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BRIEF REPORT

Porcine mast cells infected with H1N1 influenza virus release histamine and inflammatory cytokines and chemokines

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Abstract Mast cells reside in many tissues, including the lungs, and might play a role in enhancing influenza virus infections in animals. In this study, we cultured porcine mast cells from porcine bone marrow cells with IL-3 and stem cell factor to study the infectivity and activation of the 2009 pandemic H1N1 influenza virus of swine origin. Porcine mast cells were infected with H1N1 influenza virus, without the subsequent production of infectious viruses but were activated, as indicated by the release of histamines. Inflammatory cytokine- and chemokine-encoding genes, including IL-1α, IL-6, CXCL9, CXCL10, and CXCL11, were upregulated in the infected porcine mast cells. Our results suggest that mast cells could be involved in enhancing influenza-virus-mediated disease in infected animals.

Keywords Influenza virus · Mast cells · Inflammation

Influenza viruses are divided into three established genera, *Influenzavirus A*, *Influenzavirus B*, and *Influenzavirus C*, and the proposed genus *Influenzavirus D*, based on their nucleoproteins (NP) and matrix proteins (M) [20, 23]. Wild birds are major reservoirs of influenza A viruses, which include 16 hemagglutinin (HA) and nine neuraminidase (NA) antigen subtypes [4, 23]. Influenza A viruses are responsible for pandemics in humans. During the 20th century, humans experienced three pandemics in 1918, 1957, and 1968, caused by H1N1, H2N2, and H3N2 influenza viruses, respectively [11, 13, 22]. In the 21st century, novel H1N1 influenza viruses of swine origin caused pandemics, resulting in thousands of deaths [8].

Pigs are reservoirs for virus mixing and the creation of novel influenza viruses that can be efficiently transmitted to humans. It is known that pigs have both avian and human influenza virus receptors, which enables coinfection [7].

Mast cells reside in all tissues, contain granules, and regulate both innate and adaptive immune responses [5]. The abundance of mast cells at environmental interfaces enables them to respond to pathogens, similar to the response of epithelial cells and dendritic cells [1]. In addition, mast cells are located near blood vessels, lymphatics, and nerve endings, which allows them to exert diverse host effects in response to microbes [1, 5]. Mast cells are activated in two different phases, immediate degranulation, which results in the release of pre-formed mediators such as histamine, and the delayed secretion of secondary mediators, synthesized in response to activation, such as inflammatory cytokines and chemokines [1, 5, 16, 19]. Mast cell granules contain histamine, serotonin, antimicrobial peptides, and proteases [16, 19]. Upon activation, these granules are released through a calcium-dependent exocytosis pathway. Histamine is a powerful inflammatory protein that enhances vascular permeability, causes vasodilation, and induces bronchial smooth muscle contraction [16, 19]. It has been suggested that excessive inflammatory responses might enhance influenza virus pathogenesis in infected humans and animals [2, 3, 9, 10, 12, 17, 21, 22, 24].

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In this study, we investigated porcine mast cell activation in response to infection with the 2009 pandemic H1N1 influenza virus of swine origin. We cultured porcine bone marrow mast cells with IL-3 and stem cell factor. We determined if cultured porcine mast cells could support the replication of H1N1 influenza virus, release histamine, and produce inflammatory cytokines and chemokines.

The 2009 pandemic H1N1 influenza virus (A/California/07/2009) was kindly provided by the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA), and was grown in MDCK cells.

Primary porcine mast cells were cultured as described by Graham et al. [6] with some modifications. Femurs from 1-day-old pigs were obtained and homogenized. Porcine bone marrow cells were obtained from homogenized femurs by centrifugation at 1500 rpm for 10 min. Cells (1×10^6/mL) were resuspended in RPMI 1640 supplemented with 1% nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamic acid, 50 mg of gentamicin per mL, 100 U of penicillin/streptomycin per mL, 20 mM HEPES, 10% FBS, and 0.1% 2-mercaptoethanol. For 5 weeks, cells were cultured with RPMI 1640 medium containing recombinant porcine IL-3 (20 ng/mL; Transplantation Biology Research Center, Massachusetts General Hospital, MA, USA) and recombinant human stem cell factor (40 ng/mL; PROSPEC, Ness Ziona, Israel). Culture medium was changed every 5 days.

Total mRNA was collected from cultured porcine mast cells (2×10^5) and control porcine bone marrow cells (2×10^5) using an RNeasy Mini Kit (QIAGEN) with DNase treatment. The mRNA expression of porcine FcεR1α and CD117, which are mast cell markers, was quantified using specific primers, which are available upon request, using SYBR-Green-based quantitative real-time polymerase chain reaction (PCR).

Viral titers (m.o.i) were determined by plaque assay with MDCK cells, agar, and neutral red dye before they were used for infection experiments.

The cultured porcine mast cells (2×10^5 cells/well) were grown in chamber slides and infected with A/California/07/2009 (H1N1) (m.o.i. = 1) for 24 h. The infected cells were fixed with 80% cold acetone for 30 min on ice. Fixed cells were incubated with rabbit anti-influenza A nucleoprotein (NP) antibody (Sino Biologicals Inc., China) for 30 min at room temperature and then stained with FITC-labeled goat anti-rabbit IgG antibody (KPL, USA). The stained cells were evaluated using a fluorescence microscope (Olympus, Japan). As control reactions, the infected cultured porcine mast cells were stained with normal rabbit serum and FITC-labeled goat anti-rabbit IgG antibody, and the uninfected cells were stained with rabbit anti-influenza A nucleoprotein (NP) antibody and FITC-labeled goat anti-rabbit IgG antibody.

Cultured porcine mast cells (2×10^5 cells/well) were grown in a 24-well plate and infected with A/California/07/2009 (H1N1) (m.o.i. = 1). Supernatants were collected at 24, 48, and 72 h postinfection (p.i.). The histamine concentration was determined using a porcine histamine (HIS) enzyme-linked immunosorbent assay (ELISA) kit (BlueGene Biotech, China). Briefly, supernatants (100 μL) and HIS conjugate (50 μL) were incubated in wells that had been pre-coated with anti-HIS antibody for 1 h at 37 °C. The wells were washed five times with 1× buffer and incubated with horseradish peroxidase (HRP) substrate (100 μL) for 15 min at 25 °C. A stop solution (50 μL) was then added to halt the reaction. The intensity of the colorimetric reaction (optical density) was measured at 450 nm in a microplate reader. This value was inversely proportional to the HIS concentration due to the fact that HIS from samples and HIS-HRP conjugate compete for anti-HIS antibody binding. A standard curve was plotted to relate color intensity to the known concentrations of standards. The HIS concentration in each sample was interpolated from this standard curve. Uninfected cultured porcine mast cells were used as a control, and the assay was performed in triplicate.

Cultured porcine mast cells (2×10^5 cells/well) in a 24-well plate were infected with A/California/07/2009 (H1N1) (m.o.i. = 1). Total RNA was collected using an RNeasy Mini Kit (QIAGEN) at 12 h p.i. The mRNA expression of porcine inflammatory cytokines and chemokines was quantified using SYBR-Green-based quantitative real-time polymerase chain reaction (PCR) with specific primers, which are available upon request.

Statistical analysis was performed using the Statistical Product and Services Solutions (SPSS) package, version 10.0 (SPSS, Cary, NC, USA). A Student's *t*-test was performed to compare values from infected and uninfected porcine mast cells. A *P*-value less than 0.05 was considered statistically significant.

To find out whether the cultured cells were porcine mast cells, we quantified mRNA expression of the porcine mast cell markers FcεR1α and CD117, since no porcine-specific antibodies for them were commercially available (Fig. 1). The expression of FcεR1α and CD117 mRNAs in the cultured cells increased up to 17- and 6-fold, respectively, compared to those in porcine bone marrow cells.

We determined if cultured porcine mast cells could be infected by the H1N1 influenza virus. Cultured porcine mast cells were infected with the 2009 pandemic H1N1 influenza virus (A/California/07/2009) with minimal essential medium (MEM) supplemented with trypsin (1 μg/mL), and supernatants were collected at 24, 48, and 72 h p.i. The presence of virus in the supernatant was tested using MDCK cells with MEM supplemented with trypsin (1 μg/mL). No virus was detected in the supernatants (data not shown), suggesting the absence of productive infection in porcine mast cells.

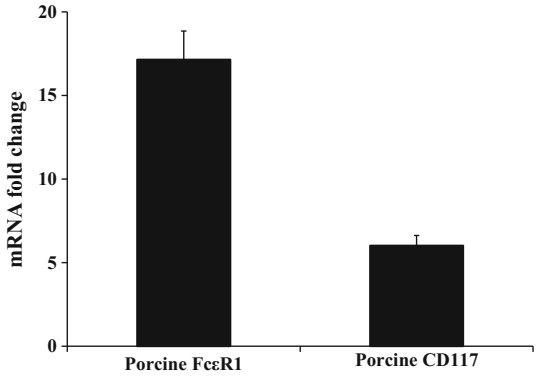

Fig. 1 Quantification of markers of mast cells in the cultured porcine mast cells. Total RNA was collected from cultured porcine mast cells or porcine bone marrow cells. The mRNAs of porcine FcεR1α and CD117, which are markers for mast cells, were quantified using SYBR-Green-based quantitative real-time PCR. The fold change in the cultured porcine mast cells was calculated based on the amount of expression in porcine bone marrow cells. The assay was performed in triplicate

We stained the infected porcine mast cells with anti-influenza-A-NP antibody and FITC-labeled secondary antibody. All infected cells were stained (Fig. 2A), whereas an isotype control (Fig. 2B) and uninfected cells were not stained (Fig. 2C).

We next determined if the infected porcine mast cells could release histamine. Cultured porcine mast cells were infected with the 2009 pandemic H1N1 influenza virus, and supernatants were collected at 24, 48, and 72 h p.i. The amount of released histamine was quantified using an anti-porcine histamine ELISA kit. Most histamine was released from infected porcine mast cells within 24 h, as no significant differences in histamine release were detected among the different time points (Fig. 2C). The values for released histamine from infected porcine mast cells at 24, 48, and 72 h were 39.7 mg/mL, 32.2 mg/mL, and 36.4 mg/mL, respectively.

We performed quantitative RT-PCR to analyze inflammatory cytokines and chemokines in infected porcine mast cells because inflammation contributes to enhancing influenza disease in infected humans and animals [17, 22, 24]. When we quantified inflammatory cytokines from porcine mast cells infected with the 2009 pandemic H1N1 influenza virus, IL-1, which is responsible for inflammation and fever, and IL-6, which is involved in inducing fever and acute-phase response, were expressed at

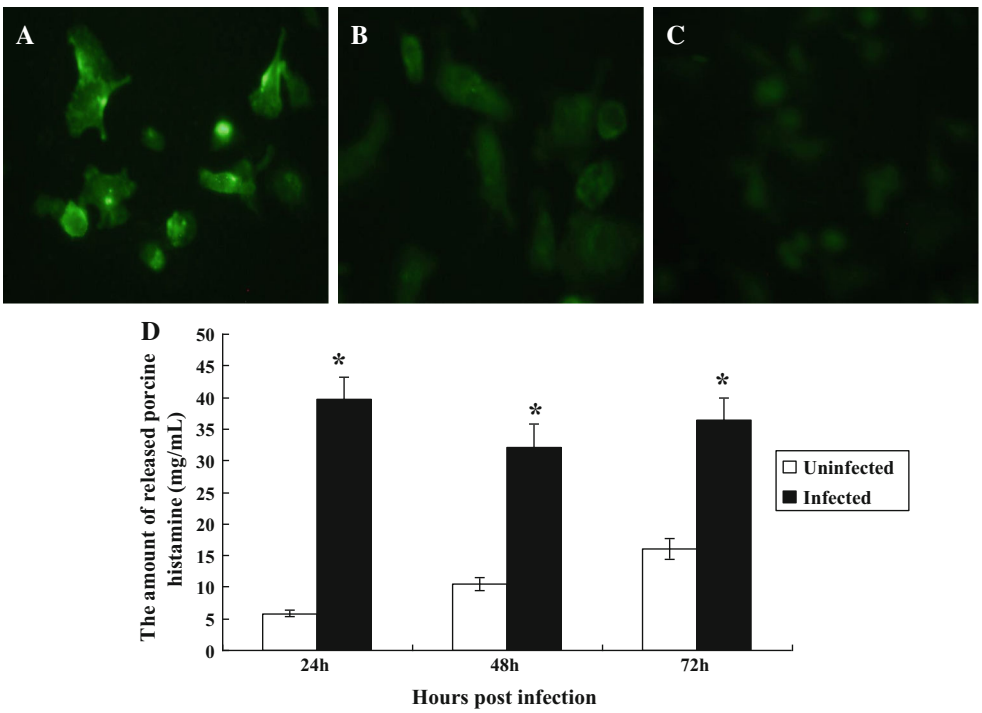

Fig. 2 Staining of infected porcine mast cells by anti-influenza-virus antibody and detection of the released histamine in the infected porcine mast cells. Cultured porcine mast cells on chamber slides were infected with A/California/07/2009, and 24 h later, they were fixed with cold 80% acetone. The fixed cells were incubated with rabbit anti-influenza A virus nucleoprotein (NP) antibody (**A**), rabbit isotype control antibody (**B**), or uninfected cells (**C**), followed by FITC-labeled goat anti-rabbit IgG antibody, and the stained cells were evaluated using a fluorescence microscope (X400). The supernatants were collected from porcine mast cells infected with A/California/07/2009 (H1N1) at 24, 48 and 72 h p.i. The amount of released histamines in the supernatants (**D**) was determined using a porcine histamine detection kit as instructed by the manufacturer. Uninfected cultured porcine mast cells were used as a control. The assay was performed in triplicate. Statistical analysis was performed to compare data from infected and uninfected porcine mast cells. *$P<0.05$

a higher level in infected porcine mast cells (2.9- and 1.6-fold, respectively) than in uninfected porcine mast cells (Fig. 3A). Among the chemokines tested, CXCL9, which is responsible for T-lymphocyte attraction; CXCL10, which is involved in attracting monocytes, macrophages, T lymphocytes, and NK cells; CXCL11, which attracts activated T lymphocytes; and CCL20, which attracts lymphocytes, were induced at a significantly higher level (29.25-, 16.6-, 20.6-fold, and 6.2-fold, respectively) in infected porcine mast cells compared to those in uninfected porcine mast cells (Fig. 3B).

Mast cells are present in all tissues and might be involved in viral pathogenesis in infected animals. We studied the infection and activation of porcine bone marrow mast cells by the 2009 pandemic H1N1 influenza virus of swine origin. Cultured porcine mast cells were infected with H1N1 influenza virus without producing infectious particles and released histamines upon infection. Infected porcine mast cells produced inflammatory cytokines and chemokines, which could cause severe inflammation in infected animals.

Our cultured cells were judged to consist mainly of mast cells, based on their morphology, but we could not confirm this by staining the cultured cells with porcine-specific antibodies for mast cell markers, since they are currently not available.

When we attempted to infect cultured porcine mast cells with H1N1 influenza virus, they became infected but did not produce infectious viral particles. This suggests that mast cells in the lungs could be infected by influenza viruses produced in lung epithelial cells. Our results are consistent with those of previous studies with primary human and mouse mast cells [6, 15]. In human mast cells infected with influenza A virus, there was no significant release of virus; however, viral genomic RNA transcription, replication, and protein synthesis were detected [15]. Mouse primary mast cells infected with human influenza viruses such as H1N1, H3N2, and human B also did not support the productive replication of these viruses [6]. However, mouse mast cells (mastocytoma cell line P815) supported the production of infectious influenza A virus, including H1N1, H5N1, and H7N2 strains [14]. This discrepancy might be due to the different origins of these cells. The porcine and human mast cells were primary cells, but the mouse mastocytoma cell line P815 was a tumor cell line.

Cultured porcine mast cells released histamines upon infection with H1N1 influenza virus. A previous study using mouse primary cells also showed that mast cells infected with influenza A virus (A/WSN/33) released histamines [6]. This suggests that influenza A virus can activate mast cells directly without the involvement of IgE

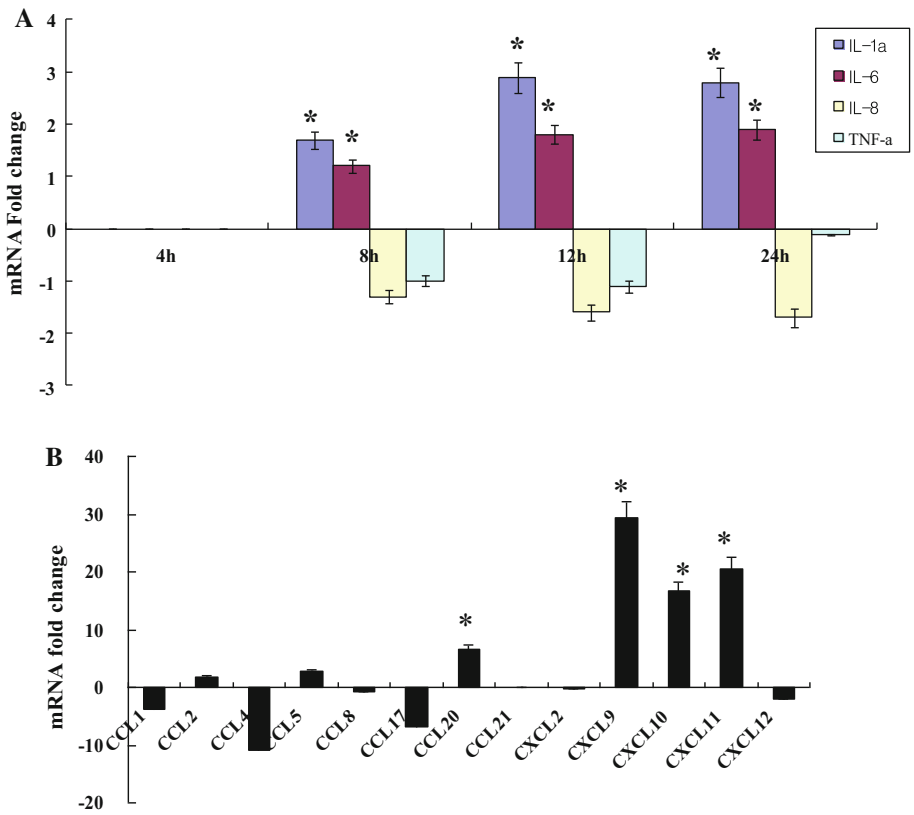

Fig. 3 Quantification of inflammatory cytokine and chemokine gene expression in infected porcine mast cells. Total RNA was collected from cultured porcine mast cells infected with A/California/07/2009 (H1N1) at 12 h p.i. The mRNAs of porcine inflammatory cytokines (**A**) and chemokines (**B**) were quantified by SYBR-Green-based quantitative real-time PCR using porcine specific primers. The assay was performed in triplicate. Statistical analysis was performed to compare data from infected and uninfected porcine mast cells. The assay was performed in triplicate. *, $P < 0.05$

antibodies. It is known that mast cells immediately release histamines and other materials stored in granules when IgE antibodies bound to their receptors (FcεRI) are cross-linked by an allergen [18].

Inflammatory cytokines and chemokines were induced in cultured porcine mast cells. The infected porcine mast cells induced expression of genes for inflammatory cytokines, such as IL-1α and IL-6, and chemokines that attract inflammatory cells, such as CXCL9, CXCL10, and CXCL11. It has been reported that mouse tumor mast cells (P815) infected with influenza A viruses (H1N1, H5N1, and H7N2) produce inflammatory cytokines (IL-6, IL-18, TNF-α) and the chemokine MCP-1 [14]. Highly pathogenic H5N1 influenza virus greatly increased the production of the inflammatory cytokines IL-1, IL-6, and TNF-α in the infected lungs of mice [2], and the 1918 H1N1 pandemic influenza virus also strongly induces IL-1, IFN-γ, and IL-6 production in the lungs of mice [17]. Canine H3N2 influenza virus induces expression of the chemokine CXCL10, which is involved in chemoattraction of monocytes and lymphocytes in the infected lungs of dogs [9].

In conclusion, cultured porcine mast cells infected with H1N1 influenza virus can be activated directly, leading to release of histamine, inflammatory cytokines, and chemokines.

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Compliance with ethical standards

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

Ethical approval This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Korean Veterinary Quarantine and Service. The animal experiments were approved by the Animal Experimental Ethics Committee at the Chungnam National University.

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