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Author/s:

Burmas, L;Lee, WS;Kelly, A;Webster, R;Esterbauer, R;Kent, SJ;Wheatley, AK;Juno, JA;Tan, HX

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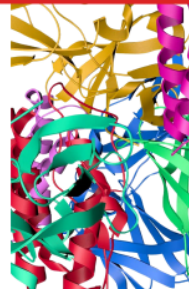
Modulation of germinal center and antibody dynamics via ipsilateral versus contralateral immunization against SARS-CoV-2

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Modulation of germinal center and antibody dynamics via ipsilateral versus contralateral immunization against SARS-CoV-2

Lauren Burmas ¹, Wen Shi Lee¹, Andrew Kelly ¹, Rosela Webster¹, Robyn Esterbauer¹, Stephen J. Kent ^{1,2}, Adam K. Wheatley¹, Jennifer A. Juno ^{1,*} and Hyon-Xhi Tan ^{1,*}

¹Department of Microbiology and Immunology, University of Melbourne, Peter Doherty Institute for Infection and Immunity, Melbourne, Victoria, Australia

²Melbourne Sexual Health Centre and Department of Infectious Diseases, Alfred Hospital and Central Clinical School, Monash University, Melbourne, Victoria, Australia

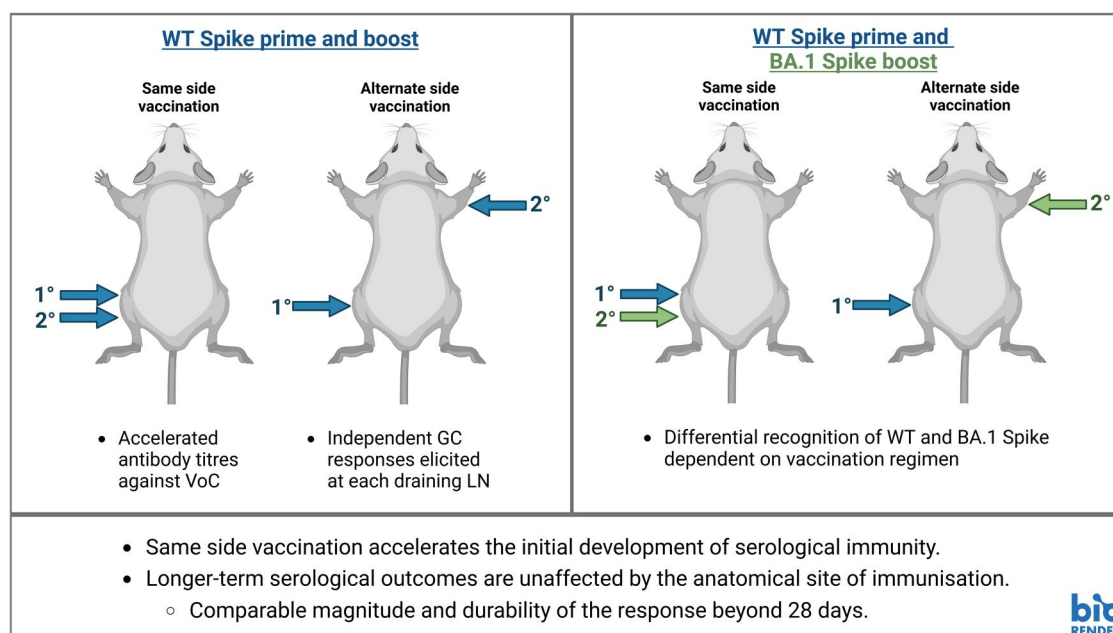
*Corresponding authors: Department of Microbiology and Immunology, University of Melbourne, Peter Doherty Institute for Infection and Immunity, Melbourne, Victoria, Australia. Emails: hxtan@unimelb.edu.au (Hyon-Xhi Tan) and jennifer.juno@unimelb.edu.au (Jennifer A. Juno).

Abstract

Human clinical trials have reported immunological outcomes can differ between ipsilateral (same side) and contralateral (alternate sides) prime-boost vaccination. However, our mechanistic understanding of how keeping or shifting the anatomical sites of immunization impacts the resultant germinal centers (GCs) and antibody responses is limited. Here, we use an adjuvanted SARS-CoV-2 spike vaccine to dissect GC dynamics in draining lymph nodes and serological outcomes following ipsilateral or contralateral prime-boost vaccination in C57BL/6 mice. Contralateral vaccination elicited independent GCs at distinct lymph nodes, where robust secondary GCs only appeared upon secondary distal vaccination, while ongoing GCs from the primary site were not boosted. In contrast, ipsilateral vaccination resulted in sustained GC activity. Ipsilateral vaccination accelerated the development of antibody titers against ancestral (wild-type [WT]), Beta, and BA.1 but were later comparable between ipsilateral and contralateral groups in terms of magnitude, durability, and neutralization capacity beyond 28 d. Using a heterologous SARS-CoV-2 WT/BA.1 spike prime-boost model, cross-reactive GC responses were generated against WT and BA.1 spike, with analogous serological and GC dynamics to our homologous model. Within the cross-reactive GC B cells, differential recognition of WT and BA.1 antigens was observed and were further compartmentalized in primary or secondary GCs, depending on ipsilateral or contralateral regimes. Collectively, maintaining a common prime-boost site augments the kinetics of memory B cell recall and transiently drive higher antibody titers, but longer-term serological outcomes are unaffected by the anatomical localization of immunization.

Keywords: B cells, germinal centers, ipsilateral/contralateral, SARS-CoV-2, vaccination

Graphical abstract



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Introduction

The generation of durable and potent neutralizing antibodies is a key mechanism by which vaccines provide protection from infectious diseases. Vaccine-specific immune responses are initiated in the draining lymph nodes (LNs) proximal to the site of administration, where ongoing germinal center (GC) reactions program the quality and magnitude of the humoral immune response. The majority of adult and adolescent vaccines are administered intramuscularly into the deltoid muscles of the upper arm.^{1,2} The injection site for a given vaccine is generally left to the discretion of the vaccinee or the physician, with a majority of individuals choosing to be vaccinated in the same nondominant arm.³ Nevertheless, for multidose vaccines, administering the second or subsequent doses of vaccine antigens to either the same or distant LN could drive divergent GC and humoral responses. A detailed understanding of how immunization site impacts the adaptive immune response is required to optimize vaccination schedules and maximize the induction of protective immunity.

Human clinical studies have reported contrasting results on the immunological outcome of ipsilateral (same side) versus contralateral (alternate sides) vaccination, with early studies suggesting that the serological impact was vaccine dependent.^{4,5} The SARS-CoV-2 pandemic has afforded a unique opportunity to assess both the immunology and real-world impact of ipsilateral versus contralateral immunization on spike (S)-specific antibody responses. A retrospective cohort of 2,678,226 participants reported increased protection against acquisition of SARS-CoV-2 in the first month following ipsilateral vaccination relative to the contralateral group,³ suggesting increased vaccine effectiveness and a clinical benefit for ipsilateral immunization. However, immunological studies have presented contrasting findings. In one study, antibody titers and CD4 T cell responses were comparable, while ipsilateral boosting resulted in higher neutralizing titers and S-specific CD8 T cell responses.⁶ In contrast, other studies showed no impact on S-specific IgG titers after 1.5 mo, but found that subjects receiving contralateral vaccination had higher titers after 8 mo of follow-up.⁷

Owing to the challenges in directly sampling human lymphoid tissues, assessment of GC dynamics has relied largely on animal models. In some reports, ipsilateral vaccination in mouse models drove higher antigen-specific IgG titers,⁸ but others have reported more qualitative changes such as increased frequencies and avidity of antigen-specific GC B cells⁹ or greater antibody breadth to SARS-CoV-2 variants.¹⁰ In the context of contralateral vaccination, antigen administered at a distal site did not alter the ongoing GC response in the primary draining LN; rather, reimmunization of the same site modulated the composition of follicular helper T and B cells in the ongoing GC.^{9,10} A detailed and concurrent longitudinal assessment of primary and secondary GC dynamics is required to clarify the currently contradictory data surrounding ipsilateral and contralateral vaccine regimens.

Here, we investigate how ipsilateral or contralateral vaccination impacts GC and serological responses to vaccination with the SARS-CoV-2 S protein. Overall, we find that primary and secondary GC dynamics were influenced by the choice of vaccination site. Ipsilateral vaccination transiently drove serological differences early postboost, suggesting that recall via local B cell memory augmented the kinetics of antibody production early

after the second dose. However, longer-term serological outcomes were ultimately unaffected by whether the immunization sites were maintained or switched. Similarly, ipsilateral vaccination with heterologous ancestral wild-type (WT)/BA.1 antigens transiently improved serological breadth and elicited cross-reactive GC B cells. These cross-reactive B cells displayed contrasting recognition of ancestral WT and BA.1 antigens, alongside distinct compartmentalization in primary or secondary GCs driven by ipsilateral or contralateral vaccination. Importantly, our findings have implications for multidose vaccination schedules, highlighting ipsilateral immunization as an optimal strategy to rapidly maximize humoral immunity on a population level during the early phases of an infectious disease outbreak.

Materials and methods

Expression of SARS-CoV-2 S proteins

Recombinant SARS-CoV-2 S or RBD proteins of WT Wuhan Hu-1, Beta, and Omicron BA.1, including a HexaPro ectodomain,¹¹ AviTag, and His-tag, were expressed in Expi293F cells (Life Technologies) and purified by Ni-NTA and size-exclusion chromatography, as previously described.¹² For use as flow cytometry probes, S proteins were biotinylated using BirA (Avidity) and labeled with the sequential addition of streptavidin R-phycoerythrin (PE) conjugate or streptavidin allophycocyanin (APC) conjugates (Thermo Fisher Scientific).

Murine immunizations and euthanasia

Mouse studies were approved by the University of Melbourne Animal Ethics Committee (nos. 21237 and 22954). For visual identification of draining LNs, 0.5% tattoo ink was coformulated at 1:1 ratio with Addavax adjuvant (InvivoGen) in a total volume of 50 μ L, as previously described.¹³ A total of 3 μ g of SARS-CoV-2 WT or Omicron variant BA.1 S protein were formulated in phosphate-buffered saline (PBS) at 1:1 ratio with Addavax adjuvant in a total volume of 50 μ L. C57BL/6 mice aged 6 to 12 wk were anesthetized to the required depth (i.e., hind legs do not move in response to light pressure) by isoflurane inhalation prior to intramuscular vaccination, with oxygen flow at 2 L/min and isoflurane vaporizer set to 3 (Stinger Anaesthetic Machine). Once anesthetized, animals are immunized with 50 μ L of vaccine to the injection site using a 29G needle (left quadriceps muscle or right deltoid muscle). Prior to tissue collection, mice were killed using CO₂ asphyxiation using a delivery of 50% of chamber volume per minute, followed by the secondary method of killing using cervical dislocation.

Enzyme-linked immunosorbent assay

IgG antibody binding to the SARS-CoV-2 S protein was assessed using enzyme-linked immunosorbent assay. The 96-well MaxiSorp plates (Thermo Fisher Scientific) were coated with 2 μ g/mL of recombinant SARS-CoV-2 RBD or S (WT, Beta, or Omicron BA.1) proteins overnight at 4 °C. Plates were washed with PBS with Tween 20 and PBS and blocked with PBS + 1% fetal calf serum (FCS) for 1 h at room temperature (RT). Serum was serially diluted (4-fold dilution, from 1:100 to 1:1,638,400) and added to the plate for 2 h at RT. Plates were washed prior to incubation with horseradish peroxidase-conjugated secondary antibody (1:20,000; anti-mouse IgG; KPL). Plates were washed before developing using TMB substrate (Sigma-Aldrich). The reaction was stopped using sulfuric acid

(0.16M), and optical density (OD) values were determined at 450 nm. Endpoint titers were determined using GraphPad Prism (v9.4.1; GraphPad Software) by assessing the intersection of 2× background with the reciprocal plasma/serum dilution on a fitted curve 4-parameter log regression.

Neutralization assay

A surrogate virus neutralization test (sVNT) utilizing SARS-CoV-2 RBD and human ACE2 interaction was performed as previously described.¹² The 96-well MaxiSorp plates were coated with 2 µg/mL of recombinant RBD protein overnight at 4 °C. Plates were washed with PBS with Tween 20 and PBS before being blocked with 1% bovine serum albumin (BSA) buffer for 1 h at RT. Plasma/serum was diluted in 0.1% BSA buffer in duplicates (3-fold dilution, 1:25 to 1:54,675). Plates were washed before plasma/serum dilutions were incubated for 1 h at RT. “No antibody” and “no RBD” controls were included to represent 100% and 0% inhibition by hACE2, respectively. Plates were washed, and 1 µg/mL of biotinylated hACE2 in 0.1% BSA buffer was added for 1 h at RT. After washing, plates were incubated with HRP-Streptavidin (Thermo Fisher Scientific; 1:10,000 dilution used) in 0.1% BSA buffer for 1 h at RT. Plates were washed before developing using TMB substrate (Sigma-Aldrich) and stopped using sulfuric acid (0.16 M), before OD values were determined at 450 nm. OD values were used to calculate % neutralization using the following formula: (“No antibody” – “Sample”) / (“No RBD” – “Sample”) × 100. Half maximal inhibitory concentration values (antibody concentration needed to reduce ACE2 binding to RBD by 50%) were determined using GraphPad Prism (v9.4.1) by constraining curves between 0% and 100% neutralization.

Live SARS-CoV-2 virus neutralization assays were performed as previously described.¹⁴ The ancestral SARS-CoV-2 (VIC01) isolate was grown in Vero cells in serum-free Dulbecco's modified Eagle's medium (DMEM) with TPCK trypsin (1 µg/mL), while the Omicron BA.1 strain was grown in Calu3 cells in DMEM with 2% FCS. Cell culture supernatants containing infectious virus were harvested on day 3 for VIC01 and on day 4 for Omicron strains, clarified via centrifugation, filtered through a 0.45 µm cellulose acetate filter, sequenced, titrated, and stored at –80 °C. In 96-well flat-bottom plates, heat-inactivated plasma samples were diluted 2.5-fold (1:20 to 1:12,207) in duplicate and incubated with SARS-CoV-2 virus at a final concentration of 2× median infectious dose at 37 °C for 1 h. Next, 30,000 freshly trypsinized HAT-24 cells in DMEM with 5% FCS were added and incubated at 37 °C. “Cells only” and “virus + cells” controls were included to represent 0% and 100% infectivity, respectively. After 46 h, 10 µL of alamarBlue Cell Viability Reagent (Thermo Fisher Scientific) was added into each well and incubated at 37 °C for 1 h. The reaction was then stopped with 1% sodium dodecyl sulfate and read on a FLUOstar Omega plate reader (excitation wavelength, 560 nm; emission wavelength, 590 nm). The RFU measured were used to calculate % neutralization with the following formula: (sample – virus + cells) ÷ (cells only – virus + cells) × 100. Half maximal inhibitory concentration values were determined using 4-parameter nonlinear regression in GraphPad Prism with curve fits constrained to have a minimum of 0% and maximum of 100% neutralization.

Flow cytometric detection of murine B cells

Mouse LNs were harvested into RF10 media (RPMI 1640, 10% FCS, 1× penicillin-streptomycin-glutamine; Life Technologies). Single-cell suspensions were prepared by mechanical dissociation through a 70 µm cell strainer. Cells were stained with Aqua viability dye (Thermo Fisher) and Fc-blocked with anti-CD16/32 antibody (clone 93; BioLegend). Cells were then labeled with PE- and APC-conjugated S probes and the following antibodies: F4/80 BV786 (BM8; BioLegend; 1:150), CD3 BV786 (145-2C11; BioLegend; 1:750), streptavidin BV786 (BD), CD45 APC-Cy7 (30-F11; BD; 1:300), CD38 PeCy7 (90; BioLegend; 1:750), GL7 AF4788 (GL7; BioLegend; 1:300), IgD BUV395 (11-26c.2a; BD; 1:300), B220 BUV737 (RA3-6B2; BD; 1:300), CD98 BV711 (H202-141; BD; 1:100), and CD138 BV421 (281-2; BD; 1:100). Cells were washed twice with PBS + 1% FCS and fixed using 1% formaldehyde (Polysciences) and acquired on a BD LSRFortessa using BD FACSDiva. Flow cytometry data were processed using FlowJo v10 (TreeStar).

Single-cell sorting and B cell receptor sequencing

Isolated lymphocytes were stained with Aqua viability dye (Thermo Fisher Scientific) and Fc-blocked with anti-CD16/32 antibody (clone 93; BioLegend). Cells were then labeled with PE- and APC-conjugated S probes and the following antibodies: F4/80 BV786 (BM8; BioLegend; 1:150), CD3 BV786 (145-2C11; BioLegend; 1:750), SA BV786 (BD), CD45 APC-Cy7 (30-F11; BD; 1:300), CD38 PeCy7 (90; BioLegend; 1:750), GL7 AF488 (GL7; BioLegend; 1:300), IgD PerCPCy5.5 (11-26c.2a; BD; 1:300), IgM BV421 (II/41; BD; 1:300), and B220 BV650 (RA3-6B2; BD; 1:300). Stained cells were resuspended in OptiMEM (Thermo Fisher Scientific) and S-positive IgD– B cells were sorted into 96-well plates using BD FACSAria III Cell Sorter and frozen before complementary DNA extraction. Murine heavy chain immunoglobulin sequencing was performed, as previously described.^{12,15} Briefly, complementary DNA was prepared using SuperScript III Reverse Transcriptase (Life Technologies) and random hexamer primers (Life Technologies). Primary and secondary polymerase chain reaction reactions were performed using HotStar Taq polymerase (Qiagen) and multiplex primers binding V-gene leader sequences or the immunoglobulin constant regions. Secondary polymerase chain reaction products underwent Sanger sequencing (Macrogen), and VDJ recombination was analyzed using IMGT¹⁶ to assess V-region identity.

Statistics

Data are presented as median ± interquartile range or range. Statistical significance was assessed by Kruskal-Wallis or Mann-Whitney *U* tests. Curve fitting was performed using 4-parameter logistic regression. All statistical analyses were performed using GraphPad Prism. Flow cytometry data was analyzed in FlowJo v10.

Results

Antigen from intramuscular injections drains only to local LNs and initiates independent GCs

We established a model of ipsilateral or contralateral intramuscular vaccination in C57BL/6 mice. Given the possibility of cross-drainage from both hindlimbs to the iliac LNs, we compared drainage patterns after immunization of the left hindlimb quadriceps and the right forelimb triceps brachii

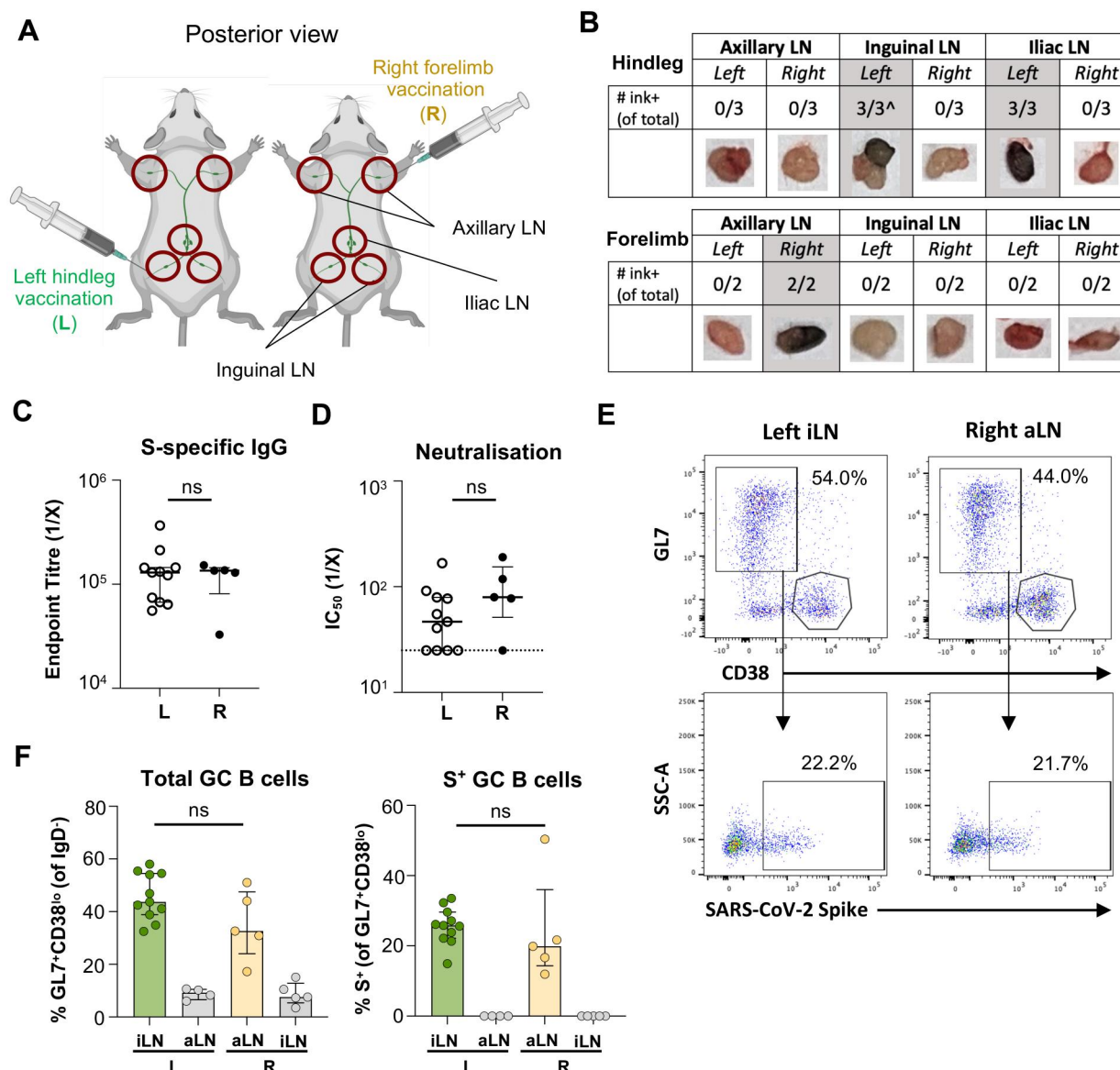


Figure 1. Validation of a mouse model for distal vaccination sites. (A) Schematic of vaccine administration and LN collection for comparison of left hindleg and right forelimb immunization. (B) Enumeration of ink-stained LNs at day 3 postvaccination in left hindleg ($n=3$) or right forelimb ($n=2$) of immunized animals. [^] Full or partial ink staining. (C) S-specific IgG or (D) sVNT neutralizing titers at day 14 postvaccination with 3 μ g SARS-CoV-2 S protein formulated with Addavax (left hindleg, $n=11$; right forelimb, $n=5$). The dashed line indicates limit of detection. (E) Representative staining of GC B cells ($GL7^+CD38^{lo}$) or MBCs ($CD38^+GL7^{lo}$), and S-specific GC B cells in vaccine-draining LNs at day 14 postvaccination. (F) Total and S-specific GC B cell frequencies at day 14 postvaccination (left iLN, $n=11$; left aLN, $n=4$; right aLN, $n=5$; right iLN, $n=5$). Bars/lines indicate median with interquartile range. Statistics assessed by Mann-Whitney test. ns, not significant; SSC-A, side scatter area.

(Fig. 1A). Three days after intramuscular injection with a mock vaccine coformulated with tattoo ink (as in Barber-Axthelm et al.),¹³ LNs draining these injection sites were collected and imaged for ink staining (Fig. 1B). Hindlimb-injected mice showed tattoo ink accumulation in the left iliac and inguinal LNs (iLNs), as previously reported¹³ but not the right iLNs or axillary LNs (aLNs). In contrast, forelimb-injected mice displayed tattoo ink accumulation only in the right aLNs.

We next assessed whether each injection site elicited comparable serological and GC responses 14 d after vaccination with SARS-CoV-2 ancestral (WT) S protein. Immunized mice exhibited similar levels of anti-S IgG (Fig. 1C) and neutralizing titers (determined by sVNT) (Fig. 1D) irrespective of injection site. Similar total and S-specific (S^+) GC B cell ($GL7^+CD38^{lo}$) responses were detectable in both LNs (left iLN or right aLN) (Fig. 1E, F; gating in Fig. S1A), with no

evidence of antigen-specific immune responses at distal sites (Fig. 1F; Fig. S1B). Overall, left hindleg and right forelimb vaccination represents a tractable model to compare immunological responses to ipsilateral and contralateral immunization, with no cross-drainage of antigen and comparable site-specific vaccine responsiveness.

Ipsilateral immunization maintains the primary GC and rapidly boosts serum antibody titers

To assess the impact of immunization site on humoral immunity, mice were immunized with SARS-CoV-2 WT S antigen 14 d apart either ipsilaterally (twice immunized on the left hindleg) or contralaterally (sequential left hindleg and right forelimb immunization) (Fig. 2A). In this model, the first vaccine dose establishes “primary” GCs in the iLNs. Any GCs established by the second vaccine are referred to as

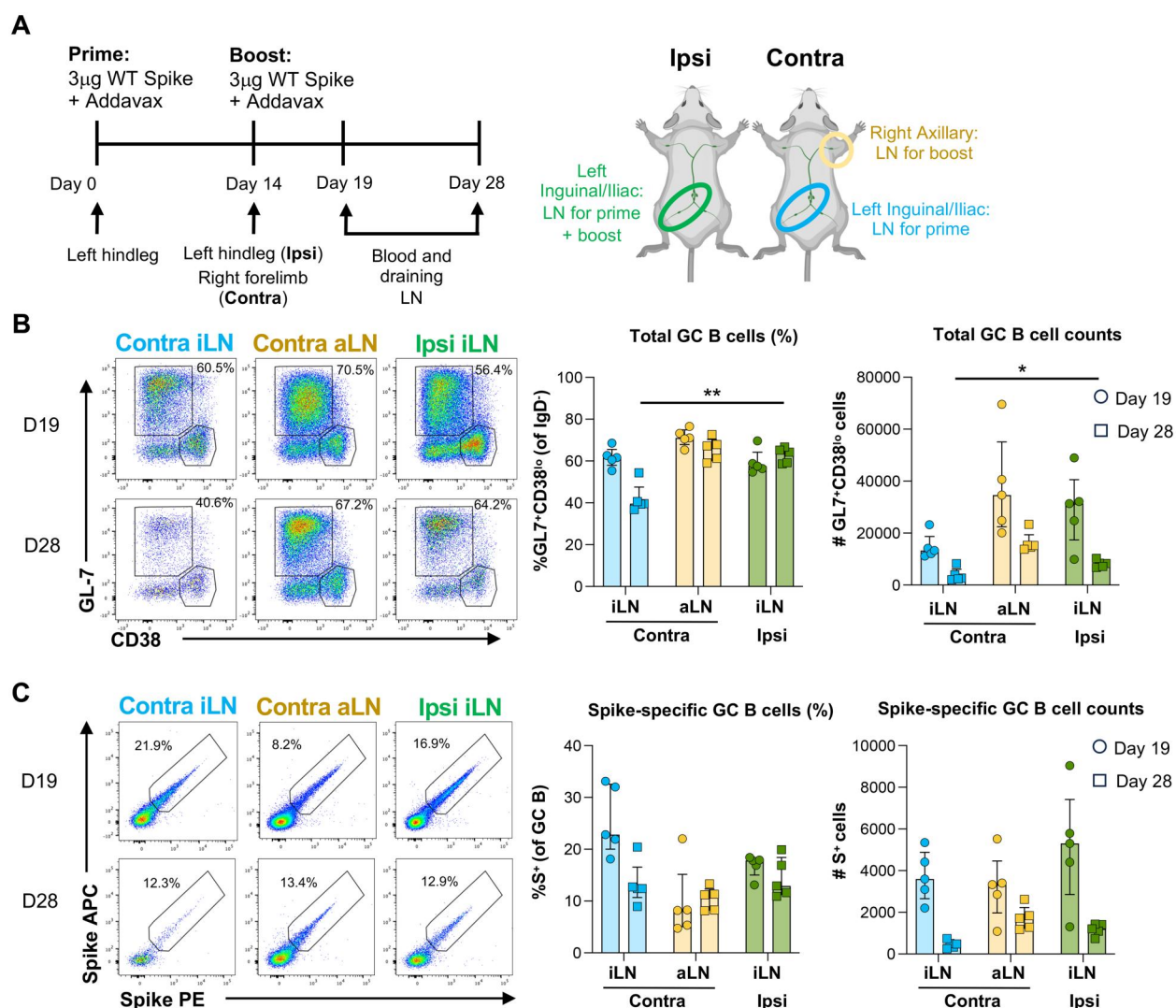


Figure 2. GC dynamics following ipsilateral or contralateral prime-boost vaccination. (A) C57BL/6 mice received 2 doses of SARS-CoV-2 S protein 2 wk apart either ipsilaterally ("ipsilateral") or contralaterally ("contralateral"). Blood and draining LNs were collected at day 19 or 28. Inguinal and iliac LNs draining the left hindleg injection site were pooled (iLNs; green or blue), and the right aLN draining the forelimb injection site was analyzed separately (aLN; yellow). (B) Representative staining and quantification of total GC B cells (GL7⁺CD38⁺) or (C) S-specific GC B cells at days 19 and 28 ($n = 5$ per group). Bars indicate median and IQR. * $P < 0.05$, ** $P < 0.01$, statistics assessed by Mann-Whitney test.

"secondary" GCs, either in the aLNs (contralateral group) or the iLNs (ipsilateral group). GC B cells and memory B cells (MBCs) (CD38⁺GL7^{lo}) were assessed at days 19 and 28 (gating in Fig. S2A).

Following contralateral vaccination, the primary GC response (contralateral iLNs) contracted both in frequency and absolute number between days 19 and 28 (Fig. 2B), while a robust secondary GC was seeded in the aLNs (contralateral aLNs) (Fig. 2B). At day 28, but not at day 19, we observed higher frequency (median 39.6% in contralateral iLNs versus 64.2% in ipsilateral iLNs at day 28) and number (median 2685 cells in contralateral iLNs versus 8,323 cells in ipsilateral iLNs at day 28) of total GC B cells in the iLNs of the ipsilateral group compared with the iLNs of the contralateral group (Fig. 2B), suggesting that the provision of antigen twice to the same site may possibly affect total GC B cell dynamics through sustained GC responses. In total, however, contralateral vaccination resulted in a greater number of GC B cells

across all LNs at both day 19 (median 13,284 cells in contralateral iLNs + 34,632 cells in contralateral aLNs versus 31,359 cells in ipsilateral iLNs) and day 28 (median 2,685 cells in contralateral iLNs + 15,259 cells in contralateral aLNs versus 8,323 cells in ipsilateral iLNs) (Fig. 2B).

Analysis of S-specific GC B cells highlighted that, despite similar overall GC size, there were lower frequencies and counts of S-binding B cells in the contralateral secondary GCs (contralateral aLNs) compared with the ipsilateral GCs at day 19 (median 8.2% versus 17.8%) (Fig. 2C). By day 28, S⁺ GC B cell frequencies and counts were comparable across these sites (median 11.2% in contralateral aLNs, 12.9% in ipsilateral iLNs) (Fig. 2C). Notably, the low frequency of S⁺ GC B cells in the contralateral aLNs at day 28 was only observed in the context of a secondary GC; primary vaccination at that site elicited substantially higher frequencies of S-specific GC B cells (median 19.9%) (Fig. 1E, F), suggesting that antibody feedback from the initial vaccination could be

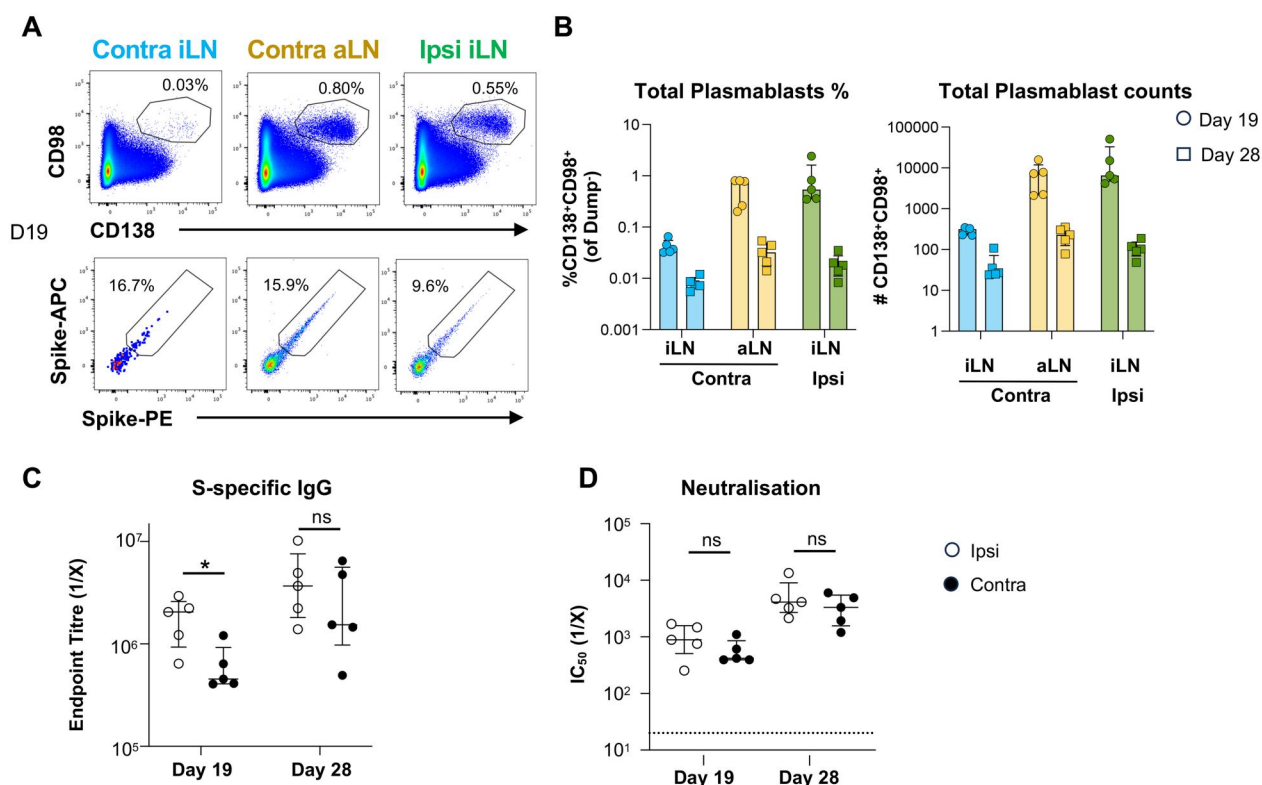


Figure 3. Plasmablast and serological responses to ipsilateral and contralateral vaccination. (A) Representative frequencies of total (CD98⁺CD138⁺) and S-specific plasmablasts at day 19 in draining LNs. (B) Total plasmablast frequencies at day 19 and 28 ($n = 5$ for all groups, except $n = 4$ for contralateral iLN at day 28). (C) S-specific serum IgG titers at days 19 and 28 ($n = 5$ per group). (D) sVNT neutralizing titers at days 19 and 28 ($n = 5$ per group). Bars/lines represent median and interquartile range. Statistics assessed by Mann-Whitney test. * $P < 0.05$. D, day; ns, not significant.

influencing the distal secondary GCs.^{17–19} Similar frequencies of S⁺ MBCs were established in all LNs at the day 19 and 28 time points (Fig. 52B).

The second vaccine dose elicited similar frequencies and counts of total plasmablasts (CD98⁺CD138⁺) at day 19 in both draining LNs (contralateral aLNs and ipsilateral iLNs) (Fig. 3A, B); however, anti-S IgG titers were significantly higher in ipsilaterally boosted animals (median 2.05×10^6 versus 0.45×10^6 ; $P = 0.02$) (Fig. 3C). By day 28, titers were generally similar between both groups ($P = 0.55$) (Fig. 3C). The higher levels of binding antibodies at day 19 in the ipsilateral group did not correspond to increased neutralizing activity, with sVNT titers at both time points comparable between groups (Fig. 3D). Collectively, these data suggest that primary and secondary GC dynamics differ based on choice of vaccination site, and ipsilateral immunization drives a more rapid increase in IgG titers compared with contralateral immunization.

The quality and durability of the antibody response is comparable between contralateral and ipsilateral vaccine regimens

Ipsilateral and contralaterally vaccinated animals were longitudinally sampled over 121 d to assess the durability of anti-S IgG (Fig. 4A) and neutralizing (Fig. 4B) titers. No significant differences in serological responses between the 2 groups were observed, suggesting that early changes in GC and antibody kinetics did not markedly impact long-term serological outcomes. Nonetheless, considering that antigen availability is critical in driving somatic hypermutation (SHM) and affinity maturation within the GC, provision of antigen into

existing GCs during ipsilateral immunization may elevate antibody maturation without impacting IgG titers.^{20,21} Therefore, we assessed qualitative aspects of the response by examining serum antibody breadth against SARS-CoV-2 variants and SHM within S-specific B cells.

Serum endpoint IgG titers were generated against WT, Beta, or Omicron BA.1 RBDs to assess the breadth of the humoral response. At day 19, ipsilateral immunization induced significantly higher titers against the WT RBD compared with contralateral boosting ($P = 0.02$), and proportionately higher titers to Beta and BA.1 ($P < 0.001$ and $P = 0.009$, respectively) (Fig. 4C). At day 28, WT and BA.1 serum titers were similar between groups, with only Beta-specific titers remaining significantly higher in ipsilateral-immunized animals ($P = 0.005$) (Fig. 4D), and by day 121, binding titers were comparable across all RBDs (Fig. 4E).

To assess the gain of somatic mutations within B cell receptors, S-specific GC B cells and MBCs were single-cell sorted and sequenced for heavy chain immunoglobulin genes. At days 42 and 98, V-gene somatic mutation frequencies were comparable across all vaccination conditions (Fig. 4F). Taken together, ipsilateral immunization resulted in accelerated boosting of S-specific antibodies at day 19 without substantially impacting long-term serum titers, antibody breadth, or SHM as compared with contralateral-vaccinated mice.

Heterologous boosting with a SARS-CoV-2 BA.1 variant antigen alters the specificities of B cells in both primary and secondary GCs

Previous studies show that ipsilateral sequential vaccination with heterologous influenza HA (H1 and H3) antigens

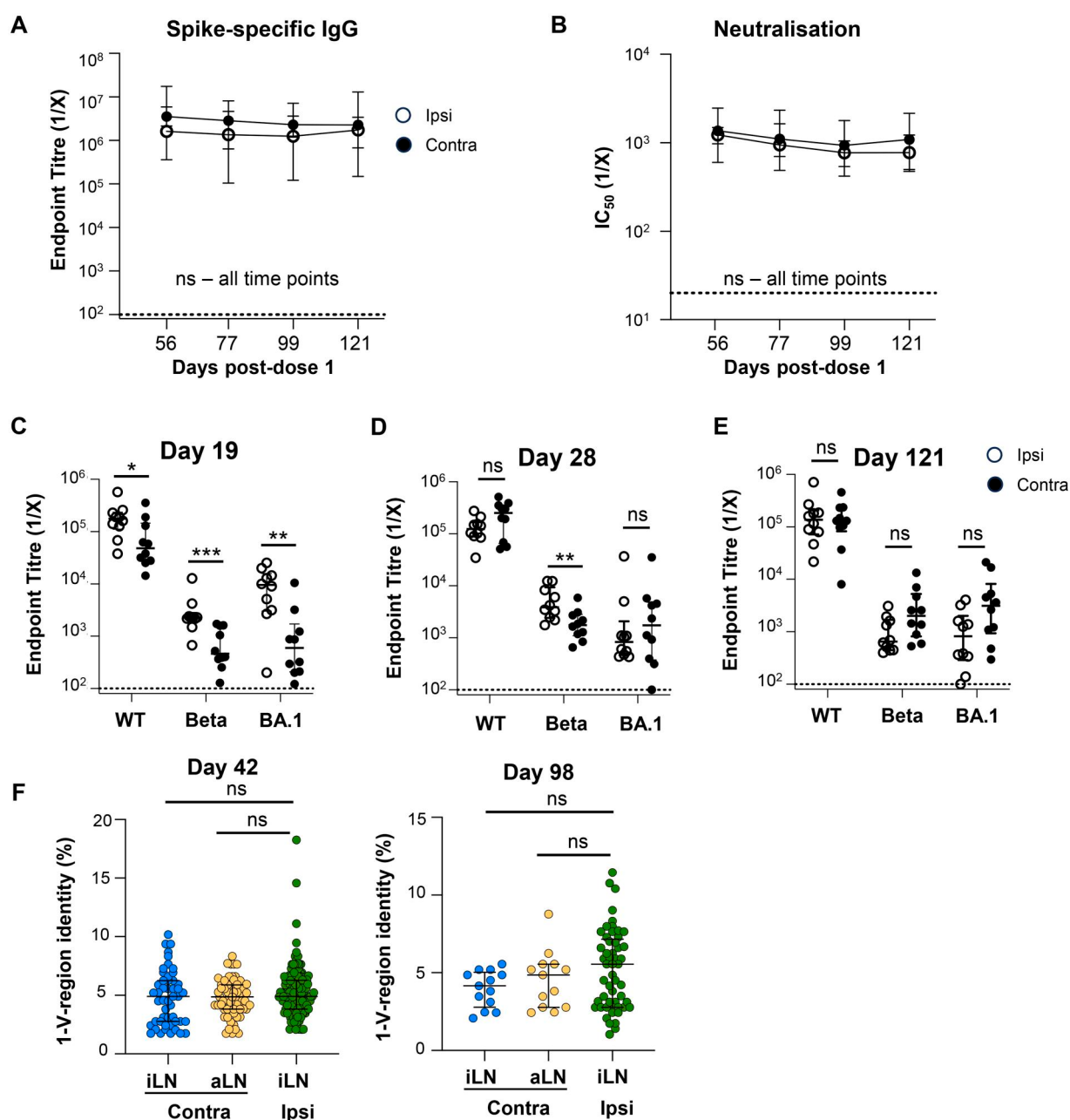


Figure 4. Durability, breadth, and SHM of the humoral response following immunization. (A) S-specific IgG and (B) sVNT neutralizing titers in serially sampled animals after ipsilateral (open circles) or contralateral (closed circles) immunization ($n = 10$ per group). (C–E) Serum antibody titers were measured against SARS-CoV-2 RBD antigens from WT, Beta, or BA.1 strains. Animals were serially sampled at (C) day 19, (D) day 28, or (E) day 121 ($n = 10$ per group). (F) V-gene somatic mutations were quantified in heavy chain immunoglobulin sequences of IgD⁺ S-specific B cells from LNs at days 42 and 98. Points indicate median with interquartile range. Statistics assessed by (A–E) Mann-Whitney or (F) Kruskal-Wallis test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. IC₅₀, half maximal inhibitory concentration; ns, not significant.

elicited a higher proportion of GC B cells with cross-reactive (H1⁺H3⁺) specificities and greater fold increase in serum IgG titers against H1 and H3.⁹ Therefore, we assessed if boosting ipsilaterally or contralaterally with a heterologous SARS-CoV-2 BA.1 variant S would influence the B cell and humoral responses in mice primed with WT S (Fig. 5A). Immunization seeded robust GC responses within all LNs by day 19, with contraction evident by day 28 in the contralateral group ($P < 0.0001$ for day 19 vs day 28 in both contralateral iLNs and aLNs) (Fig. 5B). S-specific B cells were identified using WT or BA.1 S probes (Fig. 5C). In the contralateral group,

the total frequency of S-specific GC B cells was significantly reduced in the secondary GC (contralateral aLNs) at day 19 ($P = 0.003$ vs contralateral iLNs, $P = 0.001$ vs ipsilateral iLNs) (Fig. 5C), similar to our prior observations with homologous boosting. Within S-specific GC B cells, most were cross-reactive (WT⁺BA.1⁺), with a smaller population of WT monospecific cells (WT⁺BA.1⁻) and minimal evidence for the generation of BA.1 monospecific cells (WT⁻BA.1⁺) (Fig. 5C). The low frequency of S-specific GC B cells in contralateral aLN secondary GCs was reflected by both a paucity of WT⁺BA.1⁻ (Fig. S3A) and WT⁺BA.1⁺ cross-reactive cells

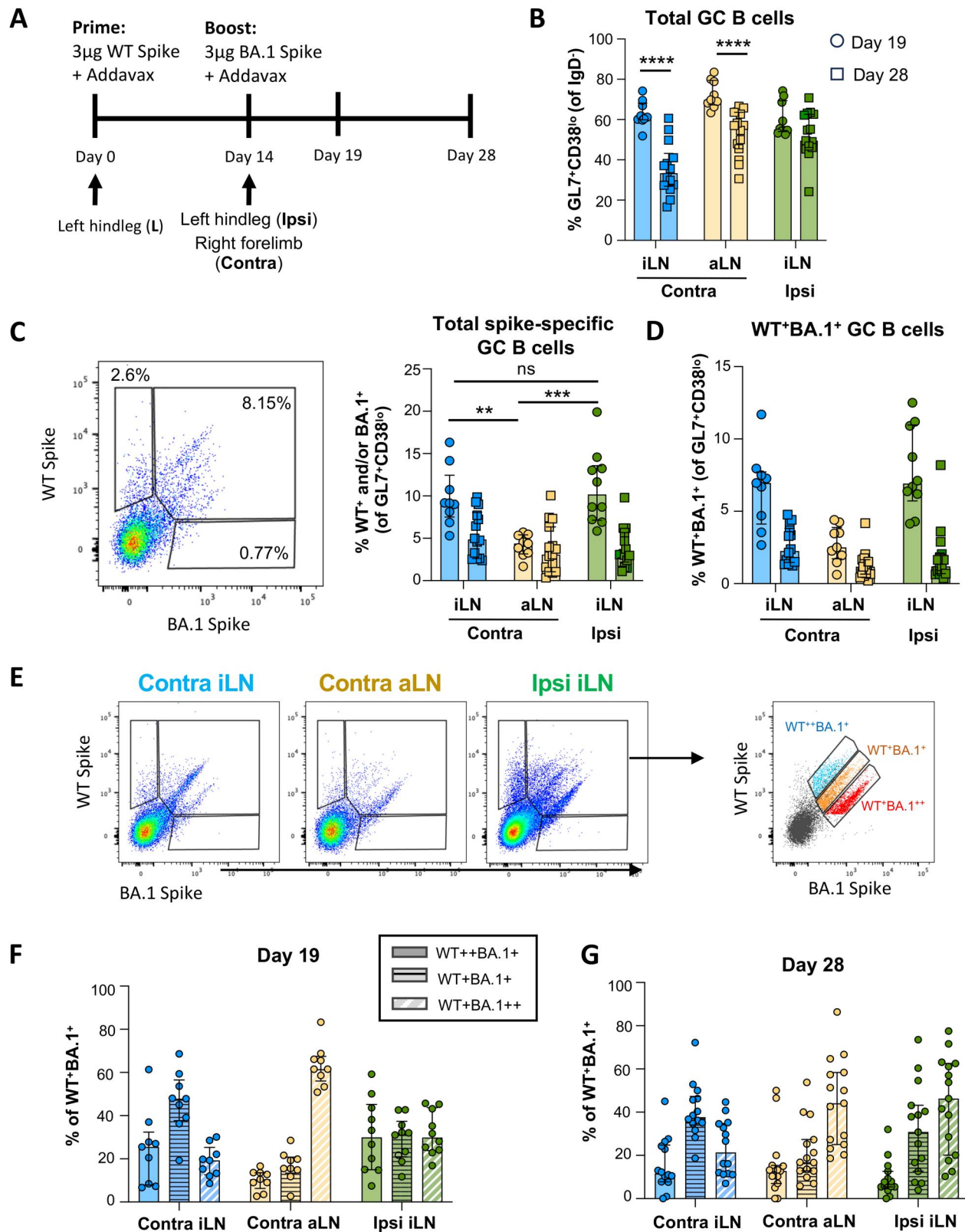


Figure 5. GC dynamics following ipsilateral or contralateral heterologous vaccination. (A) C57BL/6 mice received a SARS-CoV-2 WT S prime vaccination followed by a BA.1 S boost 2 wk apart either ipsilaterally or contralaterally. Blood and draining LNs were collected at day 19 or 28. Quantification of (B) total GC B cells (GL7⁺CD38^{lo}), (C) total S-specific B cells (WT⁺ and/or BA.1⁺), or (D) cross-reactive (WT⁺BA.1⁺) GC B cells at days 19 ($n = 9$ per group) and 28 ($n = 15$ per group). (E) Representative staining and (F-G) quantification of distinct cross-reactive GC B cell populations comprising WT⁺⁺BA.1⁺ (WT biased), WT⁺BA.1⁺ (intermediate), or WT⁺BA.1⁺⁺ (BA.1 biased) at days 19 ($n = 9$ per group) and 28 ($n = 15$ per group). Bars indicate median and interquartile range. Statistics assessed by (B) Mann-Whitney or (C) Kruskal-Wallis test. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ns, not significant.

(Fig. 5D). S-specific MBCs were generally comparable across groups. At day 28, the frequencies of WT monospecific or WT⁺BA.1⁺ cross-reactive MBCs were similar between contralateral- and ipsilateral-vaccinated animals (Fig. S3B). Cross-reactive B cells formed the major population of S-specific MBCs, with few BA.1 monospecific cells identified.

Interestingly, we noted that within the WT⁺BA.1⁺ gate, at least 3 distinct populations of S-specific cells could be identified based on differential binding to the WT and BA.1 probes (Fig. 5E), which we gated as WT⁺⁺BA.1⁺ (WT biased), WT⁺BA.1⁺ (intermediate), or WT⁺BA.1⁺⁺ (BA.1 biased). At day 19, each LN exhibited a unique pattern of GC B cell specificities (Fig. 5F), with the primary contralateral iLNs dominated by WT⁺BA.1⁺ cells, the secondary contralateral aLNs dominated by BA.1-biased responses, and the ipsilateral iLNs exhibiting comparable proportions of all 3 populations. At day 28, these hierarchies were maintained in the contralateral iLNs and aLNs (Fig. 5G), while in the ipsilateral iLNs, WT⁺BA.1⁺⁺ became predominant over time, suggesting a changing of the composition of the GC B cell population to favor the heterologous booster antigen. With BA.1-biased populations emerging equally by day 28 in ongoing (ipsilateral) or de novo (contralateral) secondary GCs, this also suggests that activation of BA.1-biased GC B cells from naïve precursors are not inhibited by WT-biased GC populations previously established by the ipsilateral primary vaccine.

Taken together, heterologous antigen boosting, irrespective of contralateral or ipsilateral vaccination, elicited a predominant cross-reactive GC B cell population against WT and BA.1 S, with analogous GC dynamics to the prior homologous vaccination. Within cross-reactive primary and secondary GC B cells, differential recognition of WT and BA.1 antigens emerges upon ipsilateral or contralateral vaccination, influenced by the antigen most recently drained within the GC.

Ipsilateral delivery of heterologous booster antigen improves serological breadth

In the context of heterologous antigen boosting, we saw clear yet transient alteration in the balance of antigen specificities of GC B cells within the vaccine draining LNs. We therefore longitudinally assessed anti-WT and anti-BA.1 RBD binding IgG and neutralizing antibody titers after ipsilateral or contralateral vaccination. At day 19, ipsilateral vaccination elicited significantly higher anti-WT ($P < 0.05$) and anti-BA.1 ($P < 0.05$) RBD IgG titers, but these titers became comparable by day 28 (Fig. 6A), consistent with the ipsilateral homologous vaccination model (Fig. 4C, D). These animals were longitudinally bled to assess the kinetics of anti-WT and anti-BA.1 RBD IgG over time up to day 121 (Fig. 6B). Significantly higher anti-WT titers were observed at most time points for ipsilaterally vaccinated animals, with the exception of day 99, while anti-BA.1 titers were comparable in titers at all time points up to day 121. Notably, irrespective of injection site, boosting with a BA.1 antigen increased overall anti-BA.1 titers at day 28 compared with homologous WT boosting (Fig. S3C). Neutralizing activity against live WT or BA.1 virus in the ipsilateral group at day 28 was comparable between ipsilateral or contralateral heterologous WT/BA.1 S-vaccinated animals (Fig. 6C). Overall, we found that ipsilateral immunization drives a transient increase in anti-BA.1 antibodies early after heterologous boosting but with

minimal impact on long-term serological and neutralizing outcomes.

Discussion

Our study details GC dynamics and serological responses following ipsilateral or contralateral prime-boost vaccinations with SARS-CoV-2 S. Overall, maintaining or alternating immunization sites during multidose immunization regimens appears to minimally impact longer-term serological and neutralizing responses. However, ipsilateral vaccination can drive accelerated antibody production in the short term and alter the balance of specificities of GC B cells draining vaccination sites. Our data align with clinical studies demonstrating higher neutralizing antibody titers at 2 wk and increased vaccine effectiveness within the first month postboost in large cohorts.^{3,6} Specifically, our findings suggest that this increased protection may be a result of accelerated antibody production induced by ipsilateral vaccination. Collectively, this suggests that in settings of early outbreaks in which rapid boosting of humoral immunity is paramount, an ipsilateral regimen would be favored in multidose immunization schedules.

Our observation that long-term antibody titers are comparable after ipsilateral or contralateral boosting is consistent with recent fate mapping studies of serum antibody responses. In the context of homologous prime-boost vaccination, most serum antibody elicited by the boost is derived from MBCs established during primary antigen exposure, with little contribution from de novo responses in secondary GCs.²² Therefore, it follows that the location in which secondary GCs are established (the original LN or a distal site) does not markedly impact serological responses postboost. What does modulate the response, however, could be the presence of antigen-specific B and/or T cells residing in local LN niches. The accelerated serological response in ipsilateral vaccination likely stems from specialized MBCs resident within the subcapsular niche of antigen-experienced LNs, which rapidly differentiate into plasma cells upon reencountering antigen.²³ Complementing this, memory CD4 T cells resident within secondary lymphoid organs are poised to facilitate rapid formation of secondary GCs upon revaccination.^{24,25} These secondary GC responses in ipsilateral vaccination furthermore arise in LNs that have undergone extensive and persistent stromal architecture remodeling,²⁶ establishing an augmented environment for subsequent immune responses, even to unrelated antigens.¹⁰ Nevertheless, we find that ipsilateral boosting is not associated with increased SHM, similar to observations by others.⁹ The comparable levels of SHM and cross-recognition of SARS-CoV-2 S variants across both ipsilateral and contralateral groups favors a model in which ipsilateral antigen delivery predominantly establishes secondary GCs in the LN, rather than “refueling” any ongoing primary GCs.

Repeated boosting with a variant antigen allows for secondary GCs to produce de novo antibody responses to mutated epitopes, bypassing feedback from pre-existing antibodies derived from the WT S priming.¹⁷ The serological benefit of ipsilateral boosting with heterologous prime-boost antigens (WT followed by BA.1) was similar to homologous WT vaccination, observable at day 19 but not at day 28. Mechanisms underlying the transient superiority of ipsilateral boosting likely comprise recall of LN-resident cross-reactive

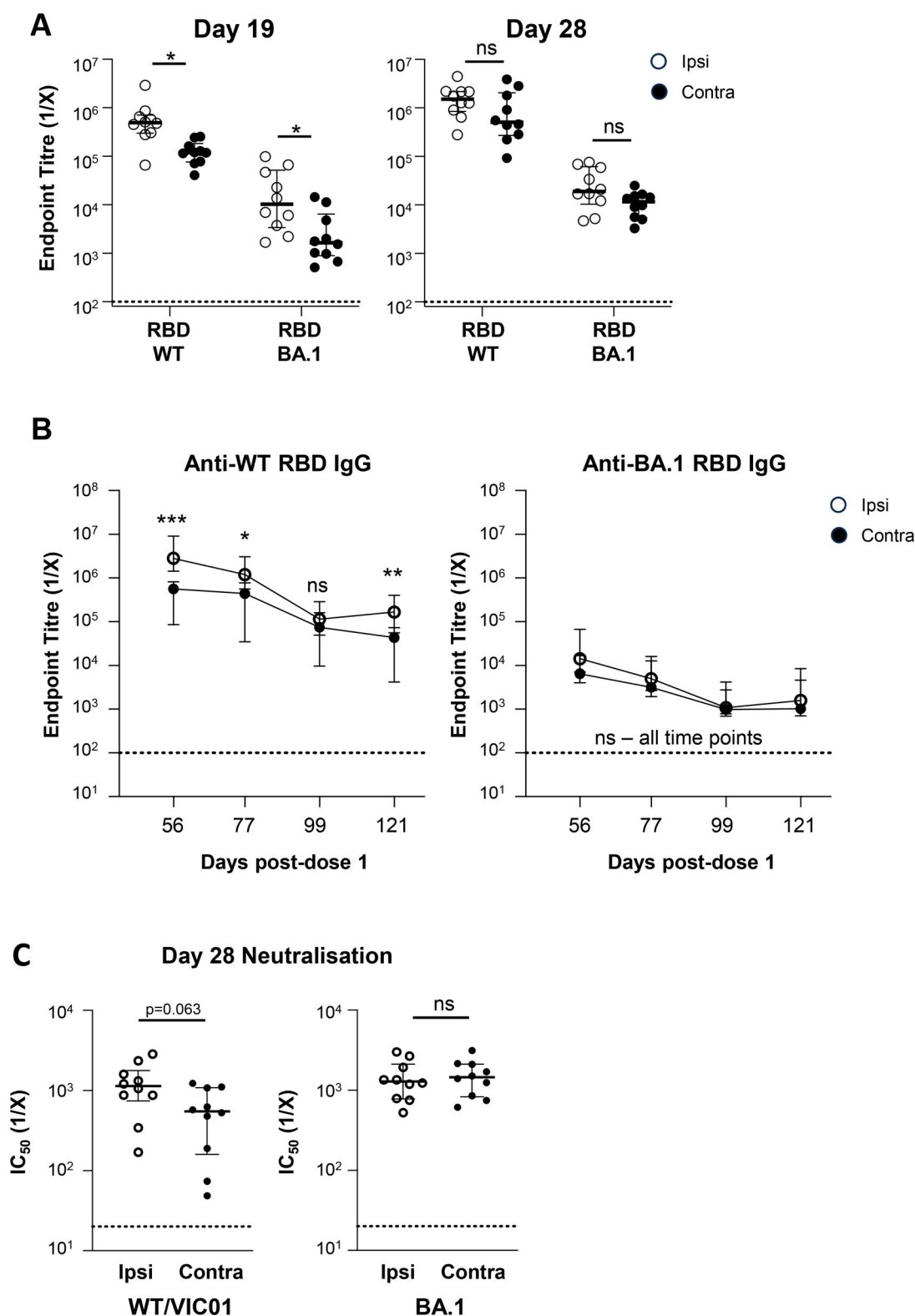


Figure 6. Humoral durability and breadth following ipsilateral or contralateral heterologous vaccination. (A) RBD-specific IgG titers (WT or BA.1) of animals serially sampled at days 19 and 28 ($n = 10$ per group; representative of 3 experiments) and (B) an independent group longitudinally sampled up to day 121 ($n = 10$ per group). (C) Live virus neutralizing titers against SARS-CoV-2 WT (VIC01) or Omicron BA.1 for an independent set of animals at day 28 after ipsilateral (open circles) or contralateral (closed circles) immunization ($n = 10$ per group). Points indicate median with interquartile range. Statistics were assessed by Mann-Whitney test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. IC_{50} , half maximal inhibitory concentration; ns, not significant.

B cells and/or T cells residing in the previously primed LN niche. Our dual-probe staining supports this, with ipsilateral immunization resulting in higher cross-reactive B cell

frequencies in secondary GCs early after boosting, mirroring findings by others utilizing heterologous influenza HA antigens.⁹ Like the serological dynamics, differences in GC cross-

reactive B cells were transient and converged at later time points. However, within this population, we found that immunization sites could further influence de novo GC B cell composition, with ipsilateral vaccination eliciting both WT⁺BA.1⁺ and WT⁺BA.1⁺⁺ GC B cells, while contralateral vaccination highly biased for WT⁺BA.1⁺⁺ GC B cells. Overall, the indistinguishable long-term antibody profiles across ipsilateral or contralaterally vaccinated animals could ultimately be driven by the equal sum of all B cell specificities that are established either within the same LN (i.e., ipsilateral iLN) or dispersed across 2 distinct LNs (i.e., contralateral iLN and aLN).

Antibody feedback from a primary response has been demonstrated to modulate subsequent GCs elicited, with antigen-specific B cells highly suppressed in secondary GCs upon boosting.^{17,19,22} While we did not observe substantial inhibition of S-specific GC B cells in secondary GCs of distal draining LNs (contralateral aLNs) by the primary antibody response, these population were reduced compared with a primary GCs, highlighting analogous inhibition by primary-derived antibodies on the participation of GC B cells during recall. A potential factor underlying this difference in suppression could be the immunization interval used, in which a prolonged interval allowing the maturation of serological responses would negatively modulate secondary GCs to a larger degree.¹⁹ In the contralateral group, the establishment of robust secondary GCs in the aLNs by day 5 postboost (earlier than primary GC kinetics in the iLNs) also suggests that factors including circulating antibody, MBCs, or CD4 T cells can coordinate to accelerate the establishment of de novo GCs.

In summary, our findings suggest that the choice of immunization site in a prime-boost vaccination regimen can influence early serological responses and composition of secondary GCs. In line with several other studies, our findings demonstrate that overall, an ipsilateral approach elicits more favorable humoral response compared with the contralateral vaccination, which warrants further studies into optimization of vaccination strategies to improve efficacy in humans.

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Author contributions

L.B., S.J.K., A.W., J.A.J., and H.-X.T. designed the study and experiments. L.B., W.S.L., R.W., J.A.J., and H.-X.T. performed experiments. A.K. and R.E. expressed and validated key reagents. L.B., J.A.J., and H.-X.T. analyzed the experimental data. L.B., J.A.J., and H.-X.T. wrote the manuscript. All authors reviewed the manuscript.

Supplementary material

[Supplementary material](#) is available at *The Journal of Immunology* online.

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Conflicts of interest

The authors declare no competing interests.

Data availability

The data underlying this article will be shared on request to the corresponding author.

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