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ORIGINAL RESEARCH

EPS T14 from *Bacillus licheniformis* Prevents Infection of Human Nasal Epithelial Cells by Respiratory Viruses

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Background: Respiratory viral infections are a leading cause of severe diseases and mortality; therefore, novel treatments effective for their prevention are highly requested. Here, we identified a broad-spectrum antiviral activity of a natural exopolysaccharide, EPS T14, purified from a marine thermotolerant strain of *Bacillus licheniformis* strain T14.

Methods: The effects on human normal nasal epithelial cells (HNEpCs) following treatment with EPS T14 was evaluated at different time points and with increasing concentration of compound. To assess the antiviral properties, viability of HNEpCs treated with EPS T14 was analysed following infection with different respiratory viruses.

Results: Neither toxicity nor pro-inflammatory properties were observed in vitro on HNEpCs treated with EPS T14 up to high concentrations, thus ensuring its safety. Cell culture-based assays revealed that treatment of HNEpCs with EPS T14 (used at 400ug/mL) results in efficient prevention of cell infection by different respiratory viruses through physically hindering the entry of the viruses via cell surface receptors. Interestingly, in addition to this prophylactic antiviral activity, EPS T14 also shows a long-lasting efficacy by inhibiting viral spread in the cell culture. Finally, combination of EPS T14 with a hypertonic saline solution shows a synergistic antiviral activity.

Conclusion: EPS T14 can exert both prophylactic and therapeutic antiviral activity by blocking viral attachment to cellular receptors and could therefore represent a promising antiviral agent for preventing infections by different respiratory viruses.

Keywords: exopolysaccharides, infection prophylaxis, nasal epithelium, respiratory virus, viral infection, EPSs

Introduction

Despite successes of preventive measures and vaccinations, respiratory tract viral infections still have a significant impact on morbidity and mortality worldwide causing seasonal epidemics or unexpected pandemics. Although respiratory viruses are commonly responsible for a relatively mild and self-limiting syndrome, they have the potential to determine fatal illness with a mortality rate estimated in 2.7 million deaths.[1] Accordingly, the recurrent outbreaks of respiratory infections by common viruses or by potentially lethal viruses previously unknown, have recently raised concerns.

Several antiviral drugs have been found to be effective against respiratory viruses and therefore used as treatment options for severe respiratory infections; however, occurrence of side effects or emerging drug-resistant strains may limit their use. Therefore, the search of new agents with broad-spectrum antiviral effects is nowadays highly required for both preventing and treating respiratory viral diseases.

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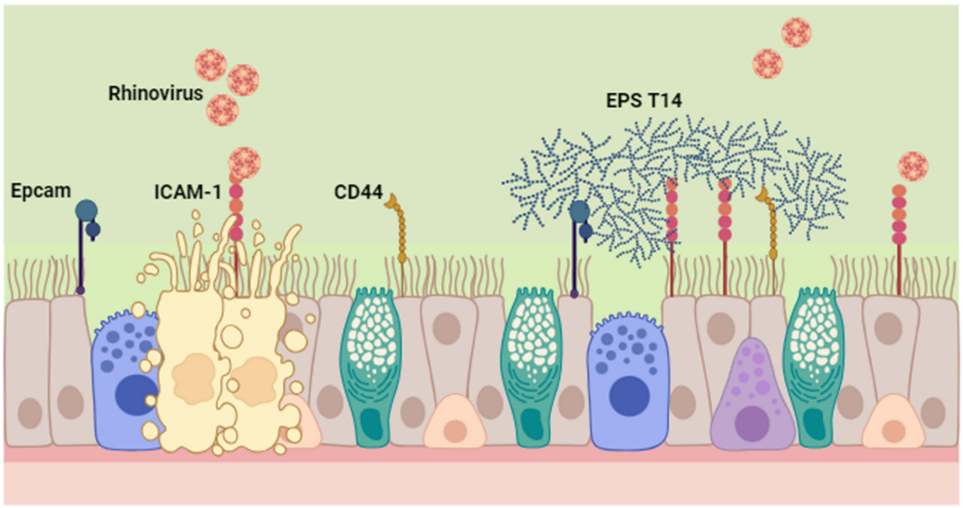
Graphical Abstract



The use of metabolic products derived from bacteria, as exopolysaccharides (EPSs), represents a promising approach for the treatment of viral infection.[2] EPSs are organic polymeric compounds with a composition highly heterogeneous and variable, that include carbohydrates (polysaccharides, as hexoses, pentoses, or amino sugars), proteins, lipids,[3,4] nucleic acids,[5] humic substances,[6] phosphates and sulphates.

Bacteria can release EPSs to the surroundings for several purposes, including protection against environmental stresses, hostile conditions as change in pH or temperature, enzymes or antibiotics and also to ensure interaction among bacteria.[7,8] Moreover, EPSs are essential for the initial bacterial adhesion and for biofilm formation on natural and abiotic surfaces.[9]

Depending on their chemical structure, EPSs were reported to possess physical, chemical and biological properties that can confer them certain antimicrobial and antiviral activities. Many studies have reported antiviral effects of EPSs against a wide range of viruses, such as herpes simplex type 1 (HSV-1) and 2 (HSV-2),[10,11] influenza virus,[12] rotavirus.[13]

However, the antiviral mechanisms of different EPSs vary widely, from virus inactivation, inhibition of viral entry or replication, to activation of the immune system[14] and strictly depend on EPSs intrinsic properties and related-bacterial producers. EPS from *L. plantarum* LRCC5310 showed antiviral activity against extracellular Rotavirus, interfering with its attachment to MA104 cells in vitro or hinder its replication via enhancing intestinal barrier function in vivo.[13] On the other hand, *L. delbrueckii* reduces viral replication and regulates inflammatory response stimulating the expression of the antiviral (IFN)-β by immune cells.[15]

Lately, marine microorganisms have gained interest as promising source of therapeutic compounds with ascertained antiviral activities.[16,17] In particular, marine organisms that live in extreme environments, *eg* in shallow hydrothermal areas, characterized by high temperatures and unusual chemical conditions, can produce new unexploited metabolites owing unique chemical composition and physicochemical properties. In this context, EPS T14, produced by *Bacillus licheniformis* strain T14, isolated from the fluid emitted from a shallow hydrothermal vent (at the sampling site the water T° was 50 °C and the pH was 5.42) off the Island of Panarea in the Eolian Archipelago, Italy. This compound has been previously reported as one of few molecules derived from marine bacteria that can exert antiviral and immunomodulatory effects against herpes simplex virus type 2.[18]

In the current study, we investigated the ability of water-soluble EPS T14, to prevent the infection of human normal nasal epithelial cells (HNEpCs) by a wide range of common respiratory tract viruses as: *RhinoVirus, AdenoVirus-2, Human Beta CoronaVirus OC43* and *Influenza A virus H1N1*, all able to efficiently infect human nasal epithelial cells. Our data revealed that EPS T14 can exert both prophylactic and therapeutic antiviral activity against several respiratory

viruses. Moreover, we uncovered one of the possible mechanisms of action of EPS T14 that rely on blocking the viral attachment to cellular receptors, which results in attenuation of viral infectivity. These findings can open interesting possibilities for the use of EPS T14 in preventing respiratory infections and for several other biomedical applications.

Materials and Methods

Cell Line Infection, Viruses and Compound

Normal human nasal epithelial (HNEpCs) cells (PromoCell, Cat. Num. C-12620) were seeded in 96-well flat-bottom plates (25,000 cells/well) and cultured until 80–90% of confluence with the specific serum-free basal medium (BEBM™ Bronchial Epithelial Cell Growth Basal Medium -CC-3171, Lonza) supplemented with additional factors to support cell growth and proliferation (BEGM™ Bronchial Epithelial Cell Growth Medium SingleQuots™ Supplements and Growth Factors -CC-4175). Cells were washed with phosphate buffered saline (PBS) and infected following recommended instructions for each virus. HNEpCs treated with the same conditions and reagents as the infected cells, but not exposed to the virus were used as controls (Mock-infected cells).

Human Influenza A Virus H1N1 (VR-1469™), Human Adenovirus 2 (VR-846™), Human RhinoVirus-16 (VR-283™), Human Beta Coronavirus OC43 (HCoV-OC43) were purchased from the American Type Culture Collection, ATCC. For viral propagation, Influenza A viruses were inoculated into HNEpCs at 35 °C, 5% CO2, in the presence of Roswell Park Memorial Institute (RPMI) 1640 Medium (Euroclone) supplemented with 1 mm HEPES (Gibco 15630–080), 0.125% bovine serum albumin (BSA) Fraction V (Gibco, 15260–037) and 1 µg/mL N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated Trypsin (Sigma-Aldrich, 4370285). Human Adenovirus 2, Human RhinoVirus-16 or Human Beta Coronavirus OC43 were propagated into HNEpCs in the presence of complete BEBM Medium in a humidified 5% CO2 atmosphere at 37°C or 33°C depending by virus growth condition. HNEpCs were infected with each viral strain at a multiplicity of infection (MOI) of 0.001 or 1 for 2 hours, rocking every 20–30 minutes to redistribute inoculum. After PBS washing to remove unabsorbed virus, mock-infected or infected cells were cultured for 48h or 5 days.

EPS T14, produced by the marine thermophilic *Bacillus licheniformis* T14 isolated from a shallow vent off Panarea Island (Italy) was purified and characterized as previously described. Briefly, bacteria were growth in bioreactor under optimal growth conditions (temperature 50°C, pH 8 and NaCl 5%) at the beginning of the stationary growth phase. Following 48h, EPS was recovered from culture medium, purified by gel chromatography and characterized for its physicochemical properties.[18,19] The product of fermentation undergoes a step of centrifugation. This step allows to isolate: 1) the biomass that will be removed; and 2) the supernatant that contains all the by-products of fermentation. Following different steps of filtration and ultrafiltration necessary to isolate EPS T14, the compound underwent a last step of lyophilization. For culture experiments, EPS T14 was resuspended in PBS and filtrated with 0.22um filter (Merk) before use.

Cell Culture-Based Antiviral Assay

To evaluate EPS T14 toxicity on HNEpCs, cells were treated with increasing concentrations of EPS T14 (from 50ug/mL up to 800ug/mL) for 2h at 37°C, 5% CO2 for 48h or 5 days. Cells cultured in medium alone or treated with cytotoxic 0.1% Saponin buffer were exploited as positive and negative controls of viability, respectively. For time-of-addition experiments, cells were cultured as follow: in the pre-treatment assay, HNEpCs were treated with EPS T14 (400ug/mL) for 2 h and then infected with each viral strain, following the above-mentioned inoculum procedures, at 1 MOI for 48h-culture or at 0.001 MOI for 5 days-culture; alternatively, HNEpCs were treated with EPS T14 for 2h, infected (at 0.001 MOI) and following washing to eliminate unabsorbed virus, the compound was added back once (*Adding*) or every day (*Daily replace*) in unchanged medium for 5 days. For synergic experiments, cells were treated with hypertonic solution at 2.7% NaCl alone or in combination with EPS T14 and then infected at 0.001 MOI for 5 days. In the post-treatment assay, HNEpCs were infected at 1 MOI, then treated with EPS T14 (400ug/mL) for 2 h and cultured for 48h. Unless otherwise indicated, in each step, cells were washed twice with PBS to remove non-bound compound or unabsorbed viruses. Mock-

infected or infected HNEpCs were cultured in complete BEBM Medium at suitable temperature, to allow replication of each virus and used as controls.

Flow Cytometric Analysis

To assess surface expression of ICAM-1, Epcam and CD44, HNEpCs were seeded in 96 well flat bottom and grown until confluence; cells were detached by using 0.05% trypsin solution for 3 minutes at 37°C, PBS-washed, then collected in FACS Polypropylene tubes to avoid clumps or aggregates. Cells were treated with EPS T14 (400ug/mL) for 2h, PBS-washed and then stained with anti-ICAM-1 (BD Biosciences), anti-Epcam (BD Bioscience) or anti-CD44 (Beckman Coulter) for 15 minutes at room temperature in the dark and analysed by flow cytometry. Staining of cells not treated with EPS-T14 or Fluorescence Minus One (FMO) were employed as positive and negative controls, respectively.

Intracellular staining for cytokine detection was performed in HNEpCs cultured in 96-well flat plates in complete BEBM Medium until confluence and then stimulated with EPS T14. Cells treated with IL-1β were employed as positive control.

To analyse the pro-inflammatory effects of EPS-T14, activation of dendritic cells (DCs) and the production of IL-6 and IL-8 cytokines by both DCs and HNEpCs was analysed. DCs were generated from peripheral blood monocytes cultured in the presence of rh-IL-4 (20 ng/mL; Miltenyi Biotec) and rh-GM-CSF (25 ng/ mL; Sargramostim, Leukine) in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 µg/ mL) for 6 days. DCs were treated with EPS-T14 (400ug/mL) and level expression of activation markers CD83 and CD40 was analysed following 24h by flow cytometry. DCs treated with TLR4-ligand LPS (1ug/mL) were used as positive control.

IL-6 and IL-8 production was analysed on HNEpCs and DCs following 24h treatment with EPS T14 (400ug/mL) by flow cytometry intracellular analysis as previously described.[20] Briefly, detached-HNEpCs and DCs were collected, PBS-washed, fixed in 1% paraformaldehyde for 10 minutes on ice in the dark, permeabilized with 0.1% saponin buffer (Sigma-Aldrich) and stained with the mAbs anti-IL6 and anti-IL8 (BD Bioscience) for 30 minutes in the dark at room temperature. Cells were then collected and analysed by flow cytometry. Cells stimulated with IL-1β (50 ng/mL, Miltenyi Biotec) were used as positive control.[21]

To assess cell viability, HNEpCs cultured under different experimental conditions were collected, PBS-washed and stained with the red-fluorescent dye TO-PRO3 (Sigma-Aldrich) for 10 minutes at room temperature and immediately analysed by flow cytometry. Cell viability was evaluated on both in suspension and adherent HNEpCs. The latter were previously detached by using 0.05% trypsin solution for 3 minutes at 37°C.

Statistical Analysis

Paired Student *t* test or one-way ANOVA test or linear regression test were applied to determine statistical significance. A p-value <0.05 was considered statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). GraphPad Prism software (GraphPad Software) was used for statistical analysis.

Results

EPS T14 from B. Licheniformis T14 Does Not Induce Cytotoxicity or Activation in Either Normal Nasal Epithelial Cells or Dendritic Cells

To assess whether EPS T14 could exert cytotoxic effects on HNEpCs in vitro, we treated cells with increasing concentration ranging from 50 to 800ug/mL for 48h.

Cell viability following EPS exposition was assessed by flow cytometry staining with the far red-fluorescent nuclear dye TO-PRO3, that represents a useful marker for identifying cell death. Our results revealed that viability of HNEpCs remained unaffected following EPS T14 treatment, as demonstrated by the low percentage of TO-PRO3 positive cells regardless of the concentrations employed (Figure 1A). Similar results were also obtained prolonging cell cultures up to 5 days (Figure 1B), thus indicating the safety of EPS T14 in cell culture.

EPSs derived from several bacterial strains have been reported to possess immunostimulatory properties, such as the ability to promote cell activation and pro-inflammatory cytokines production.[22–24] In order to establish whether EPS T14

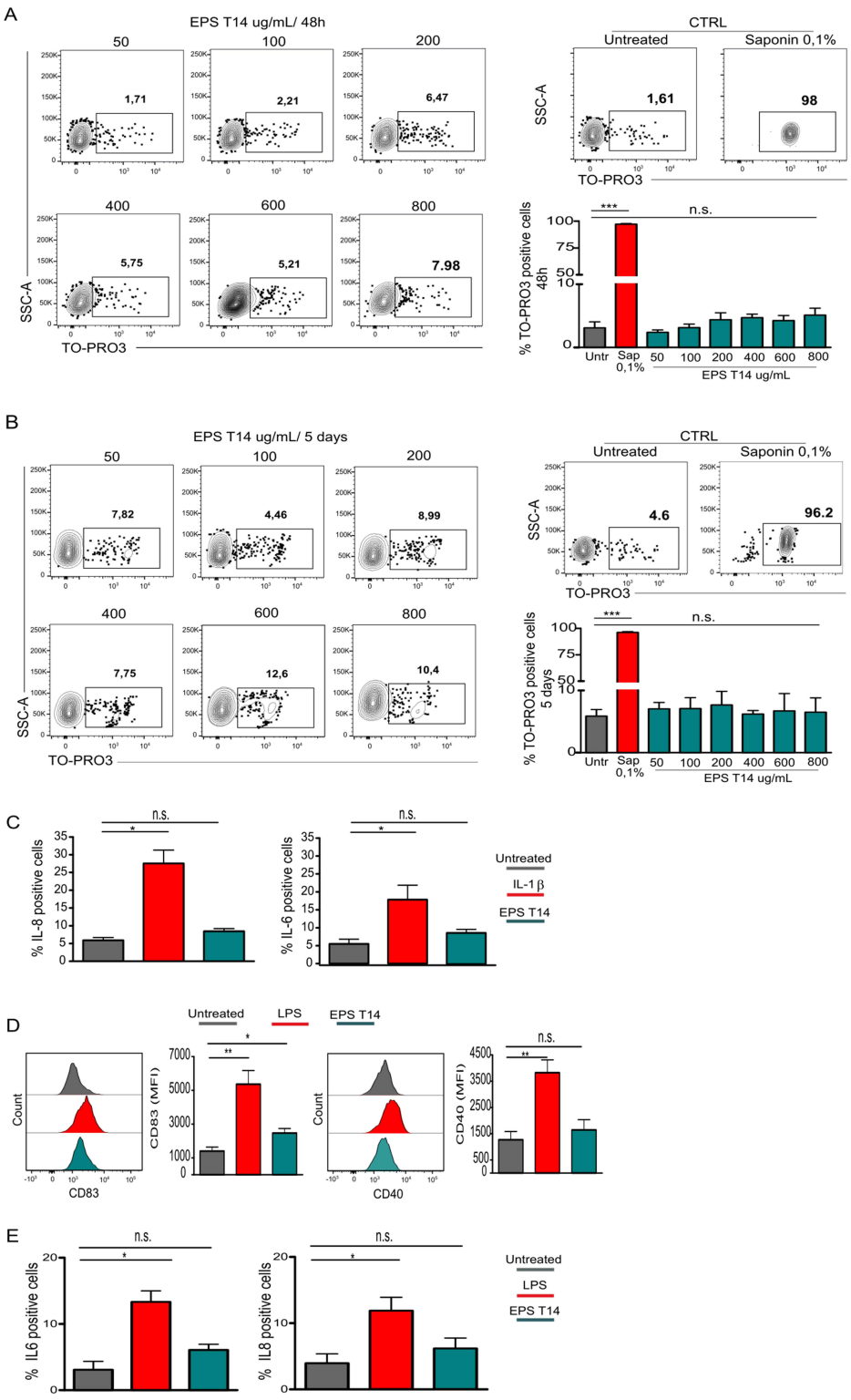


Figure 1 EPS T14 does not induce cytotoxicity or functional activation of both HNEpCs and DCs. Representative dot plots and relative statistical analysis show viability of HNEpCs cultured for 48h (**A**) or 5 days (**B**) with EPS T14 at different concentrations, assessed by flow cytometry. Bars represent mean values ± SEM of TO-PRO3 positive HNEpCs obtained in five independent experiments. Cells cultured in medium alone or treated with Saponin 0.1% were used as controls. (**C**) IL-6 and IL-8 production by HNEpCs following 24h of stimulation with EPS T14 (400ug/mL) was assessed by flow cytometry intracellular staining. Cells unstimulated or stimulated with IL-1β were employed as negative and positive control, respectively. Bars represent mean values ± SEM of the percentage of cytokine producing-HNEpCs obtained in four independent experiments. (**D**) Histograms represent expression of DC activation markers, CD83 and CD40, assessed on DCs treated with EPS T14 (400ug/mL) for 24h. DCs treated with TLR4-ligand LPS were employed as positive control. Bars represent mean values ± SEM of MFI of indicated markers. (**E**) IL-6 and IL-8 production by DCs treated with EPS T14 (400ug/mL) was assessed by intracellular staining. DCs treated with TLR4-ligand LPS were employed as positive control. Bars represent mean values ± SEM of positive cells. *p<0.05; **p<0.01; ***p<0.001.

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treatment might have the potential to induce biological activities on HNEpCs, their production of Interleukin (IL)-6 and IL-8 pro-inflammatory cytokines were analysed following exposure to EPS T14. Following 24h of cell culture, no cytokine production by EPS T14-treated HNEpCs was observed (Figure 1C). Moreover, at the mucosal site, the inflammatory process can also depend by the activation of DCs.[25] These latters, as professional antigen presenting cells, are equipped with a wide repertoire of receptors for the recognition of pathogen associated molecular patterns (PAMP) able to trigger a pro-inflammatory events. Therefore, to understand whether EPS T14 can be recognized as a potential PAMP, the functional profile of DCs was analysed following their exposure to the compound. DCs stimulated by EPS-T14 did not show expression of typical activation markers, CD83 and CD40, and no IL-6 and IL-8 production was observed (Figure 1D and E). Altogether these results indicate that EPS T14 does not exert either a direct pro-inflammatory effect on HNEpCs nor an indirect activation of flogosis via DC stimulation.

EPS T14 Prevents Viral Infection of Human Normal Nasal Epithelial Cells by Blocking Viral Entry

It has previously been reported that bacteria-derived EPSs can be effective against a wide range of viruses.[26] Thus, to examine whether EPS T14 could exert antiviral activity and in which step of infection it could be more effective, the compound was added to HNEpCs culture at different time points, i.e., before (pre-treatment), or after (post-treatment) infection with *RhinoVirus*, one of the main viruses affecting the respiratory tract, used at high multiplicity of infection (1 MOI) (Figure 2A). The progression of infection was monitored following 48h of culture by evaluating the percentage of TO-PRO3 positive cells. Data from our experiments indicated that EPS T14 pre-treatment resulted in significant protection of HNEpCs from *RhinoVirus* infection, as demonstrated by the low percentage of TO-PRO3 positive cells, while a less protective effect was observed when cells were treated with EPS T14 after the viral inoculum (Figure 2B).

Considering the prophylactic efficacy showed by EPS T14 against *RhinoVirus* infection, we tested whether similar effects could be obtained against other pathogenic respiratory tract viruses. Thus, EPS T14-pretreated HNEpCs were infected with *Human AdenoVirus-2, Human Beta CoronaVirus OC43 or Influenza A H1N1* virus (each used at 1 MOI), and cell viability was analysed by flow cytometry following 48h of culture. Under these experimental conditions, pre-treatment with the EPS T14 significantly prevents cell infection by each virus analysed (Figure 2C).

These results demonstrate that EPS T14 can significantly protect HNEpCs from infection by a large spectrum of viruses, and thus it might be effective for prophylactic treatment of several respiratory viral infections.

EPS T14 Limits the Availability of Host Cell Receptors for Viral Binding

It has been described that EPSs exert their activity by several mechanisms of action,[27,28] e.g., by impeding viral binding to their cellular receptors.[29,30] Thus, we hypothesized that EPS T14 could exert its antiviral activity by physically covering HNEpCs surface membrane and thus preventing viral binding to the specific entry receptor. To verify this hypothesis, we took advantages from specific monoclonal antibodies and flow cytometry to examine, on HNEpC cell membrane, the expression of ICAM-1, an adhesion molecule representing the main receptor involved in *RhinoVirus* entry into the cells.[31] The detection of ICAM-1 on HNEpCs significantly decreased following EPS T14 treatment, suggesting that the compound can hide *RhinoVirus* surface receptor, preventing the attachment of the virus to the target cells and thus limiting its infectivity (Figure 3). Moreover, to assess whether EPS T14 masking effect was random or targeting specific receptors, we also analysed the expression of epithelial cell adhesion molecule (Epcam) and CD44, non-kinase transmembrane glycoproteins, both representing constitutive molecules on cell surface. The concomitant lower expression of Epcam and CD44 observed in our experimental setting, suggests that EPS T14 attachment to the cells occurs randomly and not selectively, likely due to electrostatic and chemical bindings. Our data suggest that EPS T14 might intrude viral entry process, limit viral adsorption and the subsequent onset of infection, thus providing a mechanism of action by which EPS T14 can exert its antiviral activity.

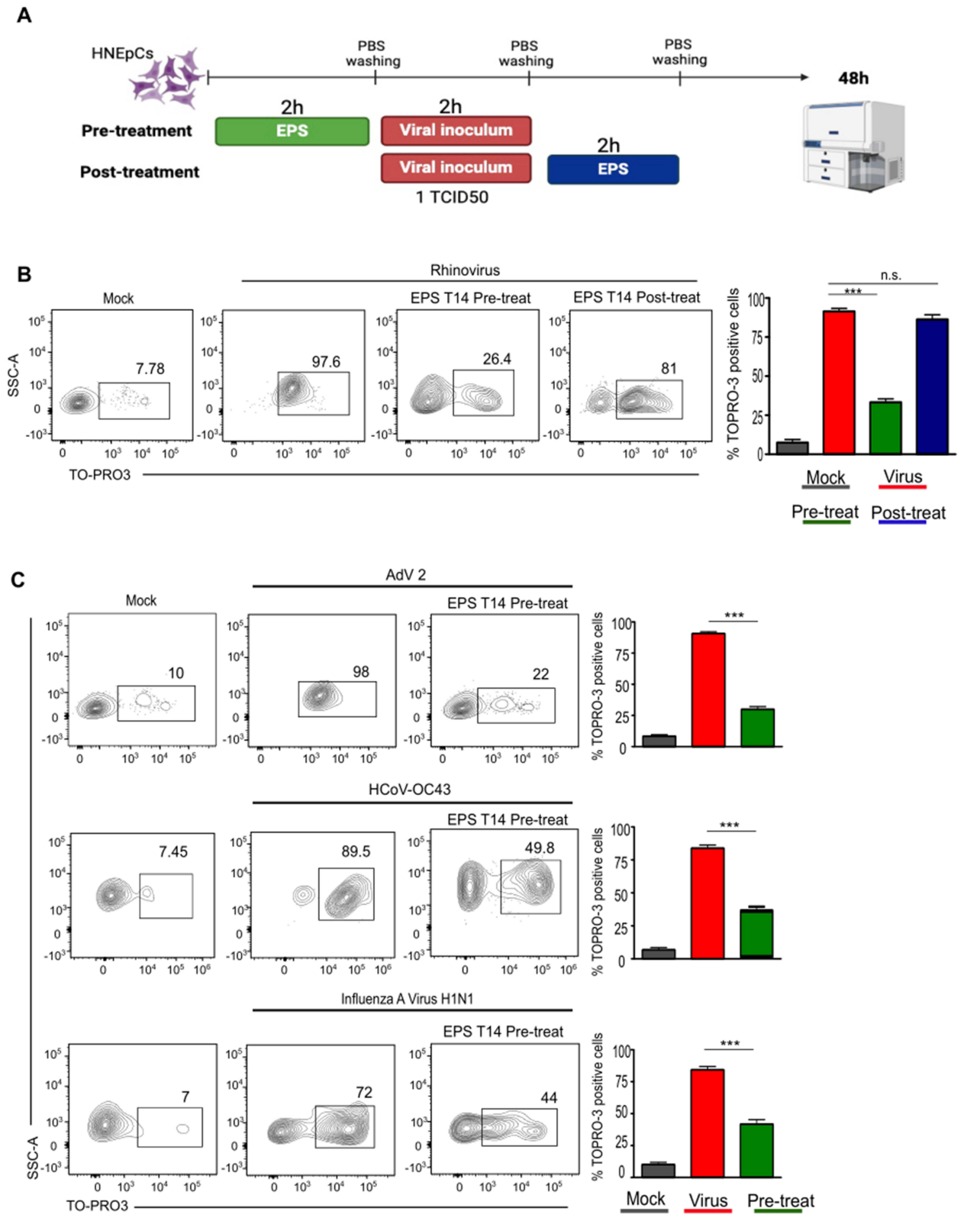

Figure 2 Treatment with EPS T14 protects human nasal epithelial cells from respiratory tract viral infections. (**A**) Schematic representation of EPS administration schedule and viral infection created with Biorender. (**B**) Representative dot plots and statistical analysis show 48h-viability of HNEpCs treated with EPS T14 (400ug/mL) before (pre-treat) or following (post-treat) infection with *RhinoVirus*, assessed by flow cytometry. Bars represent mean values ± SEM of the percentage of TO-PRO3-positive HNEpCs obtained in five independent experiments. (**C**) Representative dot plots and statistical analysis show 48h-viability of HNEpCs pre-treated with EPS T14 (400ug/mL) before infection with *AdV-2, HCoV-OC43 or Influenza A H1N1*, assessed by flow cytometry. Bars represent mean values ± SEM of the percentage of TO-PRO3-positive HNEpCs obtained in five independent experiments. Mock-infected or infected cells were employed as controls. ***p<0.001.

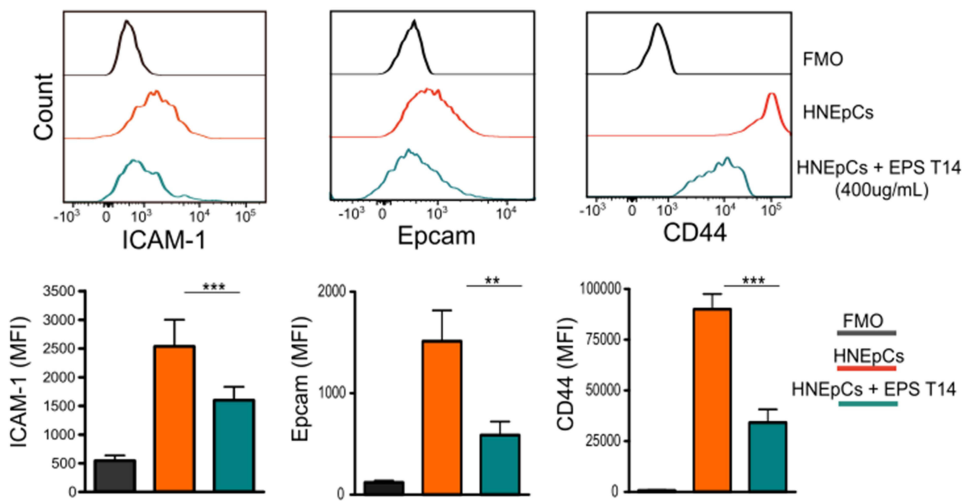

Figure 3 EPS T14 prevents viral binding to host cell receptors. Histograms and related statistical analysis show level of ICAM-1, Epcam and CD44 detected by fluorochrome-conjugated specific monoclonal antibodies on HNEpCs. Analysis was performed after 2h following EPS T14 addition. Bars represent mean values ± SEM of mean fluorescence intensity (MFI) for each molecule. **p<0.01; ***p<0.001.

EPS T14 Exerts Long-Lasting Antiviral Activity on Human Normal Nasal Epithelial Cells

Spread of infection relies on the production of new viral particles from infected cells, which can then propagate infection in neighbouring cells. Therefore, we tested whether EPS T14 could also exert antiviral activity on HNEpCs by inhibiting the progression of viral spreading. To address this issue, EPS T14 pre-treated-HNEpCs were infected using lower MOI for each respiratory viruses (0.001 MOI) and cell culture was extended up to 5 days to allow viral spread.

Pre-treatment with EPS T14 before the viral inoculum results in long-lasting protection of non-infected cells to secondary infections due to the release of virions (Figure 4). Based on this data, we further investigated whether, along with pre-treatment, a consistent presence of EPS T14 into culture could boost the already observed antiviral effect. To

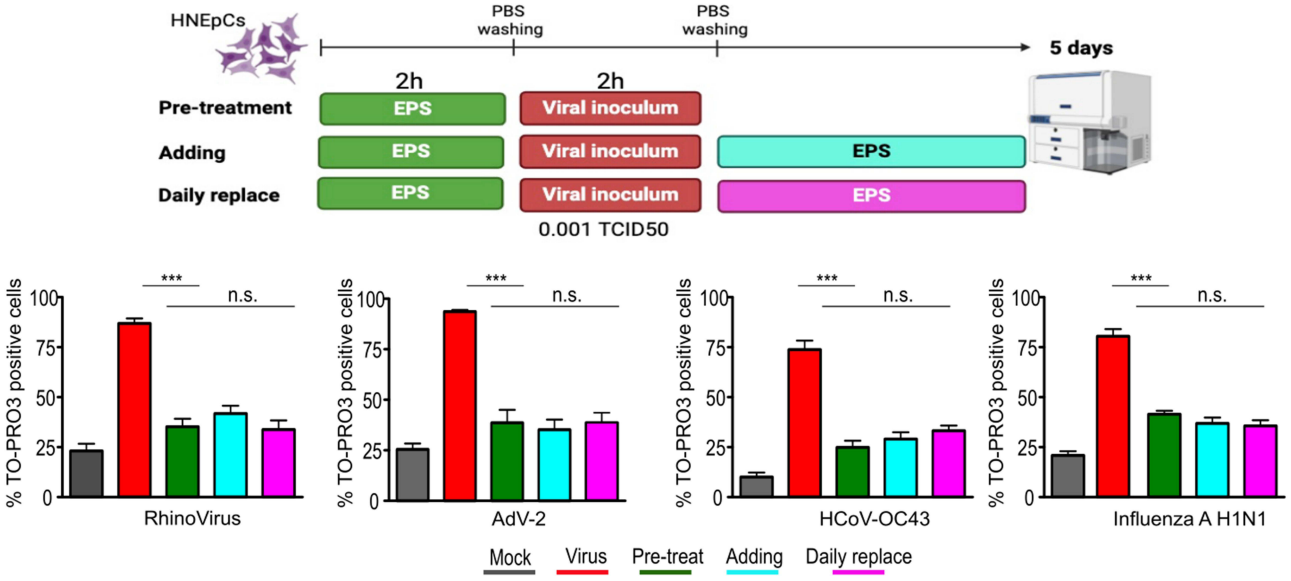

Figure 4 Pre-treatment with EPS T14 exerts an effective and long-lasting antiviral activity preventing viral spreading. Schematic representation of EPS administration schedule and viral infection created with Biorender (upper). Viability of HNEpCs treated with EPS T14 before viral inoculum (at 0.001 MOI for each virus) was assessed by flow cytometry following 5 days of culture; where indicated, EPS T14 were re-added once at the beginning of culture or daily replaced for 5 days in unchanged culture medium (lower). Bars represent mean values ± SEM of the percentage of TO-PRO3-positive HNEpCs obtained in five independent experiments. Mock-infected or infected cells were employed as controls. ***p<0.001.

this aim, following viral inoculum, EPS T14 was: 1) added back once; or 2) daily replaced for 5 days in unchanged culture medium. However, further addition of EPS T14 did not result in a statistically significant increase of cell protection (Figure 4), suggesting that EPS T14 administrated before infection is stable in culture and sufficient for exerting antiviral effects.

Combined Treatment of EPS T14 and Hypertonic Saline Solution Improves Antiviral Efficacy

It has been previously reported that hypertonic NaCl solution can exert some protective effect against different viruses.[32] Thus, we investigated whether EPS T14 and NaCl solution 2.7% can exert a synergistic effect in preventing the infection of HNEpCs by respiratory viruses. To address this issue, cells were pre-treated with EPS T14 in combination with Hypertonic solution 2.7% NaCl, infected with high 1 MOI by *RhinoVirus, AdenoVirus, Human beta CoronaVirus or Influenza A H1N1* and cell viability was analysed following 48h of culture. Although hypertonic saline solution can exert a distinct antiviral activity on its own, the treatment with EPS T14 alone was consistently more effective in cell protection, as demonstrated by the lower number of TO-PRO3-positive dead cells compared to hypertonic solution. Nevertheless, the combination of EPS T14 and hypertonic saline solution shows a significant synergistic effect which result in higher protection of HNEpCs. (Figure 5).

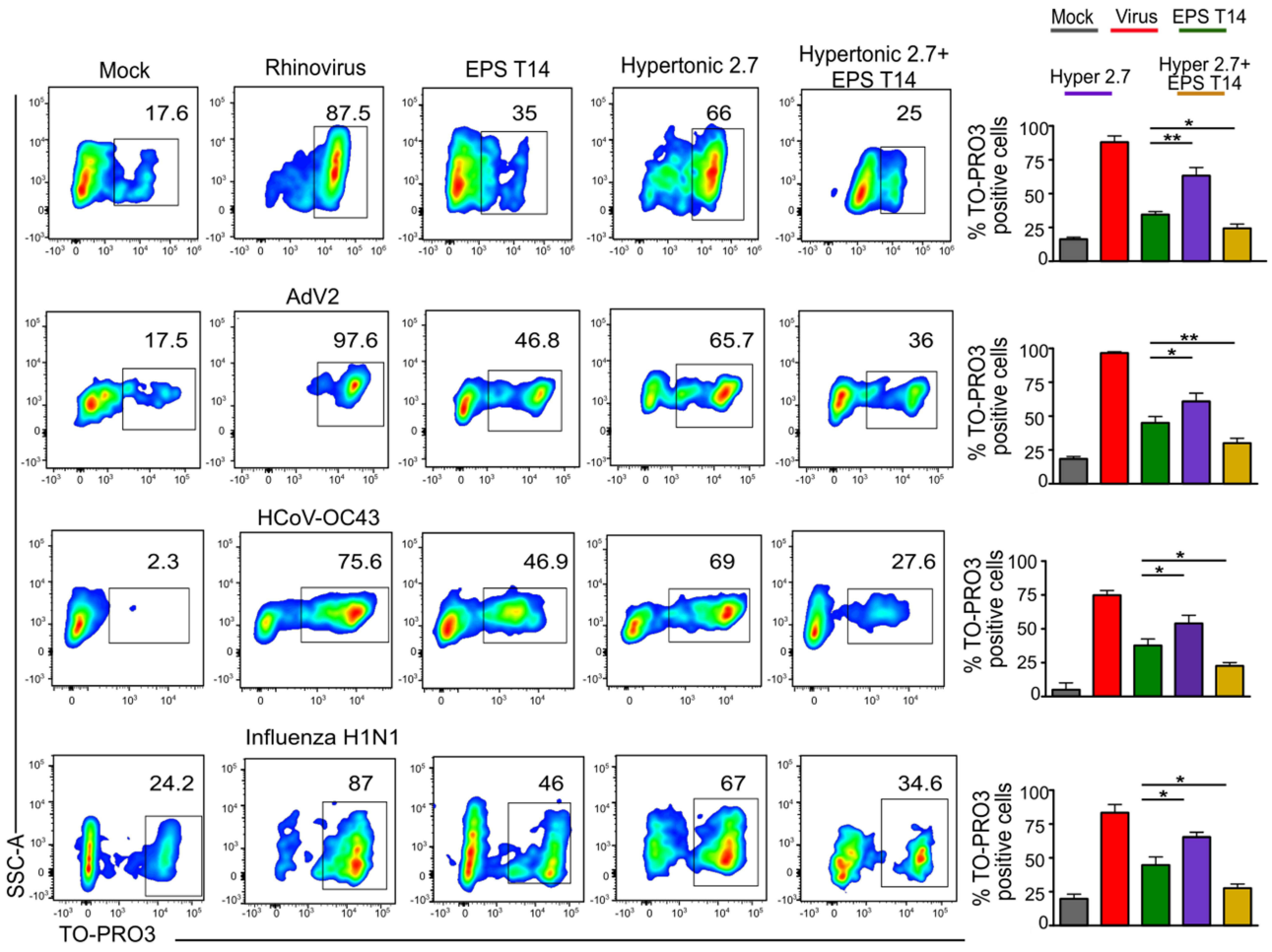

Figure 5 EPS T14 and hypertonic saline solution exhibit synergistic antiviral effect on infected human nasal epithelial cells. Representative dot plots and related statistical analysis show 48h-viability of HNEpCs treated with EPS T14 (400ug/mL), 2.7% hypertonic NaCl solution or their combination, before infection with *RhinoVirus, AdenoVirus, Human beta CoronaVirus, or Influenza A H1N1* (1 MOI), assessed by flow cytometry. Bars represent mean values ± SEM of the percentage of TO-PRO3-positive HNEpCs obtained in four independent experiments. Mock-infected or infected cells were employed as controls. *p<0.05; **p<0.01.

Discussion

In the last decades, microbial-derived EPSs gained attention as new emerging antiviral products for the treatment of infections of the respiratory and gastrointestinal tract, as well as cancer and allergic diseases.[33–35]

Although their protective mechanisms are often not fully understood, EPS properties seem to rely on their unique chemical and physical structures, depending, in turn, by bacterial species producing EPSs. Thus, considering that EPS properties are strictly strain-specific, the search for new strains is nowadays of great interest. The diversity of marine microorganisms, still largely unexplored, offers great opportunities to discover new molecules. For instance, marine-derived EPSs isolated from deep-sea hydrothermal vents were reported to be efficient against HSV-1,[19] and HSV-2.[26]

Here, we successfully observed the antiviral properties of EPS-T14, derived by the thermophilic *B. licheniformis* T14.[18] After assessing the absence of toxicity for human normal nasal cells, even at the highest concentrations, we showed that EPS T14 has efficient and broad antiviral activity against a series of viruses commonly causing respiratory tract infections, such as *RhinoVirus, Influenza type-A, Human CoronaVirus* and *AdenoVirus type-5*.

By using different treatment schemes, our results suggested that EPS T14 can display a protective effect, particularly when used before the viral inoculum. Similarly, levan product, produced by a novel honey *Bacillus subtilis* isolate, was reported to possess antiviral activity against pathogenic avian influenza HPAI, H5N1 (RNA virus) and adenovirus type 40 (DNA virus), when cells were pre-treated 1h before inoculation, while it had no effects when used simultaneously to virus inoculum or after viral adsorption.[36]

Although, in our setting, the post-treatment with EPS T14 resulted in weak cell protection to infections, it has been described that EPS 26a, produced by *Lactobacillus* spp. strains, can completely suppressed viral infection only when added to cells at the end of viral adsorption period.[37] These conflicting data could rely on the compound structural diversity, which could significantly influence the antiviral mechanisms. Indeed, some compounds can act as virucidal agents by directly damaging the virions, impeding several steps of viral infection, as adsorption, viral entry or the late stages of viral replication, or wrapping the virions via electrostatic interactions.[26]

However, based on our current results, we favour the hypothesis that the antiviral mechanism of EPS T14 relies on covering the cell membrane leading to the production of a dense network structure that can mask host cell molecules used for viral entry. More specifically, EPS layer might shield specific adhesion factors on the cell surface and electrostatically interfere with the binding to receptors, thus hindering the adhesion process and the recognition mechanisms which are required for stable adherence of viruses on cells. The lower detection of the constitutively molecule ICAM-1, exploited as rhinoviral entry receptor, could confirm our assumption regarding the masking properties of EPS T14. Supporting this hypothesis, following a short-term treatment of EPS T14 (2h), we observed the reduction of ICAM-1 expression, which, given the short time of treatment, is likely due to a physical coverage by EPS T14 rather than to its internalization for recycling. Accordingly, we failed to detect a higher intracytoplasmic level of ICAM-1 following EPS-T14 treatment (not shown). Therefore, EPS T14 might intrude viral entry process, thus limiting viral adsorption and the subsequent onset of infection. Importantly, EPS T14 attachment to the cells seems to occur randomly, indicating that EPS T14 is not able to cover selectively specific molecules/receptors on cell surface. Although in the current work we did not assess the expression of host receptors entry for each viruses analysed, the above proposed antiviral mechanism of EPS T14 may likely be applied to the other viral infections.

It has been described that several EPSs can also exert antiviral effect by activating immune cells and inducing them to secrete cytokines.[14] In this regard, an EPS produced by the marine thermotolerant *Bacillus horneckiae* was shown to possess antiviral activity against HSV-236 and HSV-110 via stimulation of inflammatory cytokines gene transcription on immune cells.[38] However, EPS T14 does not display properties able to affect either immune or non-immune cells, as it is not effective in terms of activation and production of inflammatory cytokines in both nasal epithelial cells and monocytes derived-DCs, thus underlying the absence of any alleged pharmacological activity. Taken together, our data suggest that EPS T14 can limit the infection of human nasal epithelial cells by several respiratory viruses and this ability can last up to 5 days, at least in vitro, thus ensure a long-lasting protection. Moreover, the improved antiviral efficacy observed with EPS T14 and hypertonic solution in combination could pave the way for new combinatorial treatment. Considering these

properties, EPS T14 could represents a promising antiviral agent for the prevention of common respiratory infectious diseases.

Conclusion

EPS T14 displays antiviral activity against several respiratory viruses by preventing viral binding to host cell receptors. Its viral prophylactic ability is not specific and therefore it might be exploited against different variants or different viruses, for instance when distinct respiratory viruses are circulating concurrently or even in the absence of a suitable antimicrobial therapy. Microorganisms producing EPSs, as *Bacillus licheniformis* strain T14, represent renewable resources for the production of these compounds, which represents an advantage for medical applications. Conversely, a main disadvantage in the use of this type of compound could be their poor bioavailability at lower airways level, although adequate administration strategies, such as oropharyngeal or intranasal, should lead to a protective effect. Safety and broad-spectrum of the antiviral activity of EPS T14 would make it valuable for either prophylactic or therapeutic use in several infectious respiratory diseases. Thanks to its biocompatibility and functional properties, EPS T14 might therefore represent an interesting additional tool not only for reducing viral infections but also for suitable future clinical applications as scaffolds, drug delivery systems, coating materials for medical devices, and surgical sealants. However, in this study, EPS T14 properties were tested only for infections of human epithelial cells of the upper airways caused by viruses commonly affecting the respiratory tract; whether a similar antiviral efficacy might be observed also in the context of infections by viruses with different tropism and different tissue targets, still remains to be further investigated.

Abbreviations

EPS, exopolysaccharides; HNEpCs, human nasal epithelial cells, DCs, dendritic cells; MOI, multiplicity of infection.

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Author Contributions

All authors made a significant contribution to the work reported, from conception, study design, execution to acquisition of data, analysis and interpretation; all authors took part in drafting, revising or critically reviewing the article; endorsed the final version of manuscript for publication; have agreed on the choice of the journal for submission; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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