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Epithelial control of the human pDC response to extracellular bacteria

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Plasmacytoid pre-dendritic cells (pDCs) are specialized in responding to nucleic acids, and link innate with adaptive immunity. Although the response of pDCs to viruses is well established, whether pDCs can respond to extracellular bacteria remains controversial. Here, we demonstrate that extracellular bacteria such as *Neisseria meningitidis*, *Haemophilus influenzae*, and *Staphylococcus aureus* activate pDCs to produce IFN-α, TNF-α, IL-6, and to upregulate CD86 expression. We observed that pDCs were present within tonsillar crypts and oro-nasopharyngeal epithelium, where they may contact extracellular bacteria, in situ. Tonsil epithelium-conditioned supernatants inhibited IFN-α, TNF-α, and IL-6 triggered by the direct contact of *N. meningitidis* or *S. aureus* with pDCs. However, pDC priming of naive T cells was not affected, suggesting that tonsil epithelium microenvironment limits local inflammation while preserving adaptive immunity in response to extracellular bacteria. Our results reveal an important and novel function of pDCs in the initiation of the mucosal innate and adaptive immunity to extracellular bacteria.

Keywords: Cytokines · Extracellular bacteria · Human mucosa · Innate immunity · Plasmacytoid pre-dendritic cell

See accompanying Commentary by Rescigno

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Introduction

Mucosal surfaces are the main sites of interaction between microbes and the host. In general, microbes are considered intracellular when they can replicate or reside inside the host cells. This is the case for viruses, and a number of invasive bacteria, such as *Mycobacteria*, *Rickettsia*, *Chlamydia*, and *Shigella* [1]. Rather, extracellular microbes colonize mostly cell surfaces. *Neisseria meningitidis, Haemophlius influenzae*, and *Staphylococcus aureus* are extracellular bacteria responsible for diseases ranging from

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local inflammation to life-threatening septicemia and meningitis. The major location of these bacteria is the human mucosa [2–4]. The presence of *N. meningitidis* at the oro-nasopharynx mucosa was associated with the induction of a naturally acquired immunity able to activate T and B cells [5,6] but the underlying mechanisms by which this response is initiated remain largely unknown.

Plasmacytoid pre-dendritic cells (pDCs) play a major role in innate antimicrobial immunity [7,8]. They recognize principally nucleic acids from microbes through endosomal and cytosolic receptors [9,10]. Upon stimulation, pDCs produce large amounts of type-I interferons, IL-6, TNF-α, and upregulate surface costimulatory molecules [7,11]. Although pDCs as well as type-I interferons have an important function in antiviral immunity, their role in the response to extracellular bacterial infection remains highly controversial [9,12]. Here, in an effort to reconstruct the mucosal microenvironment present in the initial steps of antibacterial immunity, we used human primary cells and address the following questions: (i) Can pDCs respond to extracellular bacteria in a mucosal context? (ii) Which is the role of the epithelium in this response and the subsequent T-cell priming?

Results

Extracellular bacteria activate pDCs to produce IFN-α, TNF-α, and IL-6 and increase CD86 expression

We evaluated the direct effect of whole live extracellular bacteria on pDC. Freshly purified pDCs from human blood were cultured for 24 h, and supernatants were collected to quantify cytokine secretion. As expected, pDCs stimulated with CpG secreted high levels of IFN-α, TNF-α, and IL-6 secretion (Fig. 1A). When pDCs were stimulated with the Gram-negative extracellular bacteria *N. meningitidis* we observed a significant induction of all three cytokines: mean of 10,000 pg/mL of IFN-α, 700 pg/mL of TNF-α, and 6000 pg/mL of IL-6, as compared with pDCs cultured with medium (Fig. 1A). We then measured by flow cytometry the expression of the costimulatory molecule CD86 on the surface of pDCs. We observed that the MFI and the frequency of CD86 positive cells increased by twofold on *N. meningitidis*–stimulated pDCs compared to untreated cells (Fig. 1B). These results demonstrate that *N. meningitidis* directly activate human primary blood pDCs.

In order to determine the specificity of the response to *N. meningitidis,* we challenged pDCs with *Haemophilus influenzae,* another Gram-negative bacteria, and the Gram-positive bacteria *S. aureus,* which share the ability to colonize the oro-nasopharynx mucosa. Similarly to *N. meningitidis*, both bacteria induced an increase in cytokine secretion and CD86 expression on pDCs. Stimulation with Gram-positive *S. aureus* triggered a stronger pDC response compared with stimulation with Gram-negative *N. meningitidis* and *H. influenzae* (Fig. 1C). Flow cytometry analysis of DAPI-negative cells showed that differences were not due to changes in pDC viability (Supporting Information Fig. 1). Together, our results demonstrate that extracellular bacteria activate human pDCs and that the intensity of the response is dependent on the nature of the bacteria.

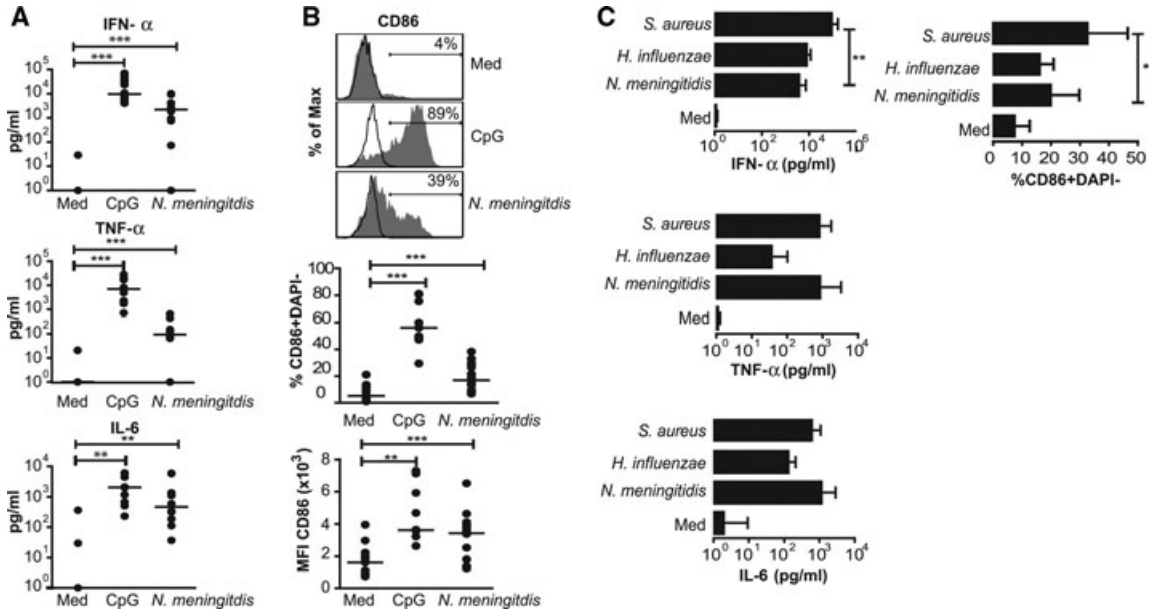

Figure 1. Extracellular bacteria activate human primary pDCs to secrete IFN-α, TNF-α, and IL-6 and increase CD86 expression. (A) Blood pDCs were stimulated with CpG, extracellular bacteria *N. meningitidis* (MOI 10) or Medium (Med) for 24 h. IFN-α, TNF-α, IL-6 were quantified in the supernatants by Flex Set CBA. Each symbol represents an individual donor and the bars represent the medians. (B) Expression of CD86 on the surface of pDCs was monitored by flow cytometry. The staining profile of CD86 and isotype-matched control are shown by shaded and open histograms, respectively (top). Inset values are the percentage of $CD86^+$ pDCs. The MFI (bottom) and the frequency (middle) from 14 independent donors were quantified; bars represent the medians. (C) The response of pDCs to *N. meningitidis* was compared with the response to other extracellular bacteria: Gram-negative *H. influenzae* and Gram-positive *S. aureus* (MOI 10). IFN-α, TNF-α, IL-6 secretion, and CD86 expression in pDCs are shown as mean ± SD of results pooled from 10 independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, two-tailed Student's *t*-test.

pDC interactions with extracellular bacteria

In order to go deeper in the understanding of the interaction of pDCs with extracellular bacteria, we analyzed whether pDCs were able to internalize the microbes. To this end, we performed inside-out experiments detailed in the *Material and methods*. We incubated freshly purified pDCs from blood with *S. aureus* labeled with biotin-streptavidin Alexa 488 staining. After 1 h, the cells were washed and stained with streptavidin-Alexa 647, which allowed us to discriminate between internalized bacteria, fluorescent only in Alexa-488, and cell-surface adhered bacteria, double positive for Alexa-488 and Alexa-647. We analyzed single cell suspensions by flow cytometry. Our results show that pDCs were able to internalize fluorescent bacteria (Fig. 2A). The quantification of two independent donors demonstrated that after 1 h, 5–8% of pDCs internalized bacteria whereas 6–12% of pDCs were associated with bacteria at their cell surface (Fig. 2B). It has to be pointed out that in these experiments the estimation of internalized bacteria may be underestimated since the pDCs that have bacteria inside and at their surface will appear in the double-positive gate. We confirmed these results using cell stream analysis (Amnis), which allowed us to quantify and visualize at the same time the cells. We confirmed that pDCs, which internalized bacteria, were fluorescent only in Alexa-488 whereas pDCs carrying surface bacteria were also fluorescent also for Alexa-647 (Fig. 2C). Finally, to obtain definitive proof that each bacterium was indeed inside a pDC, we performed fluorescent microscopy using spinning disk confocal microscopy. Bacteria are visualized in green and the cells stained to CD123 antibody in red (Fig. 2D). Furthermore, an orthogonal view is shown. We next wanted to determine whether pDC activation required that extracellular bacteria be alive. To this end, we stimulated pDCs with heat-killed bacteria, and compared cytokine secretion of these cells with that of cells activated by live bacteria. Our results show that viable bacteria were not required to activate pDCs. We even observed a slight increase in the production of cytokines and the maturation triggered by dead bacteria compared to live bacteria (Supporting Information Fig. 2).

The tonsil mucosal epithelium as a site of pDCs and extracellular bacterial interaction

Mucosal surfaces are the main place of microbial entrance into the host. pDCs have been previously described in the perifollicullar T-cell area of human mucosa, especially in tonsils [13, 14]. We evaluated whether pDCs may also be present in the mucosal epithelium by performing immunohistochemistry of frozen human tonsil sections and stained with BDCA-2, a specific marker for human pDCs. Tonsils were obtained from children with obstructive sleep apnea. As expected, we observed the presence of BDCA-2$^+$ cells around the germinal center, corresponding to the T-cell area of tonsils. Importantly, we also observed BDCA-2$^+$ cells within the stratified and the pseudo-stratified epithelium (Fig. 3A). These results showed that pDCs were present within oro-nasopharyngeal epithelium and tonsillar crypts, suggesting that they may contact extracellular bacteria in situ.

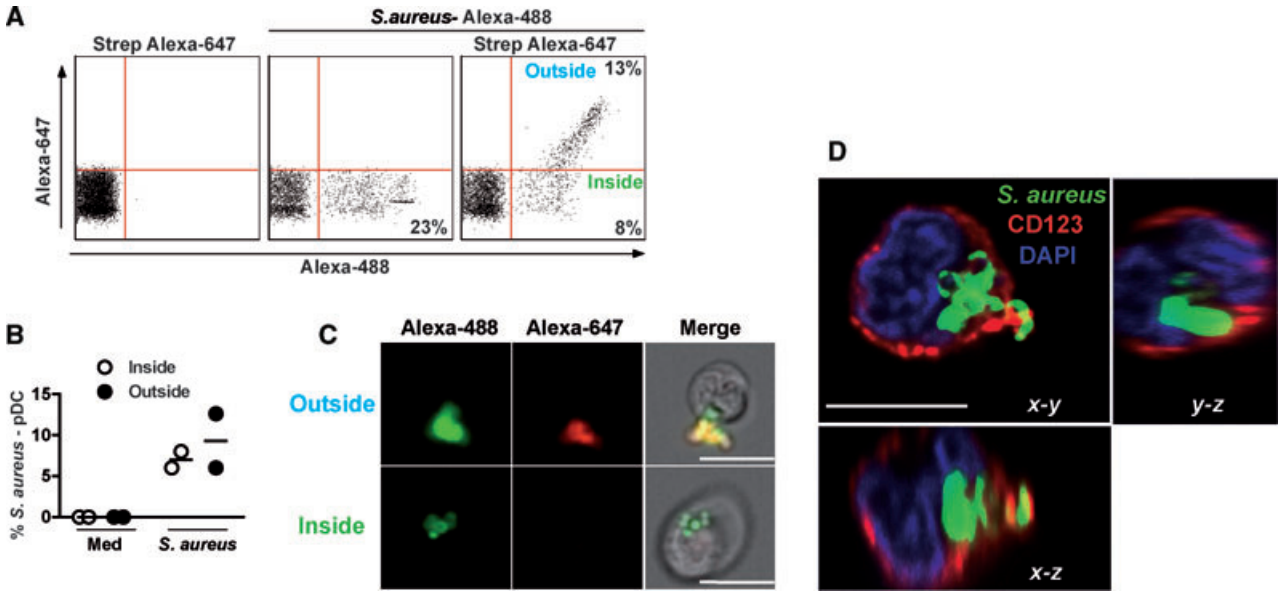

Figure 2. Human pDCs are able to internalize extracellular bacteria. Biotinylated *S. aureus* was stained with Streptavidin-Alexa-488 and added to blood pDCs. (A–C) After 1 h, surface bacteria were distinguished from internalized ones by adding Streptavidin-Alexa-647 and analyzing cells by flow cytometry and AMNIS Image Stream. (A) A representative dot plot of pDCs stimulated with medium (Med) or *S. aureus*-Alexa 488 without and with Alexa-648 is shown. The percentage of cells in the outside and inside region is depicted. (B) Quantification of inside-outside bacteria from two independent experiments is shown as the percentage of bacteria-associated pDCs. (C) A representative image of a pDC gated from the outside (top) and inside (bottom) region as defined in (A) is shown. Scale bar: 7 μm. (D) Confocal microscopy analysis of pDCs with internalized bacteria. Z-section and orthogonal view of pDCs (red), *S. aureus* (green), and the nucleus (blue). Scale bar: 5 μm. Data shown are representative of two independent experiments performed.

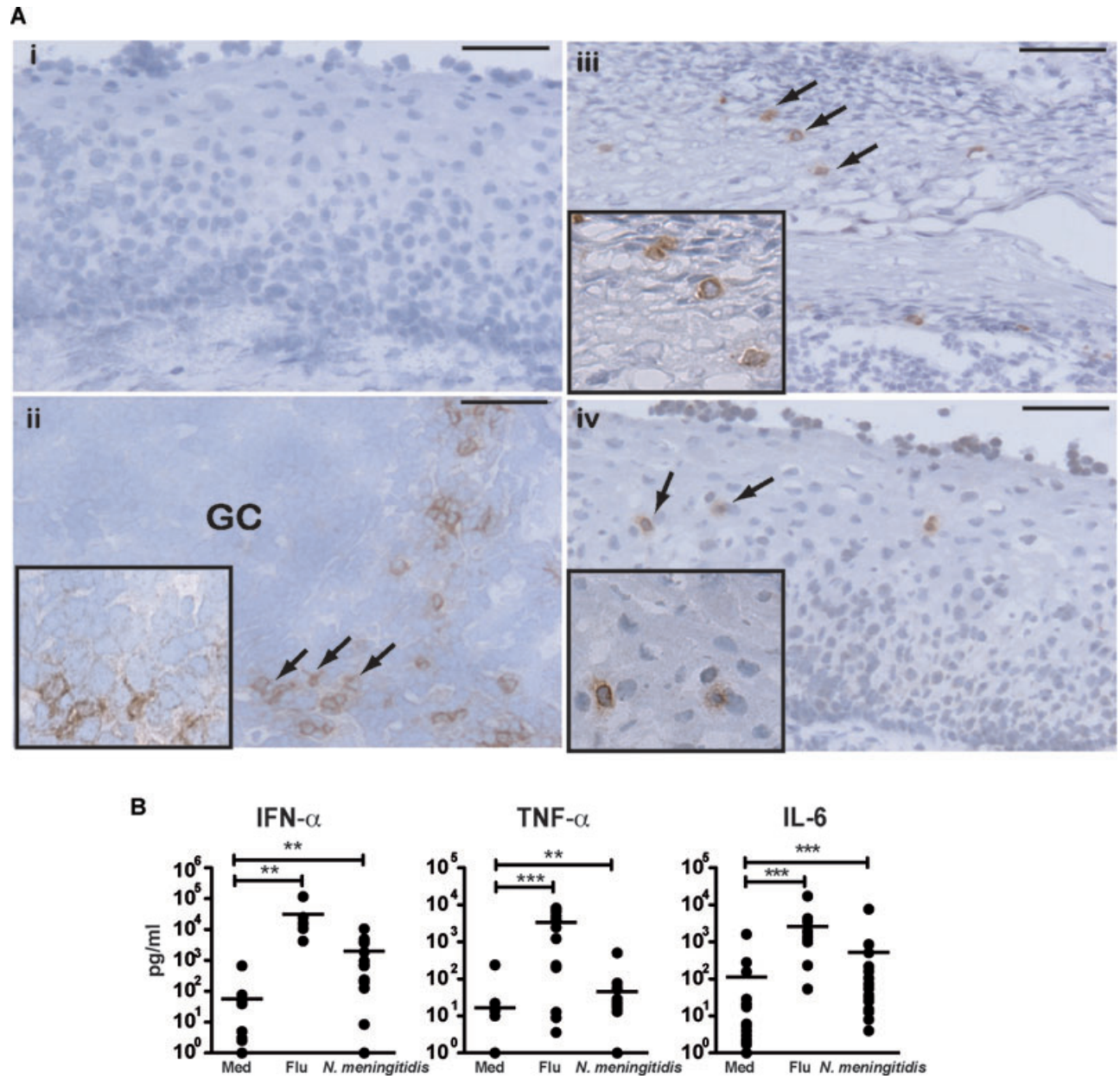

Figure 3. pDCs are localized in the tonsil epithelium and are activated by extracellular bacteria. (A) Immunohistochemistry was performed in frozen tonsil sections with (i) isotype control or (ii–iv) BDCA-2 antibody (arrows) in (ii) germinal centers (GC), (iii) stratified epithelium, and (iv) pseudostratified epithelium. Inserts are magnification of the same region. Scale bar: 50 μm. Data shown are representative of two independent experiments performed. (B) pDCs from tonsils were stimulated with Flu (10^6 PFU/mL), *N. meningitidis* (MOI 10), or Medium (Med) for 24 h. IFN-α, TNF-α, IL-6 were quantified in the supernatants by Flex Set CBA. Each symbol represents an individual donor and the bars represent the medians. Data shown are pooled from 12 independent experiments performed. $^{**}p < 0.01$, $^{***}p < 0.001$, two-tailed Student's *t*-test.

As pDCs from blood may differ from those present in tonsils, we wanted to assess whether extracellular bacteria can also activate tonsil pDCs. We measured the cytokines secreted by pDCs isolated from human tonsils put directly in contact with *N. meningitidis*. Similarly to blood pDCs we observed a significant increase of IFN-α, TNF-α, and IL-6 compared with the untreated condition (Fig. 3B). We also observed that pDCs treated with influenza virus (Flu) induced a stronger cytokine secretion compared with bacteria-treated pDCs. These results demonstrate that extracellular bacteria activate human pDCs regardless of their anatomical location.

The tonsillar epithelial microenvironment inhibits bacteria-induced cytokine production by pDCs

In order to reconstitute the physiological conditions existing in the mucosa at the time of pDC interaction with extracellular

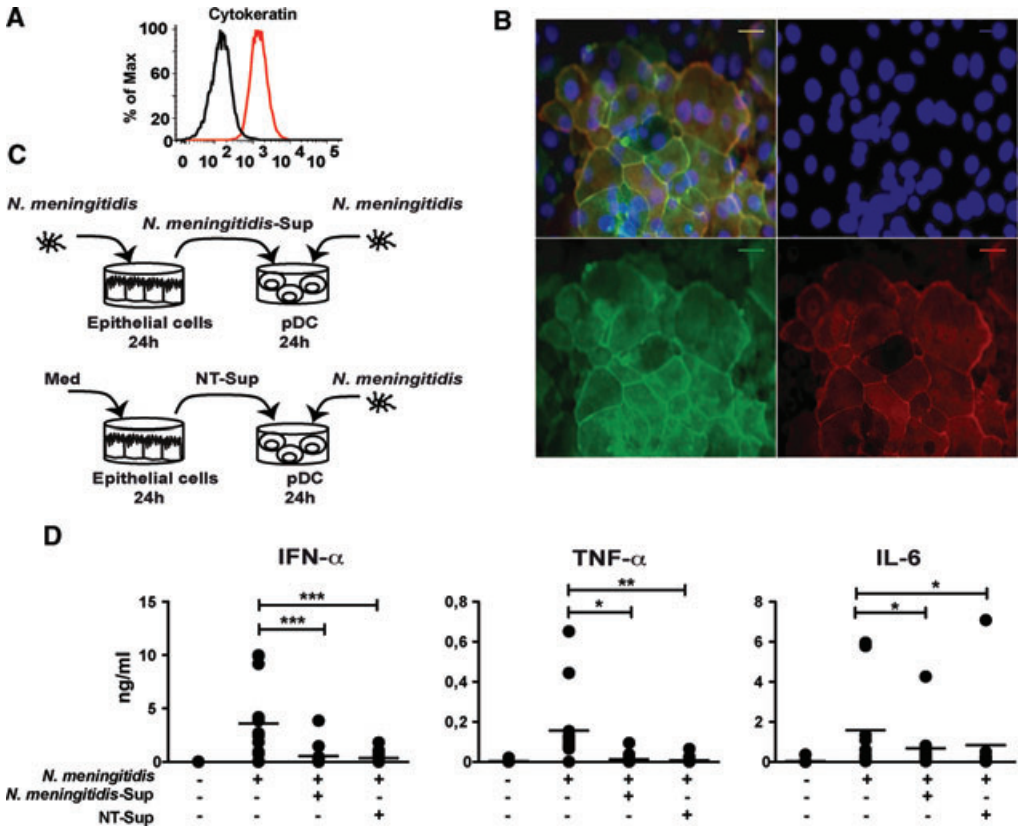

Figure 4. Soluble factors from tonsil epithelium inhibit the cytokines secreted by pDCs in response to extracellular bacteria. Epithelial cell culture was obtained after 10 days of culture from tonsil explants. (A) Cytokeratin expression was assessed by flow cytometry. Open histogram represents the isotype control, red line histogram is the specific staining for cytokeratin. (B) The expression of tight junction proteins was evaluated by immunofluorescence microscopy. Occludin (green), ZO-2 (red), the nucleus stained with DAPI (blue) and the merge are shown. Scale bar: 30 μm. (A, B) Data shown are representative of three experiments performed. (C) Strategy used to test the effect of the tonsil epithelium on the pDC response to extracellular bacteria. Epithelial cells were stimulated with *N. meningitidis*, or medium. After 24 h, *N. meningitidis*-Sup, and NT-Sup were, respectively, generated. Supernatants were added to pDCs at the same time as *N. meningitidis*. (D) The effect of *N. meningitidis*-Sup or NT-Sup on the cytokine secreted by pDCs after 24 h of stimulation with *N. meningitidis* was assessed. Each symbol represents an individual donor and bars represent the medians. Data shown are pooled from 10–14 independent experiments performed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-tailed Student's *t*-test.

bacteria, we generated a primary culture of human tonsil epithelial cells described in the *Material and methods*. The lineage and homogeneity of the primary cell culture were confirmed by flow cytometry. We observed that all the cells were positive for cytokeratin, a specific marker of epithelial cells (Fig. 4A). Furthermore, we confirmed the functionality of the cells by assessing the expression of the tight junction proteins ZO-2 and Occludin by fluorescence microscopy. Proteins were expressed at the edge of the cells confirming their ability to form functional epithelial cells (Fig. 4B).

To assess the effect of tonsil epithelia microenvironment on pDC response to extracellular bacteria, we collected supernatants from human tonsil epithelial cells stimulated with *N. meningitidis* (*N. meningitidis*-Sup) or with medium (NT-Sup). Supernatants were then added to freshly purified blood pDCs at the same time as the bacteria (Fig. 4C). We observed that *N. meningitidis*-Sup significantly inhibited the secretion of the proinflammatory cytokines produced by *N. meningitidis*-activated pDCs (Fig. 4D). IFN-α and TNF-α were reduced by sixfold, whereas IL-6 was reduced by twofold. We determined that this inhibition was not due to the interaction of the bacteria with the epithelium since NT-Sup inhibited pro-inflammatory cytokines triggered by *N. meningitidis* to the same extent as *N. meningitidis*-Sup. No significant difference was observed when the medium used to generate the epithelial cells was added to *N. meningitidis*-activated pDCs (data not shown). This demonstrated that the inhibition of the cytokines produced by pDCs was an intrinsic property of the tonsil epithelium microenvironment rather than induced by bacteria–epithelium interaction. In order to determine whether this inhibition was specific to *N. meningitidis*, we used the same strategy with *S. aureus* stimuli, which induced the strongest pDC response among the bacteria used in this study (Fig. 1C). We observed that similarly to *N. meningitidis*, *S. aureus*-Sup and NT-Sup strongly inhibited IFN-α, TNF-α, and IL-6 secreted by *S. aureus*-activated pDCs (Supporting Information Fig. 3A). Using Flu instead of bacteria, we observed that NT-Sup inhibited IFN-α production by stimulated pDCs (Supporting Information Fig. 3B). These results demonstrate that independently of the nature of stimulus, the epithelium exerts

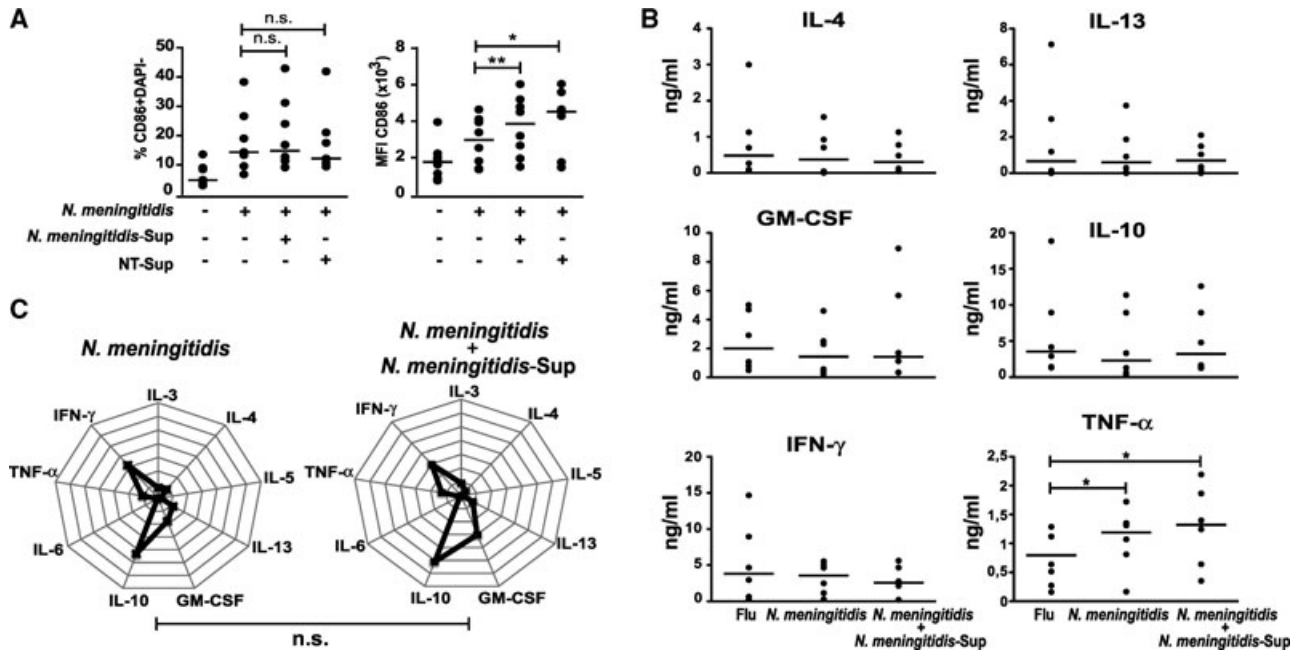

Figure 5. pDCs retain their ability to prime naive T cells in the presence of epithelial cell supernatants. (A) CD86 expression was monitored by flow cytometry on pDCs after 24 h of stimulation. Each symbol represents an individual donor and the bars represent the median. (B) pDCs stimulated for 24 h with *N. meningitidis* in the absence or presence of *N. meningitidis*-Sup were cocultured with allogeneic $CD4^+$ naive T cells. At day 6, polyclonal activation of T cells was performed and Th cytokine profile released in the supernatant was analyzed after 24 h by Flex Set CBA. (C) Spider graph shows the mean secretion for the indicated cytokines from 6 independent donors. Scale for each cytokine reaches up to 7000 pg/mL. Data shown are pooled from six independent experiments performed. * $p < 0.05$, ** $p < 0.01$. n.s. = nonsignificant differences, two-tailed Student's *t*-test.

the same inhibitory effect on the innate cytokines secreted by microbial-activated pDCs.

The tonsillar microenvironment does not affect T-cell activation and polarization by pDCs

The secretion of cytokines by pDCs is related to their innate immune functions. However, pDCs are also important in activating adaptive immunity through the priming of T cells [7, 11, 15]. We showed above that pDCs stimulated with extracellular bacteria increased the costimulatory molecule CD86. Consequently, we wondered whether the epithelial microenvironment, besides inhibiting cytokine secretion, could also impair the maturation of pDCs. We did not observe inhibition in CD86 expression when *N. meningitidis*-Sup or NT-Sup was added to *N. meningitidis*-activated pDCs. On the contrary, we observed a significant increase in the MFI of CD86 (Fig. 5A). Similar results were obtained using *S. aureus* instead of *N. meningitidis* (data not shown). These results suggested that extracellular bacteria-activated pDCs in a tonsil microenvironment, although diminished in their innate cytokine production, may retain the ability to prime naive T cells. To test this hypothesis, we cocultured allogeneic naive $CD4^+$ T cells with pDCs previously stimulated by *N. meningitidis* in the presence or absence of *N. meningitidis*-Sup. We observed that the presence of *N. meningitidis*-Sup did not affect the ability of pDCs to induce naive T helper (Th) cell proliferation (data not shown). Cytokine produced by pDCs during T-cell encounter may also modulate T-cell polarization. We determined the Th profile by measuring the cytokines secretion and related transcription factors by T cells after 6 days of coculture with pDCs, and after polyclonal restimulation. We compared the Th profile induced by *N. meningitidis*–activated pDCs, with that induced by Flu-activated pDCs. We observed that *N. meningitidis*-activated pDCs induced high levels of IL-10 and IFN-γ, and lower levels of GM-CSF, IL-13, TNF-α, and IL-4 (Fig. 5B). A significant increase in TNF-α secretion was observed in *N. meningitidis* condition compared to Flu (1072 pg/mL induced by *N. meningitidis* versus 666 pg/mL induced by Flu). We also observed that IFN-γ tended to be higher with Flu stimulation than with *N. meningitidis* stimulation although differences were not statistically significant (Fig. 5B). When we analyzed the effect of the *N. meningitidis*-Sup on Th cytokine profile induced by *N. meningitidis*–activated pDCs, we did not observe any significant differences (Fig. 5C). It was recently described that human monocytes can direct a Th17 polarization in response to microbial stimuli, such as *Candida albicans* and *S. aureus* [16]. However; in our coculture experiments we did not detect production of IL-17 in any of the conditions tested (data not shown). These results established that the Th cytokine profile induced by *N. meningitidis* through pDCs was independent of the epithelia microenvironment. We also analyzed the expression of GATA-3, T-bet, RORC, and Foxp3 transcription factors in the cocultured T cells, which described Th2, Th1, Th17, and T regulatory cells, respectively. We did not observe significant differences in the

expression of the different transcription factors when epithelia supernatant was added to *N. meningitidis*–activated pDCs. Only a slight increase in GATA-3 and RORC expression was detected when Flu was used to stimulate pDCs compared to *N. meningitidis* (Supporting Information Fig. 4). These results corroborate our previous observation that the epithelial microenvironment did not modify the Th profile trough *N. meningitdis*–activated pDCs.

In summary, our results demonstrated that extracellular bacteria activate pDCs, and suggest that at the mucosa, the inflammatory response may be attenuated by the surrounding epithelium without impairing the capacity of pDCs to induce an effective adaptive immune response.

Discussion

The pDC response to extracellular bacteria has been barely explored. Some articles claim that human pDCs could not directly respond to extracellular bacteria [17, 18]. The activation of pDCs by *S. aureus* has been reported to require the presence of an IgG-mediated memory response [19]. Another study showed that pDC activation by paraformaldehyde-fixed *Escherichia coli, Yersinia pestis*, and *S. aureus* required the presence of myeloid DCs [18]. A contradictory report showed that *Streptococcus pyogenes* is able to directly activate pDCs, increasing costimulatory molecules and TNF-α secretion [20]. However, in this study IFN-α secretion, the main marker of pDC activation, was not detected [7, 11]. Such controversy may depend on the strains and status (live versus killed) of bacteria used [17]. Another source of discrepancy may reside in the purity of pDCs since some of the previous articles used enriched pDCs instead of pure pDCs [19]. Our results provide definitive evidence that direct contact with whole live extracellular bacteria is sufficient to activate pDCs, and did not require the addition of specific antibodies or myeloid DCs as was the case in other studies [18, 19]. Furthermore, we observed a strong induction of IFN-α, in contrast with results obtained using *S. pyogenes* [20].

N. meningitidis–associated diseases remain a major problem of public health. The protective immune response generated naturally to the bacteria is initiated in the oro-nasopharynx mucosa [5, 21]. However, the mechanisms associated with this early response remain largely unknown. In this context, previous studies showed that *N. meningitidis* induce cytokine secretion by myeloid DC using monocyte-derived DC (MoDC) model [22, 23]. Our results introduce for the first time a new role for pDCs during the immune response to *N. meningitidis*. pDCs may be a key player in the initiation of a protective innate and acquired immune response.

The presence of pDCs at the perifollicullar T-cell area of lymphoid tissues such as tonsils has been largely described [13, 14]. Here, we demonstrated that pDCs are also present in the tonsillar epithelium. Our observations are in line with recent studies showing that a population of tonsil pDCs can be attracted to inflamed epithelium through CCR6 [24]. Mucosal epithelia are major sites of host–microbe interaction, including extracellular bacteria. This is striking for *N. meningitidis*, which live in tight interaction with the epithelium as previously reported [25, 26]. The presence of pDCs in the epithelium strengthens the possibility that under physiological condition these cells may directly contact extracellular bacteria at the human mucosa.

Bacteria have evolved different strategies to escape host immunity [27]. One of these involves the active suppression of the inflammation by bacteria such as nonpathogenic *Bacteroides thetaiotaomicron,* which prevent NFκB activation of the gut epithelium [28]. Here, we observed that the oro-nasopharynx epithelium inhibited IFN-α, TNF-α, and IL-6 triggered by extracellular bacteria. However, this inhibition was independent of the presence of the bacteria since untreated epithelium supernatants inhibited proinflammatory cytokines to the same extent as the supernatants from extracellular bacteria-treated epithelium. Several factors such as IL-10, TGF-β, and PGE2 can be produced by the epithelium and were previously described to inhibit IFN-α from activated pDCs [12, 17, 29]. However in our epithelial cell model, we did not detect the presence of these factors (data not shown), suggesting that other soluble factors are responsible for this function.

How a protective immune response to extracellular bacteria is initiated and modulated at the mucosal surface is unknown. The importance of the epithelium in controlling both innate and adaptive immune response has been previously shown in the gut, where the epithelium can induce the switch of Th response through modifying MoDC function. Although the epithelia microenvironment inhibits inflammatory cytokines, in our system it did not modify the capacity of pDCs to prime and polarize naive T cells after bacterial encounter. These results suggest that an effective acquired immunity to extracellular bacteria could be initiated by pDCs at the mucosal surface. Recently a major role of pDCs in the T-cell–independent IgA production by B cells was observed in mice [30]. This supports the idea that pDCs participate in several mechanisms involved in the triggering of antibacterial mucosal immunity.

In conclusion, in our study we provide definitive evidence that human pDCs can directly respond to a diversity of extracellular bacteria, which shed new light on the host–pathogen interaction in these infections. Furthermore, we provide a strong physiopathological basis for a pDC-driven pathway to initiate adaptive immunity to extracellular bacteria without causing a major destructive inflammation at the mucosal level. Our results suggest that pDC-targeting adjuvants may improve the efficacy of vaccines for the prevention of extracellular bacteria-associated diseases, such as meningococcemia, which remains a major public health challenge.

Materials and methods

Primary samples

Buffy coats were obtained from healthy adults donors at the Saint-Antoine Crozatier Blood Bank through an approved

convention with the Institut Curie. Tonsils were recovered from Hôpital Necker-Enfant malade. The study was approved by the Institut Curie Institutional Review Board, and was performed according to national regulatory rules. All patients and healthy donors gave informed consent for research use of their biological material in accordance with the declaration of Helsinki.

Bacterial strains

Bacteria were all from clinical isolates. *H. influenzae* and *S. aureus* were grown on Chocolate Agar (BD). *N. meningitidis* 8013 clone 12, serogroup C was grown on GCB agar plate (Difco) containing Kellog's supplements. Bacteria were grown overnight at 37°C, 5% CO_2. Bacteria were allowed to grow until reaching an $o.d._{(600\ nm)}$ between 0.1 and 0.5, corresponding to the growth exponential phase in culture medium. An equivalence of o.d. of 0.1 = 100 × 10^6 bacteria/mL was assumed.

pDC purification

pDCs were isolated from human peripheral blood or tonsil by using the pre-DC enrichment kit (Miltenyi Biotec) followed by cell-sorting to lineage$^-$CD11c$^-$CD4$^+$ cells by flow cytometry as previously described [7]. Purity was higher than 98%.

pDC culture

pDCs were cultured during 24 h at 10^6/mL in the presence of CpG-C 1 μM or 10 MOI of indicated bacteria. After 1 h of incubation with bacteria, penicillin 100 U/mL, and streptomycin 100 μg/mL was added to all the conditions. When mentioned, supernatants were added to pDCs in a 1:1 ratio. pDC viability and maturation was assessed by flow cytometry by staining the cells with DAPI and anti-human CD86 antibody (BD Bioscience), respectively. The secretion of IFN-α, TNF-α, and IL-6 was measured by Cytometric Beads Array (CBA) Flex Set (BD Bioscience).

Inside-out experiments

S. aureus growth as previously indicated were incubated 1 h on ice with 2 mg/mL of biotin (Pierce) and washed extensively. Bacteria were then incubated 30 min with streptavidin Alexa-488 (Molecular Probes) 1:50. After washing, stained bacteria were added to pDCs in a ratio of 10:1 in v-bottom 96-well plates. After 1 h of incubation the cells were carefully washed and then incubated with streptavidin Alexa-647 to detect noninternalized bacteria. The cells were analyzed by flow cytometry and cell stream technology (Amnis), using Flow Jo and IDEAS software, respectively.

For microscopy assays, pDCs were incubated with Alexa-488-biotinilated *S. aureus* and then seeded in poly-lysine–coated coverslides. After 1 h, the cells were fixed with PFA 2%. Cells were analyzed by spinning-disk confocal microscopy at 100× using Metamorph software.

Immunohistology

Frozen human tonsils sections were stained with mouse anti-human BDCA-2 (DENDRITICS) or the corresponding isotype control followed by the horse anti-mouse (Vector Laboratories) biotinylated polyclonal antibodies. The staining was revealed using a Vectastain ABC peroxidase system (Vector Laboratories).

Human tonsil epithelial cells

Primary culture from human tonsil was generated by using a modified protocol described elsewhere [31]. Briefly, tonsil explants were cultured with DMEM-F12 medium (GIBCO BRL) supplemented with 10% FBS and antibiotics. Medium was replaced by defined keratinocyte serum–free medium plus keratinocyte supplement (GIBCO BRL). Cells were trypsinized when colonies reached 1 cm in diameter and were assessed for cytokeratin expression by flow cytometry using anti-pan-cytokeratin-FITC (SIGMA). For infection assays, confluent cells were grown in antibiotic-free medium and stimulated with 10 MOI of indicated bacteria. After 24 h, supernatants from the apical side of the cells were centrifuged, filtered, and stored at −80°C until use. For immunofluorescent assays, cells grown on collagen-coated coverslides were fixed with PFA 4% for 20 min and permeabilized for 5 min with 0.1% Triton X-100. Cells were incubated with primary antibody ezrin antiserum (A kind gift from Paul Mangeat) ZO-2 or Occludin (Zymed), diluted in PBS containing 0.3% serum albumin bovin for 1 h. DAPI (0.5 μg/mL) were added to Alexa conjugated secondary antibody (Invitrogen, France) diluted 1:200 in PBS containing 0.3% BSA for 1 h. After several washes, coverslides were mounted in Vectashield mounting reagent (Vector Laboratories, Eurobio ABCys, France).

pDC T-cell coculture

As previously described [15], CD4$^+$ naive T cells from human blood were sorted as CD4$^-$CD45RA$^+$CD25$^-$CD45RO$^-$ by flow cytometry. Purity was higher than 98%. Naive CD4$^+$ T cells were cultured for 6 days with allogeneic pDCs at a 5:1 ratio. Supernatants were collected after 24 h of anti-CD3/CD28 microbeads (Dynal) restimulation. Human IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, TNF-α, IFN-γ secretion were measured by CBA Flex Set (BD Biosciences).

Statistical analysis

Cytokine production and CD86 expression were compared for each culture condition using a two-tailed Student's *t*-test. Statistical significance was retained for p values <0.05.

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Abbreviations: **Flu**: influenza virus · **HTEC**: human tonsil epithelial cell · **pDC**: plasmacytoid pre-dendritic cell

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