Microparticles from human the lower airway show inhibitory activity against respiratory syncytial virus

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Abstract
Airway microparticles (MPs) have been shown previously to inhibit influenza virus by trapping virions on their surface through their surface viral receptor. It was hypothesized that airway MPs may carry most of the epithelial cell surface molecules, including receptors for respiratory viruses, and may be able to inhibit various respiratory viruses. We show here that MPs from human bronchoalveolar lavage (BAL) can inhibit respiratory syncytial virus (RSV). Those MPs stained positive for the RSV receptor, CX3CR1. Furthermore, incubating the MPs with a monoclonal antibody against CX3CR1 reduced the anti-RSV activity. These data indicate that MPs can contribute to respiratory innate antiviral defense.

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Respiratory syncytial virus (RSV) is a common human-specific virus that is capable of causing severe respiratory illnesses, especially in young children. There is no efficacious vaccine or antiviral treatment available. RSV can modulate specific immune responses and evade innate immune responses. Complex interactions between viral factors and host adaptive and innate responses allow the virus to cause infection without inducing solid protective immunity [4]. Understanding the innate defense against this virus may help to improve treatment and prevention. We therefore tested whether respiratory tract MPs contribute to the innate defense against RSV.

We first tested whether human BAL MPs carry an RSV receptor. A number of molecules have been described as candidates for RSV receptors, including heparan sulfate, TLR4, nucleolin, and CX3CR1 [5, 6]. Among these, CX3CR1 has been shown to be the viral entry receptor in human primary differentiated airway epithelial cells [5]. To test whether this receptor is accessible on MPs in human airway secretion, human BAL samples were stained with anti-CX3CR1 and annexin V and analyzed by flow cytometry. The BAL samples were collected from individuals who underwent bronchoscopy and BAL for investigation of suspected lung cancer. BAL was performed on non-lesional lung segments. Collection of the samples was approved by the Ethics Committee of the Faculty of Medicine Siriraj Hospital under protocol COA No. Si 191/2016. All of the human subjects provided written informed consent. The majority of gated particles stained positive with annexin
A  Size gating for MP

B  Apoptotic body staining by PI

C  MP with CX3CR1
Fig. 1 CX3CR1 staining of BAL samples. After collection, BAL samples were immediately transported on ice to the laboratory and centrifuged at 1,500 × g at 4°C for 5 minutes to precipitate cells and cell debris. The BAL supernatant was then stored in aliquots at -80°C. BAL samples were blocked with 3% bovine serum albumin (BSA; Sigma Aldrich, USA) at room temperature for 30 minutes, incubated with 1 μg of anti-CX3CR1 mouse monoclonal antibody (Santa Cruz, USA) or isotype control at 37°C for 60 minutes, washed with PBS, and centrifuged at 4°C at 13,000 × g for an hour. The pellet was stained with goat anti-mouse Alexa Fluor 488 (Molecular Probes) at 37°C for 60 minutes and then washed with PBS and centrifuged at 4°C at 13,000 × g for an hour. The pellet was stained with annexin V PE in annexin V buffer at room temperature for 15 minutes. Stained MPs were transferred to a Falcon tube containing 50,000 particles of 1.34 μM latex beads (SPHERO fluorescent particles) as a size marker. The MP signal was confirmed by adding 0.1% Triton X-100 to confirm the MP incubated with 0.1% Triton X-100 to confirm the MP population by flow cytometry. X-100 to confirm the MP population by flow cytometry. Because the gating was done using 1.34-μM latex beads as a size marker, most of the particles in the gate had a size range that was compatible with MPs. To distinguish MPs from apoptotic bodies, we double-stained the BAL samples with annexin V and propidium iodide. BAL samples showed positive staining for annexin V only, whereas a control for the apoptotic body from the supernatant of H2O2-treated cells showed double-staining for both annexin V and propidium iodide (Fig. 1C). We therefore concluded that the majority of the events in the dot plot in Fig. 1 represent MPs. Although there was some variation in the levels of CX3CR1-positive staining among BAL samples, the number of MPs positive for CX3CR1 ranged from 181.41 to 849.03 (510.12 ± 282.96) particles/µl (Table 1). This indicates that the RSV receptor, CX3CR1, is available on the surface of MPs from human BAL and may be able to trap RSV virions and inhibit infection. We have previously shown that the majority of MPs in BAL are derived from respiratory epithelial cells, as determined by their staining for keratin sulfate and surfactant protein D [3]. However, only around 16% of MPs stained positive for CX3CR1. This is probably because CX3CR1 is expressed only on ciliated epithelial cells, while MPs can be released from ciliated, non-ciliated, and alveolar epithelial cells.

With the presence of CX3CR1 on BAL MPs, it is likely that they may be able to bind and trap RSV virions and inhibit infection. Ten human BAL samples were tested for anti-RSV activity. Figure 2A shows representative data from the 10 BAL samples in duplicate. Undiluted BAL samples showed around 80% inhibitory activity against RSV, and serially diluted samples showed declining inhibitory activity in a dose-response fashion. However, the anti-RSV activity of BAL samples did not show a significant correlation with the number of CX3CR1-positive MPs, indicating that MP was not the only anti-RSV mechanism of BAL.

Table 1 Counts of particles in individual BAL samples. BAL samples were stained with annexin V and anti-CX3CR1. The stained samples were incubated with 0.1% Triton X-100 to confirm the MP population by flow cytometry.

| BAL no. | Absolute count | Triton-X-100-treated | Annexin V and CX3CR1 positive |
|---------|---------------|---------------------|-----------------------------|
|         | n1            | n2                  | n1                          | n2 |
| 1       | 627 (681)     | 643 (15%)           | 697 (16%)                   | 16 (0.5%) |
| 2       | 207 (259)     | 211 (9%)            | 269 (11%)                   | 4 (0.2%)  |
| 3       | 119 (244)     | 123 (8%)            | 253 (10%)                   | 3 (0.2%)  |
| 4       | 635 (731)     | 649 (15%)           | 741 (19%)                   | 13 (0.4%) |
| 5       | 9 (3.3)       | 222 (10%)           | 193 (9%)                    | 8 (0.3%)  |
| 6       | 297 (211)     | 311 (13%)           | 223 (10%)                   | 13 (0.4%) |
| 7       | 723 (931)     | 737 (19%)           | 934 (12%)                   | 13 (0.4%) |
| 8       | 814 (884)     | 828 (24%)           | 888 (25%)                   | 14 (0.4%) |
| 9       | 438 (365)     | 462 (16%)           | 381 (15%)                   | 24 (0.5%) |
| 10      | 811 (827)     | 826 (24%)           | 831 (24%)                   | 15 (0.5%) |

V, which can stain MPs, exosomes, and apoptotic bodies. Because the gating was done using 1.34-μM latex beads as a size marker, most of the particles in the gate had a size range that was compatible with MPs. To distinguish MPs from apoptotic bodies, we double-stained the BAL samples with annexin V and propidium iodide. BAL samples showed positive staining for annexin V only, whereas a control for the apoptotic body from the supernatant of H2O2-treated cells showed double-staining for both annexin V and propidium iodide (Fig. 1C). We therefore concluded that the majority of the events in the dot plot in Fig. 1 represent MPs. Although there was some variation in the levels of CX3CR1-positive staining among BAL samples, the number of MPs positive for CX3CR1 ranged from 181.41 to 849.03 (510.12 ± 282.96) particles/µl (Table 1). This indicates that the RSV receptor, CX3CR1, is available on the surface of MPs from human BAL and may be able to trap RSV virions and inhibit infection. We have previously shown that the majority of MPs in BAL are derived from respiratory epithelial cells, as determined by their staining for keratin sulfate and surfactant protein D [3]. However, only around 16% of MPs stained positive for CX3CR1. This is probably because CX3CR1 is expressed only on ciliated epithelial cells, while MPs can be released from ciliated, non-ciliated, and alveolar epithelial cells.

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BAL may contain various soluble antiviral factors. To provide further evidence of the anti-RSV activity of MPs, MPs and exosomes were partially purified from BAL...
Fig. 2 Antiviral activity of BAL samples against RSV and fractionation of BAL. RSV subgroup A (Long strain, ATCC VR-26, USA) was propagated in Hep-2 cells in 1× minimum essential medium (MEM) supplemented with 2% fetal bovine serum (FBS) at 37°C for 4-7 days to produce the viral stock, which was titrated and stored in aliquots at -80°C. (A) Ten BAL samples were serially diluted twofold in medium and incubated with 50 TCID₅₀ of RSV at 37°C for an hour. The mixture was inoculated onto Hep-2 cells and incubated at 37°C for 3 days. The Hep-2 cells were pre-seeded overnight in a 96-well tissue culture plate with 2.5 × 10⁴ cells per well. The level of viral infection was measured using an ELISA assay with a specific monoclonal antibody against the viral fusion glycoprotein (Abcam, USA) on the inoculated cells after fixing with 80% cold acetone for an hour at 4°C and blocking endogenous peroxidase activity with 3% hydrogen peroxide for 30 minutes at room temperature. The results were derived from three independent experiments performed in duplicate using 10 individual BAL samples (mean ± SD). Statistical significance was determined by t-test (*, p < 0.05; **, p < 0.01). (B) Anti-RSV activity and the amount of MP after high- (13,000 × g for 1 hour) and ultra-high- (100,000 × g for 1 hour) speed centrifugation of BAL samples. The anti-RSV activity and flow cytometry were conducted using a pool of the 10 BAL samples. The data represent two independent experiments in duplicate.
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Samples by serial centrifugation. Figure 2B shows the anti-RSV activity and MP content of fractions after the serial centrifugation of pooled BALs. Almost all MPs were purified in the 13,000 g fraction. Both the 13,000 g and 100,000 g pellets showed anti-RSV activity, suggesting that both MPs and exosomes could inhibit RSV infection, while the pellet washes did not have any anti-RSV activity, indicating that the inhibitory activity in the pellet was not due to residual supernatant. The soluble fraction in the 100,000 g supernatant also contained anti-RSV activity, indicating that BAL contains soluble factors that are capable of inhibiting RSV infection. A number of soluble antiviral factors have been reported to exhibit anti-RSV activity. Beta-defensin has been shown to inhibit RSV, whereas there are conflicting data regarding the role of surfactant protein A in RSV infection [7–9].

We further tested whether the anti-RSV activity of MPs was brought about by the presence of the viral receptor CX3CR1 on the MPs. To do this, BAL and MP preparations were preincubated with an anti-CX3CR1 monoclonal antibody or isotype control before incubating with the virus. Preincubating BAL and MP with anti-CX3CR1 significantly reduced the RSV inhibitory activity (Fig. 3), whereas exosome and soluble fractions did not show any reduction of anti-RSV activity by anti-CX3CR1. This indicates that CX3CR1 on MP contributed to the MP anti-RSV activity. Since preincubation with anti-CX3CR1 did not completely abolish the anti-RSV activity, it is possible that there were other mechanisms for the anti-RSV activity. For example, other surface molecules on MPs might also bind to RSV virions and contribute to the inhibitory activity.

MPs are released from the cell surface and therefore inherit cell surface molecules from the plasma membrane of their cellular origin. Most MPs in BAL are derived from respiratory epithelial cells. Those cells carry receptors for various respiratory viruses on their surface. These receptors are likely to be transferred to the MP surface, making them capable of trapping viruses specific for those receptors. Here, we show that BAL MPs can inhibit RSV in a receptor-dependent manner. This further supports the hypothesis that MPs contribute to the innate antiviral defense against various respiratory viruses.

Fig. 3 Decrease in anti-RSV activity of BAL and MP by preincubation with anti-CX3CR1. Three BAL samples and purified MPs were pre-incubated with 1 µg of anti-CX3CR1 or isotype control at 37°C for 1 hour. The anti-CX3CR1 antibody concentration was selected by titrating the antibody by flow cytometry. The mixture was incubated with 50 TCID₅₀ of RSV at 37°C for an hour. After incubation, the mixture was inoculated onto Hep-2 cells and incubated at 37°C for 3 days. The level of viral infection was measured using an ELISA assay. The data are representative of three independent experiments performed in duplicate. Statistical significance was determined by t-test (*, p < 0.05; **p < 0.01).
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Declarations

Conflict of interest  The authors declare no conflicts of interest.

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