Antigen-specific B-cell receptor sensitizes B cells to infection by influenza virus

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Influenza A virus-specific B lymphocytes and the antibodies they produce protect against infection1. However, the outcome of interactions between an influenza haemagglutinin-specific B cell via its receptor (BCR) and virus is unclear. Through somatic cell nuclear transfer we generated mice that harbour B cells with a BCR specific for the haemagglutinin of influenza A/WSN/33 virus (FluBI mice). Their B cells secrete an immunoglobulin gamma 2b that neutralizes infectious virus. Whereas B cells from FluBI and control mice bind equivalent amounts of virus through interaction of haemagglutinin with surface-disposed sialic acids, the A/WSN/33 virus infects only the haemagglutinin-specific B cells. Mere binding of virus is not sufficient for infection of B cells: this requires interactions of the BCR with haemagglutinin, causing both disruption of antibody secretion and FluBI B-cell death within 18 h. In mice infected with A/WSN/33, lung-resident FluBI B cells are infected by the virus, thus delaying the onset of protective antibody release into the lungs, whereas FluBI cells in the draining lymph node are not infected and proliferate. We propose that influenza targets and kills influenza-specific B cells in the lung, thus allowing the virus to gain purchase before the initiation of an effective adaptive response.

Memory B lymphocytes contribute to the protective immune response to flu infection by producing immunoglobulins that bind and neutralize the virus2. The lung of an exposed individual contains influenza-specific memory B cells that bind virus, differentiate into plasma cells and secrete either immunoglobulin G (IgG) or IgA, locally reducing the spread of virus3,4. However, the fate of virus-specific B cells that encounter live influenza virus remains unknown.

The low frequency of antigen-specific B cells has hampered analysis of the interactions between live virus, flu antigens and the primary B cells specific for them5. To detect influenza-virus-specific B cells, we used sortase-mediated labelling to install Alexa 647 fluorophore onto the haemagglutinin (HA) protein6. Virus was disrupted with detergent, HA–Alexa 647 was purified by immunoprecipitation and dialysed to form fluorescent flu micelles (Extended Data Fig. 1a–d). These flu micelles did not stain splenocytes from uninfected mice, but did stain a small number of CD19+ cells in spleens of mice infected with influenza and boosted multiple times with A/WSN/33 in incomplete Freund’s adjuvant (Extended Data Fig. 1e).

Figure 1 | FluBI mice obtained by somatic cell nuclear transfer from the nucleus of an HA-specific IgG2b heavy chain/light chain rearrangement. a. A B6.129F1 mouse was infected intranasally with A/WSN/33 and immunized intraperitoneally at days 7, 14 and 21 post-infection with disrupted A/WSN/33 in incomplete Freund’s adjuvant. Splenocytes were collected at day 28 post-infection, and stained with anti-CD19 serum, FluBI serum (FluBI), or serum from wild-type or FluBI mice, before intranasal challenge with A/WSN/33. b. Represetative flow cytometry plots of naïve (nms) or A/WSN/33-infected mice (Anti-WSN) stained with monoclonal anti-M2, FluBI serum (FluBI), or serum from uninfected (nms) or A/WSN/33-infected mice (Anti-WSN). Immunoprecipitates were analysed by SDS–PAGE and autoradiography. All panels were from the same gel; anti-WSN panels shown are from a shorter exposure time. c. A/WSN/33 virus was incubated with the indicated serums before infection of MDCK cells or RAW macrophages. At 2 h.p.i., cells were stained with [35S]cysteine/methionine for 2 h before lysis. Lysates were immunoprecipitated with monoclonal anti-M2, FluBI serum (FluBI), or serum from uninfected (nms) or A/WSN/33-infected mice (Anti-WSN). Immunoprecipitates were analysed by SDS–PAGE and autoradiography. All panels were from the same gel; anti-WSN panels shown are from a shorter exposure time. d. A/WSN/33 virus was incubated with the indicated serums before infection of MDCK cells or RAW macrophages. At 2 h.p.i., cells were labelled with [35S]cysteine/methionine for 2 h, lysed and immunoprecipitated with anti-WSN serum. e. BALB/c mice received 100 μl of serum intravenously from wild-type or FluBI mice, before intranasal challenge with A/WSN/33 (2 × 107 plaque-forming units (p.f.u.) per mouse). n = 5; error bars, s.d.

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LETTER

doi:10.1038/nature12637

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**Figure 2** | Influenza virus targets B cells for infection through the BCR.

a, CD40-activated OBI and FluBI B cells and MDCK cells were incubated with A/WSN/33 at a multiplicity of infection (MOI) of 1.0 for 30 min on ice, washed, and transferred to 37 °C in RPMI (0.2% BSA). At 2 h.p.i., cells were labelled with [35S]cysteine/methionine for 2 h, immunoprecipitated with anti-WSN serum or anti-M2 antibody (inset), digested with peptide N-glycosidase F (PNGase F), and analysed by SDS–PAGE and autoradiography. b, MDCK cells and CD40-activated OBI or FluBI B cells were incubated with A/WSN/33 or A/Udorn/307/1972 (H3N2) virus and analysed as in a. c, CD40-activated OBI or FluBI B cells were incubated with HA–SRTwtOBI virus for 30 min on ice and analysed by cytofluorometry. d, CD40-activated OBI or FluBI B cells or MDCK cells were incubated on ice for 30 min with HA–SRT virus modified with a 17-mer peptide containing the OBI epitope (OBI) or a mutant version that no longer binds to OBI (OBI*). Infection was analysed as in a. e, CD40-activated OBI or FluBI B cells were infected with A/WSN/33 at a MOI of 1.0 and labelled with [35S]cysteine/methionine at 4 h.p.i. for 4 h. Released virus particles were recovered by adsorption to chicken erythrocytes and analysed by SDS–PAGE and autoradiography.

by immunoprecipitation from lysates of [35S]cysteine/methionine-labelled, A/WSN/33-infected MDCK cells (Fig. 1c). The antibody retrieves HA0 and its cleavage products10 HA1 and HA2. FluBI IgG2b antibody purified from hybridomas generated from FluBI/Rag2−/− splenocytes gave similar results (Methods). The serum from FluBI mice neutralizes A/WSN/33 in vitro (Fig. 1d) and in vivo (Fig. 1e). Cytofluorometry of B-cell populations in lymph node, spleen and bone marrow from FluBI mice showed a complete absence of B-1a B cells, and other B-cell subsets were near-normal in distribution and number (Extended Data Fig. 4). As shown for OBI mice16, the presence of a functionally rearranged γ/2 heavy chain locus does not compromise B-cell development, despite the deletion of the μ, δ, γ3 and γ1 constant regions in FluBI mice.

To determine the fate of HA-specific B cells upon encounter with virus, we obtained B cells from the FluBI mouse and from OBI mice, whose B cells produce an IgG1 specific for ovalbumin. Before infection, we activated cells overnight with anti-CD40 to improve biosynthetic labelling, used to assess viral antigen synthesis. At 2 h post infection (h.p.i.) FluBI B cells infected with A/WSN/33 synthesize vastly more HA, nucleoprotein (NP) and M2 protein (inset) than OBI B cells (Fig. 2a). In FluBI B cells, the levels of NP were comparable to those obtained from A/WSN/33-infected MDCK cells, an indication that replication of A/WSN/33 in FluBI B cells is robust (Fig. 2a and Extended Data Fig. 5). Neither FluBI nor OBI B cells were infected by the closely related strain A/Puerto Rico/8/1934 (H1N1) or with A/Udorn/307/1972 (H3N2) (Fig. 2b and Extended Data Figs 6, 7).

The increased levels of antigen detected in FluBI B cells might result from improved binding of virus via BCR–HA interactions. To measure virus binding, we incubated anti-CD40-activated B cells with sortase-modifiable (HA–SRT) Alexa 647-labelled virus (HA–SRTAlexa 647) and measured bound virus by cytofluorometry. Virus bound equally well to FluBI and OBI B cells (Fig. 2c). To determine whether increased susceptibility of FluBI B cells to infection is indeed BCR-dependent, we generated HA–SRT virus, transacylated at the carboxy terminus of HA1 with a synthetic 17-residue ovalbumin peptide that comprises the epitope recognized by the ovalbumin-specific OBI B cells (HA–SRTmtOBI). This virus should now also bind to the BCR expressed on the surface of OBI B cells. For comparison we labelled HA–SRT virus with a mutant version of the OBI 17-mer peptide (HA–SRTmtOBI) no longer recognized by the OBI IgG1. We then exposed FluBI and OBI B cells to either HA–SRTmtOBI or HA–SRTmtOBI virus (Fig. 2d). As expected, the two HA–SRT viruses infected FluBI B cells equally, regardless of the identity of the peptide epitope installed. In contrast, only HA–SRTmtOBI infected OBI B cells. The level of infection in OBI B cells exposed to HA–SRTmtOBI was similar to that seen in FluBI B cells. The presence of a BCR that recognizes HA, native or modified to impart BCR reactivity, thus causes susceptibility to influenza infection. Mere adsorption of virus to the cell surface through interactions with sialic acids may therefore not suffice to gain entry11–13, and interaction with an internalizing receptor is important14–17. Antigen-occupied BCRs are indeed efficiently internalized18, especially when engaged by a multivalent ligand, and could thus improve virus entry and infection. Do infected B cells produce virus or virus-like particles (VLPs)? We infected OBI and FluBI B cells with A/WSN/33 and biosynthetically labelled them 4–6 h.p.i. We incubated the culture supernatants with protein-G agarose. IP, recovered from supernatants using protein-G agarose. IP, and transferred to 37 °C for 18 h, stained with anti-CD19 and 7-aminoactinomycin D, and analysed by flowcytometry. Live cells were calculated as (no. GFP positive cells/no. GFP negative cells/no. GFP positive cells at time 0) × 100. Error bars are s.d. of triplicate cultures. We also looked for secreted antibody from A/WSN/33-exposed B cells (Fig. 3a). OBI cells showed no change in their ability to secrete
euthanized at 3 or 6 days post-infection. Cells were collected from spleen, mediastinal lymph nodes (MSLN) and lungs. a. Plots are gated on FGFγ−transfected cells. Dilution of violet dye indicates proliferation.

Figure 4 | B cells can be infected with A/WSN/33 in the lungs, but not in the draining lymph node. Naïve B cells from OBI;MHCII−GFP and FluBI;MHCII−GFP mice were stained with Celltrace violet, mixed in a 1:1 ratio, and transferred intravenously into C57BL/6 mice (10⁷ total cells per recipient). Mice were inoculated intranasally with live or ultraviolet-irradiated A/WSN/33. Mice were euthanized at 1, 2 or 3 days post-infection. By day 3 post-infection, no FlubI antigen-positive B cells were found in either location. Co-transferred OBI cells were present in the lungs at 3 days post-infection, but more proliferation of FluBI cells in the lungs at 3 days post-infection than animals that received live virus (Fig. 4a; day 3 lungs live 2.7 ± 0.43% n = 6 versus day 3 lungs irradiated 6.5 ± 1.3% n = 5; P = 0.015).

To detect intracellular influenza viral proteins as an additional measure of infection, we generated HA- and NP-specific heavy-chain-only antibody fragments (VHHs) from an influenza-immunized alpaca (Extended Data Fig. 9). These small, single-domain VHHs are C-terminally modified with an LPETG motif for direct coupling with TAMRA using sortase19 and detect flu antigens in FluBI B cells infected in vitro (Extended Data Fig. 10). In mice infected with live A/WSN/33 we observed HA- and NP-positive, infected FlubI B cells in the lungs (Fig. 4b, c). Although virus is reportedly delivered to the mediastinal lymph nodes by dendritic cells, no flu-antigen-positive B cells were found in the mediastinal lymph nodes (Fig. 4b). Not unexpectedly, mice exposed to irradiated A/WSN/33 lacked flu-antigen-positive B cells in either location. Co-transferred OBI cells were present in the lungs of infected mice, but were HA- and NP-negative.

To determine whether infection of lung-resident flu-specific B cells affects antibody production in vivo, we measured in bronchoalveolar lavage (BAL) fluid and serum by ELISA for A/WSN/33 reactivity using horseradish peroxidase-coupled anti-IgG2b. ND, not detected. P values were determined using a two-sided t-test with Bonferroni correction. NS, not significant.
stronger and more rapid initial response than live virus, consistent with the ability of live, but not irradiated virus to kill FluBI B cells. Serum levels of flu-specific IgG2b were equivalent in mice receiving live versus irradiated virus, indicating that loss of flu-specific IgG2b is restricted to the lung bronchoalveolar space.

FluBI cells, specific for haemagglutinin and secreting a neutralizing antibody, themselves succumb to infection mediated by the surface-disposed BCR. The rapid death of A/WSN/33-specific FluBI cells provides respite for the virus at the lung epithelium, a site to which antigen-specific B cells are recruited in the course of infection and where they remain as sentinels thereafter. Infection and killing of a fraction of the rare antigen-specific B cells impairs the kinetics of the memory response, and confers an advantage to the virus with its replication cycle measured in hours. The ability of a pathogen to exploit this mode of entry and eliminate the initial wave of the very B cells capable of counteracting the infection is an efficient means of ensuring a window for replication and horizontal transmission. It is unlikely to be limited to influenza virus.

METHODS SUMMARY
Sortase labelling. HA–SRT virus (a derivative of A/WSN/33, ref. 6) was incubated with Sortase A (150 μM) and the indicated nucleophile (500 μM) in sortase labelling buffer with 0.2% BSA at 37 °C for 1 h, resulting in site-specific labelling of HA on the intact virus particle. Labelled virus was concentrated over a 20% sucrose cushion. For flu micelles, HA–SRT mutants were disrupted with detergent, immunoprecipitated with anti-Alexa 647 and dialysed to form HA-enriched micelles. For OB1 epitope labelling, HA–SRT was labelled with OB1 (GGGFDKLPGFSI GGGK) or OB2 (GGGGDKLPAGASIEAGGGK).

Hybridoma production. FluBI or Rag2−/− spleen cells were fused with NS-1 cells. The resulting hybridomas were screened for A/WSN/33 reactivity by ELISA using horseradish peroxidase-coupled anti-IgG2b.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 24 May; accepted 4 September 2013.

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METHODS

Reagents. Anti A/WSN/33 serum was generated by infecting BALB/C mice with A/WSN/33 (2 x 10⁸ p.f.u. per mouse). Anti-M2 antibody (14C2) was purchased from Santa Cruz Biotechnology. OBI peptides were provided by the MIT biopolymers facility. The amino acid sequence that comprises the OBI epitope, as described²⁷,²⁸, is as follows: GGGFSDKLPFGDSEIAQGGK. The mutant sequence that fails to bind to the OBI antibody is as follows (substitutions denoted in bold): GGGFSDKLP AGGAEIAQGGK. Sortase was expressed in Escherichia coli and purified as described²⁴. The GGGK–Alexa 647 peptide used to label HA–SRT was provided by M. Witte. Anti IgG–FITC antibody (A1101) was purchased from Molecular Probes. Chicken erythrocytes (CRBCs) were purchased from Lampire Biological Laboratories. Express™ protein labelling mix was purchased from Perkin Elmer. Methionine and cysteine-free RPMI, OptiMEM, HEPES buffer and non-essential amino acids (NEAA) were purchased from LifeTechnologies. Endoglycosidase H (EndoH) and PNGase F were purchased from New England Biolabs. Protein-G agarose and EDTA-free protease inhibitor cocktail were purchased from Roche Diagnostics.

Virus propagation and infection. A/Puerto Rico/8/1934 (H1N1) and A/Udorn/307/1972 (H3N2) viral stocks were a gift from X. Zhuang. MDCK cells and RAW cells were originally obtained from the ATCC, and are tested for mycoplasma every 3–6 months. A/WSN/33 and HA–SRT virus²⁴ were propagated in MDCK cells grown in Optimem and supplemented with 1 µg/ml L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)–treated trypsin. Titres of A/WSN/33 and HA–SRT virus stocks were determined by plaque assay on MDCK cells. For the plaque assay, MDCK cells were cultured in 24-well dishes until sub-confluent. Cells were washed twice in PBS supplemented with Ca²⁺ and Mg²⁺ (PBS+) and infected with tenfold serial dilutions of virus in PBS+ supplemented with 0.25% BSA for 1 h at room temperature. Cells were washed once in PBS+ then overlaid with plaque media (1×MEM, 0.25% BSA, 0.8% agar, 0.5 µg/ml trypsin–TPCK) and placed at 37 °C. After 24 to 48 h, the agar overlay was removed and the cells were fixed with 3% paraformaldehyde and permeabilized using PBS 0.5% NP-40. Influenza plaques were stained using monoclonal antibody against NP conjugated to FITC then visualized and quantified by fluorescent microscopy. Labelled HA–SRT virus was quantified using haemagglutination assay against a standard containing 1.5% (w/v) chicken erythrocytes (18 K cells) in PBS. For all in vitro experimental infections virus was diluted in PBS+/BSA(0.25%) and supplemented with 1 µg/ml TPCK–treated trypsin. In the case of MDCK cells, cells were trypsinized and infections carried out in suspension. Virus and cells were incubated on ice for 30 min. Cells were washed with PBS+, and resuspended in DMEM with 0.2% BSA, 100 mM HEPES and NEAA (or RPMI with 0.2% BSA, 100 mM HEPES and NEAA in experiments where B cells were used). Sortase labelling of HA–SRT virus. HA–SRT virus was incubated with sortase A (150 µM) and the indicated nucleophile (500 µM) in sortase labelling buffer (100 mM Tris pH 7.4, 150 mM NaCl, 10 mM CaCl₂) supplemented with 0.2% BSA at 37 °C for 1 h. The labelled virus was then concentrated over a 20% sucrose cushion and stored at -20 °C. The sortase labelling buffer contained with 0.2% BSA at 37 °C for 1 h. For intracellular staining, cells were fixed and permeabilized using Cytofix/Cytoperm (BD) according to the manufacturer’s instructions.

Flu-specific ELISAs. High-binding 96-well microtitre plates (Costar) were coated overnight at 4 °C with A/WSN/33 (2 x 10⁸ p.f.u. ml⁻¹) in PBS. Plates were washed 3 times with wash buffer (PBS, 0.05% Tween-20), blocked with 10% feta bovine serum for 1 h at room temperature, washed 3 times, and incubated with samples. Bronchoalveolar lavage (BAL) fluid samples were collected by inserting a 24 gauge catheter into an incision in the trachea, filling the lungs with 1 ml PBS and recovering 0.7–0.8 ml of lavage fluid. BAL fluid samples were used neat. Samples were used at 1:10 dilution. Flubin antibody purified from hybridoma supernatants was used as standard. Plates were incubated with samples at room temperature for 2 h, washed 5 times, and incubated with HRP-coupled anti-lgG secondary reagent for 1 h. Plates were washed 7 times, and detected using 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate (Sigma).

Bronchoalveolar lavage and immune serum. Monoclonal anti M2 antibody was used at 2.5 µg/ml dilution. For all experiments where B cells and MDCK cells were compared side by side, MDCK cells were cultured during the experiment using RPMI media. For intracellular staining, cells were fixed and permeabilized using Cytofix/Cytoperm (BD) according to the manufacturer’s instructions.
buffer. Immunoprecipitates and materials adsorbed onto chicken red blood cells were analysed by SDS–PAGE and autoradiography.

**Production of FluBI hybridoma.** Spleen cells from FluBl/Rag2−/− mice were stimulated with 40 μg ml−1 LPS and 20 ng ml−1 IL4 for 5 days, and were fused with NSObc12 cells (a gift from B. Diamond) and selected in medium supplemented with 20% heat-inactivated FCS and hypoxanthine aminopterin thymidine (HAT) and grown in 10% CO2 for 3 weeks, before transfer of positive clones to hypoxanthine thymidine (HT) supplemented medium containing 10% heat-inactivated FCS. Resulting hybridomas were screened for A/WSN/33 reactivity by ELISA using HRP-coupled anti-IgG2b or anti-Igk (Southern Biotech) secondary antibodies for detection.

**Statistics.** Centre values are mean. *P* < 0.05 defined as significant. Standard two-sided *t*-test was used throughout unless otherwise noted. Sample size was based on variability from pilot studies.

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Extended Data Figure 1 | Flu micelles stain HA-specific B cells. a, Schematic for preparation of glycoprotein micelles from HA–SRT-Alexa 647 virus. b, Immunoprecipitation of HA–Alexa 647 with anti-Alexa 647 monoclonal antibody. Triton X100-disrupted virions were incubated with 400 µg anti-Alexa 647 overnight and HA–Alexa 647 was then recovered using protein G-Sepharose. Bound proteins were eluted with 0.1 M glycine pH 2.8. W, wash; E, elution. c, Typhoon image of the fractions obtained from a linear sucrose gradient after 20 h centrifugation (107,900 g). d, Fraction 8 from the sucrose gradient was concentrated and sucrose-depleted by centrifugation over a 30 kDa filter (Amicon UltraCel). The preparation was stained with phosphotungstate and examined by transmission electron microscopy (×150,000 magnification). e, Splenocytes from mice infected with A/WSN/33 or control mice were stained with anti-CD19 and HA–Alexa 647 micelles and analysed by cytofluorometry. Plots are representative of 6 mice per group.
Extended Data Figure 2 | FluBI antibody is of the IgG2b subclass. ELISA plates were coated with A/WSN/33-infected MDCK cell lysate and exposed to 1:100 diluted serum from a single C57BL/6 (wt), FluBI, FluBI;Rag2−/−, or wild-type mouse infected with A/WSN/33. Plates were washed and probed with isotype-specific secondary antibodies. Uninfected wild-type mice have flu-reactive antibodies of the IgM subclass. Flu-specific IgE was not detected in any sample. Error bars are s.d. of samples analysed in triplicate.
Extended Data Figure 3 | Sequence of the VDJ and VJ segments of the FluBI antibody. Genomic DNA was prepared from tails of FluBI mice. The heavy and light chain rearrangements were first identified by amplifying and sequencing of the segments with degenerate primers: for heavy chain: forward 5'-ARGCCTGGGRCTTCAGTGAAG-3' and reverse 5'-AGGCTCTGAGATCCCTAGACAG-3'; for light chain: forward 5'-GGCTGCAGSTTCAGTGGCAGTGGRTCWGGRAC-3' and reverse 5'-ATGCGACGTCAACTGATAATGAGCCCTCTCC-3'. Then the full sequences of the rearranged heavy and light chain segments were obtained using specific primers: forward 5'-TTACTGAGCACACAGGACCTC-3' and reverse 5'-AGGCTCTGAGATCCCTAGACAG-3'; for light chain: forward 5'-CAGCCCATATTCTCCCATGT-3' and reverse 5'-ATGCGACGTCAACTGATAATGAGCCCTCTCC-3'. Amplified products were agarose gel-purified and sequenced. Sequences were aligned to the NCBI mouse V, D and J genes using IgBlast. Sequences were deposited in GenBank (accession numbers KF419287 and KF419288).
Extended Data Figure 4 | FluBI mice lack B-1a B cells, but show near-normal development of follicular B cells. Cells were isolated from spleen, lymph node (LN, pooled mesenteric and cervical), peritoneal cavity and bone marrow of FluBI, FluBI Rag2−/− or C57BL/6 mice. Erythrocytes were lysed and cells were stained with the indicated antibodies and 7-AAD viability dye. LN plots were gated on total live cells. All other populations were gated on CD19+ live cells. Numbers indicated the percentage of cells in the indicated gates. B-1a B cells (CD5+) are absent and B-1b B cells (CD5−CD11b+) are reduced in the peritoneal cavity of FluBI and FluBI Rag2−/− mice. Plots are representative of 5 mice per group.
Extended Data Figure 5 | FluBI B cells are infected by A/WSN/33. CD40-activated OBI or FluBI B cells were incubated with A/WSN/33 virus at an MOI of 1.0 for 30 min on ice. Cells were then washed and incubated at 37 °C in RPMI (0.2% BSA). At 2 h.p.i., cells were fixed, permeabilized and stained with anti-IgG and TAMRA-conjugated anti-NP (VHH54, derived from alpaca; see Extended Data Fig. 9). a, Cells were visualized by confocal microscopy. b, Cells from a were scored as VHH54-positive or -negative. Error bars represent s.d. of positive cells counted per field (3 fields counted; ~200 total cells were counted per group).
Extended Data Figure 6 | Antibody secreted by FluBI B cells does not cross-react with other strains of influenza virus. ELISA plates were coated with A/WSN/33 (H1N1), A/Udorn/307/1972 (H3N2) or A/Puerto Rico/8/1934 (H1N1) overnight at 4°C. Plates were then washed, blocked with 10% fetal bovine serum and exposed to FluBI hybridoma supernatant or WSN-infected serum at the indicated dilutions. Bound antibody was detected using horseradish peroxidase-coupled anti-IgG2b secondary reagent.
Extended Data Figure 7 | FluBI B cells are not infected with A/Puerto Rico/8/1934 virus in vivo. C57BL/6 mice were administered $5 \times 10^6$ MHCII–GFP$^+$ FluBI B cells 2 h before intranasal infection with $2 \times 10^5$ p.f.u. per mouse of either A/WSN/33 (WSN) or A/Puerto Rico/8/1934 (PR8). Mice were euthanized 3 days post-infection, and lung resident cells were stained with anti-CD19 and TAMRA-conjugated VHH68 (anti-HA) or TAMRA-conjugated VHH52/54 (anti-NP). a, Representative plots gated on CD19$^+$ cells. b, Quantification of flu-antigen positive cells as shown in a. $n = 3$. Error bars are s.d. p = 0.06 using two-sided t-test.
Extended Data Figure 8 | Proliferating FluBI cells in the mediastinal lymph node are plasmablasts. a, Mediastinal lymph node cells from day 6 post live infection mice described in Fig. 4 were analysed by confocal microscopy. GFP$^+$ cells displayed a morphology consistent with plasmablasts. b, MSLN cells from day 6 post live infection mice described in Figure 4 were analysed by cytofluorometry. Proliferating (violet low) cells were B220$^{low}$ and CD138$^+$.
Extended Data Figure 9 | Alpaca-derived VHHS recognize HA and NP from A/WSN/33. a, An alpaca was immunized with ethanol-fixed influenza virus. Phage display libraries were constructed from selectively amplified VHH-specific complementary DNA using peripheral blood lymphocytes as starting material, and panned twice against sortase labelled influenza HA–SRT diluted virus bound to streptavidin coupled beads. VHH sequences obtained from specific binders were expressed with a sortase recognition motif to allow direct conjugation of biotin or fluorophores. b, VHH54 and VHH68 conjugated directly to agarose beads were used to precipitate lysates of A/WSN/33 infected, [35S]cysteine/methionine-labelled MDCK cells.
Extended Data Figure 10 | Flu-specific VHHs can stain infected FluBI B cells. B cells from OBI or FluBI mice were cultured for 24 h in RPMI containing anti-CD40 (1 µg ml⁻¹) before exposure to A/WSN/33. OBI B cells, FluBI B cells and MDCK cells were incubated with A/WSN/33 at an MOI of 1.0 for 30 min on ice, washed once with PBS, and transferred to 37 °C in RPMI (0.2% BSA). At 5 h post infection, cells were washed, permeabilized, fixed and stained using TAMRA-conjugated flu-specific VHHs (1 µg in 50 µl). Infected MDCK cells were analysed in parallel as a positive control. Cells were analysed by cytofluorometry using a BD Fortessa.