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New Insights into the Mechanisms of Action of Mechanical Bacterial Lysates in the Treatment of Respiratory Tract Diseases

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Bacterial lysates are widely employed in the treatment of respiratory tract infections. In this paper we show that human airway epithelial cells can directly recognize bacterial lysates obtained by mechanical lysis and, as a consequence, undergo a significant cell proliferation and up-regulation and *de novo* expression of different adhesion molecules involved in cell-cell junctions and cell-matrix adhesions, which represents critical structures for maintaining the epithelial barrier.

Key words: *respiratory tract diseases, treatment, bacterial lysates, mechanism of action.*

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Bacterial lysates have been introduced in the prevention of common human infections since the beginning of the past century. The original aim of a therapeutic approach based on bacterial-derived antigens was the induction of a specific adaptive immune response against pathogens invading different organs, in particular upper and lower respiratory tract infections.

Despite the wide use of this prophylactic approach and several evidences of efficacy [1] few information are yet available regarding the fine mechanisms of action of bacterial lysates. It is conceivable that bacterial antigen-based drugs should be able to mediate the maturation of dendritic cells (DCs) because of the presence, in the bacterial lysate, of a suitable amount of structures acting as ligands of the Pattern Recognition Receptor system expressed on circulating monocyte and immature DCs [2]. Indeed, it has been shown that the oral administration of chemically lysed bacteria of the respiratory tract was able to mediate the maturation of dendritic cells [3].

Likewise, we have previously shown similar results employing polyvalent mechanical bacterial lysate (PMBL), a commercial drug containing a mixture of microbial bodies obtained from mechanically killed bacteria, which included *Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus pyogenes, Hemophilus influenzae*, *Moraxella catarrhalis* and *Klebsiella pneumoniae*. In particular, we demonstrated a higher capacity of the bacterial strain mixture versus single bacterial strains to induce DC maturation, both in terms of expression of an activating phenotype and secretion of relevant cytokines and chemokines on different DC subsets, including monocyte-derived DC, circulating myeloid DCs and plasmacytoid DCs [4].

Remarkably, we have previously demonstrated that the weak naturally induced IgA secretion at salivary level could be significantly increased by the stimulation of mouth mucosa immunity with a Polyvalent Mechanical Bacterial Lysate (PMBL) [5].

Nevertheless, despite the clear evidence of DC activation and of a *de novo* secretion of anti-bacterial body IgA, the mechanisms of action of bacterial lysates appears to mainly rely on innate immune response [6] and, at present, still appears only partially clarified.

Because bacterial lysates are mainly employed in the treatment of respiratory tract infections, we should start to consider the complexity of the innate response in the airway epithelium, which represents the first point of contact for inhaled foreign organisms. The protective arsenal of the airway epithelium is provided in the form of physical barriers and a vast array of receptors and antimicrobial compounds that constitute the innate immune system. Many of the known innate immune receptors, including the Toll-like receptors and nucleotide oligomerization domain-like receptors, are expressed by the airway epithelium, which leads to the production of proinflammatory cytokines and chemokines that affect microorganisms directly and recruit immune cells, such as neutrophils and T cells, to the site of infection. The release of soluble factors by the airway epithelium activated by pathogens, and presumably by bacteri-

al lysates, can also affect the mucosal physical barrier, which itself represents part of the airways innate immunity.

In this paper we show that human airway epithelial cells can directly recognize bacterial lysates obtained by mechanical lysis and, as a consequence, undergo a significant cell proliferation and up-regulation and *de novo* expression of different adhesion molecules involved in cell-cell junctions and cell-matrix adhesions, which represents critical structures for maintaining the epithelial barrier.

Material and Methods

Bacterial Lysates. As bacterial lysates, the PMBL Ismigen® (Lallemand Pharma) was employed. Ismigen® represents a mixture of 13 lyophilized bacterial strains: *S. aureus, S. viridans, S. pneumoniae* (6 strains), *S. pyogenes, K. pneumoniae, K. ozaenae, M. catarrhalis* and *H. influenzae*. For our experiments, the bacterial lysate was resuspended in PBS at 5mg/ml, then added to the cell cultures at the final concentration of 100 µg/ml.

Cell cultures. The human NSCLC cell line A549 was purchased from Interlab Cell Line Collection of IRCCS AOU San Martino-IST-Istituto Nazionale per la Ricerca sul Cancro and cultured in RPMI medium supplemented with FBS 10% and penicillin/streptomycin. Human normal bronchial epithelial cells (HBEpiCs, Cat. No. 3210) were purchased from Sciencell and cultured in Human Bronchial Epithelial Cell Medium (HBEpiCM, Cat. No. 3211) supplemented with Bronchial Epithelial Cell Growth Supplements (HBEpiCGS, Cat. No. 3262) and penicillin/streptomycin solution (P/S, Cat. No. 0503).

Flow Cytometry. The following monoclonal antibodies to human proteins were used. From Miltenyi: APC-conjugated anti-EpCAM (dil: 1:100). From BD Biosciences: PE-conjugated anti-CD54 (ICAM-1, dil: 1:100), APC-conjugated anti-CD54 (ICAM-1, dil: 1:100), PE-conjugated anti-EpCAM (dil: 1:100), BV421-conjugated anti-CD324 (E-Cadherin, dil 1:50), BV421-conjugated anti-Ki67 (dil: 1:100). Intranuclear staining with BV421-conjugated anti-Ki67 was performed using the Fix/Perm buffer set by Miltenyi according to manufacturer's indications. Samples were then acquired using FACSCanto II cytometer (Becton Dickinson, Mountain View, CA) and data analyzed by FlowJo software.

Multispectral-imaging flow cytometry. For each experiment, cell lines (A549 and HBECs) were stained with relevant surface (EpCAM, ICAM-1) and intranuclear markers (Ki67) and suspended in 100 µl cold PBS in 1.5 ml Eppendorf tubes. Then, cells were analyzed by ImageStreamx (Amnis, Seattle, WA), a multispectral flow cytometer combining standard microscopy with flow cytometry. Up to 100 cells/s were acquired, with simultaneously acquisition of six images of each cell, including bright field, scatter and multiple fluorescent images. We used the integrated software INSPIRE to run the ImageStreamx. Before running the samples, the ImageStreamx was calibrated using SpeedBeads. Samples were acquired in the following order: unlabeled, single-color fluorescence controls, and finally, the experimental samples. Samples were always left on ice.

At least 10.000 cells/experimental sample and 2.000 cells/single-color control were acquired for each sample. Images were analyzed using IDEAS image-analysis software (Amnis).

Results and Discussion

Pathogens at a variety of mucosal surfaces enter epithelial cells to invade underlying tissues. This pathogenic mechanism is counteracted by epithelial exfoliation. In the distal mammalian gut that is continuously exposed to microbes, the epithelium is short-lived (turnover time 5 days). In the lungs that are only intermittently exposed to microbes, the epithelium is long-lived (turnover time 180 days) and epithelial shedding only occurs during infection or injury [7]. Thus, epithelial cells proliferation in the airways should be important for both prevention of pathogen invasion and for regeneration and maintenance of mucosal barrier integrity. Either NSCLC cell line A549 or the normal human bronchial epithelial cells (HBEpiCs) were cultured in the presence of bacterial lysates (Ismigen®, Lallemand Pharma) at the concentration of 100 µg/ml. After 24 h cells were harvested and analyzed for the expression of the

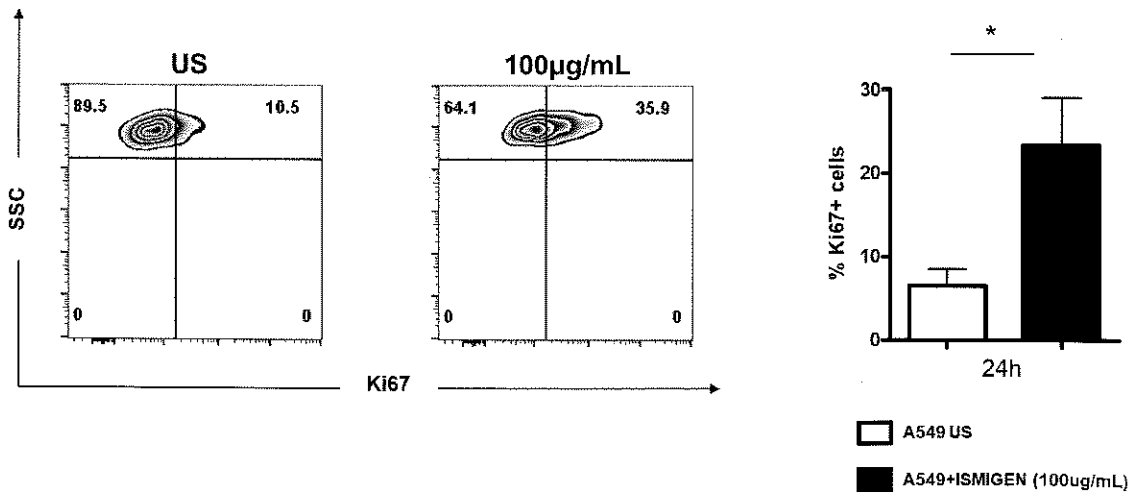

Fig. 1. **Lung epithelial cells proliferate in the presence of mechanical bacterial lysates.** Both normal and neoplastic epithelial cells express Ki67 upon contact with bacterial lysates. Data obtained with A549 cells are shown. US: unstimulated cells.

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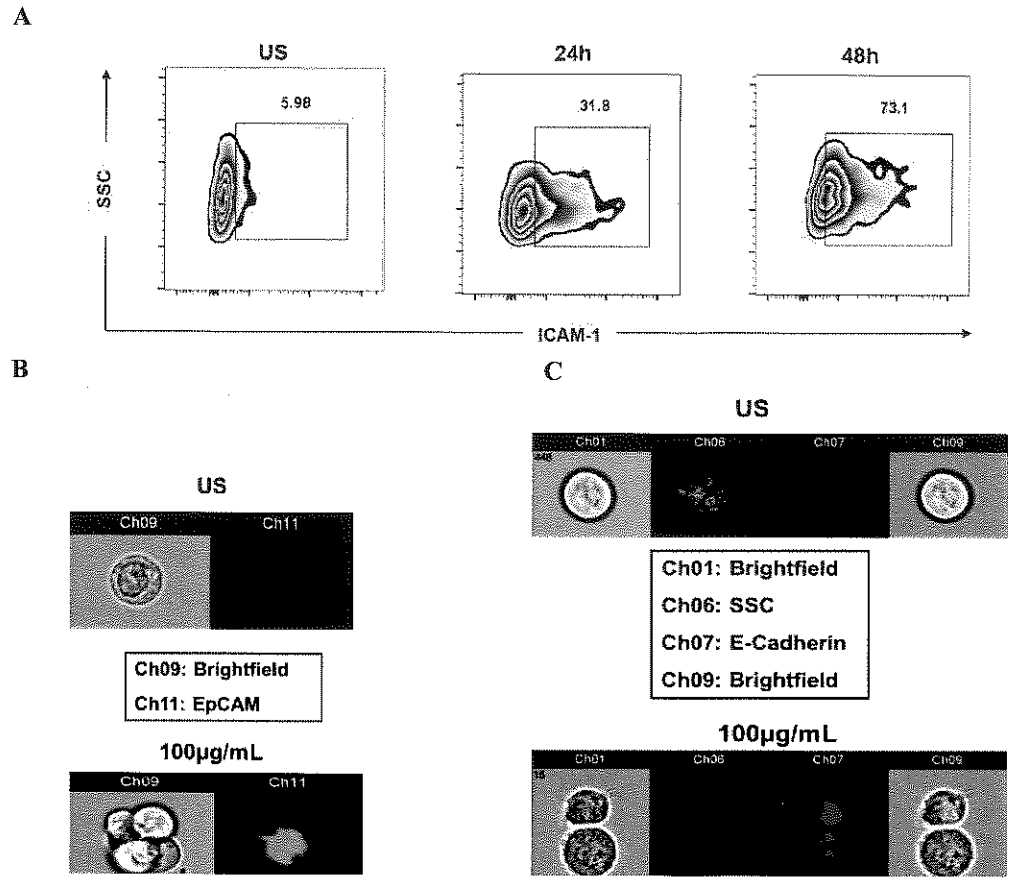

Fig. 2. **Bacterial lysates induce on human normal bronchial epithelial cells the expression of molecules involved in cell-cell junctions.** Up-regulation of ICAM-1 on bronchial epithelial cells is time-dependent; flow cytometry dot plot shows cell side scatter (SSC) versus ICAM-1 expression at two different intervals of time (A). Multispectral imaging flow cytometry shows that polyvalent mechanical bacterial lysates (PMBL) induces cellular aggregates and polarization of EpCAM (B) and E-Cadherin (adherens junctions) (C) on human bronchial epithelial cells.

proliferation marker Ki67 and of different adhesion molecules. Both in neoplastic and normal lung epithelial cells Ki67 was upregulated in the presence of bacterial lysates (Fig. 1).

Airway mucosal cells serve also as mechanical barriers to microbial entry. However, microbes have developed numerous strategies for crossing this barrier by passing between cells, entering and passing through cells. Conversely, since it is comprised of living cells, the epithelial barrier is capable of plasticity in its ability to resist microbial penetration. Indeed, epithelial barrier functions are modulated both by pathogens and as part of the host response, presenting a dynamic situation during infection. Clearly, a similar scenario can be envisaged when administered bacterial components come in contact with airway epithelial cells.

With the aim of investigating whether mechanical bacterial lysates contained in Ismigen® can improve inducible barrier function, we analyzed the expression of adhesion molecules involved in cell-cell junctions. Following culture of HBEpiCs in the presence of bacterial lysates, we observed up-regulation of ICAM-1, EPCAM and E-Cadherin on epithelial cells (Fig. 2). Remarkably, bacterial lysates induced and stronger physical association among epithelial cells (Fig. 2, panel B, C).

The efficacy of the lung epithelium in contrasting pathogen invasion has been often neglected relative to its roles in signaling to leukocytes and acting as a mechanical barrier. Similarly, the role of airway epithelium in mediating the beneficial effects of bacterial lysates-based drugs has been so far underestimated. This neglect is due in part to the requirement for stimulation before the antimicrobial capabilities of the lung epithelium become apparent, which would be overcome by the administration of bacterial lysates, and in part to being overshadowed by the dazzling array of antimicrobial activities displayed by the wide variety of human leukocytes.

Better understanding of inducible lung epithelial innate resistance in the presence of bacterial lysates is likely to lead to insight into the mechanisms of action of PMBL, in particular if loco-regionally administered, and on their ability to manipulate resistance therapeutically, not only to infections but also in the control of other inflammatory respiratory tract diseases, such as asthma and chronic obstructive pulmonary disease.

In these latter pathological conditions, the ability of PMBL to maintain the integrity of epithelial barrier and, at the same time, boost mucosal tissue regeneration could play a fundamental role in the control of the diseases.

References

1. Cazzola M., Anapurapu S., Page C.P. Polyvalent mechanical bacterial lysate for the prevention of recurrent respiratory infections: A meta-analysis // *Pulm Pharmacol Ther.* 2012. Vol. 25. # 1. P. 62–68.
2. Baral P., Batra S., Zemans R.L., Downey G.P., Jeyaseelan S. Divergent functions of Toll-like receptors during bacterial lung infections // *Am J Respir Crit Care Med.* 2014. Vol. 190. # 7. P. 722–732.
3. Zelle-Rieser C., Ramoner R., Bartsch G., Thurnher M. A clinically approved oral vaccine against pneumotropic bacteria induces the terminal maturation of CD83+ immunostimulatory dendritic cells // *Immunol Lett.* 2001. Vol. 76. #1. P. 63–67.
4. Morandi B., Agazzi A., D'Agostino A., Antonini F., Costa G., Sabatini F., Ferlazzo G., Melioli G. A mixture of bacterial mechanical lysates is more efficient than single strain lysate and of bacterial-derived soluble products for the induction of an activating phenotype in human dendritic cells // *Immunol Lett.* 2011. Vol. 138. #1. P. 86–91.
5. Rossi G.A., Peri C., Raynal M.E., Defilippi A.C., Risso F.M., Schenone G., Pallestrini E, Melioli G. Naturally occurring immune response against bacteria commonly involved in upper respiratory tract infections: analysis of the antigen-specific salivary IgA levels // *Immunol Lett.* 2003. Vol. 86. #1. P. 85–91.
6. Clement C.G., Evans.S.E., Evans C.M., Hawke D., Kobayashi R., Reynolds P.R., Moghaddam S.J., Scott B.L., Melicoff E., Adachi R., Dickey B.F., Tuvim M.J. Stimulation of lung innate immunity protects against lethal pneumococcal pneumonia in mice // *Am J Respir Crit Care Med.* 2008. Vol. 177. # 12. P. 1322–1230.
7. Evans S.E., Xu Y., Tuvim M.J., Dickey B.F. Inducible innate resistance of lung epithelium to infection // *Annu Rev Physiol.* 2010. Vol. 72. P.413–435.

Meeting Calendar

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