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Evaluating the abundance of cross-reactive influenza hemagglutinin and neuraminidase-targeted memory B cells

By

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Background

Influenza is a contagious respiratory disease in humans that poses one of the major challenges to public health globally. The influenza viruses are categorized into four types, type A (genus *influenzavirus A*), type B (genus *influenzavirus B*), type C (genus *influenzavirus C*), and type D (genus *influenzavirus D*). Notably, types A and B are responsible for the seasonal epidemics of influenza disease around the world, commonly known as "flu". These epidemics occur annually and affect populations worldwide, leading to high morbidity and mortality associated with respiratory illnesses[1]. According to the World Health Organisation (WHO), seasonal influenza viruses — including the H1N1 and H3N2 influenza A viruses, as well as influenza B viruses — account for approximately 3–5 million severe cases and 290,000 – 650,000 deaths each year worldwide[2]. Additionally, avian-originated influenza A viruses, such as H5N1 and H7N9 subtypes, have been implicated in significant numbers of zoonotic infections. These viruses can reassort their genes and occasionally cross the species barrier between animals and humans, resulting in an antigenic shift that may precipitate pandemic outbreaks[3].

The clinical manifestations associated with influenza viral infection can range from mild respiratory symptoms that are confined to the upper respiratory tract — such as fever, sore throat, cough, nasal discharge, headache, muscle pain, and malaise — to severe conditions, including life-threatening pneumonia owing to the direct influenza viral infection or secondary bacterial infection of the lower respiratory tract[4,5]. Furthermore, the virus can also affect cardiovascular function, the central nervous system, and other organ systems[6].

All influenza viruses are enveloped viruses containing a negative-sense, segmented, single-stranded RNA genome. Specifically, influenza A and influenza B viruses possess eight RNA

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[1] Centers for Disease Control and Prevention. (2021). Types of Influenza Viruses. *Centers for Disease Control and Prevention*. https://www.cdc.gov/flu/about/viruses/types.htm

[2] World Health Organization. (2023). Influenza (Seasonal). Who.int; *World Health Organization*: WHO. https://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal)

[3] Krammer, F. (2019). The human antibody response to influenza A virus infection and vaccination. Nature Reviews Immunology, 19(6), 383–397. https://doi.org/10.1038/s41577-019-0143-6

[4] Centers for Disease Control and Prevention. (2022). Clinical Signs and Symptoms of Influenza. *Centers for Disease Control and Prevention*. https://www.cdc.gov/flu/professionals/acip/clinical.htm

[5] Javanian, M., Barary, M., Ghebrehewet, S., Koppolu, V., Vasigala, V., & Ebrahimpour, S. (2021). A brief review of influenza virus infection. *Journal of Medical Virology, 93*(8), 4638–4646. https://doi.org/10.1002/jmv.26990

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segments, which encode essential viral components including RNA polymerase subunits, viral glycoproteins (haemagglutinin [HA] and neuraminidase [NA]), viral nucleoprotein (NP), matrix protein (M1), membrane protein (M2), the nonstructural protein NS1 and nuclear export protein (NEP) (Fig. 1)[7]. Among these, the viral glycoproteins are the most important surface protein as they are the main antigenic targets. HA has a globular 'head' and a 'stalk' domains. It facilitates viral entry into host cells, while NA is a sialidase that plays a key role in the virus release from the infected host cells[8].

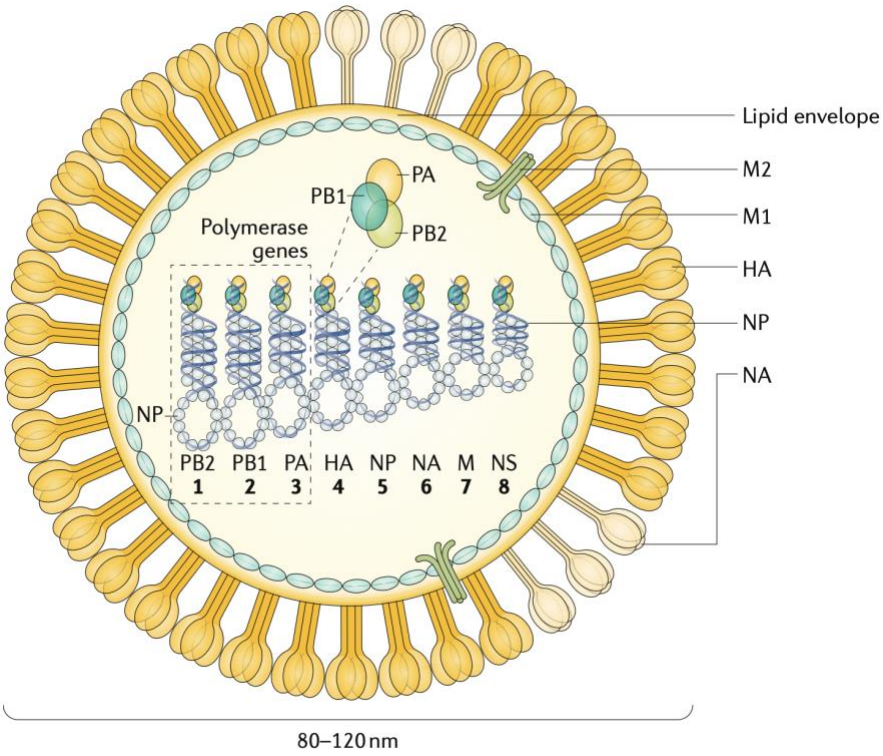

Figure 1. Influenza A and B virion structure [9].

Taking Influenza A virus as an example, this class of viruses can be further classified into different subtypes based on the 2 major surface glycoproteins. Currently, there are 19 known HA antigen subtypes (H1-H19) and 11 NA antigen subtypes (N1-N11), with three HA and two NA subtypes coming from bat influenza A-like viruses[10]. The various combinations of HA and NA subtypes can form diverse strains, shaping strain-specific antibody production in the host immune system due to the exposure of unique epitopes of different strains. In contrast,

[7] Bouvier, N. M., & Palese, P. (2008). The biology of influenza viruses. *Vaccine*, *26 Suppl 4*(Suppl 4), D49-53. https://doi.org/10.1016/j.vaccine.2008.07.039
[8] Benton, D. J., Wharton, S. A., Martin, S. R., & McCauley, J. W. (2017). Role of Neuraminidase in Influenza A(H7N9) Virus Receptor Binding. *Journal of Virology*, *91*(11). https://doi.org/10.1128/JVI.02293-16
[9] See 6
[10] See 1

influenza C and D viruses have only seven RNA segments and are less likely to cause substantial disease in humans. However, influenza C viral infections can still lead to influenza-like illness and hospitalizations in some cases, particularly among children [11].

Upon influenza viral infection in humans, the host immune system elicits a multi-tiered defense, beginning with the innate immune response, which provides the first line of defense. This initial defense will further trigger downstream inflammatory responses as well as activate respiratory mucosal immunity in the mucosal tissue of the airway[12]. While these early responses are crucial for immediate protection, adaptive immunity — comprising T cells, B cells and antibodies — plays the most critical role in viral clearance, especially during the later stages of infection[13].

Among the adaptive immune cells, $CD4^+$ and $CD8^+$ T cells are instrumental in eradicating virus-infected cells. $CD8^+$ cytotoxic T cells kill the infected cells directly while $CD4^+$ helper T cells aid in infected cell elimination and contribute to the B cell activation and subsequent antibody production in order to neutralize viral particles[14]. The adaptive immunity also generates the antigen-specific immunological memory that protects against future reinfection by the same strain.

In the first exposure to the influenza viruses, naive B cells are activated by recognizing the viral antigens and interacting with cognate CD4+ T cells (Fig. 2). This activation results in two B cell differentiating fates. The majority of the activated B cells rapidly differentiate into short-lived plasmablasts, which can provide an immediate source of virus-specific antibodies. Initially, IgM is first produced, followed by class switching to antibodies with higher affinity such as IgG or IgA. The primary role of the short-lived plasmablasts is to raise the serum antibody titers to protective levels quickly after the infection, therefore preventing severe damage by the viruses.

Meanwhile, the remaining portion of the activated B cells migrate to the B cell follicles within secondary lymphoid tissues, where they undergo germinal center (GC) reactions. During the

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[11] See 6

[12] Iwasaki, A., & Pillai, P. S. (2014). Innate immunity to influenza virus infection. *Nature Reviews Immunology*, *14*(5), 315–328. https://doi.org/10.1038/nri3665

[13] See 6

[14] Chen, X., Liu, S., Goraya, M. U., Maarouf, M., Huang, S., & Chen, J.-L. (2018). Host Immune Response to Influenza A Virus Infection. *Frontiers in Immunology*, *9*(320). https://doi.org/10.3389/fimmu.2018.00320

GC reactions, the T-follicular helper cell activated B cells undergo somatic hypermutation and clonal selection to produce affinity matured antibodies, with enhanced specificity and affinity. In humans, only a small fraction of the B cells will become long-lived plasma cells, which ultimately reside in the bone marrow and provide a continuous source of antibodies. These long-lived plasma cells maintain long-term serum antibody levels to provide long-term protection against recurrent influenza infections. Another fraction of the activated B cells from the germinal center become memory B cells (MBCs). These MBCs do not secrete antibodies, but they remain in the peripheral blood for immune surveillance. Once they encounter the same antigens, they can rapidly be re-activated and differentiate into plasmablasts that produce antibodies with high affinity as well as more MBCs to prepare the body for recurrent infection with the same viral strain[15,16].

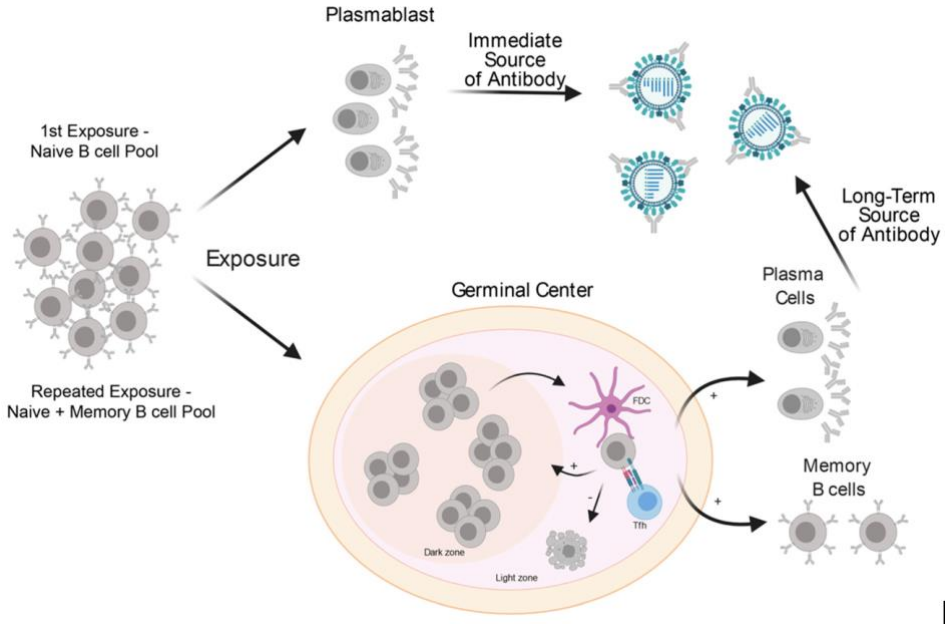

Figure 2. B cells activation pathway after influenza viral exposure[17].

The antibodies produced from plasma cells can interfere with different stages of the viral life cycle (Fig. 3). HA antibodies can bind to the HA head domain to block viral attachment to the host cells. Additionally, HA stalk-targeted antibodies can block the fusion of viral and endosomal membranes, locking HA in a pre-fusion conformation and halting viral entry. Anti-

[15] Guthmiller, J. J., Utset, H. A., & Wilson, P. C. (2021). B Cell Responses against Influenza Viruses: Short-Lived Humoral Immunity against a Life-Long Threat. *Viruses*, *13*(6), 965. https://doi.org/10.3390/v13060965

[16] Inoue, T., & Kurosaki, T. (2023). Memory B cells. *Nature Reviews Immunology*, 1–13. https://doi.org/10.1038/s41577-023-00897-3

[17] See 14

NA antibodies can inhibit the NA activity to prevent viral exit from the host cells to limit further infection[18]. MBCs produce can be activated to produce antibodies with a broader reactivity compared to the steady-state serum antibodies. This makes MBCs capable of responding to antigenically drifted or shifted viral strains. However, this process is not fast enough to prevent initial illness during infection. Therefore, establishing a steady-state serum antibody level with a broad repertoire and cross-reactivity is a key strategy of the universal influenza vaccine development[19].

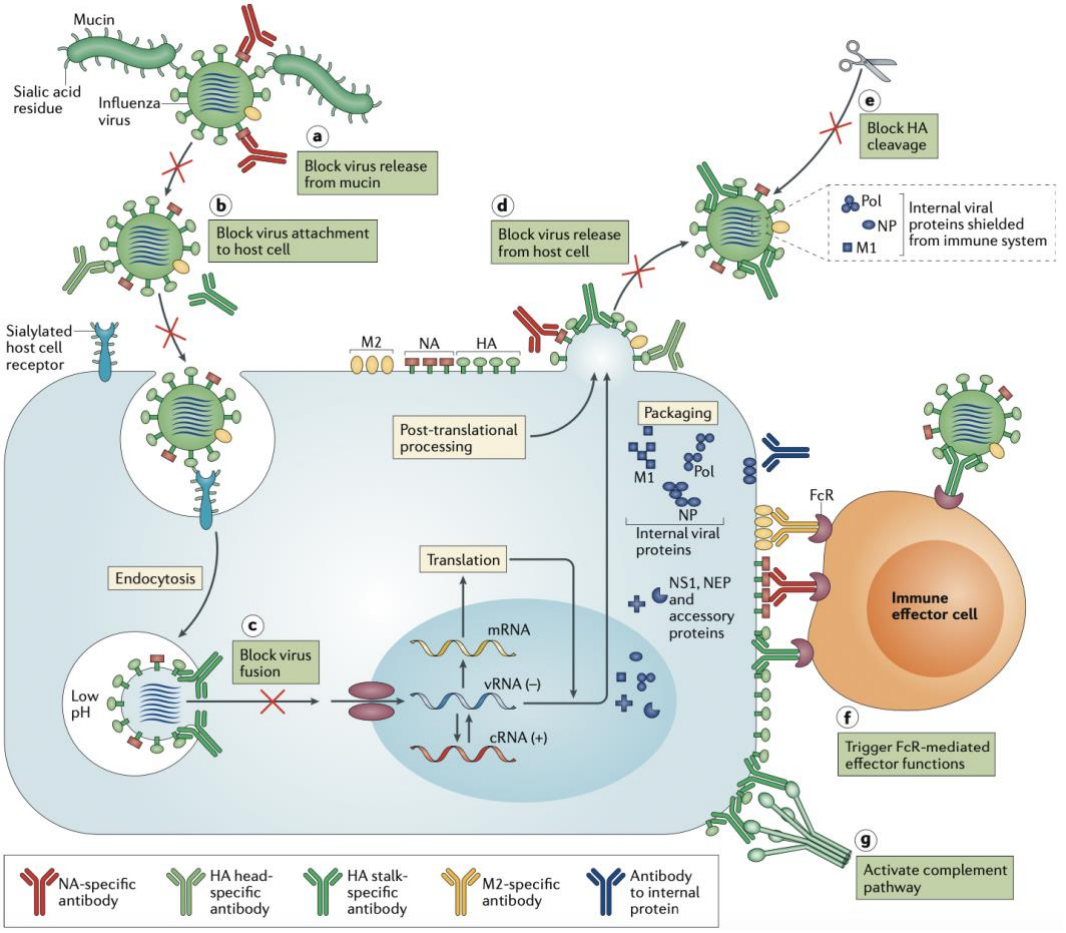

Figure 3. The mechanism of antibodies against influenza virus[20].

In vaccine development, the concepts of immunodominance and cross-reactivity are fundamental for understanding immune responses. Immunodominance refers to the prevalence of the immune system for a specific epitope over others, resulting in an immune pressure that

[18] See 4
[19] See 14
[20] See 10

drives the positive selection of strain-specific antibodies against these epitopes[21,22]. A classic example of immunodominance was demonstrated in 1987, when Johansson and his team found that, there was an immunodominance of HA over NA after influenza virus vaccination in the mouse model, resulting in low-level immune responses against NA. This skewing of antibody responses toward immunodominant epitopes, such as HA, poses a major challenge in influenza vaccine development that exhibits frequent antigenic variation[23]. Therefore, the identification of immunodominance in antibody responses against different epitopes or different viral targets in the host is critical for effective vaccine development[24]. On the other hand, cross-reactive antibodies refers to the ability of an antibody to recognize and bind to two or more similar molecules that share one or more identical epitopes[25]. For example, anti-HA antibodies are often cross-reactive within and across HA subtypes due to the conserved nature of the HA stalk[26]. In addition, research has also revealed that anti-NA antibodies conferred greater cross-reactivity than anti-HA antibodies, suggesting the inclusion of NA glycoproteins in seasonal influenza vaccines could enhance the protection effectiveness[27,28,29].

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[21] Sriharshita Musunuri, Payton, & Kim, P. S. (2024). Bringing immunofocusing into focus. *Npj Vaccines*, *9*(1). https://doi.org/10.1038/s41541-023-00792-x

[22] Lindahl, G. (2021). Subdominance in Antibody Responses: Implications for Vaccine Development. *Microbiology and Molecular Biology Reviews*, *85*(1). https://doi.org/10.1128/mmbr.00078-20

[23] Angeletti, D., Kosik, I., Santos, J. J. S., Yewdell, W. T., Boudreau, C. M., Mallajosyula, V. V. A., Mankowski, M. C., Chambers, M., Prabhakaran, M., Hickman, H. D., McDermott, A. B., Alter, G., Chaudhuri, J., & Yewdell, J. W. (2019). Outflanking immunodominance to target subdominant broadly neutralizing epitopes. *Proceedings of the National Academy of Sciences*, *116*(27), 13474–13479. https://doi.org/10.1073/pnas.1816300116

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[25] Actor, J. K. (2012). Humoral Immunity. *Elsevier EBooks*, 17–24. https://doi.org/10.1016/b978-0-323-07447-6.00003-x

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However, despite the growing interest in NA-targeting vaccine development, there is a significant gap in the understanding of the population immunity to NA, the antigenic epitopes, and the mechanisms of protection[30,31,32]. Moreover, the relationship between plasma antibodies and the profile of the circulating MBCs remains unclear.

Data from others and ourselves have shown that age is an important factor in an individuals' influenza immune history[33]. Studies on antibody landscapes showed that children have a narrow breadth of influenza antibody reactivity compared to adults, while older adults have complex immune history due to repeated exposures to multiple strains, including pandemic strains[34,35]. Meanwhile, studies have shown that individuals who were exposed to specific influenza strains in their early life may have a more robust immune response to the same strains later in their life[36]. Besides, our lab have previously shown that older adults have high levels of antibodies that cross-react to avian NA due to exposures to avian-origin viruses during the 1957 and 1968 influenza pandemics[37]. However, whether this is reflected in the memory B cell compartment is unknown.

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[35] Edler, P., Lara, Aban, M., Wille, M., Spirason, N., Deng, Y.-M., Carlock, M. A., Ross, T. M., Juno, J. A., Rockman, S., Wheatley, A. K., Kent, S. J., Barr, I. G., Price, D. J., & Marios Koutsakos. (2024). Immune imprinting in early life shapes cross-reactivity to influenza B virus haemagglutinin. *Nature Microbiology*, *9*(8), 2073–2083. https://doi.org/10.1038/s41564-024-01732-8

[36] King, S. M., Bryan, S. P., Hilchey, S. P., Wang, J., & Zand, M. S. (2023). First Impressions Matter: Immune Imprinting and Antibody Cross-Reactivity in Influenza and SARS-CoV-2. *Pathogens*, *12*(2), 169. https://doi.org/10.3390/pathogens12020169

[37] Liang, Z., Lin, X., Sun, L., Edwards, K. M., Song, W., Sun, H., Xie, Y., Lin, F., Ling, S., Liang, T., Xiao, B., Wang, J., Li, M., Leung, C.-Y., Zhu, H., Bhandari, N., Varadarajan, R., Levine, M. Z., Peiris, M., & Webster, R. (2024). A(H2N2) and A(H3N2) influenza pandemics elicited durable cross-reactive and protective antibodies against avian N2 neuraminidases. *Nature Communications*, *15*(1). https://doi.org/10.1038/s41467-024-49884-9

Purpose

The aim of my experiment is to determine the abundance and specificity of the anti-HA and NA antibodies in plasma compared to those produced from *in vitro* stimulated MBCs. Additionally, the relationship between the plasma antibody and the antibody produced by the MBCs , reflecting the size of memory B cell population, was explored as well to provide insight into the immune response process.

This project was performed in collaboration with Dr. Lin Xia, a postdoc in the laboratory. The stimulation and ELISPOT was performed by Dr. Lin, while I performed the ELISA using the plasma and MASCs. I performed all the analysis in this report.

Methodology and procedures

Workflow. The biobanked human peripheral blood mononuclear cell (PBMC) samples were retrieved and used in an in vitro stimulation assay according to a published protocol (Fig. 4)[38]. Briefly, freshly thawed cryopreserved PBMCs were rested overnight at 37 °C and 5% CO_2 in 50-ml tubes. After that, 1ml of freshly prepared stimulation cocktail consisting of the TLR7/8 agonist R848, IL-2, and IL-10 (with 2-mercaptoethanol) in R10 medium was added to PBMCs at 1 x 10^6 cells/ml each well on a 24-well plate to induce activation, proliferation, and differentiation of MBCs into memory-derived antibody-secreting cells (MASCs) on day 0. Stimulation was performed for 6 days at 37 °C and 5% CO_2. MASCs were then pooled, and washed for the ELISpot assay. The supernatant was collected as MBC-derived polyclonal antibodies (MPAbs), which were secreted by the MASCs, on day 10. The titers of the MPAbs in the supernatant and the antibodies in the plasma samples of the same individual were then analyzed and compared by ELISA.

[38] Nguyen-Contant, P., A Karim Embong, Topham, D. J., & Sangster, M. Y. (2020). Analysis of Antigen-Specific Human Memory B Cell Populations Based on In Vitro Polyclonal Stimulation. *Current Protocols in Immunology*, *131*(1). https://doi.org/10.1002/cpim.109

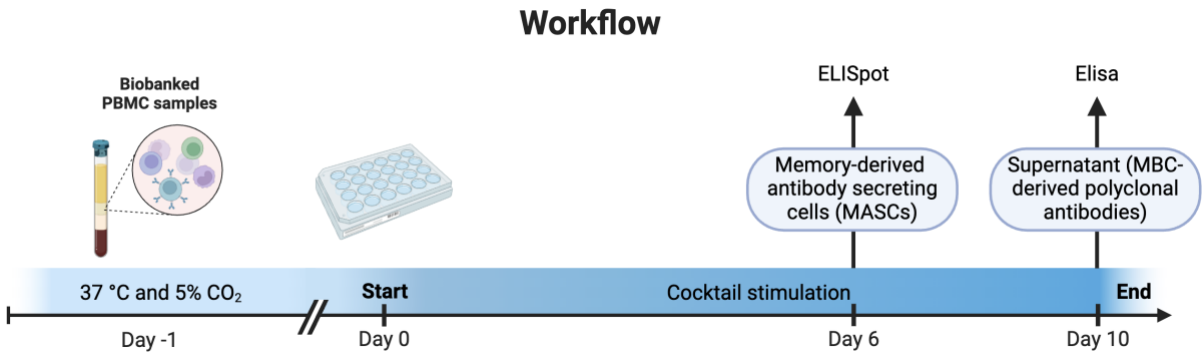


Figure 4. Generation of MASCs and MPAbs workflow.

Sample. Human biobanked PBMC samples in different age groups were collected between 2023-2024 from the Hong Kong Red Cross donors. Four individual samples were selected for this experiment, as detailed in Table 1.

Sample ID	Year of Birth	Age	Sex
1797	1981	43	F
9016	1964	60	F
26	1961	63	M
9708	1959	65	F

Table 1. Sample information.

Strain selection. A total of 11 influenza A strains, comprising 6 hemagglutinin (HA) subtypes and 5 neuraminidase (NA) subtypes, were selected as representatives based on prototypical strains of geographical and temporal importance. The specific strains tested are summarized in Table 2.

Glycoprotein	Subtype	Strain name	Host
HA	H1N1	A/Michigan/45/2015 (MI15)	Human
	H3N2	A/Aichi/2/1968 (AI68)	Human
	H3N2	A/Hong Kong/45/2019 (HK19)	Human
	H9N2	A/Hong Kong/1073/99 (HK99)	Human
	H7N9	A/Anhui/1/2013 (AH13)	Human
	H2N2	A/Japan/305/1957 (JP57)	Human
NA	H1N1	A/Michigan/45/2015 (MI15)	Human

	H3N2	A/Aichi/2/1968 (AI68)	Human
	H9N2	A/Chicken/Hong Kong/G9/97 (HK97)	Chicken
	H7N7	A/Netherlands/219/2003 (NL03)	Human
	H7N9	A/Anhui/1/2013 (AH13)	Human

Table 2. Strain information.

ELISpot assay. Recombinant human-origin and avian-origin HA and NA glycoproteins of different subtypes listed in Table 2 (available from Sinobiological Ltd) were coated onto a MultiScreen$_{HTS}$ 96-well filter plate with a polyvinylidene fluoride (PVDF) membrane filter at 1 µg/well overnight at 4 °C. Control wells were coated with either H1, H3 (as antigen-positive controls), and media only. Positive control wells were coated with goat anti-human IgG antibody. Negative wells were coated with PBS only. Plates were blocked for two hours at room temperature with PBS supplemented with 5% fetal bovine serum (Lonsera). After removing the blocking solution, MASC cell suspension on day 6 was added to ELISpot plates and incubated overnight at 37°C with 5% CO_2. The cell suspension was then removed and plates were washed six times with phosphate-buffered saline (PBS) supplemented with 0.05% Tween-20 (PBS-T). 1 µg/ml Alkaline phosphatase AffiniPure goat anti-human IgG was added to each well and the plates were incubated at room temperature for 2 hours. The plates were washed six times with PBS-T, followed by the removal of the plastic base of the plates and submergence of the plates in a tub of PBS-T for 1 hour. After removing the PBS-T and replacing the plastic bases, 100 µl of prepared AP substrate (Vector® Blue AP Substrate Kit) was added to each well and incubated at room temperature until the development of blue spots was optimal. The number of blue spots indicated the size of strain-specific MASC populations. When the development was done, the plastic bases were removed again and both sides of the plates were washed under cold tap water. The plates were then air-dried for 2 hours. After drying, the plates were scanned and the spots were counted using a CTL immunospot reader.

ELISA. Recombinant human-origin and avian-origin HA and NA glycoproteins of different subtypes listed in Table 2 were coated onto a 96-well plate at 100µl/well overnight at 4 °C. Negative wells were coated with coating buffer only. Plates were washed three times with PBS-T and blocked for two hours at room temperature with PBS supplemented with 5% fetal bovine serum (Lonsera). After removing the blocking solution, 50 µl of 2-folded serial diluted sera was added at a starting dilution of 1:200 for plasma antibodies and 1:8 for MPAbs in duplicate

wells and incubated for two hours at 37 °C. Sera were then removed and plates were washed three times with PBS-T. 50 µl of HRP-conjugated detection antibody in 5% FBS-PBS-T was introduced to each well at 0.05 µg/ml and the plates were incubated for 1 hour at 37 °C. The secondary antibody was then removed and the plates were washed three times with PBS-T, and 50 µl/well of TMB substrate was added. After 10 min, 50 µl/well of 0.5 M H_2SO_4 was added to stop the reaction and the absorbance was read at 450 nm using an ELISA microplate reader. The end-point titer was calculated as the reciprocal dilution that gave a positive/ negative optical density readout ratio of >2.

Statistical analysis. Statistical analyses were performed using GraphPad Prism (version 10), while correlations were performed by using the built-in analysis algorithm in Prism. For antibody titers, samples that did not meet the assay detection threshold were assigned a value half of the starting dilutions, i.e., human sera with no detectable signal in ELISA were assigned a titer of 100 for plasma samples and 4 for MPAb samples. Antibody titers were log-transformed for statistical testing. For the correlation of plasma antibody titers and MPAbs titers, nonparametric Spearman correlation analysis between the two data sets was performed using the rankings of the titers, i.e. the highest titer was ranked as 1 while the lowest titer was ranked as 11 (if any). For the correlation of MASC populations and plasma Ab titers as well as the MASC populations and MPAbs titers, the Pearson correlation analysis between the two data sets was performed. Statistical significance between two data sets was determined by a two-tailed test, $p < 0.05$ was considered significant.

Ethics statement. This study using samples from the Hong Kong Red Cross received ethical approval from the Institutional Review Board of the University of Hong Kong (Ref: UW 23-268).

Principal results

High H3N2 specificity in derived MASCs (done by Dr. Lin)

ELISpot analysis was employed to assess the magnitude and specificity of derived MASCs against the selected strains (Fig. 5) and the corresponding MASC frequency was shown in Figure 6. Due to the strong background noises of the staining, the numbers of spots counted by the software were adjusted before the analysis. Individual no. 1797 exhibited specificity against H1(MI15), H1(AI68), H3(HK19), H7(AH13), N2(AI68), and N2(HK97). Similarly, MASCs from individual no. 9016 showed significant specificity against H3(AI68), H3(HK19),

H2(JP57), N2(AI68), and N2(HK97). For individual no. 26, there was prominent reactivity of MACSs against H1(MI15), H3(AI68), H3(HK19), H7(AH13), N2(AI68), and N2(HK97). Last but not least, MASCs from individual no. 9708 demonstrated a notable reactivity against H1(MI15), H3(AI68), H3(HK19), N2(AI68), and N2(HK97). Taken together, all MASC samples from the four individuals demonstrated strong cellular specificities against H3 from AI68 and HK19 strains as well as N2 from AI68 and HK97 strains. Therefore, it is suspected that the MBC population against the H3N2 was relatively dominant across the four individuals.

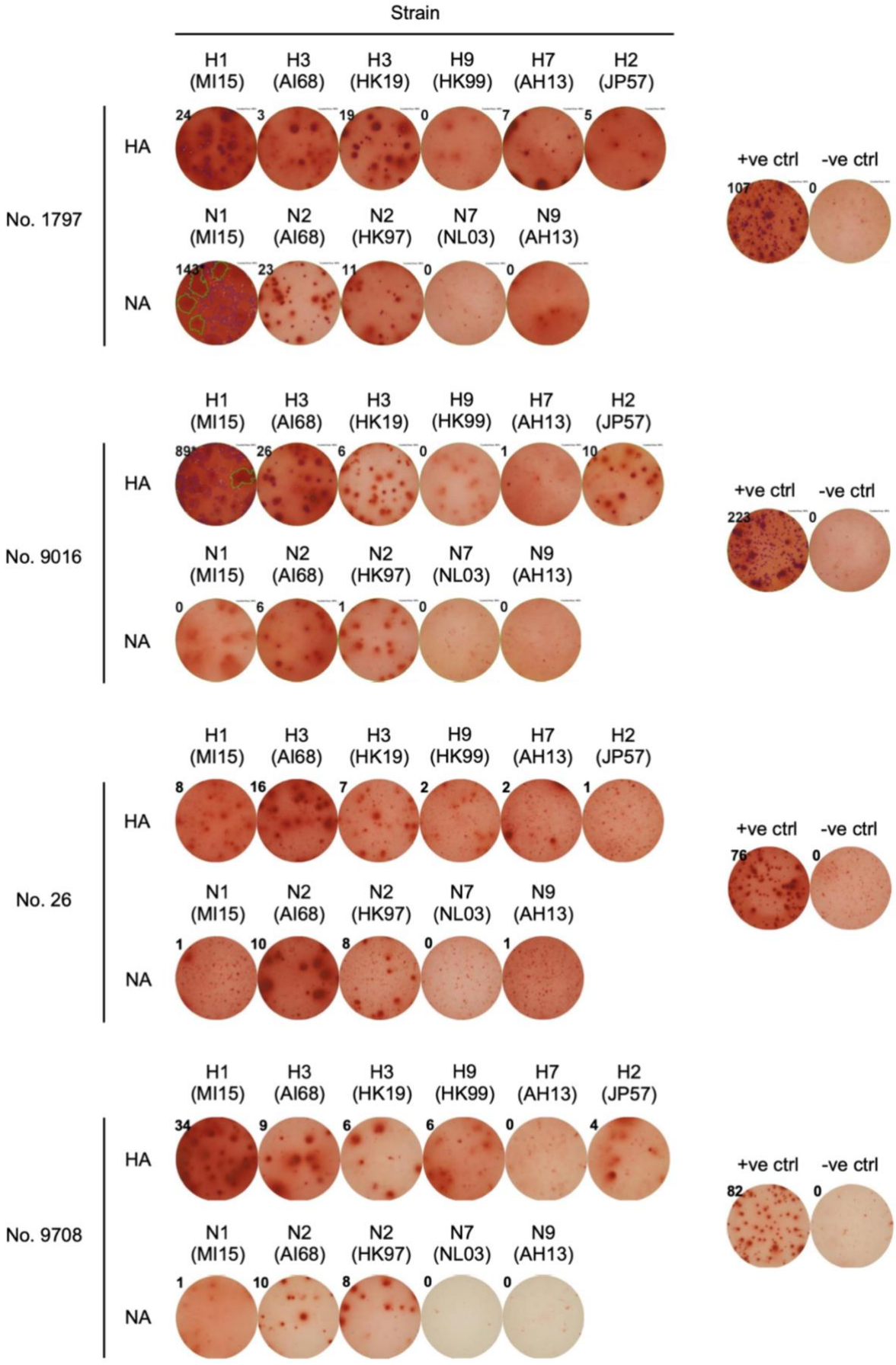


Figure 5. ELISpot results from MASCs against 11 strain antigens.

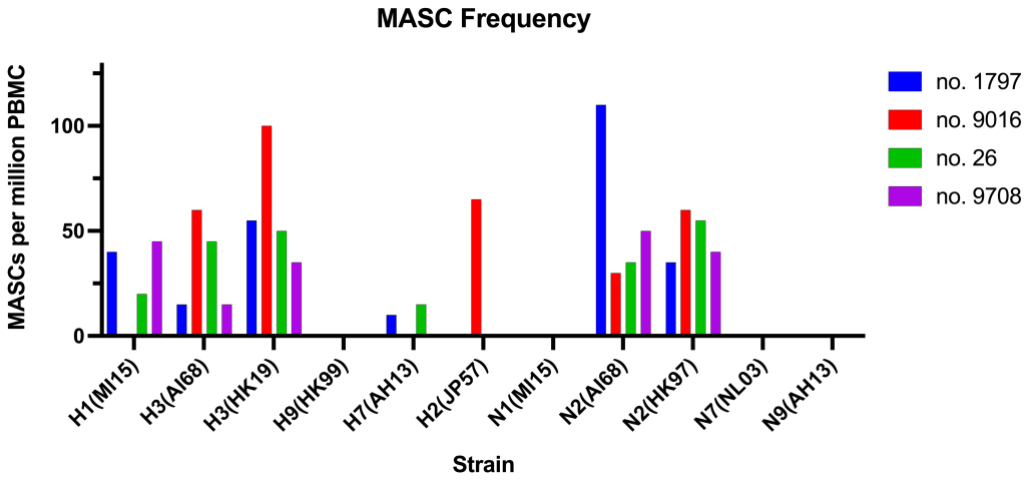


Figure 6. The frequency of MASCs derived from per million PBMC.

Positive correlation of the plasma antibody titers and MPAb titers

Next, the plasma antibody titers and MPAbs titers was assessed by ELISA (Fig. 7). This analysis aimed to provide insights into the secretion profile of the MASCs, which is also a measure to reflect the size of the specific MBC population, and ascertain if they relate to the antibody profile in the plasma samples. In general, the titers of the HA-targeted antibody were relatively higher than that of the NA-targeted antibody in both plasma and supernatant samples. In addition, the antibody specificity profiles against the different strains were similar between plasma and supernatant samples, with some exceptions noted in the titers of N2(AI68) and N2(HK97) antibodies in individual no. 1797 and no. 9708. In these individual samples, high plasma N2(AI68) and N2(HK97) Ab titers but undetectable N2(AI68) and N2(HK97) MPAb titers were observed.

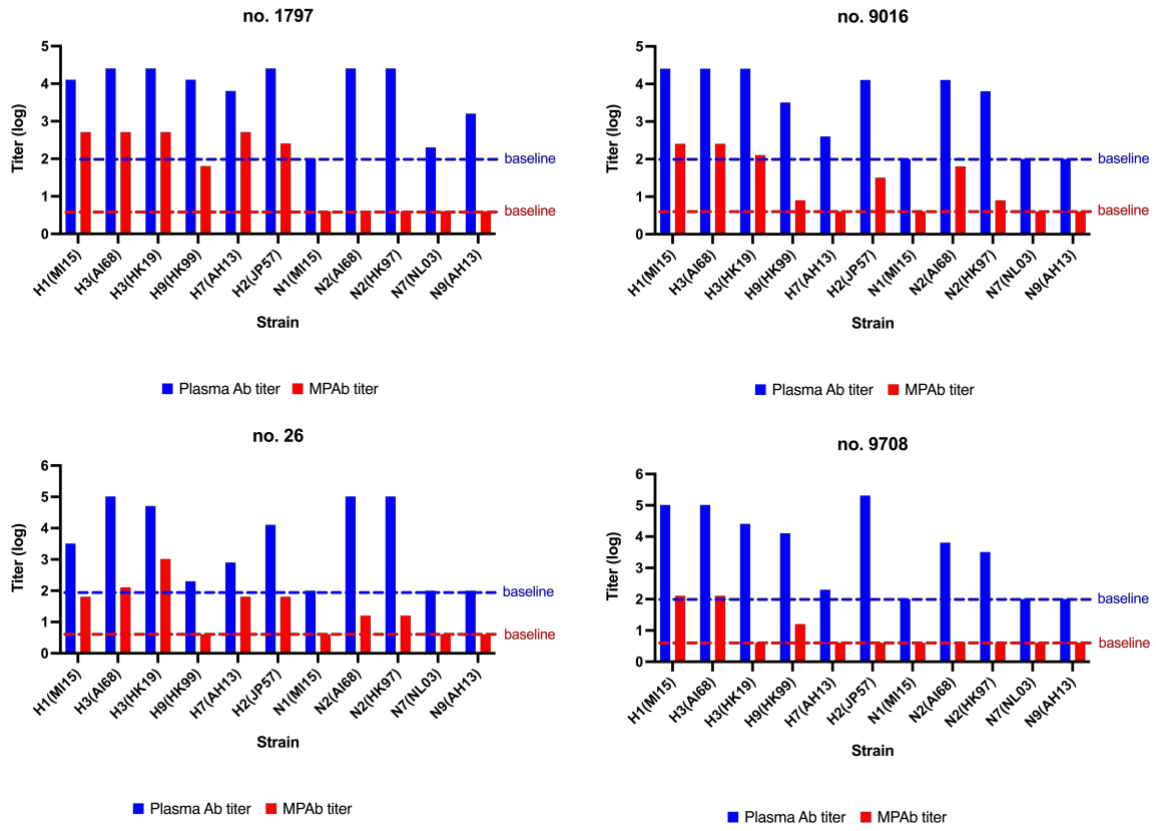


Figure 7. Antibody levels analysis from plasma and supernatant samples against 11 strains by ELISA. The baseline is the detectable antibody threshold.

Following the Spearman correlation analysis of the titer rankings of the plasma antibodies and MPAbs, a significant positive correlation was observed in no. 9016 samples with high statistical significance (r=0.9738, 95% CI=0.8951 - 0.9936, p<0.0001) (Fig. 8). Antibody abundance could be identified by recognizing the correlations in the lower left and upper right quadrants of the graph. The lower left quadrant represents high plasma Ab titers as well as high MPAb titers against the same strain, while the upper right quadrant represents low plasma Ab titers as well as low MPAb titers against the same strain. In no. 9016 samples, H1(MI15), H3(AI68), H3(HK19), and N2(AI68) abundance were the highest as their correlation points were located in the lower left quadrant. Most of the remaining correlation points were located in the upper right quadrant, indicating that a lower plasma Ab titer corresponded to a lower MPAb titer.

After pooling the rankings of all four individuals, antibody abundance of all individuals were identified as shown in Figure 9. Notable correlations were found between plasma Ab titers and MPAb titers for H3(AI68) strain across all subjects. Conversely, in the upper right quadrant,

strong correlations were observed between plasma Ab titers and MPAb titers for H9(HK99) N1(MI15), N7(NL03), and N9(AH13) strains among all individuals. The antibody titers against these four strains were both low in plasma and supernatant samples. In summary, the antibody profiles observed in both plasma and supernatant samples show a good correlation for human influenza strains, such as H3(AI68).

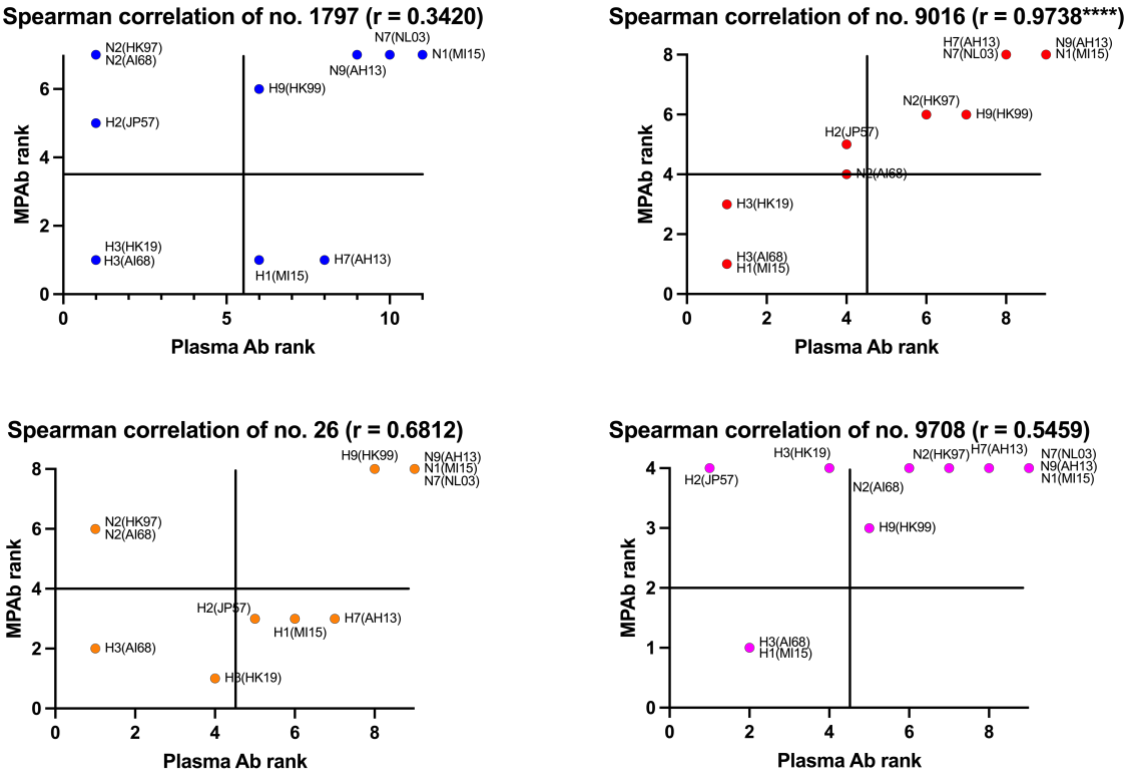


*Figure 8. Spearman correlation of the rankings of plasma Abs and MPAbs titers. The highest titer was ranked as 1 while lowest titer was ranked as 11. **** refers to p (two-tailed) <0.0001.*

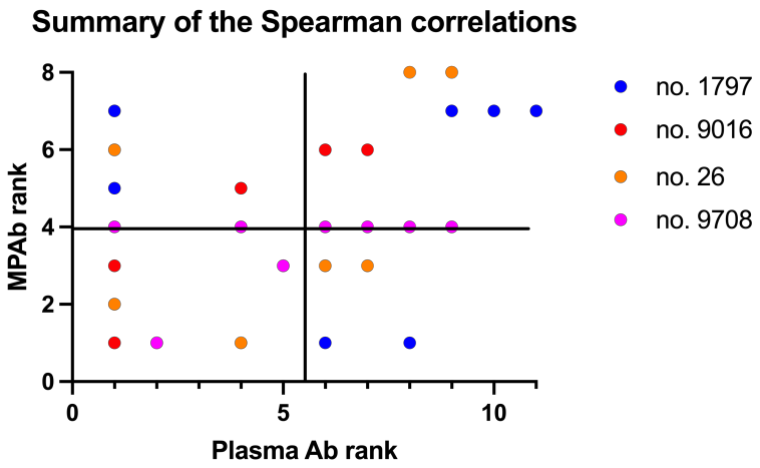


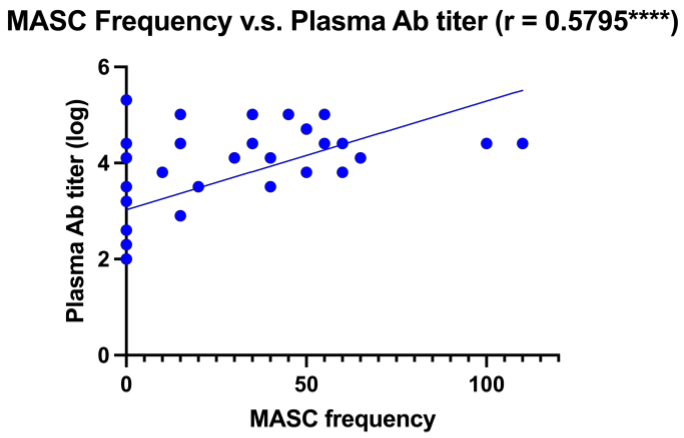
Figure 9. Summary of the Spearman correlations of all individuals.

The magnitude of MBC reactivity to HA and NA antigens closely parallels the pattern of plasma antibody secretion

Upon comparing the ELISpot and ELISA results, there was a consistency between plasma Ab titers and MPAb titers. For the no.1797 sample, the MASCs showed high specificities towards H3(AI68) and H3(HK19), along with elevated plasma Ab and MPAb levels. Also, low specificities against H9(HK99), N1(MI15), N7(NL03), and N9(AH13) in MASCs were related to the low antibody levels. Similarly, for the no.9016 sample, the MASCs showed high specificities against H3(AI68), H3(HK19), and N2(AI68), coupled with elevated antibody titers. Meanwhile, low specificities against H7(AH13), H9(HK99), N1(MI15), N7(NL03), and N9(AH13) in MASCs samples were also related to the low antibody levels. In the case of no.26 sample, the MASCs showed high specificities against H3(AI68) and H3(HK19) antigens and high antibody titers as well. At the same time, low specificities against H9(HK99), N1(MI15), N7(NL03), and N9(AH13) in MASCs were also related to the low antibody levels. Likewise, sample no.9708 exhibited high specificities against H1(MI15) and H3(AI68) antigens in MASCs, with elevated antibody titers. Conversely, low specificities against H7(AH13), H9(HK99), N1(MI15), N7(NL03), and N9(AH13) in MASCs were also related to the low antibody levels. Taken together, there was a high MASCs specificity and elevated antibody levels to H3(AI68) as well as a low MASCs specificity and low antibody levels to H9(HK99), N1(MI15), N7(NL03), and N9(AH13) among all individuals. It is interesting to note that, despite the high frequency of MASC targeting N2 antigens among all individuals, only individuals no. 9016 and no. 26 had detectable levels of anti-N2 MPAbs secreted from the MASCs. This observation suggests that a high MASC population against N2 antigens does not

necessarily correlate with elevated production of anti-N2 antibodies, indicating a reduced functionality or efficacy of the MASCs in producing anti-N2 antibodies.

The correlations between MASC frequency and both plasma Ab levels and MPAb levels were subsequently analyzed. After pooling data from all four individuals together, statistically significant positive correlations were observed. Notably, the correlation between MASC specificity and plasma Ab titers ($r=0.5795$, 95% CI=0.3380 - 0.7494, $p<0.0001$) was stronger compared to that between MASC specificity and MPAb titers ($r=0.3209$, 95% CI=0.02276 - 0.5666, $p=0.0359$) (Fig. 10). The stronger positive correlation between MASC specificity and plasma Ab levels further indicates the potential link between MBC specificity and the pattern of plasma antibody secretion in human body. However, the weaker correlation with MPAb levels may suggest that the functionality or efficacy of MBCs in producing certain antibodies could be limited or impaired.



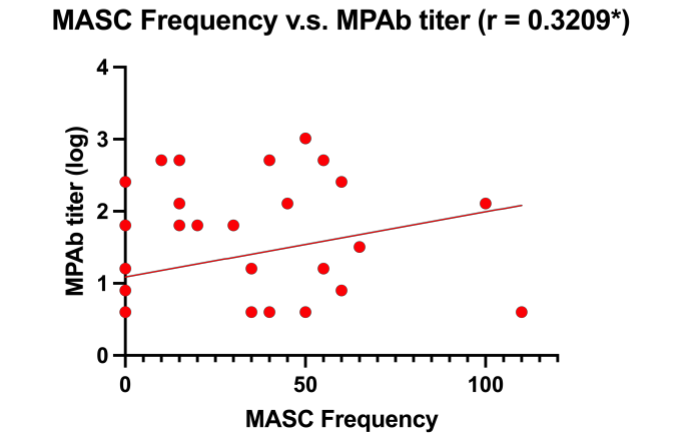


*Figure 10. Relationship between MASCs frequency, plasma Ab titers and MPAb titer. * refers to p (two-tailed) <0.05, **** refers to p (two-tailed) <0.0001.*

Discussion

The experimental findings suggest that the magnitude of MBC population reactivity to HA and NA antigens was closely parallel to the pattern of plasma antibody secretion, as evidenced by the similarity in the antibody profiles between the MACs specificity, plasma samples, and MPAb samples. The ELISpot assay revealed a high level of specificity against H3N2 antigens across all four individuals, suggesting that the MBC population targeting H3N2 was relatively dominant, indicating a potentially strong immune response directed towards the specific viral strains. In addition, a positive correlation between plasma antibody titers and MPAb titers was found, indicating a parallel antibody profile between the plasma samples and MPAb samples. All of the four individuals shared the specificity against H3(AI68). Furthermore, the correlation analysis revealed a statistically significant positive relationship between MASC specificity and both plasma Ab levels and MPAb titers. However, the correlation was stronger for plasma Ab

titers compared to MPAb titers, suggesting that while plasma antibody profile may be affected by the MBC specificity, the efficacy of MBCs in producing certain antibodies may be limited or impaired in some cases. This was also illustrated in our study that the anti-N2 antibodies were only detected in individuals no. 9016 and no. 26, despite a high MASC frequency targeting N2 antigens in all individuals. Cate et.al. also reported that elderly immunized with standard doses of influenza vaccine only exhibited modest levels of NA-specific antibodies. However, administration of a higher vaccine dosage could result in significantly increased antibody levels and elevated mean post-vaccination anti-NA titers[39]. Therefore, our findings highlight the importance of sufficient dosage in NA-targeted influenza vaccine development in order to enhance anti-NA antibody immunogenicity.

Limitation

This experiment only provides a preliminary idea of the potential association between MBCs and plasma antibody profiles. The generalizability is limited due to the small sample size. The comparisons of antibody profiles across different age and sex groups were not feasible also due to the small sample size. In addition, a technical problem was encountered with the A/Michigan/45/2015 strain, which has also been reported in previous research as well.

Conclusion

This study shows a positive correlation between MASC specificity and both plasma Ab levels and MPAb titers, suggesting a potential link between MBCs and plasma antibodies. In addition, the significance of anti-NA antibodies has also been pointed out, highlighting the need to focus on NA-targeted vaccine development in order to achieve optimal NA titers in the body.

Personal reflection

Understanding the full picture of immunology was challenging. To address the difficulty, I engaged in an extensive literature review and sought guidance from my professor and mentor Linxia, who helped me clarify my confusion and consolidate a better understanding of this topic. Reflecting on this experiment, I do acknowledge that initiating the research earlier and

[39] Cate, T. R., Rayford, Y., Niño, D., Winokur, P., Brady, R., Belshe, R., Chen, W., Atmar, R. L., & Couch, R. B. (2010). A high dosage influenza vaccine induced significantly more neuraminidase antibody than standard vaccine among elderly subjects. *Vaccine*, *28*(9), 2076–2079. https://doi.org/10.1016/j.vaccine.2009.12.041

incorporating a larger sample size in the experiment could lead to more robust and insightful results, thereby further enhancing the learning outcomes.

I would like to express my sincere gratitude to Prof. Sook-San Wong for her invaluable support and guidance throughout my project. Her insights and encouragement have been instrumental in shaping my research. I also extend my heartfelt thanks to Dr. Xia Lin for her guidance and contributions, which greatly enhanced the quality of my work. I would also like to thank Miss Zaolan Liang, Miss Cheryl Leung, and Miss Iris Li for their mentorship as well as Dr. Yaqin Sarah Bai, Mr. Check Long Chow, Miss Cheng Xiao, Mr. Lewis Siu, Miss Lisa Touyon and Miss Yihan Lin for their support in the lab. These people made a significant impact on my academic journey, and I am truly grateful for their assistance.

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