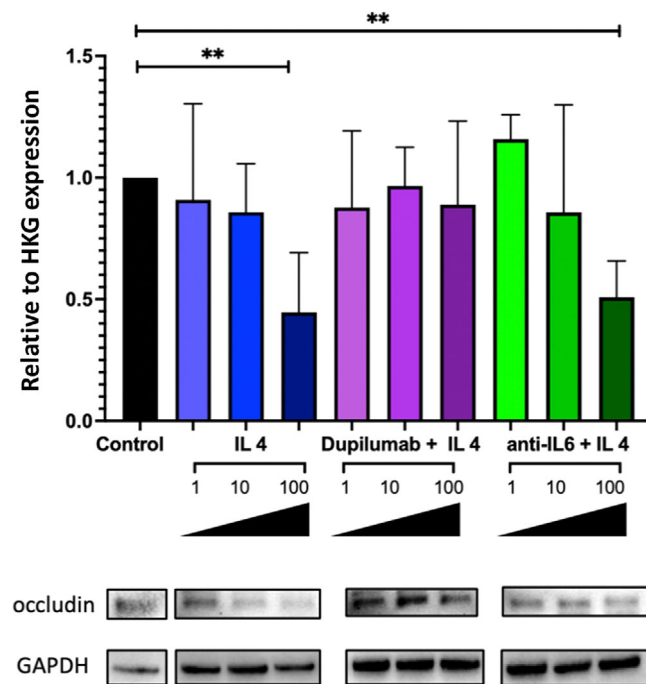


FIGURE 1 Interleukin (IL)-4 reduced the expression of occludin in human nasal epithelial cells (HNEC) and dupilumab counteracted this effect. Occludin ($n = 5$) relative protein expression was analyzed by Western blotting using glyceraldehyde 3 phosphate dehydrogenase (GAPDH) as House Keeping Gene (HKG) to normalize protein expression. The y-axis represents the relative expression normalized to HKG. Comparisons were made between groups. $**p = 0.0079$. Statistical analyses were performed using Wilcoxon test. Error bars correspond to the standard error of the mean.

controls ($p > 0.05$; Figure 3A). Experiments were pursued as described in Section 2 using the following conditions: dupilumab alone, anti-IL-6 alone, IL-4 alone, IL-4 with dupilumab, and IL-4 with anti-IL-6 (Figure 3B). The baseline TEER measurement across all culture wells used for the analysis was $448 \pm 123 \Omega/\text{cm}^2$ ($n = 6$, 72 wells; Figure 3B) prior to cytokine exposure. After 48 h of cytokine exposure, only IL-4 (1 ng/mL) exposure demonstrated a significant decrease in TEER (77.5% of baseline values, $379 \pm 85 \Omega/\text{cm}^2$ at 48 h vs. $489 \pm 65 \Omega/\text{cm}^2$ at baseline, $p = 0.041$; Figure 3B). In the presence of IL-4 with a mAb (dupilumab and anti-IL-6), no decrease in TEER was observed at all concentrations tested.

3.4 | IL-4 decreased the ciliary beating efficiency in HNEC and dupilumab counteracted this effect

A significant decrease was observed with 1, 10, and 100 ng/mL of IL-4 exposure in comparison to control wells

($n = 6$, 84 wells, 0.264 ± 0.09 mPa, 0.216 ± 0.08 mPa, and 0.317 ± 0.11 mPa vs. 0.461 ± 0.16 mPa, $p = 0.03$, 0.012, and 0.04, respectively; Figure 4A). No significant change was observed at 48 h for IL-13 at all concentrations tested when compared with unstimulated controls (Figure 4B). Experiments were pursued as described in Section 2 using the following conditions: dupilumab alone, anti-IL-6 alone, IL-4 alone, IL-4 with dupilumab, and IL-4 with anti-IL-6 ($n = 6$, 72 wells; Figure 4C). The mean ciliary index was 0.542 ± 0.24 mPa, 0.557 ± 0.20 mPa, and 0.501 ± 0.72 mPa, for control, dupilumab alone, and anti-IL-6 alone, respectively. IL-4 stimulation significantly reduced the mean ciliary efficiency index at all concentrations tested when compared with the unstimulated control (solid line, Figure 4C). Dupilumab significantly counteracted the effect of IL-4 on ciliary beating for each concentration tested and anti-IL-6 had no effect (dotted line, Figure 4C).

3.5 | IL-4 decreased the wound repair rate in HNEC and dupilumab counteracted this effect

As shown in Figure 5A, IL-4 seemed to decrease the WRR from 18 h post-stimulation onwards while no significant effect on the WRR was observed with IL-13 ($n = 3$, 24 wells, no statistical analysis; Figure 5A,B). Experiments were pursued as described in Section 2 using the following conditions: dupilumab alone, anti-IL-6 alone, IL-4 alone, IL-4 with dupilumab, and IL-4 with anti-IL-6 ($n = 6$, 72 wells; Figure 5C). In each condition tested, the initial wound areas were similar (213 ± 51 pixels for unstimulated controls vs. 220 ± 55 pixels for IL-4 stimulated wells, $p = 0.493$; vs. 206 ± 73 pixels for dupilumab stimulated wells, $p = 0.889$; and vs. 211 ± 37 pixels for anti-IL-6 stimulated wells, $p = 0.974$). No effect on the WRR was observed with IL-4 (1 ng/mL) (Figure 5D). The stimulation of HNEC with IL-4 (10 ng/mL) decreased the WRR at 42 and 48 h (Figure 5E). A decrease in the WRR was also observed with IL-4 (100 ng/mL) at 24, 42, and 48 h (Figure 5F). The decrease in WRR observed after stimulation by IL-4 (10 and 100 ng/mL) was normalized by dupilumab (Figure 5E,F). The WRR of the 100 ng/mL IL-4 with dupilumab stimulated group was significantly higher than the IL-4 (100 ng/mL) stimulated group ($68.3 \pm 17.3\%$ vs. $46.9 \pm 18.4\%$ at 24 h, $p = 0.022$; $96.3 \pm 6.4\%$ vs. $76.7 \pm 18.9\%$ at 42 h, $p = 0.006$; $99.0 \pm 2.4\%$ vs. $85.7 \pm 17.0\%$ at 48 h, $p = 0.010$, respectively; Figure 5F). Dupilumab alone and anti-IL-6 alone had no effect on WRR (Figure 5D–F).

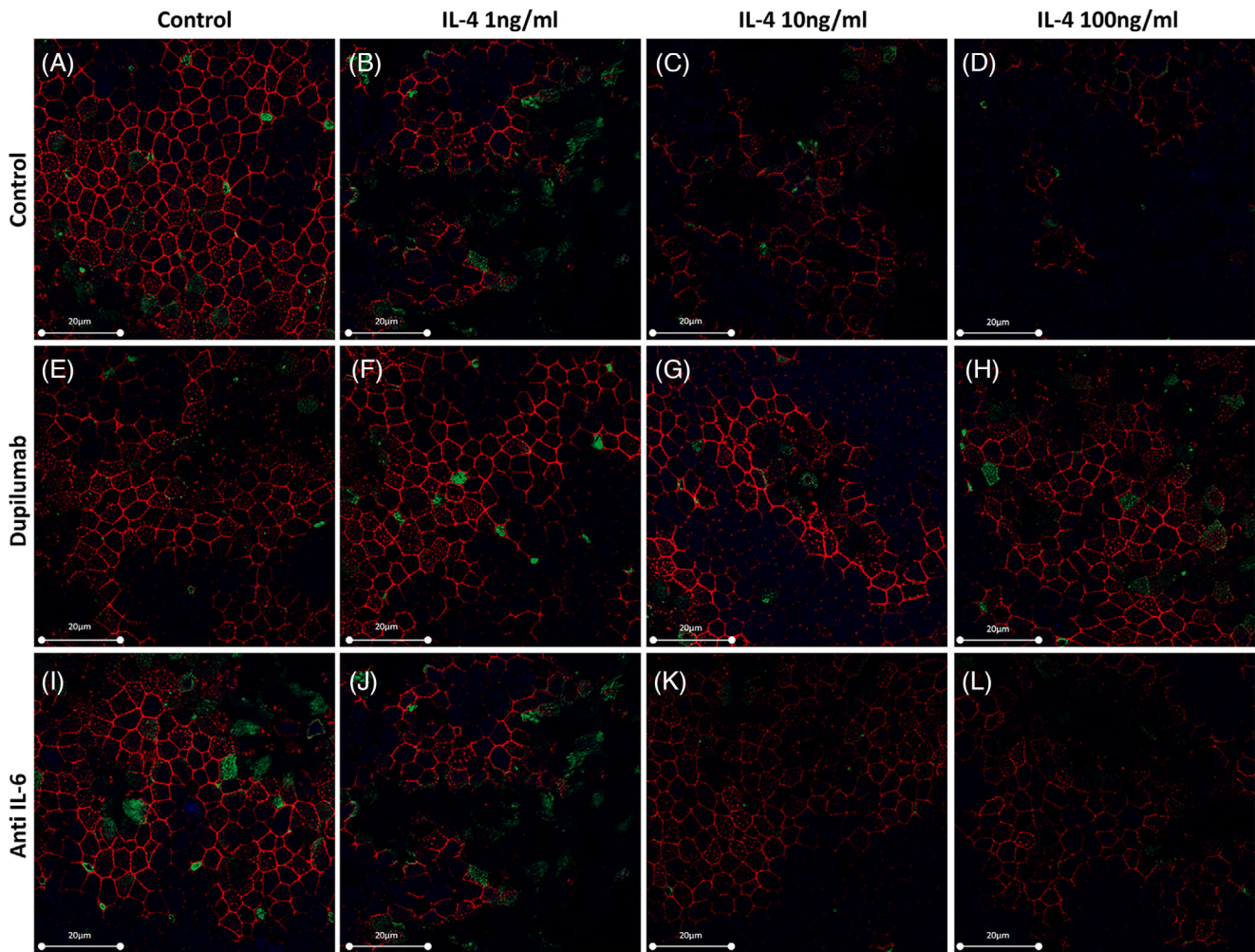


FIGURE 2 Interleukin (IL)-4 reduced the expression of zonula-occludens (ZO-1) in human nasal epithelial cells (HNEC) and dupilumab counteracted this effect. In order to test the effect of IL-4, and dupilumab on tight junction (TJ), various conditions were tested: IL-4 alone, dupilumab alone, IL-4 with dupilumab, anti-IL-6 alone, and IL-4 with anti-IL-6. Air–liquid interface (ALI) cultures of HNEC were stimulated on day 21 with various concentration of IL-4 (1, 10, and 100 ng/mL) during 48 h ($n = 8$). ZO-1 (red) expression after immunolabeling, actin fiber labeling (green), and nuclear 4',6-diamidino-2-phénylindole (DAPI) labeling (blue) are shown without any stimulation, control well (A), after stimulation with IL-4 alone at 1 ng/mL (B), 10 ng/mL (C), and 100 ng/mL (D), after stimulation with dupilumab alone (E), and dupilumab with IL-4 at 1 ng/mL (F), 10 ng/mL (G), and 100 ng/mL (H), and after stimulation with anti-IL-6 alone (I), and anti-IL-6 with IL-4 at 1 ng/mL (J), 10 ng/mL (K), and 100 ng/mL (L). To focus on the TJ belt, observations were made at the apical pole of the cell, DAPI and actin labeling cannot be quantified. The analysis was performed on ZO-1 protein expression, which is one of TJ protein complex.

4 | DISCUSSION

The present results show that IL-4 but not IL-13 had a significant effect on different epithelial functions: IL-4 decreased ZO-1 and occludin expression, TEER, ciliary beating efficiency, and WRR. Dupilumab alone had no effect on HNEC but specifically counteracted the effect of IL-4 on all epithelial functions tested supporting our main hypothesis.

In the present study, the three increasing concentrations of IL-4 and IL-13 used to analyze the dose–response effect were chosen based on previous *in vitro* studies using other cytokines.^{11,34,39} Conversely to the reported decrease

in CBF and impairment in lateral cell contacts induced by IL-13,¹⁶ no effect was observed herein. As such, and based on the results from Wise et al. who reported no effect of IL-13 on TEER,²³ we thus chose IL-4 as the working cytokine to test the effect of dupilumab in HNEC. Although IL-4 and IL-13 both belong to the same family of cytokines sharing the IL-4R α subunit, the present study highlighted their different functional properties.¹² These may be due to the specific and complex interactions of each cytokine with their common receptor¹² but also to the multiple and different signaling pathways that are activated following their interaction with the other receptor subunit.⁴⁰ *In vivo*, IL-4 is known to be a regulator

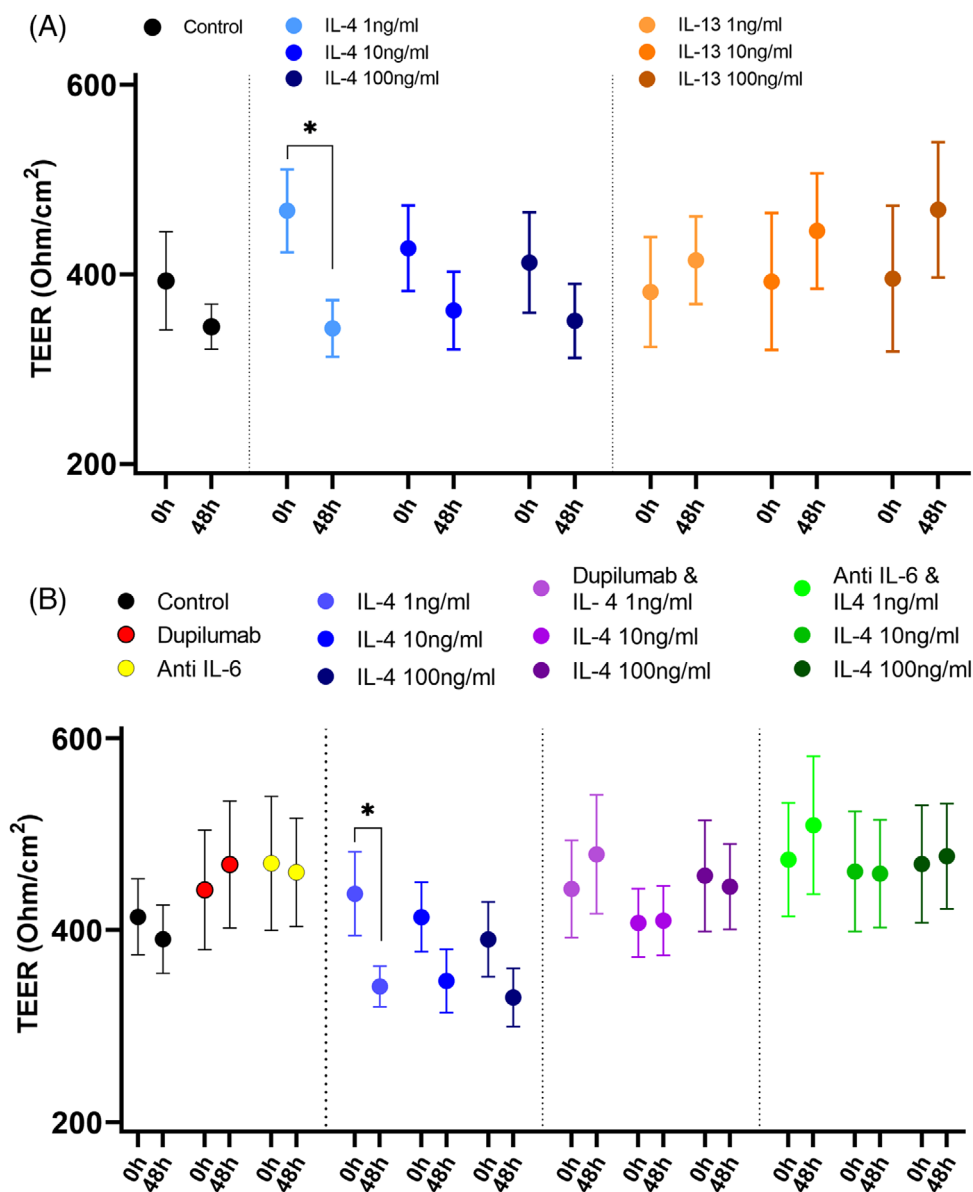


FIGURE 3 Interleukin (IL)-4 but not IL-13 decreased transepithelial electrical resistance (TEER) in human nasal epithelial cells (HNEC). In order to test the effect of IL-4, IL-13, and dupilumab on TEER, various conditions were tested: IL-4 alone, IL-13 alone, dupilumab alone, IL-4 with dupilumab, anti-IL-6 alone, and IL-4 with anti-IL-6. Air-liquid interface (ALI) cultures of HNEC were stimulated on day 21 with various concentrations of IL-4 or IL-13 (1, 10, and 100 ng/mL) during 48 h and compared to unstimulated cultures. TEER measurements (expressed in Ω/cm^2) were performed in unstimulated controls (black), IL-4 alone at 1 ng/mL (light blue), 10 ng/mL (blue), and 100 ng/mL (dark blue), IL-13 alone at 1 ng/mL (light orange), 10 ng/mL (orange), and 100 ng/mL (dark orange), IL-4 with dupilumab (light purple, purple, and dark purple), and IL-4 with anti-IL-6 (light green, green, and dark green). IL-4 at 1 ng/mL exposure demonstrated a significant decrease in TEER, whereas IL-13 had no effect ($n = 8$). Effect of IL-4 was counteracted by dupilumab and anti-IL-6 ($n = 6$). (A) $*p = 0.05$ and (B) $*p = 0.041$. Statistical analyses were performed using Wilcoxon test. Error bars correspond to the standard error of the mean.

of T2 lymphocyte differentiation and class switching of B cells (IgG1 to IgE),⁴¹ which is in line with its direct in vitro effect on several epithelial functions reported herein.

CRSwNP is the result of a chronic nasal inflammatory response triggered by airway epithelial disruption regardless of the initial environmental insult.^{2,5,11,42,43} This insult could increase cytokine expression in nasal polyps.¹⁰ The TJ proteins, including occludin and ZO-1, are crucial in

ensuring the efficacy of the epithelial barrier function.^{5,44} Both IL-4 and IL-13 are supposed to decrease the expression of TJ proteins in vitro²³ and therefore, contribute to the alteration of the epithelial barrier described in CRSwNP.^{23,24} However, we only observed this under IL-4 stimulation, in accordance with Wise et al.²³ Similarly, a decrease in TEER was observed with IL-4 but not IL-13. Then, mucociliary clearance, which represents a key

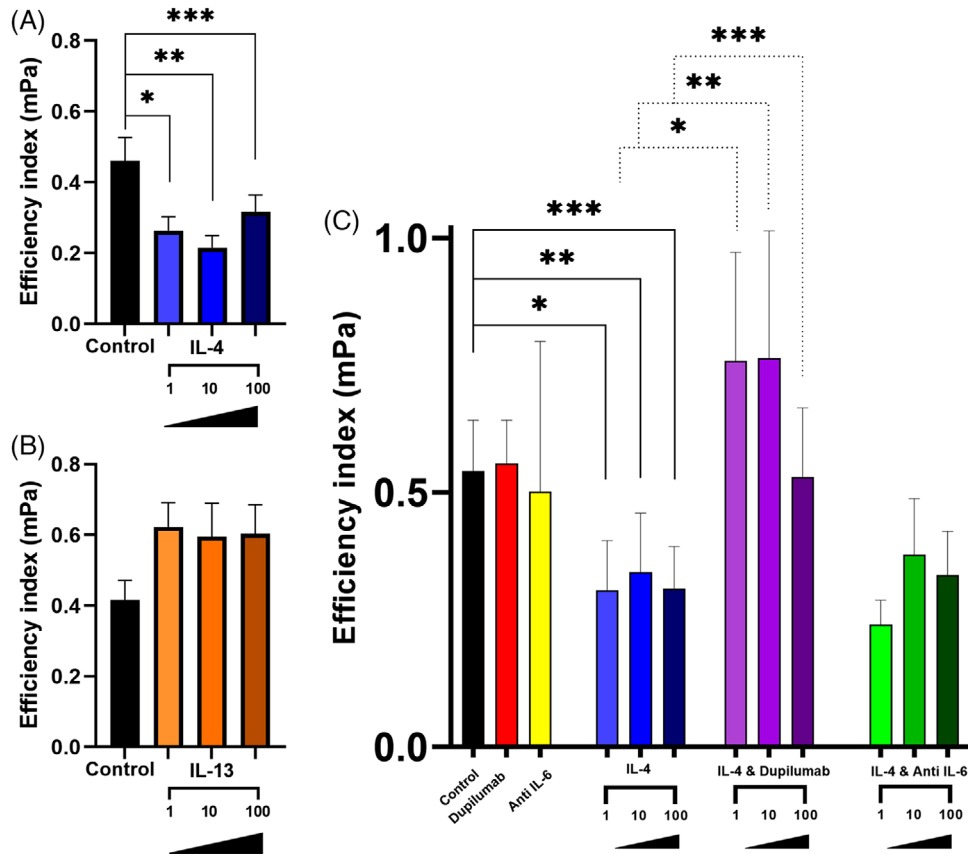


FIGURE 4 Interleukin (IL)-4 decreased the ciliary beating efficiency and dupilumab in vitro counteracted this effect. In order to test the effect of IL-4, IL-13, and dupilumab on ciliary beating efficiency, various conditions were tested: IL-4 alone, IL-13 alone, dupilumab alone, IL-4 with dupilumab, anti-IL-6 alone, and IL-4 with anti-IL-6. The ciliary beating efficiency (mPa) was evaluated after 48 h of IL-4 (light blue, blue, and dark blue) (A) and IL-13 (light orange, orange, and dark orange) (B) stimulation at various concentrations (1, 10, and 100 ng/mL) and compared with unstimulated controls (black; $n = 6$). Stimulation of human nasal epithelial cells (HNEC) with IL-4 alone at 1, 10, and 100 ng/mL decreased ciliary beating efficiency ($*p < 0.05$). The ciliary beating efficiency (mPa) was then evaluated after 48 h of stimulation with IL-4 alone (light blue, blue, and dark blue), IL-4 with dupilumab (light purple, purple, and dark purple), and IL-4 with anti-IL-6 (light green, green, and dark green) at various concentrations of IL-4 for each group (1, 10, and 100 ng/mL) (C). Comparisons were made with unstimulated controls (black; $n = 6$, $*p = 0.005$, $**p = 0.03$, and $***p = 0.007$). The effect of IL-4 was counteracted specifically by dupilumab (respectively, $*p = 0.003$, $**p = 0.016$, and $***p = 0.003$) but not by anti-IL-6. Statistical analyses were performed using Wilcoxon test. Error bars correspond to the standard error of the mean.

step in the defense capacity of the airway epithelium, could also be altered in CRSwNP.^{16,17,35,45} Previous studies demonstrated that IL-4 and IL-13 both significantly reduced CBF^{17,35} and that IL-13 reduced cell differentiation in HNECs from CRSwNP patients.¹⁷ The hypothesis is that a decrease in ciliary function could contribute to the persistence of pathogens at the apical pole of epithelial cells. In the present study, IL-4 but not IL-13 significantly reduced the mean ciliary efficiency index when compared to controls. These results differ from those reported by Jiao et al. who found an effect of IL-13 on CBF when studying the proportion of ciliated cells,¹⁷ a parameter that is less specific than the efficiency index for CRSwNP.^{34,36,37} In another study, the RNA sequencing of sinus epithelial brushings from CRSwNP patients found an increase in tuft cell transcripts and a decrease in ciliated cells after

IL-13 stimulation, but the ciliary beating efficiency per se was not studied.²¹ As TJ alterations are directly correlated with cell migration and epithelial wound healing,^{46–48} we expected results similar to those described by Wise et al., that is an effect with IL-4 but not IL-13.²³ A link between a decreased WRR and a decreased ciliary beating efficiency could be drawn under the hypothesis of a complete loss of usual function of the epithelium when exposed to an inflammatory environment, herein characterized by IL-4 exposure.

A defective epithelial barrier with decreased tissue resistance and decreased expression of TJ proteins⁵ is one of the numerous hypothesis underlying the CRSwNP pathogenesis.⁴⁹ Therefore, restoring the integrity of the epithelial barrier becomes a key element in this disease. In the present study, dupilumab specifically restored all

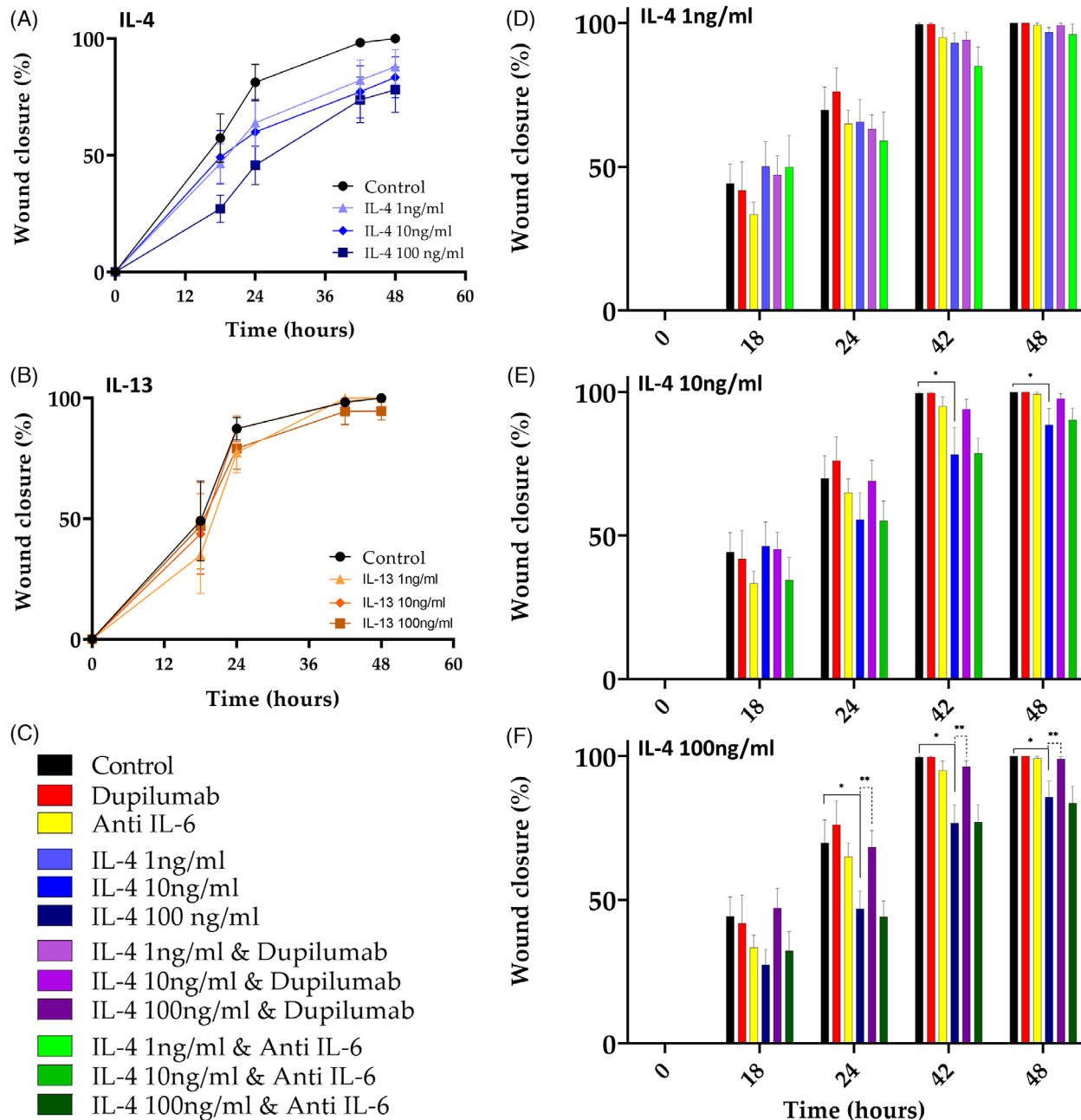


FIGURE 5 Interleukin (IL)-4 decreased the wound repair rate (WRR) in vitro and dupilumab counteracted this effect. In order to test the effect of IL-4, IL-13, and dupilumab on the repair rate, various conditions were tested: IL-4 alone, IL-13 alone, dupilumab alone, IL-4 with dupilumab, anti-IL-6 alone, and IL-4 with anti-IL-6. The repair rate was evaluated as the proportion of the wounded area closed at each time point for IL-4 and IL-13 stimulated wells (either 1, 10, or 100 ng/mL) and compared to the one of unstimulated control wells, first for IL-4 alone ($n = 3$) (A) and IL-13 alone ($n = 3$) (B) to test their effect on human nasal epithelial cells (HNEC) (no statistical analysis performed). Then, experiments were pursued with IL-4 alone, dupilumab alone, anti-IL-6 alone, IL-4 with dupilumab, and IL-4 with anti-IL-6 ($n = 5$) (C–F). Statistical analyses were performed using Wilcoxon test. Error bars correspond to the standard error of the mean. No effect on the WRR was observed with IL-4 alone at 1 ng/mL (D). At 42 and 48 h, the 10 ng/mL IL-4 alone stimulated group had a lower repair rate than the unstimulated control group ($76.7 \pm 18.9\%$ vs. $99.5 \pm 1.4\%$ at 42 h, $p = 0.001$; $88.6 \pm 16.9\%$ vs. $100.0 \pm 0.0\%$ at 48 h, $p = 0.013$) (E). When HNEC were stimulated with IL-4 at 10 ng/mL with dupilumab, the WRR was normalized (E). At 24, 42, and 48 h, the 100 ng/mL IL-4 stimulated group had a lower repair rate than the unstimulated control group ($46.9 \pm 18.4\%$ vs. $69.8 \pm 23.9\%$, $p = 0.037$ at 24 h; $76.7 \pm 18.9\%$ vs. $99.5 \pm 1.4\%$ at 42 h, $p = 0.001$; $85.7 \pm 17.0\%$ vs. $100.0 \pm 0.0\%$ at 48 h, $p = 0.001$). When HNEC were stimulated with IL-4 at 100 ng/mL with dupilumab, the WRR was normalized and the 100 ng/mL IL-4 with dupilumab stimulated group had a higher repair rate than the 100 ng/mL IL-4 alone stimulated group ($68.3 \pm 17.3\%$ vs. $46.9 \pm 18.4\%$ at 24 h, $p = 0.022$; $96.3 \pm 6.4\%$ vs. $76.7 \pm 18.9\%$ at 42 h, $p = 0.006$; $99.0 \pm 2.4\%$ vs. $85.7 \pm 17.0\%$ at 48 h, $p = 0.010$, for IL-4 at 100 ng/mL with dupilumab vs. IL-4 100 ng/mL alone, respectively) (F). *Statistical significance between control and IL-4 stimulated wells ($p < 0.05$); **between IL-4 and IL-4 with dupilumab stimulated wells.

epithelial functions (TJ protein expression, ciliary beating efficiency, and WRR) altered by IL-4; its effect on TEER measurements, however, was not specific. We could suggest that in vitro, a decreased TEER under IL-4 exposure is counteracted in the presence of mAbs, whether they are specific (dupilumab) or not (anti-IL-6), partly due to the sensitivity of HNEC to their environment.

Among the limitations of the present study, the lack of data regarding goblet cell secretions under IL-13 stimulations could be raised; the latter however has already been well described in the literature.¹⁶ Another limitation could be the lack of nice dose response curves with IL-4 stimulation, which was unexpected. Indeed, the concentrations chosen were close to the one found in vivo, but the response is complex, keeping in mind that cytokines have paracrine effects. In addition, IL-4 was tested alone and a synergistic effect with other cytokines should be considered. However, since no effect of IL-13 was identified, we decided not to test the epithelial functions under the co-exposition of IL-4 and IL-13. Furthermore, addressing the embryological distinction between septal and ethmoidal mucosae is conceivable, but unlikely to significantly impact our results. The common use of septal mucosae or inferior turbinate biopsies as controls in unraveling CRSwNP pathogenesis reinforces this assertion.^{50–55} Finally, our choice to focus on CRSwNP patients could be a limitation, but since dupilumab is only authorized in these patients, it did not appear relevant to study patients with CRSsNP or allergic rhinitis.

5 | CONCLUSION

In conclusion, dupilumab, in vitro, maintained epithelial integrity by counteracting the effects of IL-4 on the epithelial barrier (increased epithelial permeability, decreased ciliary beating efficiency, and decreased WRR). These findings suggest that the efficacy of dupilumab is underlined by its capacity to prevent the alteration of epithelial functions, one of the key features of CRSwNP pathogenesis.

AUTHOR CONTRIBUTIONS

Conceptualization: Emilie Bequignon, Laure Favot, Maxime Fieux, Stéphane Tringali, André Coste, Jean Claude Lecron, Florent Carsuzaa, and Yvan Bellanger. *Data curation:* Maxime Fieux, Florent Carsuzaa, and Yvan Bellanger. *Formal analysis:* Emilie Bequignon, Maxime Fieux, Laure Favot, Sophie Bartier, Xavier Dufour, Matthieu Binaud, Bruno Louis, Florent Carsuzaa, and Yvan Bellanger. *Methodology:* André Coste, Emilie Bequignon, Maxime Fieux, Florent Carsuzaa, and Yvan Bellanger. *Project administration:* André Coste, Bruno

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CONFLICT OF INTEREST STATEMENT

M.F., E.B., F.C., X.D., and A.C. have received honoraria from Sanofi and GlaxoSmith. Remaining authors declare they have no conflicts of interest in relation to this work.

DATA AVAILABILITY STATEMENT

Data supporting reported results can be found in the Laboratoire Inflammation Tissus Epithéliaux et Cytokines (UR15560), Université de Poitiers (86000 Poitiers), and the Laboratoire de l'Institut Mondor de Recherche Biomédicale (INSERM U955, CNRS EMR 7000, 93000 Créteil).

ETHICS STATEMENT

The study was approved by the Institutional Ethics Committee on Human Experimentation of the University Hospital of Poitiers and received the agreement of the French “Ministère de l'Enseignement Supérieur et de la Recherche” (DC-20193746, December 2019). Regarding primary culture of human nasal epithelial cells, all the patients had given informed consent, and the study was approved by the local ethics committee (CPP IDF X 2016-01-01).

CONSENT FOR PUBLICATION

Informed consent was obtained from all subjects involved in the study.

DATA, MATERIALS, AND/OR CODE AVAILABILITY

No additional data are available.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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