Virologica Sinica 37 (2022) 619–622

Contents lists available at ScienceDirect

Virologica Sinica

journal homepage: www.keaipublishing.com/en/journals/virologica-sinica
www.virosin.org

Letter

Development of functional antibodies against influenza B virus by activation-induced cytidine deaminase in hybridoma cells

Yuanzhi Chen [a,1], Chenguang Shen [a,b,*,1], Jing Chen [a], Junyu Chen [a], Fentian Chen [a], Limin Zhang [a], Xue Liu [a], Siyuan Chen [a], Sen Xue [a], Yongliang Liu [a], Jixian Tang [a], Quan Yuan [a], Yixin Chen [a,*], Wenxin Luo [a,*], Ningshao Xia [a]

[a] National Institute of Diagnostics and Vaccine Development in Infectious Diseases, State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, School of Public Health & School of Life Science, Xiamen University, Xiamen 361102, China
[b] Biosafety Level-3 Laboratory, School of Public Health, Southern Medical University, Guangzhou 510515, China

Dear Editor,

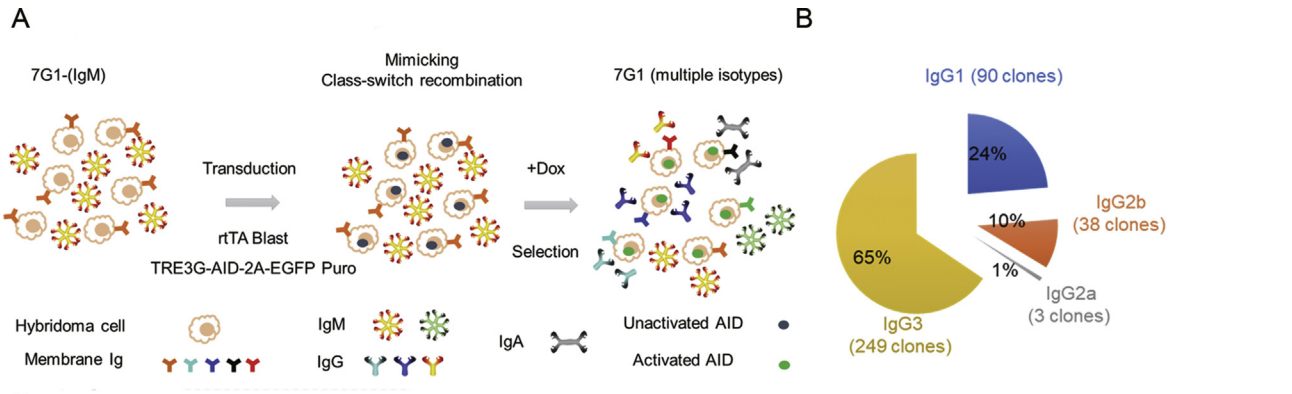
Influenza B viruses (IBVs) have circulated among humans for more than 80 years. Seasonal influenza virus epidemics caused by two IBV lines (Victoria and Yamagata) and influenza A virus have considerable effects on public health globally and result in approximately 290,000–650,000 annual influenza-attributed deaths (Caini et al., 2015; Pan et al., 2015). Currently, the most effective countermeasures against influenza B virus infections are influenza virus vaccines (Subbarao and Matsuoka, 2013). However, these vaccines have limited efficacy because only strain-matched humoral immune responses are induced, while the globular head domain of viral hemagglutinin (HA) continues to evolve rapidly. In addition, few anti-influenza drugs are available, and the treatment window for administering the available drugs is small (Subbarao and Matsuoka, 2013; van de Sandt et al., 2015). A universal drug that targets both influenza B virus lineages remains a public health priority. Conferring passive immune protection using broadly neutralizing antibodies (bnAbs) may be a promising alternative viral infection treatment (Wilson and Andrews, 2012; Corti and Lanzavecchia, 2013; Walker and Burton, 2018).

We previously generated a panel of mouse anti-IBV mouse myeloma Sp2/0 hybridoma cells, among which several IgM antibodies were produced to target the receptor-binding site of influenza B and block viral infection with great breadth and potency. One of these antibodies, 7G6-IgM, was described in our previous study (Shen et al., 2019), and the other is 7G1-IgM, which is described in the present study. On the basis of a "cloning free" approach, previous studies have elegantly demonstrated that forced expression of activation-induced cytidine deaminase (AID) in hybridoma cells can induce somatic hypermutation (SHM) and heavy-chain class-switch recombination (CSR) of antibodies (Iglesias-Ussel et al., 2006; Stavnezer et al., 2008; Su et al., 2014). We used this strategy to generate different subtypes of anti-IBV IgG antibodies from 7G1 hybridoma cells and to obtain effective functional IgG antibodies against IBVs in mice. A schematic illustration of this strategy is shown in Fig. 1A. To control the expression of AID, a tetracycline-inducible AID (Tet-On) expression cassette (pLenti-CMVTRE3G-hAID-F2A-eGFP-Puro), which contains a tetracycline response element promoter (CMV-TRE3G), was constructed in house, and 7G1 hybridoma cells were forced to express rtTA after lentiviral transduction with the pLenti-CMV-rtTA-Blast vector (a gift from Eric Campeau, Addgene plasmid # 26429) (Supplementary Fig. S1). The cells were then selected in complete medium containing blasticidin (10 μg/mL) and puromycin (5 μg/mL) for 10 days to generate stable 7G1-TetOn-hAID cells, which were then incubated with 500 ng/mL doxycycline to induce the expression of enhanced green fluorescent protein (eGFP), corresponding to the inducible expression of human AID. Photographs were taken 2 and 4 days after incubation with doxycycline, and the eGFP fluorescence was observed (Supplementary Fig. S2). After continuous induction of AID for an additional two weeks, the cells were incubated with goat anti-mouse IgG antibody Fluor 568, and a significant IgG-positive signal was observed. Next, $eGFP^{+}$ and membrane IgG^{+} cells were enriched via flow cytometry (BD Aria III) (Supplementary Fig. S3). After single-cell sorting by flow cytometry, the cells were then seeded into twenty 96-well plates at one cell/well without doxycycline in the medium. After two weeks of culture, anti--B/Florida/4/2006 hemagglutinin (HA) IgG-positive wells were selected by an ELISA assay.

We successfully generated 380 cell clones, including 249 IgG3 subtype cell clones, 90 IgG1 subtype cell clones, 38 IgG2b subtype cell clones and 3 IgG2a-subtype cell clones (Fig. 1B). We next examined whether the increases in CSR were associated with SHM in the V region of 7G1; however by sequencing, no mutations were found in the representative

* Corresponding authors.
E-mail addresses: a124965468@smu.edu.cn (C. Shen), yxchen2008@xmu.edu.cn (Y. Chen), wxluo@xmu.edu.cn (W. Luo).
[1] Yuanzhi Chen and Chenguang Shen contributed equally to this work.

https://doi.org/10.1016/j.virs.2022.03.009
Received 21 September 2021;
Available online 22 March 2022



C

MN

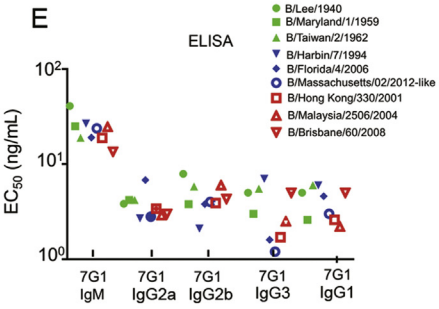
Virus		IgM	7G1-IgG2a			7G1-IgG2b				7G1-IgG3				7G1-IgG1			
			249	173	106	294	325	218	210	126	44	258	286	80	27	209	17
Ancestral	B/Lee/1940	80	80	160	160	160	160	40	40	160	80	80	80	80	80	40	20
	B/Maryland/1/1959	0	20	0	0	10	10	0	10	40	20	10	10	10	20	10	10
	B/Taiwan/2/1962	320	320	160	320	160	160	80	40	160	320	160	160	160	160	80	80
Yamagata	B/Harbin/7/1994	0	0	0	0	320	0	0	0	80	320	40	10	20	20	20	20
	B/Florida/4/2006	1280	640	320	1280	1280	640	80	80	640	640	320	160	160	80	320	320
	B/Massachusetts/02/2012-like	320	320	160	1280	640	320	80	80	320	320	160	320	160	80	160	160
Victoria	B/Hong Kong/330/2001	1280	1280	640	640	640	640	160	160	128	640	320	320	640	320	320	320
	B/Malaysia/2506/2004	640	320	320	640	640	320	40	160	160	640	320	640	160	80	80	40
	B/Brisbane/60/2008	640	320	320	320	640	320	80	80	640	320	320	320	320	320	320	160

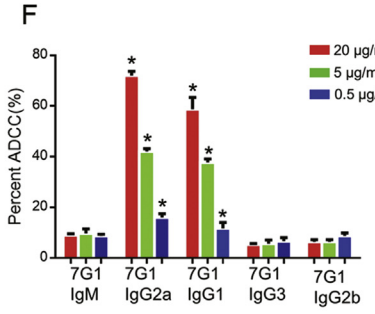
HI

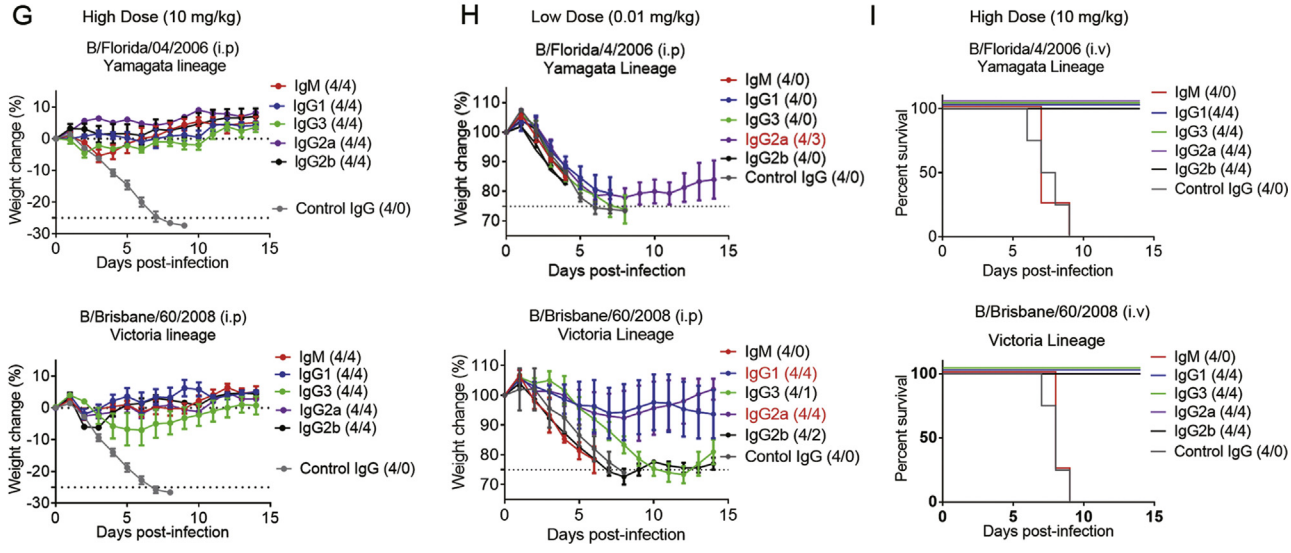
Virus		IgM	7G1-IgG2a			7G1-IgG2b				7G1-IgG3				7G1-IgG1			
			249	173	106	294	325	218	210	126	44	258	286	80	27	209	17
Ancestral	B/Lee/1940	80	40	80	80	80	160	40	20	80	40	80	80	40	80	40	20
	B/Maryland/1/1959	0	20	0	0	20	20	0	20	40	20	20	20	10	10	10	10
	B/Taiwan/2/1962	320	160	160	320	160	160	80	40	160	320	160	160	160	40	20	80
Yamagata	B/Harbin/7/1994	0	0	0	0	320	0	0	0	80	320	40	20	20	10	20	20
	B/Florida/4/2006	1280	640	320	640	640	640	80	40	160	320	320	160	160	80	320	320
	B/Massachusetts/02/2012-like	160	320	160	1280	320	320	40	80	320	160	160	320	160	80	160	160
Victoria	B/Hong Kong/330/2001	640	1280	640	640	640	640	160	80	1280	640	320	320	640	320	320	320
	B/Malaysia/2506/2004	320	160	160	640	640	320	40	160	160	320	160	640	160	80	80	40
	B/Brisbane/60/2008	160	320	320	320	640	320	80	40	640	320	320	320	320	320	320	160

D

Virus		7G1-IC_{50} (μg/mL)									
		MN					HI				
		IgM	IgG1	IgG2a	IgG2b	IgG3	IgM	IgG1	IgG2a	IgG2b	IgG3
Ancestral	B/Lee/1940	4.42	4.42	4.42	4.42	4.42	4.42	8.84	4.42	8.84	4.42
	B/Maryland/1/1959	>50	17.68	>50	17.68	8.84	>50	35.36	>50	35.36	8.84
	B/Taiwan/2/1962	0.55	0.55	1.1	1.1	0.55	0.55	1.1	2.21	1.1	0.55
Yamagata	B/Harbin/7/1994	>50	17.68	>50	1.1	4.42	>50	17.68	>50	1.1	4.42
	B/Florida/4/2006	0.07	0.55	1.1	1.1	1.1	0.07	2.21	1.1	1.1	1.1
	Massachusetts/02/2012-like	0.55	1.1	0.55	0.55	0.55	0.55	1.1	0.55	1.1	0.55
Victoria	B/Hong Kong/330/2001	0.28	2.21	1.1	4.42	1.1	0.28	2.21	1.1	4.42	1.1
	B/Malaysia/2506/2004	0.55	0.55	0.55	4.42	8.84	0.55	0.55	0.55	4.42	8.84
	B/Brisbane/60/2008	0.78	1.1	1.1	1.1	2.21	0.78	1.1	1.1	1.1	2.21







(caption on next page)

Fig. 1. Class-switch recombination was mimicked in an IgM hybridoma cell line to develop effective functional IgG antibodies against influenza B virus. **A** Schematic illustration of the strategy used in this study. A Tetracycline-triggered activation-induced cytidine deaminase (AID) was constructed and transducted into 7G1-IgM hybridoma cells. Anti-FluB hemagglutinin (HA; B/Florida/4/2006)-positive IgG clones were isolated after "on-demand" heavy-chain class-switch recombination. **B** The number of class-switch recombination IgG isotypes obtained from IgM were isolated and determined in this study. Mouse hybridoma cell culture supernatants were collected and detected via the mouse immunoglobulin isotyping ELISA kit. **C** Hemagglutination inhibition (HI) and microneutralization (MN) of mouse 7G1-IgM antibody and three or four representative monoclonal antibodies (mAbs) of each IgG isotype. 1×10^5 of each hybridoma cells were cultured in the cell culture plate for 72 hours, and the cell culture supernatant was collected, serial 2-fold dilutions of the supernatant were mixed with an equal volume of virus for the assay. The neutralization titer was the highest cell culture supernatant dilution that was negative for hemagglutination. The HI titer was the highest cell culture supernatant dilution that was negative for hemagglutination. The red color labeled clones IgG2a-294, IgG3-126 and IgG3-44 were able to neutralize more influenza virus variants compared to the parental IgM antibody. The values are color-filled: red, strong reactivity; yellow, moderate reactivity; green, weak reactivity. **D** Hemagglutination inhibition (HI) and microneutralization (MN) activities of mouse 7G1-IgM antibodies and representative 7G1-IgG antibodies. The values are the averages of three independent experiments. Values below 50 μg/mL are color-filled: red-orange shades, strong reactivity; yellow shades, moderate reactivity; beige-light blue shades, weak reactivity; values >50 show no reactivity. **E** Binding of the indicated antibodies to nine strains of the three influenza B lineages. EC_{50} (ELISA-based half-maximal effective binding concentration) greater than 10^4 ng/mL were considered to be negative. **F** Antibody-dependent cell-mediated cytotoxicity (ADCC) activity of 7G1-IgM and representative 7G1-IgG antibodies against B/Florida/4/2006 virus stain-infected MDCK cells. MDCK cells infected by influenza B viruses at an MOI of 10 were used as target cells and mouse NK cells were used as effector cells in the ADCC assay. The indicated antibodies were tested at 20 μg/mL, 2 μg/mL and 0.5 μg/mL. The bars represent the mean ± SEM. $^*P < 0.05$, compared to the control IgM group. The experiment was performed 3 times with mouse NK cells from 3 different mice. One representative dataset is presented in this study. **G-I** Comparison of the therapeutic efficacies of 7G1-IgM and representative 7G1-IgG antibodies in mice. Body weight changes (**G** and **H**) of BALB/c mice (n = 6 per group) treated intraperitoneally with the indicated antibodies (1 mg/kg or 0.01 mg/kg) 24 h after lethal challenge with MA-B/Florida/4/2006 or B/Brisbane/60/2008 at a 50% mouse lethal dose (MLD50) of 25. This experiment was repeated three times. The body weight curves represent the mean and 95% confidence intervals. Statistical analysis was performed with log-rank test ($^*P < 0.05$, compared to the control IgG-treated group). Prophylactic efficacy of the dose of 10 mg/kg IgM and IgG antibodies against lethal challenge with 25 MLD50 of MA-B/Florida/4/2006 or MA-B/Brisbane/60/2008 by intravenous antibody administration (**I**). The numbers in the diagonal brackets indicate the numbers of mice infected comparing to mice survived. The survival curves of BALB/c mice (n = 4 per group) treated with antibodies (10 mg/kg) 1 day before lethal challenge are shown. This experiment was repeated three times; Log-rank test was used to assess the significance ($^*P < 0.05$) of survival outcome. The control IgG is a mouse IgG1 monoclonal antibody against the HA of the influenza A virus generated by our laboratory. The ancestral virus strains represent the strains that were isolated before 1980 when the influenza B virus did not evolve into two lineages Yamagata and Victoria.

clones. We compared the *in vitro* microneutralization (MN) and hemagglutination inhibition (HI) activities and the breadth of reactivity of the representative antibodies in the cell culture supernatant obtained from different antibody subtype cultures using a panel of nine representative influenza B virus strains derived from three distinct lineages. The IgM subtype antibody did not show MN or HI activity against the two ancestral influenza B virus strains; however, several IgG subtype antibodies exhibited MN and HI activity against all the influenza B viruses tested. The red color labeled clone IgG2a-249, IgG2b-294, IgG3-126 and IgG3-44 were able to neutralize more influenza virus variants compared to the parental IgM antibody (Fig. 1C). Next, four representative antibodies from each subtype were selected and purified from mouse ascites for further evaluation. The purified IgG1, IgG2b and IgG3 subtype antibodies showed HI and MN activity against all the viruses tested, the IgG2a subtype antibody showed HI and MN activity against most of the virus strain, and IgM did not show reactive activity against one ancestral and one Yamagata virus strains (Fig. 1D). In the binding test, all antibodies reacted specifically with all nine tested viruses derived from the three influenza B lineages. The four IgG subtype antibodies revealed significantly lower half-maximal effective concentration (EC_{50}) values than the IgM antibody against the tested influenza virus strains (Fig. 1E). We also evaluated the antibody-dependent cell-mediated cytotoxicity (ADCC) activity of each antibody against B/Florida/4/2006. IgG2a and IgG1 subtype antibodies demonstrated high ADCC activity; in contrast, all the other antibodies demonstrated no ADCC activity against B/Florida/4/2006 (Fig. 1F). We finally evaluated the *in vivo* antiviral activity of each antibody using two mouse-adapted influenza B virus strains with a mouse model. A dose-ranging study was carried out by intraperitoneal or intravenous administration after intranasal virus challenge with MA-B/Florida/4/2006 or B/Brisbane/60/2008 at a 50% mouse lethal dose (MLD50) of 25. Ten milligrams per kilogram of each antibody administered intraperitoneally completely protected the mice against infection with viruses from the two lineages, and increased body weight was observed at the end of the study (Fig. 1G). In contrast, all the mice intraperitoneally administered 0.01 mg/kg IgM 7G1 died within 8 days of infection, and at this dose, each IgG 7G1 subtype showed a complete or partial therapeutic effect against the two influenza B viruses in mice (Fig. 1H). A single dose of 0.01 mg/kg IgG2a 7G1 protected 75% mice against the Yamagata influenza B virus B/Florida/04/2006 infection, in contrast, all the other classes of 7G1 and control IgG failed to protect any mice infected with this virus. A single dose of 0.01 mg/kg IgG2a and IgG1 7G1 protected 100% mice against the Victoria influenza B virus B/Brisbane/60/2008 infection, and the same dose of IgG3 7G1 protected 25% of mice and IgG2b protected 50% of mice against the infection. All the mice treated with control IgG died within eight days of infection. IgG2a 7G1 showed the best *in vivo* antiviral activity (Fig. 1H). When the infected mice were administered antibodies intravenously with 10 mg/kg, all the IgG subtypes showed protective potency against influenza B virus (Fig. 1I).

In this study, additional mutations in *V* genes were not found in either the heavy or light chains. This outcome may be a result of the frequency of CSR is higher than that of SHM (Iglesias-Ussel et al., 2006), and *V* genes may be insensitive to AID. For obtaining details on frequencies, a large number of cells should be sequenced. Long-term AID expression may lead to higher frequencies of CSR and SHM (Iglesias-Ussel et al., 2006). However, an "on-demand" or "Tet-on AID-expression" system is better for obtaining individuals or families of class-switched monoclonal antibodies from hybridomas. Our strategy can also be used to generate IgM hybridomas from IgG hybridomas with the same or mutated *V* genes. It is relatively difficult to develop bnAbs against influenza virus due to the high variability of HA; fortunately, the strategy used in this study shows the potential to further broaden the reactivity spectrum of neutralizing antibodies against influenza virus. Additionally, this strategy can be extended to the development of antibodies against other human pathogens. In a recent anti-COVID-19 antibody study, researchers found that engineering an IgM-neutralizing antibody from its IgG version can improve efficacy, reduce resistance and simplify the prophylactic and therapeutic treatment of COVID-19 (Ku et al., 2021; Yerabham and Ho, 2021). Forced expression of AID can facilitate the isolation of different class-switch variants from anti-SARS-CoV-2 hybridoma cells without antibody engineering or expression. Interestingly, in the present study, 65% of the IgG-switching clones were IgG3, and 24% were IgG1. The frequencies of IgG2b (10%) and IgG2a (1%) were much lower than the frequency of IgG1/3. Isotype convergence seems unlikely as we do not have enough clones. One possible reason is that the IgG3-constant gene is closer to the IgM-constant gene on the chromosome of the heavy chain, and they may be followed by IgG1, IgG2b, and IgG2a (Stavnezer et al., 2008). However, it is interesting to consider the reason that the

frequencies of IgG3 and IgG1 isotypes were increased. This study observed that 7G1 of different classes displayed slightly different reactive spectrums against IBV and provided different protection efficiency in the mice model, which may be the reason that different antibody classes have different Fc structures and thus have different Fc-based functions. For example, IgG1 and IgG2a have strong Fc-mediated ADCC activity, while other types of antibodies have no such activity. We also observed differences in antiviral activity from different sub-clones with the same IgG isotypes *in vitro*. However, additional mutations in *V* genes were not found in all these antibodies. Forced expression of AID in the hybridomas may lead to additional mutations in other genes and affect the binding activity of the antibodies. To our knowledge, a variety of immune cells and cytokines are involved during the natural SHM in the germinal centers. This *in vitro* model does not fully emulate the complexity of the human immune system or the human *in vivo* microenvironment. Future studies are needed to screen more clones, harvest some of the hybridoma cells after a prolonged incubation and develop some *in vivo* model to figure out the mechanism.

In summary, this methodology may represent a general strategy to enhance antibody properties with “on-demand” CSR, and even SHM, without the need for antibody engineering or recombinant protein expression. Moreover, our strategy is convenient for investigating the functions of different isotypes of a therapeutic antibody during early drug discovery and development. We hope to generate an AID “on-demand” myeloma fusion partner cell line in the future study.

Footnotes

This work is funded by the National Natural Science Foundation of China (31870925), Shenzhen Science and Technology Innovation Commission for Research and Development Project (JCYJ20190809183205622) and Guangdong Basic and Applied Basic Research Foundation (2020A1515010368).The authors declare that they have no conflicts of interest. All *in vivo* studies were performed in accordance with Institutional Animal Care and Use Committee guidelines and were approved by the Ethics Committee of Xiamen University Laboratory Animal Center.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2022.03.009.

References

Caini, S., Huang, Q.S., Ciblak, M.A., Kusznierz, G., Owen, R., Wangchuk, S., Henriques, C.M., Njouom, R., Fasce, R.A., Yu, H., Feng, L., Zambon, M., Clara, A.W., Kosasih, H., Puzelli, S., Kadjo, H.A., Emukule, G., Heraud, J.M., Ang, L.W., Venter, M., Mironenko, A., Brammer, L., Mai le, T.Q., Schellevis, F., Plotkin, S., Paget, J., Global Influenza, B.S., 2015. Epidemiological and virological characteristics of influenza b: results of the global influenza b study. Influenza Other Respir. Viruses 9 (Suppl. 1), 3–12.

Corti, D., Lanzavecchia, A., 2013. Broadly neutralizing antiviral antibodies. Annu. Rev. Immunol. 31, 705–742.

Iglesias-Ussel, M.D., Fan, M., Li, Z., Martin, A., Scharff, M.D., 2006. Forced expression of aid facilitates the isolation of class switch variants from hybridoma cells. J. Immunol. Methods 316, 59–66.

Ku, Z., Xie, X., Hinton, P.R., Liu, X., Ye, X., Muruato, A.E., Ng, D.C., Biswas, S., Zou, J., Liu, Y., Pandya, D., Menachery, V.D., Rahman, S., Cao, Y.A., Deng, H., Xiong, W., Carlin, K.B., Liu, J., Su, H., Haanes, E.J., Keyt, B.A., Zhang, N., Carroll, S.F., Shi, P.Y., An, Z., 2021. Nasal delivery of an igm offers broad protection from sars-cov-2 variants. Nature 595, 718–723.

Pan, Y., Zhang, Y., Yang, P., Qian, H., Shi, W., Wu, S., Cui, S., Zhang, D., Wang, Q., 2015. Epidemiological and phylogenetic characteristics of influenza b infection in severe acute respiratory infection cases in beijing, 2014 to 2015. Medicine (Baltim.) 94, e2399.

Shen, C., Zhang, M., Chen, Y., Zhang, L., Wang, G., Chen, J., Chen, S., Li, Z., Wei, F., Chen, J., Yang, K., Guo, S., Wang, Y., Zheng, Q., Yu, H., Luo, W., Zhang, J., Chen, H., Chen, Y., Xia, N., 2019. An igm antibody targeting the receptor binding site of influenza b blocks viral infection with great breadth and potency. Theranostics 9, 210–231.

Stavnezer, J., Guikema, J.E., Schrader, C.E., 2008. Mechanism and regulation of class switch recombination. Annu. Rev. Immunol. 26, 261–292.

Su, Y.C., Al-Qaisi, T.S., Tung, H.Y., Cheng, T.L., Chuang, K.H., Chen, B.M., Roffler, S.R., 2014. Mimicking the germinal center reaction in hybridoma cells to isolate temperature-selective anti-peg antibodies. mAbs 6, 1069–1083.

Subbarao, K., Matsuoka, Y., 2013. The prospects and challenges of universal vaccines for influenza. Trends Microbiol 21, 350–358.

van de Sandt, C.E., Bodewes, R., Rimmelzwaan, G.F., de Vries, R.D., 2015. Influenza b viruses: not to be discounted. Future Microbiol 10, 1447–1465.

Walker, L.M., Burton, D.R., 2018. Passive immunotherapy of viral infections: 'Super-antibodies' enter the fray. Nat. Rev. Immunol. 18, 297–308.

Wilson, P.C., Andrews, S.F., 2012. Tools to therapeutically harness the human antibody response. Nat. Rev. Immunol. 12, 709–719.

Yerabham, A., Ho, M., 2021. A novel igm intranasal intervention against sars-cov-2. Antib. Ther. 4, 171–174.