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. Conceiving 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-CMV-TRE3G-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 at 20% well plate on 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

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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. 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 IgG group. The experiment was performed 3 times with mouse NK cells from 3 different mice. One representative dataset is presented in this study. G 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 MA-B/Brisbane/60/2008 at a 50% mouse lethal dose (MLD50) of 25. This experiment was repeated three times. The body weight changes represent the strains that were isolated before 1980 when the influenza B virus did not evolve into two lineages Yamagata and Victoria.
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 in vitro influences in antiviral activity from different sub-clones with the same IgG isotypes.

To our knowledge, a variety of immune cells and cytokines are involved during the natural SHM in the germinal 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

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