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PhD in Clinical and Experimental Immunology

Bacterial lysate enhances protective mucosal immunity via increased expression of antimicrobial peptides

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Sommario

Abstract

Polyvalent mechanical bacterial lysate (PMBL) has been reported to be effective in the prevention of common respiratory tract infections. PMBL are produced through a process that preserves the structure of the bacterial antigens and because of their immunogenic capabilities, increasing importance is given to its mechanism of action, not yet fully understood. Respiratory tract epithelial cells constitute a front-line physical barrier between the organism and the environment. They are able to sense pathogen associated molecular patterns and secrete a wide spectrum of protective factors. By using primary *normal* Human Bronchial Epithelial Cells (HBEpiCs), we observed that PMBL can improve epithelial barrier integrity via induction of adhesion molecules. Along with adhesion molecules, PMBL had a significant effect on epithelial cell proliferation increasing the expression of the autocrine growth factor Amphiregulin (AR). Moreover, treatment with PMBL promoted *ex-novo* gene expression of human beta-defensin 2 (HβD-2) on HBEpiCs and conferred them a direct antimicrobial activity. Epithelial cells are structural components of mucosal immunity that include also dendritic cells (DCs) and innate lymphoid cell-type 3 (ILC3s). PMBL induced IL-23 and IL-1β secretion by DCs that, in turn, activate ILC3s to produce IL-22, cytokine primarily involved in the induction of antimicrobial peptides (AMPs). Interestingly, IL-23 produced by DCs can also activate epithelial cells and lead to a boost of IL-22 production by ILC3s. Remarkably, HβD-2 and LL-37 AMPs can be induced in the saliva of normal subjects after administration of PMBL. Altogether, these results indicate that PMBL administration might support a critical barrier-protective immune pathway that originates from, and is orchestrated by airway epithelial cells and could be therapeutically exploited for the prevention of airway infections.

Introduction

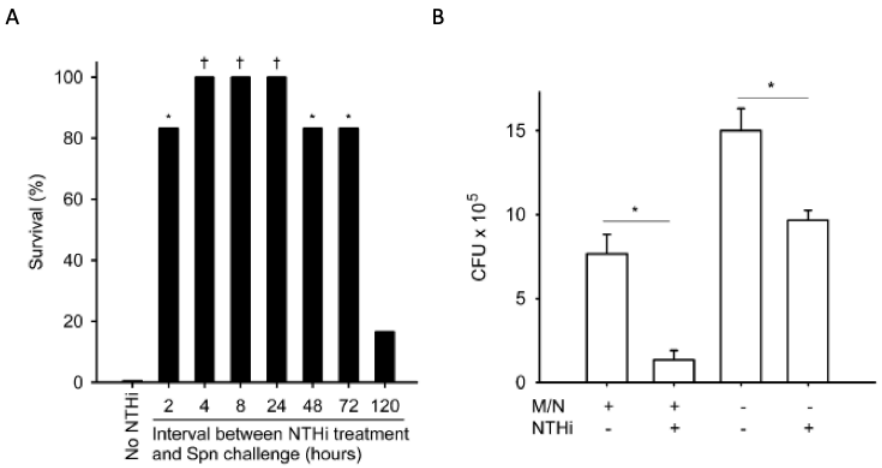
Polyvalent Mechanical Bacterial Lysate

Respiratory tract infections represent one of the most common causes of human disease in terms of morbidity, mortality, and economic cost worldwide. Usually, respiratory system is considered as a sterile environment, by which pass air breathing. On the contrary, airway system is constantly exposed to airborne pathogens and environmental particles. Respiratory diseases are one of the main causes of human death around the world: <<*About 65 million people suffer from chronic obstructive pulmonary disease (COPD) and 3 million die from it each year, making it the third leading cause of death worldwide. About 334 million people suffer from asthma, the most common chronic disease of childhood affecting 14% of all children globally. Pneumonia kills millions of people annually and is a leading cause of death among children under 5 years old. Globally, 4 million people die prematurely from chronic respiratory disease*>> **(1)**. At the moment, a large spectrum of different therapeutic options is available, essentially based on administration of antibiotics. Beside these, bacterial lysates have obtained a progressively larger consensus in the medical practices, since they seem preventing upper and lower respiratory tract infections in both pediatric and elder ages **(2)**. Bacterial lysates are mixtures of bacterial antigens derived from different microbial species. Antigens are obtained using a chemical (PCBL) or mechanical (PMBL) cellular lysis. PMBL are produced through a process that preserves the structure of bacterial antigens and because of their immunogenic capabilities, they are commonly used as immunomodulators able to induce a range of effects on the immune system **(3,4)**. These effects include enhanced expression of adhesion molecules on monocytes and neutrophils, activation of dendritic cells, T and B lymphocytes, IgA secretion, as well as the production of antibodies directed against administered bacterial antigens. For example, Ismigen™, a PMBL of 13 different bacterial strains, has been reported to induce the activation of DCs, leading to a significantly higher expression of co-stimulating membrane molecules (CD80, CD83, CD86). Furthermore, another study revealed that this PMBL activated IL-2 receptor (IL-2R)

on both B and T lymphocytes and promote cytokine synthesis (IL-2, IL-10, IL-12, IFN-γ) in immune competent cells, thus significantly regulating immune responses. Although these important evidences, the immunologic mechanism of action of bacterial lysates is not yet clearly determined.

Epithelial cell barrier function

In this regard, an interesting result was obtained in a murine model treated with an aerosolized lysate of ultraviolet-killed nontypeable (unencapsulated) *Haemophilus influenzae* (NTHi), then challenged with a lethal dose of live Streptococcus pneumonia **(5)**. Upon exposition of mice to NTHi lysate, complete protection against Pneumococcal infection is recorded when the treatment is performed between 4 and 24 hours before bacterial infection (**Figure 1A**). The observed effect is due to innate immune mechanisms, because it occurs too rapidly for an adaptive immune response. In particular, it was associated with an induction of protective response that did not depend on neutrophils, mast cells and alveolar macrophages, suggesting the presence of another cellular effector of this response (**Figure 1B**).

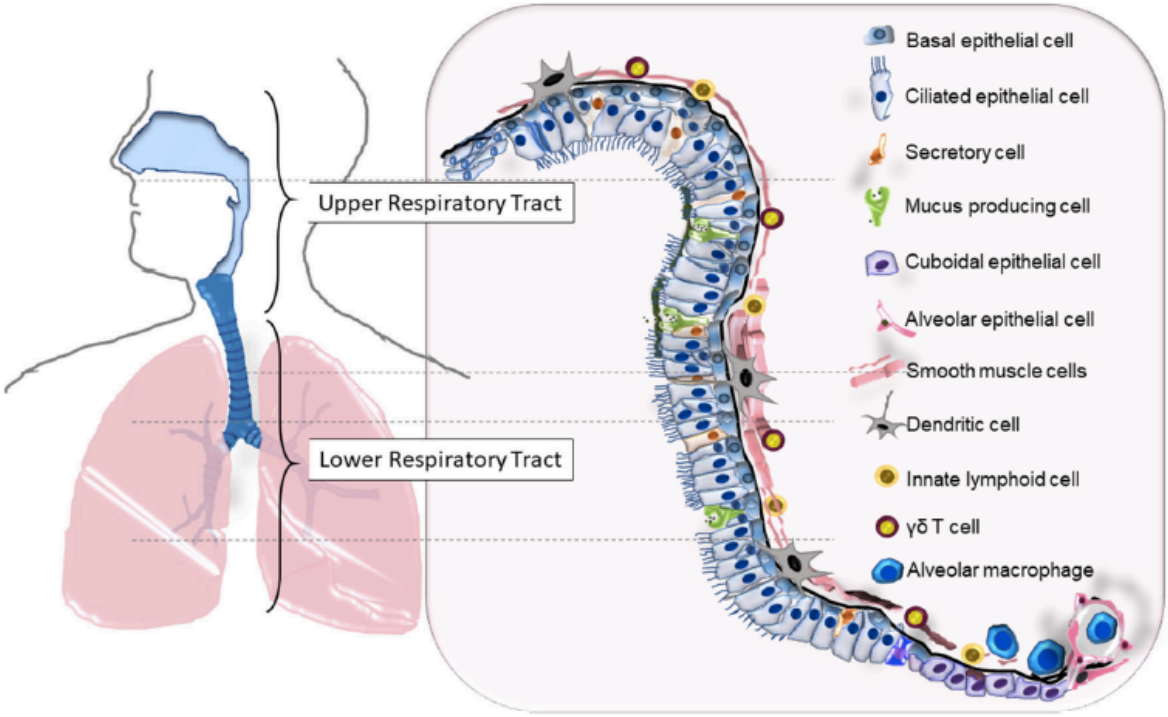


(Clement CG et al., Am J Respir Crit Care Med, DOI: 10.1164/rccm.200607-1038OC)

Figure 1. Supporting evidences of Mucosal Innate Immunity induced by bacterial lysate

Indeed, the very first step in the immune-activation resulting from PMBL is the interaction of the microbial particles with epithelial cells. The mucosal surfaces of the respiratory tracts are covered by a layer of epithelial cells that are responsible for sensing and promoting an host immune response

(6,7). The respiratory system is composed by upper (nasal passages, pharynx, larynx) and lower (trachea, bronchial tree, lungs) tracts with a whole mucosal surface area over than 70 m^2 **(8,9)**. Epithelium represents the protective coverage of inner and external mucosa and the first contact site of environmental particles and microbes (**Figure 2**). Mechanically, airway epithelium is composed by a continuous layer of basal, goblet, ciliated cells and mucus, determining a consistent physical barrier. In particular, the epithelial structural configuration is maintained by a large number of cell adhesion molecules (CAMs), involved in cellular junctions. The coexistence of dynamic physical structures together with active mechanisms of immune defense makes epithelium the first line defense of innate immune system. First, to preserve mucosal homeostasis and integrity, epithelial cells have the ability to reinforce barrier integrity improving the tightness of cellular junctions or regulating their proliferation. In particular, specialized cellular compartments require cell-to-cell adhesions or "synapses", based on interaction between CAMs, to preserve their structure and function **(10)**. For example, human mucosa need a way through which immune cells can transmigrate the blood or tissue and exit toward sites of inflammation. This way is represented by the dynamic adhesion between leukocytes and epithelial/endothelial cells (immunologic synapse). As well, the structural and functional distribution of cells in mucosal epithelium is due to cell-to-cell contact (epithelial synapse).



(LeMessurier KS et al., Front. Immunol. doi: 10.3389/fimmu.2020.00003)

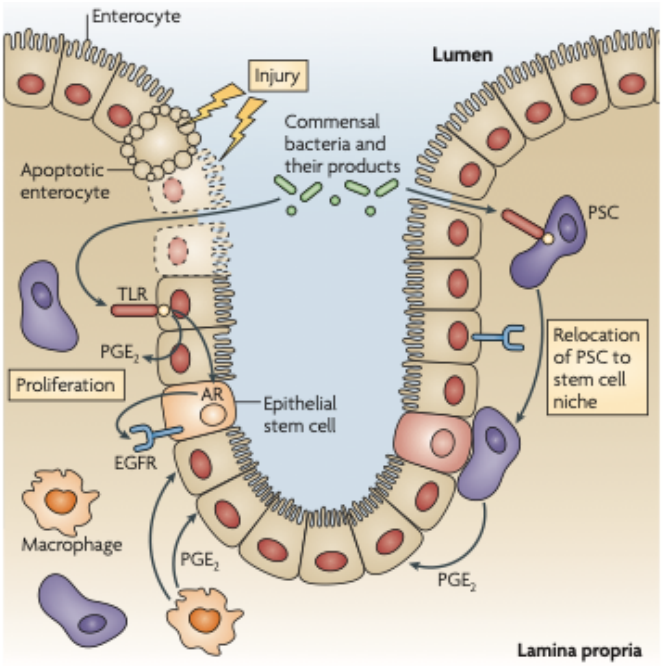
Figure 2. Structure of airway epithelium

One of the main adhesion molecules involved in immunologic synapse is the intercellular adhesion molecule-1 (ICAM-1/CD54). It is a surface glycoprotein, constitutively expressed at low levels on hemopoietic and endothelial cells, fibroblasts and different epithelia (e.g., bronchial, kidney, urinary tract, and skin). Its expression is upregulated during inflammation by proinflammatory cytokines (e.g. TNF-α, IL-1, and IFN-γ) and bacterial components such as LPS **(11,12)**. Relevant studies showed that ICAM-1 expression on epithelial cells promotes neutrophils and lymphocytes transmigration to the site of inflammation **(13-15)**, facilitating the beginning of local immune response.

In the context of epithelial synapse, Adherens Junctions (AJs), one of the main apical adhesive structures, maintain cellular tightness by homophilic interactions of E-Cadherin molecules **(16)**. E-cadherin is one of the classical cadherins, together with the neural cadherin (N-Cadherin), placental cadherin (P-Cadherin), and the vascular endothelial cadherin (VE-Cadherin). E-Cadherin is a transmembrane glycoprotein involved in Ca2+-dependent cellular junctions. The highly conserved intracellular domain of E-Cadherin is conjugated with a complex of α-, β- and p120-catenins **(17)**.

The interaction of E-Cadherin with this intracellular complex modulate its association with the cytoskeleton, its transport and recycling, and morphological polarity of epithelial cells **(18)**. Thus, the primary function of E-Cadherin is to stabilize intercellular adhesion between adjacent cells, preserving the integrity of epithelium. Moreover, the stability of AJs prevents alteration and disruption of epithelial layer, maintaining tissue homeostasis and improving survival of the organism **(19)**.

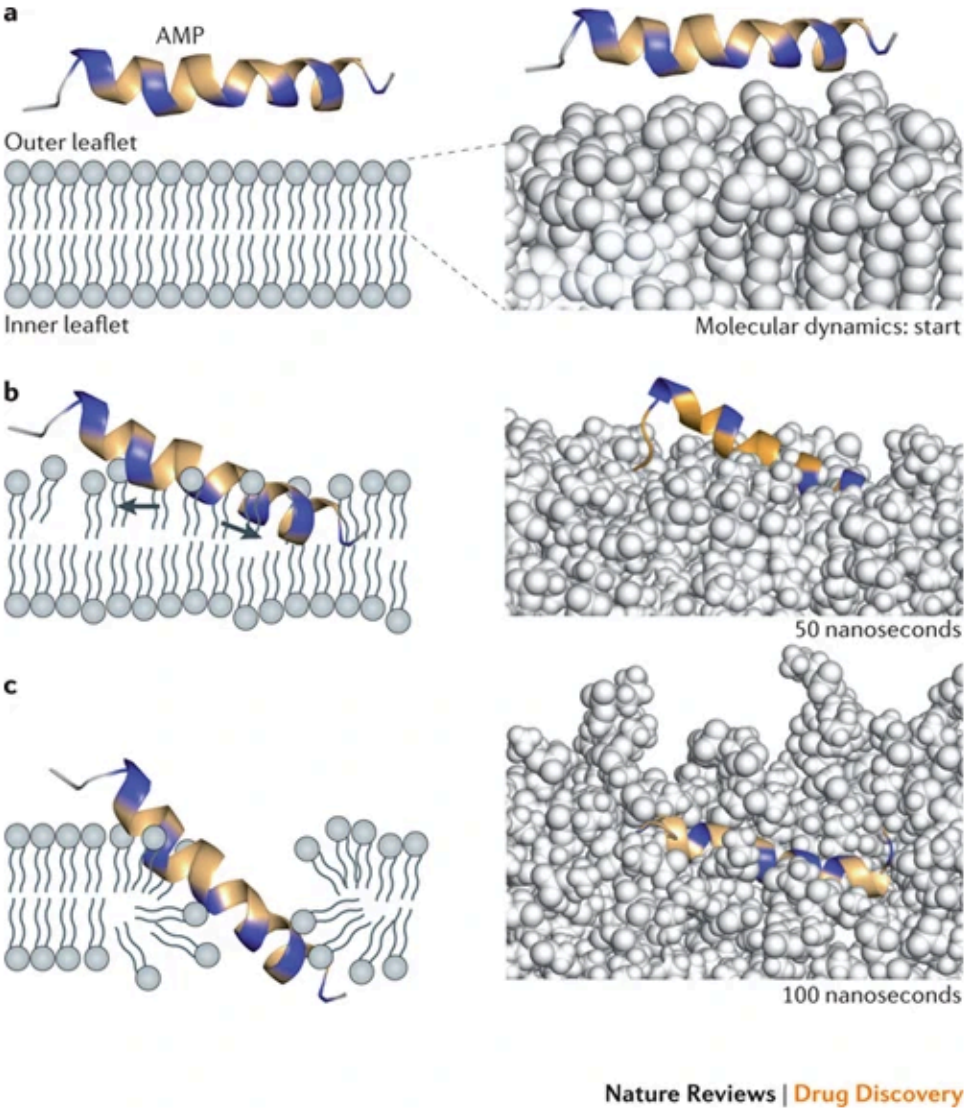
Another important mechanism to maintain epithelial barrier integrity is due to the regulation of proliferation. In normal conditions, the muco-ciliary clearance preserves epithelial integrity, actively contrasting the microbial colonization. When the mucus layer is disrupted, epithelial injuries, derived by a direct interactions between epithelial cells and microbes or microbial compounds, determines the activation of host defense mechanism (**Figure 3**). In particular, baso-lateral TLRs of epithelial cells interact with microbial compounds, determining the secretion of autocrine proliferative factors, such as Amphiregulin (AR), an Epidermal Growth Factor Receptor ligand, promoting epithelial cell turn over and proliferation. **(20,21)**



(Abreu, M. T., Nature Reviews Immunology, doi:10.1038/nri2707)

Figure 3. Involvement of bacteria in induction of epithelial cell proliferation

Beside the physical mechanisms of host immune defense, epithelial cells have also the capability to directly kill microbial agents through the production of antimicrobial peptides (AMPs) (**Figure 4**).



(Christopher D. Fjell et al., Nature Reviews Drug Discovery, doi:10.1038/nrd3591)

Figure 4. AMPs mechanism of action.

Small cationic AMPs are synthetized and stored as pre-pro-peptides by epithelial cells and neutrophils. To become functional, AMPs are cleaved before secretion to obtain a cationic charge able to induce electrostatic interactions with anionic fragments of microbial membrane, determining the formation of ion pores and membrane disruption. The major lung epithelial AMPs are defensins and cathelicidins. Defensins are cationic peptides with a molecular mass of 3.5–4.5 kDa, that contain

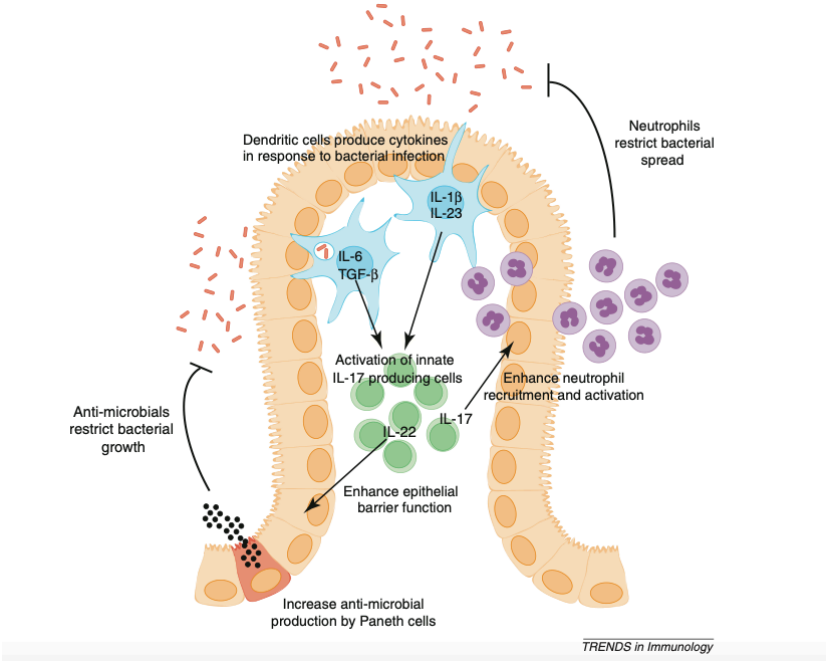
six cysteine residues to form three characteristic intramolecular disulfide bridges. The disulfide bridge arrangement determines three different classes of defensins: α-defensins, β-defensins, and θ-defensins. β-defensins are the only class of defensins produced by epithelial cells. β-defensins are cationic peptides with a broad-spectrum antimicrobial activity. They are composed by 36–42 amino-acids and can be constitutive (e.g. HBD-1) or inducible (e.g. HBD-2) in different mucosal sites (skin, saliva, lung, intestine, kidney, urogenital tract) **(22-24)**.

About cathelicidins, they have an N-terminal domain (*cathelin*), involved in protein binding, and a C-terminal domain with antimicrobial activity. In human, we can find only a propeptide (CAMP or hCAP-18) which is cleaved to the active antimicrobial peptide, LL-37. This AMP shows an amphipathic α-helical structure able to bind lipopolysaccharides or teichoic acids of bacterial membrane. It is supposed that interaction between the hydrophobic components of helix and the lipid bilayer promotes distortion and disruption of the membrane, causing microbial death. **(25).**

Mucosal Innate Immune Response

All these evidences of an active antimicrobial role of epithelial cells demonstrate that epithelium is not a wooden barrier: it has plasticity and actively participates to innate immune response, by sensing the presence of bacterial components. Moreover, innate immune signaling derived from intact bacteria or microbial fragments in contact with epithelial cells, beyond mucus layer, produces a cascade reaction, by which resident leukocytes are enrolled for immune reaction to clear pathogens, promoting tissue repair **(26).** As intestinal mucosa, airway system is constantly exposed to environmental factors and microbial agents, derived from air breathing. In this scenario, tissue-resident DCs play a pivotal role in recognition of pathogen-associated molecular patterns (PAMPs) to modulate mucosal immune response **(27)**. In particular, DCs are able to sample beyond the lumen of mucosa that, in normal conditions, is covered by a thick layer of mucus, full of antimicrobial proteins and secretory IgA. In case of inflammation and mucosal injury, derived from bacterial

colonization, DC sampling results in their maturation and activation. Thus, activated DCs are able to orchestrate the local innate immune response, producing a large number of cytokines and chemokines to recruit innate immune cells **(28)**. In particular, mucosal DCs show to be a relevant source of IL-23 and IL-1b, the main soluble factors involved in activation of innate lymphoid cells-type 3 (ILC3s) **(29-31) (Figure 5)**.



(SJ Rubino et al., Trends in Immunology, doi:10.1016/j.it.2012.01.003)

Figure 5. DCs and ILC3s cross-talk

Innate lymphoid cells (ILCs) are a newly described heterogeneous population, representing the innate counterpart of CD4+ T-Helper cells **(32)**. Indeed, ILCs lack of rearranged antigen receptors and have no demonstrated cytotoxic capacity, showing to be able to produce cytokines. Conventionally, ILCs are distinguished into three classes by their expression of transcription factors and cytokines and ILC3s are the main players of mucosal immune system. ILC3s express the transcription factor RORγt and represent a great innate source of IL-22 **(33)**. In contrast to many other soluble factors, IL-22 exerts its effect on nonhematopoietic epithelial cells and fibroblasts in tissues, such as lung. IL-22 is involved in induction of epithelial-derived antibacterial proteins, such as β-defensin 2, in proliferation of epithelial cells and epithelial repair after injury. In mucosal immunity, upon microbial infections and epithelial injury, IL-23 represents a potent inducer of IL-22 production **(34)**.

Aim of the study

Epithelial cells represent the first mucosal barrier and they are able to directly sense and response to bacterial stimuli (**Figure 6**).

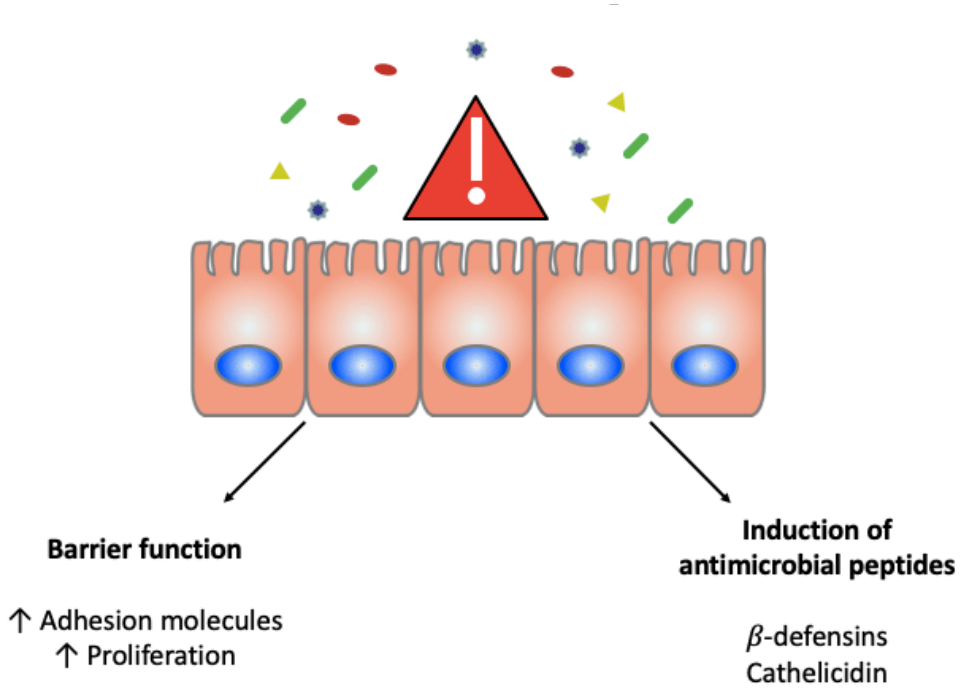


Figure 6. Host defense mechanism of epithelial cells

To this reason, PMBL, that include bacterial components derived by several strains, may activate epithelial cell-mediated innate immune response against pathogens. In particular, considering the capability of epithelium to contrast microbial infections, we investigated whether PMBL is able to modulate epithelial cell adhesion, proliferation and, more important, to enhance the production of antimicrobial agents. Also, we evaluated the capability of PMBL to induce the production of DC-derived IL-23, licensing the production of IL-22 by ILC3s, the main soluble factor involved in maintenance of mucosal integrity and production of AMPs. Therefore, we evaluated the capability of PMBL to reinforce in-vivo mucosal barrier, quantifying the expression of AMPs on saliva samples derived from healthy volunteers, upon sublingual administration of Ismigen™.

Results

PMBL stimulates epithelial cell adhesion and proliferation.

Microbial invasion can be facilitated by an alteration of epithelial barrier integrity, mainly regulated by junction proteins. To analyze whether PMBL can improve airway epithelial barrier functions, we threated a primary cell line of *normal* Human Bronchial Epithelial Cells (HBEpiCs) for 24 hours with PMBL and then assessed the expression of the adhesion molecules CD54 (ICAM-1) and CD324 (E-Cadherin).

ICAM-1 is constitutively expressed at low level on epithelial cells, but it can be upregulated in the early phase of bacterial infections **(11)**. PMBL treatment increased ICAM-1 expression on HBEpiCs and a clear image of this upregulation was obtained by Image Stream cytometry analysis (**Figure 7A**).

Concerning E-cadherin, flow cytometry analysis revealed a mild upregulation of E-cadherin on HBEpiCs upon stimulation with PMBL, but immunofluorescence assay revealed a different distribution of this adhesion molecule. In particular, epithelial cells left untreated showed an intracellular and surface distribution of E-cadherin, whereas PMBL treatment induced a clear polarization to cell membrane (**Figure 7B**). In this regard, E-cadherin should be expressed on plasma membrane to be functional for cellular adhesion, while it can be partially internalized for recycling or degradation **(18)**.

The homeostasis of airway barrier is also based on the regulation of epithelial proliferation **(21)**. To address whether PMBL can promote epithelial proliferation, we evaluated epithelial expression of Ki67 marker upon PMBL stimulation and we observed a higher frequency of $Ki67^{+}$ HBEpiCs (**Figure**

7C), compared to unstimulated condition. Mechanistically, epithelial cell proliferation can be induced by the production of autocrine factors, such as Amphiregulin (AR) and HBEpiCs stimulated with PMBL for 6h showed an induction of AR gene expression (**Figure 7D**). Altogether, these results suggest an active role of PMBL on epithelial cell function, that can lead to an improvement of barrier integrity.

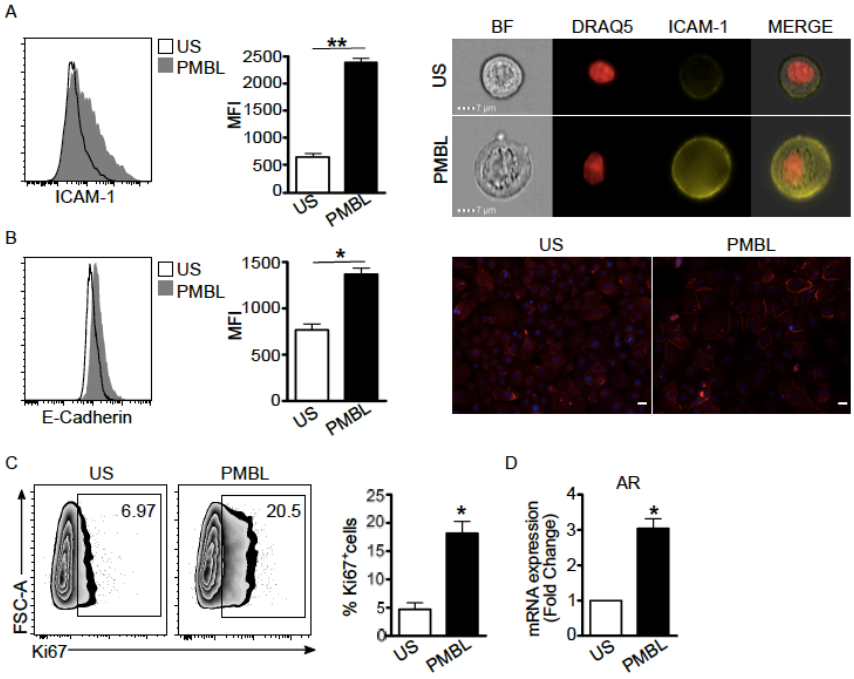


Figure 7. PMBL increased the expression of adhesion molecules and proliferative factors on HBEpiCs. (A) Surface expression of ICAM-1 was assessed on HBEpiCs stimulated or not for 24 hours with PMBL by flow cytometry and Image Stream cytometry. (*Left*) Bars represent mean fluorescence intensity (MFI) values ± SEM of ICAM-1 from three independent experiments. (*Right*) Comparative Brightfield (BF), DRAQ5 (nucleus), ICAM-1 and MERGE profile of HBEpiCs are shown. Scale bar = 7 µm. **(B)** Analysis of E-cadherin expression on HBEpiCs stimulated or not for 24 hours with PMBL by flow cytometry and immunofluorescence assay. (*Left*) Bars represent MFI values ± SEM of E-Cadherin from three different experiments. (*Right*) Fluorescence Microscopy analysis of E-Cadherin expression. Representative images of three independent experiments are shown. Scale bar = 20 µm. **(C)** Proliferation was assessed on HBEpiCs stimulated or not for 24 hours with PMBL by intranuclear Ki67 staining. Representative dot plots of three independent experiments show the percentage of $Ki67^+$ cells. Bars represent mean values ± SEM of $Ki67^+$ HBEpiCs. **(D)** AR gene expression was measured by RT-PCR on HBEpiCs stimulated or not with PMBL for 6 hours. Data derived from three different experiments. Bars represent Fold Change values ± SEM of AR mRNA expression of HBEpiCs. $*P < .05$, $**P < .01$; Student *t*-test.

PMBL enhances epithelial antimicrobial activity via release of AMPs.

Administration of PMBL has been reported to prevent recurrent respiratory tract infections (rRTI) **(35)**. Epithelial cells can sense PAMPs and, along with forming a physical barrier against infection, can exert a direct antimicrobial activity by producing antimicrobial peptides (AMPs). Human beta

defensin-2 (HBD-2) is a major antimicrobial peptide produced by many types of epithelial cells and it is transcriptionally inducible by various proinflammatory agents, such as cytokines and bacteria.

We, therefore, investigated whether epithelial cells can respond to PMBL treatment by augmentating antimicrobial defenses and both RT-PCR and gel electrophoresis analyses revealed *ex-novo* growing induction of HBD-2 gene expression on HBEpiCs, between 6h and 24h of stimulation with PMBL (**Figure 8A**). Then, we assessed the direct antimicrobial activity of epithelial cells treated with PMBL by performing an antimicrobial sensitivity testing, after conditioning with epithelial cell derived supernatants. In agreement with *ex-novo* expression of HBD-2, supernatants of PMBL-stimulated epithelial cells significantly reduced S. Aureus colony-forming units (CFU) (**Figure 8B**). Thus, PMBL is able to induce the production of epithelial AMPs that might support mucosal "Stimulated Innate Resistance (StIR)" to microbial infections **(24)**.

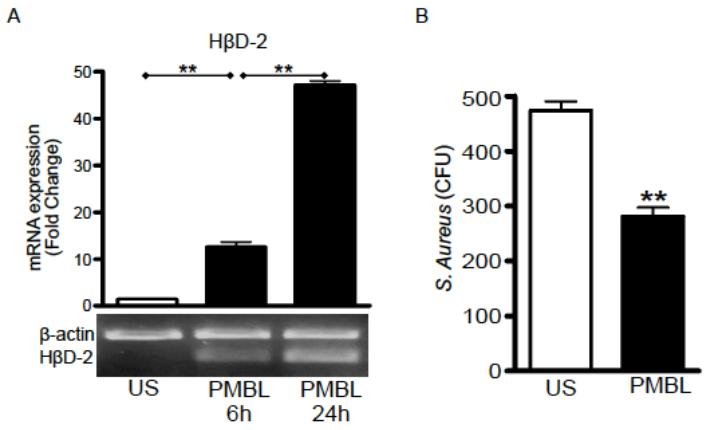


Figure 8. PMBL induced *ex-novo* gene expression of HBD-2 on HBEpiCs and promoted their direct antimicrobial activity on live *S.Aureus*. **(A)** HBD-2 gene expression was measured by RT-PCR and gel-electrophoresis on HBEpiCs stimulated or not with PMBL for 6 and 24 hours. Data derived from three different experiments. Bars represent Fold Change values ± SEM of HBD-2 mRNA expression of HBEpiCs **(B)** Antimicrobial sensitivity testing was performed by conditioning with HBEpiC culture supernatants 100 CFU/mL of live *S. Aureus* for 5 hours. The experiment was performed in triplicate. Bars represent the number of CFU ± SEM of *S. Aureus* bacteria. (**P < .01; Student t-test).

PMBL promotes IL-23-dependent epithelial cell activation licensing mucosal IL-22 production.

Epithelial cells are structural components of mucosal immune network, physically related to other innate immune players, such as dendritic cells (DCs) and innate lymphoid cells-type 3 (ILC3s) **(34, 36)**.

Production of IL-23 by DCs supports innate IL-22 secretion and binding of IL-22 to the IL-22 receptor expressed on the epithelium can have multiple effects, including the enhanced secretion of antimicrobial peptides. The administration of PMBL induced the maturation of DCs (**Figure 9A**). Along with maturation markers, we observed that PMBL, but not other maturation stimuli, can induce on DCs the expression of CD103 and Langerin (**Figure 9B**), both typical markers of DCs preferentially localized within the epithelial layer. Moreover, PMBL-stimulated DCs produced high amounts of both IL-23 and IL-1b (**Figure 9C**) that supported in turn IL-22 production by ILC3s (**Figure 9D, *upper panel***). Although the epithelium has been mainly thought to "passively" receive cytokine signals and transduce them into an antimicrobial response, we here observed that airway epithelial cells boosted IL-22 produced by ILC3s (**Figure 9D, *middle panel***), becoming a crucial active player in orchestrating mucosal IL-22 production. It was recently reported that IL-23R signaling on intestinal epithelial cells can support protective mucosal IL-22 production. In line with this report, in the presence of IL-23 blocking antibody, airway epithelial cells lose the capability to support IL-22 production by ILC3s (**Figure 9D, *lower panel***), thus clearly suggesting that epithelial cell licensing of mucosal IL-22 production is mediated by IL-23. Altogether, these findings revealed that PMBL can also promote an indirect induction of AMPs by supporting mucosal IL-22 production.

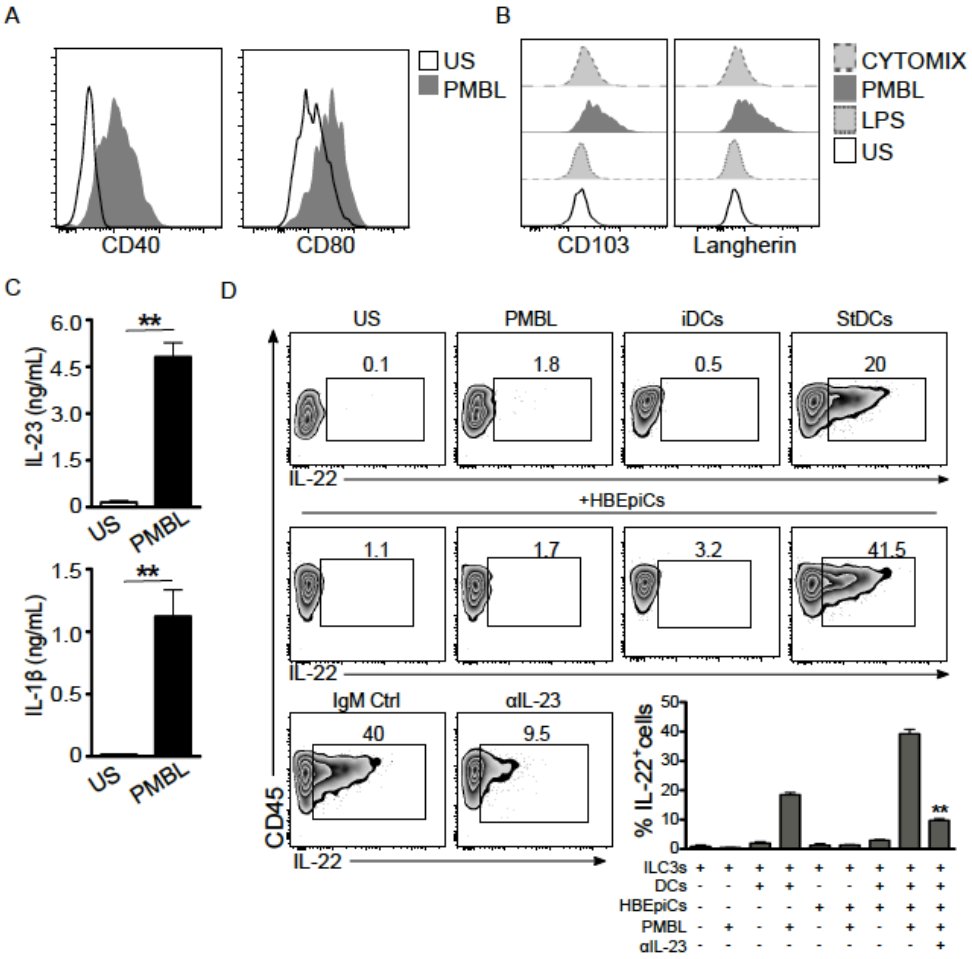


Figure 9. PMBL activated epithelial cells via IL-23 produced by DCs, resulting in a boost of IL-22 secreted by ILC3s. (A) Expression of costimulatory/activation molecules CD40 and CD80 on unstimulated DCs and DCs stimulated 24 hours with PMBL was assessed by flow cytometry. A representative multiple overlay histogram of three different experiments is shown **(B)** CD103 and Langerin expression were assessed on DCs left untreated or stimulated with PMBL, LPS, or CYTOMIX (TNF-α+IFN-γ) by flow cytometry. One representative experiment out of three is shown. **(C)** IL-23 and IL-1b concentration was assessed by ELISA assay in supernatants of DCs stimulated with PMBL for 48 hours and of unstimulated DCs, as control. Bars represent the mean values ± SEM of IL-23 (*left*) and IL-1b (*right*) concentration. **(D)** intracellular expression of IL-22 was assessed on ILC3s upon overnight stimulation in the indicated culture conditions. **(*Upper panel*)** ILC3s alone were stimulated with PMBL or supernatant of DCs cultured 48 hours with/without PMBL. Also, (***middle panel***) ILC3s were co-cultured with HBEpiCs at ratio 1:5 and stimulated in the same conditions. In specific experiments, (***lower panel)*** DC supernatants were incubated with IL-23 blocking antibody, before stimulation of ILC3/HBEpiC co-culture. One representative experiment of three is shown in every panel. Data from all the experiments are summarized in the bars as percentage ± SEM of IL-22$^+$ ILC3s. (**P < .01; Student t-test).

Expression of AMPs on human saliva samples upon administration of PMBL.

PMBL (Ismigen™) is a sublingually delivered formulation able to activate local mucosal immunity. Thus, we investigated whether PMBL administration could induce in normal subjects a local production of AMPs. To address this issue, we collected human saliva samples in 8 healthy volunteers under basal conditions, after sublingual administration of 1 tab of Ismigen™ for consecutive 10 days and for the next 20 days (**Table 1**). By using ELISA technique, we quantified the amount of inducible

HBD-2 and LL-37, respectively belonged to the group of b-defensins and cathelicidins. Significant increased levels of these AMPs following bacterial challenge were obtained in 5/8 volunteers. In particular, after PMBL administration, AMP concentration increased, reaching significant higher level at day 6 for HBD-2 (**Figure 10A**) and at day 10 for LL-37 (**Figure 10B**). This effect seems to be specific since once PMBL administration is stopped, both HBD-2 and LL-37 levels went progressively down. Altogether, these results confirm that one of the relevant mechanisms of action of PMBL relies on the induction of antimicrobial peptides which can prevent colonization of host tissues by a range of pathogens at nanomolar concentration.

Table 1. Main characteristics of healthy volunteers.

	Healthy volunteers *n* = 8
Age	26-53
Gender	4M/4F
Control condition	BS
Days of PMBL (Ismigen) tabs administration	10 days
Interval between each tab	24 hours
Days without treatment	20 days
Time of the study	30 days

M = male; F = female; BS = baseline;

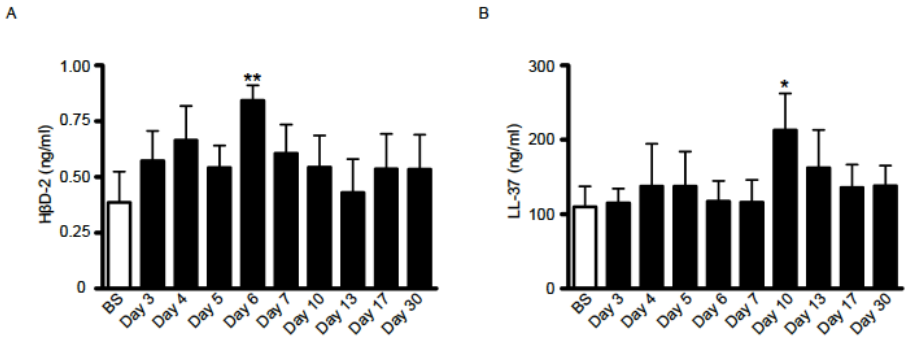


Figure 10. Sublingual administration of PMBL improved salivary concentration of AMPs in healthy subjects. (A) HBD-2 and **(B)** LL-37 detection by ELISA assay in saliva samples of healthy subjects, at baseline (BS), during the administration of PMBL (Ismigen™) (Day3-Day10) and, during the next twenty days (Day13-Day30). Data of each protein derived from 5 of 8 healthy subjects. Bars represent mean values ± SEM of HBD-2 and LL-37 concentrations, respectively (*P < .05, **P < .01; Student t-test).

Discussion

The spread of antibiotic resistance among bacteria responsible for common respiratory infections has prompted calls for an improvement or a reduction of antibiotic usage **(37,38)**. The use of antibiotics was significantly reduced thanks to the treatment with PMBL™, representing a valid immunization

approach in fighting the spread of antibiotic resistance. Thanks to its administration route (sublingual), PMBL treatment can favor the activation of mucosal immune-surveillance, an important first line of defense. In the present study, we demonstrated that PMBL improves a critical barrier-protective immune pathway by promoting a number of independent epithelial cell functions. Stimulation with PMBL can improve epithelial barrier integrity via both induction of adhesion molecules and proliferation. In addition, epithelium activated by PMBL shows a direct antimicrobial activity, since secreted HBD-2, an epithelium-specific antimicrobial protein. This change in epithelial cell functional capability is extremely relevant in defense against bacterial responsible for common respiratory infections and viral pneumonia. Indeed, AMPs are able to rapidly kill a broad range of infective agents and mechanisms that reduced antimicrobial protein expression resulted in microbial invasion and pathogenicity. Proof of this concept is provided by transgenic overexpression of HD-5 expression in Paneth cells that, enhancing microbial killing and host defense, made mice resistant to an oral challenge with virulent Salmonella typhimurium **(39)**. We also establish that lung epithelial cells require co-stimulation by DCs to reinforce their effector response. Although DCs could not be stimulated by the lysate to directly kill extracellular bacteria, they can instruct epithelial cells to express antimicrobial proteins, through local signaling via IL-22 dependent mechanisms **(40)**. Our results demonstrated that DCs stimulated with PMBL expressed CD103 and Langerin and secreted high amounts of both IL-23 and IL-1b inducing, in turn, IL-22 production by ILC3s. In line with this observation, intestinal tissue-resident lamina propria dendritic cells (LPDCs) expressing CD103 rapidly produced high amounts of IL-23 following bacterial flagellin administration and drove IL-22-dependent RegIIIγ production **(41)**. Interestingly, it has been reported that Broad-spectrum antibiotic administration along with killing commensal bacteria in the gut, decreases Toll-like receptor (TLR)-dependent mucosal expression of antimicrobial factors, such as the bactericidal C-type lectin RegIIIγ, leading to a predisposition to VRE colonization (**42,43**). Therefore, strategies to enhance the expression of antimicrobial peptides may be effective against increasingly antibiotic-resistant bacterial pathogens. In light of the PMBL's mechanism of action described here, its

administration in the setting of antibiotic treatment could thus restore AMPs expression in airway mucosal barrier and restrict colonization of pathogens. Although the epithelium has been mainly thought to "passively" receive cytokine signals and transduce them into an antimicrobial response, we here observed that epithelium represents a crucial modulatory factor of mucosal immune response stimulated by PMBL. In particular, IL-23 dependent activation of airway epithelial cells leads to a boost of IL-22 production by ILC3s, thus actively orchestrating antimicrobial and regenerative processes in the mucosa. The relevance of this protective pathway has been recently highlighted in a mouse model of intestinal inflammation where deficiency of IL-23R expression in intestinal epithelial cells resulted in an impaired mucosal IL-22 induction, reduced antimicrobial peptide expression, and an expansion of flagellated bacteria that lead mice to succumb to DSS colitis **(44)**. Finally, after having investigated the mechanisms by which PMBL promote antimicrobial expression, we translated our *in vitro* results into human subjects. We demonstrated that PMBL administration have the ability to induce HBD-2 and LL-37 production in the saliva of normal subjects. However, identification of other antimicrobial peptides as well as the protection they confer against subsequent pathogen challenges represent relevant issues that should be addressed in order to acquire a therapeutic value in patients. In summary, our studies reveal great functional plasticity of airway epithelial cells upon PMBL stimulation. We gained more insights into the nature of innate immune defenses triggered by this complex mixture of bacterial antigen, identifying a protective immune pathway orchestrated by airways epithelial cells. Therapeutic manipulation of this pathway may be possible especially in clinical settings where antimicrobial and regenerative processes mediated by IL-22 secretion represent important protective factors.

Material and Methods

Polyvalent Mechanical Bacterial Lysate

Polyvalent Mechanical Bacterial Lysate (PMBL) is provided by Lallemand Pharma. PMBL contains a blend of 13 inactivated bacterial strains of common pathogens involved in infections of the upper and lower respiratory system (*S. aureus, S. viridans, S. pyogenes, K. pneumoniae, K. ozaenae, H. influenzae, N. catarrhalis and S. pneumoniae*). For the experiments, the bacterial lysate was resuspended in PBS at 5 mg/ml, then added to the cell cultures at the final concentration of 100 µg/ml.

Healthy volunteers and Study Design

Saliva samples were obtained from 8 healthy volunteers aged between 26-53 years. All subjects were not affected by oropharyngeal inflammation and were not taking any medication at the beginning of the study. The study was approved by the institutional ethics committees of IRCCS Polyclinic Hospital San Martino, Genova (587/2020 - DB id 11025) and all participants gave written informed consent according to the Declaration of Helsinki. Volunteers assumed 1 tab of the commercial formulation of PMBL (Ismigen™) every 24 hours for 10 consecutive days. Saliva samples were obtained at baseline (BS), at six timepoints during Ismigen™ administration (Day 3/Day 4/Day 5/Day 6/Day 7/Day 10) and at further three timepoints along the next twenty days (Day 13/Day 17/Day 30). No secondary effects were reported by volunteers during the study.

Samples Collection

Peripheral blood was obtained from healthy donors and mononuclear cells (PBMCs) were separated over Ficoll density gradient centrifugation (30 minutes, 25°C, 2100 rpm).

Tonsils tissues were obtained from patients undergoing tonsillectomy.

Saliva samples were collected from healthy volunteers and processed as previously described **(45)**. Briefly, we planned to collect saliva between 8–10 a.m. We asked subjects to refrain from eating,

drinking or oral hygiene procedures for at least 1h prior to sampling. The subjects rinsed mouth out well with sterile-filtered distilled water and, 10 minutes later, approximately 5 mL of saliva were collected into pre-cooled 50 mL sterile tubes. Collection tubes were put into ice for up to 2 hours or immediately spinned for 30 seconds and then centrifuged at 3000 rpm for 10 minutes at 4°C, to pellet debris and mucoid components. Protease Inhibitor Cocktail (Sigma-Aldrich, Catalog Number: #P8340) was added to aliquots of 500 µL of saliva (dil. 1:500) and then samples were immediately stored at -80°C.

All the samples were collected at the Immunology Unite of IRCCS Polyclinic Hospital San Martino, Genova.

Cell isolation and culture

For generation of DCs, monocytes were isolated from PMBCs by adherence to culture flasks for 45 min at 37°C in 5% CO_2. Subsequently, non-adherent cells were removed by washing with PBS. The resulting monocytes were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 ng/ml human recombinant granulocyte–macrophage colony-stimulating factor (rGM-CSF) (Sargramostim) and 20 ng/ml human recombinant IL-4 (rIL-4) (Miltenyi Biotec). Resulting DCs were collected after 6 days.

ILC3s were sorted and expanded as previously described. Briefly ILC3s were FACS-sorted from tonsil as LIN^{neg} (CD3, CD14, CD19, CD34, CD80 and BDCA2), $CD127^{high}$ $CRTH2^{neg}$ $CD117^{+}$ $NKp44^{+}$ cells and stimulated in 96-well round-bottom plates with irradiated PBMCs and irradiated 721.221 tumor cells in the presence of PHA (1 ug/ml, Life Technologies), IL-2 (100U/ml, proleukin; Chiron) and IL-7 (10 ng/ml, Miltenyi). Cells were cultured in RPMI 1640 medium supplemented with 1% human AB serum. Cytokines were replaced every 2–3 days.

Primary Cell Lines

Normal Human Bronchial Epithelial Cells (HBEpiC, Catalog Number: #3210) were purchased from ScienCell (ScienCell Research Laboratories, San Diego, California). HBEpiCs were cultured at 37°C in a 5% CO_2 incubator using a serum-free Bronchial Epithelial Cell Medium complemented with designed growth supplements (BECM, Cat. No. 3211, ScienCell Research Laboratories), following manufacturer's instructions.

Cell Stimulation

In our experiments, HBEpiCs were grown to confluence in multiwell flat-bottom plate and stimulated with PMBL at the final concentration of 100 μg/ml. To evaluate the expression of ICAM-1 and E-Cadherin, HBEpiCs were stimulated or not with PMBL for 24 hours and then analyzed by different immunofluorescence assays.

To evaluate HBEpiC proliferation, cells were starved overnight in basal medium and then stimulated for 24 hours with PMBL in supplement medium. Then, cells were harvested and analyzed for the expression of Ki67 marker. In addition, cells were stimulated with PMBL for 6 hours and cell pellets were harvested to analyze the gene expression of AR.

To assess the expression HBD-2, HBEpiCs were stimulated with PMBL for 6 and 24 hours, and then cell pellets were harvested to perform HBD-2 gene expression by both RT-PCR and gel-electrophoresis analyses.

To evaluate the direct antimicrobial activity of epithelial cells, HBEpiCs were cultured in antibiotic-free supplemented medium and stimulated with 100 ug/mL of PMBL or left untreated for 24 hours. Then, HBEpiC supernatants were collected to perform an antimicrobial sensitivity testing on live bacteria.

To assess DC activation, moDCs were stimulated or not with PMBL for 48 hours and, then, both cells and culture supernatant were collected to evaluate expression of maturation markers and quantify cytokines, respectively. Alternatively, DC culture supernatants, collected in the same experimental conditions, were used to stimulate IL-22 production by ILC3s. In selected experiment, along with PMBL, DCs were stimulated with LPS (1 ug/ml) or CYTOMIX (IFN-γ 1000 U/mL; TNF-α 100 ng/mL).

To evaluate IL-22 production on ILC3s, IL-22 expression was analyzed on ILC3s stimulated overnight with culture supernatants of DCs stimulated with PMBL or left untreated, in the presence of Golgi Stop and Golgi Plug (BD Bioscience). To evaluate the effect of HBEpiCs on IL-22 secretion, HBEpiCs were added to ILC3s (ratio 1:5) cultured in the above mentioned experimental conditions. In selected experiments, IL-23 blocking antibody (50 ug/mL) (Human IL-23 p19 Antibody, Catalog Number: #AF1716 R&D systems) was added to DC supernatant before ILC3s/HBEpiCs co-culture.

Flow cytometry

The following monoclonal antibodies (mAbs) were used in this study: anti-CD40 PE (clone 5C3, BD Biosciences), anti-CD45 (clone 2D1, BD Biosciences), anti-CD80 PE-Cy5 (clone L307.4,BD Biosciences), anti-CD80 FITC (clone L307.4, BD Biosciences), anti-CD103 BV421 (clone Ber-ACT8, BioLegend), anti-Langerin PE (clone DCGM4, Beckman Coulter), anti-BDCA2 FITC (clone AC144, Miltenyi), anti-CD3 FITC (clone BW264/56, Miltenyi), anti-CD14 FITC (clone TÜK4,

Miltenyi), anti-CD34 FITC (clone AC136, Miltenyi), anti-CD19 VioBright FITC (clone LT19, Miltenyi), anti-CD117 BV421 (clone YB5.B8, BD Biosciences), anti-NKp44 PE (clone 2.29, Miltenyi), anti-CRTH2 PerCP-Cy5.5 (clone BM16, Biolegend), anti-CD127 PE/Dazzle 594 (clone A019D5, Biolegend), anti-ICAM-1 PE (clone HA58, BD Biosciences) and anti-E-Cadherin BV421 (clone 67A4, BD Biosciences). For surface markers, cells were stained for 30 minutes at 4°C.

For intracellular staining, upon surface staining, cells were fixed and permeabilized (Cytofix/Cytoperm Kit, BD Biosciences) according to the manufacturer's protocol and incubated with anti-IL-22 e-fluor660 (clone 22URTI, Invitrogen) for 30 minutes at room temperature.

For Intranuclear staining of Ki67 marker, fixation/permeabilization procedures, performed according to manufacturer's protocol (eBioscence), were followed by incubation with anti-Ki67-BV421 mAb (clone B56) purchased from BD Bioscences.

Dead cells were excluded by staining with LIVE/DEAD FIXABLE Aqua Dead dye (Invitrogen). Sample acquisition was performed on Gallios (Beckman Coulter) or MACSQuant (Miltenyi-Biotec) flow cytometers. Data were analysed by FlowJoVX (Tree Star Inc) software.

Image Stream cytometry

HBEpiCs were stained with anti-ICAM-1 and nuclear dye DRAQ5 and then analysed using Amnis Image Stream flow cytometer. After acquisition of focused single cells, we generated a compensation matrix, based on single-stained controls, performing our analysis with IDEAS 3.0 software. Briefly, we merged all acquisition files to apply a common gating strategy on $DRAQ5^{+}$ cells, thus evaluating simultaneously ICAM-1 expression in every experimental conditions. Fluorescence intensity of each channel was normalized respect to unlabeled control. Finally, we showed images of single cells,

comparing the profile of Brightfield (Ch01), ICAM-1 (Ch03), DRAQ5 (Ch11) and channel merge (Ch01+Ch03+Ch11).

Immunofluorescence assay

Adherent HBEpiCs were washed twice prior of fixation in 2% paraformaldehyde (30 minutes, RT) and permeabilization in 0.1% Triton (10 minutes, RT). Staining with primary mAb mouse anti-human E-cadherin (clone 67A4, Santa Cruz biotech) was performed in PBS-BSA 1% (45 minutes, RT). Likewise, we stained with secondary mAb Alexa Fluor 594 goat anti-mouse IgG1 (30 minutes, RT). Finally, HBEpiCs were counterstained with DAPI using ProLong® Gold Antifade Mountant (Life Technologies). Day after, 20x images were captured using an ApoTome microscope with AxioCam (Karl Zeiss, Thornwood, NY, USA).

RT-PCR and gel electrophoresis

mRNA was extracted from pellets of HBEpiCs by using RNeasy MicroKit (QIAGEN, Catalog Number: #74004,). cDNA was synthesized by using Quantitect Reverse Transcription Reagents (QIAGEN, Catalog Number: #205311,). AR (Hs00950669_m1 - ThermoFisher Scientific) and HBD-2 gene expression (Hs00175474_m1 - Thermofisher Scientific) was assayed by qPCR (Quant Studio DX real-time PCR system, ThermoFisher Scientific). mRNA content was normalized to b-actin expression. Mean relative gene expression was determined by using DDCT method.

The product of HBD-2 gene amplifications was verified through size determination, resolving it on 3% agarose gel stained with ethidium bromide.

Microbial culture conditions

Staphylococcus aureus bacteria, used for the antimicrobial sensitivity testing, was a clinical strain obtained from our *in-house* culture collection kept at the University of Messina. Bacteria were grown in Mueller Hinton Broth (MHB; Oxoid, Milan, Italy) and Tryptic Soy Agar (TSA; Oxoid, Milan, Italy) at 37°C for 18-24 hours.

Antimicrobial sensitivity testing

Starting from a standard concentration of bacterial suspension (10^8 CFU/mL) determined with a spectrophotometer, we diluted bacteria in HBEpiC supernatants to a final concentration of 100 CFU/mL. Then, samples were incubated in a thermostat shaker at 37°C. After 5 hours of conditioning, different dilutions of samples were plated in TSA plates, then incubated at 37°C for 18-24 hours. The analysis was performed by counting the bacterial CFU.

ELISA assay

Concentrations of cytokines (IL-23, IL-1b) and Amps (HBD-2, LL37) were measured on supernatants of DC cultures and saliva samples, respectively using validated commercially available ELISA kits. We used TMB ELISA kits for IL-23 (Catalog Number: #EH3270, FineTest), IL-1b (Catalog Number: #BGK01584, Biogens), and LL-37 (Catalog Number: #HK321-02, HycultBiotech) and ABTS ELISA kit for HBD-2 (Catalog Number: #900-K172, PEPROTECH).

Briefly, we set for each assay a standard curve, obtained by 7 dilutions of standard protein plus blank condition. First step, 100 µL of properly diluted standards or samples (Targets) were added to coated wells in duplicate and incubated at room temperature. Next steps provided the sequential use of diluted Biotinylated Antibody (Detection Ab), Avidin/Streptavidin-HRP (peroxidase) conjugate, then a properly substrate (ABTS or TMB) to start 20-30 minutes of colorimetric reaction. Only for TMB ELISA, peroxidase reaction was stopped with Stop Solution after no more than 30 minutes. Finally, optical density (OD) for color development was monitored at a specific wavelength for each ELISA

kit using AD200 Plate Reader (Beckman Coulter). Protein concentration of our samples was derived by standard curve of each plate.

Statistical analysis

A paired Student t test was used to evaluate statistical significance. $P < .05$ was considered statistically significant. Statistics were calculated using GraphPad Prism 6 software.

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