

Supplementary Information for

Neutrophils promote VLA-4-dependent B cell antigen presentation and accumulation within the meninges during neuroinflammation

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Movie S1

Supplementary Information Text

Materials and Methods

Mice

C57BL/6 (B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). $IA\beta^{bstop^{flox/flox}}$ mice (1) were used to express MHCII in a cell-specific manner. To generate constitutive and inducible MHCII expression by MOG-specific B cells we first crossed $IA\beta^{bstop^{flox/flox}}$ mice to IgH^{MOG} mice (2). $CD19-B^{MHCII}xIgH^{MOG}$ mice were generated by crossing the $CD19^{Cre}$ (The Jackson Laboratory) to $IA\beta^{bstop^{flox/flox}}xIgH^{MOG}$ mice as described (3). Tam- WT^{APC} mice were generated by crossing the $UBC^{Cre/ERT2}$ mice (The Jackson Laboratory) with $IA\beta^{bstop^{flox/flox}}$ animals, resulting in progeny that express MHCII in all expected wild-type (WT) professional APCs after oral gavage with tamoxifen (Tam). $CD20^{Cre/ERT2}$ mice (4) were supplied by Dr. Mark Shlomchik and crossed to $IA\beta^{bstop^{flox/flox}}xIgH^{MOG}$ mice to generate Tam- B^{APC} mice which express MHCII exclusively by B cells enriched for MOG specificity following oral gavage with Tam. Additionally, Tam- B^{APC} mice were crossed to mice with the $\alpha 4$ integrin locus flanked by LoxP sites ($itga4^{flox/flox}$ mice), generously provided by Dr. Thalia Papayannopoulou (5). In these mice, oral gavage with Tam simultaneously induces MHCII expression while abrogating VLA-4 expression in all B cells.

EAE

Encephalitogenic CD4 T cell lines were prepared from the spleens of congenic $Thy1.1^{+}$ or $CD45.1^{+}$ B6 mice immunized with 200 μ g myelin oligodendrocyte glycoprotein residues 35-55 (MOG_{35-55}) (GenScript; Piscataway, NJ) in CFA (Sigma-Aldrich; St. Louis, MO) made with 5mg/ml heat-killed *Mtb* H37Ra (BD; San Diego, CA) in incomplete Freund's adjuvant (BD). After 14 days post immunization, MOG_{35-55} -specific CD4 T cells were purified using a 30 ml syringe by filtering splenocytes through sterile 0.6g nylon wool (Polysciences, Inc.; Warrington, PA) to remove macrophages and B cells. Eluted cells were incubated with J11D and 3.155

antibody supernatants (6) for 1 hour before rinsing and resuspending in 10% Low Tox Rabbit Complement (Cedarlane Labs; Burlington, NC) to induce complement-mediated cell lysis of B cells and CD8 T cells. Red blood cells were removed by Ficoll (Sigma-Aldrich) gradient and the remaining CD4 T cells were washed with RPMI media supplemented with 10% fetal calf serum (GE Life Sciences; Logan, UT). On days 0 and 7, CD4 T cells were cultured *in vitro* with irradiated WT splenocytes, 10% 11B11 anti-IL-4 supernatant, 10U/ml IL-2 supernatant, 10U/ml IL-12p70 (eBioscience; San Diego, CA), and 10 μ g/ml MOG₃₅₋₅₅. On Day 14 CD4 T cells and irradiated WT splenocytes were cultured with only 10U/ml IL-2 supernatant and 10 μ g/ml MOG₃₅₋₅₅. Before each re-stimulation and prior to adoptive transfer, apoptotic cells and debris were removed by Ficoll gradient and CD4 T cells were then washed in media. CD4 T cells were re-suspended in HBSS (Sigma-Aldrich) and transferred into naive recipients by retro-orbital injection of 5x10⁶-7.5x10⁶ encephalitogenic CD4 T cells to induce passive EAE.

Active EAE was induced by immunization with 150 μ g of hMOG protein generated by the protein core at Children's Hospital of Philadelphia in CFA, followed by i.p. injection of 200ng pertussis toxin (Enzo Life Sciences) on day 0 and day 2 post immunization (7). Mice were observed daily and clinical scores were determined by a five point scoring system, as follows: 0 - no disease; 1 - limp tail; 2 - mild hind limb paresis; 3 - severe hind limb paresis; 4 - paraplegia; 5 - moribund or dead. Anti-CXCR2 treatment paradigm was performed as follows. On day 19 post T cell transfer, recipient mice were randomly assigned to three groups and either dosed with 250 μ l of control polyclonal rabbit serum, dosed with anti-CXCR2 serum, or left untreated as controls. Animals received additional serum doses every other day for a total of four doses. All groups were treated with Tam on day 21 post-T cell transfer and were scored daily for signs of EAE.

Flow Cytometry

Spleens were dissociated with frosted glass slides to make single cell suspensions, which were then filtered and subjected to red blood cell lysis. Mice were perfused with 25-30 mL of ice-cold PBS prior to isolation of CNS tissue. Mononuclear cells were purified separately from

homogenized brains and spinal cords by centrifugation for 30 min in 30% Percoll (GE Healthcare) solution as previously reported (1). Spinal cord meninges were carefully peeled from vertebrae after the collection of spinal cord parenchyma and digested in 3 ml Meninges Digest Media (0.1mg/ml DNase (Sigma-Aldrich), 0.2mg/ml Collagenase P (Sigma-Aldrich), and 0.8mg/ml Dispase II (Sigma-Aldrich) in HBSS (Gibco; Dublin, Ireland)) for 20 min at 37°C while shaking. After digestion, the cell suspension was filtered into excess media and rinsed. Cells were incubated at 4°C with the anti-Fc receptor blocking antibody 2.4G2 and washed with FACS buffer prior to the addition of staining antibodies in FACS buffer (1.0% bovine serum albumin (Fisher Bioreagents; Waltham, MA) with 0.05% sodium azide (Sigma-Aldrich) in PBS (Gibco)). The following anti-mouse antibodies were purchased from BioLegend (San Diego, CA): anti-GL7 clone GL7 (FITC), anti-CD19 clone 1D3 (PE), anti-CD19 clone 6D5 (Allophycocyanin), anti-IgM clone RMM-1 (Allophycocyanin), anti-I-A/I-E clone M5/114.15.2 (AF700), anti-I-A/I-E clone M5/114.15.2 (Pacific Blue), anti-CD138 clone 281-2 (Allophycocyanin-Cy7), anti-IgD clone 11-26c.2a (Pacific Blue), anti-CD49d clone R1-2 (PE), anti-mouse IgG2b kappa isotype clone RTK4530 (PE), anti-CD4 clone GK1.5 (BV421), anti-CD4 clone GK1.5 (FITC), anti-CD11b clone M1/70 (BV510), anti-CD11b M1/70 (PE-Cy7), anti-CD11b clone M1/70 (PerCP-Cy5.5), anti-Ly-6G/C clone RB6-8C5 (PE-Cy7), anti-CD8a clone 53-6.7 (Allophycocyanin-Cy7), anti-CD45 clone 30-F11 (Pacific Blue), anti-CD45 clone 30-F11 (BV510), anti-CD45.2 clone 104 (PerCP-Cy5.5), anti-CXCR2 clone SA044G4 (PE), anti-CD62L clone MEL-14 (Pacific Blue), anti-B220 clone RA3-6B2 (PerCP-Cy5.5). The following antibodies were purchased from BD Biosciences (San Jose, CA) anti-Ly-6G/C clone RB6-8C5 (PE), anti-Ly6C clone A1-21 (PerCP-Cy5.5), anti-Ly6G clone 1A8 (FITC), anti-B220 clone RA3-6B2 (PE-CF594), anti-CD19 clone 1D3 (Allophycocyanin), anti-CD90.1 clone OX-7 (FITC), anti-B220 clone RA3-6B2 (FITC). Anti-CD11c clone N418 (AF700) was purchased from eBioscience (San Diego, CA).

After staining, cells were fixed with 2% paraformaldehyde in FACS buffer with the exception of experiments in which CXCR2 expression was examined. Following two washes, cells were run on a Gallios (Beckman Coulter; Indianapolis, IN). Data was analyzed using FlowJo software version 8.5.2 (Treestar; Ashland, OR) with debris and remaining erythrocytes excluded according to their forward and side scatter location, after which doublets were excluded.

Imaging

Mice were sacrificed and perfused with 25 mL ice cold PBS followed by 20 mL ice-cold 4% paraformaldehyde (Sigma-Aldrich). CNS tissue was extracted while still encased in the skull and vertebral column then fixed in 4% paraformaldehyde for more than 12 hours. Spinal cord vertebra were decalcified in 6% tri-chloroacetic acid (Sigma-Aldrich) changed daily for 5 days, washed with PBS, and then dehydrated in 30% sucrose (Sigma-Aldrich) in PBS for 48 hours. Spinal cords in decalcified bone were embedded in optimal cutting temperature (OCT) media (TissueTek; Torrance, CA) and 8-10µm thick sections were cut using a Leica CM1900 cryostat (Buffalo Grove, IL). Sections were stained with the following primary antibodies: rat anti-mouse VCAM-1 (BioLegend), rat-anti mouse Ly-6G (BioLegend), Syrian hamster anti-mouse podoplanin (AngioBio; San Diego, CA), rabbit anti-KI-67 (Abcam; Cambridge, MA), rabbit anti-Laminin (ThermoFisher Scientific; Waltham, MA), rat anti-mouse B220 (BD Biosciences), and rabbit anti-human CD3 (Dako; Santa Clara, CA). The following secondary antibodies were used: goat anti-rat AF555 (Invitrogen; Carlsbad, CA), goat anti-hamster AF647 (Life Technologies; Carlsbad, CA), goat anti-rabbit AF647 (Invitrogen) and goat anti-rabbit AF488 (Life Technologies). Slide covers were mounted with Fluoroshield Mounting Medium with DAPI (Abcam) to detect nuclei. Slides were examined by light microscopy using a Nikon 90i motorized upright digital microscope with camera and Metamorph software (Molecular Devices; San Jose, CA).

Imaging using iDISCO was performed as reported (8) with modifications for the spine. Briefly, spinal cord samples were cut transversely at the thoracic vertebra and pre-treated with

methanol (Sigma-Aldrich) to dehydrate and rehydrate for at least two days at each step in the protocol. Immunolabeling was performed in 10% normal goat serum (EMD Millipore; Burlington, MA), with 1.0% Triton-X100 (Sigma-Aldrich) in PBS with the following anti-mouse primary antibodies: rat anti-B220 (BD Biosciences), rabbit anti-CD3 antibodies (Dako), rabbit anti-Lyve-1 (Abcam), rabbit anti-Ki67 (Abcam), and hamster anti-podoplanin (AngioBio Co.). Goat anti-rat AF555 (Invitrogen), goat anti-hamster AF647 (Life Technologies), goat anti-rabbit AF488 (Life Technologies), and goat anti-rabbit AF647 (Invitrogen) were used as secondary antibodies. Tissues were dehydrated with methanol, rinsed in DiChloroMethane (Sigma-Aldrich) followed by DiBenzyl Ether (Sigma-Aldrich) for at least 5 days prior to imaging. Cleared spinal cord tissue was imaged with an inverted Leica DMI8 laser scanning confocal microscope, tile scanned with a 4x Leica objective (dry) at room temperature. Images were prepared with Bitplane (Zurich, Switzerland) Imaris 3D/4D Image Analysis software in collaboration with the Washington University Center for Cellular Imaging.

Statistical Analyses

All data are reported in the text as mean \pm the standard error of the mean (SEM) unless otherwise indicated. Mann-Whitney U-tests were used to compare CSF concentrations and cellular infiltrates. Time-to-EAE-onset incidence curves were compared by Log-rank (Mantel-Cox) tests. All group effects for day of onset were compared via analysis of variance (ANOVA) with Tukey's test for multiple comparisons when assumptions were not violated. Differences in EAE severity were compared by calculating the mean area under the curve. All statistical analyses were completed using PRISM 7 software (GraphPad; La Jolla, CA).

Supplemental Figures

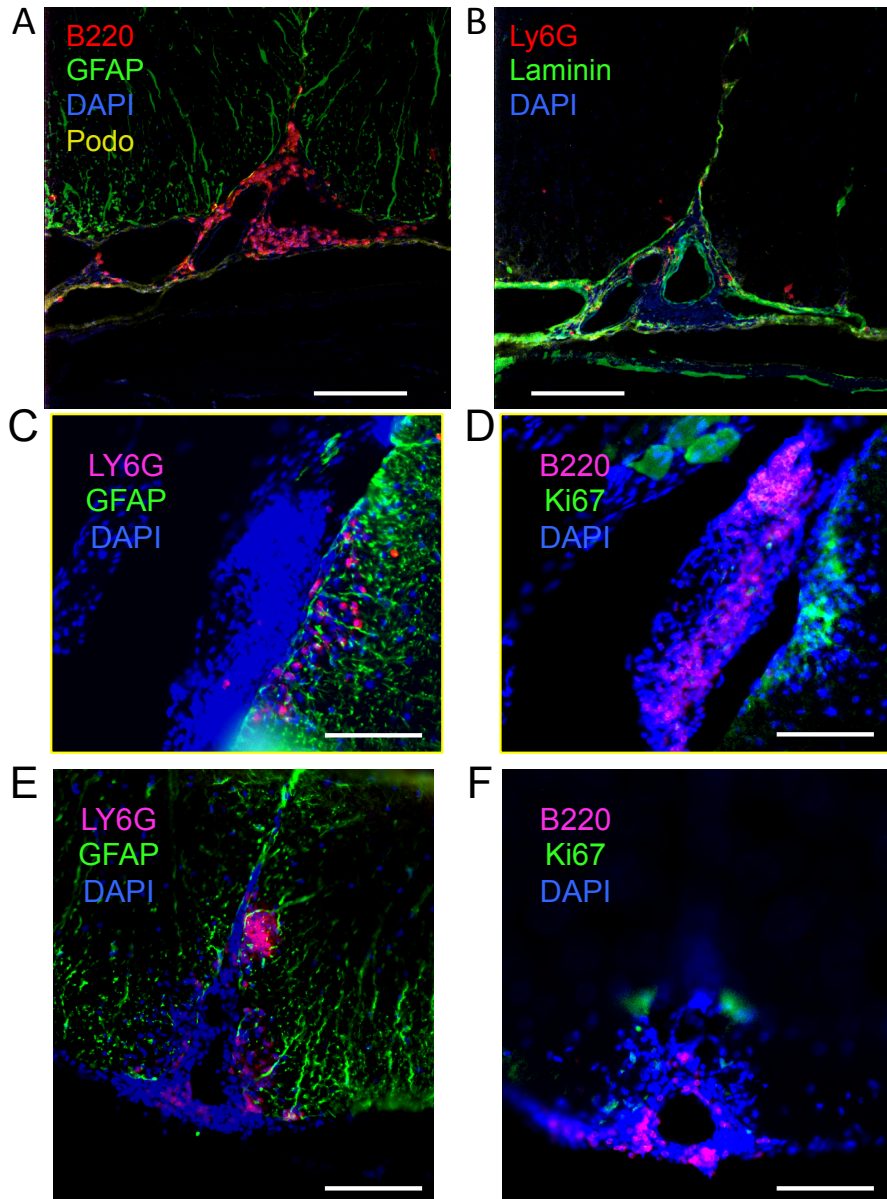


Figure S1. PMN infiltration precedes B cell infiltration in B cell-mediated EAE.

Serial sections of decalcified Tam-B^{APC} spinal cord stained for (A): B220 (red), GFAP (green), DAPI (blue), and podoplanin-1 (yellow); (B): Ly6G (red), Laminin (green), DAPI (blue); (C, E): Ly6G (magenta), GFAP (green), DAPI (blue); and (D, F): B220 (magenta), Ki67 (green), DAPI (blue). Scale bars = 100µm.

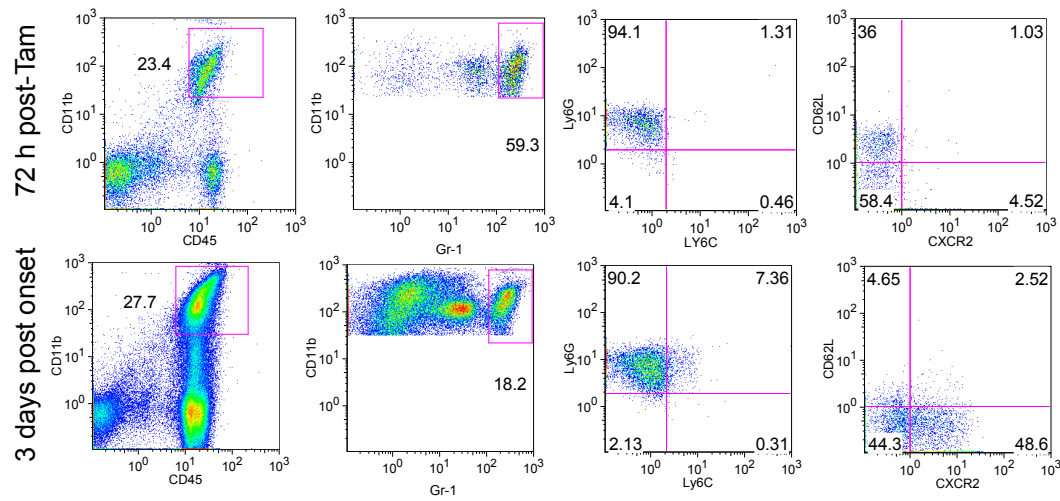


Figure S2. Dynamic CXCR2 expression by PMNs during EAE.

(A) Representative flow cytometry for surface marker expression on PMNs isolated from the spinal cord meninges of Tam-B^{APC} collected at 72 h post Tam (top) and 3 days post EAE onset (bottom). Graphs are representative of $n = 4-5$ mice pooled from 2 separate experiments.

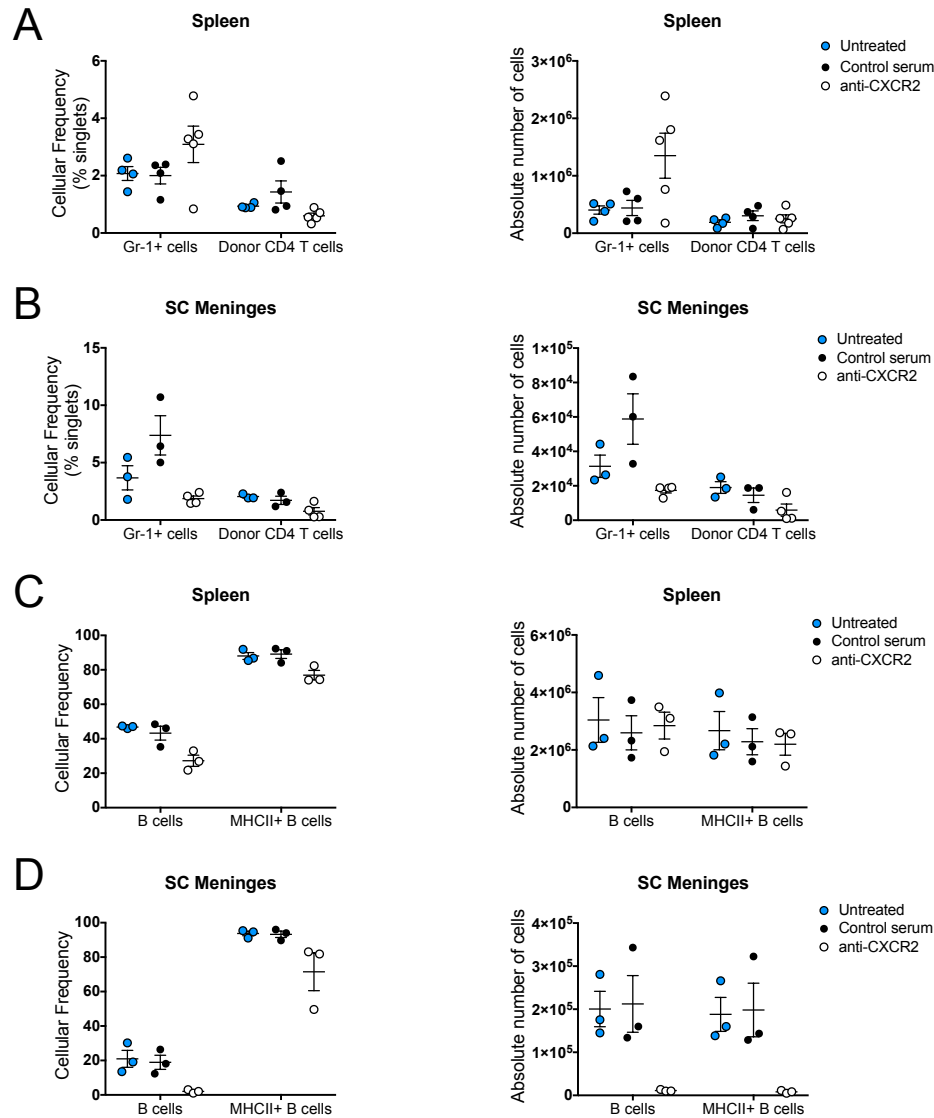


Figure S3. Granulocyte recruitment to the CNS is necessary for formation of B cell clusters and is critical for pathogenic B cell antigen presentation in EAE.

Flow cytometric analysis shows the mean \pm SEM of the frequency (left) and absolute number (right) immune cell spleen (A, C) and spinal cord meninges (B, D) of Tam-B^{APC} mice from untreated groups (blue circles), control serum groups (black circles) and anti-CXCR2 recipients (white circles) harvested either 18 days post Tam (A, B) or 3 days post EAE onset (C, D). Data for (A&B) is from one experiment with $n = 4$ mice/group and data for (C&D) is from one experiment with $n = 3$ mice/group.

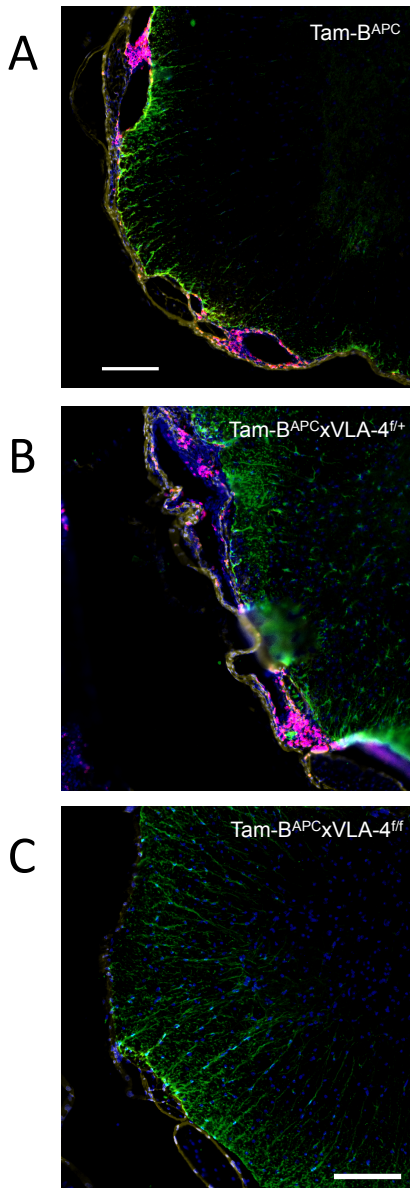


Figure S4. B cell access to the sub-arachnoid space is necessary for B cell antigen presentation to support passive EAE.

Decalcified spinal cords from Tam-B^{APC} mice (A), Tam-B^{APC} x VLA-4^{f/+} (B), and Tam-B^{APC} x VLA-4^{f/f} mice (C) stained for left: B220 (pink), GFAP (green) podoplanin-1 (yellow) and DAPI (blue), imaged at 10x magnification. Scale bar = 100μm. Images are representative of n = 5 mice/genotype, sacrificed at 3 days post EAE onset.

Table S1. B cell deletion of VLA-4 expression results in protection from EAE.

Recipient	Recipient VLA4 genotype	EAE Incidence
B6 (WT)	WT	13/13
CD19-B ^{MHCII} _x IgH ^{MOG}	VLA4 ^{flox/+}	21/25
	VLA4 ^{flox/flox}	0/20
Tam-B ^{APC}	VLA4 ^{flox/+}	22/25
	VLA4 ^{flox/flox}	0/17

Movie S1. Confocal microscopy of an optically cleared spinal column from a Tam-B^{APC} mouse in harvested 3 days post EAE onset. Auto-fluorescent bone and soft tissue (green) contrasted with B220+ (red) B cells.

References

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