

RESEARCH ARTICLE

Treatment with broadly neutralizing influenza antibodies reduces severity of secondary pneumococcal pneumonia in mice

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Secondary bacterial pneumonia is a frequent complication of influenza, associated with high morbidity and mortality. We hypothesized that treatment with neutralizing influenza A antibody AT10_002 protects against severe secondary pneumococcal infection in a mouse model of influenza A infection. Influenza A (H3N2) virus-infected male C57Bl6 mice were treated intravenously with either AT10_002 or a control 2 days postinfection. Seven days later, both groups were infected with *Streptococcus pneumoniae* and killed 18 hours later. Mice receiving AT10_002 showed less loss of bodyweight compared with controls (+1% vs -12%, $P < .001$), lower viral loads in bronchoalveolar lavage fluids (BALFs) (7 vs 194 RNA copies per μL ; $P < .001$), and reduced bacterial outgrowth in lung homogenates (3.3×10^1 vs 2.5×10^5 colony-forming units per mg; $P < .001$). The treatment group showed lower pulmonary wet weights, lower cell counts, and lower protein levels in BALF compared with controls. Treatment with AT10_002 was associated with lower levels of tumor necrosis factor- α , interleukin (IL)-6, cytokine-induced neutrophil chemoattractant (KC), and interferon- γ in BALF and lower IL-6 and KC in lung homogenates. Treatment with anti-influenza antibody AT10_002 is associated with reduced weight loss, viral load, bacterial outgrowth, and lung injury in a murine model of secondary pneumococcal pneumonia following influenza infection.

KEYWORDS

AT10_002, broadly neutralizing antibodies, influenza virus, murine model, secondary infections, *Streptococcus pneumoniae*

1 | INTRODUCTION

The annual influenza epidemics have a high burden on society, with an estimated 250 000 to 500 000 influenza-related deaths worldwide.¹ Secondary bacterial pneumonia is a frequent complication and an important cause of both seasonal and pandemic influenza-associated mortality.² Although influenza virus infection commonly

leads to mild and self-limiting disease, it can cause dysregulation of the innate and adaptive immune response, increasing susceptibility to bacterial infection and contributing to disease severity.³⁻⁶ Secondary bacterial infection is most frequently caused by *Streptococcus pneumoniae* and *Staphylococcus aureus*,^{6,7} though in critically ill patients Gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, are often also implicated.^{8,9}

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Postinfluenza pneumonia is associated with a high mortality, and there is a paucity of data as for the optimal approach of antiviral and antibacterial treatments.^{2,10} Hence, much effort is directed toward the prevention of influenza infection and disease severity. Seasonal influenza vaccination is associated with a reduction of hospitalizations for pneumonia, as well as with reduced mortality following admission.¹¹⁻¹⁴ However, influenza vaccine efficacy is limited when the seasonal vaccine composition does not precisely match the actual circulating strains, as has been observed in the 2014-2015 season.¹⁵ Once influenza virus infection has been established, there are indications that early treatment with neuraminidase inhibitors reduces the risk of hospitalization and lower respiratory tract complications, such as pneumonia.¹⁶⁻²⁰ However, influenza variants resistant to neuraminidase inhibitors may emerge, potentially complicating their future use.²¹⁻²³

Broadly neutralizing influenza antibodies are a promising new treatment option. These antibodies target conserved regions of the surface glycoprotein hemagglutinin (HA), thereby blocking infection and replication of multiple influenza A virus subtypes.^{24,25} Influenza A antibody AT10_002 targets HA of group 2 viruses and has been shown to neutralize multiple influenza H3 and H7 variants *in vitro*.²⁶ In the current study, we hypothesized that treatment with influenza antibody AT10_002 reduces disease severity in a mouse model of secondary pneumococcal infection following influenza virus infection.

2 | MATERIALS AND METHODS

2.1 | Mice

All experiments were approved by the Animal Care and Use Committee of the Academic Medical Center, University of Amsterdam (Amsterdam, the Netherlands). Male C57Bl6 mice (± 25 g) were obtained from Charles River Nederland B.V. (Leiden, the Netherlands) and maintained at animal biosafety level 2.

2.2 | Experimental infection protocol

Mice were briefly anesthetized by inhalation of 3% isoflurane and intranasally inoculated with 400 median tissue culture infective dose (TCID₅₀) of influenza A/Hkx/31 (H3N2) in a volume of 50 μ L phosphate-buffered saline. On day 2 after inoculation, mice were injected in the tail-vein with antibody AT10_002 ($n = 8$) (kindly provided by AIMM Therapeutics B.V., Amsterdam, the Netherlands) in a dose of 15 mg/kg. AT10_002 is a human antibody derived from memory B cells of influenza-vaccinated individuals.²⁶⁻²⁸ Controls received an isotypic nonbinding control anti-CD20 antibody (Rituximab, Roche, Grenzach-Wyhlen, Germany) ($n = 7$) in the same volume. On day 7 after influenza virus infection, both groups were intranasally inoculated under isoflurane anesthesia with *S. pneumoniae*, type 3 (ATCC 6303, Rockville, MD) in a dose of 5×10^3 colony-forming units (CFU) suspended in 50 μ L saline (0.9%). Mice were killed 18 hours after pneumococcal

infection. Bodyweight was measured at baseline, 3 times during the course of the infection, and at sacrifice.

2.3 | Exsanguination and organ processing

Mice were anesthetized intraperitoneally with 7.5 μ L/g bodyweight of a mixture of ketamine (16.8 mg/mL) and dexmedetomidine (27 μ g/mL) in sterile saline (0.9%). Mice were bled by heart puncture. Blood was collected in EDTA tubes, centrifuged at 800g for 10 minutes at 4°C, and plasma was stored at -80°C for further analysis. The right lung lobes were harvested and homogenized in 4 volumes of sterile saline using an Omni-TH Tissue Homogenizer (Wilten Instruments, Etten-Leur, the Netherlands). The left lung was used for bronchoalveolar lavage (BAL) and the right lung was used to determine wet weight. BAL fluids were obtained by flushing the left lung 3 times with 0.5 mL sterile saline (0.9%). Supernatant was obtained after centrifugation at 260g for 10 minutes at 4°C, in which the total amount of cells was counted using a Z2 Coulter Particle Counter (Beckman-Coulter Corporation, Miami, FL) and the total protein levels were measured (Oz Biosciences, Marseille, France).

2.4 | Viral load measurement

Nucleic extractions were performed on 50 μ L BAL fluid obtained after sacrifice using the Roche MagNA Pure Total Nucleic Acid Kit on a MagNA Pure 96 instrument (Roche Diagnostics, Penzberg, Germany). A semiquantitative reverse transcription-polymerase chain reaction was performed with the LightCycler 480 (Roche Diagnostics, Penzberg, Germany), using a validated protocol for influenza A.²⁹ Cycle threshold (C_t) values above 40 were considered negative; viral load estimates were calculated based on C_t values.

2.5 | Bacterial outgrowth

Lung homogenates were diluted in serial 10-fold dilutions in sterile saline (0.9%) and plated on blood-agar plates. CFUs were counted after 12-hour incubation at 37°C with 5% CO₂.

2.6 | Cytokine and chemokine assays

Lung homogenates were diluted with equal volumes of lysis buffer (300 mM sterile saline [0.9%], 30 mM Tris, 2 mM MgCl₂, 2 mM CaCl₂, 1% [v/v] Triton X-100, 20 ng/mL Pepstatin A, 20 ng/mL Leupeptin, 20 ng/mL Aprotinin, pH 7.4) and placed on ice for 30 minutes. After centrifugation at 680g for 10 minutes at 4°C, supernatants were obtained and stored at -80°C. The following cytokines and chemokines were measured in lysates by enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D Systems, Abingdon, UK): interleukin 6 (IL)-6, tumor necrosis factor (TNF)- α , interferon (IFN)- γ , and cytokine-induced neutrophil chemoattractant (KC).

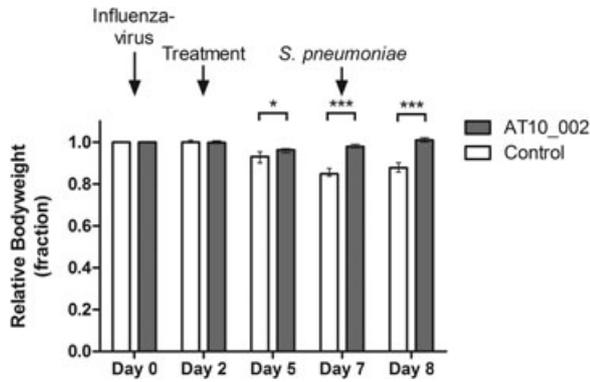


FIGURE 1 Relative bodyweight of mice treated with AT10_002 influenza antibodies vs controls, in a mouse model of influenza infection and secondary pneumococcal infection

2.7 | Statistical analysis

Data are expressed as median and interquartile range, unless stated otherwise. Results of the intervention group were compared to those of the control group using Mann-Whitney *U* tests. $P < .05$ was considered to represent a statistical significant difference.

3 | RESULTS

3.1 | Influenza antibody treatment reduces bodyweight loss during influenza and secondary pneumococcal pneumonia

All mice survived until exsanguination. Mice receiving influenza antibodies exhibited less loss of bodyweight compared with the control group on day 5 (−4% [interquartile range, −3% to −5%] vs −7% [−5% to −10%], $P = .029$) on day 7 after influenza infection (−2% [−1% to −3%] vs −15% [−13% to −16%], $P < .001$), as well as following

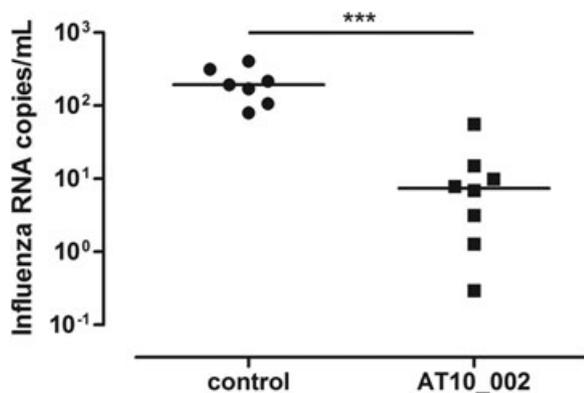


FIGURE 2 Viral loads in bronchoalveolar lavage fluids of influenza-infected mice with secondary pneumococcal pneumonia treated with AT10_002 influenza antibodies vs control. Measurements were performed after sacrifice at 18 hours after pneumococcal infection

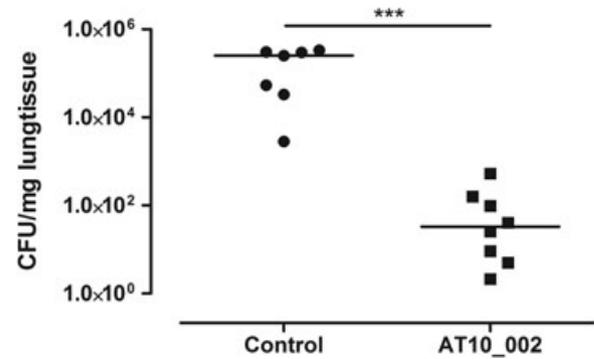


FIGURE 3 Bacterial outgrowth in lungs of influenza-infected mice with secondary pneumococcal pneumonia treated with AT10_002 influenza antibodies vs control. Measurements were performed after sacrifice at 18 hours after pneumococcal infection. CFU, colony-forming units

secondary pneumococcal infection (+1% [0% to 2%] vs −12% [−9% to −14%], $P < .001$) (Figure 1).

3.2 | Influenza antibody treatment reduces viral load and pneumococcal outgrowth

Treatment with influenza antibodies resulted in lower viral loads in BAL fluids compared with the control group (7 [2–14] vs 194 [107–314] RNA copies/ μ L, $P < .001$) (Figure 2), as well as almost a 4 log reduction in CFUs of *S. pneumoniae* in lung homogenates compared with controls (3.3×10^1 vs 2.5×10^5 CFUs/mg, $P < .001$) (Figure 3).

3.3 | Influenza antibody treatment reduces lung injury after secondary pneumococcal pneumonia

Mice receiving AT10_002 influenza antibodies exhibited less pulmonary edema, as measured by a lower lung wet weight compared with

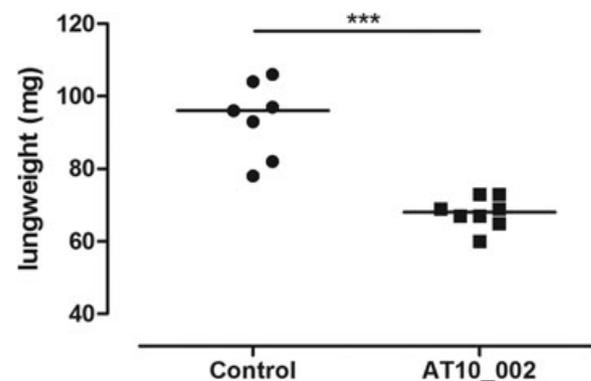


FIGURE 4 Wet lung weight in influenza-infected mice with secondary pneumococcal pneumonia treated with AT10_002 influenza antibodies vs control. Measurements were performed after sacrifice at 18 hours after pneumococcal infection

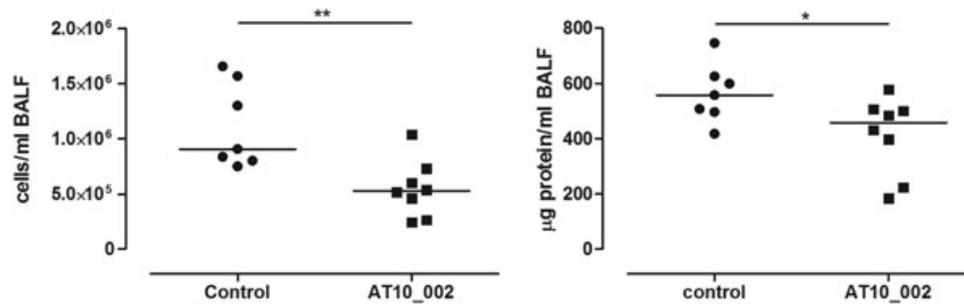


FIGURE 5 A,B, Total cell count and protein levels in BALF in influenza-infected mice with secondary pneumococcal pneumonia treated with AT10_002 influenza antibodies vs control. Measurements were performed after sacrifice at 18 hours after pneumococcal infection. BALF, bronchoalveolar lavage fluid

the control group (68 [66 to 72] vs 96 [82 to 104] mg; $P < .001$) (Figure 4). Treatment also resulted in lower total cell count (53×10^4 [31×10^4 to 70×10^4] vs 91×10^4 [80×10^4 to 156×10^4] cells/mL; $P = .004$) and protein levels in bronchoalveolar lavage fluid (BALF) (458 [267 to 505] vs 558 [496 to 625] µg/mL; $P = .04$) compared with the control group (Figure 5A,B). In addition, lower levels of TNF- α , IL-6, KC, and IFN- γ in BALF after pneumococcal infection were observed in treated mice compared with the control group (TNF- α : 24 [20–46] vs 375 [507–284] pg/mL; $P < .001$; IL-6: 12 [6 to 46] vs 490 [320 to 1251] pg/mL; $P < .001$; KC: 74 [22 to 111] vs 583 [459 to 781] pg/mL; $P < .001$; and IFN- γ : 90 [16 to 345] vs 509 [306 to 864] pg/mL; $P = .004$, respectively) (Figure 6A–H). In lung homogenates, IL-6 and KC were also lower compared to the control group (153 [139 to 181] vs 1160 [795 to 4253] pg/mL; $P < .001$ and 644 [502 to 921] vs 7066 [4560 to 12 825] pg/mL; $P < .001$, respectively), whereas no difference was observed for TNF- α (1538 [1382 to 1781] vs 1338 [1273 to 2080] pg/mL; $P = .397$). Of note, levels of IFN- γ in lung homogenates were higher in mice receiving AT10_002 compared with controls (1136 [910 to 1250] vs 838 [681 to 916] pg/mL; $P = .014$).

4 | DISCUSSION

The current study is the first to investigate the effect of broadly neutralizing influenza antibody treatment in a murine postinfluenza bacterial infection model. Treatment with AT10_002 significantly reduces loss of bodyweight, viral load, and bacterial outgrowth. This effect was associated with reduction of lung injury, as demonstrated by lower wet lung weight, lower cell count, and lower protein levels in BALF. Moreover, lower BALF levels of pro-inflammatory cytokines were observed in the treatment group.

The observed treatment effect may be attributed to a less-severe course of influenza infection, thereby retaining the ability of the host to prevent or combat secondary infection. The current study could not distinguish between lung injury caused by influenza infection and bacterial infection. However, mice receiving AT10_002 had lower viral loads and already exhibited less loss of bodyweight before inoculation with *S. pneumoniae*, suggesting a

less-severe primary influenza infection. It is well established that the virus, bacteria, and host interact with each other in the development of secondary bacterial pneumonia. Influenza virus infection can impair the host response against bacterial invaders in several ways, including an increased adherence and invasion of bacteria due to virus-induced damage of the epithelium^{30,31} and decreased mucociliary velocity and bacterial clearance.³² Furthermore, the innate host response can be dysregulated, including decreased phagocytosis of neutrophils^{33,34} and desensitization of alveolar macrophages.³⁵ The adaptive response can also be impaired during severe influenza infection, which may increase the susceptibility of the host to bacterial superinfection.³⁶

There were distinct effects of AT10_002 on IFN- γ levels, a key cytokine mediator in antiviral immunity. IFN- γ inhibits viral replication directly and has numerous immunomodulatory effects, including promoting Th0 differentiation to Th1 cells, which leads to an adaptive response. In postinfluenza pneumonia however, IFN- γ appears to suppress the host response, possibly by inhibiting phagocytosis of bacteria.³⁷ In our study, we found lower IFN- γ in BALF but higher IFN- γ in lung homogenates of mice receiving antibody treatment compared with controls. We hypothesize that a lower IFN- γ response in the lung compartment is the result of a lower viral load, which might have led to less epithelial damage and increased clearance of bacteria. We are however unsure as to how to interpret the high IFN- γ level in lung homogenates. This may suggest a more adequate systemic host response to bacterial infection, a treatment effect prior to bacterial infection, or a specific binding in the assay.

The current study has several limitations. AT10_002 antibody treatment was highly effective when administered 2 days after influenza infection; however, it remains to be determined whether treatment at a later time point or during secondary bacterial infection is equally beneficial. In addition, survival experiments are needed to study the effects of AT10_002 treatment on mortality. These were not performed as the focus of this project was to study bacterial loads and markers of inflammation and lung injury. Furthermore, adverse effects of AT10_002 are unknown and should be closely monitored when tested in a clinical trial.

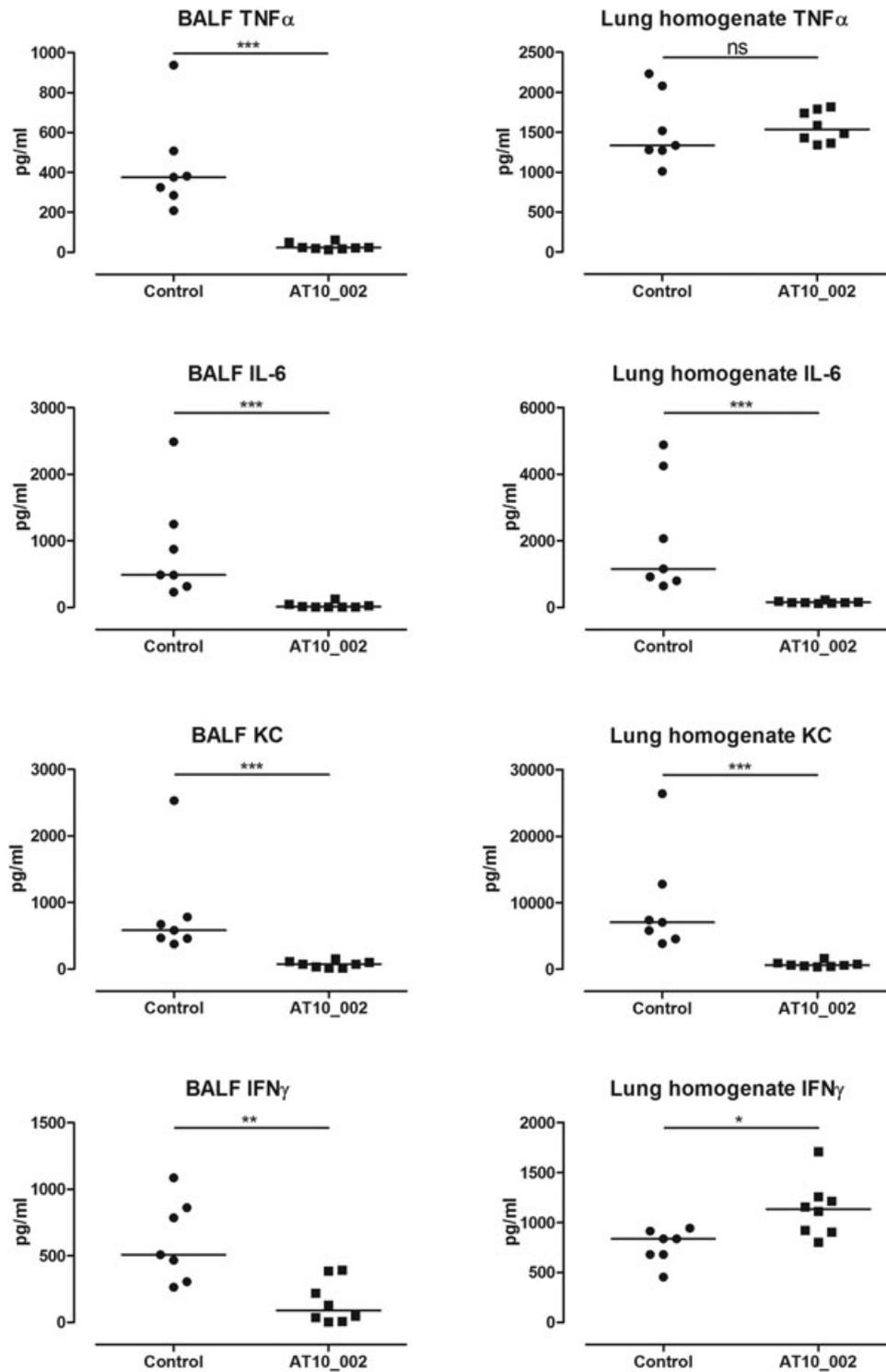


FIGURE 6 A-H, Pulmonary cytokines and chemokines in BALF and lung homogenate influenza-infected mice with secondary pneumococcal pneumonia treated with AT10_002 influenza antibodies vs control. Measurements were performed after sacrifice at 18 hours after pneumococcal infection. BALF, bronchoalveolar lavage fluid; IFN, interferon; IL, interleukin; KC, cytokine-induced neutrophil chemoattractant; TNF, tumor necrosis factor

In conclusion, we show that early treatment with influenza antibody AT10_002 significantly reduced weight loss, lung injury, bacterial outgrowth, and inflammation in a mouse model of influenza infection, followed by secondary pneumococcal pneumonia. Currently, several phase 2 studies are ongoing with similar broadly neutralizing HA antibodies in primary influenza infection.²⁵ Results from these studies will help determine the significance of our promising findings.

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CONFLICTS OF INTEREST

The authors declare no potential conflicts of interest.

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