

Complement C5 Activation during Influenza A Infection in Mice Contributes to Neutrophil Recruitment and Lung Injury

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Abstract

Influenza virus A (IAV) causes annual epidemics and intermittent pandemics that affect millions of people worldwide. Potent inflammatory responses are commonly associated with severe cases of IAV infection. The complement system, an important mechanism of innate and humoral immune responses to infections, is activated during primary IAV infection and mediates, in association with natural IgM, viral neutralization by virion aggregation and coating of viral hemmagglutinin. Increased levels of the anaphylatoxin C5a were found in patients fatally infected with the most recent H1N1 pandemic virus. In this study, our aim was to evaluate whether targeting C5 activation alters inflammatory lung injury and viral load in a murine model of IAV infection. To address this question C57Bl/6j mice were infected intranasally with 10^4 PFU of the mouse adapted Influenza A virus A/WSN/33 (H1N1) or inoculated with PBS (Mock). We demonstrated that C5a is increased in bronchoalveolar lavage fluid (BALF) upon experimental IAV infection. To evaluate the role of C5, we used OmCI, a potent arthropod-derived inhibitor of C5 activation that binds to C5 and prevents release of C5a by complement. OmCI was given daily by intraperitoneal injection from the day of IAV infection until day 5. Treatment with OmCI only partially reduced C5a levels in BALF. However, there was significant inhibition of neutrophil and macrophage infiltration in the airways, Neutrophil Extracellular Traps (NETs) formation, death of leukocytes, lung epithelial injury and overall lung damage induced by the infection. There was no effect on viral load. Taken together, these data suggest that targeting C5 activation with OmCI during IAV infection could be a promising approach to reduce excessive inflammatory reactions associated with the severe forms of IAV infections.

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Introduction

The fast activation of the complement system (CS) after a microorganism infects a potential host is an important step in clearance of many pathogens [1]. On the other hand, anaphylatoxins like C3a and C5a, products of the CS cascade, are commonly involved in exacerbated inflammatory reactions that can cause direct harm to the host following infections [2,3,4]. Influenza A virus (IAV), an eight segmented, single stranded, negative-sense RNA virus that belongs to the *Orthomyxoviridae* family, [5], is known to activate the CS [6]. CS activation during infection is thought to be important for an effective immune response and virus neutralization in association with natural IgM antibodies in primary infections [7]. Indeed, IAVs have developed

mechanisms of evading CS within the host, as shown by the anti-C1q action of influenza protein M1 [8]. The pathology caused by IAV is due mainly to excessive inflammatory responses that culminate in lung damage and acute respiratory distress [9].

Extensive deposition of C4d was found in lungs of fatal cases of influenza caused by IAV pandemic strains, 2009 H1N1 and also 1957 H2N2 [10]. Furthermore, increased levels of C3a and C5a had been found in bronchoalveolar lavage fluid (BALF) of mice infected with highly pathogenic avian Influenza H5N1 but not following seasonal IAV infection [11]. The anaphylatoxin C5a is a strong chemoattractant for neutrophils and activates these cells to generate oxidative burst, release of enzymes and phagocytosis (reviewed by [12]). C5a is also able to activate macrophages and

endothelial cells and to promote vascular leakage [12]. All these inflammatory responses in which C5a is involved are important for IAV-associated pneumonia. We and others have previously described a direct relation between severity of inflammation and lethality [13,14]. In addition, CD8⁺ T cell activation during influenza infection requires C5a that acts as a chemoattractant for T lymphocytes [15,16]. Increased levels of C5a found in BALF of severe cases of patients infected with IAV reinforce the idea that C5a is involved in influenza induced lung pathology [6]. Therefore, we hypothesized that preventing activation of C5 during IAV infection could decrease the magnitude of inflammation.

The co-evolution of pathogens and their hosts has generated a plethora of molecules that increase pathogen survival [17]. We previously described a 17 kDa lipocalin protein derived from salivary gland of the soft tick *Ornithodoros moubata*, OmCI (also known as coversin and rEV576). OmCI binds to C5 and prevents conformational changes that lead to C5a generation and formation of the membrane attack complex (MAC) [18,19]. OmCI is also reported to capture the inflammatory mediator leukotriene B₄ (LTB₄) in a binding pocket and to have a neutralizing effect [20,21]. The protein OmCI has been proven active against a variety of species (mouse, rat, guinea pig, pig and human) which has enabled it to be studied in a wide range of animal models including asthma, immune complex lung disease [20], coronary and renal ischaemia-reperfusion injury and sepsis (unpublished data) and in models of autoimmune peripheral neuropathy [21,22,23]. Phase 1 clinical trials of coversin are currently in progress.

In this study, we used OmCI to investigate the inflammatory response to IAV infection in mice and demonstrate that reducing C5 activation decreases IAV mediated inflammation and pathology without increasing viral titer within the lung.

Materials and Methods

Ethics Statement

All animal experiments were approved by CETEA/UFMG animal ethics committee (203/08), according to Brazilian national guidelines on animal work.

Virus

The mouse adapted virus Influenza A/WSN/33 (H1N1)-herein named WSN - was used to infect mice as previously described [13]. Stock virus was produced in chicken eggs and passed once again in eggs and then cultured at a m.o.i of 0.001 in MDCK (Madin-Darby Canine Kidney) cells grown at 37°C and 5% CO₂ for 72 hours in complete Dulbeccos modified Eagle Medium (DMEM; SIGMA) with 1 mM sodium pyruvate, 4.5 mg/ml L-glucose, 100 U/ml penicillin and 100 µg/ml streptomycin, supplemented with 5% heat inactivated fetal calf serum (FCS; CULTILAB; Brazil). Cell culture supernatants were collected and clarified by low speed centrifugation. Virus aliquots were frozen at -80°C until use. Viral stocks were titrated on MDCK cell monolayers in standard plaque assays using agarose overlay in complete DMEM with 2% FCS. Viral inoculum was prepared by diluting the stock virus in sterile phosphate buffered saline (PBS).

Animal infections, and treatment with OmCI and Zileuton

Male 8–10 weeks old C57BL/6J mice were maintained in pathogen free conditions at Laboratório de Imunofarmacologia (UFMG/Brazil). Prior to infection, mice were anesthetized with ketamine/xylazine (60 mg/kg, 4 mg/kg, respectively; Syntec,

Brazil) and then received, via intranasal administration, 10⁴ PFU of Influenza WSN virus in sterile PBS or PBS only (Mock group) in a total volume of 25 µL. Infected mice (5 mice per group) were bled and euthanized 1, 3 and 6 days after infection to obtain Bronchoalveolar Lavage Fluid (BALF) and harvest lungs.

In order to evaluate the effects of OmCI during IAV infection, mice were treated 30 minutes prior to infection with 250 µg OmCI diluted in 200 µL of PBS via intraperitoneal injection and received further daily injections of 200 µg OmCI between days 1 to 5. Animals in the vehicle group were injected with 200 µL PBS each day. Weight loss was assessed daily. Five mice per group were euthanized 1, 3 and 6 days after infection to assess leukocyte infiltration into the airways and in lungs, levels of inflammatory mediators in lungs or in BALF, cell free double stranded DNA (cfDNA) in BALF, histopathological changes and viral load in lungs.

The 5-lipoxygenase enzyme (5-LOX) inhibitor Zileuton (Zyflo, Cornerstone Therapeutics In, USA) was used to study the role of leukotrienes during IAV infection. 5-LOX is solely responsible for an early step in the synthesis of leukotrienes. A group of three mice received Zileuton from day zero to day five post-infection and another group of five mice received a combination of Zileuton and OmCI in the same period. Zileuton was given at a dose of 30 mg/kg, diluted in 5% absolute ethanol (Synth, Brazil) in a solution of Carboxymethyl Cellulose 0.5% (Synth, Brazil), via oral gavage in 200 µL.

Bronchoalveolar lavage and tissue extraction

At indicated time points, infected mice were anesthetized with a solution of 150 mg/kg of ketamine and 10 mg/kg of xylazine and blood was collected by the brachial plexus. Blood was allowed to clot and centrifuged, for collection of serum, which was frozen for further analysis. Once death was confirmed, a 1.7 mm catheter was inserted into the trachea and bronchoalveolar lavage (BAL) performed. To this end, the bronchoalveolar compartment was flushed twice with 1 mL aliquots of PBS going back and forth three times to acquire leukocytes recruited to the airways [24]. After centrifugation, the pellet was used to derive total and differential cell counts and for FACS analysis (see below). After BAL and perfusion of lungs with 5 mL of PBS in order to remove circulating blood, the right lobes were removed and frozen for quantification of Myeloperoxidase (MPO) to assess neutrophil infiltration, inflammatory mediators and plaque assay to measure viral load. Left lobes were fixed in formalin for histological examination.

Measurement of MPO, inflammatory mediators and total protein

Lung tissue (100 µg) was homogenized in PBS containing antiproteases, as previously described [25]. MPO levels in cell pellets were assayed using a solution of 1.6 mM tetramethylbenzidine and 0.1 mM H₂O₂ as previously described [25]. Supernatants were used to assess the concentrations of IFN-γ, CXCL1 and C5a by ELISA using DuoSet kits from R&D Systems, in accordance with the manufacturer's instructions. BALF was also used to quantify C5a, IFN-γ and CXCL1 levels and serum was used to quantify C5a levels using the same ELISA assay. Total protein quantification based on Bradford's method (Bio-Rad Protein Assay) was performed on BALF to measure protein leakage to airways.

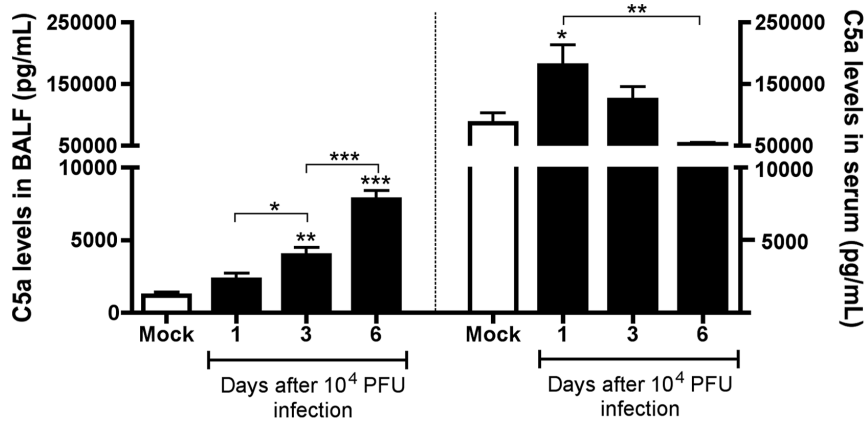


Figure 1. Effects of IAV infection on C5a levels in BALF (left) and serum (right). C57BL/6J mice were infected intranasally with 10^4 PFU of Influenza A/WSN/33 H1N1, or received PBS intranasally (Mock group). At the indicated time points after infection 5 mice per group were euthanized, blood was collected and BAL performed. Serum and BALF concentrations of C5a were measured by ELISA. Data are presented as Mean \pm SEM. *, ** and *** for $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively, when compared to Mock group or indicated group (One-way ANOVA, Newman-Keuls Multiple Comparison test).

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cfDNA quantification

As an indirect way to assess NETs and cell death, cfDNA was quantified in cell-free BALF using Quant-iT PicoGreen dsDNA quantification kit (Invitrogen) in accordance with the manufacturer's instructions. Briefly, the BALF samples and a DNA standard curve (from 1 ng/mL to 1 μ g/mL) were diluted in TE buffer and then mixed and incubated for 5 minutes with Quant-iT PicoGreen reagent at room temperature before measuring fluorescence (480 nm for excitation and 520 nm for emission). Samples were compared to the standard curve and the results expressed in μ g/mL.

Flow cytometric analysis of leukocyte populations and cell death

Leukocytes collected from airways of non-infected and infected mice at day 6 after IAV infection were used to analyze cell death by Annexin V staining and Propidium Iodide (PI) incorporation (FITC Annexin V apoptosis detection kit I, BD Pharmingen), according to the manufacturer's instructions. The populations of macrophages, neutrophils and lymphocytes were analyzed by staining with fluorescent monoclonal antibodies against CD3 (PE-Cy7, BD Biosciences, USA), CD4 (APC, BD Biosciences, USA), CD8 (PerCP, Biolegend, USA), F4/80 (PE-Cy7, eBioscience) and GR1 (Alexa 488, Biolegend). Stained cells were acquired in BD LSRFortessa cell analyzer (BD Biosciences, USA) and analyzed using FlowJo software (Tree Star, Inc., USA).

Histological analysis

Formalin-fixed left lobes of lungs were dehydrated gradually in ethanol, embedded in paraffin, cut into 4 μ m sections (3 sections per lung), stained with Hematoxylin and Eosin (H&E) and examined under light microscopy by a pathologist blind to the experimental procedure. All 3 sections of all left lobes were examined and an average score assigned to each lung [26]. The maximum score was 23 and the following parameters were evaluated: airway inflammation (4 points), vascular inflammation (4 points) and parenchymal inflammation (5 points), epithelial injury (5 points) and overall neutrophilic infiltration (5 points).

Plaque assay

Lungs were collected in sterile conditions, weighed and homogenized in PBS. The viral load in lungs were assessed on MDCK cell monolayers, in standard plaque assays using agarose overlay in complete DMEM with 2% FCS. Briefly, MDCK cell monolayers, in 6 well plates, were incubated for 60 minutes with serial 10-fold dilutions of lung homogenates at 37°C and 5% of CO₂, when the agarose monolayer was added. After additional 72 hours of incubation, agarose overlays were removed, the cells were fixed in formalin and stained with crystal violet. The viral titer was expressed as plaque forming unit (PFU) per gram of tissue.

Statistical analysis

All data are presented as the mean \pm SEM and were analyzed using One-way analysis of variance followed by Newman-Keuls post-test to compare different groups. Unpaired t test was used to compare two groups. Statistical significance was set as $P < 0.05$ and all graphs and analysis were performed using Graph Pad Prism 4 software.

Results

Local complement activation after Influenza virus A (IAV) infection

Complement activation following IAV infection has been found to be correlated with clinical disease severity [6,10,27]. Thus, aiming to assess complement activation in our system, we infected C57BL/6J mice with 10^4 PFU of the mouse-adapted Influenza WSN virus. We collected blood, performed bronchoalveolar lavage (BAL) and lungs were harvested 1, 3 and 6 days after infection. We found significantly increased C5a levels in BALF of IAV infected animals from the third to the sixth day of infection, peaking at day 6 after infection (Figure 1, left). By contrast, C5a levels in lung homogenates after infection were similar to mock group at all time points after infection (data not shown), and serum levels of C5a increased at the first day of infection and decreased at day six (Figure 1, right).

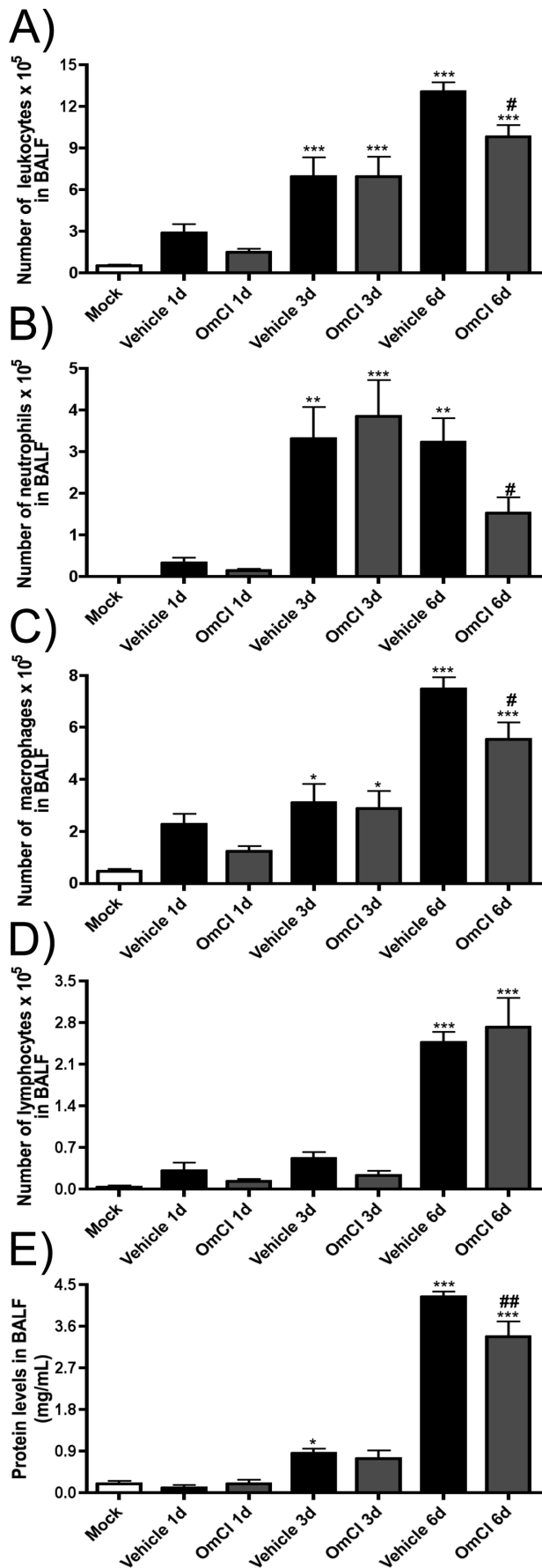


Figure 2. Effects of C5 activation inhibitor OmCI during IAV infection on leukocyte transmigration and protein leakage.

C57BL/6J mice were infected intranasally with influenza or Mock infected as described in Figure 1. OmCI treated mice received the protein prior to IAV infection and daily thereafter, while vehicle group received PBS, until one day before the indicated time point. At the 1, 3 and 6 days after infection, mice were euthanized and BAL performed. A) Total number of leukocytes, B) number of neutrophils and C) number of macrophages, D) number of lymphocytes recovered from the airways; E) total protein quantification in BALF. Data are presented as Mean \pm SEM. *, ** and *** for $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively, when compared to Mock group; # and ## for $p < 0.05$, $p < 0.01$ respectively, when compared to Vehicle group sampled on the same day (One-way ANOVA, Newman-Keuls Multiple Comparison test). doi:10.1371/journal.pone.0064443.g002

Treatment with OmCI decreases C5a levels, neutrophil and macrophage accumulation and protein leakage after IAV infection

In order to evaluate a possible role of C5a for the inflammatory responses caused by IAV infection, infected mice were treated with the protein OmCI that binds to C5 and prevents generation of C5a and formation of the membrane attack complex (MAC). Infection with IAV induced an intense influx of leukocytes (Figure 2A) in the airway spaces that peaked at day 6 after infection. The inflammatory infiltrate was predominantly composed of neutrophils (Figure 2B) that were present in high number from the third day after infection and macrophages (Figure 2C) that peaked at day six. Lymphocytes were found in significantly elevated numbers only 6 days after infection (Figure 2D). Protein levels in BALF, a sign of increased vascular permeability [28], were increased from the third day after infection and greatly increased by day 6 (Figure 2E). There was also significant infiltration of neutrophils in lungs of vehicle treated, infected mice as assessed by MPO quantification (Figure 3A) or histopathological analysis (Figure 3B–E; asterisks).

When IAV-infected mice were treated with OmCI, the total number of leukocytes (Figure 2A), neutrophils (Figure 2B) and macrophages (Figure 2C) were reduced in BALF when compared to vehicle treated group at day 6 after infection. This was also confirmed when leukocyte types were evaluated by flow cytometry (data not shown). The number of lymphocytes in BALF of OmCI-treated mice was similar to that of vehicle treated mice (Figure 2D). Protein leakage at day six after IAV infection was reduced in OmCI treated mice, when compared to vehicle group (Figure 2E). Inhibition of neutrophil influx into the airways of OmCI-treated animals was not associated with decrease in accumulation of these cells in lung parenchyma, as assessed by MPO levels (Figure 3A) and histopathological analysis (Figure 3B–E). Both OmCI treated and control mice inoculated with IAV displayed similar weight loss ($23.1\% \pm 6.2\%$ versus $22.5\% \pm 4.6\%$, respectively).

IAV infection induced a significant increase in levels of C5a (Figure 4A) and the chemokine CXCL1 (Figure 4B) in BALF and levels of CXCL1 (Figure 4C) and IFN- γ (Figure 4D) in lung homogenates. After OmCI administration into IAV-infected mice, levels of CXCL1 (Figure 4C) and IFN- γ (Figure 4D) were similar to those found in the vehicle group, while C5a levels were partially reduced (Figure 4A). There was very significant inhibition of CXCL1 levels in BALF in OmCI treated mice 3 days after infection (Figure 4B).

Effects of OmCI treatment on lung damage caused by IAV infection

Histopathological analysis of lung slides showed that, after 6 days of IAV infection, there was significant inflammatory infiltration in peribronchiolar and perivascular areas in addition

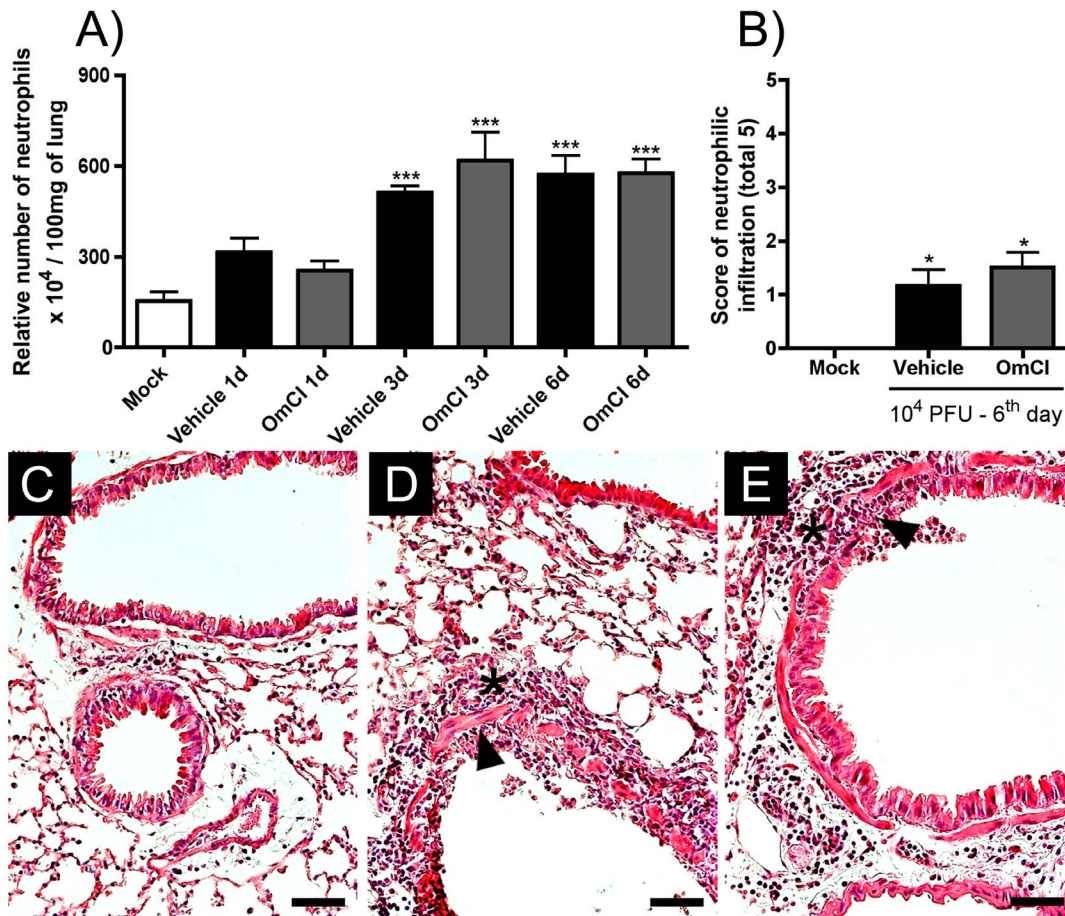


Figure 3. Neutrophil accumulation in lung parenchyma of vehicle and OmCI treated mice. C57BL/6J mice were infected and assigned to treatment groups as in Figure 2. At 1, 3 and 6 days after infection, mice were euthanized, lungs harvested and MPO assayed, to measure neutrophil accumulation in tissue. A) Relative numbers of neutrophils in lungs. At day 6, lungs were harvested for assessment of neutrophil infiltration by analysis of H&E stained lung slides. B) Pathologic score (0–5) of neutrophil accumulation in lungs performed by a pathologist. Representative slides of H&E stained lungs of a C) mock mouse; D) vehicle mouse; E) OmCI treated mouse. Asterisks indicate areas with neutrophils infiltration and arrowheads indicate bronchial epithelial damage. Data are presented as Mean ± SEM. * and *** for p<0.05 and p<0.001, respectively, when compared to Mock group (One-way ANOVA, Newman-Keuls Multiple Comparison test). Bars represent 100 µm. doi:10.1371/journal.pone.0064443.g003

to bronchial epithelial damage and parenchymal inflammation. Focal inflammatory infiltrates composed of mononuclear cells and neutrophils were visualized in the septa and alveolar lumen (Figure 5D, E, F) while normal lungs of PBS instilled mice (Mock group) did not present these alterations (Figure 5A, B, C). Remarkably, the overall appearance of the lung of OmCI-treated animals was better than that of vehicle-treated animals and this was reflected in an overall amelioration of pathologic scores (Figure 5G, H, I, J). We also used a score system (Figure 5J) which measures individual airways (a), vascular (v) and parenchymal inflammation (p) as well as neutrophil infiltration (asterisks) and epithelial injury (arrowheads, Figure 3 D, E). In all the evaluated parameters vehicle-treated IAV infected group had a higher score compared to Mock group, but OmCI-treated IAV infected group presented reduced vascular and parenchymal inflammation and also lower epithelial injury. Thus, overall pathologic scores, on a 23 points scale, were reduced in OmCI (8.0±1.1) when compared to vehicle treated group (14.5±1.2), Figure 5 J.

Effects of OmCI treatment on cfDNA and cell death caused by IAV infection

NETs are composed of DNA fibers and cytoplasmic proteins and released by neutrophils during infections to kill bacteria, fungi or protozoa [29]. However, NETs may also contribute to tissue damage [30]. The quantification of cell free double-stranded DNA (cfDNA) in BALF is an indirect way to quantify NETs [31] and showed that IAV infection leads to increased cfDNA content from day 3 that continues to increase to day 6 after infection (Figure 6A). Treatment with OmCI was associated with significant reduction in cfDNA levels in BALF at day 6 but not day 3 after infection (Figure 6A).

Cell free DNA can also be found as a result of late apoptosis or necrosis [32]. So, we analyzed whether OmCI treatment affected cell death known to be induced by IAV infection [33]. We analyzed Annexin V binding and PI incorporation in leukocytes recovered from the airways. Indeed, IAV infection caused increased numbers of apoptotic (Annexin V+ PI-, Figure 6B), but also necrotic (or late apoptotic, Annexin V+ PI+, Figure 6C) leukocytes. OmCI treatment resulted in diminished number of necrotic (Figure 6C) but not apoptotic cells (Figure 6B).

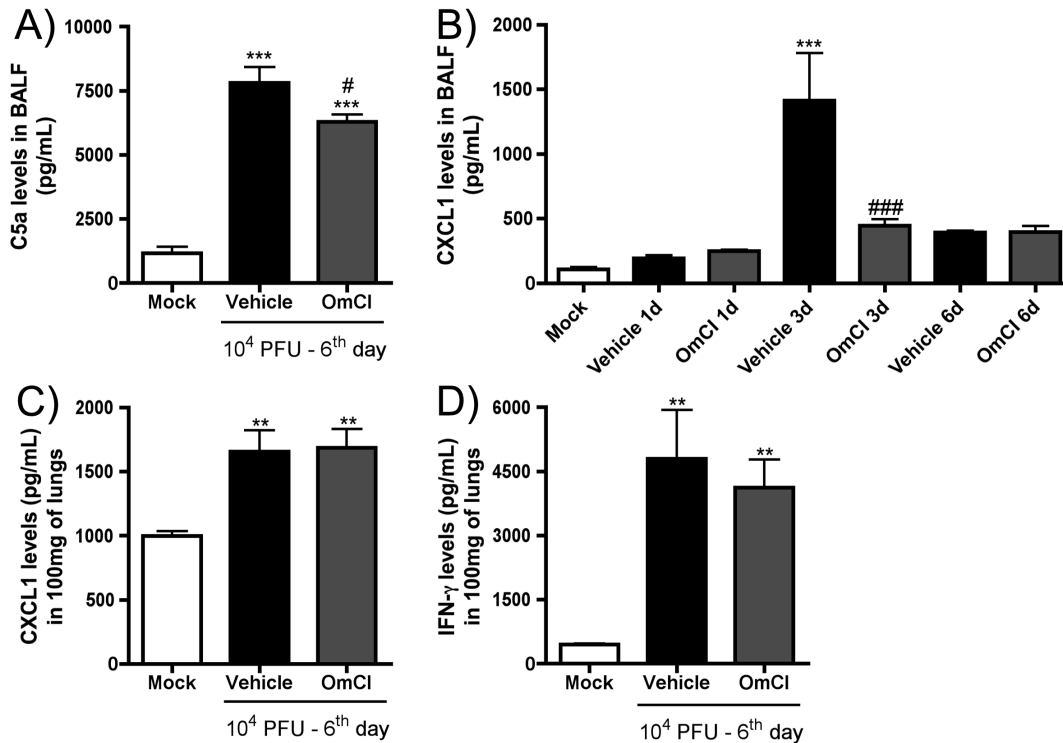


Figure 4. Inflammatory mediator levels after IAV infection. C57BL/6J mice were infected and assigned to treatment groups as in Figure 2. At 1, 3 and 6 days after infection, mice were euthanized, BAL performed and lungs harvested. BALF concentrations of C5a (A), CXCL1 (B) and pulmonary concentrations of CXCL1 (C), and IFN- γ (D) were measured by ELISA. Data are presented as Mean \pm SEM. ** and *** for $p < 0.01$ and $p < 0.001$ respectively, when compared to Mock group; # and ### for $p < 0.05$ and $p < 0.001$ respectively, when compared to vehicle group sampled on same day (One-way ANOVA, Newman-Keuls Multiple Comparison test). doi:10.1371/journal.pone.0064443.g004

OmCI treatment does not alter the titer of IAV

Complement is a crucial host system that is activated during influenza virus infection and plays an important role in viral clearance [34]. The anaphylatoxin C5a, which is highly chemoattractant to neutrophils [12], is also chemoattractant to T lymphocytes [15] and is important for effective anti-IAV activity mediated by CD8⁺ T cells [35]. Although differential cell counts showed the same number of lymphocytes in vehicle and OmCI treated mice at day 6 after infection (Figure 2 E), further analysis of lymphocyte populations by FACS showed that CD8⁺ T cells, that are increased in the airways after IAV infection are present in lower numbers in OmCI treated mice (Figure 7A). This difference is not found in CD4⁺ T cells subset (data not shown). However, despite reduction in CD8⁺ T cells, viral titers in lungs of mice infected with IAV was not altered by treatment with OmCI (Figure 7B), which suggests viral clearance was unaffected by OmCI.

Treatment with Zileuton, a 5-LO inhibitor, does not alter IAV-associated pulmonary inflammation

In order to assess whether the LTB₄ binding ability of the protein OmCI accounts for its properties during IAV infection, we used Zileuton, a 5-LOX inhibitor, alone or in combination with OmCI. If LTB₄ binding by OmCI accounts for the anti-inflammatory activity of OmCI in this model, Zileuton should inhibit IAV associated pulmonary inflammation as effectively as OmCI. After IAV infection, Zileuton treatment or combination of Zileuton and OmCI did not change leukocyte recruitment to the airways (Figure 8A, B) or viral loads (Figure 8D), when compared

to vehicle treated mice. The combined treatment of Zileuton and OmCI, but not Zileuton alone, induced similar effects to that of OmCI treatment in cfDNA levels (Figure 8C), which indicates that LTB₄ might not be involved in OmCI effects.

Discussion

Medical concern about IAV pandemics and epidemics faces the challenge of new viral influenza strains that may become resistant to antivirals [36]. For this reason, anti-inflammatory strategies targeting excessive activation of the innate immune system may be a useful adjunctive therapy against IAV infection [13,37]. We demonstrate here that an arthropod derived inhibitor of C5 activation decreases the recruitment of neutrophils and macrophages to the alveolar space and reduces consequent epithelial damage and lung pathology in IAV infected mice. We also show that reduction of lung inflammation by OmCI did not reduce viral clearance.

A recent paper from O'Brien and colleagues (2011) detected increased C3a and C5a levels in BALF of mice infected with a highly pathogenic H5N1 avian Influenza virus, VN/1194 but not in BALF of animals inoculated with low virulent seasonal or 2009 pandemic viruses [11]. Using the mouse adapted IAV strain A/WSN/33, that mimics a model of severe infection [38] to study innate immune response to the virus we found an increase in C5a levels during the course of infection. O'Brien and colleagues (2011) showed that C3 plays a protective role during IAV infection using a C3 KO mouse strain that showed more severe lung inflammation, weight loss and increased viral load than wild type mice. The protective role of C3 in IAV infection might be explained by the

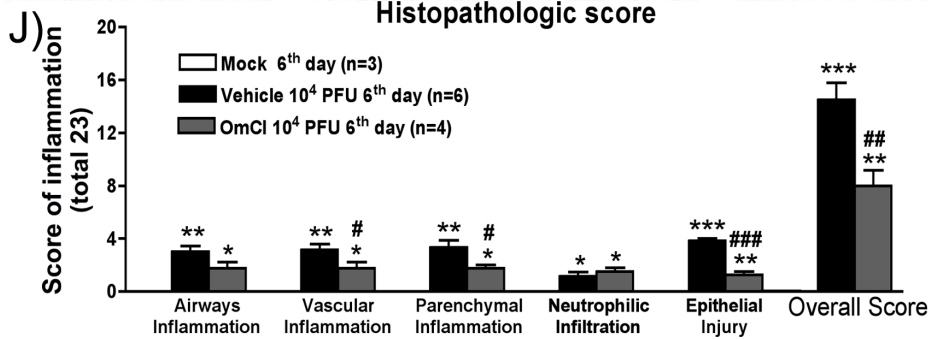
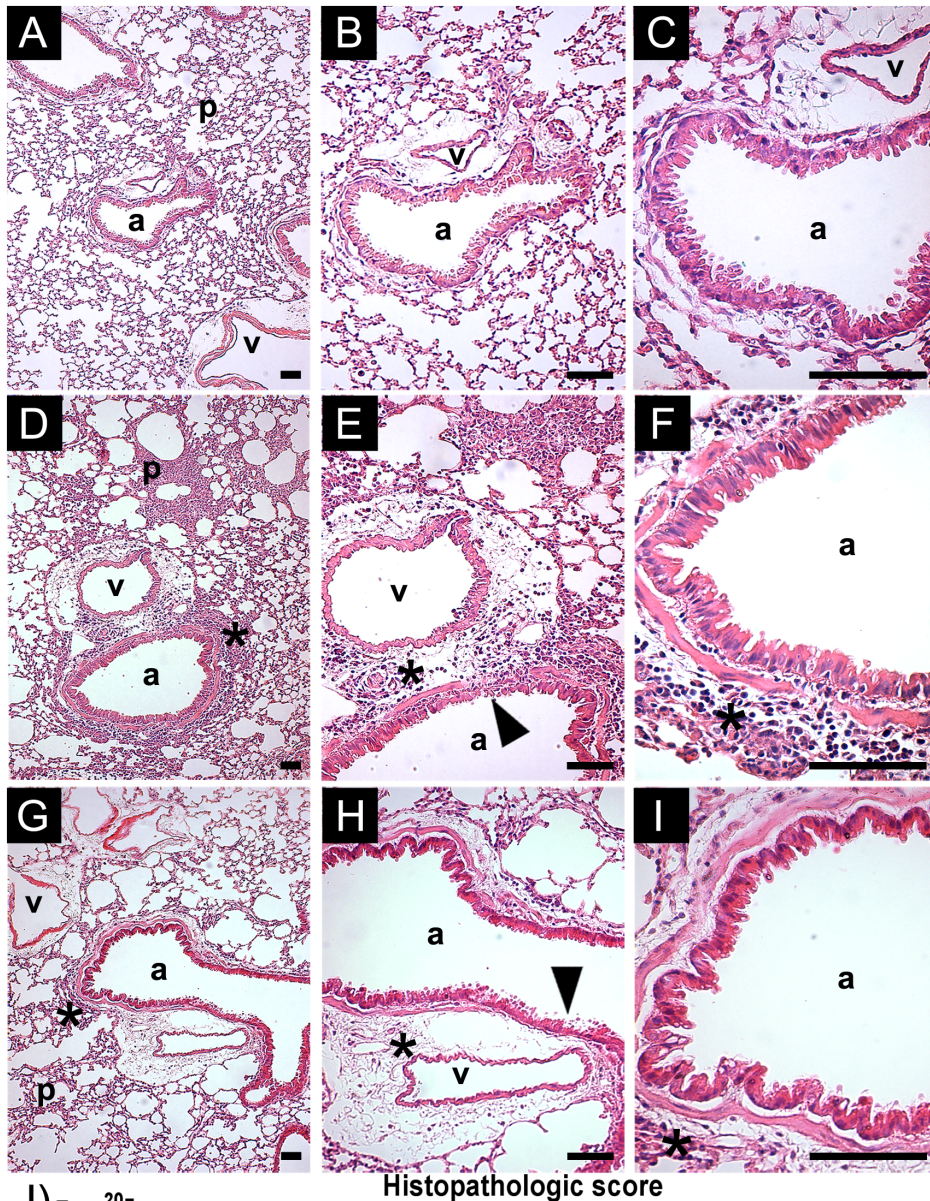


Figure 5. Histopathologic changes caused by IAV are reduced in OmCI treated mice. C57BL/6J mice were infected and assigned to treatment groups as in Figure 2. At the sixth day after infection, mice were euthanized and lungs harvested. Representative slides of H&E stained lungs of mock (A, B, C); vehicle (D, E, F); and OmCI treated mice (G, H, I). “p” represents parenchyma, “v” represents vessels and “a”, airways. Asterisks indicate foci of inflammatory infiltrates and arrowheads indicate areas of epithelial injury. (J) Histopathological score (maximal of 23) evaluated airway, vascular, parenchymal inflammation, neutrophilic infiltration and epithelial injury. Data are presented as Mean ± SEM. *, ** and *** for p<0.05, p<0.01 and p<0.001 respectively, when compared to Mock group; # and ## for p<0.05, p<0.01 respectively, when compared to Vehicle group (One-way ANOVA, Newman-Keuls Multiple Comparison test). Bars represent 100 µm. doi:10.1371/journal.pone.0064443.g005

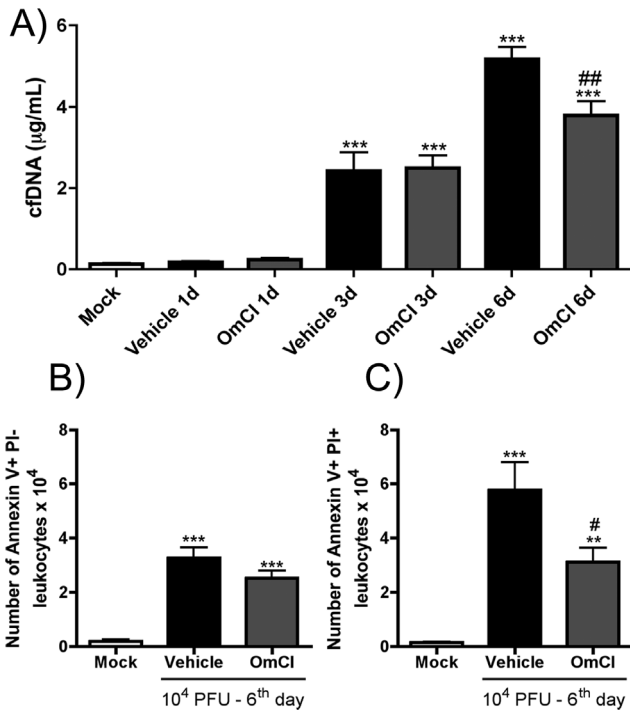


Figure 6. Effects of OmCI on cfDNA levels and number of dead cells in BALF after IAV infection. C57BL/6J mice were infected and assigned to treatment groups as in Figure 2. At 1, 3 and 6 days after infection, mice were euthanized and BAL was performed. A) Cell free DNA (cfDNA) levels were measured in BALF by the Quant-iT PicoGreen dsDNA quantification kit. At six days after infection, leukocytes recovered from airways of Mock, Vehicle and OmCI treated mice were analyzed for cellular death by Annexin V and PI incorporation. B) Total apoptotic leukocytes, Annexin V+ PI-, in BALF; C) Total necrotic or late apoptotic leukocytes, Annexin V+ PI+, in BALF. Data are presented as Mean ± SEM. ** and *** for p<0.01 and p<0.001 respectively, when compared to Mock group; # and ## for p<0.05, p<0.01 respectively, when compared to Vehicle group (Kruskal-Wallis test, Dunn's Multiple Comparison post-test). doi:10.1371/journal.pone.0064443.g006

ability of C3a to down-regulate regulatory T cell population and antigen-specific Th17 cells observed in a model of allergic lung inflammation [39]. Furthermore, in a model of IgG immune-complex deposition, C3 deficient mice developed marked lung injury that diminished with C5a blockade [40], suggesting that C5a has a more pathogenic role during inflammation than C3 and its products. This pathogenic role of C5a can be explained by its greater ability to stimulate chemotaxis, granule release and superoxide production in neutrophils than C3a even in low concentrations [41]. The activation of macrophages and endothelial cells by C5a to promote vascular leakage [12], and consequently protein accumulation in the airways, may also explain the protection found after OmCI treatment. Moreover, *in vitro* studies demonstrated that C5a, in association with GM-CSF, is able to induce the release of NETs [42]. It was recently reported that NETs are produced during IAV infection and are associated with alveolar damage in IAV induced pneumonitis [43]. We observed that IAV-induced cfDNA, an index of NETs formation, was reduced after OmCI treatment (Figure 6), and is coincident with reduced lung damage after infection in OmCI treated mice (Figure 5). It is of note that OmCI treatment also reduced number of dead cells in the BAL of infected animals. As dead cells may directly contribute to an increase in cfDNA, the effects of OmCI

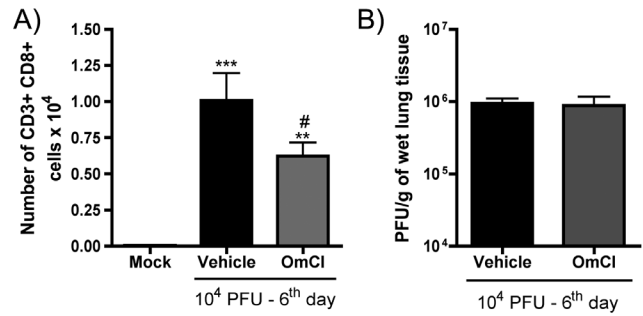


Figure 7. Number of CD8+ T cells and pulmonary viral load after IAV infection and OmCI treatment. C57BL/6J mice were infected and assigned to treatment groups as in Figure 2. A) Numbers of CD3+ CD8+ cells recovered by BAL and analyzed by FACS are reduced in OmCI treated group 6 days after infection. ** and *** for p<0.01 and p<0.001 respectively, when compared to Mock group; # for p<0.05, when compared to Vehicle group (Kruskal-Wallis test, Dunn's Multiple Comparison post-test). B) Viral titers 6 days after infection in lungs homogenates shown are not changed between vehicle and OmCI treated groups, as assessed by MDCK plaque formation (Unpaired t test). doi:10.1371/journal.pone.0064443.g007

on cell death may also contribute to the decrease of this parameter, in addition to any possible effect on NET formation.

We found reduced neutrophil and macrophage transmigration and reduced protein leakage in alveolar spaces but no change in the number of neutrophil in lungs of OmCI treated mice (Figures 2 and 3). Accumulation of neutrophils in different lung compartments requires different mechanisms, and the migration of neutrophils to the airways after crossing the epithelial layers is known to be critical for the induction of lung injury [44]. For example, we have previously shown that, in a model of pulmonary fibrosis induced by bleomycin, the blockade of CXCR2, the chemokine receptor involved in neutrophil chemotaxis, led to reduced neutrophil number in BALF and protection from lung fibrosis, but there was still neutrophil accumulation in lungs at a later time point [25]. Blockade of neutrophil and macrophage influx into the airways, accompanied by lower protein leakage by OmCI treatment with consequent reduction in NETs formation, cellular death and epithelial damage may explain the beneficial effects of the protein during IAV infection.

OmCI, also known as coversin and rEV576 is a recombinant small protein belonging to the lipocalin super-family [19] and has a circulating half-life of 30 hours when bound to C5 [18]. It is derived from a native protein discovered in the saliva of the soft tick *Ornithodoros moubata* where its function appears to be to assist the parasite to feed without provoking a host immune response. OmCI has been found to prevent in a dose-related manner the cleavage of complement C5 of all species that it has been tested, including mouse, rat, guinea pig, pig and human [18,19,23].

The use of complement antagonists during viral infections faces the challenge of possible impairment of pathogen clearance due to ineffective immune responses against the virus [45]. Reduced levels and activity of specific T CD8 cells were found in mice treated with a C5aR antagonist [35] and C3 deficient mice presented reduced T cell activity and increased viral levels after IAV infection [34]. Nevertheless, here we showed that partially reducing C5a levels through the use of a C5 binding protein during IAV infection indeed reduced CD8+ T cells, but did not affect host response against the virus. Normal viral loads and IFN-γ levels were found in OmCI treated mice, suggesting that the ability of the host to deal with the virus was unaffected. However,

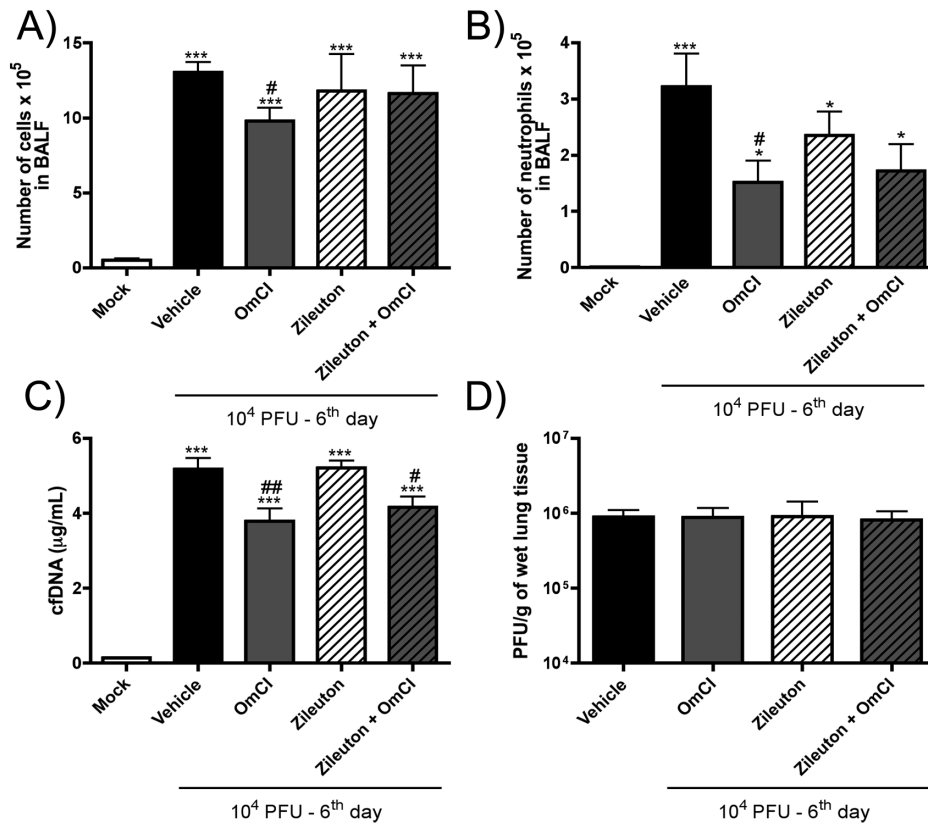


Figure 8. Leukocyte recruitment and cfDNA levels in BALF after IAV infection upon OmCI and Zileuton treatment. C57BL/6J mice were infected intranasally with 10^4 PFU of Influenza A/WSN/33 H1N1, or received PBS intranasally (Mock group). The 5-LO inhibitor Zileuton (30 mg/kg) was given alone or in combination with OmCI. Mice received the treatment prior to the infection and daily until day 5 after infection, while vehicle group received PBS both, via i.p, Zileuton was given by oral gavage. At the sixth day after infection, mice were euthanized, BAL performed and lungs were harvested. A) Total number of leukocytes; B) number of neutrophils; C) cfDNA levels measured in BALF. Data are presented as Mean \pm SEM. * and *** for $p < 0.05$ and $p < 0.001$ respectively, when compared to Mock group; # and ## for $p < 0.05$, $p < 0.01$ respectively, when compared to Vehicle group (One-way ANOVA, Newman-Keuls Multiple Comparison test). doi:10.1371/journal.pone.0064443.g008

inflammation and tissue injury caused by the infection were reduced by OmCI treatment. The concept that there are mediators which contribute to tissue injury but not to host resistance may be useful from a therapeutic point of view [46]. Indeed, our group has provided several proof of concept studies demonstrating the beneficial role of blocking certain aspects of inflammation for the outcome of infection in several animal models, including those caused by bacteria [47], IAV [13], other viruses [48] or protozoan [49].

The molecule CD59a, is a natural regulator of the MAC. It was demonstrated that CD59a KO mice infected with IAV present exacerbated lung inflammation associated with enhanced activation of MAC, excessive neutrophil numbers and death, despite increased virus specific CD4 T cell responses [50]. This latter finding supports our studies by suggesting that excessive C5 activation may cause unwanted inflammation in the context of IAV infection. Furthermore, the latter study suggests that the ability of OmCI to reduce MAC formation [19] may contribute to the observed effects in our model, in addition to an effect on C5a production.

Besides its action in preventing C5 cleavage, OmCI has a neutralizing effect over LTB₄ [20,23]. This property of OmCI could potentially contribute to its effects on IAV infection, as LTB₄ is a potent neutrophil chemoattractant in its own right. However, treatment of mice with an inhibitor of 5-LO, the

enzyme responsible for LTB₄ formation, showed that this pathway appears to have little effect in the context of IAV infection. A recent study showed that treatment of mice with a human C5a agonist protected mice from lethality caused by IAV infection by enhancing neutrophil, dendritic cell and NK cell populations [51]. The authors used a small synthetic polypeptide from C5a, called EP67, engineered to eliminate the anaphylactic activity of C5a, because it lacks the ability to bind C5aR (CD88) on neutrophils but can directly activate C5aR on other cells such as APC. Therefore, the authors used a pharmacological strategy to enhance the innate anti-viral immune responses without enhancing viral-associated inflammation. We used a compound which prevents C5a formation, which was associated with decreased neutrophil influx and associated damage. Therefore, although these studies are apparently contradictory, both molecules – EP67 and OmCI – have been engineered to cause reduced inflammation with enhanced (EP67) [51] or unaltered (OmCI) anti-viral immunity. It is possible that the role of complement in preventing excessive inflammation during IAV infection may be virus strain specific or dependent on the particular host. Only studies in humans naturally infected with different strains and subtypes could address this possibility. Notably, other studies using different therapeutic agents have reported benefits associated with decreasing excessive cell influx – macrophages, neutrophils, DCs, CD4 and CD8 T cells – during influenza virus infections (reviewed by [52]).

Therefore, the hypothesis that decreasing unwanted inflammation during IAV infection may be beneficial is potentially of interest but needs to be addressed adequately in clinical trials in humans.

The involvement of C5 in many diseases could be studied in a wide range of animal models including asthma, immune complex lung disease, coronary and renal ischemia-reperfusion injury (unpublished data) and in models of autoimmune peripheral neuropathy, using the treatment with OmCI [21,22]. Phase I clinical trials of coversin are currently in progress. If it proves to be safe and effective in blocking the terminal common pathway of the complement cascade in humans there is a possibility that it may prove to be a useful therapeutic agent in the treatment of lung inflammation caused by IAV infection.

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Author Contributions

Conceived and designed the experiments: CCG WW RCR BR MN MMT. Performed the experiments: CCG RCR LPT MAR AVM JCAF. Analyzed the data: CCG RCR LPT MAR. Contributed reagents/materials/analysis tools: WW AVM MN BR JCAF. Wrote the paper: CCG WW MN MMT.

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