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C5a receptor1 inhibition alleviates influenza virus-induced acute lung injury

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ABSTRACT

Influenza A virus is an important human pathogen that causes 3 to 5 million severe cases of influenza worldwide each year. An aberrant innate immune response, particularly hypercytokinemia, is thought to play an important role in the disease, although the pathogenesis of severe influenza virus infection remains unclear and no specific and efficacious immunotherapy is available. This study reports dysregulated complement activation in mice after infection with A/Puerto Rico/8/34 (PR8). C5aR1-deficient mice and mice treated with an anti-C5aR1 antibody were used as models to study the C5a-C5aR1 axis during acute lung injury (ALI) induced by influenza virus infection. The results showed that blocking the C5a-C5aR1 axis alleviated ALI by inhibiting endothelial cell activation and dampening the host immune response (i.e., reduced TNF-α, IL-1β, IL-6, IP-10, MCP-1, IL-12p70, and IFN-γ concentrations in plasma), particularly CTL-mediated immunopathology. Furthermore, blockade of the C5a-C5aR1 axis inhibited viral replication in lung tissue. Taken together, the results indicate that the C5a-C5aR1 axis plays an important role in the outcome of ALI induced by influenza virus infection and that regulation of complement activation, particularly the C5aR1 inhibition, is a promising intervention and adjunctive treatment.

1. Introduction

Influenza viruses are important human pathogens that cause substantial seasonal and pandemic morbidity and mortality [1]. Clinically, patients with severe influenza virus infection present with bilateral pulmonary infiltration and hypoxaemia and often die from acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) accompanied by hypoxaemic respiratory failure [2]. Influenza-mediated respiratory injury results from a combination of intrinsic viral pathogenicity and a robust host innate immune response; although such a response often clears the virus, it can exacerbate lung injury [3,4]. Therefore, the high mortality associated with influenza virus infection might be due to an overly exuberant immune response or to impaired viral clearance [1,5,6].

The complement system is an important bridge between the innate and adaptive immune systems and is vital for defence against invading pathogens. The complement system is regulated precisely to balance/control the host immune response. Dysregulated and aberrant complement activation contributes to the pathogenesis of many inflammatory and immunological diseases [7]. The central effector molecules that cause complement-dependent inflammation include the complement-derived peptides C3a and C5a, which promote an inflammatory response by binding to their cognate receptors C3aR and C5aR (C5aR1 or CD88). Although there are two C5aR receptors, C5aR1 and C5a receptor-like 2(C5L2), C5a binding to C5aR1 play important role in promoting inflammatory response during effector phase of allergic, infectious and autoimmune diseases [8,9]. C5a plays an important role in acute and chronic inflammation by activating the lipoxygenase pathway of arachidonic acid metabolism in neutrophils and monocytes; these cells then secrete various cytokines/chemokines that trigger vasodilation and extravasation of both cells and fluid [10,11]. In addition, C5a induces expression of TNF-α and interleukin (IL)-1β mRNA and protein, the major regulators of adhesion molecule expression by monocytes and macrophages, both of which play a major role in acute inflammatory responses [12]. Furthermore, C5a acts as a chemotactic factor for leukocytes by binding to its receptor C5aR; it also stimulates release of IL-8 from bronchial epithelial cells, mononuclear cells and macrophages, which again triggers migration of leukocytes to

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the site of injury [13,14]. Therefore, complement inhibition especially targeting C5a is a promising treatment/prevention of inflammatory diseases and serious infections [15–17].

Here, we examined the role played by the C5a-C5aR1 axis in mediating lung injury induced by influenza virus and the efficiency of C5aR1 inhibition in alleviating ALI. Hence, we examined immune responses and lung damage in virus-infected C5aR1 knockout mice ($C5aR1^{-/-}$) and wild-type (WT) mice treated (or not) with an anti-C5aR1 antibody. The results showed that blockade of the C5a-C5aR1 axis ameliorated inflammatory responses and ALI, suggesting that aberrant activation of the complement cascade, particularly C5a, plays a vital role in mediating ALI after influenza virus infection. Regulation of complement activation, particularly the C5aR1 inhibition, is a promising intervention and adjunctive treatment.

2. Materials and methods

2.1. Ethics statement

All procedures involving animals were approved by the Institutional Animal Care and Use Committees of the Laboratory Animal Center, State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology (permit number: BIME 2016–0015). Animal studies were carried out in strict accordance with the recommendations set out in the Guide for the Care and Use of Laboratory Animals.

2.2. Mice and viruses

Six-week-old wild-type (WT) female BALB/c mice (Laboratory Animal Center, Academy of Military Medical Science, Beijing, China) and $C5aR1^{-/-}$ mice (C·129S4(B6)-*C5ar1*^{TM1Cge}/J, the Jackson Laboratory), which target mutation of complement component 5, receptor1, were maintained in a pathogen-free facility and housed in cages containing sterilised feed and drinking water. A/Puerto Rico/8/34virus (PR8), provide by State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, was grown and titrated in embryonated chicken eggs. The PR8 virus stock was serially diluted to determine the 50% lethal dose in mice.

2.3. Experiment design

The mice were divided into the three groups: WT mice infected with PR8 ($n = 18$), $C5aR1^{-/-}$ mice infected with PR8 ($n = 18$), WT mice administered i.v. with anti-C5aR1 monoclonal antibody (600 μg/kg) 4 h after PR8 virus infection (HM1076, HycultBioTech, The Netherlands) ($n = 18$). All mice were challenged intranasally with ten 50% lethal doses of PR8 virus. On Days 3 and 5 post-virus challenge, plasma and lung tissues from five mice per group were collected and virus titres and cytokine levels were measured. Tissue sections were also subjected to histopathology and immunohistochemistry (IHC). The remaining eight mice per group were monitored daily for 14 days post-infection (p.i.) for signs or symptoms of disease [18].

2.4. Measurement of viral titres in lung tissues

Lung tissues were obtained from infected mice after euthanasia at the indicated times. Tissues were homogenised in minimal essential medium (MEM) plus antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Life Technologies Corporation, NY) to generate 10% (w/v) suspensions of lungs. Virus titres were determined by $TCID_{50}$ as described [19] except L-1-tosylamide-2-phenylethyl chromethyl ketone (TPCK)-trypsin (Sigma-Aldrich, St. Louis, MO), was added into the medium (2 μg/ml) and 0.5% chicken erythrocytes were used in hemagglutination confirming cytopathic effect (CPE) endpoint.

2.5. Detection of C5a concentration, myeloperoxidase (MPO) and intercellular cell adhesion molecule (ICAM)-1 in plasma

The plasma concentration of C5a on Days 0, 3 and 5 and MPO activity, ICAM-1 concentration on Day 5 was measured using mouse enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, CA, USA; Hycult Bio Tech, The Netherlands; R&D Systems, MN, USA respectively) following manufacturer instructions.

2.6. Measurement of inflammatory cytokines in plasma

Plasma samples were collected on Days 3 and 5 post-challenge and the concentrations of interferon (IFN)-γ, IL-1β, IL-6, tumor necrosis factor (TNF)-α, IL-10, IL-12p70, interferon-inducible protein (IP)-10, monocyte chemotactic protein (MCP)-1 and cytokine-induced neutrophil-attracting chemokine (KC) were measured using a MILLIPLEX $_{MAP}$ kit (Merck-Millipore Corporation, MA, USA).

2.7. Immunohistochemical analysis of C5aR1, macrophage, neutrophils and PR8 haemagglutinin (HA)

Formalin-fixed, paraffin-embedded lung tissue samples were sectioned (4 μm thick) and stained with antibodies specific for C5aR1, neutrophils (Santa Cruz Biotechnology, Paso Robles, CA, USA), macrophages (Abcam, Cambridge, MA, USA) and anti-influenza A H1N1 (A/Puerto Rico/8/34) haemagglutinin (HA; Sino Biological Inc. China). After an overnight incubation, bound antibodies were detected using a standard streptavidin-biotin detection system (Beijing Zhongshan Biotechnology Co., Ltd., China). Semi-qualitative analysis was conducted as previously described [20].

2.8. Detection of C5aR1 expression at the transcription level

Total RNA was isolated from lung tissue, and RT-PCR was performed using primers specific for C5aR1 (forward: 5′-caccacgaaacgtgaagttcaaat-3′; reverse: 5′-ggccgtgttatcgtgattatcc-3′). Relative expression of C5aR1 in lung tissue was analysed using the $2^{-\triangle\triangle CT}$ method [21].

2.9. Histological analysis of lung damage

Mice were monitored daily after virus challenge, and symptoms were scored as previously described [18]. Lung tissue was excised on Days 5 and fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. Sections were cut, stained with haematoxylin and eosin, and examined under a light microscope. Semi-quantitative assessment of lung injury was performed by assessing degeneration and necrosis of the bronchi and bronchiolar epithelium, infiltration by inflammatory cells, degeneration and collapse of alveoli, expansion of parenchymal walls, haemorrhage, and interstitial oedema [22].

2.10. Statistical analysis

All statistical analyses were performed using the GraphPad Prism Program (version 5.01; GraphPad Software, Inc.). Student's *t*-test with Welch's correction was used to compare the number of viral RNA copies in lung tissue and expression of MPO, ICAM-1, sickness score and inflammatory cytokines/chemokines levels. C5a concentration and C5aR1 relative expression were analysed by one-way ANOVA with Dunnett's post-test. Survival curves were analysed by Kaplan-Meier survival analysis and the log-rank test. p values < 0.05 were considered statistically significant.