



# Influenza-binding antibodies immobilise influenza viruses in fresh human airway mucus

*To the Editor:*

Airway mucus is thought to serve as a dynamic filter that can trap respiratory viruses in the dense mucin network [1], and quickly eliminate them along with airway mucus from the respiratory tract. Nevertheless, viruses must penetrate airway mucus to infect the airway epithelium, and we and others have found that various viruses can readily penetrate physiological mucus gels [2–4]. We were particularly interested in whether airway mucus can serve as a barrier against influenza viruses, due to the prevalence and health burden of seasonal outbreaks. It has been suggested that airway mucus protects against influenza by presenting sialylated “decoys” on mucins that bind to influenza haemagglutinin and trap the virions in mucus. Alternatively, we have shown that antibodies in cervicovaginal mucus can trap viruses *via* multiple low-affinity Fc–mucin bonds between antibodies on the virus surface and mucins, akin to a Velcro patch [4]. To gain insight into how airway mucus might serve as a barrier against influenza, we characterised the mobility of influenza virions and virus-like particles (VLPs) in human airway mucus using real-time high-resolution multiple particle tracking.

We collected undiluted human airway mucus from the endotracheal tubes of healthy adult patients intubated for general anaesthesia during elective surgery [5] outside the typical flu season months. This mucus probably represents a mixture of mucus secreted locally in the trachea as well as mucus from the bronchi/bronchioles that has been transported by mucociliary clearance. Since wild-type influenza virions with an internal fluorophore are exceedingly challenging to prepare, we decided to measure the translational motions of wild-type H1N1 (influenza A/PR/8/34) and H3N2 (influenza A/Victoria/3/75) viruses fluorescently tagged with amine-reactive Alexa Fluor dye (Life Technologies, Carlsbad, CA, USA) which exhibited no appreciable affinity to mucins [6, 7]. We quantified virus motions using multiple particle tracking [8] and found that the movements of both H1N1 and H3N2 virions were strongly and uniformly hindered in most airway mucus specimens (figure 1a, online supplementary movies S1 and S2). Virions appeared as uniform punctate spots in airway mucus, indicating that they were trapped in airway mucus as individual virions rather than as agglutinates. Nearly all virions (>80% in all samples, on average ~95%) moved less than their diameter (~100 nm) over the course of 20-s movies, and virions were slowed ~3000–4000-fold in airway mucus compared to theoretical speeds in water. In contrast, comparably sized latex nanoparticles coated with 2 kDa polyethylene glycol (PS-PEG) to minimise mucoadhesion [9, 10] exhibited rapid diffusion in the same airway mucus specimens (figure 1a, online supplementary movie S3;  $p < 0.05$ ), suggesting that influenza was trapped by adhesive interactions with mucins or other mucus components.

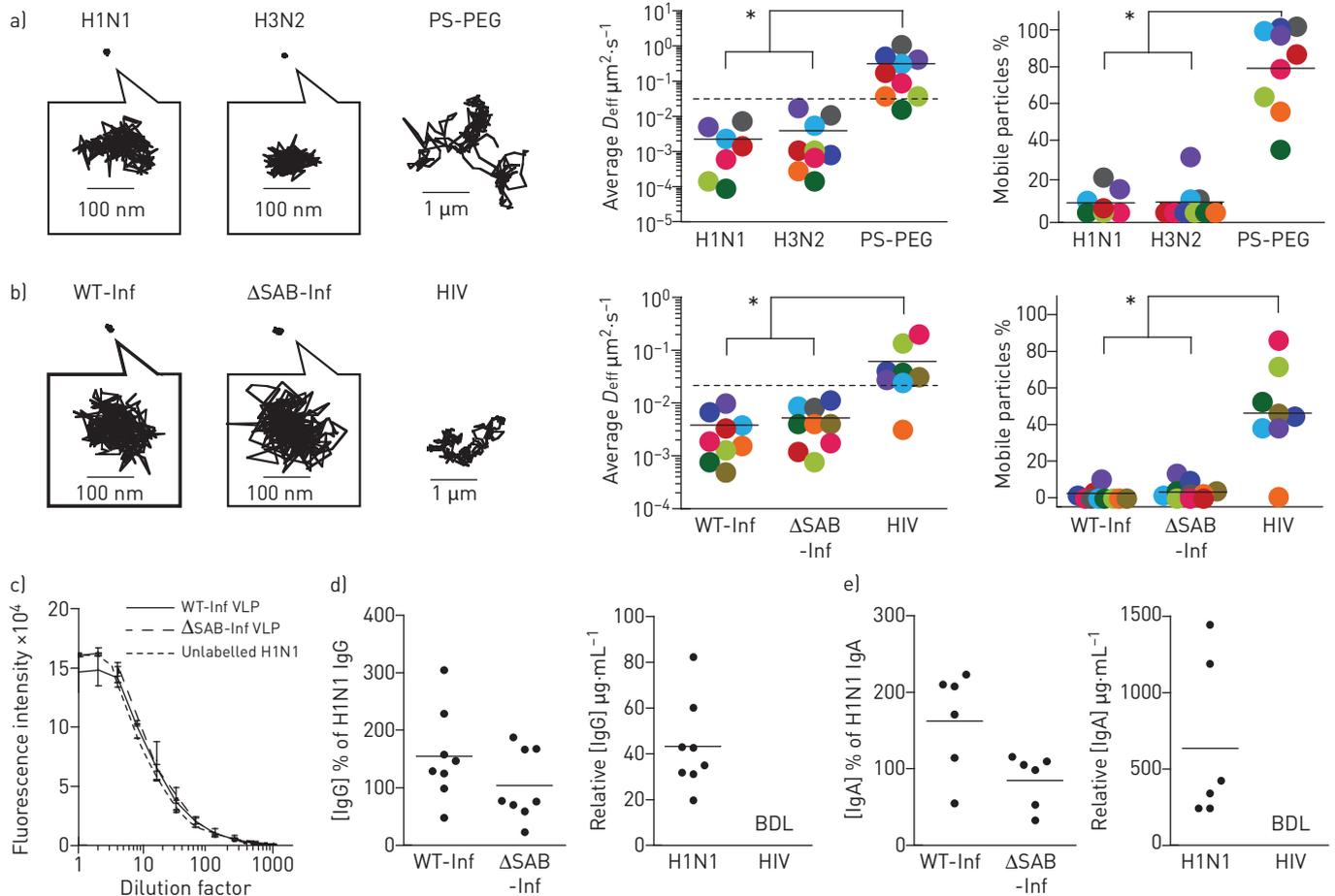
To investigate whether trapping of influenza in airway mucus can be attributed primarily to haemagglutinin binding to mucin-associated sialic acid, we prepared VLPs fluorescently labelled internally using HIV-1 GAG-mCherry capsid proteins in the core, and expressing both neuraminidase and haemagglutinin from H1N1 (influenza A/PR/8/34) (WT-Inf), or the same neuraminidase and haemagglutinin that has the sialic acid-binding domain deleted ( $\Delta$ SAB-Inf) and hence cannot bind directly to mucins [11]. Interestingly, both WT-Inf and  $\Delta$ SAB-Inf were trapped in airway mucus to a similar extent as H1N1 and H3N2, with roughly 98% of WT-Inf and 97% of  $\Delta$ SAB-Inf immobilised in airway mucus and average diffusivities ~1700- and ~1100-fold lower than expected speeds in buffer, respectively (figure 1b, online supplementary movies S4 and S5). Virus trapping was not associated with steric obstruction or impaired neuraminidase function, as WT-Inf and  $\Delta$ SAB-Inf VLPs both exhibited



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**Common influenza viruses are immobilised in fresh human airway mucus, probably by influenza-binding antibodies** <http://ow.ly/yxu0305bqh6>

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**FIGURE 1** Characterisation of the diffusion of and specific antibodies against viruses and virus-like particles (VLPs) in human airway mucus. Diffusion rates of a) H1N1 and H3N2 influenza viruses and 100 nm polyethylene glycol-coated latex nanoparticles (PS-PEG) and b) WT-Inf,  $\Delta$ SAB-Inf and HIV VLPs in human airway mucus. Representative trajectories are presented for particles exhibiting effective diffusivities within  $\pm 1$  SEM of the ensemble average at a time scale of 0.2667 s. Ensemble geometric average effective diffusivity ( $D_{eff}$ ) at a timescale of 0.2667 s and fraction of mobile particles are plotted for distinct samples (indicated by different colours) with averages indicated by solid lines. Nine distinct samples were used to compare the mobility of H1N1, H3N2 and PS-PEG in airway mucus, and another 10 samples were used to compare the mobility of WT-Inf,  $\Delta$ SAB-Inf and HIV VLPs. Airway mucus sample volumes were sufficient for at least two or three aliquots, allowing direct comparisons between different conditions. Data represent the ensemble average of between seven and 10 independent experiments per particle type, with  $n \geq 40$  particles per frame on average ( $n \geq 95$  particle traces tracked) for each experiment. \*:  $p < 0.05$ , based on a paired t-test where  $D_{eff}$  values are log-transformed. c) Neuraminidase activity of WT-Inf and  $\Delta$ SAB-Inf VLPs compared to unlabelled H1N1 virus adjusted to the same concentration. Data are presented as mean  $\pm$  SEM of  $n=3$  independent measurements. Amounts of d) IgG and e) IgA that bind WT-Inf and  $\Delta$ SAB-Inf VLPs, relative to the amounts of IgG and IgA that bind whole H1N1 virions, and amounts that bind whole H1N1 virions and HIV-VLPs determined using polyclonal anti-H1N1 IgG purified from IVIG using whole H1N1 virions and commercial anti-haemagglutinin IgA, respectively. Data represent  $n=6-8$  different human airway mucus specimens per condition and an average of two to four independent experiments. BDL: below detection limit.

comparable size (measured using Nanosight; Malvern, Malvern, UK) and neuraminidase activity (assessed using NA-Fluor influenza neuraminidase assay kit; Life Technologies) as wild-type influenza (figure 1c). These results demonstrate that influenza virus can be trapped in human airway mucus without haemagglutinin binding to mucin sialic acids, and suggest that the inherent neuraminidase activity of influenza virus is insufficient to overcome physiological human airway mucus.

Using whole-virus ELISA assays, we detected substantial quantities of endogenous IgG and IgA against influenza in airway mucus (data not shown), as well as against both WT-Inf and  $\Delta$ SAB-Inf VLPs (figure 1d, e). This leaves open the possibility that influenza-specific antibodies in airway mucus may immobilise virions by cross-linking the antibody-virus complex to mucus constituents, such as mucins. We sought to measure virus and VLP mobility in airway mucus devoid of antibodies; however, we were not able to adequately remove Ig by dialysis, possibly due to membrane clogging, and mucus secretions isolated from air-liquid interface cultures of bronchial epithelial cells did not produce a sufficiently rigid matrix to immobilise mucoadhesive latex nanoparticles. We also attempted to “saturate” the mucus-antibody barrier by mixing  $>20$ -fold more unlabelled than labelled influenza viruses into airway mucus prior to adding labelled viruses, and still observed no discernible difference in the trapping of the labelled

influenza viruses. Therefore, we investigated whether the lack of binding antibodies in mucus correlates to greater virus mobility by tracking HIV VLPs that were prepared similarly to the influenza VLP, but expressing HIV YU2 gp160. We found no detectable HIV-binding IgG or IgA in airway mucus (figure 1d, e), and HIV VLPs exhibited markedly greater diffusivity in airway mucus (figure 1b, online supplementary movie S6;  $p < 0.05$ ), with >45% of HIV VLPs classified as mobile and ~10-fold higher ensemble effective diffusivity than WT-Inf and  $\Delta$ SAB-Inf. HIV VLP mobility was similar to that of PS-PEG in the same airway mucus samples (data not shown).

Together, these results demonstrate that influenza virus can be trapped in human airway mucus without binding to sialic acids on mucins, in good agreement with the evidence that human influenza viruses possess haemagglutinin that preferentially binds  $\alpha$ 2,6-linked sialic acids on the airway epithelium rather than  $\alpha$ 2,3-linked sialic acids on mucins [12, 13]. Trapping of influenza in human airway mucus can probably be attributed to the presence of influenza-binding antibodies that can cross-link individual virions to the mucus mesh network. Importantly, adhesive interactions between the array of pathogen-bound antibodies and mucus gel provide a universal strategy that enables the otherwise relatively nonadaptive and nonspecific biochemistry and microstructure of mucus secretions across different mucosal surfaces to be fortified with adaptive antibodies against an ever-changing spectrum of pathogens. Effective influenza vaccines remain elusive [14], and there is emerging evidence that systemic neutralising antibody titres may not correlate well with protective efficacy [15]. Our findings suggest that it may be important to assess vaccine efficacy based on the quantities of influenza-binding antibodies induced and secreted into mucus. Alternatively, in high-risk populations (e.g. teachers and healthcare workers), topical delivery of influenza-binding antibodies may help reduce the odds of becoming sick as well as the viral load reaching the airway epithelium.

Mucus is often viewed as a passive rather than dynamic and adaptive barrier against pathogens, and is consequently overlooked in most studies of mucosal infection. The consistent and extensive trapping of influenza in airway mucus collected from endotracheal tubes suggests that airway mucus can serve as an effective diffusional barrier against respiratory viruses, and influenza is unlikely to transmit efficiently in the trachea. Instead, we speculate that infections probably initiate in parts of the sinus and oral cavity least protected by mucus or most exposed to impinging virions, such as the soft palate [16]. It is also possible that viruses may infect in smaller conducting airways where airway mucus barrier properties may not be as robust. However, we have not yet characterised the barrier properties of mucus coating the lower airways (bronchi/bronchioles) with respect to the ability of influenza-binding antibodies to immobilise virions in mucus. In either scenario, once initial infection is established, viruses may spread to other parts of the airways *via* intercellular spreading [17].

Heterogeneity is a hallmark of the transport of nanosized entities in mucus, such as particles and viruses. Both donor–donor variation and intrasample variation in the mesh spacing (pore sizes) of the mucin network are possible due to differences in mucin or water content across samples or locally within a particular sample. Nevertheless, by using aliquots of the same samples to make direct comparisons between different conditions, we found that influenza viruses and VLPs were consistently trapped or slowed, while PS-PEG and HIV VLPs were significantly faster. Because we did not observe rapid influenza mobility in any of the airway mucus specimens, we cannot correlate a breakdown of the mucus barrier to potential elevated susceptibility to influenza infections. While influenza is a common infection, the number of people who are susceptible to and acquire influenza infection is relatively low at any moment in time; indeed, despite high rates of exposure and constantly evolving viral strains, seasonal influenza attack rates typically vary from only 5% to 20% [18, 19]. Future studies that greatly expand the sample pool to hundreds of airway mucus specimens may capture an adequate number of specimens from susceptible individuals to reveal potential differences in the barrier properties of airway mucus against influenza.

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## References

- 1 Fahy JV, Dickey BF. Airway mucus function and dysfunction. *N Engl J Med* 2010; 363: 2233–2247.
- 2 Lai SK, Hida K, Shukair S, *et al.* Human immunodeficiency virus type 1 is trapped by acidic but not by neutralized human cervicovaginal mucus. *J Virol* 2009; 83: 11196–11200.
- 3 Olmsted SS, Padgett JL, Yudin AI, *et al.* Diffusion of macromolecules and virus-like particles in human cervical mucus. *Biophys J* 2001; 81: 1930–1937.
- 4 Wang YY, Kannan A, Nunn KL, *et al.* IgG in cervicovaginal mucus traps HSV and prevents vaginal herpes infections. *Mucosal Immunol* 2014; 7: 1036–1044.
- 5 Schuster BS, Suk JS, Woodworth GF, *et al.* Nanoparticle diffusion in respiratory mucus from humans without lung disease. *Biomaterials* 2013; 34: 3439–3446.
- 6 Lai SK, Wang YY, Hida K, *et al.* Nanoparticles reveal that human cervicovaginal mucus is riddled with pores larger than viruses. *Proc Natl Acad Sci USA* 2010; 107: 598–603.
- 7 Schuster BS, Kim AJ, Kays JC, *et al.* Overcoming the cystic fibrosis sputum barrier to leading adeno-associated virus gene therapy vectors. *Mol Ther* 2014; 22: 1484–1493.
- 8 Wang YY, Nunn KL, Harit D, *et al.* Minimizing biases associated with tracking analysis of submicron particles in heterogeneous biological fluids. *J Control Release* 2015; 220: 37–43.
- 9 Lai SK, O’Hanlon DE, Harrold S, *et al.* Rapid transport of large polymeric nanoparticles in fresh undiluted human mucus. *Proc Natl Acad Sci USA* 2007; 104: 1482–1487.
- 10 Wang YY, Lai SK, Suk JS, *et al.* Addressing the PEG mucoadhesivity paradox to engineer nanoparticles that “slip” through the human mucus barrier. *Angew Chem Int Ed Engl* 2008; 47: 9726–9729.
- 11 Steel J, Lowen AC, Wang TT, *et al.* Influenza virus vaccine based on the conserved hemagglutinin stalk domain. *MBio* 2010; 1: 00018–10.
- 12 Gagneux P, Cheriyan M, Hurtado-Ziola N, *et al.* Human-specific regulation of  $\alpha$ 2-6-linked sialic acids. *J Biol Chem* 2003; 278: 48245–48250.
- 13 Matrosovich M, Klenk HD. Natural and synthetic sialic acid-containing inhibitors of influenza virus receptor binding. *Rev Med Virol* 2003; 13: 85–97.
- 14 Nicoll A, Sprenger M. Low effectiveness undermines promotion of seasonal influenza vaccine. *Lancet Infect Dis* 2013; 13: 7–9.
- 15 Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, *et al.* Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N Engl J Med* 2009; 361: 2209–2220.
- 16 Lakdawala SS, Jayaraman A, Halpin RA, *et al.* The soft palate is an important site of adaptation for transmissible influenza viruses. *Nature* 2015; 526: 122–125.
- 17 Roberts KL, Manicassamy B, Lamb RA. Influenza A virus uses intercellular connections to spread to neighboring cells. *J Virol* 2015; 89: 1537–1549.
- 18 Chowell G, Miller MA, Viboud C. Seasonal influenza in the United States, France, and Australia: transmission and prospects for control. *Epidemiol Infect* 2008; 136: 852–864.
- 19 Chunara R, Goldstein E, Patterson-Lomba O, *et al.* Estimating influenza attack rates in the United States using a participatory cohort. *Sci Rep* 2015; 5: 9540.

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