Supporting Information for

Immunofluorescence staining of live lymph node tissue slices

Benjamin D. Groff*, Andrew W.L. Kinman*, Jacob F. Woodroof,
and Rebecca R. Pompano†
University of Virginia

Contents:

Supporting Movie Captions:

Movie S1: Data from confocal Z-stacks of a lymph node slice stained with Alexa Fluor-647-labelled anti-B220 (whole antibody). The top of the slice is marked as 0 µm.

Movie S2: Data from confocal Z-stacks of a lymph slice stained with Alexa Fluor-555-labelled anti-B220 $F(ab')_2$. The top of the slice is marked as 0 µm.

Supporting Tables and Figures:

Table S1: List of antibodies used for experimental procedures.

Specificity	Isotype	Clone	Vendor
Anti-mouse/human CD45R/B220 - FITC	Rat IgG2a, k	RA3-6B2	Biolegend, #103206
Anti-mouse/human CD45R/B220	Rat IgG2a, k	RA3-6B2	BioLegend, #103202
Anti-KLH (Isotype Control)	Rat IgG2a, k	RTK2758	BioLegend, #400502
Anti-mouse CD3Ɛ	Armenian Hamster IgG	145-2C11	BioLegend, #100302
Anti-mouse Lyve 1 – eFluor 660	Rat IgG1, k	ALY7	eBiosciences, #50-0443-82
Anti-mouse Lyve 1 – eFluor 570	Rat IgG1, k	ALY7	eBiosciences, #41-0443-80
Anti-mouse CD16/32	Rat IgG2a, λ	93	BioLegend, #101302
Anti-mouse CD69 – Alexa Fluor 647	Armenian Hamster IgG	H1.2F3	BioLegend, #104518

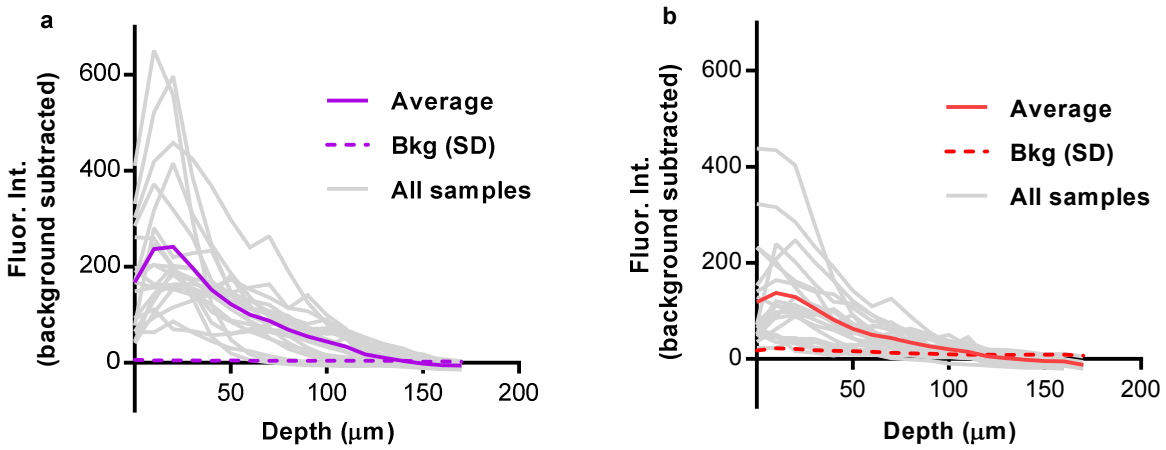

Figure S1. Antibody and fragment signal in live lymph node slices as a function of depth. Slices were stained with **(a)** Alexa Fluor 647-labelled whole IgG and **(b)** Alexa Fluor 555-labeled F(ab')$_2$ fragment for B220. For each tissue slice, the average fluorescent intensity of the B220+ region was determined at multiple positions as a function of depth. The top of the slice was defined as 0 μm. 20 positions were measured (grey lines), representing seven stained slices. In separate unstained slices, background autofluorescence was measured at each depth. The autofluorescent signal was averaged across 18 positions representing six unstained slices, and the mean autofluorescence (background) was subtracted from the intensity of each stained image as a function of depth. Dotted lines indicate one standard deviation (SD) of autofluorescence background as a function of depth, and solid colored lines indicate the average background-subtracted intensity (averaged across all 20 positions). For the plots shown in Figure 1h, the average and background-SD lines were normalized to the maximum intensity of the average line for each staining reagent.

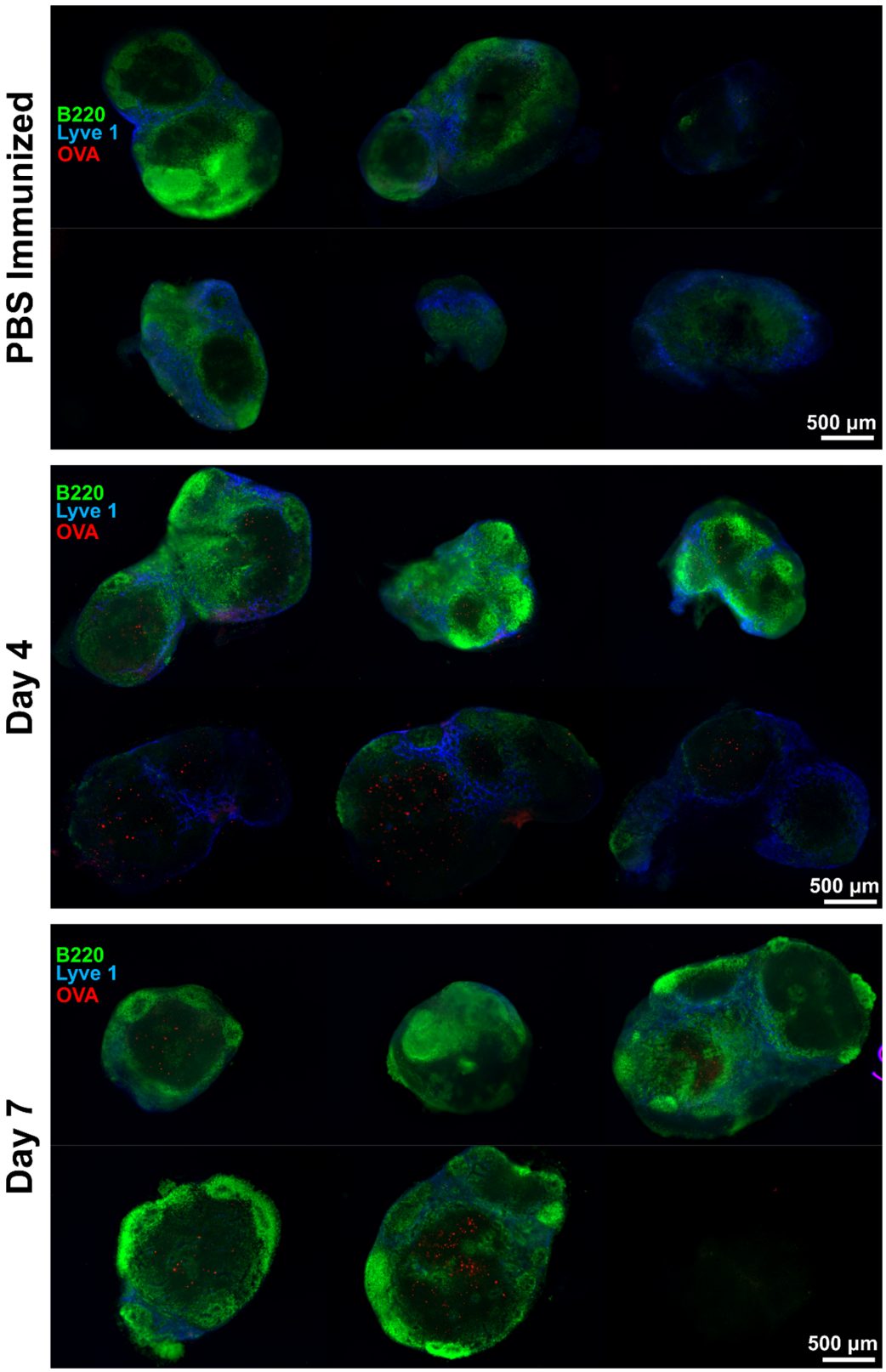

Figure S2: Lymph node slices are heterogeneous, with a wide distribution in shape and organization. Lymph node slices are from the vaccination experiment shown in Figure 1. Mice were immunized with

CFA and OVA-Rhodamine, or PBS, and then slices were collected at day 4 and 7. Slices were stained with anti-B220 FITC and anti-Lyve 1 eFluor 660. Six images per condition were randomly selected for this display, out of ~ 20 total per condition, excluding 1 slice that exhibited abnormally high autofluorescence. Brightness and contrast were adjusted uniformly for all images, which results in some appearing dark while others appear bright.

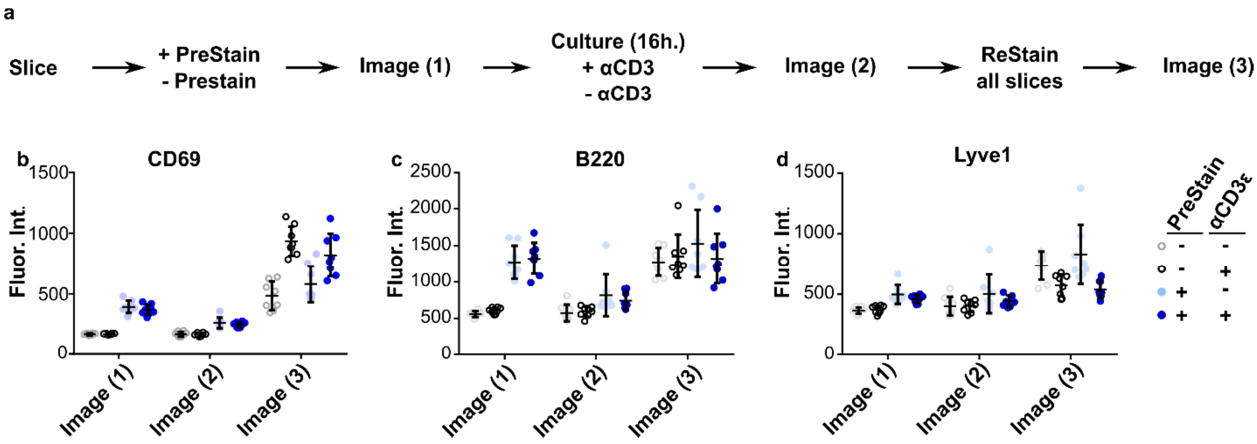

Figure S3. Immunostain fades over time and B220 and Lyve 1 do not change significantly after stimulation with αCD3Ɛ. Slices were stained with a cocktail mix of anti-CD69 Alexa Fluor 647, anti-Lyve1 eFluor 570, and anti-CD45R/B220 FITC. **(a)** Experimental timeline for the PreStaining, stimulation (16 hour culture), and ReStaining of the same lymph node slice to determine change in surface marker presentation over time. Note that Image (1) and Image (3) are referred to as 0 Hour and 16 Hour, respectively, in the main text. Fluorescent intensity for **(b)** anti-CD69 Alexa Fluor 647 (repeated from main text for clarity), **(c)** anti-CD45R/B220 FITC, and **(d)** anti-Lyve1 eFluor 570 is shown for each imaging step listed in the timeline. Data is compiled over 2 replicate experiments, N=8 per condition, error bars denote standard deviation.

Supporting Methods:

Immunization: Rhodamine-conjugated chicken egg ovalbumin (Rhod-OVA) was prepared by reacting 1 mg/mL OVA (Ovalbumin, InvivoGen, San Diego, CA) with 10x molar excess NHS-Rhodamine (Fisher Scientific). The reaction was performed for 1 hour at 25 °C. Excess unreacted fluorophore was removed via centrifuge filtration using a 50 kDa molecular weight cut off (MWCO) filter (Fisher Scientific), spinning at 14,000 G for 3-5 minutes, repeatedly adding PBS until excess dye was removed. A solution of 1.2 mg/mL Rhod-OVA in PBS was mixed 1:1 v/v with Complete Freund's Adjuvant (CFA; Invivogen) and vortexed for 30 min to generate an emulsion. Mice were injected subcutaneously with 25 µL per site, in the left and right shoulders, flanks, and right rear hock (125 µL total per mouse), to ensure drainage to each of the skin-draining LNs and the right popliteal LN. Control animals were vaccinated with PBS only. LNs were harvested 4 and 7 days after vaccination, sliced, and immunostained with anti-B220 FITC and anti-Lyve-1 eFluor 660 (lymphatics).

Conjugation of antibodies: Antibodies that were not available with the required fluorophore were conjugated in house using fluorophores with N-hydroxy succinimidyl ester (NHS) to amine conjugation chemistry. NHS fluorophores (Fisher Scientific) were mixed with 50 µg of 1 mg/mL or 0.5 mg/mL antibody at molar excess of 15X or 30X respectively. The reaction was performed for 1 hour at 25 °C. Excess unreacted fluorophore was then removed via centrifuge filtration using a 50-kDa molecular weight cutoff filter (EMD Millipore Amicon Ultra 0.5 mL, Fisher Scientific), spinning at 14,000 G for 3-5 minutes, repeatedly adding PBS until excess dye was removed. Labeling ratio was measured by UV-Vis on a ND1000 nanodrop spectrophotometer (Fisher Scientific) at 280 nm and λ_{max} of fluorophore. Fragments were conjugated in a similar manner.

Antibody fragmentation: Anti-CD45R/B220 F(ab')$_2$ antibody fragments were prepared by digestion with pepsin enzyme (Immobilized Pepsin Agarose Resin, Fisher Scientific). Pepsin resin in 20 mM sodium

acetate buffer, pH 4.0, was mixed at 10% w/w with antibody at 1 mg/mL and incubated for 5 hours at 37 °C while shaking. Reaction was stopped by removing pepsin resin using a 10-µm pore centrifugal filter (Pierce spin column, Fisher Scientific). Antibody fragments were buffer exchanged into PBS using a 50 kDa MWCO filter. Analysis of fragments was performed via non-reducing SDS PAGE (NuPage 4-12% Bis-Tris, Fisher Scientific) with Commassie stain (Commassie R250, Fisher Scientific).

Cytokine ELISA: A high-binding plate (Corning Costar 96 well ½ area, #3690; Fisher Scientific) was coated with 1 µg/mL anti-IFNγ XMG1.2 or 4 µg/mL anti-TNFα 6B8 (BioLegend) in PBS overnight at 4°C. Wells were blocked for 2 hours with 1% BSA and 0.05% Tween-20 (Fisher Scientific) in PBS (block solution). Serial dilutions of IFNγ and TNFα cytokines (Peprotech, Rocky Hill, NJ) and sample supernatant dilutions were prepared in a 1:1 v/v mixture of block solution and complete media. These samples were added to the plate and incubated for 2 hours, then washed. Streptavidin-conjugated anti-IFNγ R46A2 or anti-TNFα MP6-XT22 antibodies (BioLegend) were added to the plate at 0.5 µg/mL and 0.25 µg/mL, respectively, in block solution. All washing steps were performed with 0.05% Tween-20 in PBS. Plates were developed using TMB substrate (Fisher Scientific) and absorbance values were read at 450 nm on a plate reader (CLARIOstar; BMG LabTech, Cary, NC). To determine concentration of sample solutions, calibration curves were fit in GraphPad Prism 6 with a sigmoidal 4 parameter curve (Eq. (1)), where X is log of concentration, Y is absorbance, min and max are the plateaus of the sigmoidal curve on the Y axis, and HillSlope describes the steepness of the slope.

$$Y = min + \frac{max - min}{1 + 10^{(Log(IC50 - X) * HillSlope)}}$$