

Role of macrophage migration inhibitory factor in influenza H5N1 virus pneumonia

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Summary. – The severe and often fatal disease in humans and birds caused by H5N1 influenza viruses has been attributed to aberrant pulmonary inflammatory responses. We investigated the role of macrophage migration inhibitory factor (MIF), a proinflammatory cytokine and a pivotal regulator of innate immunity, in H5N1 influenza virus pneumonia in murine model. We found increased MIF mRNA levels in the lungs and MIF protein levels in the serum of infected mice. Although the inhibition of MIF action by isoxazolone-1 (ISO-1) did not render mice more resistant to the lethality of infection, it caused a significant reduction in pulmonary inflammatory cytokines interleukin-1 beta (IL-1 β), IL-6 and tumor necrosis factor alpha (TNF- α) and chemokine interferon-inducible protein-10 (IP-10). These results indicate the involvement of MIF in inflammatory responses to H5N1 influenza virus infections by induction of pulmonary inflammatory cytokines and chemokines, and suggest that pharmacotherapeutic approaches targeting MIF may hold promise for the treatment of H5N1 influenza virus pneumonia.

Keywords: MIF; H5N1 influenza virus; mice; pneumonia; isoxazolone-1

Introduction

Recent outbreaks of H5N1 influenza virus infections had important health and economic consequences and raised concerns that a new influenza pandemic would occur in the near future. The H5N1 virus was first recognized to infect humans in 1997, when avian strains were transmitted directly from birds to humans in Hong Kong, causing 18 cases of human respiratory disease inclusive of 6 deaths

(Bender *et al.*, 1999). Since late 2003, the H5N1 virus has spread across Asia to the Middle East, Europe and Africa, causing outbreaks of disease and death in poultry, mammals, and humans. As of March 2009, approximately 411 laboratory-confirmed human cases of H5N1 virus infection was reported by the WHO, with a human fatality rate above 60%. As in the 1997 outbreak, the clinical manifestations of H5N1 virus infection have typically been fever, respiratory distress, leukopenia, and lymphopenia before progressing to primary viral pneumonia complicated by acute respiratory distress syndrome (Chan *et al.*, 2005). Elevated levels of serum cytokines and chemokines accompanied these clinical manifestations. The occurrence of this “cytokine storm” has been proposed to contribute to the increased severity of the disease caused by the H5N1 virus (de Jong *et al.*, 2006). In support of this hypothesis, the H5N1 virus was found to elicit substantially higher expression of proinflammatory chemokines and cytokines, particularly TNF- α , IL-1 β , IL-6, IP-10, monokine induced by IFN- γ (MIG), monocyte chemoattractant protein-1 (MCP-1),

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Abbreviations: BBB = blood-brain barrier; IFN = interferon; IL-1 β = interleukin-1 beta; IL-6 = interleukin-6; i.n. = intranasally; IP-10 = IFN-inducible protein-10; ISO-1 = isoxazolone-1; MCP-1 = monocyte chemoattractant protein-1; MIF = macrophage migration inhibitory factor; MIG = monokine induced by IFN- γ ; p.i. = post infection; RANTES = regulated upon activation, normal T cell expressed and secreted protein; TNF- α = tumor necrosis factor alpha

and regulated on activation, normal T cell expressed and secreted protein (RANTES) (de Jong *et al.*, 2006; Chan *et al.*, 2005).

MIF is a potent proinflammatory cytokine with broad upstream actions in the inflammatory cascade (Calandra *et al.*, 1994; Mitchell *et al.*, 2002). Once released, MIF exerts critical autocrine and paracrine activating effects, including the induction of inflammatory cytokines and chemokines (Mitchell *et al.*, 2002; Sampey *et al.*, 2001). Recent studies have confirmed the involvement of MIF in a number of inflammatory and autoimmune diseases originally studied in animals (Lolis *et al.*, 2001; Morand *et al.*, 2005). The increased levels of MIF in certain pathological conditions may be indicative of its involvement in those diseases.

Within the lungs, MIF has been shown to be constitutively expressed in bronchial epithelial cells, alveolar macrophages, alveolar endothelium, and in monocytes and eosinophils isolated by bronchial alveolar lavage (Donnelly *et al.*, 1997). In a clinical study, MIF levels were found to be significantly elevated in bronchoalveolar fluids obtained from acute respiratory distress syndrome patients (Donnelly and Bucala, 1997; Donnelly *et al.*, 1997), which indicates that this cytokine may play an important role in lung diseases. MIF was first identified as a T cell-derived lymphokine (Bernhagen *et al.*, 1993), and is currently considered a critical mediator of the inflammatory cascade and therefore of the innate immune response (Lue *et al.*, 2002). It exhibits a broad range of immunostimulatory and proinflammatory activities. As a proinflammatory cytokine, MIF antagonizes the action of glucocorticoids and functions as a pivotal activator of innate responses, and induces alveolar cells/macrophages to secrete TNF- α , IL-1 β , and IL-6 (Bernhagen *et al.*, 1994; Calandra *et al.*, 2003), which are widely considered relevant to the pathogenesis of H5N1 virus disease. Elevated levels of MIF are deleterious in sepsis and shock (Calandra *et al.*, 2000), and several reports have suggested involvement of MIF in virus pathogenesis. For example, among dengue virus-infected individuals, higher MIF serum levels were found in the patients with hemorrhagic fever compared to those with a milder form of disease (Chen *et al.*, 2006). The patients suffering from chronic Hepatitis B virus and West Nile virus infection also exhibited elevated MIF serum levels (Zhang *et al.*, 2002; Arjona *et al.*, 2007). *In vitro* studies have shown that Human cytomegalovirus and influenza virus infections induce MIF production (Arndt *et al.*, 2002; Bacher *et al.*, 2002). Nevertheless, the mode of involvement of MIF in virus infection remains unknown.

The aim of this study was to investigate whether MIF influences the H5N1 virus immunopathogenesis in mice, particularly the pneumonia, by assaying MIF expression in the lungs and serum, and by following the effect of ISO-1,

an inhibitor of MIF (Al-Abed *et al.*, 2005) on the virus replication, induction of cytokines and chemokines in the lungs, and loss of body weight.

Materials and Methods

Virus. The influenza virus A/Chicken/Harbin/01/2003(H5N1), further abbreviated as Harbin/01, was used. A stock virus was propagated in the allantoic cavity of 10-day-old embryonated chicken eggs at 37°C for 48 hrs, and the allantoic fluid was harvested, aliquoted, and stored at -70°C. Infectious virus titer (TCID₅₀/ml), based on CPE was assayed in MDCK cells. The entire work with this virus was carried out in a BL3 biocontainment facility.

Infection and ISO-1-treatment of mice. SPF female BALB/c mice (18–20 g body weight), purchased from the Experimental Animal Center, Academy of Military Medical Sciences, Beijing, were lightly anesthetized with CO₂ and inoculated intranasally (i.n.) with 100 TCID₅₀ of Harbin/01 virus in 50 μ l. Control mice received UV-inactivated virus or PBS. The infected mice were then injected intraperitoneally with 3.5 mg of ISO-1 (Merck) per kg weight in 200 μ l and the injections were repeated daily for 7 days. Control mice were given PBS instead of ISO-1. The morbidity, mortality and body weight were monitored daily. To monitor virus titers and levels of cytokines and chemokine IP-10 in the serum and the lungs, the mice were euthanized and whole lungs and serum samples were collected every 3 hrs until 12 hrs and then every 6 hrs until 72–108 hrs post infection (p.i.). The samples were frozen and stored at -70°C until assayed, while lung samples for histological and immunochemical analyses were fixed in a buffered 10% formalin. For each time interval a group of 3 mice was used.

Real-time RT-PCR for cytokine and chemokine mRNAs. For determination of levels of mRNAs of various cytokines total RNA was prepared from frozen lung samples using TRIzol reagent (Invitrogen, USA) and RNeasy Mini kit (TaKaRa, P.R. China). cDNA was synthesized using oligo-dT primers and Superscript II reverse transcriptase (Invitrogen, USA), and amplified by real-time PCR using specific oligonucleotide primers (Table 1) and the ABI Prism 7000 Sequence Detection System (Applied Biosystems, USA). The mRNAs for MIF, IL-1 β , IL-6, IP-10, TNF- α , RANTES, and MIG were normalized using β -actin as standard (Awandare *et al.*, 2006; Overbergh *et al.*, 2003). Results were expressed as mean \pm SD values from at least 3 samples.

ELISA of MIF. Frozen sera were thawed, clarified by centrifugation and assayed for MIF by ELISA using a commercial kit (USCNLIFE Co., USA). The assay sensitivity was 156 pg of MIF per ml. Results were expressed as mean \pm SD values from at least 3 samples.

Histological and immunochemical analyses. Fixed lung specimens were paraffin-embedded, sectioned at 4 mm, and mounted on slides. A part of slides were stained with hematoxylin and eosin for light microscopy. Other slides were stained with antibodies to CD3, CD68, and MIF (Abcam, UK).

Statistical analysis was performed using the one-way ANOVA followed by the Bonferroni multiple-comparison test. Differences with $P \leq 0.05$ were considered significant.

Table 1. Primers used in real-time PCR

| Gene | Primer (5'-3') | PCR product (bp) | Acc. No. |
|---------|--------------------------------|------------------|-----------|
| β-actin | TGACAGGATGCAGAAGGAGA (F) | 131 | HNM007393 |
| | GCTGGAAGGTGGACAGTGAG (R) | | |
| MIF | CTCCGTGCCAGAGGGGTTTC (F) | 130 | HNM010798 |
| | GGGATCGTTTCGTGCCGCTA (R) | | |
| IL-1β | TCCAGGATGAGGACATGAGCAC (F) | 105 | NM_008361 |
| | GAACGTCACACACCAGCAGGTTA (R) | | |
| MIG | CCGAGGCACGATCCACTACA (F) | 117 | M34815 |
| | TCTAGGCAGTTTGTATCTCCGTTT (R) | | |
| TNF-α | AAGCCTGTAGCCCACGTCGTA (F) | 122 | NM_013693 |
| | GGCACCAGTGTGGTTGTCCTTTG (R) | | |
| IL-6 | CCACTTCACAAGTCGGAGGCTTA (F) | 112 | NM_031168 |
| | GCAAGTGCATCATCGTTGTTTCATAC (R) | | |
| IP-10 | GTCCGCTGCAACTGCATCCATA (F) | 135 | M33266 |
| | CTGCTCATCATTCTTTTCATCGTG (R) | | |
| RANTES | CTGCTGCTTTGCTACCTCTCCC (F) | 156 | M77747 |
| | TATTCTTGAACCCACTTCTTCTCTG (R) | | |

Results and Discussion

Effect of virus infection on MIF expression in mice

Monitoring of the MIF mRNA expression in the lungs of virus-infected mice revealed three peaks at 6, 24, and 60–78 hrs p.i., the first peak coinciding with the start of virus invasion of the lungs (Fig. 1a). As for the MIF protein, its serum levels were similarly elevated in virus-infected mice, while in uninfected mice it was barely detectable (Fig. 1b). These data correspond to a widely accepted view that a massive H5N1 virus replication in the lungs results in long-lasting induction of MIF in this organ and consequently in fatal outcome of infection. They are also in accord with the finding that cytokines are not induced by an inactivated virus (Chan *et al.*, 2005).

Immunohistochemistry of the lungs of virus-infected mice showed that bronchiolar epithelial cells contained large amounts of MIF at 24 hrs p.i. and confirmed its localization in the lung tissue (Fig. 2b). MIF was also found in alveolar epithelial cells and intercellular space at 36 hrs p.i. (Fig. 2c). The staining for CD3 and CD68 demonstrated a more intense pulmonary inflammatory cell infiltration of the lungs at 36 hrs p.i., predominantly with T lymphocytes (Fig. 2d) and macrophages (Fig. 2e).

Altogether these data indicate that (i) the H5N1 virus upregulates the MIF expression during initial and middle stages of the infection, and (ii) the elevated MIF expression enhances the inflammatory cell infiltration of the lungs.

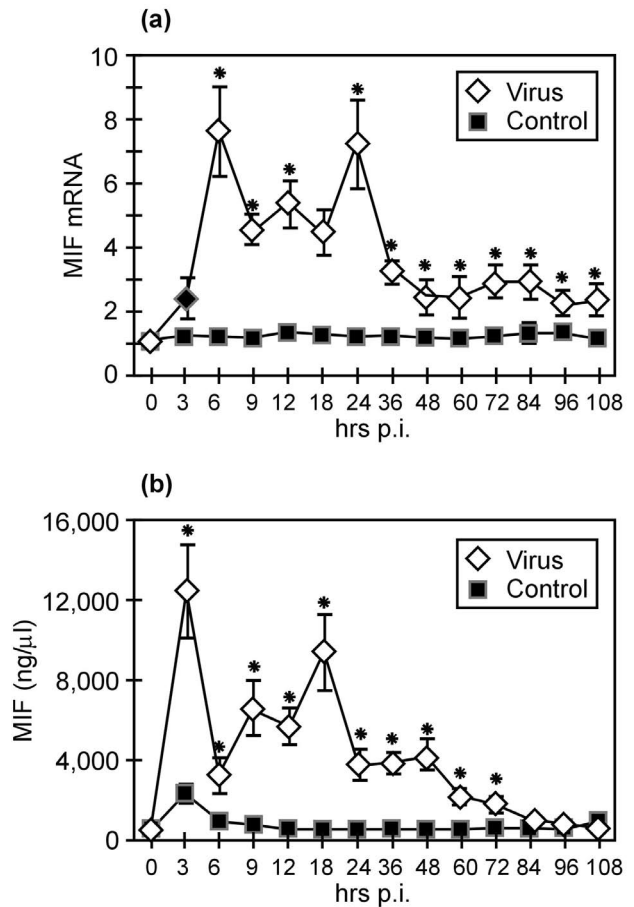


Fig. 1

Effect of virus infection on MIF expression in mice
MIF mRNA in the lungs (a) and MIF protein in the serum (b).

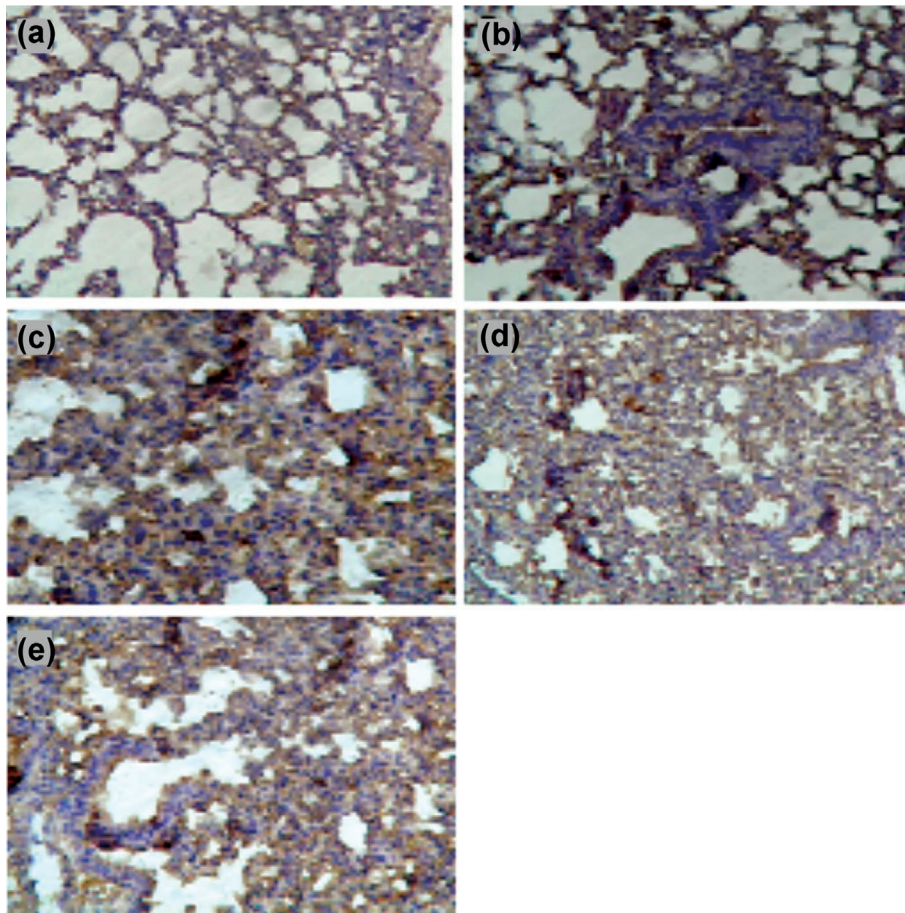


Fig. 2

Immunohistochemistry of the lungs of virus-infected mice

MIF in control (a) and infected lungs (b, c). CD68 in macrophages (d) and CD3 in T lymphocytes (e) in infected lungs. Magnification 25x.

Effect of ISO-1 on virus replication and induction of cytokines and chemokine IP-10 in the lungs of infected mice

Previous studies have suggested that a high virus load and the resulting intense inflammatory responses are central to influenza H5N1 pathogenesis (de Jong *et al.*, 2006). Furthermore, the H5N1 virus elicits substantially higher expression of inflammatory chemokines and cytokines as compared with H1N1 or H3N2 viruses (Chan *et al.*, 2005). To address the question whether the markedly elevated MIF influences viral titers and inflammatory cytokine secretion in the lungs of mice infected with the H5N1 virus, we examined the effect of ISO-1, an inhibitor of proinflammatory activity of MIF, in such a model.

The lungs of virus-infected ISO-1-treated mice were assayed at various times p.i. for virus titers and levels of mRNAs for cytokines IL-1 β , IL-6, and TNF α , and chemokines IP-10, MIG and RANTES. ISO-1-untreated mice served as controls. The data showed that viral infection resulted in high virus titers in the lungs between days 1 to 7 p.i. with a peak at day 5 (Fig. 4a), without a significant effect of ISO-1. These results are consistent with the previous ones showing that MIF does not directly affect virus replication (Kimura *et al.*, 2006).

As for the levels of mRNAs for IL-1 β , IL-6, TNF α , and IP-10 in the lungs of virus-infected mice, ISO-1 reduced them at 72 hrs p.i. about 2- to 3-fold as compared with controls (Fig. 3a-d). Interestingly, the levels of mRNAs for TNF- α and IL-1 β (the cytokines important for the pathogenesis of H5N1 influenza viruses (Chan *et al.*, 2005))

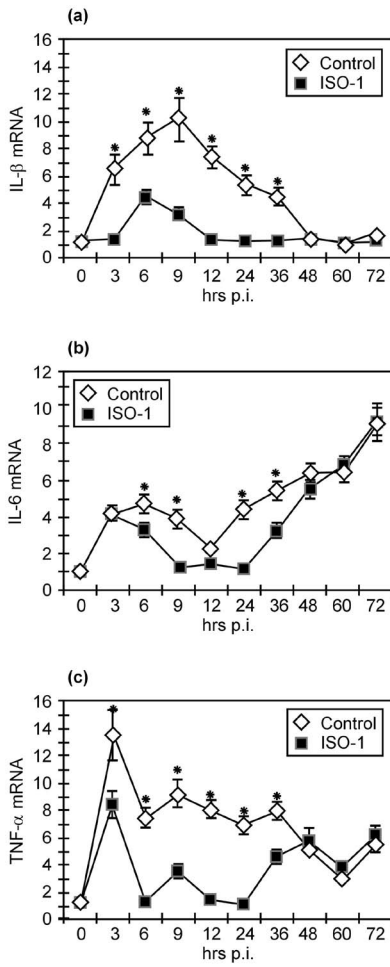


Fig. 3

Effect of ISO-1 on the induction of cytokines and chemokine IP-10 in the lungs of virus infected mice

IL-β (a), IL-6 (b), TNF-α (c), IP-10 (d), RANTES (e), and MIG (f).

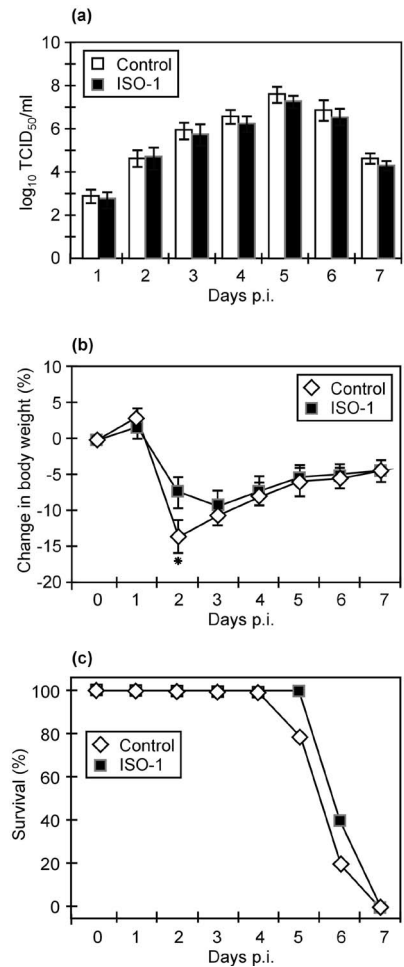


Fig. 4

Effects of ISO-1 on virus titers in the lungs (a), body weight (b), and survival (c) of virus-infected mice

were significantly reduced at the time of the virus entry into the lungs. However, the levels of mRNAs for RANTES and MIG were not affected by ISO-1 (Fig. 3e,f). As for the effect of virus infection alone, IP-10, IL-1β, IL-6, TNFα, RANTES, and MIG mRNA levels were substantially higher in virus-infected mice as compared with uninfected controls (data not shown). All these data further support the placement of the MIF action upstream of the host inflammatory response resulting from pathogene invasion (Calandra *et al.*, 1994, 2000).

The effect of ISO-1 on morbidity, mortality, and body weight of infected mice

To assess whether the inhibition of MIF by ISO-1 renders mice more resistant to H5N1 virus infection, as this inhibi-

tion can diminish the expression of inflammatory cytokines and chemokines, ISO-1-treated virus-infected mice were monitored for morbidity, mortality, and body weight in comparison with ISO-1-untreated virus-infected controls. The virus (100 TCID₅₀) was highly lethal for 6- to 8-week-old BALB/c mice, which quickly lost weight (Fig. 4b) and succumbed between days 6 and 7 p.i. (Fig. 4c). In contrast, ISO-1 caused a reduction of the weight loss at day 2 p.i. (Fig. 4b). However, ISO-1 did not render the mice more resistant to virus lethality (Fig. 4c).

The histology of the lungs of ISO-1-treated virus-infected mice revealed a weaker inflammatory response (Fig. 5b) as compared with control (Fig. 5c). The virus infection alone caused mainly epithelial injury and interstitial pneumonia. The effect of ISO-1 consisted of fewer diffuse areas with small patches of interstitial pneumonia containing a smaller

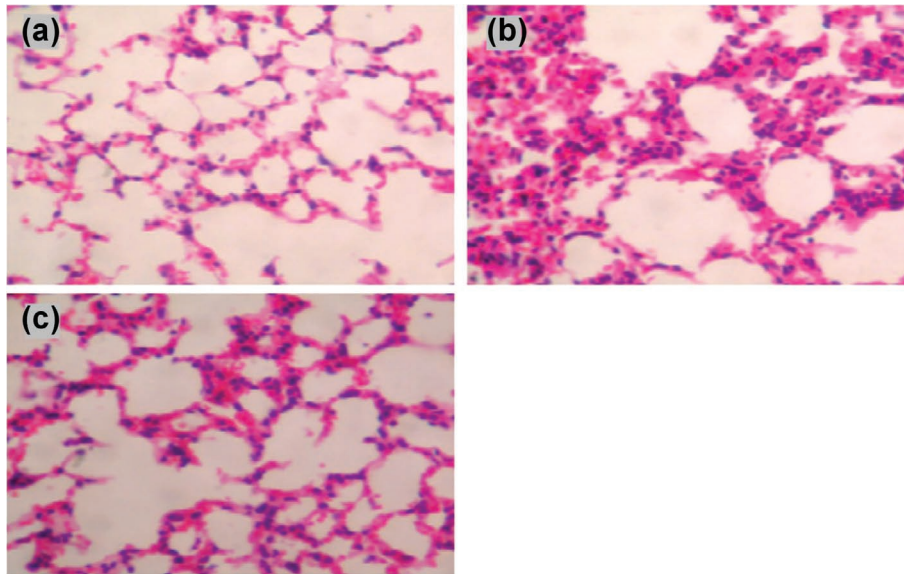


Fig. 5

Effect of ISO-1 on the inflammatory response in the lungs of virus-infected mice

Uninfected mice (a), infected mice (b), and ISO-1-treated infected mice (c). Magnification 25x.

number of inflammatory cell infiltrates in the alveoli and around the bronchioles. These results showed that ISO-1 protected mice from the lung pathology mediated by H5N1 virus infection to a limited extent only.

Summing up, although we did not observe a difference in survival rate between the ISO-1-treated and untreated mice, our data strongly suggest that MIF enhances the H5N1 virus pathogenicity by inducing the production of downstream inflammatory cytokines and chemokines, chiefly TNF- α , IL-1 β , IL-6, and IP-10, which are widely considered to contribute to the pathogenesis of the H5N1 virus disease.

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