Native human monoclonal antibodies with potent cross-lineage neutralization of influenza B viruses

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Abstract

Although antibodies that effectively neutralize a broad set of influenza viruses exist in the human antibody repertoire, they are rare. We used a single cell screening technology to identify rare monoclonal antibodies (mAbs) that recognized a broad set of influenza B viruses (IBV). The screen yielded 23 mAbs with diverse germ line origins that recognized hemagglutinins (HAs) derived from influenza strains of both the Yamagata and Victoria lineages of IBV. Of the 23 mAbs, three exhibited low expression in a transient transfection system, four were neutralizers that bound to the HA head region, eleven were stalk-binding non-neutralizers, and five were stalk-binding neutralizers with four of these five representing unique antibody sequences. Of these four unique stalk-binding neutralizing mAbs, all were broadly reactive and neutralizing against a panel of multiple strains spanning both IBV lineages as well as highly effective in treating lethal IBV infections in mice at both 24 and 72 hours post-infection. The mAbs in this group were thermostable and bound different epitopes in the highly conserved HA stalk region. These characteristics suggest these mAbs are suitable for consideration as candidates for clinical studies to address effectiveness in treating of IBV infected patients.
Introduction
Influenza B viruses (IBV) have two antigenically differentiated lineages, termed Yamagata and Victoria (1, 2), which differ enough to warrant the inclusion of two IBV-specific components in quadrivalent influenza vaccines (3). Vaccination against IBV is only a partially effective means of protection as IBV is subject to antigenic drift among circulating strains prevalent in successive seasons necessitating annual vaccine reformulation. Influenza vaccine effectiveness can be further compromised when trivalent vaccines are used, which only have a single IBV-specific component and fail to cover both IBV lineages (4-6). While IBV does not cause pandemics, as there are no known naturally occurring non-human IBV reservoirs permitting extensive antigenic shift, in some years IBV can be the predominant circulating influenza strain in certain geographic regions (4, 7, 8) and IBV infections can be as severe or even more severe than infections due to influenza A viruses (IAV) (9). IBV can cause serious life-threatening infections in pediatric populations. For example, in the 2010/2011 season IBVs caused 25% of the influenza cases while being the cause of 38% of the pediatric deaths (10). The marketed anti-influenza neuraminidase inhibitor oseltamivir is less effective in treating IBV infections than IAV infections (11), highlighting the imperative for the development of new effective anti-IBV treatments to treat this prevalent and sometimes deadly infection.

Several attempts to define strain-independent epitopes on the hemagglutinin (HA) surface glycoprotein of influenza viruses have been made for vaccine candidates or therapeutic monoclonal antibodies (mAbs) (reviewed in (12)). Neutralization of
viruses from both Group 1 and Group 2 strains of IAV by single cross-reactive mAbs have been reported (13, 14), although generally at lower overall potency than group-specific mAbs (15-17). These investigative efforts have focused attention on the stalk region of the HA protein which is essential for viral-cell fusion and is more conserved than the HA globular head domain. For the fusion site in the stalk to be functional, the HA0 primary protein must first be activated by proteolytic cleavage into HA1 and HA2 subunits, which remain coupled by disulfide bonds, thereby facilitating the conformational change of HA necessary for fusion of the viral and cellular membranes (18). The ability of mAbs to block the conformational change of HA at the low pH of the endosomal compartment appears to be the key attribute of stalk-binding neutralizing mAbs responsible for preventing influenza viral infectivity (19).

Many of the mAbs to the HA stalk region have been derived from in vitro phage display antibody libraries (16, 17, 20). Alternatively, in the case of IAV, high quality mAbs were obtained directly from memory B cells derived from people vaccinated against or naturally infected with influenza virus (13, 15, 21). This natural repertoire of affinity-matured mAbs has provided effective immunity against influenza in model systems, making them attractive as a source for therapeutic candidates. Human mAbs in general have low failure rates in Phase 1 clinical trials (22) and native human mAbs may have even lower risk having been produced safely in at least the one human from whom it was cloned. Memory B cells are of particular interest for their potential to be an enriched source of B cells that display reactivity against
multiple strains of influenza virus encountered over decades. However, blood
samples from human donors can vary widely in their frequency of high-quality
neutralizing antibodies for a particular pathogen, and the frequency can be low (15).

The Cellspot™ technology provides the ability to screen for rare human antibodies
and has been used to generate clinical candidate mAbs against respiratory syncytial
virus (23), cytomegalovirus (24), a bacterial target implicated in antibiotic resistance
(25) and immune regulating mAbs (26). This approach uses antigen independent
stimulation of B cells to induce secretion of IgG which is captured as a microscopic
footprint around the cell (~150 µm in diameter). Millions of these footprints (i.e. cell
spots), each comprising a single mAb, are then probed in parallel with multiple
antigens on distinguishable fluorescent beads. A computerized microscope tabulates
the antigen specificity for the mAb in each B cell footprint by counting the number of
each type of captured bead (up to ~10,000 beads can bind to one cell spot,
providing a dynamic range of ~1.5 logs for each analyte). Following isolation of the
rare B-cells with favorable specificity profiles, heavy and light chains are cloned by
single cell RT-PCR and mAbs are expressed by transient transfection in HEK293
cells. The complete assay is conducted on a time scale compatible with the limited
in vitro lifetime of human B-cells, thereby enabling isolation of the mRNAs encoding
the mAb heavy and light chain variable regions from rare favorable cells.
Applying the Cellspot™ technology to HA from widely divergent IAV subtypes yielded strain-independent mAbs to Group 1 and to Group 2 IAVs (15). When expressed as an intact recombinant IgG1, these native human anti-IAV mAbs reliably recapitulated the binding properties observed in the primary assay. We now report the application of this technology to isolate mAbs that bind and neutralize IBV with the aim of discovering new antiviral agents.

Results

Our aim was to discover anti-IBV mAbs that have the following qualities: (i) broadly-reactive against circulating IBV strains by targeting the conserved HA stalk region; (ii) high affinity binding in vitro; (iii) high potency in vivo (as assessed in a murine IBV infection model); (iv) suitable for human use; and (v) structurally stable.

Primary screen To achieve these goals, we applied the CellSpot™ technology to anonymized human blood samples. To identify broadly-reactive anti-IBV mAbs, we used HA antigens from representative members of the two major lineages of IBV strains: B/Florida/04/2006 (Yamagata lineage) and B/Malaysia/2506/2004 (Victoria lineage) (27). Eight blood donors were surveyed with a total of approximately 2.5 million memory B cells screened in both dedicated IBV screens and as controls in screens for other antigens. Memory B cell (CD19+/CD27+) abundance was measured and adjusted to equal levels in each screen. In the primary screen, most blood samples had a frequency of memory B cells secreting a mAb against IBV HA at an average frequency of 2 in 10,000, which was about 10-fold higher than we
previously found when screening against IAV HAs. Of these anti-IBV mAbs, about half showed cross-reactivity to HAs from both B/Florida/04/2006 and B/Malaysia/2506/2004 strains. For further characterization, 23 mAbs were expressed in HEK293 cells and confirmed as being double-positive for both IBV HAs.

**Secondary screens.** Of the 23 initial antibody clones examined, 20 were produced at sufficiently high levels (at least 20 mg/L) in a mammalian HEK293 transient expression system to warrant further investigation. The germline origins for the sequences of these 20 mAbs are diverse, indicating that the CellSpot process surveys a wide sampling of the natural human repertoire (Table 1). All of the 20 mAbs discovered in this study are derived from germline sequences different from the previously characterized IBV-specific mAbs: 5A7 (28); and CR8033, CR8071, and CR9114 (14). These 20 antibodies were further tested for their ability to neutralize two IBV strains (B/Victoria/2/1987 and B/Yamagata/16/1988) that were different from the strains from which the HAs used in the CellSpot screen were derived. Eleven mAbs failed to neutralize one or both of these two IBV strain isolates and were dropped from further consideration.

For IAVs, it is generally more difficult to generate escape mutants against mAbs that bind the conserved stalk region of HA than to the head region (29). Therefore we sought to identify pan-reactive anti-IBV mAbs that bound to the conserved stalk region. Of the nine remaining broadly-reactive mAbs, four were found to exhibit...
inhibitory activity in a hemagglutination inhibition (HI) assay (using the same strains used for the neutralization screen). A positive reading in the HI assay depends on the interaction of the globular head of HA with sialic acid receptors on the surface of red blood cells (30). Therefore these four HI-positive mAbs were considered presumptive HA head-binding mAbs and were removed from further consideration.

Of the remaining final 5 lead candidate mAbs (TRL845, TRL847, TRL848, TRL849, TRL854), most have unique heavy and light chain sequences and likely arose from independent B cell lineages. The exceptions are mAbs TRL845 and TRL847 (derived from the same donor) that are closely related at the sequence level (Table 2). Identification of two related clones in the final 5 lead candidates was indicative of reproducibility of the CellSpot assay and the subsequent screening process.

**Binding constant determination.** Affinities for these 5 mAbs for HA of B/Florida/04/2006 were measured using the ForteBio Octet™ biosensor assay (Figure 1) with TRL845 and TRL849 being the highest affinity candidates, with \( K_D \) values ≤200 pM. The broad reactivities of the 5 mAbs against different IBV strains were illustrated by binding to HAs from 3 Yamagata and 3 Victoria strains (Table 3). The HA of IBV is sufficiently different from that of IAV that the previously described high affinity mAbs TRL053 and TRL579 (15), directed against the HAs of the H1N1 and H3N2 IAV subtypes, respectively, show negligible cross-reactivity to IBV and vice-versa.
Thermal stability measurements. Thermal stabilities of the 5 mAbs were assessed by differential scanning fluorimetry (31). The mAbs were slowly heated from 25°C to 99°C in the presence of the fluorescent dye SYPRO Orange for which fluorescence increases as the dye binds to hydrophobic residues exposed when the protein unfolds. As shown in Figure 1, the $T_m$ for all of the mAbs was 57°C or above.

Interestingly, even though TRL845 and TRL847 are derived from the same germline sequences and share a high degree of overall sequence identity, TRL847 is significantly more thermostable. The biphasic melting curve seen for most of the mAbs is a common phenomenon, with heavy and light chains differing in their stability (32).

Epitope mapping. The epitopes for 4 independent mAbs (excluding TRL847) were determined using the Chemical Linkage of Peptides onto Scaffolds (CLIPS™) technology (33), comprising >6,500 linear and constrained synthetic peptide fragments (5-30 residues long) derived from the HA stalk sequence of B/Lee/40/1940. In addition, two published IBV stalk-binding mAbs, 5A7 (28) and CR9114 (14) were similarly analyzed. Figure 2 shows surface and backbone trace representations of the trimeric IBV HA structure with the binding peptide segments indicated in red. Despite the fact that the heavy and light chains of all the mAbs analyzed in the CLIPS study shown in Figure 2 were derived from completely different germline sequences, convergent patterns of peptide recognition were observed in some cases. For example, TRL845 and TRL854 displayed nearly indistinguishable binding patterns. Also, TRL849 and CR9114 recognized a related
set of peptides even though they are very divergent at the sequence level and they exhibit widely different in vivo activities.

To investigate the extent of HA sequence conservation for each mAb epitope, the entropy scores at each position were determined and mapped against an alignment of all currently available full-length IBV HA sequences isolated from human subjects (2543 HA sequences) (Figure 3). The epitopes of the four unique neutralizing stalk-binding mAbs obtained in this study, as well as two anti-IBV HA mAbs from the literature (14, 28), all mapped to HA sequences with low entropy scores within the HA alignment indicating that all the mAbs recognize epitopes that are comprised of highly conserved elements within the HA structure. This high degree of sequence conservation at the HA epitope sites responsible for binding to the mAbs suggests that the mAbs are likely to bind and neutralize the vast majority of IBV strains circulating in the human population.

The only epitope corresponding to a HA segment encompassing a residue position with an entropy score above 16 (on a scale of 1 to 232) is the first epitope of mAb TRL848 (HA residues S₅₄H₅₅F₅₆A₅₇N₅₈L₅₉K₆₀). In this case the high entropy position is HA sequence position 55 (Score = 100), where there is either a histidine or a phenylalanine for Victoria and Yamagata strains, respectively. Since TRL848 binds tightly to strains from both lineages (Table 3), residues other than position 55 within this epitope must be largely responsible for binding the TRL848 mAb. The other
epitope segments display very low entropy scores at each of the corresponding HA residue positions (see Figure 3 panel B).

In vitro neutralization. The IBV neutralizing activities of mAbs TRL845, TRL848, TRL849 were titrated to determine IC₅₀ values in a plaque-formation assay performed on MDCK cells (34) infected with either B/Florida/04/2006 (Yamagata lineage) or B/Malaysia/2506/2004 (Victoria lineage) strains (Table 4). mAb TRL849 was additionally tested against three more strains and found to neutralize the Victoria lineage B/Nevada/3/2011 strain as well as two older IBV strains (B/Great Lakes/1954, B/Taiwan/2/1962), whose isolations pre-date the Yamagata/Victoria lineage divergence (1, 2).

In vivo activity. The mAbs were tested for their ability to protect mice from weight loss and lethality in a BALB/c mouse model of IBV (35). mAbs were administered at 1 mg/kg by the intranasal route 24 h after infection with IBV strains B/Florida/04/2006 (Yamagata) or B/Malaysia/2506/2004 (Victoria) (Figure 4). AUC analyses of the weight loss profiles indicate that treatments with all TRL mAbs and mAb 5A7 (28) were significantly differentiated relative to PBS treatments (P <0.0001) and all treatments resulted in 100% survival (survival log-rank P<0.005). In contrast, CR9114 (14) treatments against both infections were less effective with substantial weight losses observed, with only 3 and 0 mice surviving for B/Florida and B/Malaysia infections, respectively.
A dose-ranging experiment using TRL849 to treat B/Florida/04/2006 infection was performed (Figure 5). AUC analyses of the weight loss profiles indicate that all mAb treatments were significantly differentiated relative to PBS treatments (P <0.0001). All mAb-treated mice survived (survival log-rank P<0.005). Mice treated IP with TRL849 at 1.0 mg/kg at 24 h post-infection were fully protected with <10% transient weight loss, while mice receiving 0.1 mg/kg experienced >20% weight loss before recovering. Intranasal administration improved the potency with 0.1 mg/kg at 24 h providing full protection from lethality and <10% weight loss. The increased potency of intranasal administration of neutralizing mAbs can be attributed to increased levels of virus neutralization locally at the apical side of the site of infection resulting in a lower viral burden (36, 37). When the 1.0 mg/kg treatment was administered at 72 h post-infection by either route of administration, transient weight loss of ~10% was observed with full protection from lethality.

Discussion

Compared to mice immunized for a hybridoma antibody discovery effort, the human donors of the B cells that gave rise to the antibodies in this study were likely exposed to multiple IBV strains over time. While this situation might be expected to give rise to broadly cross-reactive anti-IBV antibodies, this outcome might also be considered unexpected, considering demonstrations that prior exposure can limit the responses to new variants, i.e. “original antigenic sin” (38). These two concepts might be reconciled considering a hierarchy of responses over time which still allow for the generation of broadly reactive antibodies (39).
Because the globular head region of HA is highly immunogenic, the frequency of high affinity HA-specific antibodies reactive to the conserved stalk region epitopes was markedly lower requiring a high throughput technology to achieve a comprehensive screen of the human repertoire. As shown in Table 1, the set of 23 mAbs isolated in this study using the CellSpot technology are highly diverse being derived from many different human germline antibody sequences. The subset of 20 mAbs we analyzed in detail is also notable in that several distinct classes are represented including eleven non-neutralizers that bound to the stalk region and nine neutralizers, with 4 HI-positive mAbs (presumably binding to the head region) and 5 to the stalk region. The fact that the set of 20 mAbs does not include any non-neutralizers to the head region likely reflects the screening process requirement that the mAbs must bind to HAs from both B/Florida/04/2006 (Yamagata) and B/Malaysia/2506/2004 (Victoria lineage) representing divergent IBV strains. This screening constraint may have eliminated mAbs binding to non-conserved HA head sites that do not perform an essential viral function (such as binding to sialic acid on the host receptor) resulting in the absence of head-binding mAbs that are unable to neutralize the virus.

The multiplexed screening strategy employed in this study was designed to enrich for mAbs that bound to conserved sites on the HAs from diverse IBV strains. Mapping of the epitope data from the top four unique neutralizing mAbs to an alignment of the HA sequences derived from 2543 unique IBV human isolates
confirmed that these mAbs bind to very highly conserved IBV HA stalk residues (Figure 3). Consistent with its essential role in the viral fusion event with the host cell, the HA stalk region does not tolerate many mutations, making the discovery of mAbs that bind to the HA stalk an attractive and successful strategy for isolating broadly neutralizing anti-influenza virus antibodies (29, 40). Even though mutant IAV viruses with certain amino acid substitutions in the HA stalk region have been isolated that allow escape from neutralization by stalk-binding mAbs, these IAV escape variants exhibited decreased viral fitness (41). Vaccine strategies that focus the immune response to the HA stalk region by employing a series of chimeric HA antigens in which the stalk region is held constant while the head region of the antigen is varied in successive booster doses has been a successful strategy in animals studies for IAV (42) and IBV (43). Stable “headless” HA vaccine molecules consisting of only the stalk region can also elicit broadly neutralizing antibodies to IAV (44). However, the ability of mAbs to bind to the HA stalk by itself may not ensure a neutralization phenotype, as evidenced by the eleven non-neutralizing HI-negative binders isolated in this study, some of which presumably bind to conserved epitopes on the HA stalk given the initial selection criterion of binding HA from both lineages. Targeting other regions besides the stalk can be productive, as recent work has shown that broadly cross-reactive anti-IBV mAbs can be generated in mice to the conserved regions of the sialic-acid binding region of the HA head (45) as well as the neuraminidase (46).
The human-derived HA stalk-binding antibodies isolated in this study bind HAs from a broad range of IBV strains (Table 3) and also show potent neutralization activities against IBV strains isolated from humans over many years (Table 4). They also show excellent activities in \textit{in vivo} murine models of influenza infection (Figures 5 and 6) against both IBV lineages, with results that compare favorably to activities observed for broadly neutralizing stalk-binding mAbs against IBV that have been previously described, notably 5A7 (28) and CR9114 (14). Four of the five mAbs in this study exploit a varied set of epitopes in the HA stalk as evidenced by their unique peptide signatures in the CLIPS analysis (Figure 2 and Figure 3) which correspond to highly conserved IBV HA residues (Figure 3). The results of the CLIPS analysis for 5A7 are in excellent agreement with the previously published epitope map on the IBV stalk region for 5A7 determined by HA truncations and site-directed mutagenesis (28), which underscores the validity of the epitope mapping results obtained with the CLIPS methodology (33). While in some cases there are some shared residues in the CLIPS peptide signatures with the previously described mAbs 5A7 and CR9114, the germline sequences of all the mAbs included in this analysis (Figure 2 and Table 1) are highly divergent among themselves, with each mAb representing a unique solution to binding to the IBV HA stalk and achieving viral neutralization. For instance, while TRL849 and CR9114 share some (but not all) of the same epitope space as determined by the CLIPS analysis (see Figures 2 and 3), their germline origins are completely different and their \textit{in vivo} activity profiles are highly differentiated, with TRL849 being much more efficacious against viral challenges by both Yamagata or Victoria lineage IBV strains (Figure 4).
Clearly the human antibody repertoire is a rich source capable of giving rise to diverse HA-binding antibodies that are able to neutralize IBV. The mAbs discovered in this study exhibit a combination of broad reactivity across IBV lineages, high potency, marked efficacy at 72 hours post-infection, high thermal stability that generally correlates with ease of manufacturing, and the safety advantages of being derived directly from humans. These attributes make the novel mAbs suitable for use in investigative studies to assess their effectiveness in combating morbidity and mortality due IBV infections.
Materials & Methods

**Single B cell mAb Discovery Technology (CellSpot™).** Leukopaks were obtained from a total of 8 anonymized donors under informed consent approved by Stanford’s Institutional Review Board (Stanford Blood Center; Stanford, CA). Peripheral Blood Mononuclear Cells (PBMCs) were prepared by standard methods and individual memory B cells (CD19⁺/CD27⁺) assayed following stimulation to proliferate and differentiate using a cocktail of mitogens and cytokines as previously described (23-26). The cells were distributed in 96 well microplates at ~200 memory B cells/well. From 10 mL of blood, ~100,000 memory B cells begin a course of dividing and secreting IgG lasting ~10 days. After 5 days, an aliquot of each well was transferred to a replicate microplate whose surface had been coated with an anti-Ig capture antibody; after gently spinning down the cells to the surface, a secreted IgG footprint of each cell was collected for 5h and the cells removed. These purified products of single cells were probed using homologous HA proteins from different strains conjugated using sodium cyanoborohydride to distinguishable fluorescent beads (6 types having different ratios of embedded red and green fluorophores). Lack of binding to beads coated with bovine serum albumin was used as a specificity counter-screen. This assay format provides replicates in the primary screen (sibling cells derived from the original positive cell in the master plate), which assures a low false-positive rate. After identifying wells with B cells secreting a mAb meeting the selection criteria (typically 1 or 2 wells per plate), the corresponding wells of the master plate were distributed at limiting dilution across new microplates. The secreted footprint was again collected, but without removal of the cells before assay.
Although the signal in this secondary assay was weaker than in the primary assay, the assay is non-destructive and thus the encoding mRNAs for heavy and light chains could be amplified by single cell RT-PCR. After cloning into the pTT5 vector (47), recombinant antibodies were produced in HEK293 Freestyle cells (Thermo Fisher Scientific, Waltham, MA) by transient transfection and purified using Protein A (Mab Select Sure, GE Healthcare; Pittsburgh, PA). The control mAbs 5A7 and CR9114 mAbs were similarly produced by cloning synthetic DNAs encoding the VH and VL sequences (as described (14, 28)) into recombinant expression vectors.

**ELISA assay.** For analysis of recombinant antibody binding, 96-well microplates were coated with 2 μg/ml HA overnight in PBS at 4°C. HA proteins were purchased from Sino Biological for B/Florida/04/2006 (cat#11053-V08H) and from Protein Sciences for B/Malaysia/2506/2004 (cat#/2506/04), and from Immune Tech for B/Brisbane/60/2008 (IT-003-B2TMp), B/Victoria/2/1987 (IT-003-B6p), B/Massachusetts/02/2012 (IT-003-B19pTMp), and B/Wisconsin/1/2010 (IT-003-B7pTMp). The next day, plates were blocked with 3% BSA/PBS and then incubated for 1 h with serial dilutions of the mAb starting at 5 μg/ml (30 nM) in 0.5% BSA/PBS-T. After washing with PBS-T, HRP-conjugated anti-human kappa was added for 45 min. Plates were then washed with PBS-T and developed with SureBlue TMB substrate (KPL Inc, Gaithersburg, MD).

**Virus neutralization assay.** IBV neutralizing activity of mAbs was titrated to determine 50% inhibitory concentration values (IC₅₀) in a 72 h plaque-formation
assay on MDCK cells (ATCC, CCL-34) as described (34). The day before the assay, one 12-well plate per test antibody was seeded with 2x10^5 MDCK/well in MEM/ 5% FBS/ 1% L-glutamine. Pre-titered virus stocks were rapidly thawed and diluted in MEM/1% L-glutamine and 1 μg/ml TPCK trypsin (Worthington; Lakewood, NJ), vortexed and kept on ice. Antibodies were added to the virus at concentration ranging from 0-20 μg/ml, vortexed and incubated at 37°C for 1 h. Cell monolayers were washed 3 times with PBS and 100 μl MEM/1% L-glutamine and 1 μg/ml TPCK trypsin added to each well before adding 200 μl of the pre-incubated virus/antibody preparation. After 2 h at 37°C the supernatant was aspirated, the monolayer was washed once with PBS and a 1.2% Avicel RC-581 (FMC Bio-polymer Inc.; Philadelphia, PA) overlay containing 1 μg/ml TPCK trypsin in MEM, 20mM HEPES, 2mM L-glutamine, 0.075% NaHCO3 and 100 units/ml penicillin G/ 100 μg/ml Streptomycin/ 0.25 μg/ml amphotericin B, was applied for 72 h at 37°C/ 5% CO2. The plaques were visualized by fixing and staining with crystal violet. First, the Avicel overlay was aspirated and monolayers gently washed with PBS. Monolayers were then fixed by applying 1 ml of ice cold 80%/20% methanol/acetone for 10 minutes. Stock solutions of 0.13% (w/v) crystal violet were prepared in deionized water containing 5% methanol/11% formaldehyde. Working stocks of crystal violet were made by diluting the stock solution 1:2 in PBS. After monolayer fixation, enough crystal violet working solution was added to a well to just cover the cells and allowed to stain for 10 to 20 min. Wells were rinsed in distilled deionized water and the average number of plaques counted from duplicate wells, expressed as a percentage of plaques in uninhibited (100% infected) control wells. IC50 values were
calculated using the log (inhibitor) versus response non-linear regression analysis method within the Prism7™ software package (GraphPad Software, Inc; La Jolla, CA).

Hemagglutination Inhibition Assay.
The hemagglutination inhibition (HI) assay was performed as described (30) using strains B/Victoria/2/1987 and B/Yamagata/16/1988.

Thermal stability assay. The melting temperature ($T_m$) of the mAbs were determined by differential scanning fluorimetry (31). Reactions were run in a StepOne Plus instrument (Life Technologies; Carlsbad, CA). Antibodies were diluted to 2 µg/mL into PBS containing 1X SYPRO Orange (Life Technologies; Carlsbad, CA) in a final volume of 20 µL. Reaction tubes were heated for 2 min at 25°C followed by a 1% ramp rate (~0.8°C/minute) to 99°C followed by 2 min at 99°C. Fluorescent intensity of the emission signal was monitored over the course of the incubation using the filters for ROX dye (Thermo Fisher Scientific, Waltham, MA). Melting temperatures were determined by curve fitting the first derivative of the fluorescence data in Microsoft Excel and confirmed using the Protein Thermal Shift software (v1.0, Life Technologies; Carlsbad, CA) using the derivative $T_m$ method. All plots were made using Prism software (GraphPad Software, Inc; La Jolla, CA). $T_m$ values were determined from the average of 3 or more experiments, each run in duplicate.
Affinity measurement. A stock solution of mAb at 600 nM was diluted to 5.0 and 0.5 nM in PBS pH 7.4 and affinities were measured with a ForteBio Octet™ biosensor model QK (Pall Corporation; Menlo Park, CA). Streptavidin-coated sensors were dipped into biotinylated HA antigen (1 mg/ml in PBS, pH 7.4) and after a wash step with the mAbs at a concentration of 250 nM. The resulting on and off rates measured yielded the $K_D$ by standard methods (48).

Epitope mapping. Using the Chemically Linked Peptides on Scaffolds technology (CLIPS™; Pepscan B.V.; Lelystad, Netherlands) a set of >6,500 peptides covering linear, discontinuous and conformational epitopes (including single loops, double-loops, triple loops, sheet-like folds, helix-like folds and combinations thereof) were designed and synthesized as previously reported (33) using HA2 from influenza B/Lee/40/1940. ELISA binding assays were used to identify mAb binding sites, which were visualized by projection onto the structure of influenza B HA (PDB entry 4NRL, B/Lee/40/1940).

Bioinformatics. All unique human-derived full-length IBV HA sequences deposited as of December 31, 2017 in the Influenza Research Database (IRD; http://www.fludb.org) were obtained (2543 unique HA sequences). Using the IDB set of analysis tools (http://www.viprbrc.org), the HA sequences were aligned and an analysis was performed to determine a positional entropy score (related to the Shannon entropy) at each aligned sequence position. The entropy scores at each HA residue position range from zero (100% conservation) to 232 (random
distribution of all twenty amino acid residue types). These values were used to assess the conservation at each of the IBV HA residue positions making up the epitopes recognized by the anti-IBV mAbs.

Mouse model. Female BALB/c mice were ordered from Jackson labs and were 6 to 8 weeks old at the time of the experiments. Mice (n=5 per group) were anaesthetized with a ketamine/xylazine mixture and subsequently infected intranasally using 50 µL of PBS containing 3xLD$_{50}$ of either B/Florida/04/2006 (4,500 pfu) or B/Malaysia/2506/2004 (13,000 pfu). Mice were treated at 24 or 72 h post-infection with 0.1 or 1.0 mg/kg body weight of mAb in PBS delivered as a 0.1 mL bolus intraperitoneal (IP) injection or 0.05 mL intranasal (IN) instillation into anaesthetized mice. Mice were monitored for death, morbidity and weight loss and date of death was recorded for 14 days.

Statistical Analysis. All statistical analyses and plot generation were made using Prism7™ software (GraphPad Software, Inc; La Jolla, CA). Area under the curve (AUC) analysis was used to assess statistical relationship between the percent weight loss profiles of the different treatments. Areas under the weight loss curves were measured using the AUC functionality of Prism7 software. The statistical relationship associated with any two weight loss curves was assessed by calculating the mean AUC, standard error and degrees of freedom for each pair and performing the unpaired t-test. Survival data were analyzed by the log-rank test.
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TABLE 1  Characterization of the native human anti-HA IBV mAbs

| mAb classification | mAb     | Expression in HEK293* | Hi Activitya | Neutralization in MDCK cellsb | Predicted binding site and mode | HC germlinec | LC germlinec |
|--------------------|---------|-----------------------|--------------|-------------------------------|--------------------------------|--------------|--------------|
|                    | TRL798  | -                     | nd           | nd                            | nd                             | IGHV1-2*02  | IGKV1-6*01  |
|                    | TRL834  | -                     | nd           | nd                            | nd                             | IGHV1-69*12 | IGKV1-44*01 |
|                    | TRL851  | -                     | nd           | nd                            | nd                             | IGHV1-51*01 | IGKV2D-28*01|
| Low Expression     | TRL809  | *                     | +/-          | +                             | +                              | head, neut  | IGHV3-30-3*02 | IGKV4-1*01 |
|                    | TRL823  | *                     | +            | +                             | +                              | head, neut  | IGHV3-43*02  | IGKV1-39*01 |
|                    | TRL832  | *                     | +            | +                             | +                              | head, neut  | IGHV4-59*02  | IGKV3-1*01  |
|                    | TRL833  | *                     | +            | +                             | +                              | head, neut  | IGHV4-31*03  | IGKV3-1*01  |
| HA Head neutralizing | TRL784  | *                     | -            | -                             | -                              | stalk, no neut | IGHV1-69*12 | IGKV3-21*02 |
|                    | TRL799  | *                     | -            | -                             | -                              | stalk, no neut | IGHV1-2*02  | IGKV2D-28*01|
|                    | TRL811  | *                     | -            | -                             | -                              | stalk, no neut | IGHV3-30*13 | IGKV1-17*01 |
|                    | TRL812  | *                     | +/-          | +/-                           | +/-                           | stalk, no neut | IGKV3-33*01 | IGKV3-15*01 |
|                    | TRL813  | *                     | -            | -                             | +/-                           | stalk, no neut | IGKV3-73*01 | IGKV1-5*03  |
|                    | TRL835  | *                     | -            | -                             | -                              | stalk, no neut | IGKV3-51*01 | IGKV2D-28*01|
|                    | TRL837  | *                     | +/-          | -                             | +                              | stalk, no neut | IGKV3-69*12 | IGKV3-20*01 |
|                    | TRL841  | *                     | -            | -                             | -                              | stalk, no neut | IGKV3-30*18 | IGKV3-15*01 |
|                    | TRL842  | *                     | -            | -                             | -                              | stalk, no neut | IGKV3-30*18 | IGKV3-21*02 |
|                    | TRL846  | *                     | -            | -                             | -                              | stalk, no neut | IGKV3-23*04 | IGKV3-28*01 |
|                    | TRL855  | +                     | nd           | -                             | -                              | stalk, no neut | IGKV3-74*01 | IGKV3-21*02 |
| HA Stalk non-neutralizing | TRL845  | *                     | -            | -                             | +                              | stalk, neut  | IGKV3-48*02  | IGKV1-9*01  |
|                    | TRL847  | *                     | -            | -                             | +                              | stalk, neut  | IGKV3-48*02  | IGKV1-9*01  |
|                    | TRL848  | *                     | -            | -                             | +                              | stalk, neut  | IGKV3-31*03  | IGKV3-15*01 |
|                    | TRL849  | *                     | -            | -                             | +                              | stalk, neut  | IGKV3-31*03  | IGKV3-15*01 |
|                    | TRL854  | *                     | -            | -                             | +                              | stalk, neut  | IGHV1-2*02  | IGKV2D-28*01|
| HA Stalk neutralizing | TRL845  | *                     | -            | -                             | +                              | stalk, neut  | IGKV3-48*02  | IGKV1-9*01  |
|                    | TRL847  | *                     | -            | -                             | +                              | stalk, neut  | IGKV3-48*02  | IGKV1-9*01  |
|                    | TRL848  | *                     | -            | -                             | +                              | stalk, neut  | IGKV3-31*03  | IGKV3-15*01 |
|                    | TRL849  | *                     | -            | -                             | +                              | stalk, neut  | IGKV3-31*03  | IGKV3-15*01 |
|                    | TRL854  | *                     | -            | -                             | +                              | stalk, neut  | IGKV3-2*02  | IGKV2D-28*01|
| Previously Published mAbs* | 5A7     | +                     | nd           | nd                            | nd                            | stalk, neut  | IGKV3-33*01 | IGKV1-47*02 |
|                    | CRB033  | nd                    | nd           | nd                            | nd                            | head, neut   | IGKV3-9*01  | IGKV3-20*01 |
|                    | CRB070  | nd                    | nd           | nd                            | nd                            | head, neut   | IGKV1-18*01 | IGKV1-47*01 |
|                    | CR9114  | +                     | nd           | nd                            | nd                            | stalk, no neut | IGHV1-69*06 | IGKV1-44*01 |

a Neutralizing activity of mAbs.

b HI activity of mAbs.

c Predicted binding site and mode.
a Expression in HEK293: expression level >20 mg/liter (+) and ≤<20 mg/liter (-)
b Hemagglutination inhibition activities against strains B/Victoria/2/1987 and B/Yamagata/16/1988 at effective mAb concentrations 1.5 µg/ml (+), 200 µg/ml (+/-), and >200 µg/ml (-)
c In vitro neut: in vitro neutralization IC_{50} values against strains B/Victoria/2/1987 and B/Yamagata/16/1988 at mAb concentrations ≤10 µg/ml (+) and >10 µg/ml (-)
d Germline families for heavy chain (HC) and light chain (LC) are listed using IMGT nomenclature (www.imgt.org).
e mAb 5A7 is described in (28) and the CR mAbs in (14).

nd: not determined.
Table 2. Lead mAb variable region sequences

| mAb   | Heavy chain gene | HCDR1       | HCDR2       | HCDR3          | Light chain gene | LCDR1   | LCDR2   | LCDR3   |
|-------|------------------|-------------|-------------|----------------|------------------|---------|---------|---------|
| TRL845| IGHV3-48*02      | GFTFCRYI    | ISDTSRTI    | ARDPDPFVRAFDS  | IGKV1-9*01       | QLISSY  | AAS     | PPLNSYPIT |
| TRL847| IGHV3-48*02      | GFTFSRFSD  | ISDTGRTI    | ARDPDPFVRAFDS  | IGKV1-9*01       | QVISSY  | AAS     | QQLTTYPPIT |
| TRL848| IGHV3-30*18      | GFSLWTSG    | MSYDETKK    | AKPRLDYLFHADDS | IGLV3-21*02      | YIGSKS  | DDS     | CQVWETSEDLWV |
| TRL849| IGHV4-31*03      | GGSSNGGYYH  | IYRGST      | ARMPLNYDLLTYIGAFDL | IGKV3-15*01   | QSVNRN  | DAS     | QQDKWPPG  |
| TRL854| IGHV1-2*02       | GYTFAYH     | INPSNGAT    | ATDIVVERASLGGNFSYGMDSV | IGKV2D-28*01 | QSSLHSNGYNH | LAS      | MQSLQTSIT  |

The amino acid sequences (in single letter code) of the three complementarity-determining-regions of the mAb heavy and light chains (HCDR1-3 and LCDR1-3, respectively) are listed in the Table. The IMGT designations of the germline antibody sequences are listed for the heavy and light chains for each mAb.
Table 3. Binding of anti-HA stalk neutralizing mAbs to influenza A and B virus HAs

| Influenza A and B virus HA | anti-IBV TRL845 | TRL847 | TRL848 | TRL849 | TRL854 | anti-IAV TRL053 | TRL579 |
|---------------------------|----------------|--------|--------|--------|--------|----------------|--------|
| IBV - Victoria Lineage HA | +++            | +++    | +++    | +++    | +++    | -              | -      |
| B/Brisbane/60/2008         | +++            | +++    | +++    | +++    | +++    | -              | -      |
| B/Malaysia/2506/2004       | +++            | +++    | +++    | +++    | +++    | -              | -      |
| B/Victoria/2/1987          | +++            | +++    | +++    | +++    | +++    | -              | -      |
| IBV - Yamagata Lineage HA | +++            | +++    | +++    | +++    | +++    | -              | -      |
| B/Florida/4/2006           | +++            | +++    | +++    | +++    | +++    | -              | -      |
| B/Massachusetts/02/2012    | +++            | +++    | +++    | +++    | +++    | -              | -      |
| B/Wisconsin/1/2010         | +++            | +++    | +++    | +++    | +++    | -              | -      |
| IAV (H1N1) - Group 1 HA   | -              | -      | -      | -      | -      | +++            | -      |
| A/California/07/2009       | -              | -      | -      | -      | -      | -              | +++    |
| IAV (H3N2) - Group 2 HA   | -              | -      | -      | -      | -      | -              | +++    |
| A/Sydney/05/1997           | -              | -      | -      | -      | -      | -              | +++    |

The ELISA signals are represented as "-" (negative; i.e. background level) or "+++" (strongly positive; i.e. corresponding to 18 to 21 times background level). Anti-IAV control mAbs are previously described antibodies TRL053 and TRL579 (15).
Table 4. Neutralization IC_{50} values for mAbs against IBV strains

| Influenza B Virus     | IC_{50} a (95% CI b) |
|-----------------------|----------------------|
| Lineage               | Strain               | TRL845    | TRL848 | TRL849    |
| Yamagata              | B/Florida/04/2006    | 8.2 (6.1 – 11.3) | 4.7 (2.9 – 8.0) | 9.7 (6.7 – 14.6) |
| Victoria              | B/Malaysia/2506/2004 | 1.2 (0.2 – 6.6) | 1.1 (0.2 – 5.1) | 1.4 (0.4 - 5.1) |
|                       | B/Nevada/03/2011    | -         | -      | 1.7 (0.3 – 9.4) |
| Pre-Yam/Vic           | B/Taiwan/2/1962     | -         | -      | 1.5 (0.3 – 8.9) |
| Lineage Split         | B/Great Lakes/1954  | -         | -      | 25.7 (7.0 - x^c) |

IC_{50} values were determined from data obtained from the plaque-formation assay performed on MDCK cells (34).

a IC_{50}, 50 percent inhibitory concentration (micrograms per milliliter)
b 95% CI, 95 percent confidence interval (micrograms per milliliter)
c The upper limit of the 95% CI for the TRL849 IC_{50} against B/Great Lakes/1954 is undefined due to insufficient data for concentrations above the IC_{50} value.
Figures

Figure 1. Thermostabilities and affinities of lead mAbs. Melting temperatures ($T_m$ in degrees Celsius) of the mAbs were determined by binding of SYPRO™ Orange fluorescent dye (Thermo Fisher). The first derivative of the melting curve is plotted to highlight the $T_m$. The observed biphasic $T_m$s for the mAbs are due to the separate contributions of the heavy and light chains. Dissociation constants ($K_d$) were measured with a ForteBio Octet™ biosensor.

Figure 2. Epitopes determined by CLIPS™ analysis mapped onto IBV trimeric HA structure. IBV-specific mAbs (2 from the literature and 4 from this study, with germline families noted) all bind to the HA stalk region, with the specific peptide sequences recognized by each mAb listed (numbering scheme based on HA from B/Lee/40/1940). Those amino acids are shown in red using a space-filling representation for the surface, with a ribbon representation for the backbone. For clarity, the mAb-binding residues are only shown on one of the three monomers that make up the HA homotrimer. Graphic images were made using the PyMOL Molecular Graphics System (Schodinger LLC).

Figure 3. mAb epitopes map to HA segments with low sequence position diversity scores. The entropy scores (Score) at each HA residue position for the full length and expanded views of HA segments 1-100 and 300-500 are shown in panels A, C, and D respectively. The segments of HA residues corresponding to the mAb
epitopes (listed in panel B and Figure 2) are illustrated by colored rectangles in
panels C and D, following the coloring scheme indicated in panel B. Panel B lists the
CLIPS peptide sequences that define each epitope and the corresponding entropy
scores at each residue position.

Figure 4. mAbs exhibit in vivo activity against both Yamagata and Victoria
lineage strains. mAbs TRL845, TRL848, TRL849, 5A7, and CR9114 were tested
for efficacy in a mouse influenza challenge model against B/Florida/04/2006
(Yamagata lineage – panel A) and B/Malaysia/2506/2004 (Victoria lineage – panel
B). At 24 hours after intranasal infection using a viral inoculum of 3xLD50/mouse,
each mAb was administered at 1 mg/kg mAb via the intranasal route (n=5). Percent
reductions in body weights are shown with error bars set at one standard deviation.
dpi; days post infection. **** P <0.0001, ** P <0.005

Figure 5. mAb TRL849 exhibits in vivo activity when administered at 24 or 72
hours post-infection. A single treatment with TRL849, either intranasal (IN) or
intraperitoneal (IP), was administered at the indicated doses at either 24 (panel A) or
72 (panel B) hours post-infection with Yamagata lineage strain B/Florida/04/2006
(n=5). Percent reductions in body weights are shown with error bars set at one
standard deviation. dpi; days post infection. **** P <0.0001.
Figure 1. Thermostabilities and affinities of lead mAbs. Melting temperatures (Tm in degrees Celsius) of the mAbs were determined by binding of SYPRO™ Orange fluorescent dye (Thermo Fisher). The first derivative of the melting curve is plotted to highlight the Tm. The observed biphasic Tms for the mAbs are due to the separate contributions of the heavy and light chains. Dissociation constants (Kd) were measured with a ForteBio Octet™ biosensor.
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