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# The presence of monocytes enhances the susceptibility of B cells to highly pathogenic avian influenza (HPAI) H5N1 virus possibly through the increased expression of α2,3 SA receptor

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ABSTRACT

The highly pathogenic avian influenza (HPAI) H5N1 virus causes severe systemic infection in avian and mammalian species, including humans by first targeting immune cells. This subsequently renders the innate and adaptive immune responses less active, thus allowing dissemination of the virus to systemic organs. To gain insight into the pathogenesis of H5N1, this study aims to determine the susceptibility of human PBMCs to the H5N1 virus and explore the factors which influence this susceptibility. We found that PBMCs were a target of H5N1 infection, and that monocytes and B cells were populations which were clearly the most susceptible. Analysis of PBMC subpopulations showed that isolated monocytes and monocytes residing in whole PBMCs had comparable percentages of infection (28.97 ± 5.54% vs 22.23 ± 5.14%). In contrast, isolated B cells were infected to a much lower degree than B cells residing in a mixture of whole PBMCs (0.88 ± 0.34% vs 34.87 ± 4.63%). Different susceptibility levels of B cells for these tested conditions spurred us to explore the B cell-H5N1 interaction mechanisms. Here, we first demonstrated that monocytes play a crucial role in the enhancement of B cell susceptibility to H5N1 infection. Although the actual mechanism by which this enhancement occurs remains in question, α2,3-linked sialic acid (SA), known for influenza virus receptors, could be a responsible factor for the greater susceptibility of B cells, as it was highly expressed on the surface of B cells upon H5N1 infection of B cell/monocyte co-cultures. Our findings reveal some of the factors involved with the permissiveness of human immune cells to H5N1 virus and provide a better understanding of the tropism of H5N1 in immune cells.



## 1. Introduction

Since the first outbreak in 1997, several attempts have been made to understand the differences between the highly pathogenic avian influenza (HPAI) H5N1 virus and other influenza virus strains. In mammalian hosts, including humans, there are several key hallmarks of infection that are of interest. First, pieces of evidence have suggested that while systemic infection with the H5N1 virus is common, infection with the less pathogenic strains is confined only to the respiratory tract [1,2]. Other hallmarks of infection include excessive cytokine production and lymphopenia which reflect attack on the immune system [1,3].

To fight invasion of a harmful virus, cells in the immune system work together to ultimately reduce viral replication and spread. Upon recognition of the virus, immune cells are rapidly recruited to the primary site of infection according to their innate migratory properties. Exploitation of these immune cells by the virus in order to facilitate its spread systemically is also feasible; for example (i) dendritic cells (DCs), have been shown to facilitate the dissemination of several viruses such as human immunodeficiency virus (HIV) and varicella zoster virus (VZV) [4,5], (ii) data suggest that neutrophils might serve as a vehicle for viral replication and transportation in avian influenza [6]. As immune cells are best equipped for controlling influenza infection, targeting immune

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cells could be a potential strategy used by the H5N1 virus to overcome the activation of an immune response and could possibly explain the unique clinical findings. H5N1 has been reported to infect, kill, and induce hyperinflammatory cytokine secretion in many types of immune cells including neutrophils, monocytes, macrophages and DCs, resulting in a range of disease severities of H5N1 infection [6–8]. However, there is limited data on H5N1 infection in primary human PBMCs and their subpopulations, particularly the lymphoid lineages. In this study, we demonstrated that human PBMCs, specifically monocytes and B cells, but not T cells were susceptible to H5N1 infection *in vitro*. In analyzing the subpopulation susceptibility, we found that only isolated monocytes were permissive to H5N1 virus, whereas, isolated B and T cells were resistant. Surprisingly, B cells were more susceptible to H5N1 infection when cocultured with monocytes. Upon infection, the interaction of B cells and monocytes promotes a high expression level of the α2,3-linked sialic acid (SA) receptors on B cells, which are thought to be critical players in supporting the increased susceptibility of B cells. Our findings suggest that monocytes play a role in enhanced B cell susceptibility to infection by the H5N1 virus via inducing the up-regulation of α2,3 SA expression on B cells *in vitro*.

## 2. Materials and methods

### 2.1. Cell culture and isolation

PBMCs were isolated from fresh whole blood collected from a healthy donor. All samples for the present study were obtained with written informed consent from all the donors as per the approval of Institutional Review Board, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Thailand. Peripheral blood was mixed 1:1 with warmed RPMI-1640 medium (Gibco, NY, USA). The mixture was gently layered on top of a warmed Ficoll solution, LymphoprepTM (Axis-Shield, Oslo, Norway). The suspension was centrifuged at 25 °C, at 1000 × g for 35 min. A layer of PBMCs was collected and washed twice with RPMI-1640 medium. PBMCs were cultured in RPMI 1640 with 10% FBS supplemented with nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, NY, USA).

PBMCs were subjected to specific cell isolation. Monocytes, B cells, and T cells were isolated from PBMCs using CD14, CD20, and CD3 MACS MicroBeads (Miltenyi Biotec, Germany), respectively following the manufacturer's instructions. Briefly, PBMCs were washed and resuspended with cold MACS buffer, then mixed with magnetic beads-labeled with anti-CD14, anti-CD20, and anti-CD3 antibodies for monocytes, B cells, and T cells respectively. After 30 min incubation at 4 °C, the cells were washed once, and then applied to a MACS column placed on a magnetic stand. The column was washed three times before isolated cells were retrieved from the column. Cell purity was confirmed by flow cytometric analysis. More than 95% purity was accepted for further experiments. Isolated monocytes, B cells, and T cells were cultured in RPMI-1640 medium with 10% FBS supplemented with nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco).

### 2.2. Virus propagation

Influenza virus strain A/open-billed stork/Nakhonsawan/BBD0104F/04 was propagated and quantified as previously described [7]. This H5N1 strain was used throughout this study. Briefly, confluent MDCK cells were inoculated with influenza virus. After 1 h of adsorption, all medium was removed and replaced with fresh MEM (Gibco, NY, USA). Supernatant was harvested when 3+ to 4+ cytopathic effect (CPE) was observed. Virus titer was quantified by a plaque assay. All procedures involved with H5N1 virus were performed in BSL3 containment.

### 2.3. H5N1 infection

#### 2.3.1. PBMCs

PBMCs were incubated with H5N1 virus at an MOI of 1 for one hour. Incubated PBMCs were then washed three times, and plated into 96-well plates. Samples from each well were collected at specific time for staining. Infected PBMCs were tagged with antibodies against CD markers of each cell population such as APC-conjugated anti-CD3, PerCP-conjugated anti-CD14 and PE-conjugated anti-CD20 antibodies (BD biosciences). To detect intracellular antigen, cells were incubated with Cytofix/Cytoperm (BD biosciences, CA, USA) for 10 min at 4 °C, and then washed with Perm/Wash (BD biosciences, CA, USA) before incubation with FITC-conjugated anti-NP antibody (Chemicon) for 30 min at 4 °C. After that, cells were washed and resuspended with 3.7% formaldehyde in PBS. The samples were analyzed using FACSCalibur (BD biosciences, CA, USA). Data was analyzed using cell quest pro software (BD biosciences, CA, USA).

#### 2.3.2. Isolated cells

$CD14^+$ monocytes, $CD20^+$ B cells, and $CD3^+$ T cells were infected with H5N1 virus at an MOI of 1 for an hour. Following absorption, cells were washed, and then plated onto 96-well plates. After 12 h of incubation, cells were harvested for intracellular staining with a FITC-conjugated anti-Influenza NP antibody (Chemicon). Samples were analyzed by flow cytometry.

### 2.4. Interaction of B cells and monocytes

#### 2.4.1. B cell and monocyte co-cultures (B/M co-cultures)

An equal number of isolated B cells and monocytes were separately incubated with virus at an MOI of 1. After washing, each cell type was co-cultured. Samples were collected at specific time points. For simultaneous staining of surface sialic acid and intracellular viral antigen, cells were first stained with PE-conjugated anti-CD20 antibody and fluorescently-labeled MAA I for 30 min at 4 °C in the dark. After washing, cells were fixed, permeabilized, and incubated with a primary antibody, mouse anti-NP antibody IgG2a (Millipore) and secondary antibody, APC-conjugated goat anti mouse IgG2a (SouthernBiotech). The samples were analyzed by flow cytometry.

#### 2.4.2. B cell and monocyte transwell co-cultures (B/M transwell)

To prevent cell–cell contact between B cells and monocytes, transwell chambers with a 0.4 um pore size membrane (Coning, NY, USA) were used. An equal number of isolated B cells and monocytes were separately absorbed with H5N1 virus at an MOI of 1 as described above. After absorption, B cells were cultured in the lower part of the chamber, while monocytes were retained in the upper compartment. After 12 h of incubation, B cells in the lower part were collected and stained with the same protocol as described above for the B/M co-cultures.

### 2.5. Lectin staining

To determine the expression level of sialic acid receptor on the cell surfaces; cells were incubated with 50 μl of FITC-labeled Sambucusnigra (SNA) lectin (2 μg/ml; specific for SAα2,6Gal) and FITC-labeled amurensis (MAA) lectin (20 μg/ml; specific for the SAα2,3Gal) for 30 min at 4 °C in the dark. The samples were analyzed using a laser scanning confocal microscope (Olympus FV10i).

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