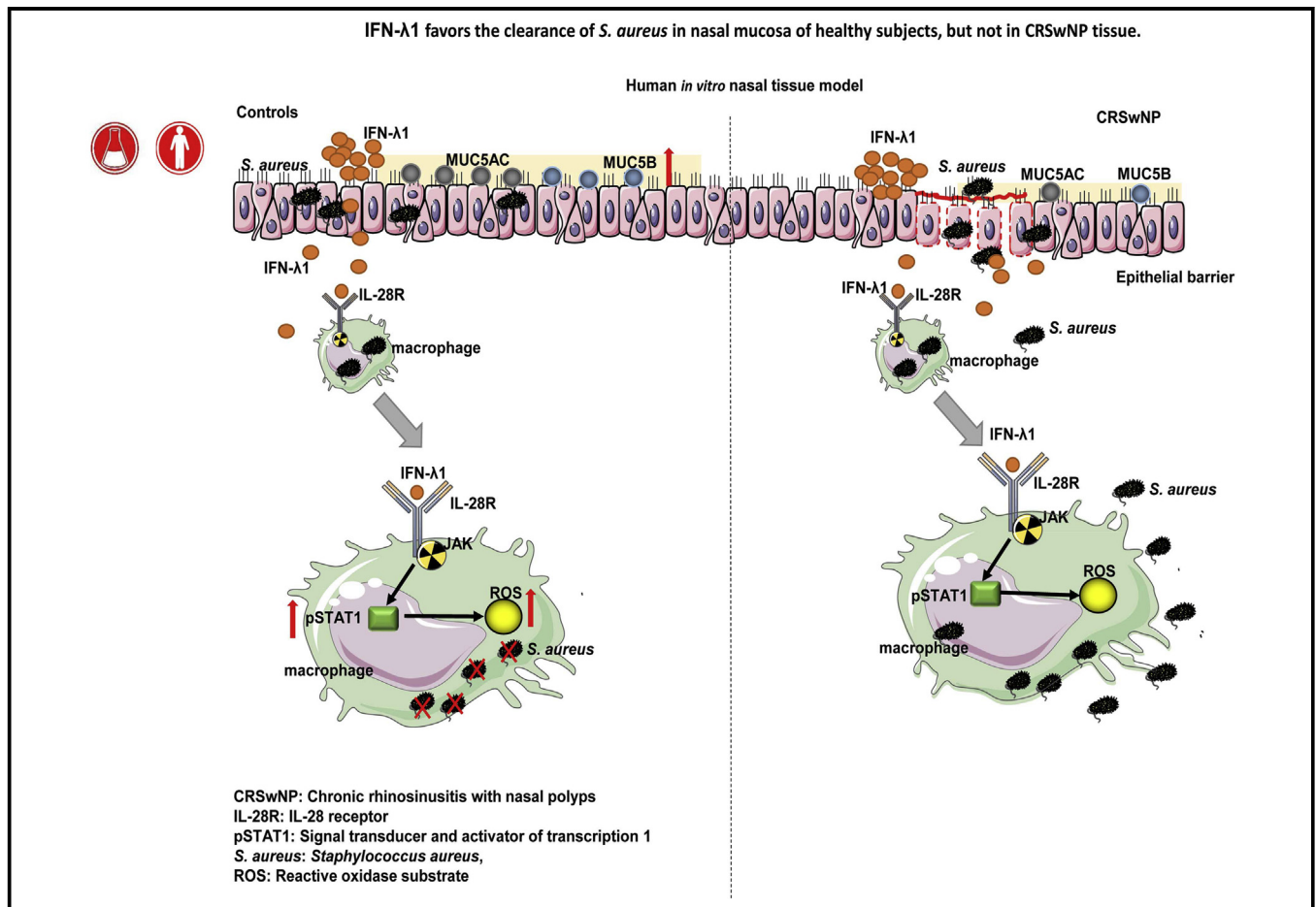


IFN- λ 1 enhances *Staphylococcus aureus* clearance in healthy nasal mucosa but not in nasal polyps



Feng Lan, MD, PhD,^{a,b,*} Hua Zhong, MD, PhD,^{b,d,*} Nan Zhang, MD, PhD,^b Sebastian L. Johnston, MD, PhD,^c Weiping Wen, MD, PhD,^d Nikos Papadopoulos, MD, PhD,^e Luo Zhang, MD, PhD,^a and Claus Bachert, MD, PhD^{b,f}
Beijing and Guangzhou, China, Ghent, Belgium, London and Manchester, United Kingdom, and Stockholm, Sweden

GRAPHICAL ABSTRACT



From ^athe Department of Otolaryngology Head and Neck Surgery, Beijing Institute of Otolaryngology, Beijing TongRen Hospital, Capital Medical University, Beijing; ^bthe Upper Airways Research Laboratory, Department of Oto-Rhino-Laryngology, Ghent University Hospital; ^cthe Airway Disease Infection Section, National Heart and Lung Institute, Imperial College London; ^dthe Otorhinolaryngology Hospital, First Affiliated Hospital, Sun Yat-sen University, Guangzhou; ^ethe Centre for Pediatrics & Child Health, Institute of Human Development, University of Manchester; and ^fthe Division of ENT Diseases, Clintec, Karolinska Institute, Stockholm.

*These authors contributed equally to this work.

Supported by grants to C.B. from the European Commission's Seventh Framework programme under grant agreement no. 260895 (PREDICTA); from the Flemish Scientific Research Board (FWO), projects 1841713N, G.039412N, and G.067512N; BOF14/GOA/019 and the Interuniversity Attraction Poles Program—Belgian State Belgian Science Policy, no. IAP P7/30; to L.Z. from the Program for Changjiang Scholars and Innovative Research Team (IRT13082), the 12th five-year science and technology support project (2014BAI07B04), and the National Natural Science Foundation of China (81630023 and 81420108009); to F.L. from the National Natural Science Foundation of China (81700887).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication September 9, 2017; revised August 28, 2018; accepted for publication September 7, 2018.

Available online November 30, 2018.

Corresponding author: Luo Zhang, MD, PhD, Beijing Institute of Otolaryngology, Department of Otolaryngology Head and Neck Surgery, Beijing TongRen Hospital, Capital Medical University, No. 17, HouGouHuTong, DongCheng District, Beijing 100730, China. E-mail: dr.luozhang@139.com. Or: Claus Bachert, MD, PhD, Upper Airways Research Laboratory, ENT Department, Ghent University, De Pintelaan 185, 9000 Ghent, Belgium. E-mail: claus.bachert@ugent.be.

The CrossMark symbol notifies online readers when updates have been made to the article such as errata or minor corrections

0091-6749/\$36.00

© 2018 American Academy of Allergy, Asthma & Immunology

<https://doi.org/10.1016/j.jaci.2018.09.041>

Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is characterized by T_H2-skewed inflammation and increased colonization by *Staphylococcus aureus*. IFN-λ1 is known for its antiviral activity, but there is little information on its antibacterial role.

Objective: We sought to determine the expression and release of IFN-λ1 from nasal mucosal tissue of healthy subjects and patients with CRSwNP on exposure to *S aureus* and assess its potential role in antibacterial defense mechanisms.

Methods: Nasal tissue from healthy subjects and patients with CRSwNP was exposed to *S aureus*, and we assessed expression of IFN-λ1, MUC5AC, and MUC5B. THP1-derived macrophages incubated with or without IFN-λ1 were assessed for uptake and killing of *S aureus* and expression of lysosomal-associated membrane protein 1 and intracellular reactive oxidase substrate (ROS), the IFN-λ1 receptor IL-28 receptor (IL-28R), and the Janus kinase/signal transducer and activator of transcription (STAT) 1 pathway by means of immunofluorescence staining.

Results: *S aureus* infection increased IFN-λ1 expression in tissue from patients with CRSwNP. IFN-λ1 (10 ng/mL) significantly decreased the number of *S aureus* colony-forming units in healthy control tissue but not in tissue from patients with CRSwNP and upregulated MUC5AC and MUC5B expression in control tissue on *S aureus* infection. IFN-λ1 stimulation increased intracellular killing of *S aureus* in THP1-derived macrophages and substantially increased lysosomal-associated membrane protein 1, IL-28R, ROS, and STAT signaling in macrophages incubated with *S aureus*. All of these effects were attenuated by blocking IL-28R and ROS activities.

Conclusions: IFN-λ1 favors clearance of *S aureus* in healthy nasal mucosa and enhances antibacterial function of macrophages through IFN-λ1–IL-28R–ROS–Janus kinase–STAT signaling pathways. (J Allergy Clin Immunol 2019;143:1416–25.)

Key words: Chronic rhinosinusitis with nasal polyps, IFN-λ1, *Staphylococcus aureus*, antibacterial activity, macrophages

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a chronic inflammatory airway disease. Nasal polyps in the Western world are mainly characterized by T_H2-biased eosinophilic inflammation.^{1–3} Patients with CRSwNP also show greater nasal carriage of *Staphylococcus aureus* compared with healthy subjects and partially express specific IgE against *S aureus* enterotoxin.⁴ Evidence also indicates that *S aureus* can directly induce T_H2 responses,⁵ and alternatively activated macrophages (M2s) in a T_H2-biased environment do not adequately phagocytose and kill *S aureus*.⁶ Thus impaired bacterial clearance can aggravate inflammation through *S aureus* colonization and contribute to persistent inflammation in patients with CRSwNP. Moreover, invasion of *S aureus* into the nasal mucosa can be facilitated as a consequence of virus-induced disruption of the nasal epithelial barrier,⁷ particularly because a high frequency of viruses, such as human rhinovirus and human respiratory syncytial virus, can be observed in nasal tissue.⁸ Unfortunately, an efficient way to eradicate both viruses and bacteria for long periods is still unavailable.

Type III interferons, a new family of interferons including IFN-λ1 and IFN-λ2/3, have been shown to be induced by viral infection and have antiviral activity similar to type I and type II

Abbreviations used

CFU:	Colony-forming unit
CRSwNP:	Chronic rhinosinusitis with nasal polyps
DAPI:	4'-6-Diamidino-2-phenylindole dihydrochloride
DPI:	Diphenyleioidonium chloride
IL-28R:	IL-28 receptor
JAK:	Janus kinase
LAMP1:	Lysosomal-associated membrane protein 1
M1:	Activated proinflammatory macrophage
M2:	Alternatively activated macrophage
MDM:	Monocyte-derived macrophage
PMA:	Phorbol 12-myristate 13-acetate
ROS:	Reactive oxidase substrate
STAT:	Signal transducer and activator of transcription
TCM:	Tissue culture medium

interferons.⁹ IFN-λ specifically interacts with a heterodimeric receptor composed of 2 chains, a specific ligand-binding chain IL-28 receptor (IL-28R) α (IL-28Ra or IFN-λR1) and an IL-10 receptor β (or IL-10 receptor 2) chain and further activates the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway.⁹ Because of preferential expression of IFN-λ receptor on airway epithelial cells, the antiviral effects of IFN-λ are most evident against pathogens targeting barrier sites without activating a systemic proinflammatory immune response.¹⁰

Several studies have investigated the association between IFN-λs and *S aureus* infection and colonization. In particular, IFN-λ plays a significant role in murine defense against nasal colonization of *S aureus*.¹¹ In IL-28R^{−/−} mutant mice influenza virus infection does not lead to an increase in abundance or diversity of nasal flora, whereas there is a significant increase in upper airway flora, including a 40-fold increase in *S aureus* in wild-type mice, suggesting the involvement of IL-28R in *S aureus* colonization.¹² Indeed, mice lacking IL-28R have significantly improved clearance of *S aureus* and consequently led to less pulmonary pathology, which was associated with reduced levels of inflammatory cytokines.¹³ IFN-λ3 has also been linked to killing of the intracellular bacterial pathogen *Listeria monocytogenes* in mice.¹⁴

To date, the role of IFN-λ in bacterial eradication has been investigated by using IL-28R^{−/−} mouse models, but information on the effect of IFN-λ in bacterial infections in human subjects is scarce. To this end, making use of previously described human nasal tissue *ex vivo* models, we developed a human mucosal tissue *S aureus* infection model to investigate the role of IFN-λ1 in innate immune responses to *S aureus*. We specifically analyzed the number of *S aureus* colony-forming units (CFUs) in patients with CRSwNP and control tissue in the presence of IFN-λ1 and investigated the underlying mechanisms through which IFN-λ1 mediated its protective activity during infections.

METHODS

Patients

Nasal polyp tissues were obtained from 26 patients with CRSwNP, and similarly, healthy control inferior turbinate mucosal tissues were obtained from 18 patients scheduled for turbinate surgery because of septal deviations or turbinate hypertrophy at the Department of Oto-Rhino-Laryngology, Ghent University Hospital. None of the patients had taken oral or nasal

TABLE I. Primer sequences used for quantitative RT-PCR

Targets	Forward (5'-3')	Reverse (5'-3')
IFNL1	TGGGAACCTGTGTCTGAGAACG	AGGGCTCAGCGCATAAATAAGGTG
MUC5AC	CCACTGGTTCTATGGCAACACC	GCCGAAGTCCAGGCTGTGCG
MUC5B	CTGCTACGACAAGGACGGAAAC	AAGGCTGTGAGCGCACTGGATG

corticosteroids for 4 weeks or antibiotics for 2 weeks before surgery. The diagnosis of CRSwNP was made according to the European Position Paper on Rhinosinusitis and Nasal polyps 2007 guidelines.¹⁵ The study was approved by the ethics committees of Ghent University Hospital, and informed consent was obtained from all patients before sample collection.

S aureus stocks

S aureus RN6390 containing the plasmid pALC1743, a pSK236-derived shuttle plasmid carrying an active *S aureus* promoter (RNAIII promoter), was chosen as the infection strain in this study. In particular, this strain does not produce *S aureus* enterotoxins A, B, C, and D (tested by using SET-RPLA kit in Trypticase Soy medium; BD, Franklin Lakes, NJ) and toxic shock syndrome toxin 1 (assayed with the TST-RPLA KT; Oxoid, Hampshire, United Kingdom).

Human nasal mucosal model

Tissue from patients with CRSwNP or control tissue from each patient was cut into small pieces of approximately 25-mm² surface area and washed 3 times in fresh tissue culture medium (TCM; RPMI 1640/Dulbecco modified Eagle medium = 1:1) with 50 IU/mL penicillin (Invitrogen, Carlsbad, Calif) and 50 µg/mL streptomycin (Invitrogen). Washed tissue explants were placed on a metal mesh triangle in a 6-well plate, and 3 mL of TCM without any antibiotics was added to each well to create an air-liquid interface before incubation at 37°C in 5% CO₂ in air overnight.

For the noninfection tissue model, tissues were transferred to 24-well plates and suspended in 2 mL of TCM containing IFN-λ1 (10 ng/mL), which was chosen as the optimal concentration based on dose-response experiments, and then incubated for 24 hours at 37°C in 5% CO₂ in air. For the *S aureus* infection model, tissue cubes were suspended in 2 mL of TCM containing 2×10^7 CFUs per well of *S aureus* and IFN-λ1 (10 ng/mL) and then incubated in 24-well plates, as indicated above. A dose of 2×10^7 CFUs per well of *S aureus* was used because we have previously demonstrated this to be optimal in both preserving the integrity and eliciting measurable immune responses in explant cultures.⁷ Tissue incubated with TCM alone was taken as the control condition. At the end of incubation, all tissues were weighed and snap-frozen in liquid nitrogen before storage at -80°C until further assessment of IFNL1, MUC5AC, and MUC5B mRNA expression by using RT-PCR; MUC5AC and MUC5B levels were determined by using immunofluorescence staining.

THP1 cell-differentiated macrophage model

For differentiation, THP1 cells from the VIB Research Center of Ghent University were cultured in RPMI 1640 medium supplemented with 10% FBS. THP1 cells differentiated to macrophages and used in further experiments to investigate the effect of *S aureus* infection in the absence or presence IFN-λ1. The effect of inhibiting IFN-λ1-mediated uptake and killing of *S aureus* by macrophages was investigated by preincubating macrophages for 1 hour with 10 µmol/L NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI; Sigma, St Louis, Mo) or IL-28R blocking peptide (2 µg/mL Santa Cruz Biotechnology, Dallas, Tex) in RPMI 1640 plus 10% FBS before incubation with IFN-λ1. More detailed information is provided in the Methods section and Fig E1 in this article's Online Repository at www.jacionline.org.

Quantitative RT-PCR

Total RNA was extracted from nasal mucosal tissue by using the RNeasy kit (Qiagen, Hilden, Germany), and 5 ng of cDNA was synthesized by using the iScript Advanced cDNA-synthesis Kit (Bio-Rad laboratories, Hercules, Calif). The mRNAs for IFNL1, MUC5AC, and MUC5B were detected by

TABLE II. Clinical data of included patients

	Patients with CRSwNP	Control subjects
No. of subjects	20	13
Age (y), median (range)	50 (25-74)	42 (28-53)
Sex, female/male	6/14	2/11
Atopy	9/11	7/6
Asthma	9/11	0/13
AERD	1/19	0/13

AERD, Aspirin-exacerbated respiratory disease.

using Sso Advanced SYBR Green Super Mix (Bio-Rad Laboratories) on the LightCycler 480 Instrument II (Roche Applied Science, Penzberg, Germany) by using the primer sequences shown in Table I. Two housekeeping genes, succinate dehydrogenase complex and elongation factor 1, were used to normalize for transcription and amplification variations among the samples. Relative quantities of mRNA expressed were calculated by using the commercially available qBasePlus software (Bio-Rad Laboratories).¹⁶

Cytokine measurements

IFN-λ1 in sample supernatants was assessed by using commercially available ELISA kits from R&D Systems (Minneapolis, Minn). All samples were processed according to the manufacturer's instructions, and cytokine levels were measured on a Bio-Plex 200 Array Reader (Bio-Rad Laboratories).

Immunofluorescence staining

Nasal tissue sections from noninfection and infection models and THP1-differentiated macrophages were immunostained with antibodies for MUC5AC (1:2000; Abcam, Cambridge, United Kingdom), MUC5B (1:200; Novus, Centennial, Colo), and lysosomal-associated membrane protein 1 (LAMP1; 1:20, R&D Systems), respectively, for 2 hours at room temperature. Mouse IgG₁ antibody (DakoCytomation, Glostrup, Denmark) and rabbit IgG antibody (Abcam) were used as isotypes. After incubation with the secondary antibody, sections were rinsed with PBS and mounted with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI; Vector Laboratories, Burlingame, Calif) for assessment of fluorescence by using a Leica confocal system (Leica, Wetzlar, Germany) linked to a DMB fluorescence microscope.

Similarly, levels of intracellular reactive oxidase substrate (ROS) in differentiated macrophages were measured by using dihydroethidium (Sigma), a cell-permeable probe that becomes fluorescent when it is oxidized by the reaction with a variety of ROS inside the cells. After fixing with 4% paraformaldehyde for 20 minutes at room temperature and permeabilizing with 0.7% Triton-100 for 10 minutes, macrophages were incubated with dihydroethidium working solution (12.5 µmol/L) in the dark for 45 minutes at 37°C. Stained cells were rinsed with PBS and mounted with DAPI. More detailed information about fluorescence intensity calculation has been provided in the Methods section in this article's Online Repository.

Western blotting

Total protein of nasal tissue sections from the noninfection and infection models and THP1-differentiated macrophages with or without *S aureus* infection was extracted by using RAPI buffer (Invitrogen) with cocktail proteinase inhibitor (Roche, Mannheim, Germany). The following antibodies were used for Western blotting: anti-phospho-STAT1 (1:1000) and anti-STAT1 (1:1000) were purchased from Cell Signaling Technology

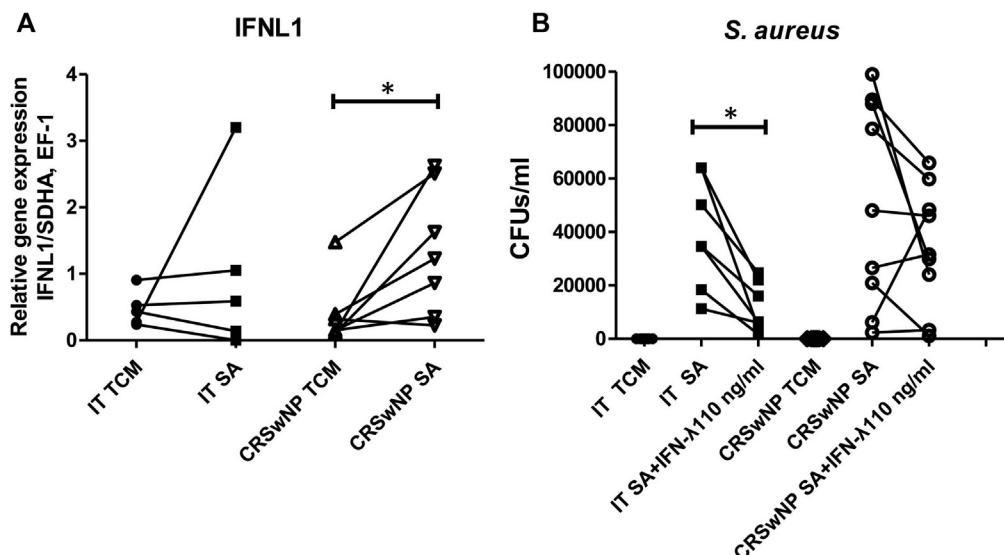


FIG 1. IFN-λ1 was induced in nasal tissue during *S aureus* (SA) infection, and the induced IFN-λ1 in healthy nasal tissue had an antibacterial role. **A**, mRNA levels of IFNL1 in nasal tissue on *S aureus* exposure. CRSwNP (n = 7); IT, inferior turbinate (n = 5). **B**, CFUs of *S aureus* estimated in human nasal mucosal tissue homogenates plated on mannitol salt agar plates. CRSwNP (n = 9); IT, inferior turbinate (n = 7). **P* < .05.

(Danvers, Mass), and anti-IL-28R antibody (1:800, sc-135101) was purchased from Santa Cruz Biotechnology. Relative band densities of the target protein to glyceraldehyde-3-phosphate dehydrogenase (Invitrogen) were estimated with Lab Image Analysis Software (Bio-Rad Laboratories).

Statistical analyses

Data were analyzed with GraphPad Prism software (GraphPad Software, San Diego, Calif). The Mann-Whitney *U* test was used for between-group comparisons, and the Friedman test was used for comparison of related samples. A *P* value of less than .05 was considered statistically significant.

RESULTS

Clinical characteristics of study groups

Table II shows clinical data of the patients with CRSwNP and control subjects from whom tissues were obtained. Both groups were similar with respect to age, and there were more male than female subjects. Around 50% of the subjects in both groups were atopic, although asthma was shown to be present only in patients with CRSwNP (45%).

IFN-λ1 can be induced in nasal tissue during *S aureus* infection but only deploys its antibacterial function in tissue from healthy control subjects and not that from patients with CRSwNP

IFN-λ1 was measured at both the mRNA and protein levels in the human nasal mucosal model after *S aureus* infection. IFNL1 mRNA expression was significantly increased in nasal polyp tissue from patients with CRSwNP infected with *S aureus* compared with noninfected tissue (*P* < .05; Fig 1, A). A similar trend was noted in IFNL1 mRNA expression in *S aureus*-infected and noninfected control turbinate tissue, although the differences in expression were not statistically significant (Fig 1, A). Protein levels of IFN-λ1 were measured by using ELISA; however, protein A resulted in unspecific binding of protein and therefore prevented interpretation of the data.

Nasal tissues were exposed to *S aureus* in the absence or presence of IFN-λ1 to investigate the role of IFN-λ1 on *S aureus* amplification during infection. The number of CFUs of *S aureus* in control turbinate tissue decreased significantly about 3-fold in the presence of 10 ng/mL IFN-λ1 compared with *S aureus* exposure in the absence of 10 ng/mL IFN-λ1 (*P* < .05; Fig 1, B). An IFN-λ1-induced decrease in CFUs of *S aureus* was not observed in tissue from patients with CRSwNP (Fig 1, B).

IFN-λ1 modulates mucin expression in healthy control subjects but not in patients with CRSwNP

In view of the bacterial clearance function of mucus during infection, MUC5AC and MUC5B, the main components of mucus, were evaluated in the human nasal mucosal model. Samples were evaluated by 2 blinded observers who quantified the mucins according to the intensity of immunofluorescence staining. MUC5AC protein was located in the epithelial layer (Fig 2, A and B), whereas MUC5B protein was mainly distributed in both the epithelial layer and the subepithelial glands (Fig 2, C-F). MUC5AC expression was markedly increased in control turbinate tissue after 24 hours in the presence of IFN-λ1 (10 ng/mL) with or without *S aureus* exposure (Fig 2, A, B, and G). *S aureus*-positive epithelial regions with a visually damaged epithelial layer had less MUC5AC expression than intact epithelial layer regions in TCM controls (Fig 2, B and G). In contrast, MUC5AC expression was not altered in the mucosa of patients with CRSwNP with *S aureus* infection in the presence of IFN-λ1 (Fig 2, B and G). IFN-λ1 upregulated MUC5B expression specifically in the epithelial layer in control turbinate mucosa with *S aureus* infection but did not alter the expression of this mucin in the mucosa in patients with CRSwNP, irrespective of *S aureus* infection (Fig 2, C-F and H).

IFN-λ1 supports *S aureus* clearance by macrophages

Differentiated macrophages (M0s) were generated from the monocyte-like cell line THP1 stimulated with 5 ng/mL phorbol

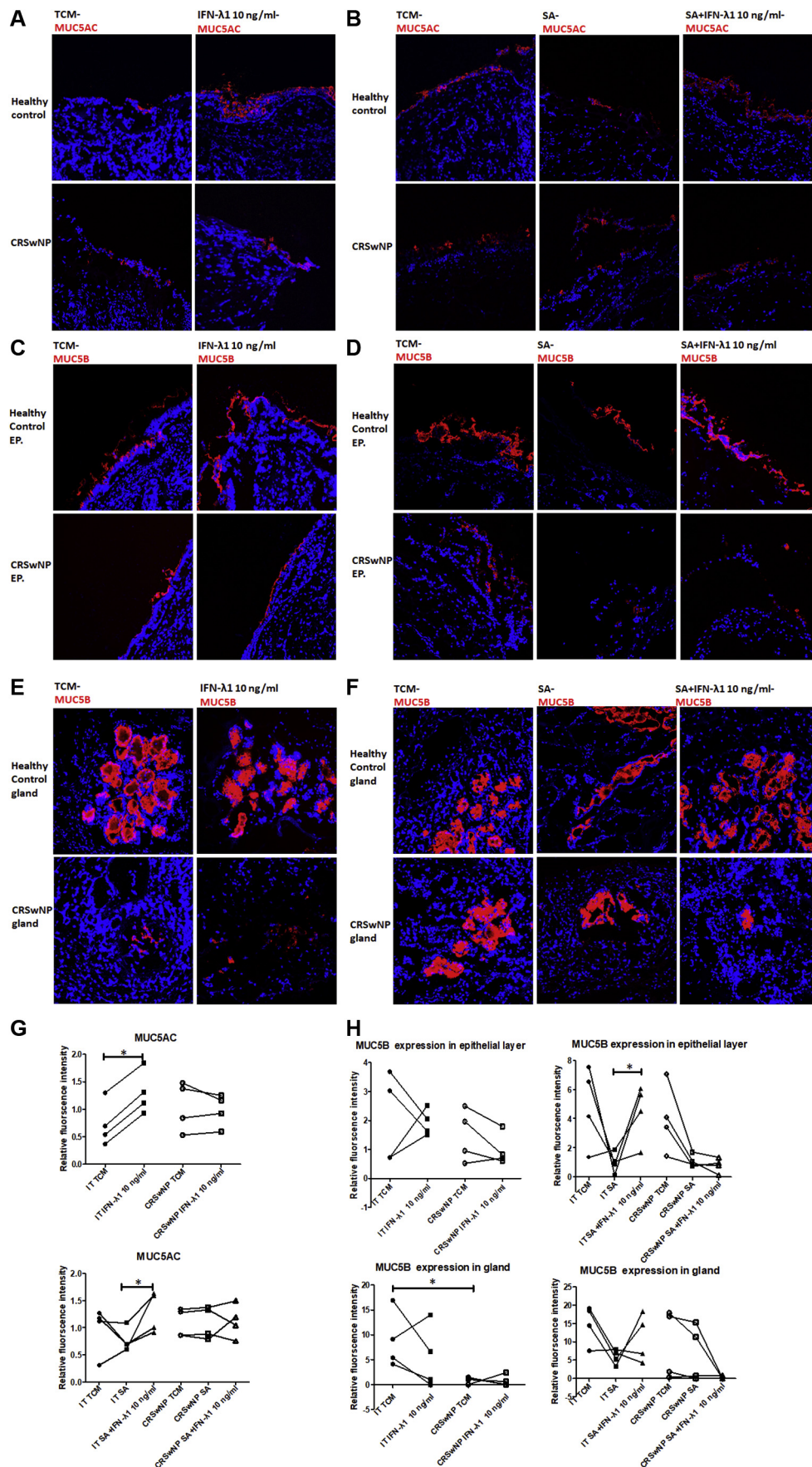


FIG 2. IFN-λ1 increased MUC5AC and MUC5B expression in healthy control tissue in the epithelial layer with or without *S aureus* exposure. **A-F**, Representative immunofluorescence confocal images of tissues from noninfection and *S aureus* infection models. **G** and **H**, Relative fluorescence intensity of MUC5AC and MUC5B expressed in the epithelial layer or submucosal gland were quantified by using ImageJ software. CRSwNP (n = 4); EP, epithelial layer; IT, inferior turbinate (n = 4). * $P < .05$, ** $P < .01$, and *** $P < .001$.

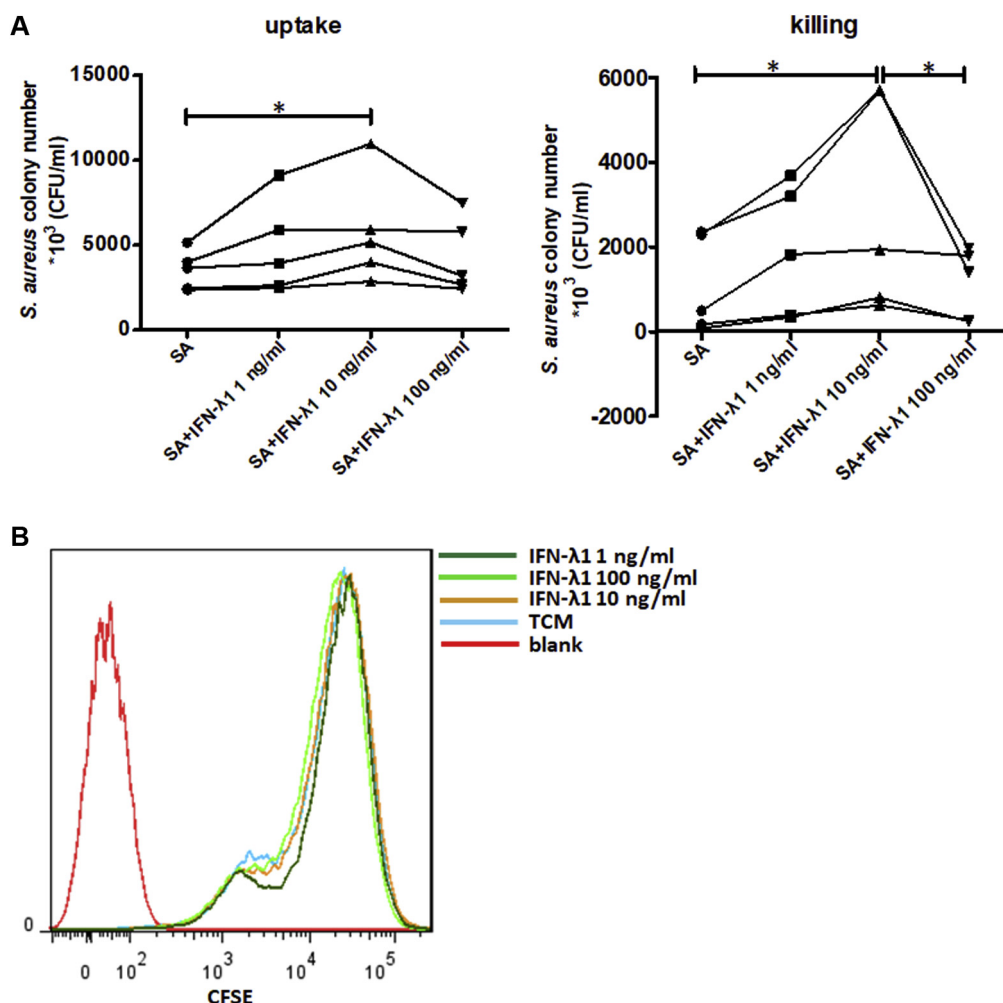


FIG 3. Uptake and killing of *S aureus* were increased in differentiated macrophages incubated with IFN- λ 1. **A**, *S aureus* (SA) CFUs counted on mannitol salt agar plates with lysates of macrophages incubated in the presence of *S aureus* with or without IFN- λ 1 (0–100 ng/mL; $n = 5$). In the same condition the number of intracellular *S aureus* CFUs minus the number of CFUs left in cells after a 6-hour incubation time was used to calculate *S aureus* killing. **B**, Representative image of THP1 cells differentiated to macrophages labeled with the Cell Trace CFSE kit and stimulated with IFN- λ 1 measured by using flow cytometry ($n = 3$). * $P < .05$.

12-myristate 13-acetate (PMA) for 3 days to further clarify the antibacterial effects of IFN- λ 1 on macrophage function during *S aureus* infection. Macrophage uptake and killing of *S aureus* was investigated at concentrations of 0 to 100 ng/mL IFN- λ 1. Preincubation of macrophages for 24 hours with IFN- λ 1 (10 ng/mL) before exposure was found to be optimal and significantly increased uptake of *S aureus* in macrophages by 48.8% ($P < .05$) and killing of *S aureus* in macrophages by 41.83% ($P < .05$) compared with no IFN- λ 1 treatment (Fig 3, A).

Macrophages were labeled with the Cell Trace CFSE kit to exclude the possibility that the observed effects of IFN- λ 1 were not due to an increase in macrophage proliferation. Assessment of labeled live cells demonstrated that macrophage proliferation was not altered by prestimulation with IFN- λ 1 (Fig 3, B), indicating that IFN- λ 1 increases macrophage phagocytosis and killing activity. Moreover, preliminary data also showed that IFN- λ 1 enhanced the microbicidal activity of human monocyte-derived macrophages (MDMs) by enhancing *S aureus* uptake and killing

(see Fig E2 in this article's Online Repository at www.jacionline.org).

Levels of 2 related macrophage markers were measured to understand the underlying mechanisms by which IFN- λ 1 mediates macrophage activation: LAMP1, a lysosomal membrane protein responsible for the integrity of lysosomes and associated with maturation of differentiated macrophages, and ROS oxidase activation, which contributes to the microbicidal function of the phagosome in macrophages. Differentiated macrophages were initially incubated with IFN- λ 1 at concentrations of 1, 10, and 100 ng/mL, of which 10 ng/mL IFN- λ 1 was found to be the optimal concentration based on ROS and LAMP1 expression in macrophages (see Fig E3 in this article's Online Repository at www.jacionline.org) and subsequently used in further studies involving *S aureus*. In this regard macrophages exposed to 10 ng/mL IFN- λ 1 demonstrated a significant increase in relative fluorescence intensity for

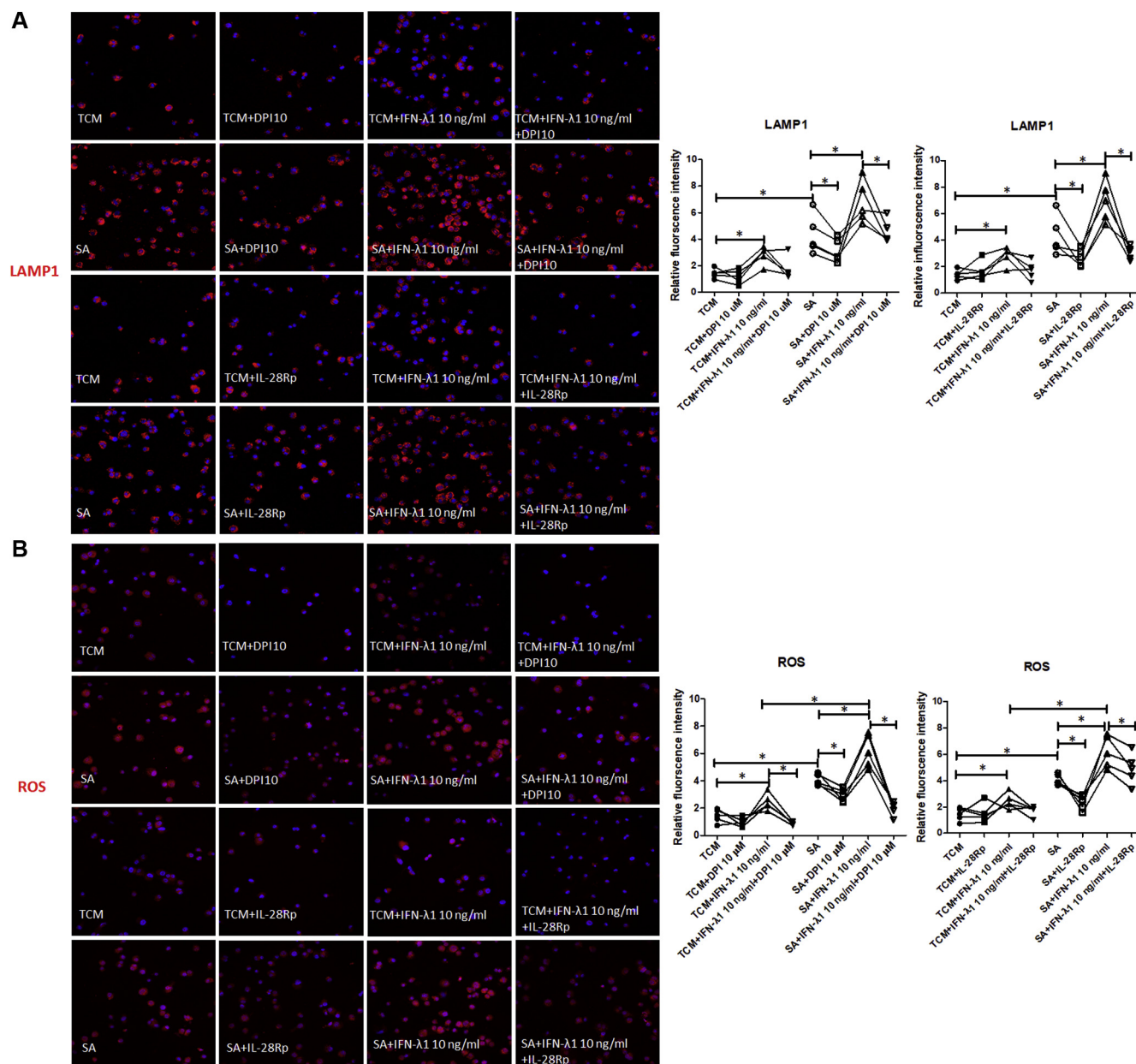


FIG 4. Blockage of IL-28R and DPI decreased ROS and LAMP1 expression. **A** and **B**, Representative images of LAMP1 and ROS staining, respectively, in macrophages incubated under different stimulatory conditions with or without IL-28R blocking (by using IL-28R peptide antibody) and ROS expression inhibition (by using NADPH oxidase inhibitor DPI). Relative immunofluorescence intensity of staining was quantified by using ImageJ software (n = 5). * $P < .05$, ** $P < .01$, and *** $P < .001$.

LAMP1 and ROS protein expression compared with the TCM control, regardless of *S aureus* infection (Fig 4, A). *S aureus* infection alone also led to a significant upregulation of ROS and LAMP1 expression (Fig 4, A and B). In the JAK-STAT pathway the degree of phosphorylation of STAT1 was significantly increased in macrophages in the presence of IFN-λ1 during *S aureus* infection (Fig 5, A).

Because IFN-λ1 exerts its effect through binding to its receptor, IL-28R, we investigated the presence of IL-28R on macrophages using Western blot analysis. Our data demonstrated that IL-28R was expressed on macrophages (Fig 5, B) and that incubation

of the macrophages with IL-28R inhibitor, IL-28R peptide antibody, and the NADPH oxidase and ROS inhibitor DPI blocked IFN-λ1-induced effects in macrophages. In particular, blocking IL-28R led to a reduction in both LAMP1 and ROS expression in IFN-λ1 plus *S aureus*-treated macrophages (Fig 4, A and B). DPI also downregulated expression of LAMP1 (Fig 4, A), suggesting that IL-28R–ROS–LAMP1 can be associated with THP1-mediated macrophage cell differentiation and maturation. Blocking IL-28R and inhibition of ROS expression contributed to a significant 3-fold decrease in phosphorylation of STAT1 in macrophages during *S aureus* infection in the presence of IFN-λ1 (Fig 5, A). Similarly, blocking

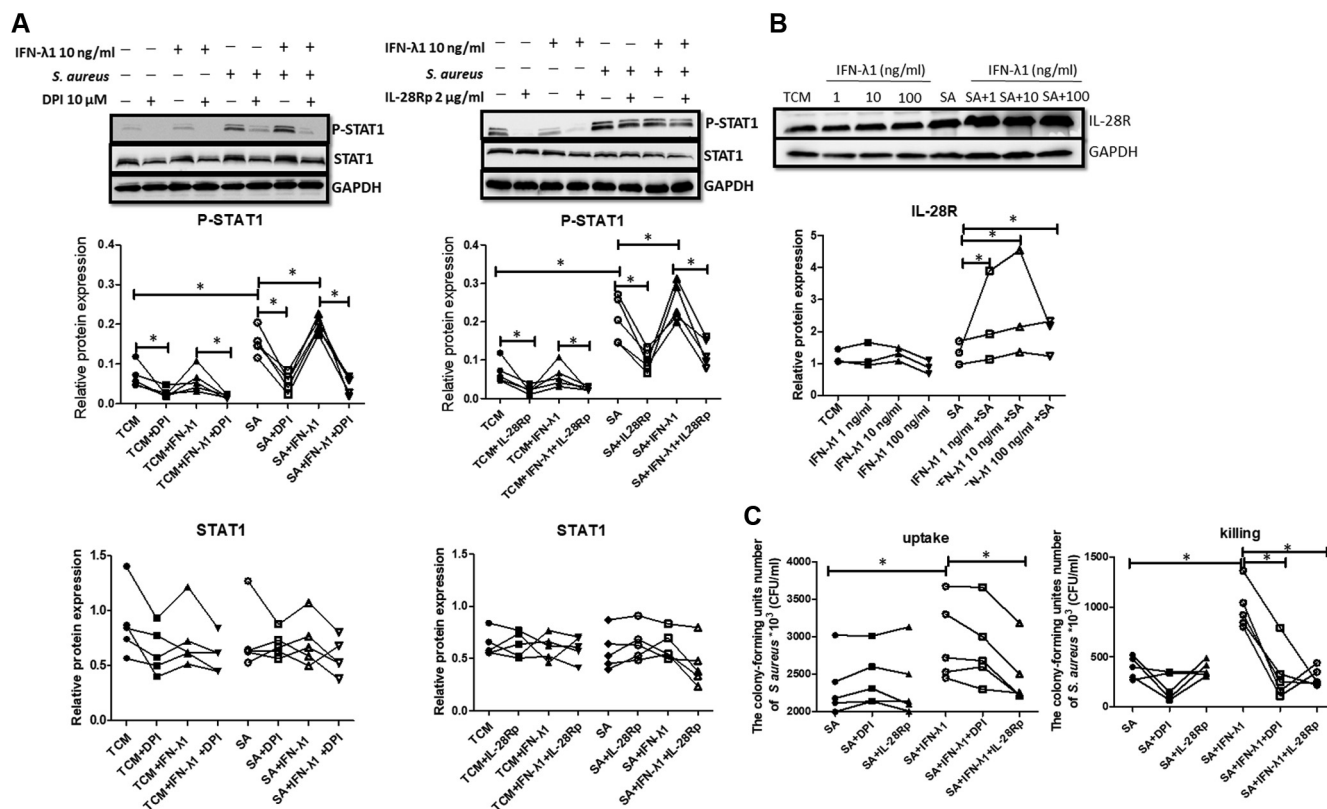


FIG 5. IFN-λ1 enhanced IL-28R expression on macrophages, and blockage of IL-28R and inhibition of ROS reduced the level of phosphorylated STAT1, resulting in attenuation of IFN-λ1-induced uptake and killing of *S aureus* (SA) by differentiated macrophages. **A**, Relative protein level of STAT1 and phosphorylated STAT1 in macrophages with IL-28R blockage and ROS inhibition; the concentration of IFN-λ1 used in the experiment was 10 ng/mL, 10 μmol/L DPI was used as an ROS inhibitor, and 2 μg/mL IL-28R peptide was used to block IL-28R (n = 5). **B**, Expression of IL-28R in macrophages with or without *S aureus* infection assessed by using Western blotting; expression of IL-28R was normalized to the housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH; n = 3). **C**, IL-28R blockage and ROS inhibition decreased the uptake and killing of *S aureus* in THP1 cell-differentiated macrophages. *S aureus* colonies were counted in macrophage lysates plated on mannitol salt agar plates (n = 5). In the same condition the number of intracellular *S aureus* CFUs minus the number of CFUs left in cells after 6 hours of incubation time was used to calculate *S aureus* killing. IL-28Rp, IL-28R peptide. *P < .05, **P < .01, and ***P < .001.

IL-28R and inhibition of ROS expression significantly decreased IFN-λ1-induced bacterial uptake and bacterial killing by macrophages (Fig 5, C).

DISCUSSION

IFN-λ plays a major role in providing the frontline defense against viruses.¹⁷ In this study we aimed to understand whether IFN-λ played a similar role against bacteria in the nasal mucosa. For the first time, we demonstrated that IFN-λ1 can be induced in nasal tissue through exposure to *S aureus* and demonstrated antibacterial activity in tissue from healthy control subjects but not tissue from patients with CRSwNP. In particular, IFN-λ1 enhanced MUC5AC and MUC5B secretion from healthy nasal mucosa on *S aureus* stimulation, keeping in accordance with the general role of mucus in bacterial clearance. Moreover, IFN-λ1 promoted the differentiation of THP1 cells into macrophages and enhanced their microbicidal activity by enhancing *S aureus* uptake and killing. Mechanistic studies underlying these IFN-λ1 functions further indicated that IFN-λ increased expression of LAMP1 and ROS proteins in the

macrophages, irrespective of the presence of *S aureus* but, when present together with *S aureus*, increased phosphorylation of STAT1 in the JAK-STAT1 pathway, the classical pathway through which IFN-λ exerts its antiviral function. Furthermore, blocking IL-28R and inhibiting ROS expression significantly decreased phosphorylation of STAT1, as well as IFN-λ1-induced bacterial uptake and bacterial killing by macrophages. Taken together, these findings led us to hypothesize that in this model IFN-λ1 deploys its antibacterial function by (1) enhancing macrophage differentiation through the IL-28R-ROS-LAMP1 signaling pathway and (2) enhancing macrophage bacterial killing through the IL-28R-ROS-JAK-STAT1 signaling pathway.

Airway mucus is a heterogeneous mixture of water, mucins, proteins, salts, and lipids. The mucus is assembled into a protective gel-like barrier to lubricate and hydrate the epithelial layer, as well as to clear the dust and exogenous luminal insults.¹⁸ However, an increase in levels of MUC5AC and MUC5B, the principal gel-forming mucins in mucus, has been observed in tissue from patients with CRSwNP,¹⁹ suggesting that accumulation of sticky and thick mucus can create a favorable

growth environment for pathogens in patients with CRSwNP. In the event of failure to clear the mucus, this might predispose to chronic bacterial infection,²⁰ and a complete loss of secreted mucins, especially MUC5B, has been shown to worsen lung inflammation.²¹ In the *ex vivo* infection model investigated in the present study, MUC5AC and MUC5B were generally not seen in tissue from patients with CRSwNP in contrast to control tissues. Here we focused on the bacterial clearance function of mucus in the infection model and demonstrated that IFN- λ 1 stimulation with or without *S aureus* infection induced MUC5AC and MUC5B in the epithelial layer in control tissue but not in tissue from patients with CRSwNP. This finding suggests that in healthy nasal tissue IFN- λ 1 can exert antibacterial function through upregulation of mucin synthesis and secretion, whereas in nasal tissue from patients with CRSwNP, IFN- λ 1 is unlikely to have a similar level of protective function, possibly because of defective mucin induction.

The effect of IFN- λ 1 was also investigated on expression of important tight junction proteins because tight junctions have been shown to be pivotal in the maintenance of epithelial barrier integrity and consequent contribution to antibacterial defense.²² A previous study also demonstrated that *S aureus* enhances the tight junction barrier integrity in healthy nasal tissue but not in nasal polyps, demonstrating that healthy and diseased tissue respond differently to *S aureus* exposure.²³ In the present study no obvious change in the expression of tight junction proteins was observed in nasal tissue in the presence of IFN- λ 1 during infection with *S aureus*, indicating that modulation of the tight junction function and epithelial integrity is unlikely to be a prominent mechanism involved in IFN- λ 1 antibacterial function, although this needs to be confirmed in larger specifically designed studies in the future.

IFN- λ activity has been shown to be highly prominent in epithelial cells in comparison with other cell types.⁹ It has also been reported that IFN- λ 1-pretreated macrophages are more responsive to IFN- γ because IFN- λ 1 enhances IFN- γ -induced IL-12p40 and TNF production during viral infection.²⁴ In line with these findings, here we demonstrate that THP1 cell-differentiated macrophages express IL-28R and are responsive to IFN- λ 1 stimulation. In the present study we focused on the influence of IFN- λ 1 on macrophage function during bacterial infection. Classically, 2 types of macrophages can be polarized from the monocyte (M0): an activated proinflammatory macrophage (M1) phenotype or an M2 macrophage phenotype, acting as a bridge between innate and adaptive immunity. M1s are activated by IFN- γ and/or LPS or TNF- α and participate in induction of a T_H1 response and prevention of pathogen persistence, whereas M2s are differentiated from M0s in the presence of IL-4, IL-10, and IL-13 and associated with the persistence of allergic disease and asthma.²⁵ We have previously demonstrated that patients with CRSwNP had increased nasal carriage of *S aureus*⁴ and a reduced capacity of macrophages to phagocytose *S aureus in vitro* compared with macrophages derived from control subjects. Moreover, patients with CRSwNP had greater numbers of M2s than control subjects, which were positively correlated with increased levels of IL-5, eosinophil cationic protein, and locally produced IgE.

The current study clearly showed that IFN- λ 1 enhanced *S aureus* killing in healthy control tissue but not tissue from patients with CRSwNP. Collectively, our findings suggested that IFN- λ 1 can affect macrophages with normal phagocytotic

function to alleviate persistent airway inflammation. To test this hypothesis, we generated macrophages from monocyte-like cell line THP1 cells and demonstrated that IFN- λ 1 indeed enhanced macrophage differentiation from the monocyte-like cell line THP1 through induction of LAMP-1, a late phagosome maturation marker, indicating delayed formation of a fully bioactive lysosome.^{26,27} Because production of ROS by the NADPH oxidase complex is a critical component of the macrophage bactericidal machinery,²⁸ we further confirmed that IFN- λ 1 mediated its antibacterial function through the IL-28R-ROS-JAK-STAT signaling pathway in the macrophage. This was supported by demonstration of (1) induction of ROS in IFN- λ 1-pretreated macrophages and (2) significantly decreased STAT1 phosphorylation and bacterial killing but not bacterial uptake through inhibition of ROS expression and blocking of IL-28R activity.

This study is somewhat limited in that the THP1-differentiated macrophages were used instead of MDMs, and the role of IFN- λ 1 was investigated on the total macrophage pool, not taking into account the potential effects of polarized M1 and M2s on outcomes. Despite this limitation, however, THP1-differentiated M1 or M2 phenotypes express markers similarly to polarized macrophages obtained from freshly isolated monocytes and provide a suitable alternative to primary monocytes. Thus whether IFN- λ 1 affects the function of M1 or M2 cells from patients with CRSwNP will be the subject of future investigations. Similarly, further investigations addressing the potential mechanisms of the deficit of macrophage activity in response to IFN- λ 1 in tissue from patients with CRSwNP are also warranted because this might lead to a better understanding of the way to reverse the deficit of macrophage function to alleviate inflammation in patients with CRSwNP.

In conclusion, for the first time, we demonstrated IFN- λ 1 favors clearance of *S aureus* in healthy nasal mucosa, but not in tissue from patients with type 2 inflammatory CRSwNP, by affecting mucin secretion and enhancing the antibacterial function of macrophages through the IL-28R-ROS-JAK-STAT1 pathway.

Key messages

- IFN- λ 1 favors clearance of *S aureus* in nasal mucosa of healthy subjects but not in nasal tissue of patients with CRSwNP.
- IFN- λ 1 enhances the antibacterial capacity of macrophages.
- IFN- λ 1 upregulates MUC5AC and MUC5B expression.

REFERENCES

1. Bachert C, Zhang N, Holtappels G, De Lobel L, van Cauwenberge P, Liu S, et al. Presence of IL-5 protein and IgE antibodies to staphylococcal enterotoxins in nasal polyps is associated with comorbid asthma. *J Allergy Clin Immunol* 2010;126:962-8, e1-e6.
2. Wang X, Zhang N, Bo Ms M, Holtappels G, Zheng M, Lou H, et al. Diversity of TH cytokine profiles in patients with chronic rhinosinusitis: a multicenter study in Europe, Asia, and Oceania. *J Allergy Clin Immunol* 2016;138:1344-53.
3. Zhang N, Van Zele T, Perez-Novo C, Van Bruaene N, Holtappels G, DeRuyck N, et al. Different types of T-effector cells orchestrate mucosal inflammation in chronic sinus disease. *J Allergy Clin Immunol* 2008;122:961-8.

4. Van Zele T, Gevaert P, Watelet JB, Claeys G, Holtappels G, Claeys C, et al. *Staphylococcus aureus* colonization and IgE antibody formation to enterotoxins is increased in nasal polyposis. *J Allergy Clin Immunol* 2004; 114:981-3.
5. Lan F, Zhang N, Holtappels G, De Ruyck N, Krysko O, Van Crombruggen K, et al. *Staphylococcus aureus* induces a mucosal type 2 immune response via epithelial cell-derived cytokines. *Am J Respir Crit Care Med* 2018;198: 452-63.
6. Krysko O, Holtappels G, Zhang N, Kubica M, Deswarte K, Derycke L, et al. Alternatively activated macrophages and impaired phagocytosis of *S. aureus* in chronic rhinosinusitis. *Allergy* 2011;66:396-403.
7. Wang X, Zhang N, Glorieux S, Holtappels G, Vaneechoutte M, Krysko O, et al. Herpes simplex virus type 1 infection facilitates invasion of *Staphylococcus aureus* into the nasal mucosa and nasal polyp tissue. *PLoS One* 2012;7:e39875.
8. Lan F, Zhang N, Gevaert E, Zhang L, Bachert C. Viruses and bacteria in Th2-biased allergic airway disease. *Allergy* 2016;71:1381-92.
9. Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat Immunol* 2003;4:63-8.
10. Lazear HM, Nice TJ, Diamond MS. Interferon-lambda: immune functions at barrier surfaces and beyond. *Immunity* 2015;43:15-28.
11. Satorres SE, Alcaraz LE, Cargnelutti E, Di Genaro MS. IFN-gamma plays a detrimental role in murine defense against nasal colonization of *Staphylococcus aureus*. *Immunol Lett* 2009;123:185-8.
12. Planet PJ, Parker D, Cohen TS, Smith H, Leon JD, Ryan C, et al. Lambda interferon restructures the nasal microbiome and increases susceptibility to *Staphylococcus aureus* superinfection. *MBio* 2016;7:e01939-15.
13. Cohen TS, Prince AS. Bacterial pathogens activate a common inflammatory pathway through IFNlambda regulation of PDCD4. *PLoS Pathog* 2013;9: e1003682.
14. Lebreton A, Lakisic G, Job V, Fritsch L, Tham TN, Camejo A, et al. A bacterial protein targets the BAH1D1 chromatin complex to stimulate type III interferon response. *Science* 2011;331:1319-21.
15. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. *Rhinology* 2012;50:1-12.
16. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol* 2007;8:R19.
17. Edwards MR, Johnston SL. Interferon-lambda as a new approach for treatment of allergic asthma? *EMBO Mol Med* 2011;3:306-8.
18. Kim JJ, Khan WI. Goblet cells and mucins: role in innate defense in enteric infections. *Pathogens* 2013;2:55-70.
19. Zhang Y, Wang X, Wang H, Jiao J, Li Y, Fan E, et al. TMEM16A-mediated mucin secretion in IL-13-induced nasal epithelial cells from chronic rhinosinusitis patients. *Allergy Asthma Immunol Res* 2015;7:367-75.
20. Randell SH, Boucher RC. University of North Carolina Virtual Lung G. Effective mucus clearance is essential for respiratory health. *Am J Respir Cell Mol Biol* 2006;35:20-8.
21. Livraghi-Butrico A, Grubb BR, Wilkinson KJ, Volmer AS, Burns KA, Evans CM, et al. Contribution of mucus concentration and secreted mucins Muc5ac and Muc5b to the pathogenesis of muco-obstructive lung disease. *Mucosal Immunol* 2017;10:395-407.
22. France MM, Turner JR. The mucosal barrier at a glance. *J Cell Sci* 2017;130: 307-14.
23. Altunbulakli C, Costa R, Lan F, Zhang N, Akdis M, Bachert C, et al. *Staphylococcus aureus* enhances the tight junction barrier integrity in healthy nasal tissue, but not in nasal polyps. *J Allergy Clin Immunol* 2018;142:665-8.e8.
24. Egli A, Santer DM, O'Shea D, Tyrrell DL, Houghton M. The impact of the interferon-lambda family on the innate and adaptive immune response to viral infections. *Emerg Microbes Infect* 2014;3:e51.
25. Genin M, Clement F, Fattaccioli A, Raes M, Michiels C. M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide. *BMC Cancer* 2015;15:577.
26. Eskelinen EL, Tanaka Y, Saftig P. At the acidic edge: emerging functions for lysosomal membrane proteins. *Trends Cell Biol* 2003;13:137-45.
27. Okai B, Lyall N, Gow NA, Bain JM, Erwig LP. Rab14 regulates maturation of macrophage phagosomes containing the fungal pathogen *Candida albicans* and outcome of the host-pathogen interaction. *Infect Immun* 2015;83:1523-35.
28. Bylund J, Brown KL, Movitz C, Dahlgren C, Karlsson A. Intracellular generation of superoxide by the phagocyte NADPH oxidase: how, where, and what for? *Free Radic Biol Med* 2010;49:1834-45.

METHODS

THP1 cell–differentiated macrophage- and MDM model

For the purpose of this study, THP1-differentiated macrophages were used because primary human macrophages are difficult to isolate in sufficient amounts from the tissue, do not proliferate in culture, and often exhibit significant phenotypic heterogeneity. In contrast, THP1 cells differentiate in the presence of PMA and can be polarized into M1s or M2s that express markers similarly to polarized macrophages obtained from freshly isolated monocytes. Furthermore, we have previously demonstrated that these are also the phenotypes found in nasal tissue,^{E1} and thus THP1-differentiated macrophages provide a suitable model to investigate mechanisms of relevant nasal macrophage phenotypes *in vitro*.

THP1 cells from the VIB Research Center at Ghent University were cultured in RPMI 1640 supplemented with 10% FBS. THP1 cells were added to a 6-well plate at 2×10^6 cells/well and differentiated into macrophages by means of incubation in the presence of 5 ng/mL PMA (Sigma-Aldrich) for 3 days.^{E2} IFN- λ 1 expression in THP1-differentiated macrophages was assessed by measuring mRNA levels of IFN- λ 1 with or without *S aureus* infection (Fig E1), and conversely, the effect of *S aureus* infection (infection model) in the absence or presence of 0 to 100 ng/mL IFN- λ 1 for 24 hours was assessed as follows: group 1, *S aureus*; group 2, *S aureus* plus IFN- λ 1, 1 ng/mL; group 3, *S aureus* plus IFN- λ 1, 10 ng/mL; and group 4, *S aureus* plus IFN- λ 1, 100 ng/mL. Similarly, macrophages not infected with *S aureus* (noninfection model) were incubated in the absence or presence of 1, 10, or 100 ng/mL IFN- λ 1, and cells with culture medium were used as controls for comparison. The effect of inhibiting IFN- λ 1-mediated uptake and killing of *S aureus* by macrophages was investigated by preincubating macrophages for 1 hour with 10 μ M of the NADPH oxidase inhibitor DPI (Sigma) or IL-28R blocking peptide (2 μ g/mL; Santa Cruz Biotechnology) in RPMI 1640 plus 10% FBS before incubation with IFN- λ 1, and then the differentiated macrophages were infected by *S aureus* in the presence of IFN- λ 1.

Macrophages were infected with *S aureus* at a multiplicity of infection of 1:5 for 15 minutes. The multiplicity of infection value was determined by simply dividing the number of macrophages added (milliliters added $\times$ cells per milliliter) by the number of bacteria added (milliliters added $\times$ CFUs per milliliter). The 15-minute incubation time and 10 ng/mL IFN- λ 1 were found to be optimal incubation conditions for further investigations of cell death and *S aureus* uptake and killing by macrophages, as assessed based on the number of *S aureus* CFUs, which were retrieved from treated macrophages after phagocytosis.^{E3}

To assess *S aureus* uptake or killing by macrophages, differentiated macrophages that had been preincubated with 1 to 100 ng/mL IFN- λ 1 for 24 hours and were divided into two equal samples, and each sample was infected with *S aureus* at a multiplicity of infection of 1:5 for 15 minutes at 37°C. At the end of this incubation, one of the infected macrophage samples was washed with PBS 3 times and lysed with 1 mL of sterile water (ice cold) on ice for 30 minutes for assessment of *S aureus* uptake. The second sample was further incubated in infection medium for a further 6 hours, and

macrophages were lysed for assessment of *S aureus* killing. Before lysis, to exclude any remaining extracellular *S aureus* on the surfaces of macrophages, 50 μ g/mL lysostaphin (Sigma) was added to macrophage samples for 30 minutes, and the supernatants of cells were collected for assessment of cell CFUs of *S aureus* by culturing on mannitol salt agar plates for 24 h at 37°C. Macrophage cell lysates were diluted 1:1000 with sterile PBS, and then 50- μ L samples were also cultured in duplicate on mannitol salt agar plates for 24 hours at 37°C for assessment of cell CFUs of *S aureus*. The number of CFUs of *S aureus* were counted and expressed as CFU/mL. Each experiment was repeated at least 4 times.

Our observation on the role of IFN- λ 1 in THP1 cell–derived macrophages was preliminarily confirmed on MDMs. To obtain MDM0 cells, peripheral PBMCs were isolated by using LymphoPrep (Axis-Shield) from blood of healthy donors. Then PBMCs were suspended in RPMI 1640 medium with 10% FBS and a 1% mixture of penicillin and streptomycin. PBMCs were seeded at a density of 5×10^6 cells per well in a 6-well culture plate for 3 hours in a humidified incubator containing 5% CO₂ at 37°C to allow monocyte adhesion. Nonadherent cells were removed, and the adherent monocytes were further incubated in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin and 10 ng/mL GM-CSF (R&D Systems) for another 7 days to obtain MDM0 cells. MDM0 cells were pretreated with 100 ng/mL LPS and 20 ng/mL IFN- γ to differentiate to MDM1 cells. MDM0 cells with 24 hours of preincubation of IFN- λ 1 at different concentrations were used for uptake and killing assessment.

Immunofluorescence staining

For the fluorescence intensity calculation in each experiment ($n = 4$), 5 fields were randomly selected and assessed by 2 independent observers. MUC5B was quantified in 5 fields each in both the epithelial and subepithelial regions ($<100 \mu$ m distance from the epithelium). Fluorescence intensity was calculated after adjusting for background signal by using ImageJ software. For the ROS fluorescence intensity calculation, stained THP1-differentiated macrophage cells were rinsed with PBS and mounted with DAPI for observation of fluorescence in 5 randomly selected fields, as for the tissue samples.

REFERENCES

- E1. Krysko O, Holtappels G, Zhang N, Kubica M, Deswarte K, Derycke L, et al. Alternatively activated macrophages and impaired phagocytosis of *S. aureus* in chronic rhinosinusitis. *Allergy* 2011;66:396-403.
- E2. Park EK, Jung HS, Yang HI, Yoo MC, Kim C, Kim KS. Optimized THP-1 differentiation is required for the detection of responses to weak stimuli. *Inflamm Res* 2007;56:45-50.
- E3. Kubica M, Guzik K, Koziel J, Zarebski M, Richter W, Gajkowska B, et al. A potential new pathway for *Staphylococcus aureus* dissemination: the silent survival of *S. aureus* phagocytosed by human monocyte-derived macrophages. *PLoS One* 2008;3:e1409.

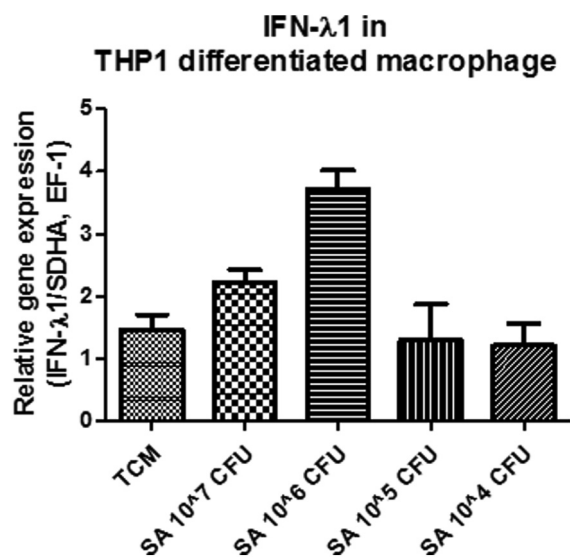


FIG E1. Relative gene expression of IFN- λ 1 in macrophages with different doses of *S aureus* exposure ($n = 3$). *SA*, *S aureus*. Expression of IFN- λ 1 was normalized by using the SDHA and EF-1 housekeeping genes.

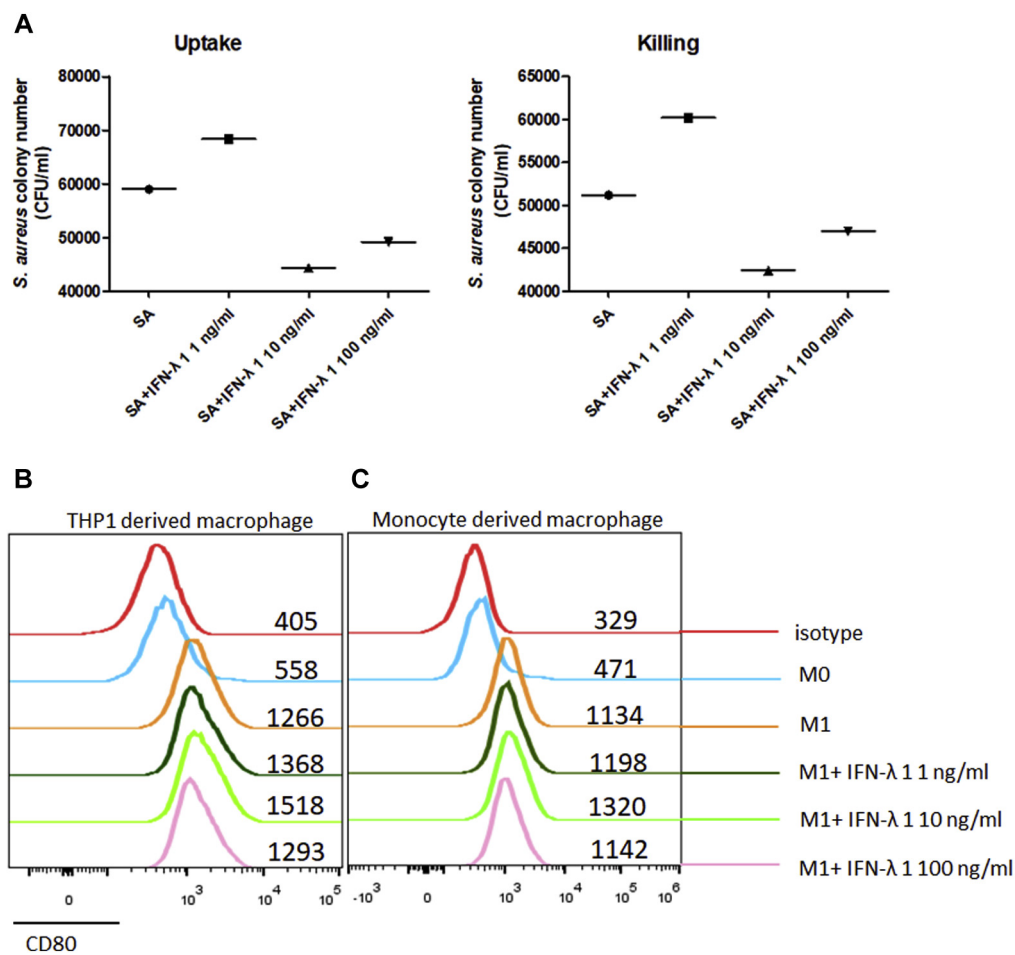


FIG E2. **A**, Uptake and killing of *S aureus* were increased in MDMs incubated with IFN-λ1 at 1 ng/mL. Uptake of *S aureus* (SA) CFUs was counted on blood plates with lysates of MDM1 cells in the presence of *S aureus* with or without 1 to 100 ng/mL IFN-λ1 (n = 1 in duplicates). In the same condition the number of intracellular *S aureus* CFUs minus the number of CFUs left in cells after 6 hours of incubation time was used to calculate *S aureus* killing. **B**, Expression of CD80 was measured on THP1 cell-derived macrophages. To obtain M1 cells, M0 cells were stimulated by 100 ng/mL LPS and 20 ng/mL IFN-γ for 24 hours. After differentiation, M1 cells were then stimulated with different concentrations of IFN-λ 1 for another 24 hours. Numbers indicate the geometric mean fluorescence intensity. **C**, Expression of CD80 was measured on monocyte-differentiated macrophages. MDM0 cells were pretreated with 100 ng/mL LPS and 20 ng/mL IFN-γ to differentiate MDM1 cells. Afterward, CD80 was measured on MDM1 cells in the presence of different concentrations of IFN-λ.

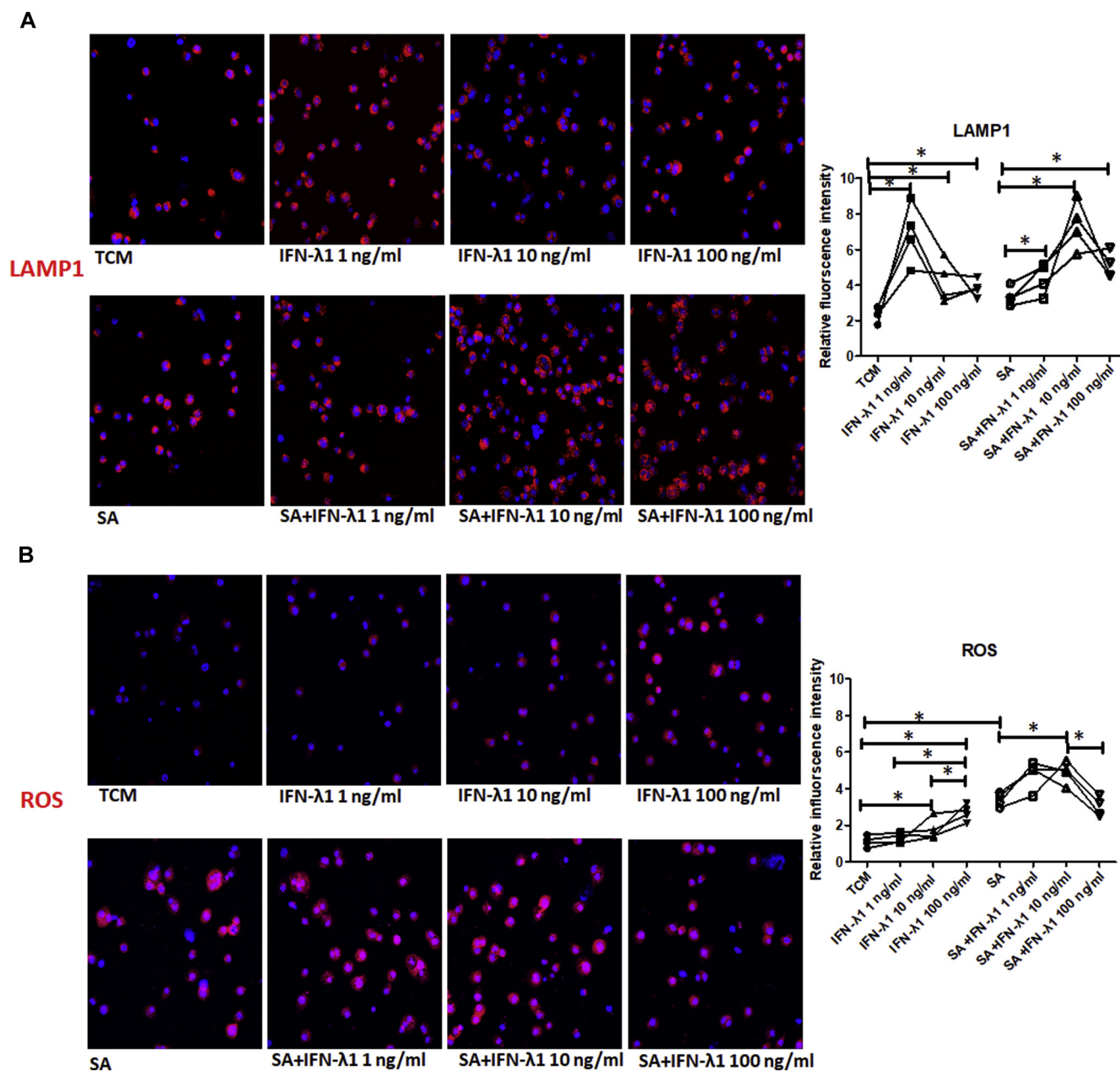


FIG E3. Expression of ROS and LAMP1 in differentiated macrophages with or without *S aureus* (SA) infection in the presence of different doses of IFN-λ1 ($n = 4$). Relative immunofluorescence intensity of staining was quantified by using ImageJ software. * $P < .05$, ** $P < .01$, and *** $P < .001$.