Supplement to "Asymmetric B cell division in the germinal center reaction"

Supporting Online Material
Materials and Methods
Figs. S1 to S2

Materials and Methods:

Mice, Immunizations, Cell Transfers: All animal work was done in accordance with Institutional Animal Care and Use Guidelines of the University of Pennsylvania. All mice were housed in specific-pathogen-free conditions prior to use. C57BL/6 wild-type, *Rag1*$^{-/-}$, and *Icam1*$^{-/-}$ mice were purchased from Jackson Laboratories. Mice were immunized intraperitoneally with 50 μg (4-hydroxy-3-nitrophenyl)acetyl coupled to OVA at a substitution ratio of 15 (NP-OVA) (Biosearch Technologies) in alum. Splenocytes were harvested for sorting prior to microscopy of germinal center B cells. For antibody production studies, control mice were immunized with alum only. Homeostatic proliferation studies were performed by transferring 1x10^7 MACS-purified CD23$^+$ naïve B cells intravenously into *Rag1*$^{-/-}$ mice, and CD19$^+$ B220$^+$ donor cells were FACS-sorted from spleens of recipient mice 4 days after transfer.

In Vitro Stimulation: CD23$^+$ naïve follicular B cells were purified by incubating splenocytes with biotinylated anti-CD23 followed by strepavidin conjugated magnetic beads. Purified cells were cultured in BAFF (100 ng/ml; R&D Systems) and stimulated with F(ab')$_2$ anti-IgM (10 μg/ml; Jackson ImmunoResearch). Some groups received either 10 μg/ml soluble or 1 μg/ml plate-bound anti-CD40 (BD Pharmingen). Stimulated cells were harvested at 36 hours post-stimulation.

Flow Cytometry and Cell Sorting: Staining with fluor-conjugated antibodies (purchased from BD Pharmingen, Ebioscience, Biolegend, and Invitrogen) for cell sorting and analysis was done in 2% FBS on 10^7 cells/ml. NP-APC was used to detect antigen-specific B cells by flow cytometry. Data were sorted or acquired on a FACSAria IIu or LSR II, respectively (BD Biosciences). Data were analyzed using FlowJo (Tree Star).

Microscopy: Germinal center B cells (B220$^+$IgD^{lo}Fas$^+$CD38^{lo}) were FACS-purified from spleens of immunized mice at the indicated time points. To acquire cytokinetic images, sorted germinal center B cells were cultured in 10 μM cytochalasin B (Sigma) and 100 ng/ml BAFF (R&D Systems) for 3 hours at 37^{o}C. Sorted cells were plated on poly-L-lysine coated coverslips in PBS, fixed with 3% paraformaldehyde, and permeabilized with 0.1% Triton-X. Antibody staining for confocal microscopy was done in 0.25% fish skin gelatin (Sigma) and 0.01% saponin (Sigma) blocking buffer overnight at 4^{o}C or for 1 hour at room temperature. Coverslips were mounted in Prolong Gold Anti-Fade Reagent with DAPI (Invitrogen). Antibodies against indicated proteins were purchased from commercial sources: PKCζ (Abcam), IL-21R (Abcam), IRF4 (Sigma), Bcl6 (BD Pharmingen), B220 (BD Pharmingen), β-tubulin (Abcam). Fluor-conjugated secondary antibodies (Invitrogen) were used for detection of primary antibodies.

Images were collected on a Perkin Elmer Ultraview ER6 spinning disk confocal system equipped with a Zeiss Axiovert 200 microscope and a 63X 1.4 NA objective in z-stacks of 0.3 μm. Images were collected using an Orca ER camera (Hamamatsu). Protein localization was not determined until after mitotic or cytokinetic events were collected. Mitotic cells were identified based on the presence of two oppositely-facing microtubule organizing centers (MTOCs). Cytokinetic cells were identified by one MTOC per daughter cell with tubulin bridge between cells. Pre-mitotic cells were identified by the presence of one MTOC. 3D images were rendered and analyzed using Volocity v.5 software (Perkin Elmer), and the sum of fluorescence in each channel was calculated on either side of the plane of division, defined as the equator bisecting the mitotic spindle or the tubulin bridge. The ratio of fluorescence between the two halves of the cell was calculated. The mean+2SD of the ratios of tubulin fluorescence was used as a cutoff to distinguish asymmetrically versus symmetrically segregated molecules. The percentage of cells that had segregated each molecule asymmetrically (% Polar) was compared between experimental groups.

ELISPOT assay: Splenocytes or bone marrow cells from immunized mice were incubated on plates coated with 10 μg/ml NP4-BSA (to detect high affinity ASCs) or NP26-BSA (to detect all ASCs). Secreted antibody was detected with biotinylated anti-IgG1 (Southern Biotech) followed by ExtrAvidin-Alkaline Phosphatase and NBT-BCIP substrate (Sigma). Spots were counted on the CTL-ImmunoSpot (Cellular Technologies).

Immunohistochemistry: Spleens in OCT (Tissue Tek) were flash frozen in 2-methyl-butane and liquid nitrogen. 8 μm sections were fixed with acetone and stained with PNA and antibodies against IgD and Thy1.2. Images were collected on a Zeiss LSM510META NLO laser scanning confocal microscope and analyzed using Volocity (PerkinElmer).

Statistical Analyses: Comparisons of the frequencies of polarity between different groups were done using the chi-squared test, due to the binary nature of the data sets (asymmetric vs symmetric). Comparisons for antibody-secreting cells (ASCs) were done using the unpaired student's t-test to compare different groups of mice.

Supplemental Figure 1

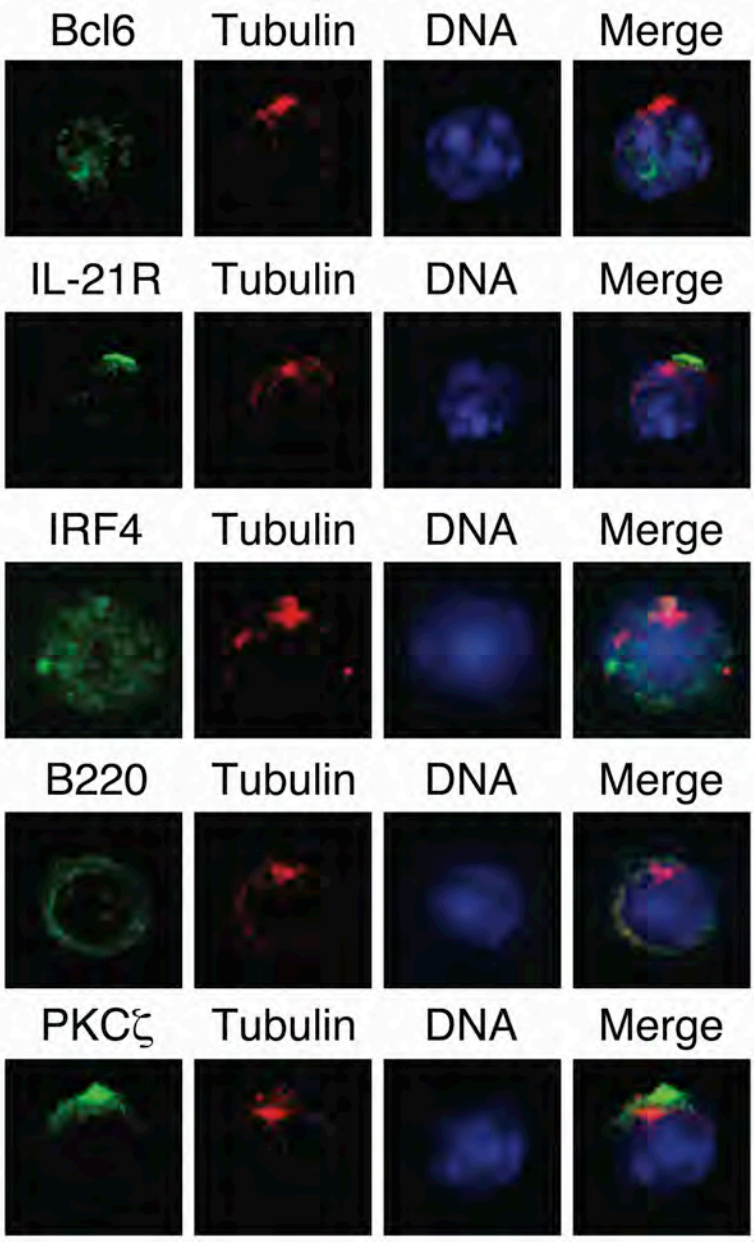

Figure S1. Polarity of pre-mitotic germinal center B cells from immunized mice. Cells were sorted from pooled spleens of 3 mice and stained for localization of Bcl6, IL-21R, IRF4, B220, or PKCζ (green), β-tubulin (red), and DNA (blue). IL-21R and PKCζ are co-polarized with the microtubule organizing center (MTOC) at the site of the putative B cell synapse in 60% and 80% of cells, respectively (n=10 cells). Bcl6 and IRF4 appear to have nuclear localization in interphase blasts. B220 appears circumferentially distributed. Similar results were obtained at d5 and d8 post-immunization.

Supplemental Figure 2

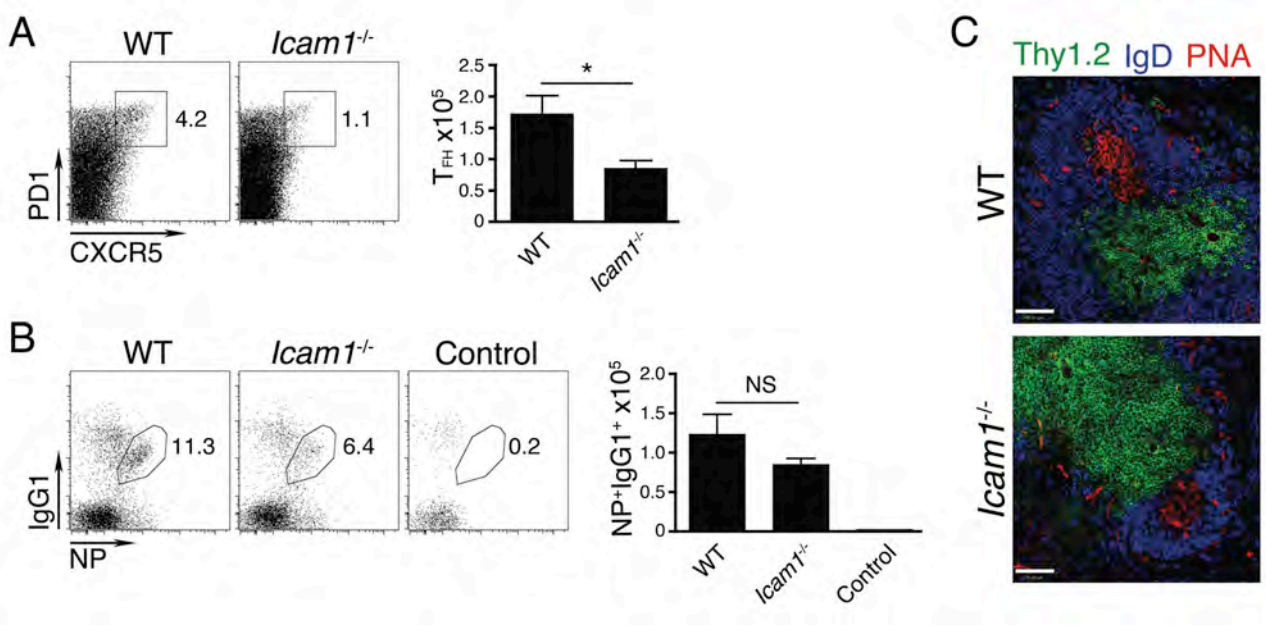

Figure S2. Relative preservation of germinal center reaction in immunized *Icam1*$^{-/-}$ mice. Analyses of wild-type (WT) and *Icam1*$^{-/-}$ mice immunized with NP-OVA in alum adjuvant, and WT mice injected with alum adjuvant alone (control) at d14 post-immunization. **(A)** Modest reduction in T_{FH} cells (PD1^{hi}CXCR5^{+} cells, gated from TCRb^{+}CD19^{-}CD4^{+}CD62L^{lo} splenocytes) in *Icam1*$^{-/-}$ mice. **(B)** Antigen-specific, isotype-switched B cells (NP^{+}IgG1^{+} cells, gated from CD19^{+}B220^{+}F4-80^{-}GR1^{-}TCRβ^{-}IgM^{-}IgD^{-} splenocytes) readily detected in *Icam1*$^{-/-}$ mice. n=6 mice/group, NS = $p>0.05$; * = $p<0.05$ by unpaired student's t-test. Result representative of 2 separate experiments.
(C) Preserved micro-anatomic architecture in immunized *Icam1*$^{-/-}$ mice. Splenic sections stained with anti-IgD, anti-Thy1.2, and PNA, to detect B cell follicles, T cell zones, and GCs, respectively. PNA-rich, IgD^{lo} germinal centers located at borders of B cell follicles and T cell zones. Scale bar denotes 70 μm.