

Oseltamivir treatment of mice before or after mild influenza infection reduced cellular and cytokine inflammation in the lung

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Background Lung inflammation is a critical determinant of influenza infection outcomes but is seldom evaluated in animal studies of oseltamivir (OS), which have focused on viral titre and survival.

Objectives To study the effects of pre- and post-infection dosing with OS on viral replication and inflammation in a mouse model of non-lethal influenza infection.

Methods BALB/c mice were infected with a laboratory-adapted H3N1 strain of influenza. In pre-dosing studies, OS was gavaged twice daily (1 and 10 mg/kg/day) from 4 hours prior to infection and continuing for 5 days (d) post-infection (p.i). In the second post-infection dosing study, dosing at 10 mg/kg/day began at 24–48 hours p.i. Mice were dissected at d3, d5 and d7 p.i. (pre-dosing study) and d5 p.i. (post-dosing study). Lung viral titres were determined by plaque assay. Bronchoalveolar lavage

fluid (BALF) was collected and used for the quantitation of inflammatory cells and mediators.

Results Pre-infection dosing of OS reduced total cells, neutrophils and macrophages in BALF. With pre- or post-infection dosing, the pro-inflammatory mediators TNF- α , IL-1 β , IL-6 and granulocyte-macrophage colony-stimulating factor, the neutrophil chemokines keratinocyte-derived chemokine and MIP-1 α and the macrophage chemokine MCP-1 were reduced in BALF. Pre-dosing with 1 mg/kg OS did not reduce viral titres, while 10 mg/kg slightly reduced viral titres at d3 and d5 p.i.

Conclusions Oseltamivir reduced the inflammatory response to influenza when given pre- or post-infection. This anti-inflammatory effect may contribute to the clinical benefit of OS.

Keywords Cytokine, inflammation, influenza, leukocyte, oseltamivir.

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Introduction

Oseltamivir (OS) is a widely used and reasonably effective drug in the treatment of influenza.^{1,2} Oseltamivir impairs the release of new influenza virions from infected cells by blocking the action of the neuraminidase enzyme.³

Most of the published studies using OS in influenza-infected mice use lethal doses of highly virulent strains such as A/H5N1,^{4,5} A/New Caledonia/20/99 (H1N1, this strain is not normally serious but was adapted in that study for greater mouse virulence)⁶ and A/NWS/33 (H1N1)⁷ and generally focus on endpoints such as death and body weight loss. Measures of inflammation such as lung inflammatory cells and cytokines are seldom evaluated. These inflammatory parameters are important as there is evidence that the often debilitating symptoms of human influenza are at least partly because of pro-inflammatory cytokines.^{8–11} These cytokines are produced by

epithelial cells as well as by resident and infiltrating leucocytes and are important for viral clearance.¹¹ Inflammatory cytokines have a major role in recruitment and activation of inflammatory cells such as neutrophils and lymphocytes, a process that must be tightly controlled to avoid immune pathology and disruption of immune homeostasis.¹² Further improvements to influenza treatment may arise through a better understanding of which aspects of the host response could be reduced without compromising host defences.¹³

The aim of this study was to develop a model of OS antiviral activity in mice infected with a milder influenza strain and to determine whether OS reduces lung inflammatory cells and cytokines in this model. We report that while OS had little impact on lung viral titres, it reduced several features of cellular and cytokine inflammation in the lung, which is likely to contribute to improved outcomes in influenza infection.

Methods

Mouse procedures: infection with influenza and dosing with oseltamivir

Specific-pathogen-free, male BALB/c mice were obtained at 8 weeks of age from the Animal Resources Centre in Perth, Australia. Mice were housed at 20°C on a 12-hours light/dark cycle in sterile microisolator cages and fed *ad libitum* with sterile chow and water. All groups were initially weight matched. All mouse procedures were approved by the University of Melbourne Animal Experimentation Ethics Committee and complied with the standards of the National Health and Medical Research Council of Australia. After arrival, mice were acclimatized for 2–3 days.

The mildly virulent H3N1 (Mem71) strain of influenza A is a genetic reassortant of A/Memphis/1/71 (H3N2) × A/Bellamy/42 (H1N1).¹⁴ The virus was grown and titrated by plaque assay in MDCK cells as previously described.¹⁵ We have previously characterized the kinetics of lung inflammation and viral replication in response to Mem71 in BALB/c mice¹⁵ (and our unpublished results). Mice were anaesthetized by methoxyfluorane inhalation (Medical Developments Australia) and infected intranasally with 10^{4.5} plaque forming units (pfu/mL) of influenza in a 50- μ l volume, diluted in serum-free, low protein medium (VP-SFM; Invitrogen, Mulgrave, Victoria, Australia).

Oseltamivir phosphate was a generous gift of Roche Pharmaceuticals. It was prepared in sterile water and administered by twice daily oral gavage, with an 8-hours gap between doses. In the initial pre-infection (prophylactic) dosing study, OS dosing at 1 and 10 mg/kg/day began 4 hours prior to infection and continued for 5 days post-infection, in line with human dosing schedules.¹⁶ Mice were dissected at d3, d5 and d7 post-infection (p.i.). Control mice were gavaged with sterile water according to the same schedule. In the second (therapeutic dosing) part of this study, dosing at 10 mg/kg/day began at 24 or 48 hours p.i. and mice were dissected at d5 p.i. Dosage of OS was adjusted for the interspecies difference in esterase activity and metabolic rates. Dosing of OS at 10 mg/kg/day was chosen as the oral bioavailability is reportedly similar to the recommended human oral dose of 75 mg twice daily.^{1,17}

Dissection of mice

At each time point of the prophylactic study, 7–8 mice/data point were studied [3–4 for bronchoalveolar lavage (BALF) and 3–4 mice for lung homogenization for viral titration]. In the therapeutic study, 6 mice were studied per data point. Mice whose lungs were collected for viral quantitation were not used for BALF collection. Collection of BALF, the usage of BALF for total and differential inflammatory cell counts and processing of mouse lungs for viral

titration by plaque assay were performed as previously described.^{15,18}

Bio-Plex Pro™ multiplex cytokine assay

Individual BALF samples were assayed using the Bio-Plex Pro™ cytokine assay (Mouse Group I custom 10-Plex Panel; Bio-Rad, Gladesville, New South Wales, Australia) for simultaneous quantitation of interleukin (IL)-1 β , IL-6, IL-10, IL-17A, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)- γ , keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-1 α , monocyte chemoattractant protein (MCP)-1 and tumour necrosis factor (TNF)- α . A high photomultiplier tube setting (according to the manufacturer) was used for cytokine detection in the range of 10–1000 pg/ml. The plate was read on a dual-laser, flow-based microplate reader (the Bio-Plex System). The data were analysed using Bio-Plex Manager™ software (version 4.1.1) with 5PL curve fitting.

Data analysis

Results are expressed as mean \pm standard deviation (SD), where *n* values represent the number of independent subjects. Data were analysed by one-way ANOVA followed by Bonferroni's multiple comparison tests using GraphPad Prism software (version 4.0). Data that were not normally distributed were log-transformed.¹⁹ **P* < 0.05 was considered to be statistically significant.

Results

OS dosing reduced the influenza-induced influx of inflammatory cells into the BALF

Oseltamivir dosing had little impact on the mild and brief weight loss due to Mem71 influenza infection (not shown), and the drug was well tolerated.

Bronchoalveolar lavage fluid from infected mice contained 12-fold more total inflammatory cells than control mice at d3 p.i. (Figure 1A). The influx of inflammatory cells, mainly macrophages and neutrophils, peaked at d3 p.i. and declined at d5 p.i. Total inflammatory cells rebounded slightly at d7 p.i., because of the typical later influx of lymphocytes.

Pre-infection dosing of OS (1 mg/kg/day) slightly reduced macrophages and neutrophils at d5 p.i. (Figure 1B, C) but slightly increased lymphocytes. A higher dose of OS (10 mg/kg/day) 4 hours before inoculation reduced the influx of neutrophils at d3 p.i. by 42.8% (Figure 1C). At d5 p.i. macrophages, neutrophils and lymphocytes were significantly reduced by 46.9%, 92.6% and 91.3%, respectively (Figures 1B–D). However, at d7 p.i., mice pre-dosed with 10 mg/kg/day OS had increased lymphocytes in BALF, although this increase was not significant (Figure 1D).

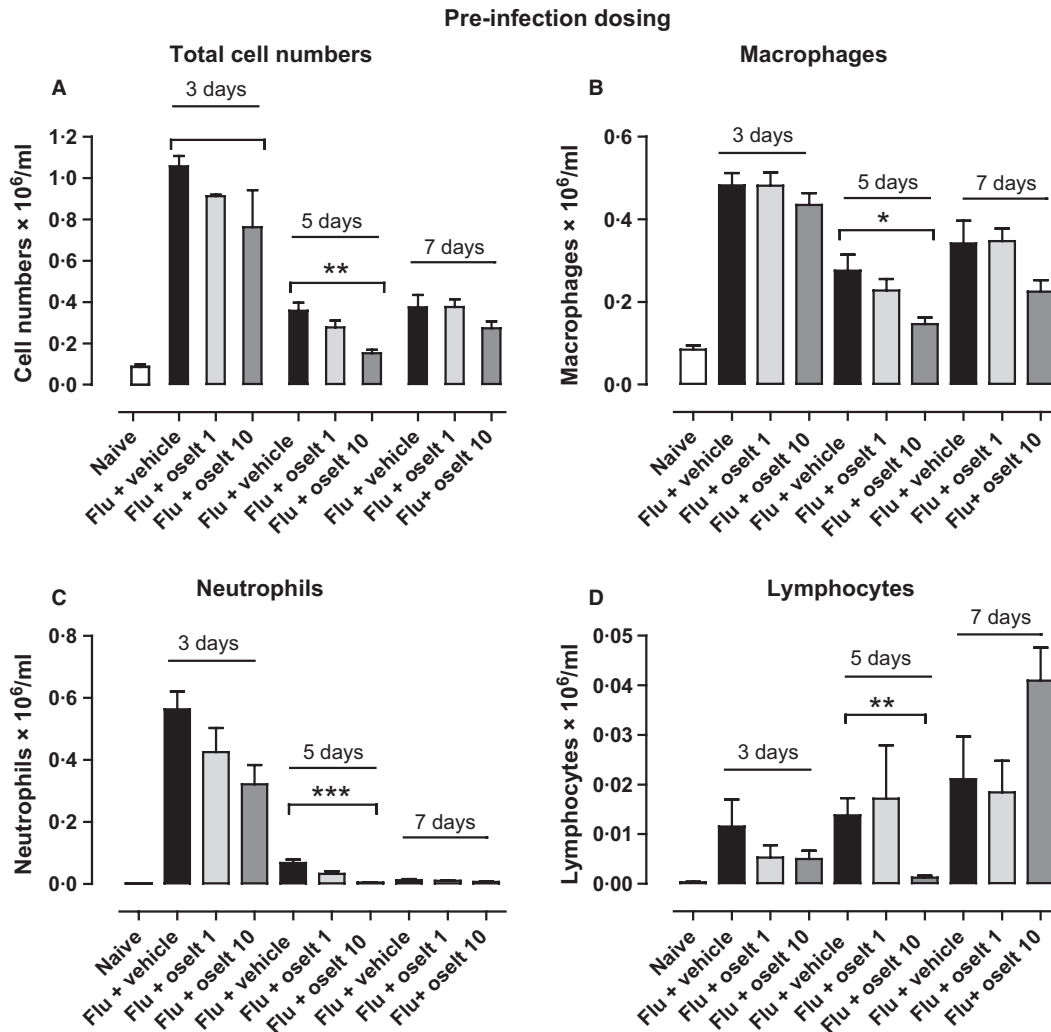


Figure 1. Cell numbers in bronchoalveolar lavage fluid in pre-infection dosing study: (A) Total cell numbers, (B) macrophages, (C) neutrophils and (D) lymphocytes at d3, d5 and d7 after influenza infection (flu). Oseltamivir (OS) (1 & 10 mg/kg/day) was given 4 hours prior to influenza inoculation and OS treatment ceased at d5 p.i. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to flu + vehicle group. (Naive $n = 9$, Flu infected $n = 4$ each).

A significant reduction of macrophages and neutrophils was also observed at d5 p.i. when OS treatment started 24 hours p.i. (Figure 2B, C). While OS treatment starting at 48 hours p.i. significantly reduced the influx of neutrophils (Figure 2C), lymphocytes in BALF were significantly increased (Figure 2D).

OS dosing had little impact on lung influenza virus titres

Virus titres (in mice dosed only with vehicle) peaked at d3 p.i. ($5.82 \pm 0.31 \times 10^6$ PFU/g of lung) and were greatly reduced at d5 p.i. ($0.57 \pm 0.18 \times 10^6$ PFU/g of lung) (Figure 3). At d7 p.i., the viral titres were below the detection limit of the plaque assay and are therefore not shown.

There was no significant effect of OS dosing (1 mg/kg/day) 4 hours before inoculation on virus titres at d3 p.i. With the administration of OS (10 mg/kg/day) 4 hours before inoculation, viral titres were slightly reduced at d3 p.i. but this was not statistically significant. There were no significant changes in the viral titres at d5 p.i. when OS (10 mg/kg/day) was administered before (Figure 3) or after (Figure 4) viral inoculation.

Influenza increases pro-inflammatory cytokines in BALF, and this is reduced by OS dosing

To study the mechanisms of lung inflammation, the d3 and d5 p.i. BALF samples from mice treated with OS

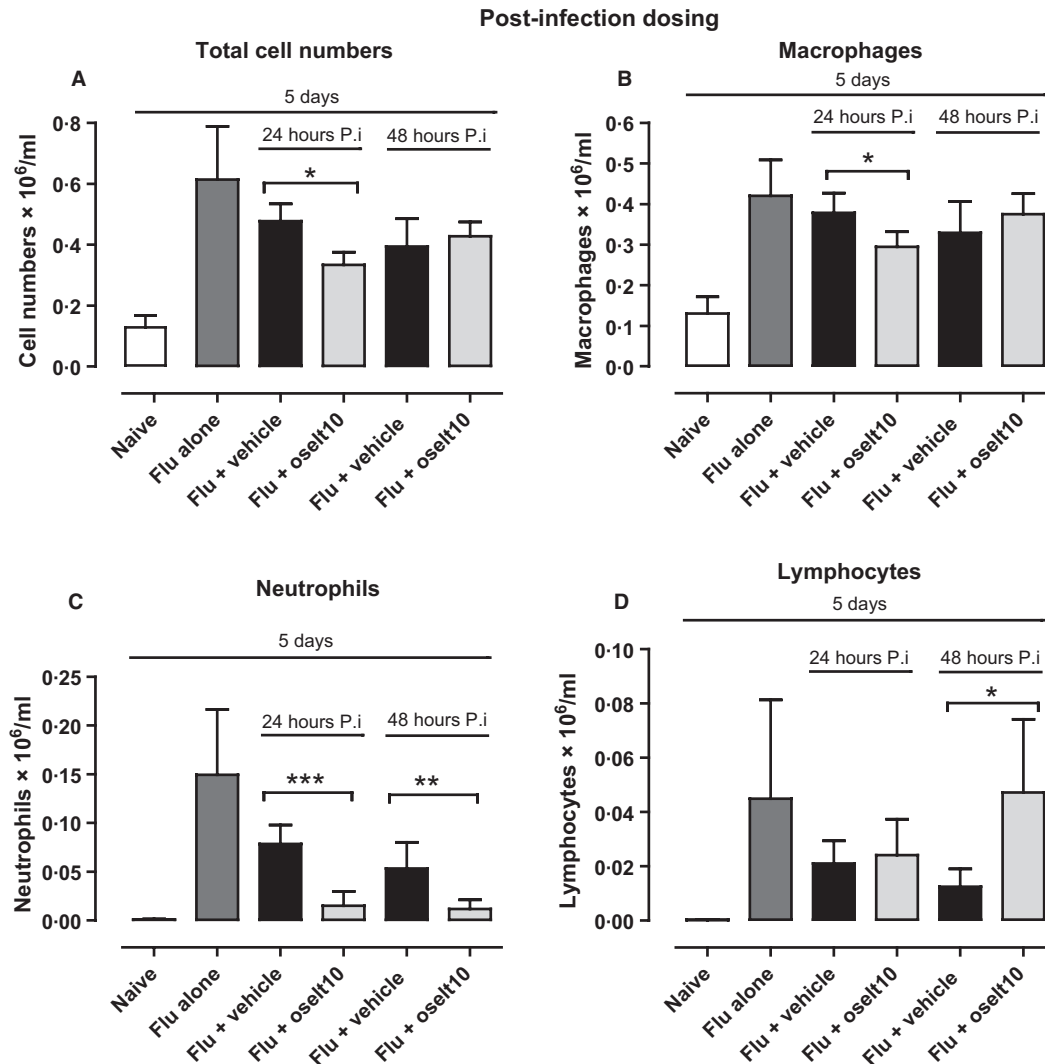


Figure 2. Cell numbers in bronchoalveolar lavage fluid at d5 p.i. in post-infection dosing study: (A) Total cell numbers, (B) macrophages, (C) neutrophils and (D) lymphocytes. Osetamivir (10 mg/kg/day) was administered at 24 and 48 hours after influenza inoculation and ceased at d5 p.i. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to flu + vehicle group. (Naïve $n = 6$, Flu infected $n = 6$ each).

(10 mg/kg/day) were chosen for in-depth cytokine and chemokine analysis with the Bio-Plex™ system. After influenza infection, IL-1 β , IL-6, GM-CSF, IFN- γ , KC, MIP-1 α , MCP-1 and TNF- α were increased at d3 and d5 p.i. (Table 1). Osetamivir before or after infection significantly reduced IL-1 β , IL-6, GM-CSF, KC, MIP-1 α , MCP-1 and TNF- α at d5 p.i. IL-10 levels at d5 p.i. were reduced by OS treatment before or after infection, but results are not shown as they were close to the limit of detection. IL-17A levels in all BALF samples were below the detection limit of the assay. The fold reduction in cytokine levels at d5 p.i. was similar with pre- or post-infection dosing. However, at d3 p.i., only TNF- α and MCP-1 were significantly reduced by pre-infection OS treatment.

Discussion

Most previous studies using OS in mice have used highly pathogenic influenza strains and focused on mortality and viral titres. The aim of this study was to characterize how OS affected viral replication and lung inflammation in a mouse model of mild influenza infection. As there is increasing evidence that the immune response contributes to the symptoms of influenza, an improved understanding of the protective and pathological aspects of the immune response may contribute to better treatments.¹³ We observed that OS reduced lung inflammation whether administered prophylactically or therapeutically. As OS reduced both cellular and cytokine inflammation but had little effect on viral titres, this raises the possibility that OS

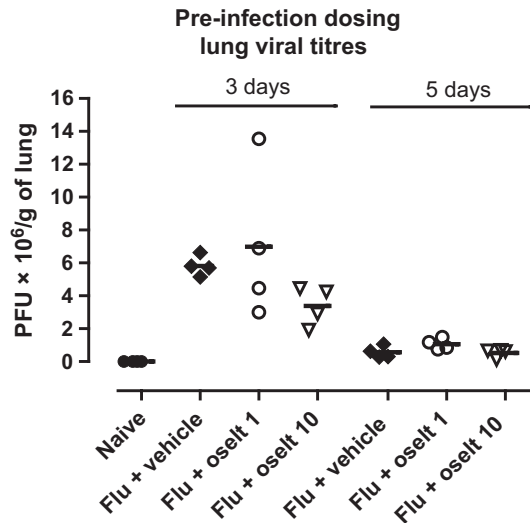


Figure 3. Dot plot of lung viral titres in pre-infection dosing study quantitated by MDCK plaque assay at d3 and d5 p.i. oseltamivir (1 and 10 mg/kg/day) was administered 4 hours prior to influenza inoculation and ceased at d5 p.i. (Naïve $n = 4$, Flu infected $n = 4$ each).

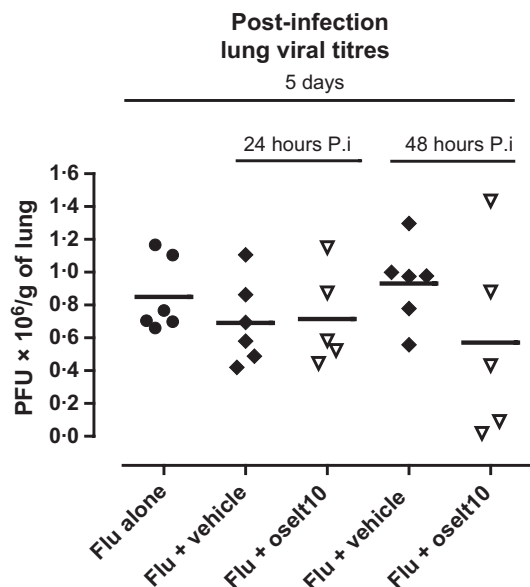


Figure 4. Dot plot of lung viral titres in post-infection dosing study quantitated by MDCK plaque assay. Oseltamivir (10 mg/kg/day) was administered at 24 and 48 hours after influenza inoculation and ceased at d5 p.i. (Naïve $n = 6$, Flu infected $n = 5-6$ each).

has intrinsic anti-inflammatory effects. However, it is also possible that the small OS-mediated differences in lung viral load in our study may underlie the reduced inflammation.

As expected, we found that OS caused little or no reduction in lung viral titres. Our novel and unexpected findings

were that OS reduced macrophages, neutrophils and lymphocytes in BALF when given before infection and macrophages and neutrophils in BALF when given post-infection. As this reduction in inflammatory cell numbers was accompanied by reduced BALF levels of key pro-inflammatory cytokines and there was no apparent worsening of infection in OS-dosed mice [as assessed by body and organ weights (not shown) as well as viral titres], we consider that OS has had a beneficial anti-inflammatory effect in this mouse model.

The major endpoints of clinical trials of OS were often symptom scores rather than viral titres, and when started promptly after symptom onset, OS treatment is associated with a reduction in duration and severity of influenza symptoms.³ Where viral titres are reported, the effects of OS are typically quite modest in both healthy adults²⁰ and elderly hospitalized patients.²¹ As symptom scores for influenza correlate with levels of inflammatory cytokines in nasal lavage,⁹ we speculate that anti-inflammatory activity of OS contributes to the overall improvement.

There is some evidence that OS may have intrinsic anti-inflammatory activity. When ferrets with a non-lethal H5N1 influenza infection were dosed with OS from 24 hours p.i., viral titres in nasal washes were not reduced but inflammation (as assessed by total cell count and protein content of the nasal washes) was consistently reduced at d3, d5 and d7 p.i.²² These ferrets also showed reduced symptoms and faster weight regain, which supports our view that less inflammation can lead to improved outcomes even if viral titres are not reduced. Moore *et al.*²³ dosed respiratory syncytial virus (RSV)-infected mice with OS and saw reduced weight loss but impaired viral clearance. RSV is unrelated to influenza and lacks the neuraminidase protein that is the main target of OS. The likely mechanism is that T-cell activation during RSV infection activates sialidase and OS reduces sialidase activity. The reduction of sialidase activity reduces the levels of the sialoglycosphingolipid GM1, which is involved in T-cell signalling through lipid rafts. Hence, OS reduces T-cell signalling, which reduces RSV pathology but delays virus clearance. Future studies of OS in influenza infections should explore whether small reductions in viral load are directly linked to reduced inflammation and the mechanism of OS-mediated modulation of inflammatory cell activation.

Early innate response cytokines (IL-1 β , IL-6, TNF- α and GM-CSF) peaked on d3 and were still detectable on d5 p.i. Cytokines such as IL-6 increase antibody production and induce the synthesis of acute-phase proteins.²⁴ IL-1 β activates the vascular endothelium, promoting access of leucocytes to the site of infection.²⁴ Granulocyte-macrophage colony-stimulating factor regulates the expansion, survival and activation state of myelo-monocytic cells lineages.²⁵ IL-6 and TNF- α are elevated in nasal wash and plasma of

Table 1. Levels of pro-inflammatory cytokines in BALF

Cytokines	Cytokine concentration (pg/ml) ± SD								
	Uninfected (n = 3)	D3 p.i.			D5 p.i.			24 hours post-infection OS dosing	
		4 hours pre-infection OS dosing				24 hours post-infection OS dosing			
		Veh (n = 4)	OS [†] (n = 4)	Veh (n = 4)	OS [†] (n = 4)	Flu only (n = 6)	Veh (n = 6)	OS [†] (n = 6)	
IL-1b	17.43 ± 2.68	65.16 ± 8.25	49.14 ± 12.92	29.13 ± 2.03	15.49 ± 2.3***	33.18 ± 5.04	28.09 ± 2.38	15.03 ± 2.94***	
IL-6	ND	63.19 ± 12.58	43.05 ± 21.4	18.14 ± 5.44	ND	42.76 ± 11.33	21.91 ± 6.17	ND	
TNF-α	23.1 ± 0.39	136.3 ± 23.14	79.35 ± 22.67*	41.79 ± 5.62	20.91 ± 2.69***	46.98 ± 7.8	44.86 ± 7.5	25.83 ± 4.09***	
IFN-γ	10.92 ± 2.77	17.1 ± 1.13	14.59 ± 3.93	16.61 ± 6.0	10.46 ± 3.78	24.73 ± 5.2	16.4 ± 5.89	13.59 ± 3.95	
MCP-1	27.7 ± 6.52	415.0 ± 55.52	166.3 ± 68.96**	87.76 ± 18.37	43.9 ± 7.2**	93.37 ± 21.74	76.84 ± 20.5	29.97 ± 10.19**	
MIP-1α	ND	45.10 ± 7.79	33.42 ± 6.11	12.67 ± 1.38	5.65 ± 1.4***	18.45 ± 1.49	15.98 ± 4.37	ND	
GM-CSF	10.42 ± 1.2	18.63 ± 1.71	14.85 ± 2.75	12.74 ± 1.84	8.57 ± 3.2	12.62 ± 0.78	10.53 ± 2.63	6.78 ± 2.08***	
KC	10.26 ± 1.78	275.9 ± 92.14	225.4 ± 140	80.17 ± 4.68	22.99 ± 5.45***	113.6 ± 36.56	89.2 ± 28.26	22.94 ± 7.97***	

Cytokine assays were carried out on BALF collected from pre-infection and post-infection studies at d3 and d5 p.i. All groups were infected with flu except for the uninfected group. All groups are treated with vehicle or oseltamivir except the flu only and uninfected groups. ND denotes not detectable, i.e. any values <10 pg/mL.

Bold values denote a significant reduction in oseltamivir-treated mice, in comparison with vehicle-treated mice. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, flu + vehicle compared with flu + OS (10 mg/kg/day) group.

[†]Oseltamivir 10 mg/kg/day.

BALF, bronchoalveolar lavage fluid; OS, oseltamivir.

humans with naturally occurring influenza,²⁰ and nasal wash levels of these two cytokines correlate with severity of respiratory and systemic symptoms, especially fever.¹⁰

Bronchoalveolar lavage fluid levels of these four early, innate response cytokines were increased by influenza infection but reduced by OS treatment pre- or post-infection. The reduction of these cytokines is probably due to reduced leucocytes, because leucocytes are a major source of these pro-inflammatory cytokines, although the respiratory epithelium also secretes many cytokines.^{11,12} It is likely that OS treatment reduced the overall number of infected cells. This reduction in cytokine levels with OS dosing did not appear to worsen infection. While these cytokines are required for clearance of influenza owing to their roles in recruitment, activation and function of immune effector cells such as macrophages and neutrophils,¹¹ this downregulation of the cytokine response could contribute to reducing influenza symptoms without adverse effects.

MCP-1 is a major macrophage chemokine of mice, and it is also induced during influenza infection in humans.¹⁰ In our study, OS-dosed mice had significantly less MCP-1 in BALF at both d3 and d5 p.i. The reduction in MCP-1 can be linked to the reduced number of macrophages in BALF as MCP-1 is expressed by monocytes/macrophages. Activated macrophages and monocytes can produce MIP-1α and KC, respectively. MIP-1α and KC recruit and activate neutrophils.^{24,25} MIP-1α, in particular, stimulates the

release of reactive oxygen species and proteases from neutrophils.^{24,25} These reactive oxygen species and proteases are non-specific immune mediators that may cause some damage to host cells. In our study, neutrophils in BALF peaked at d3 p.i., which was also the peak of MIP-1α and KC levels in BALF. The levels of MCP-1, MIP-1α and KC in BALF were reduced by OS treatment. It appears that there is a feedback loop where OS-treated mice may have less activation of macrophages which led to reduced neutrophil recruitment. This could be a key anti-inflammatory mechanism.

The lymphocyte influx into the BALF of influenza-infected mice increased gradually over d3-d7. This was determined by differential cell staining for total lymphocytes. From our previous (unpublished) work, flow cytometry of BALF from BALB/c mice infected with influenza Mem71 showed that the initial d3 lymphocyte influx mostly consists of natural killer cells. In the same prior experiment, we observed that the later influx (d7) represents the B, CD4+ and CD8+ T lymphocytes of the adaptive immune response (our unpublished observations). In the current study, the lymphocyte influx into BALF was reduced at d3 and d5 p.i. by pre-infection dosing of OS but increased at d7 with the 10 mg/kg/day dose. This was not because of a rebound of viral replication after OS dosing ceased at d5. Oseltamivir reduces influenza replication by blocking the action of neuraminidase on influenza virus,

thereby interfering with the release of infectious virus from cells. It is more likely that the retention of influenza virions on the cell surface by OS treatment may have led to prolonged presentation to dendritic cells, thereby increasing the adaptive immune response. Oseltamivir dosing after influenza infection had variable effects on lymphocyte numbers (Figure 2D). At d5 p.i., lymphocytes in BALF were reduced if dosing began at 24 hours p.i. but increased to the level of untreated mice if dosing began at 48 hours p.i. It is possible that sufficient viral replication had already occurred by 48 hours p.i. and that the delayed initiation of OS dosing could not reduce the lymphocyte response.

IFN- γ is an immunomodulatory and antiviral cytokine produced by NK, B and T lymphocytes.²⁵ IFN- γ levels in BALF were low at both d3 and d5 p.i. but were slightly reduced by OS, probably reflecting reduced numbers of lymphocytes at these times. There are conflicting reports on whether IFN- γ is required for a protective mouse immune response to influenza, with varying effects of knockouts depending on the mouse and viral strain and the immune parameters investigated.^{26–29} Hence, a small reduction in IFN- γ levels with OS dosing may be beneficial.

To our knowledge, there are no comparable mouse studies and two studies in humans on effects of OS on pro-inflammatory cytokines during influenza infection. In a small study of post-infection (naturally occurring) OS dosing in young adults, OS treatment did not significantly reduce cytokine levels in blood or nasal washes.²⁰ In a larger study with a dose range of 20–200 mg OS twice daily or 200 mg OS once daily, nasal wash levels of IL-6, TNF- α and IFN- γ were reduced by OS treatment of influenza-infected young adults.³⁰ Spleen lymphocyte numbers and activity were mostly unchanged by dosing with OS at 100 mg/kg (starting 16 hours pre-infection) in a lethal mouse influenza model.⁷ In a study where mice were infected with one of five different strains of avian H5N1 influenza and dosed with 20 mg/kg/day of OS from 4 hours pre-infection, there was no consistent effect of OS on levels of pro-inflammatory cytokines in lung homogenates.¹⁷ There was a trend for OS to reduce IFN- α (not measured in our study) and MCP-1 with two viral strains and to reduce TNF- α with one viral strain, but OS dosing slightly increased the levels of pro-inflammatory cytokines with other viral strains. A key finding of the Govorkova *et al.* study was that the A/Turkey virus that was the most lethal and the least sensitive to OS was also the one that induced the strongest cytokine responses. This reinforces the point that the cytokine response to influenza (and the effects of drugs on it) should be investigated along with viral titres, as an excessive immune response is likely to initiate tissue damage that will not be improved by antivirals that target viral replication.

Overall, we conclude that OS reduced the inflammatory response to influenza when given pre- or post-infection. This anti-inflammatory effect may contribute to the clinical benefit of OS.

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