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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).

2.6. Fluorescence microscopy

Harvested cells were washed and resuspended with 1XPBS, and 10 ul of the resuspended cells was dropped onto Superfrost Plus slides (Erie Scientific, NH, USA)f. After air drying, cells were fixed with 4% paraformaldehyde for 1 h at room temperature and then washed with 1XPBS. Cells were permeabilized with Cytofix/Cytoperm reagent (Becton Dickinson) for 2 min at 4 °C in the dark. To detect viral nucleoprotein (NP) and α2,3 SA expression on $CD20^{+}$ cells, cells were stained with an Alexa 647-conjugated anti-CD20 antibody (Biolegend, US), Alexa 548-conjugated MAAI (Biolegend, US), and a FITC-conjugated NP antibody (Chemicon, Temecula, CA). Cells were counterstained with DAPI, and washed with 1XPBS. The samples were analyzed using a laser scanning confocal microscope (Olympus FV10i).

3. Results

3.1. PBMCs and their subpopulations are susceptible to H5N1 virus infection

To determine the susceptibility of PBMCs towards H5N1 virus, we infected PBMCs isolated from healthy young adults with Open-billed/H5N1 virus. This H5N1 virus was isolated from an open-billed stork from a wetland in the middle of Thailand in 2004. The virus was of genotype Z, and all essential residues for receptor specificity and pathogenicity were confirmed to be identical to those from human isolates. Preferential binding to avian type sialic acid receptor was confirmed by a receptor binding assay (data not shown). PBMCs were susceptible to H5N1 virus, as about 8% of the total PBMCs were found to be infected at 12 h post-infection (Fig. 1A). As the percentage of infection peaked at 12 h, this time point was then used throughout the study. We also analyzed subpopulations of PBMCs by tagging H5N1-infected PBMCs with antibodies against CD14, CD20, and CD3; surface markers for monocytes, B cells, and T cells, respectively. We found that the majority of infected cells were monocytes and B cells which accounted for 22% and 34% of infected cells, respectively. T cells on the other hand were found to be relatively resistant (Fig. 1B). Thus, we demonstrated for the first time that B cells residing in whole PBMCs were highly permissive to the H5N1 virus.

To understand the mechanism of H5N1 infection in PBMCs and their subpopulations, monocytes, B cells, and T cells were isolated from fresh PBMCs using magnetic beads and were infected with Open-billed/H5N1 at an MOI of 1. After 12 h of incubation, the results showed that about 29% of the isolated monocytes were infected, while less than 1% of the isolated B cells were susceptible to H5N1 infection (Fig. 1C), which was inconsistent with the results obtained for infection of whole PBMCs in Fig. 1B. However, no matter what environment the T cells were studied in, they were found to be completely resistant to H5N1 infection (Fig. 1B and C). These findings indicated that the susceptibility of B cells was distinct under different environmental conditions. Thus, it is important to explore how factors in these different cellular conditions alter the susceptibility of B cells to infection.

3.2. The presence of monocytes could enhance the susceptibility of B cells

The drop in the percentage of infection of isolated B cells convinced us to investigate the interaction between subpopulations in PBMCs. Since both monocytes residing in the mixture of PBMCs and isolated monocytes were susceptible to H5N1 infection, we proposed that the presence of monocytes is crucial for B cell infection. To prove this hypothesis, B cells were co-cultured with monocytes (B/M co-cultures) after one hour of viral adsorption. At

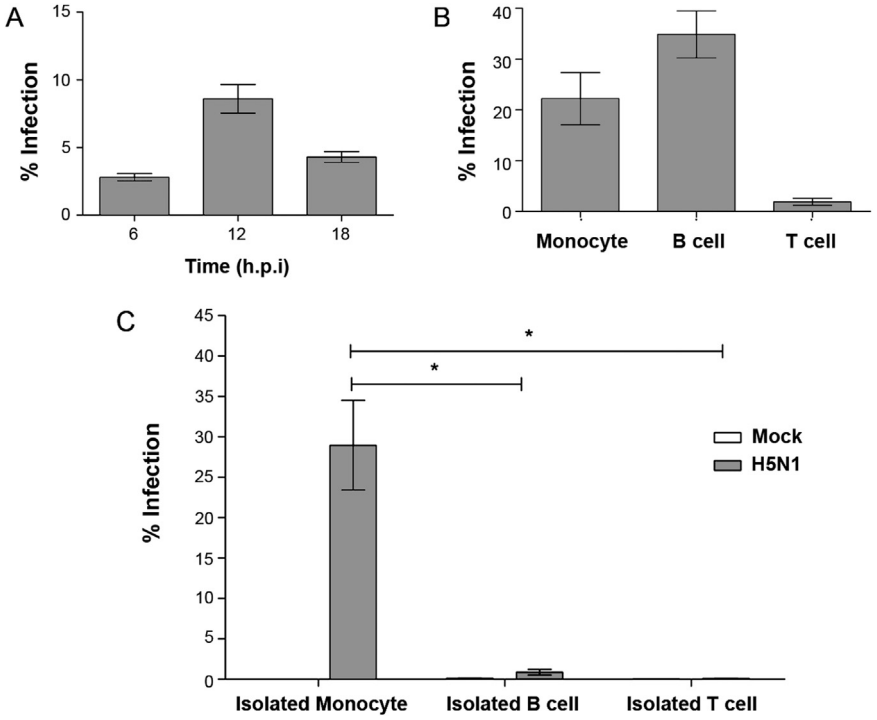

Fig. 1. Susceptibility of PBMCs and their subpopulations to H5N1 virus. PBMCs isolated from healthy donors were infected H5N1 virus at an MOI of 1. (A) The cells were collected at the indicated time point for staining of Influenza nucleoprotein (NP). (B) Infected PBMCs were stained with antibodies against specific surface markers in order to distinguish B cell (CD20), T cell (CD3), and monocyte (CD14) populations along with antibody to viral NP at 12 h post-infection. (C) CD3+ cells, CD20+ cells, and CD14+ cells were isolated from PBMCs and infected with H5N1 at an MOI of 1 for 12 h. Cells were harvested for staining of viral NP. Samples were analyzed by flow cytometry. The results were obtained from three different donors and are presented as means plus standard errors.

12 h post-infection, co-cultures were harvested for staining with a viral (NP) antigen and a B cell marker (CD20). Interestingly, B cells in B/M co-cultures were highly susceptible to H5N1 infection compared to isolated B cells; 30.50 ± 29.03% vs 1.26 ± 0.09%, respectively (Fig. 2). The percentage of infected B cells in B/M co-cultures was not significantly different from the percentage of infected B cells in whole PBMCs. We thus confirmed the importance of monocytes in promoting the susceptibility of B cells by removing monocytes from the PBMCs by using anti-CD14 microbeads. Compared to PBMCs before monocyte removal and the B/M co-cultures, the number of B cells infected with H5N1 virus significantly decreased (Fig. 2A). To explore whether the interaction between B cells and monocytes requires cell–cell contact or whether this effect is mediated by paracrine factors, a transwell system was used to prevent physical contact of these two cell types (B/M transwell). B cells and monocytes were separately absorbed with H5N1 virus at an MOI of 1 for 1 h. Following absorption, monocytes were cultured in the upper part of the transwell, while B cells were cultured in the lower part. B cells were harvested 12 h postinfection to detect viral NP antigen. We found that the susceptibility of B cells in B/M transwell cultures was less than 2%, which was comparable to isolated B cells (Fig. 2B). These results suggested that monocytes play a role in the increased susceptibility of B cells to H5N1 infection and cell-to-cell contact is required in this enhancement.

3.3. An up-regulation of sialic acid receptor on B cells upon H5N1 infection is associated with increased susceptibility

The enhanced susceptibility of B cells in B/M co-cultures led us to explore the factors associated with this high susceptibility. The expression of sialic acid receptor was measured since this receptor is a major determinant of influenza virus cellular tropism. Avian influenza strains prefer α2,3 sialic acid (SA), while human strains prefer α2,6 SA. Under normal conditions, B cells showed high levels of SNA staining, reflecting high levels of α2,6 SA expression, but low levels of MAA I staining, reflecting low levels of α2,3 SA expression (Fig 3A). It is hypothesized that an increase in α2,3 SA receptor expression might support the permissibility of B cells to H5N1 virus. To prove this hypothesis, isolated B cells, B/M co-cultures, and B/M transwell cultures were infected with H5N1 virus. After 12 h, the level of α2,3 SA expression on B cells was analyzed. We found that α2,3 SA expression was dramatically increased on B cells in B/M co-cultures upon H5N1 infection, while there was little expression on B cells of both isolated B cells and B/M transwell cultures (Fig. 3B). In contrast, we did not observe any difference in the α2,6 SA expression level on B cells in any tested conditions (data not shown). Immunofluorescence staining was done to confirm the increase of α2,3 SA expression on B cells in B/M co-cultures under infected conditions. B cells derived from isolated B cells, B/M transwell cultures, and B/M co-cultures were stained with MAAI, anti-CD20, and anti-NP for the detection of α2,3 SA, the B cell specific marker, and viral antigen, respectively. DAPI was used for nuclei staining. The results were analyzed by Fluoview Viewer software version 3.0. We observed the co-localization of α2,3 SA (red) and viral NP (green) in CD20-positive cells (yellow) only in H5N1-infected B/M co-cultures. This phenomenon was not seen in non-infected B/M co-cultures or stand-alone B cells and B/M transwell cultures in both mock and H5N1-infected conditions (Fig. 3C). The immunofluorescence staining supported the data from flow cytometry suggesting that the up-regulation of α2,3 SA expression on B cells in H5N1-infected B/M co-cultures might be responsible for the enhanced B cell susceptibility.

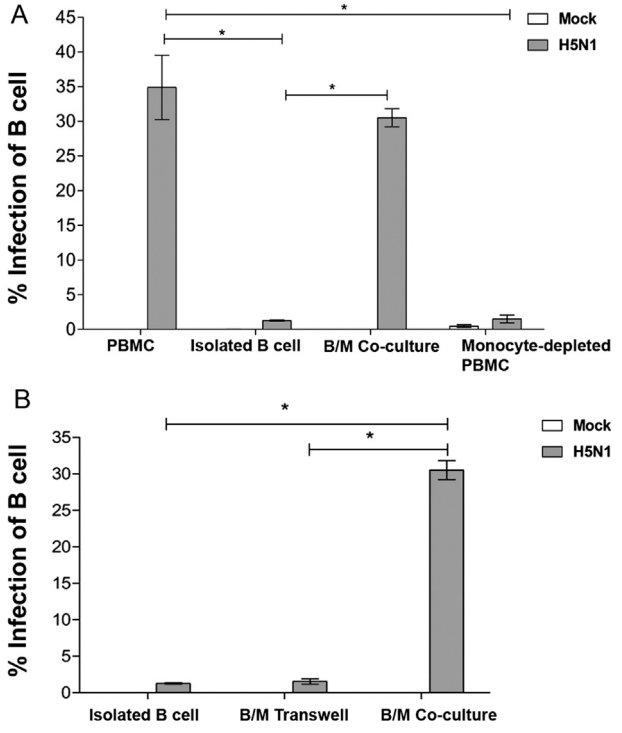

Fig. 2. Interaction of B cells and monocytes is required for enhancing B cells susceptibility. (A) Whole PBMCs, isolated B cells, B cell-monocyte co-culture (B/M co-culture), and monocyte-depleted PBMCs. (B) Isolated B cell, B cell-monocyte co-culture in a transwell system (B/M transwell), and B/M co-culture. Both (A) and (B) were infected with H5N1 at an MOI of 1 for 12 h. After that, cells were harvested for staining of CD20 and viral NP, and analyzed by flow cytometry. Percentage of infected B cells is shown. White and grey bars represent mock and H5N1 infection. The results were obtained from three different donors and are presented as means plus standard errors.

4. Discussion

The H5N1 virus has unusually high pathogenicity, which causes fatal disease in humans. Major complications from H5N1 viral infection are systemic infection, viremia, and hypercytokinemia. One typical laboratory finding in human cases of H5N1 is marked lymphopenia. Comparison of the lymphocyte count of fatal and nonfatal cases of H5N1-infected patients has shown significantly lower lymphocyte counts in fatal cases [8]. Decreased lymphocyte count is correlated with impaired viral clearance as well as a longer course of disease. We first examined the susceptibility of whole PBMCs to H5N1 and screened the majority of targeted cell populations. We found that monocytes and B cells, but not T cells in whole PBMCs were highly susceptible to H5N1 infection. There has been a study on the susceptibility of PBMCs to human influenza virus; human H3N2 virus was shown to infect primary PBMCs culture, and the majority of the infected cells were monocytes and macrophages subpopulations, while lymphocytes and purified CD3 positive cells were not infected [9]. This ability to infect B cells appears to be unique to the H5N1 virus, since very low susceptibility to human influenza viruses (H3N2, and H1N1 2009) was observed for B cells in whole PBMCs (data not shown). We also demonstrated that monocytes were required to enhance B cell susceptibility to H5N1 infection in the context of direct cell-to cell contact since separation of the cells in a transwell system did not raise the susceptibility of the B cells. Furthermore, upon complete depletion of monocytes from the PBMC culture, B cells were unable to be infected by H5N1 virus, emphasizing the role of monocytes in promoting B cell susceptibility to infection. It is possible that the interaction of monocytes with B cells could induce the expression of some proteins or receptors needed for H5N1 infection.

Sialic acid linked to glycoproteins is used by influenza viruses as

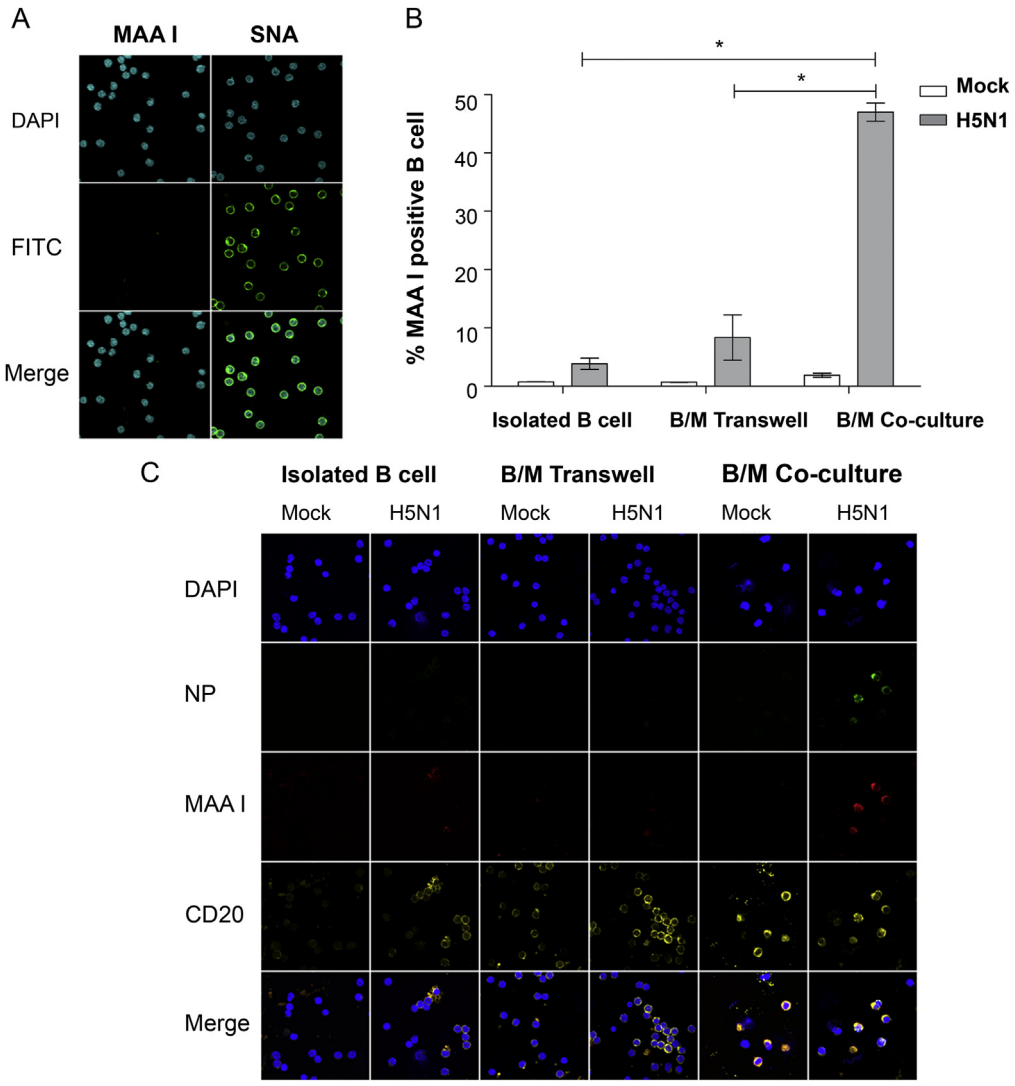

Fig. 3. Up-regulation of 2,3 sialic acid expression on B cells in B/M co-cultures upon H5N1 infection. (A) Isolated B cells were incubated with FITC-conjugated MAA I and SNA which are specific for α2,3 SA and α2,6 SA, respectively. The samples were examined on a confocal microscopy and photographed at 200× magnification. (B) Isolated B cells, B/M transwell cultures, and B/M co-cultures were infected with H5N1 at an MOI of 1 for 12 h. Cells were then collected for triple-staining of CD20, MAAI, and viral NP, and then subjected to flow cytometry. The results were obtained from three different donors and are presented as means plus standard errors. (C) Isolated B cells, B/M transwell cultures, and B/M co-cultures were infected with H5N1 at an MOI of 1. At 12 h post-infection, cells were fixed and permeabilized for staining of CD20 (yellow) and MAA I (red), then intracellularly stained of viral NP (green). Nuclei were counter-stained with DAPI (blue). Samples were analyzed by confocal microscopy and photographed at a 120× magnification. Data are representative of three separate donors.

a receptor for cell entry. Differences in receptor-binding specificity of influenza viruses can contribute to viral host range restriction. Human influenza viruses preferentially bind to α2,6 SA, while avian influenza viruses prefer to bind to α2,3 SA [10,11]. It has been reported that in ST6Gall-overexpressing Madin–Darby canine kidney (MDCK) cells, which were forced to express high levels of α2,6 SA, are more susceptible to human influenza than normal MDCK cells, which equally have both α2,6 SA and α2,3 SA on surfaces [12,13], suggesting that an increase in the sialic acid on the target cell surface can enhance the degree of viral infection. In general, B cells were capable of expressing high levels of α2,6 SA but very low levels of the 2,3 SA. Surprisingly, we found that, in the presence of monocytes, the expression of α2,3 SA on B cells was enhanced upon H5N1 infection. The increase in α2,3 SA expression on B cells could possibly provide more receptors for H5N1 to bind to on target cell surfaces. We also observed the co-localization of α2,3 SA and viral NP antigen in B cells of H5N1-infected B/M co-cultures, which confirmed that α2,3 SA on B cells was up-regulated during infected by H5N1. Since α2,3 SA staining was done following H5N1 infection, it is difficult to conclude whether increased α2,3 SA levels is the cause or the effect of enhanced B cell susceptibility. It is noted that these results were obtained from *in vitro* studies; the outcomes of infection *in vivo* may be similar to or different from our studies. Future *in vivo* investigations on H5N1 infection are needed to confirm these findings. In addition, how monocytes are affected by receptor expression on B cells should be explored.

The role of DC-SIGN in viral dissemination has been reported in H5N1 infection in DCs [14]. Studies done in sialic acid-deficient cells have shown that DC-SIGN mediated H5N1 infection is independent of the SA receptor [15]. B cells in the inactive state were capable of expressing very low levels of DC-SIGN which increased upon B cell activation [10]. The association of DC-SIGN expression on B cells with B cell susceptibility upon H5N1 infection is being investigated.

Influenza A viruses including H5N1 was shown to target and destroy lymphocytes both *in vitro* and *in vivo*, leading to lymphopenia and impaired immune system [16,17]. Our observations also supported the H5N1-associated lymphopenia. A substantial loss of B cells upon infection may contribute to poor adaptive immunity owing to incapable of producing antibodies, thus ultimately allowing delayed viral clearance and secondary infection.

In conclusion, we have demonstrated, for the first time, that the susceptibility of B cells to H5N1 virus was enhanced by the presence of monocytes, in which increased receptor expression is a possible explanation of this phenomenon. This study provides new insights into H5N1 pathogenesis by demonstrating how the virus causes lymphopenia. Targeting B cells might be the strategy that the virus uses to evade the immune system, ultimately leading to viral dissemination into non-respiratory organs. We are currently examining the mechanisms underlying the increased α2,3 SA receptor expression. The better understanding of this mechanism may lead us to new strategies for treatment of H5N1 infections.

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Transparency document

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