Potential role of high-mobility group box 1 protein in the pathogenesis of influenza H5N1 virus infection

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Summary. – During influenza A virus (IAV) (H5N1) infection, the levels of inflammatory cytokines are markedly elevated in the lungs of infected hosts. One of them, high-mobility group box 1 protein (HMGB1) functions in regulation of cellular transcription and activation of proinflammatory responses, but little is known about its role in viral infection. In this study, we attempted to address this question. Using an IAV (H5N1) – mouse model, lung tissues were analyzed for virus titer, expression of HMGB1 and other inflammatory cytokines and histopathological changes. Moreover, the effect of administration of HMGB1-specific antibody to the infected mice on these parameters was investigated. The results showed that the HMGB1 expression was induced on days 3–7 post infection (p.i.) and primarily localized to epithelial cells of alveoli and bronchioles. The HMGB1-specific antibody reduced the levels of inflammatory cytokines and chemokines and the survival rate, but did not influence the virus titer. Summing up, these data suggest that HMGB1 contributes to the pathogenesis of IAV (H5N1) infection in mice by inducing extensive inflammatory responses and severe pneumonia.

Keywords: HMGB1; influenza A virus (H5N1); pathogenesis

Introduction

Previous studies have shown that excessive cytokine responses with recruitment of inflammatory immune cells to the lung were key contributors to the high morbidity and significant mortality of the 1918 influenza and IAV (H5N1) infection (de Jong *et al.*, 2006; Kobasa *et al.*, 2007). In support of this statement, CCR2-deficient mice were protected from early pathological manifestations despite higher pulmonary titers of the influenza virus A/PR/8/34 (H1N1) strain (Dawson *et al.*, 2000). Tumor necrosis factor receptor 1 (TNFR-1)-deficient mice exhibited significantly reduced morbidity following challenge with IAV (H5N1) (Szretter *et al.*, 2007), and

other cytokines or chemokines have also been investigated (Schmitz *et al.*, 2005; Deng *et al.*, 2008). Thus, at least some of the elevated proinflammatory cytokines may contribute to the pathogenesis of IAV

HMGB1 is a 25 KDa, nonhistone, nucleosomal protein (Bianchi *et al.*, 2007). HMGB1 protein can be released passively by necrotic cells and/or actively by activated immune cells such as dendritic cells, monocytes and macrophages, into the extracellular milieu to function as a proinflammatory cytokine in response to injury, infection and inflammation (Andersson *et al.*, 2000; Messmer *et al.*, 2004; Yang *et al.*, 2005). Evidence is accumulating for the involvement of HMGB1 during the course of viral infections. It was shown to be released from cells infected with a range of viruses, including dengue virus, hepatitis C virus, HSV-2 and HIV (Kamau *et al.*, 2009; Barqasho *et al.*, 2010; Jung *et al.*, 2011); however, the involvement of HMGB1 in the IAV infection, and its contribution to the excessive inflammatory cytokine response associated with severe influenza cases, remains largely unknown. Increased levels of HMGB1 were detected in sera from patients with IAV infection and bacterial

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Abbreviations: HMGB1 = high-mobility group box 1; IAV = influenza A virus; IFN = interferon; IL-1β = interleukin 1β; IL-6 = interleukin 6; IP-10 = IFN-inducible protein 10; p.i. = post infection; RANTES = regulated upon activation, normal T-cell expressed and secreted protein; TNF-α = tumor necrosis factor α

pneumonia, and in sera from IAV (H1N1) pdm09-infected children with severe pneumonia (Kosai *et al.*, 2008; Ito *et al.*, 2011). In a mouse model of severe influenza infection, elevated HMGB1 levels were observed in bronchoalveolar lavage fluids (Van *et al.*, 2009).

In this study, the role of HMGB1 in IAV (H5N1)-infected mice was investigated. The results revealed that (1) the infection induces increased HMGB1 levels in the lungs, (2) the released HMGB1 contributes to extensive inflammatory responses and severity of pneumonia, and (3) the effect of HMGB1 can be blocked by a HMGB1-specific antibody.

Materials and Methods

Virus, cells and mice. A stock of influenza virus A/Chicken/Harbin/01/2003(H5N1), further abbreviated as Harbin/01, was propagated in the allantoic cavity of 10-day-old embryonated chicken eggs at 37ºC for 48 hr. The allantoic fluid was then harvested, aliquoted, and stored at -70ºC. Infectious virus titer ($TCID_{50}$/ml), based on CPE, was assayed in MDCK cells. The entire work with this virus was carried out in a BSL-3 biocontainment facility. SPF female BALB/c mice, 18–20 g body weight, were purchased from Beijing Experimental Animal Center (China). HMGB1-specific antibody was prepared in a rabbit immunized with *Escherichia coli*-expressed mouse HMGB1 protein.

Experiments on mice. SPF female BALB/c mice were lightly anesthetized with ether and inoculated intranasally with 100 $TCID_{50}$ Harbin/01 virus in 50 μl. Control mice received UV-inactivated virus or PBS. UV-inactivated virus, as validated by cell culture-based assay, had no effect on the hemagglutinin (HA) titer by HA assay in PBS. To further address the role of HMGB1 in virus-infected mice, virus-infected mice were injected intraperitoneally with HMGB1-specific antibody (200 μg/mouse) on days 1 and 4 p.i. Control infected mice received an equivalent volume of PBS alone. Mice were monitored daily for morbidity and mortality, and weights were measured daily throughout the infection. To monitor virus titers and levels of cytokines and chemokines in serum and lungs, the mice were euthanized and whole lungs and serum samples were collected daily until day 7 p.i. The samples were frozen and stored at -70ºC until assayed, while lung samples for histological and immunochemical analyses were fixed in buffered 10% formalin. For each time interval a group of 3 mice was used.

Real-time PCR for HMGB1 mRNA. For determination of mRNA levels of various cytokines, total RNA was prepared from frozen lung samples using TRIzol reagent (Invitrogen, USA) and RNeasy Mini kit (TaKaRa, 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 7,000 Sequence Detection System (Applied Biosystems, USA). HMGB1 mRNA titers were normalized using β-actin as a standard. Results were expressed as mean ± SD values from at least 3 samples.

Western blot analysis of HMGB1 protein. Total protein extracts of lung tissues were prepared for western blot analysis in ice-cold lysis buffer (150 mmol/l NaCl, 1% NP40, 0.5% Na deoxycholate, 0.1% SDS and 50 mmol/l Tris-HCl, pH 8.0). Proteins of the lysates were then separated by SDS-PAGE and analyzed by Western blotting with HMGB1 antibody (Abcam, USA). The blots were developed with a Western Luminescent Kit (Vigorous Biotechnology, China) and bands visualized using an X-ray film.

ELISA of serum cytokines. Frozen sera were thawed on ice and clarified by centrifugation. Using ELISA kits, concentrations of HMGB1 (ADL Company, USA), IL-1β, IL-6, TNF-α, IP-10, and RANTES (R&D Systems, USA) in serum were determined following the manufacturers' protocols. The detection limit was 2 pg/ml for HMGB1, 1.88 pg/ml for TNF-α, 1.6 pg/ml for IL-6, 2.31pg/ml for IL-1β, 2.2 pg/ml for IP-10, 2 pg/ml for RANTES. Results were expressed as mean ± SD values from at least 3 samples.

Infectious virus titration. Individual lungs were completely homogenized in 2 ml cold PBS containing 100 units/ml penicillin and 100 μg/ml streptomycin. The serial dilutions of lung samples from 10^{-1} to 10^{-10} were added onto a monolayer of MDCK cells in 96-well culture plates for 2 to 3 days. CPE was examined by light microscopy and titers of individual viral samples were calculated by the Reed-Münch method (Pandiri *et al.*, 2007).

Histopathology, immunochemical staining. Harvested lung tissues from each time point and each experimental group were fixed in 10% (v/v) buffered neutral formalin, then processed for paraffin embedding and sectioning by routine methods. A part of slides were stained with hematoxylin and eosin for light microscopy. Other slides were stained with rabbit antibody to HMGB1.

Statistical analysis. Results were expressed as mean ± SD values for each series of experiments. All analyses were performed by unpaired *t* test using the Statistical Package for Social Sciences (SPSS) Version 13.0. Results were considered statistically significant at $P < 0.05$.

Results

The virus infection induces HMGB1 expression in mouse lungs

Following viral infection, HMGB1 mRNA levels in mouse lung were slightly increased on day 3 and more markedly on day 4, with the highest level being observed on day 5 p.i. (Fig. 1a). Compared with control mice given inactivated virus, the difference was statistically significant ($P < 0.05$). HMGB1 mRNA levels started to decrease on day 6, but remained high. Western blot analysis showed that the infection resulted in a notable increase of HMGB1 expression by day 5 (Fig. 1c). Moreover, HMGB1 concentrations in the sera of infected mice were increased on day 3, with highest titers on day 5, by

which time most mice exhibited severe disease (Fig. 1b). Immunohistochemical staining showed that bronchiolar epithelial cells, alveolar epithelial cells, and intercellular spaces contained high quantities of HMGB1 (Fig. 1d). These data show that IAV (H5N1) infection positively upregulated HMGB1 expression during the middle and late phases of infection.

Blocking of HMGB1 with specific antibodies reduces the inflammatory response, morbidity and mortality in virus-infected mice

The occurrence of a "cytokine storm" has been proposed to contribute to the increased severity of disease caused by
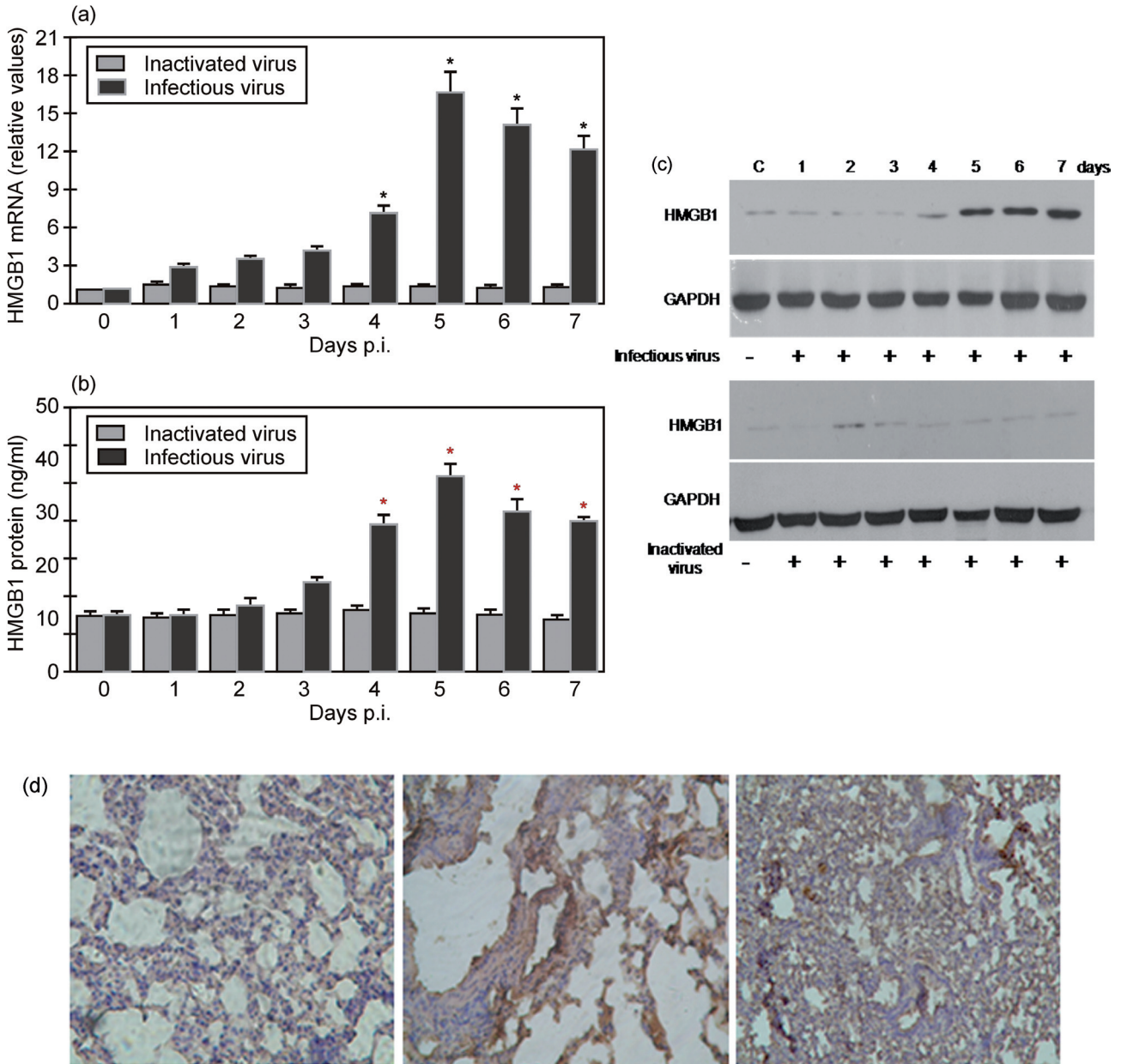

Fig. 1

The virus infection induces HMGB1 expression in mouse lungs

Expression of HMGB1 mRNA as detected by real-time RT-PCR (a), HMGB1 protein in serum as detected by ELISA (b), and HMGB1 level of lung tissues as detected by Western blot (c). HMGB1 protein detected in the lungs by immunochemical staining, magnification 25x (d). *$P < 0.05$.

IAV (H5N1) (Chan *et al.*, 2005; de Jong *et al.*, 2006). To directly examine the contribution of HMGB1 during IAV (H5N1) infection, we blocked HMGB1 using HMGB1-specific antibody to measure the levels of inflammatory cytokine (IL-1β, IL-6, TNF-α, IP-10, and RANTES), which are widely thought to be relevant to the pathogenesis of IAV (H5N1) in the mouse model. As shown in Fig. 2, the levels of IL-1β, IL-6, TNF-α, IP-10, and RANTES were significantly lower in mice treated with HMGB1-specific antibody at day 4 p.i. ($P < 0.05$).

To determine how HMGB1 alters survival in IAV (H5N1)-infected mice, virus-infected mice were treated with HMGB1-specific antibody. Body weight and pulmonary viral load were measured at various times after infection. Body weight loss was significantly more apparent in infected mice, reaching its nadir (10% decrease) by day 5 p.i. In contrast, antibody-treated infected mice continued to lose weight through day 5 (4% total decrease) and then rapidly recovered (Fig. 3b). Virus titers were high in the lungs on days 2–7 p.i., peaking on day 5 (Fig. 3a), and did not differ
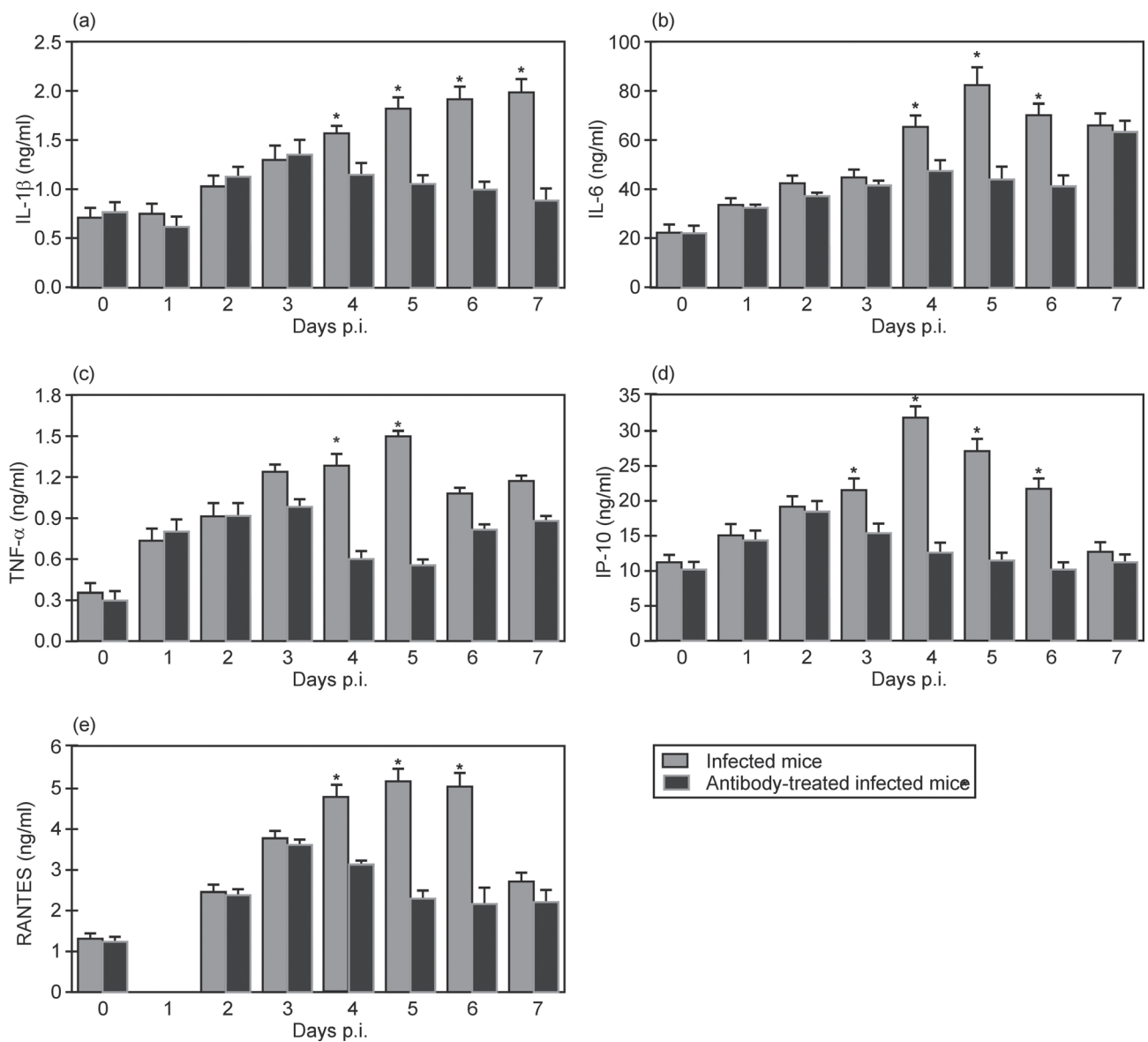

Fig. 2

HMGB1-specific antibody suppresses induction of inflammatory cytokines in virus-infected mouse lungs
IL-1β (a), IL-6 (b), TNF-α (c), IP-10 (d), and RANTES (e). *$P < 0.05$.

between infected mice and antibody-treated infected mice. All infected mice died within 6 days p.i., whereas 20% (2/10) of antibody-treated infected mice survived beyond day 12 with no subsequent deaths (Fig. 3c). These results clearly show that HMGB1 is responsible for lethal viral effects following virus infection.

Virus-infected mice displayed an initial peribronchiolar patchy pneumonia on days 2–4 p.i., with predominantly peribronchiolar lesions, progressing to fully developed bronchiolitis and bronchopneumonia by day 7 p.i. (Fig. 4). On day 5 p.i., severe interstitial pneumonia (Fig. 4b and c) with edema, thickening and inflammatory cellular infiltration of the alveolar walls was found in infected mice, compared with milder symptoms in antibody-treated infected mice (Fig. 4d and e).

Discussion

We have demonstrated that IAV (H5N1) infection causes elevated expression of HMGB1 in the lungs of mice. Pulmonary HMGB1 mRNA did not increase until day 3 p.i., and its protein levels were significantly increased on day 4 p.i., when most mice had severe disease. In mouse models of endotoxemia and sepsis, HMGB1 is first detectable in the circulation 8 hr after the onset of the lethal endotoxemia and sepsis, subsequently increasing to plateau levels from 16 to 32 hr (Wang *et al.*, 1999; Yang *et al.*, 2004). This late appearance of circulating HMGB1 precedes and parallels the onset of animal lethality from endotoxemia or sepsis. The present results, showing that the HMGB1 levels peaked in the serum on day 5 p.i., when most mice had died, support the concept that the released HMGB1 contributes to IAV (H5N1)-induced mortality, as has been postulated elsewhere (Chu *et al.*, 2003). Local or systemic production of HMGB1 is possible during influenza infections, since other viruses are known to induce HMGB1 release from infected cells (Chen *et al.*, 2004, 2008). Visualization of HMGB1 expression in the lungs of IAV (H5N1)-infected mice by immunohistochemistry also showed that the bronchiolar epithelial cells, alveolar epithelial cells, and intercellular spaces contained large amounts of HMGB1 protein from days 3–5, consistent with the observed cellular HMGB1 expression in lung tissues. Furthermore, a difference in the profile of HMGB1 induced in the lungs by infectious versus inactivated IAV (H5N1) was also observed. HMGB1 production in the lungs requires viral replication, since HMGB1 expression in mice given inactivated virus was much lower than in infectious virus-infected mice. This is similar to what has been seen with H5N1 viruses in primary human type II pneumocytes, since inactivation of H5N1 virus by ultraviolet irradiation prior to the infection of the alveolar epithelial cells abolished the induction of IP-10, INF-β, RANTES, and IL-6 (Chan *et al.*, 2005). The massive viral replication in the lungs may contribute to
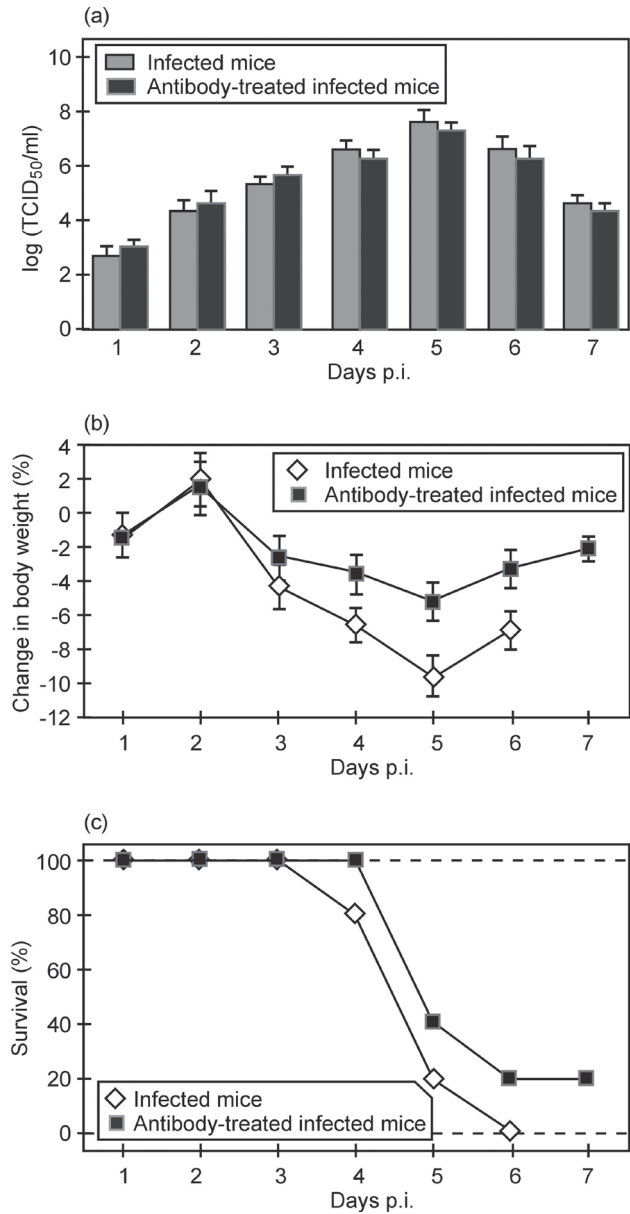
Fig. 3

HMGB1-specific antibody reduces morbidity and mortality in virus-infected mice

Infectious virus titer in the lungs (a), body weight (b) and survival time (c). *$P < 0.05$.

fatal IAV (H5N1) infection by eliciting an intense cytokine response. Given that HMGB1 functions as a late mediator cytokine and/or enhancer of proinflammatory activities of other cytokines, and that IAV (H5N1) induces HMGB1 release into the lungs, the role of HMGB1 required further investigation. IAV (H5N1)-infected mice were treated with
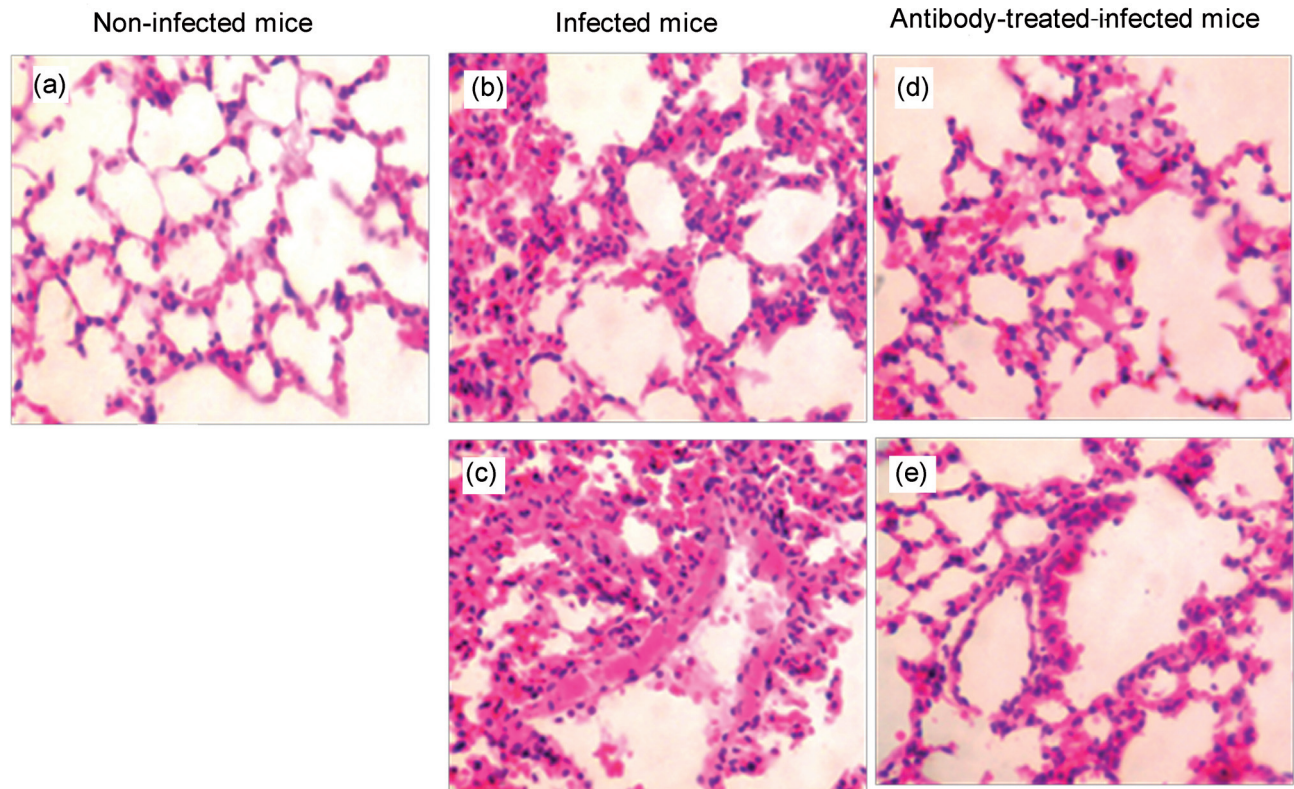
Non-infected mice   Infected mice   Antibody-treated-infected mice

Fig. 4

HMGB1-specific antibody reduces inflammatory responses in virus-infected mouse lungs
Non-infected mice (a), infected mice (b and c) and antibody-treated infected mice (d and e).

Table 1. Primers used in real-time PCR

Gene	Primer (5'to 3')	PCR product (bp)	Acc. No.
HMGB1	AAGAAGTGCTCAGAGAG GTGGAAG(F) ATAAGCCAGGATGCTCG CC(R)	235	HBC085090
β-actin	TGACAGGATGCAGAAGG AGA(F) GCTGGAAGGTGGACAGT GAG(R)	131	NM007393

HMGB1-specific antibody, which conferred significant protection against acute inflammation via inhibition of HMGB1 secretion (Kobasa *et al.*, 2007). Following virus infection, mice treated with HMGB1-specific antibody had significantly reduced levels of IL-1β, IL-6, TNF-α, IP-10, and RANTES in the lungs, reduced loss of body weight and enhanced survival to more than 7 days (P <0.05), but blocking of the HMGB1 activity had no significant effect on viral titers in the lungs during infection.

In summary, we have found that HMGB1 production in mouse lungs appears to correlate with death. This finding supports the hypothesis that HMGB1 is an important factor enhancing the inflammatory response in IAV (H5N1) infection, at least in the mouse model.

Conflict of interest. The authors declare that they have no conflicts of interest.

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