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Exploring airway biological barriers: Implications for IgG-based therapeutics in respiratory infections

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KEYWORDS

Bronchiectasis, IgG, anti-infectious, inhalation, biological barriers

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AUTHOR CONTRIBUTIONS

NHV and TS designed the study and supervised the work. GP and CP performed the experiments. SG, AS and AS provided the polyclonal IgG and data from human samples. SG, AS and AS provided expertise regarding bronchiectasis. NHV wrote the initial manuscript, and all authors contributed to the subsequent drafting and revision process.

CONFLICTS OF INTEREST

The current study is part of a collaboration between CSL Behring and CEPR, with financial commitment between the two structures. N.H.V. has been paid as a scientific advisor (Novartis, European Commission, Immune Biosolution, ANR, Alvea/Telis Bioscience) and her group has financial relationships with private-sector entities (Aerogen, Nemera, Aptar Pharma, Reysica) outside the scope of the present work.

Abstract

Despite the considerable value of prophylactic vaccines and post-exposure antimicrobials, respiratory infections represent a huge burden. The control of bacterial respiratory infections is compromised by the alarming rise in antibiotic resistance, which underscores the requirement for alternative or complementary interventions. Recent studies have demonstrated that anti-infective IgG-based therapeutics can have an important role in tackling pathogens, and that inhalation is a promising route to target respiratory infections in the lung. Here, we investigated the impact of physical and biological barriers encountered by inhaled IgG during respiratory infections. These include hyper-production and thickening of mucus, as well as bacteria embedded in biofilm. Experiments using artificial bronchiectasis-like mucus showed reduced mobility and pathogen-binding of IgG as compared to artificial mucus with healthy characteristics. Our findings highlight the reduced ability of IgG to effectively opsonize pathogens and trigger effector functions during respiratory infections, such as the ones associated to bronchiectasis. In parallel, IgG was able to disrupt *P.aeruginosa* biofilm integrity upon repeat administrations *in vitro*. These results support the use of locally applied human polyclonal IgG in infection-driven chronic lung diseases and highlight the challenges to consider for the development of inhaled IgG.

Introduction

Respiratory infections, whether they are acute or recurrent, constitute a world-leading cause of death in humans and represent a huge burden in terms of direct loss of life, health service costs, and indirect economic costs (due to staff absence from work and production losses) [1–3]. Respiratory infections are primarily attributable to bacteria and viruses, which enter the respiratory tract and affect/spread to the lungs, where the infection may become chronic or cause life-threatening pneumonia. The current therapeutic arsenal against respiratory pathogens comprises prophylactic vaccines and antimicrobials, mostly small-molecule drugs like antibiotics and antivirals [4]. Today, the rise of antimicrobial resistance complicates the management of respiratory infections. For instance, antibacterial resistance has been declared a significant public health threat worldwide by the World Health Organization, which emphasizes the requirement for alternative or complementary interventions [5,6].

Among them, IgG was successfully used in patients with bacterial and viral infections, such as preventing HIV, recurrence of *Clostridium difficile* infections and treating Ebola virus disease. For respiratory infections, several IgG-derived therapeutics have been approved and act by directly inhibiting the pathogen [7,8]. Over time, several important lessons have been learned, and it is now acknowledged that the three critical variables for the effectiveness of anti-infective IgG are dose timing and dose level to achieve a rapid therapeutic concentration at the target site and broad-spectrum coverage to reduce the risk of resistance. Anti-infective IgG-derived therapeutics are usually administered parenterally, and the challenge is to provide an effective concentration in the infected tissues rather than systemically.

In recent years, preclinical evidence indicates that direct delivery of anti-infective IgG to the lungs is more effective than its delivery by other routes to prevent and/or control respiratory infections, requiring reduced doses to achieve a therapeutic/prophylactic response with a rapid onset of action. Protection mediated by local IgG is associated with better control of the pathogen load, a fast resolution of lung inflammation and a favorable pharmacokinetic profile [9–14]. Moreover, airway delivery of IgG - by inhalation - has recently been investigated in preclinical studies with some accomplishment in preventing severe pneumonia caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [15]. Thus, delivering IgG directly to the lungs may be a promising prophylactic approach against respiratory infections in high-risk individuals.

The airway environment is modified during respiratory infections to contain and clear the pathogen. On the other hand, pathogens usually adapt to survive the host environment, counteracting treatment and colonizing the respiratory tract [16,17]. We hypothesized that the changes occurring in the airways and pathogens during respiratory infections may affect the pharmacokinetics and pharmacodynamics of endogenous IgG and anti-infective IgG-derived therapeutics delivered by inhalation. For instance, we have previously shown that the massive release of proteases into the airways and alveolar spaces during pneumonia acted as a biological barrier, degrading IgG. Moreover, we found that IgG2 were more resistant to the proteolytic airway environment associated with pneumonia [18]. To better understand the impact of airway alterations associated with respiratory infection, we investigated how the rheological alterations of mucus, as well as bacteria embedded in biofilm, may interfere with IgG binding to their cognate antigen [19,20]. As a model of endogenous IgG and IgG-based therapeutics, we used human plasma-derived IgG from healthy donors [21]. A solution of plasma-derived polyvalent IgG administered via inhalation of an aerosol produced using a nebulizer (CSL787) is currently being investigated in the clinic as a promising novel approach to prevent chronic respiratory tract infections and progression of chronic lung disease in bronchiectasis. In the Phase 1 clinical trial (NCT04643587), antibacterial effects were observed following 15 days of once daily dosing in subjects with mild bronchiectasis tested positive for the presence of ≥ 1 of 6 types of bacteria (including *Pseudomonas aeruginosa*). Bronchiectasis is a chronic, progressive lung disease marked by permanent bronchial dilation, chronic cough, sputum production, and recurrent infections. Its pathophysiology involves infection, inflammation, impaired mucociliary clearance, and structural damage, creating a self-

perpetuating cycle of lung decline. Common pathogens include viruses (e.g. rhinovirus, influenza, coronavirus) and bacteria (e.g. *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*) [22]. Immune deficiencies, especially antibody-related, increase infection risk and contribute to disease [23–26]. Even with adequate systemic replacement of IgG and IgG reaching the respiratory tract, lung inflammation and microbial imbalance persist in immunodeficient individuals [27]. In the present study, we produced artificial healthy- and bronchiectasis-like mucus and biofilm derived from *Pseudomonas aeruginosa* to better understand the impact of the physical/biological alterations associated with airway diseases on IgG. Our findings help to understand the inability of endogenous IgG to control respiratory pathogens during chronic respiratory infections. Additionally, they provide a better mechanistic understanding of inhaled IgG-based therapeutics for respiratory infections and thus support clinical trial success.

Material and methods

1. Materials

a. Antibodies and reagents

Human plasma-derived polyclonal, polyvalent IgG used in this study is manufactured by CSL Behring. The IgG subclass distribution in the product corresponds approximately to that of normal human plasma (approximate values: 69% IgG₁, 26% IgG₂, 3% IgG₃, 2% IgG₄). The IgG solution is stabilized with 250 mM L-proline at pH 4.8. Pulmozyme®, a solution for inhalation from Roche, an irrelevant human IgG1 (Rituximab) and MPC11, an irrelevant mouse IgG2b produced by BioXcell were used as controls for biofilm studies.

Specific IgG levels were detected using diagnostic ELISA kits from several providers (see Supplementary Table S1): kits from the Binding Site to detect anti-PCP IgG (MK012) and anti-*H. influenzae type b* IgG (MK016) and kits from EUROIMMUNE to detect anti-*Influenza A* virus IgG (EI 2691-9601 G), anti-*Influenza B* virus IgG (EI2692-9601 G), anti-*Parainfluenza* Virus IgG (EI 2721-9601-1 G) and anti-RSV IgG (EI 2670-9601 G). Anti-*P. aeruginosa* IgG levels against extracellular protein Exotoxin A were measured using an ELISA kit from Mediagnost. Assays were performed as described in the manufacturer's protocol.

Prior to FRAP experiments, polyvalent IgG was FITC labelled using the Pierce FITC antibody labelling kit (ThermoFisher Scientific), according to the manufacturer's instructions. After labelling, the IgG concentration was measured by spectrophotometry (Nanodrop™ 2000, ThermoFisher Scientific), using a molar extinction coefficient of 210,000 cm⁻¹M⁻¹.

b. Pathogens

The influenza A virus (IAV) and influenza B virus (IBV) strains used in this study were, respectively the PR8 (H1N1) and the Victoria strains kindly provided by Dr. Mustapha Si-Tahar (Tours University, France). *Pseudomonas aeruginosa* strain PAO1 was obtained from ATCC. The colonies' uniformity was checked by plating on Pseudomonas isolation agar (PIA) plates. Starting from a frozen stock solution at 37°C, an overnight culture of PAO1 was prepared in 10 mL of Luria broth (LB) with shaking at 220 rpm. The culture was stopped, bacteria were washed once in 1XPBS and diluted in 1XPBS, to obtain a titer of 1×10⁸ bacteria/mL.

c. Sputum samples

Human sputum samples used in this study were collected for an independent prospective study and qualified for secondary use in the field of bronchiectasis. Induced sputum from patients with bronchiectasis and healthy individuals were collected at the Royal Melbourne Hospital (ethical approval granted under reference 2019.085) according to the European Respiratory Society 2002 guidelines [28]. After collection, raw sputum material was frozen at -70°C until viscosity measurements.

d. Mucus surrogates

Artificial mucus (normal mucus) and its modified versions (fluid and viscous mucus) were formulated and prepared as described above by Dinesh et al. [29]. 250 mg of mucin from porcine stomach (type II), 200 mg of DNA from salmon sperm, 250 mg of NaCl, 110 mg of KCl, 90,5 mg of Trizma base, and 250 mg of casamino acid were dissolved in 50 mL ultra-pure water by stirring during twenty minutes. All constituents were obtained from Sigma-Aldrich. When mucins were completely solubilized, 500 µL of 0.15 M diethylenetriaminepentaacetic acid was added and the solution was stirred for four hours.

To increase the medium viscosity, 0.4% (w/v) or 0.9% (w/v) poly-acrylic acid (Sigma-Aldrich) was added to the initial formulation, resulting in fluid and viscous mucus, respectively. After stirring the media overnight, the pH was adjusted to 7.0 by adding Trizma base (1M). Finally, the media were sterilized (autoclave) and cooled to room temperature before adding 250 μ L of egg yolk emulsion (Sigma-Aldrich). All artificial mucus formulations were aliquoted and kept at 4°C.

2. Methods

a. Rheological parameters and imaging of mucus/sputum

The dynamic viscosities of artificial mucus were measured using two equipments depending on the lab location: a HAAKE RheoStress 1 rheometer (Thermo Scientific) with a plane/cone geometry C35/2° Ti (35 mm diameter; 2° angle) and the HAAKE Viscotester Iq Air rheometer (Thermo Scientific) with a double gap cylinder geometry CC27 DG/Ti. Cross-validation of the data was done with artificial mucus. Measurements were performed at 37°C. Sputum samples from patients with bronchiectasis and individuals without chronic pulmonary diseases (healthy controls) were also analysed, following the same protocol as for artificial mucus. The shear stress was obtained by increasing shear frequency range from 1 to 1500 s⁻¹. The dynamic viscosity was determined by the Ostwald de Waele model – a simplified mathematical relationship used to describe the behavior of non-Newtonian fluids - using the following equation:

$$\eta = K\dot{\gamma}^{(n-1)}$$

where K is the consistency index, $\dot{\gamma}$ is the shear rate (s⁻¹), and n is the flow index. The results were expressed in millipascals per second.

The viscoelastic modulus G^* , the complex resultant of the elastic (G') and viscous (G'') moduli, and the damping factor $\tan\delta$ have been assessed for normal, fluid and viscous mucus.

Artificial mucus and human sputum samples were observed through a transmission electron microscope (JEOL JEM-1400 Plus (120 kV), Département des Microscopies, University of Tours).

b. Fluorescence recovery after photobleaching (FRAP)

FRAP measurements were performed by mixing 2.2 μ L FITC-labelled polyclonal IgG (final concentration of 0.05 mg/mL) into 7.8 μ L of artificial mucus, placed in a microscopy slide with spacers (Invitrogen, Secure-Seal™ Spacer). FITC-labelled polyclonal IgG is gently stirred to achieve uniform distribution. A #1.5 coverslip is added on top of the spacer which is then imaged directly. FRAP experiments were conducted on a Leica SP8 confocal microscope with a 40X objective (Département des Microscopies, University of Tours). For each condition, 4 regions of interest (ROI) of 10*10 μ m were selected and bleached using an argon laser (at 458, 476, 488, 496 and 514 nm) with 100% intensity for 15 seconds. After bleaching, the fluorescence recovery was measured for 55 seconds with frames acquired every 0.148 seconds. The acquisition was repeated on 4 independent fields. Fluorescence intensity was normalised to the starting intensity. The mobile fraction of FITC-labelled polyclonal IgG in artificial mucus was determined by this equation:

$$(F_{inf}-F_0)/(F_i-F_0),$$

where F_{inf} is the maximum fluorescence intensity coated after photobleaching, F_0 is the minimum fluorescence intensity obtained after photobleaching, and F_i is the maximum fluorescence intensity before photobleaching.

The immobile fraction was calculated by subtracting the mobile fraction from the initial fluorescence intensity before photobleaching (F_i). The half-time fluorescence recovery ($T_{1/2}$) was estimated from the

recovery curves and corresponds to the time required to recover half of the fluorescence after photobleaching. Recovered fluorescence is calculated as the difference between F_i and F_0 . These experiments were repeated 6 times with IgG for each artificial mucus.

c. Diffusion of polyclonal IgG through Transwell model

Transport studies of polyclonal IgG through artificial mucus were performed in 24-well plates with Transwell® inserts (Sarstedt, PET, 0.4 μm) containing 100 μL artificial mucus. In the basolateral compartment, 500 μL of phosphate-buffered saline (PBS) was added. The experiments were carried out in duplicate for each condition and repeated at least 7 times for each artificial mucus. 100 μL of a 40 mg/mL polyclonal IgG solution were added on top of mucus surrogates to obtain polyclonal IgG at a final concentration of 20 mg/mL in the apical compartment. Wells were incubated statically at room temperature for 24 hours. Samples of 15 μL were taken in the basolateral compartment from 15 minutes to 24 hours. The initial concentration of IgG in the apical compartment and the concentration of IgG in the basolateral samples were measured using a spectrophotometer (NanoDrop™ 2000, ThermoFisher Scientific), using a molar extinction coefficient of $210,000 \text{ cm}^{-1}\text{M}^{-1}$.

In some experiments, the medium from the basal compartment (500 μL) was collected at 24 hours to analyze the diffusion of each IgG subclass through artificial mucus. Total IgG and IgG1 to 4 were measured by ELISA (Invitrogen) according to the manufacturer's instructions. For the ELISA, samples were diluted 1:75,000 for total IgG, 1:500-1:2,000 for IgG1 and IgG3, 1:10,000-1:20,000 for IgG2, and 1:10,000 for IgG4. Results were expressed as the percentage of IgG that had diffused through the mucus and they were normalized to the results obtained with normal mucus.

d. Deglycosylation

In some cases, IgG samples from the basal compartment of the Transwell experiments were digested with PNGase F under non-denaturing conditions according to the manufacturer's instructions (PNGase F Glycan Cleavage Kit, Gibco). The cleavage profile was assessed by separating 2 μg of PNGase F-digested or undigested IgG by SDS-Page (10-20% NuPAGE Tris-acetate gels, Invitrogen). After protein migration, the gels were stained with Coomassie Blue (ThermoFisher Scientific). The band intensities corresponding to PNGase F-digested or undigested IgGs were measured with GelAnalyzer software (<http://www.gelanalyzer.com>) and normalized to undigested IgG.

e. Polyclonal IgG binding to pathogens

The binding of polyclonal IgG to *Pseudomonas aeruginosa* and influenza viruses (influenza A and B viruses) was studied with artificial mucus by ELISA. Briefly, 96-well Maxisorp plates were coated with pathogens (*P. aeruginosa* or influenza viruses) overnight at 4°C. *Pseudomonas aeruginosa* O1 strain was used at a concentration of 1.10^7 cfu/well diluted in LB medium, the influenza A virus (PR8 strain) and the influenza B virus (Victoria strain) were used at 1.10^6 pfu/well diluted in PBS 1X. After washing wells with PBS 1X containing Tween 20 0.05%, the non-specific binding sites were blocked with PBS 1X containing 5% milk for 2 hours at room temperature. Polyclonal IgG diluted at 8 $\mu\text{g}/\text{mL}$ in artificial mucus were added in the 96-well plate and the plate was incubated for 1 hour at room temperature. After washing wells with PBS 1X containing Tween 20 0.05%, 50 μL of HRP donkey anti-human IgG (Jackson ImmunoResearch) diluted 1:10,000 in PBS 1X containing 5% milk was added and incubated for 2 hours, at room temperature. For the detection, 50 μL of tetramethylbenzidine substrates (Biolegend) were added in each well and the reaction was stopped by adding 50 μL of H_2SO_4 2N. Absorbance at 450 nm was analysed using a spectrophotometer. Several batches of human polyclonal IgG were tested for their binding interactions with *Pseudomonas aeruginosa* (PAO1) in normal and modified artificial mucus.

f. Antibacterial effect of polyclonal IgG

The impact of polyclonal IgG on bacterial growth was determined on *Pseudomonas aeruginosa* PAO1 in LB medium. The kinetics of bacterial growth were monitored by measuring the optical density at 600 nm, in the presence or absence of 100 µg/mL polyclonal IgG at 30°C or 37°C, under static conditions or with shaking every 20 minutes for 18 hours. Treatments were either polyclonal IgG or MPC11 at 100 µg/mL diluted in 250 mM L-proline, pH 4.8. The bacteria were treated either at the beginning of the culture (t=0h), during the exponential growth phase (t=4.5h) or during the stationary phase (t=8h). Absorbance results were normalised with PAO1 alone.

g. Effect of polyclonal IgG on biofilms derived from *Pseudomonas aeruginosa*

Biofilm formation was conducted as previously described [30]. Bacterial overnight cultures of *pseudomonas aeruginosa* (strain O1) were diluted 1:50 in LB medium and 200 µL were added to a flat 96-well tissue culture-treated plate (ThermoFisher Scientific). The outer wells were filled with 200 µL of PBS to prevent evaporation. *Pseudomonas aeruginosa* is grown under static conditions at 30°C to promote biofilm formation. Polyclonal IgG at 100 µg/mL were added to the bacterial culture at the initiation of biofilm formation, or on pre-formed biofilms (after 24 hours of culture of *pseudomonas aeruginosa* at 30°C) and biofilm biomass was quantified 6 hours after treatment. In some experiments with pre-formed biofilms, the administration of polyclonal IgG was repeated twice (at 24 hours and 30 hours). In some wells, Pulmozyme® at 50 µg/mL and MPC11 at 100 µg/mL were used as positive and negative controls. All treatments were diluted in 250 mM L-proline, pH 4.8. The biofilm biomass was assessed by crystal violet colorimetric assay as described previously [30], reading absorbance at 570 nm. Absorbance results were normalised with wells with PAO1 alone.

3. Statistical analysis

Statistical evaluation of differences between the experimental groups was determined by using one- or two-ways analysis of variance (ANOVA) followed by a Tukey's post-test (which allows comparison of all pairs of groups) when number of samples was > 5; Kruskal-Wallis followed by a Dunn's post-test when number of samples was ≤5 and Student's *t*-test was used for comparison between two groups. All tests were performed with GraphPad Prism software (version 8.0.2 for Windows, GraphPad SoftWare Inc., San Diego, CA, USA).

Results

Characterization of mucus surrogates and human plasma-derived IgG

Bronchiectasis is characterized by dysregulated immunity and impaired airway clearance, resulting in mucus accumulation, which provides a favorable environment for microbial colonization and infection. Here we show that sputum from patients with bronchiectasis is significantly more viscous than controls. We produced artificial mucus with different dynamic viscosities covering the viscosity range of sputum obtained from patients with bronchiectasis and controls (Figure 1A, [31]). As shown in Supplementary Table 1, the viscoelastic modulus G^* and the damping factor $\tan\delta$ showed a more solid-like, stiff structure in the viscous mucus as compared to the fluid or normal ones. Moreover, the qualitative features of the artificial mucus (Supplementary Figure 1) evaluated by TEM were comparable to the ones of human sputum.

We analysed the binding capacity of human plasma-derived IgG to different respiratory pathogens associated with bronchiectasis and their virulence factors. As shown in Figure 1B, polyclonal IgG bound to various epitopes of respiratory viruses (influenza B virus, parainfluenza virus influenza A virus) and bacteria such as *Pseudomonas aeruginosa*. Based on this result, we selected *Pseudomonas aeruginosa* and influenza viruses, as pathogen models to investigate the impact of both mucus and biofilm on IgG on their target antigens.

Diffusion of polyclonal IgG through mucus and binding to its cognate antigens is dependent on mucus viscosity

First, the impact of mucus viscosity on the diffusion of polyclonal IgG was assessed by FRAP imaging (Figure 2A) and a permeable membrane (Transwell) assay (Figure 2E). As shown in Figure 2B-D, the more viscous the artificial mucus, the lesser the mobile IgG fraction and the longer the fluorescent recovery half-time. In addition, the viscosity of the artificial mucus limited the quantity of polyclonal IgG diffusing in the basal compartment of the Transwell assay (Figure 2F). Interestingly, in the fluid mucus, the percentage of IgG1 and IgG3 passed into the basal compartment of the Transwell was significantly lower compared to normal mucus; this was not the case for the other IgG subclasses (Figure 3A). In the viscous mucus, all IgG subclasses were similarly retained (Figure 3A). Next, we assessed whether the glycosylation of the polyclonal IgG may influence its diffusion through mucus. Analysis of the N-glycosylation profile of the IgG in the basal compartment did not show markable modification compared to the polyclonal IgG product (Figures 3B-C).

The capacity of IgG to pass through the mucus surrogates and bind to its cognate antigens (*Pseudomonas aeruginosa* PAO1 and IAV/IBV) was analysed by ELISA (Figures 4A, C and E). As shown in Figure 4 (B, D and F), the more viscous the artificial mucus, the fewer IgG binds to *Pseudomonas aeruginosa* PAO1 and IAV/IBV. Notably, different batches of human plasma-derived IgG displayed comparable behaviors (Supplementary Figure 2).

Polyclonal IgG does not interfere with the formation of biofilms but can disrupt existing biofilms

The formation of biofilm is a key virulence factor for many bacterial-driven chronic respiratory infections [32]. We investigated whether polyclonal IgG may interfere with *Pseudomonas aeruginosa* when embedded in biofilm to mimic conditions in chronic respiratory diseases like bronchiectasis. We observed that IgG could not inhibit bacterial biofilm formation or growth when added in prophylactic setting, at the initiation of the bacterial culture (Figure 5A). This result agrees with the lack of direct impact of polyclonal IgG on *Pseudomonas aeruginosa* growth in standard liquid culture when treated at the initiation of the culture (Figure 6). On the other hand, repeated IgG treatment significantly disrupted the existing biofilm (Figure 5B), while a single treatment was insufficient (Figure 5C). IgG treatment at different time points after the initiation of the culture had no significant impact on *Pseudomonas aeruginosa* growth (Supplementary Figure 3).

Discussion

Chronic respiratory diseases and recurrent infections alter the airway environment, including changes in the protease/anti-protease balance, infiltration of inflammatory cells, hyper-production and thickening of mucus and embedment of bacterial pathogens in biofilms. Here, we show that the viscosity of the mucus and biofilms act as barriers for IgG and are important to consider for the clinical development of inhaled IgG as a therapeutic.

The airway mucus hydrogel primarily contains water (95%), respiratory mucins MUC5AC and MUC5B (2%-5%), salts, lipids, DNA, other globular proteins and cell debris. Here, we prepared artificial mucus as previously described [31], displaying qualitative features and viscoelastic properties similar to mucus/sputum obtained from healthy donors and patients with bronchiectasis. This allowed us to investigate the effects of mucus viscosity on IgG diffusion without the confounding factors such as cell debris, endogenous IgG and IgG-degrading proteases found in natural sputum [33]. Airway mucus is expected to allow the rapid diffusion of particles up to 0.2 μm and the typical dimensions of IgG are 13.7 nm (width), 8.4 nm (height) [34–36]. Although IgG are smaller than the mesh size and interact only transiently with mucins [33,36], our results show that only a fraction of polyclonal IgG diffused in all mucus surrogates. The high viscoelastic modulus (21.72 Pa), and $\tan \delta$ close to 0 (0.26) of viscous mucus indicate a solid-like structure with moderate stiffness, which may result in increased steric hindrance and resistance to flow – as seen in changes of the pore sizes of the mucus mesh in pathological conditions [36]. The normal and fluid mucus showed solid-like behaviour as well ($\tan \delta$ 0.17 and 0.29) but with mucus low viscoelastic modulus (1.33 and 1.68 Pa) indicating a weakly and soft structure, which can flow and may explain the higher IgG diffusion rates. The reduced recovery rate of polyclonal IgG after FRAP, as described elsewhere for cervicovaginal mucus and buffered physiological saline [37], indicates partial immobilization of IgG by components of the mucus from different origins [37,38].

Interestingly, the diffusion of IgG1 and IgG3 decreased significantly in the fluid mucus as compared to normal mucus. This was not expected as IgG1 and IgG3 are known to be more flexible than IgG2 and IgG4 [39,40]. In viscous mucus diffusion rates were reduced similarly for all IgG subclasses. Factors beyond hinge-mediated flexibility may contribute to IgG subclass interaction with mucus. For instance, interactions of IgG with mucus may include direct interaction with mucin fibres, depending on the Fc- and N-glycans [36]. Although all human IgG subclasses share a conserved N-glycosylation site in the Fc-region, their N-linked glycan structures differ [40]. We did not observe any striking difference in the glycosylation level of IgG after diffusion through the mucus as compared to the original product. This might be due to the limitation of the method analyzing IgG N-glycosylation, but not the composition of glycan structures. Glycan structures affect molecular weight, interactions with other molecules and effector functions, and there is a high degree of complexity and diversity of N-glycan structures present in human individuals [42], which is the source of IgG in this study. Mucins also have regions rich in cysteine residues that may interact with free sulfhydryls present in all subclasses of IgG antibodies [43,44], which may contribute to higher IgG in the more solid-like structured mucus [45–47]. Though the use of artificial mucus eliminates cofounding factors, focusing on the dynamics between IgG mobility and mucus thickness, our results may not be fully extrapolated to human airway mucus, which is more complex and different by nature [38].

During infections, pathogens are embedded in the mucus secreted by the host cells, and mucus is swept along by the ciliated epithelium in a process called mucociliary clearance, leading to the elimination of pathogens from the respiratory tract. We found that polyclonal IgG has reduced access to pathogens when the mucus gets thicker. Taken together, the immunoglobulin deficit associated with bronchiectasis [25,48], may hinder endogenous IgG diffusion into the airways and thereby hamper efficient elimination of persistent pathogens.

Management of bronchiectasis relies on antibiotics to reduce infections and treat exacerbations, but prolonged use increases antimicrobial resistance, highlighting the need for alternative anti-infective therapies. Administration of human plasma-derived IgG from healthy donors has been suggested to prevent recurrent respiratory infections [21]. When delivered intranasally, this IgG binds pathogen antigens and has prevented acute respiratory infections in mice [13,14]. To gain insight into the underlying mechanisms, we modelled conditions encountered by topically applied IgG in the RT and assessed their capacity to penetrate the mucus and bind relevant respiratory pathogens. Pathogens like influenza viruses and *Pseudomonas aeruginosa* are recognized by plasma-derived IgG. In normal mucus surrogates, polyclonal IgG bound to its cognate antigens efficiently. As IgG did not directly inhibit pathogen growth upon binding, our results suggest that IgG forms multivalent low-affinity bonds with mucins and thereby contributes to the reduction of the bacterial burden by mucociliary clearance. This mechanism has been previously described as a new function of the Fc domain of IgG - named muco-trapping – and potentially contributes to the anti-infective properties of polyvalent IgG observed *in vivo*. [36,38]. We showed that the increased viscosity of the mucus – mirroring patient sputum [49] – can limit IgG access to its cognate antigens, which may influence optimal treatment dosing and frequency. Interestingly, IgG binding to viruses was much less affected by mucus viscosity than the binding to bacteria in our assay conditions. We cannot rule out that this discrepancy is due to differences in pathogen diffusivities or depends on the different concentrations of specific anti-pathogen IgG. In future studies, it would be interesting to investigate if the muco-trapping properties of polyclonal, polyvalent IgG are modified in pathological mucus.

Biofilms are intricate colonies of bacteria embedded in a self-produced matrix. The biofilm matrix comprises a complex mixture of proteins, polysaccharides, extracellular DNA and lipids. In biofilms, bacteria are more resistant to antimicrobials (such as antibiotics) and protected from immune system effector functions [19]. Notably, *Pseudomonas aeruginosa* is a pathogen of critical concern due to its ability to form antibiotic-tolerant biofilms during chronic respiratory infections [50]. We found that plasma-derived IgG from healthy donors does not inhibit biofilm formation but – interestingly – can disrupt existing biofilms. As opposed to previous studies, our results suggest that polyclonal IgG does neither inhibit bacterial growth directly nor prevent adhesion to the culture plastic to create biofilms [51,52]. In contrast, they may destabilize the matrix-embedded bacteria [53–55] by binding either to some extracellular matrix components or to bacteria, thereby freeing bacteria from the biofilms thereby rendering them more susceptible to antimicrobials and/or opsonophagocytic killing by immune cells. In future experiments, it would be interesting to determine the specific targets responsible for this effect. Given that thick mucus can serve as a niche for biofilm formation, it would be valuable to study how polyclonal IgG interferes with *Pseudomonas aeruginosa* within biofilms produced in mucus of different viscosities.

Our findings indicate that mucus may act as a barrier for IgG, depending on its viscoelastic characteristics, limiting the diffusivity and access of IgG to its antigens. Although polyclonal IgG does not directly inhibit pathogen replication/growth, it may facilitate pathogen clearance by interacting with both pathogens and mucins through muco-trapping. In addition, it interferes with continuous biofilm production by destabilizing the biofilm matrix. Thus, inhaled human plasma-derived IgG (CSL787) is a promising prophylactic strategy to limit infections and exacerbations in chronic respiratory disease.

Bronchiectasis is increasingly recognized as an overlapping syndrome of other chronic lung diseases like COPD or asthma [56]. Reducing exacerbations may preserve lung function and improve long-term outcomes in patients with bronchiectasis. It would be interesting to analyze the clinical response to IgG-based therapeutics as a function of the viscoelastic properties of sputum collected from bronchiectasis patients.

In conclusion, our findings help to better understand the inability of IgG to control and eliminate respiratory pathogens if the mucus is too viscous. They also provide a better mechanistic understanding of inhaled IgG-based therapeutics for respiratory infections. Furthermore, the data provides a rationale for exploring higher inhaled doses or treatment frequencies to overcome the increased physical barrier represented by mucus viscosity in bronchiectasis.

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Supplementary Table S1: ELISA assays used to characterize the binding of polyclonal IgG against pathogens, which are often encountered in bronchiectasis.

Pathogen	ELISA Kit	Antigen	Cat. No.
<i>Pneumococcal capsular polysaccharide (PCP) from Streptococcus pneumoniae</i>	The Binding Site	Combined 23 most common capsule polysaccharides	MK012
<i>Haemophilus influenzae</i> Type b (Hib)	The Binding Site	Hib capsule polysaccharide	MK016
<i>Pseudomonas aeruginosa</i>	mediagnost	Exotoxin A	E15
Respiratory syncytial virus (RSV)	EUROIMMUN	Inactivated cell lysates of Vero cells infected with RS virus from strain "Long"	EI 2670-9601 G
Influenza A	EUROIMMUN	Inactivated allantois fluids of chicken embryos infected with either "Texas" (H3N2) strain, "Singapore" (H1N1) strain or "California" (H1N1, porcine Influenza	EI 2691-9601 G
Influenza B	EUROIMMUN	Inactivated allantois fluid of chicken embryos infected with "Hongkong 5/72"	EI 2692-9601-G
Parainfluenza	EUROIMMUN	Inactivated cell lysates of Vero cells infected with Parainfluenza viruses	EI 2721-9601-1 G

Figure Legends

Figure 1: (A) Dynamic viscosity of artificial mucus (n=6) and human sputum (healthy control, n=9 and bronchiectasis patients, n=10). Results are presented as box plot with the median, the minimum, the maximum and the first and third quartiles. *: p<0.05; **: p<0.01; ****: p<0.0001 with a t test test comparing healthy control and bronchiectasis patients or with a one-way Anova followed by a Tukey's post-test. **(B)** Binding capacity of polyclonal IgG to influenza A virus (IAV), influenza B virus (IBV), parainfluenza virus (PIV), respiratory syncytial virus (RSV), *P. aeruginosa* (ExoA), *Haemophilus influenzae* Type b (Hib) and Pneumococcal capsular polysaccharide (PCP) from *Streptococcus pneumoniae* was measured by ELISA. Results are expressed as the OD extinction of the sample as compared to a calibrator or a standard curve with known pathogen concentration. The data is presented as box plot with the median, the minimum, the maximum and the first and third quartiles.

Figure 2: (A) Schematic representation of FRAP assay and analysis. **(B, left panel)** Normalized fluorescence intensities over time for photobleached ROIs of FITC-labelled IgG in normal mucus (black), fluid mucus (red) and viscous mucus (blue). Results are expressed as median value of 6-8 independent experiments. **(B, central panel)** Time to recover 50% of initial fluorescence **(B, right panel)** Recovered and unrecovered fraction of fluorescence within the time scale of measurement normalized to the initial bleached fraction. Results are presented as box plots with the median, the minimum, the maximum and the first and third quartiles, representative of 6-8 independent experiments. *: p<0.05; **: p<0.01; ****: p<0.0001 with a one-way Anova followed by a Newman-Keuls post-test. **(C)** Schematic representation of Transwell transport assay. **(D)** The cumulative concentration [mg/mL] of IgG in the basolateral compartment is shown over time [hours]. Results are presented as the median, the minimum and the maximum and are representative of 10 independent experiments. ****: p<0.0001 with a one-way Anova followed by a Tukey's post-test.

Figure 3: (A) Total IgG (IgG) and IgG subclasses (IgG1-IgG4) quantification by specific ELISA in the basolateral compartment of the Transwell as described in Figure 2E for normal mucus (black), fluid mucus (red) and viscous mucus (blue). Results are presented as box plots with the median, the minimum, the maximum and the first and third quartiles, and are representative of 5 independent experiments. **: p<0.01; ****: p<0.0001 with a two-way Anova followed by a Tukey's post-test. **(B)** Representative picture of SDS-Page of PNGase-digested or undigested IgGs recovered from the basolateral compartment of the Transwell. **(C)**. Band intensity evaluation with GelAnalyzer. Results are presented as box plots with the median, the minimum, the maximum and the first and third quartiles, and are representative of 5 independent experiments, normalized to undigested IgG.

Figure 4: (A) Schematic representation of pathogen-ELISA assay using normal mucus (black), fluid mucus (red) and viscous mucus (blue). Binding capacity of polyclonal IgG to PAO1 **(B)**, IAV **(C)** and IBV **(D)**, respectively. Results are presented as the median, the minimum and the maximum and the first and third quartiles, representative of 5-7 independent experiments. *: p<0.05; **: p<0.01; ****: p<0.0001 with a one-way Anova followed by a Tukey's post-test.

Figure 5: PAO1 biofilm biomass evaluation by Crystal Violet staining 18 hours after **(A)** prophylactic treatment with polyclonal IgG at the initiation of the biofilm, or **(B)** therapeutic 6 hours post-treatment, or **(C)** therapeutic 6 and 24 hours post-treatment. Results are presented as box plots with the median, the minimum, the maximum and the first and third quartiles, and are representative of 7 independent experiments, normalized to biofilm grown in LB alone ("no treatment"). *: p<0.05; **: p<0.01 with a one-way Anova followed by a Tukey's post-test.

Figure 6: PAO1 biomass was evaluated for 18h, comparing untreated bacteria (black dots) with bacteria treated with polyclonal IgG (white squares), added at the initiation of the culture. Results are expressed as median +/- standard deviation of 2 independent experiments.

Supplementary Figure 1: Representative TEM pictures of **(A)** normal, fluid and viscous artificial mucus and **(B)** sputum from healthy controls and bronchiectasis patients. **(B)** Semi-quantitative evaluation of structural features of artificial and patient-derived mucus.

Supplementary Figure 2: **(A)** Schematic representation of PAO1-ELISA assay using normal mucus, fluid mucus and viscous mucus. **(B)** Binding capacity of 4 lots of polyclonal IgG to PAO1. Results are expressed as median +/- min/max of 3 independent experiments. *: $p < 0.05$; **: $p < 0.01$ with a two-way Anova followed by a Tukey's post-test. .

Supplementary Figure 3: PAO1 biomass was evaluated for 18h, comparing untreated bacteria (black dots) with bacteria treated with polyclonal IgG (white squares), added **(A)** 4 hours or **(B)** 8 hours after the initiation of the culture. Results are expressed as median of 3 technical replicates of 1 independent experiment.

Supplementary Figure 4: PAO1 biofilm biomass evaluation by Crystal Violet staining 18 hours after **(A)** prophylactic treatment with polyclonal IgG at the initiation of the biofilm, or **(B)** therapeutic 6 hours post-treatment, or **(C)** therapeutic 6 and 24 hours post-treatment. Results are presented as box plots with the median, the minimum, the maximum and the first and third quartiles, and are representative of 7 independent experiments, normalized to biofilm grown in LB alone ("no treatment"). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ with a one-way Anova followed by a Tukey's post-test.

Supplementary Table 1: The table includes rheological data from preparations of normal, fluid and viscous artificial mucus (n=3). The values are presented as the geometric means and s.e.m.