Supporting Information

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SI Materials and Methods

Mouse Models. The *Cd11c/eyfp* transgenic (Tg) (C57BL/6 background) was developed at The Rockefeller University (1). Wild-type C57BL/6J, C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-II), C57BL/6-Tg(TcraTcrb)425Cbn/J (OT-II), and *B6.SJL-Ptpcra-Pepcb/BoyJ* (CD45.1) mice were purchased from Jackson Laboratories. Animals were bred in The Rockefeller University facilities under 12:12 light:dark cycle with ad libitum access to chow and water. All experimental procedures were approved by The Rockefeller University Animal Care and Use Committee.

Antibodies. A sheep antivesicular stomatitis virus (VSV) antiserum (2) was generously donated by Carol Shoshkes Reiss (New York University, New York, NY) and used at 1:50,000 in immunofluorescence staining. Other antibodies used for immunofluorescence were rabbit α-mouse Iba1 (019-19741; Wako) used at 1:2,000 and rabbit α-ovalbumin (OVA; Ab1221; Abcam) used at 1:1,000. For flow cytometry the following eBioscience antibodies were used at 1:200: αCD4 (GK1.5); αCD8 (53-6.7); αCD11b (M1/70); αCD11c (N418); αCD19 (eBio1D3); αCD40 (1C10); αCD45 (30-F11); αCD45.1 (A20); αCD45.2 (104); αCD45R (RA3-6B2); αCD49b (DX5); αCD80 (16-10A1); αCD86 (GL1); αCD103 (2E 7); αCD115 (AFS98); αDEC205 (205yekta); αGr-1/Ly-6G (RB6-8C5); αLy-6C (HK1.4); αMHC I (28-14-8); αMHC II (NIMR-4); Mouse $IgG2_a$ (eBM2a); αNK1.1 (PK136); Rat IgG_1 (eBRG1); Rat $IgG2_a$ (eBR2a); Rat $IgG2_{b,k}$ (eB149/10H5); Rat $IgG2_{c,k}$ (RTK4174); Rat IgM (eBRM); αSiglec H (eBio440c); αTCRβ (H57-597). In addition, the following antibodies were also used for flow cytometry: αCD122 (TM-β-1; BD Pharmingen); αCD135 (A2F10.1; BD Pharmingen); αF4/80 (CI:A3-1; AbD Serotec); Rat $IgG2_{c,k}$ (RTK4174; Biolegend).

Viral Infection Model. The wild-type VSV LD_{50} (7,000 PFU) for intranasal administration was determined in 5- to 7-wk-old male *Cd11c/eyfp* Tg mice. All subsequent intranasal infections with wild-type VSV were carried out using the LD_{50}. Briefly, mice were lightly anesthetized with 3–4% (vol/vol) isofluorane and 5 μL VSV was applied per nare and were killed between 24 and 96 h postinfection (hpi). For experiments involving UV-inactivated VSV as a control, stock virus was split into two identical aliquots. One aliquot was then subjected to a 4 W UV lamp at 254 nm for 10 min; the other was kept on ice.

Perfusion. *Cd11c/eyfp* Tg mice were deeply anesthetized with 150 mg/kg sodium pentobarbital and transcardially perfused with 0.9% NaCl containing 1 U/mL heparin, followed by 4% (wt/vol) paraformaldehyde in 100 mM phosphate buffer. Brains were removed, postfixed overnight, and immersed in 100 mM PB containing 30% sucrose. Whole brains and olfactory bulb (OB) were embedded in optimal cutting temperature compound (Sakura Finetek) and sectioned at 40 and 60 μm, respectively, on either a Microm HM 440E Sliding Microtome (Thermo Scientific) or Cryostat CM3050S (Leica), and stored at −20 °C in cryoprotectant solution (50% ethylene glycol; 50% glycerol) for later immunofluorescence staining.

Immunofluorescence. Free-floating sections from perfused brains were blocked for one hour using 0.1% Triton in 0.1 M TBS plus either normal species specific serum or BSA and incubated for 1 to 2 d at 4 °C with primary antibodies. Sections were then rinsed and incubated for 1 h at room temperature with either Cy3 fluorescently labeled secondary antibodies of the appropriate species diluted 1:400 (Jackson ImmunoResearch) or Alexa-657 fluorescently labeled secondary antibodies diluted 1:1,000 (Invitrogen). For staining with the VSV antiserum, 1:500 dilutions of biotin-conjugated secondary antibodies followed by streptavidin-conjugated horseradish peroxidase and Alexafluor-647-conjugated tyramide signal amplification as per the manufacturer's protocol (Invitrogen). Fluorescent micrographs were acquired on a LSM510 confocal Zeiss Axioplan microscope (Carl Zeiss Microimaging) or a Nikon Eclipse 90i epifluorescent microscope (Nikon).

Flow Cytometry and FACS. OB cells from whole-cell suspensions were washed, treated with anti-mouse CD16/32 F_c block (eBioscience), stained for CD45, CD11b, and DAPI (Sigma-Aldrich), and sorted in a FACS Aria Flow Cytometer (BD Bioscience) at the Rockefeller Flow Cytometry Resource Center. Similar procedures were performed for flow cytometric analysis with the exception that Molecular Probes Live/Dead Fixable Aqua (Invitrogen) was used in place of DAPI and cells were fixed with the BD Fix/Perm kit (BD Bioscience). Fixed cells were analyzed with a BD LSR-II FACS analyzer using FACS Diva software. Postacquisition data analysis was carried out using FlowJo software (TreeStar).

Splenic Dendritic Cell Isolation. Whole spleens from *Cd11c/eyfp* Tg mice were also incubated with collagenase D (Roche), dispase II (Roche), and DNase (Invitrogen) to yield single-cell suspensions. This suspension was then treated with ACK lysis buffer (Invitrogen), washed in PBS, and enriched by centrifugation using a PBS and 30% BSA step gradient (Sigma Aldrich). Cells were washed with PBS, treated with anti-mouse CD16/32 F_c block, and stained for B220 (CD45R) and DAPI. Splenocytes were sorted in a FACS Aria Flow Cytometer (BD Bioscience) to isolate dendritic cells (DC).

T-Cell Isolation. Single-cell suspensions prepared from spleens and lymph nodes dissected from OT-I or OT-II mice were rinsed in RPMI medium 1640 (Invitrogen) supplemented with 1% penicillin/streptomycin (Invitrogen), 1% L-glutamine (Invitrogen), 0.5 μM β-mercaptoethanol, and 5% FCS (Sigma-Aldrich). The lysis of red blood cells was performed for 1 min at room temperature with ACK lysis buffer (Invitrogen) and the cells were counted and resuspended at 10^7 cells/mL. After washing, cells were stained with either an OT-I antibody mixture (αB220, αCD4, αF4/80, αMHC-II, and αNK1.1) or an OT-II antibody mixture (αB220, αCD8, αF4/80, αMHC-II, and αNK1.1) for 30 min at 4 °C. OT-I or OT-II T cells were subsequently obtained by negative selection using anti-rat IgG Dyna-beads (Invitrogen). Antibodies were obtained from ATCC hybridomas raised against B220 (RA3–6B2), CD4 (GK1.5, TIB 207), CD8 (53.6.7 TIB 105), F4/80 (HB 198), MHC class II (TIB120), and NK1.1. After negative selection, OT-I or OT-II T cells were stained for 10 min at 37 °C with carboxyfluorescein succinimidyl ester (CFSE) and seeded at 3×10^4 cells per well in 96-well, round-bottom plates.

Antigen Presentation Assays. Consequently, sorted populations were cocultured with 30,000 enriched OT-I or OT-II T cells at either a 1:5 or 1:30 ratio, and incubated at 37 °C in 5% CO_2 for 3–4 d with or without 5–25 μM OVA, 250 pM OT-I peptide (SIINFEKL), or 100 nM OT-II peptide (ISQAVHAAHAEINEAGR). Cells were then stained with Molecular Probes Live/Dead Fixable Aqua (Invitrogen) and antibodies against T-cell specific markers (αCD3ε, αCD8/CD4, and αTCR $V_\alpha 2$); pro-

liferation was assessed by CFSE dilution within the T-cell population using a LSR-II flow cytometer after gating for live, $CD3\varepsilon^+$, $CD8\alpha^+$ (for OT-I) or $CD4^+$ (for OT-II), and TCR $V_\alpha 2^+$ cells.

Cytokine Profiling. Supernatants obtained from antigen presentation assays were separated into aliquots and stored at −20 °C. Thawed supernatants were subsequently analyzed using a BD Cytometric Bead Array Mouse $T_H1/T_H2/T_H17$ Cytokine Kit (BD Bioscience). Samples were processed according to the manufacturer's protocol and analyzed immediately on a LSR-II flow cytometer. FCS 2.0 files were analyzed using FCAP Array v1.0.1 software (BD Bioscience).

Radiation Chimeras. Radiation chimeras restored with bone marrow were generated by methods described in previously published protocols (3, 4). Briefly, 3-wk-old, male host CD45.1 or Cd11c/eyfp (CD45.2) Tg animals were maintained for 1 wk on antibiotics (Sulfatrim; Test Diet) before lethal cobalt irradiation with two doses of 550 cGy, delivered over 4 min with a 3-h interval between the two doses. Within an hour following the second dose of irradiation, host animals were reconstituted by tail vein injection with 7–10 million bone marrow cells from sex-matched *Cd11c/eyfp* Tg or CD45.1 donors, respectively. Irradiated mice, restored with bone marrow, were maintained on antibiotics for 2 wk, and allowed to reconstitute for a total of 4 wk.

EdU Incorporation Assays. *Cd11c/eyfp* Tg mice were intranasally infected with either VSV or HBSS vehicle alone. The mice subsequently received intraperitoneal injections of 50 mg/kg EdU (5-ethynyl-2′-deoxyuridine) once daily at 24, 48, and 72 hpi (5). After 96 hpi, mice OB were prepared for single-cell suspension as described above, and subsequently stained with CD45, CD11b, and Live/Dead Fixable Aqua. Finally, EdU incorporation was assessed using the Click-iT EdU Flow Cytometry Assay Kit (Invitrogen) according to the manufacturer's specifications.

Statistics. Most statistical analysis was carried out using GraphPad Prism 5 v5.03 (GraphPad Software). *P* values were generated using either an unpaired, one- or two-tailed *t* test or a one-way ANOVA with post hoc Tukey tests for pairwise comparisons. Error bars represent SEM. FCAP Array v1.0.1 software was used for statistical analysis of cytometric bead array standard curves and concentration determinations.

1. Lindquist RL, et al. (2004) Visualizing dendritic cell networks in vivo. *Nat Immunol* 5: 1243–1250.
2. Little LM, Zavada J, Der CJ, Huang AS (1983) Identity of HeLa cell determinants acquired by vesicular stomatitis virus with a tumor antigen. *Science* 220:1069–1071.
3. Kennedy DW, Abkowitz JL (1997) Kinetics of central nervous system microglial and macrophage engraftment: Analysis using a transgenic bone marrow transplantation model. *Blood* 90:986–993.
4. Priller J, et al. (2001) Targeting gene-modified hematopoietic cells to the central nervous system: Use of green fluorescent protein uncovers microglial engraftment. *Nat Med* 7:1356–1361.
5. Zeng C, et al. (2010) Evaluation of 5-ethynyl-2′-deoxyuridine staining as a sensitive and reliable method for studying cell proliferation in the adult nervous system. *Brain Res* 1319:21–32.

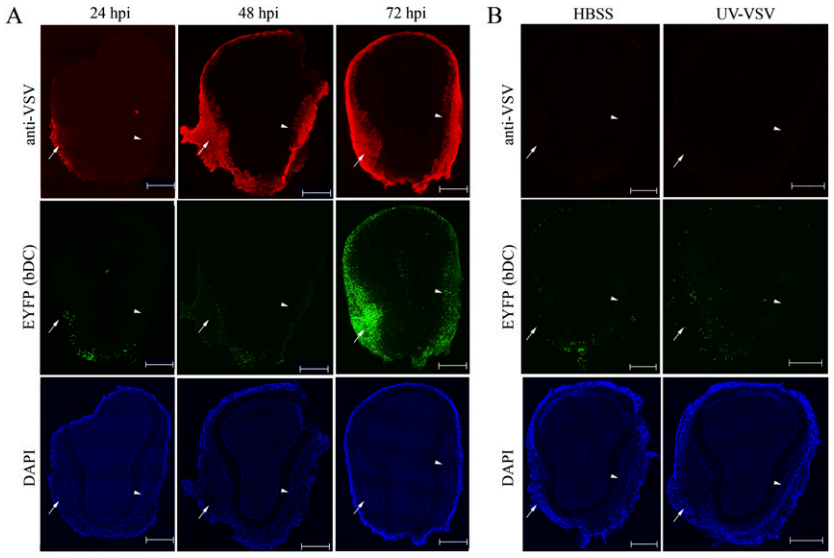

Fig. S1. Brain DC (bDC) respond to VSV-infected areas in the OB after 72 hpi. (*A*) Paraformaldehyde fixed OB sections were taken from mice intranasally infected with VSV at 24, 48, and 72 hpi. Tissue was stained with an anti-VSV antiserum (red) and examined by epifluorescence microscopy to visualize cells expressing EYFP (green); DAPI (blue) was used for anatomical reference. Viral antigens begin to penetrate the olfactory nerve layer (ON, arrows) by 24 hpi and have spread throughout the glomerular layer (GL, arrowheads) by 48 hpi. $EYFP^+$ cells are heavily recruited to VSV-infected areas by 72 hpi. (*B*) Vehicle alone and UV-inactivated VSV at 72 hpi neither exhibit $EYFP^+$ cell accumulation nor VSV antigens within the ON and GL. Representative images from three experiments with $n = 3$. (Scale bars, 500 μm.)

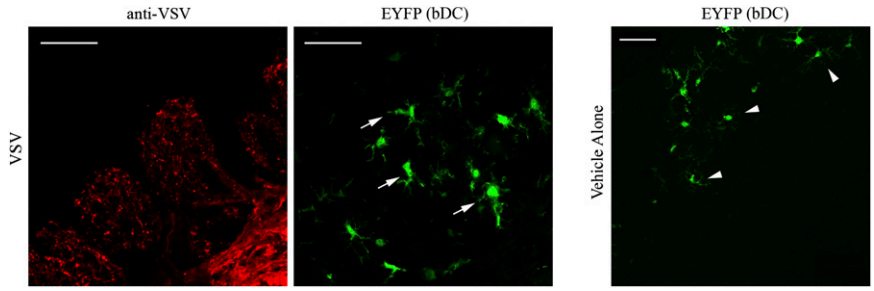

Fig. S2. EYFP$^+$ cells responding to the VSV infection possess an activated morphology. Confocal evidence that bDC around the site of VSV infection at 96 hpi have a stout deramified morphology commonly associated with activated microglia (arrows), but those from HBSS-treated mice at the same area and time point have thin highly branched dendrites (arrowheads). Representative images from three experiments $n = 3$. (Scale bars, 50 μm.)

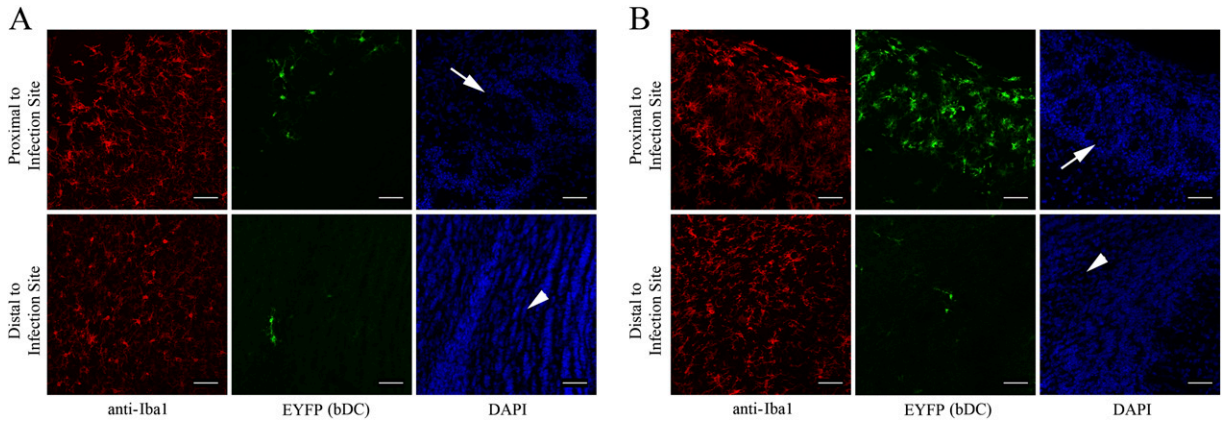

Fig. S3. Unlike bDC, microglia do not alter their distribution pattern in response to the VSV infection. Confocal *z*-stack analysis of (*A*) uninfected and (*B*) 96 hpi tissue visualizing cells with colocalized EYFP (green) and Iba1 antigen (red). Areas proximally associated with an active VSV infection mostly show Iba1$^+$ cells colabeled with EYFP; this staining pattern represents bDC. Conversely, areas distally associated with an active VSV infection mostly show Iba1$^+$ cells without the EYFP reporter; this staining pattern represents microglia. Arrows represent OB glomerular layer, arrowheads represent granule cell layer. Representative images from three experiments $n = 3$. (Scale bars, 50 μm.)

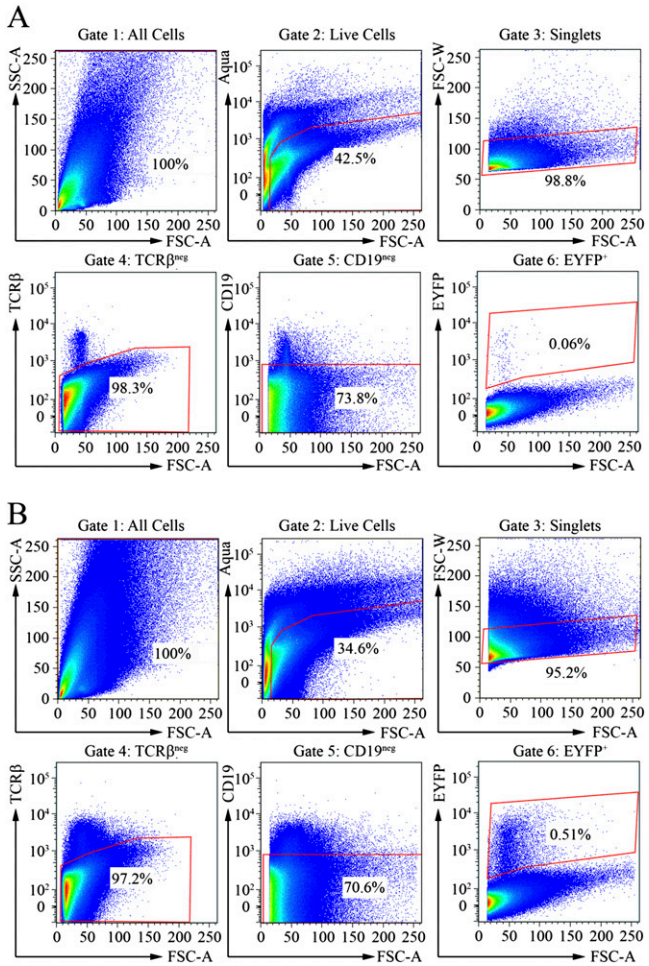

Fig. S4. Representative gating strategy used to identify the EYFP^{+} population at 96 hpi. Gating live cells of all size and complexity to exclude T and B lymphocytes in the OB of (*A*) HBSS-treated and (*B*) VSV-infected *Cd11c/eyfp* Tg mice at 96 hpi. Representative images from three experiments.

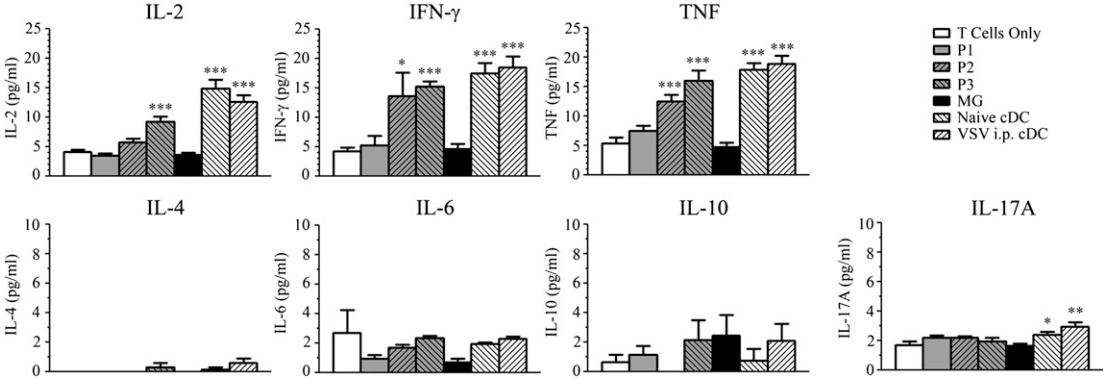

Fig. S5. P2 and P3 direct a T_H1 cytokine profile in ex vivo T lymphocyte cultures. The levels of proinflammatory cytokines IL-2, IFN-γ, and TNF are elevated in CD4^{+} OT-II T-cell receptor (TCR)-restricted proliferating T-cell cultures, in response to P2 and P3 bDC antigen presentation. IL-2, IL-4, IL-6, IFN-γ, TNF, IL-10, and IL-17A levels were assessed in cultured supernatants, after 4-d stimulation, using cytometric bead arrays, and were analyzed using BD FCAP Software v1.0.1; error bars represent SEM of six replicates repeated three times. *P value$_{vs.\ T\text{-cell only}} < 0.05$, **$P$ value$_{vs.\ T\text{-cell only}} < 0.01$, ***$P$ value$_{vs.\ T\text{-cell only}} < 0.001$.

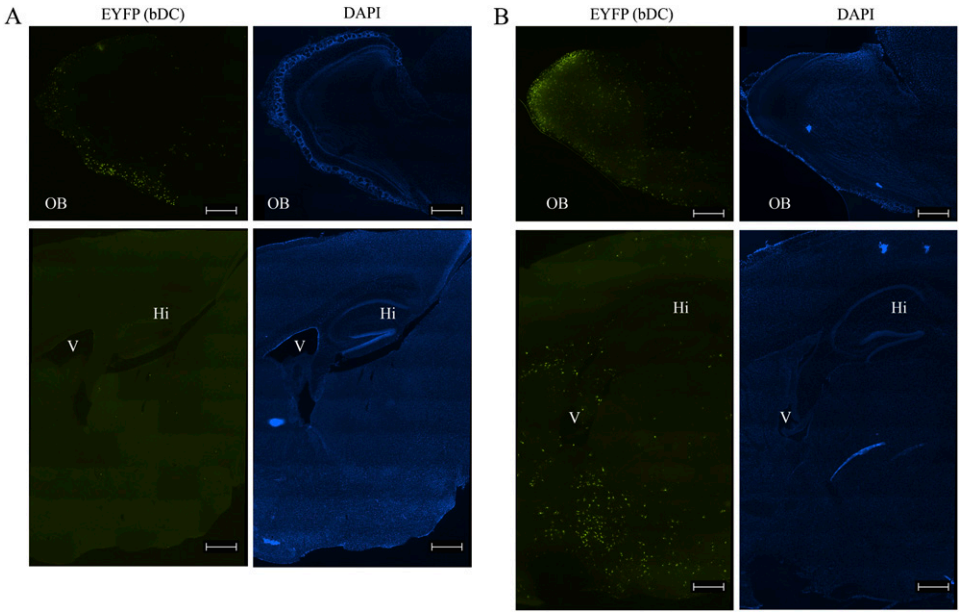

Fig. S6. Peripherally derived P2 only responds to areas associated with VSV infection. (*A*) In the CD45.1 host replaced with EYFP$^+$ (CD45.2) bone marrow chimeras EYFP$^+$ cells represent P2. In these chimeras, EYFP$^+$ cells appear only in the OB, they do not appear in other areas of the brain associated with bDC in the steady state, such as near the ventricles. (*B*) In contrast, in the EYFP$^+$ (CD45.2) hosts replaced with CD45.1 BM EYFP$^+$ cells represent P1. In these chimeras, not only is there an accumulation of the EYFP$^+$ cells in the OB, but this radio-resistant population appears throughout the brain in areas associated with bDC presence in the steady state. H and V denote hippocampus and ventricle, respectively. Representative images from three experiments n = 5–6. (Scale bars, 500 μm.)

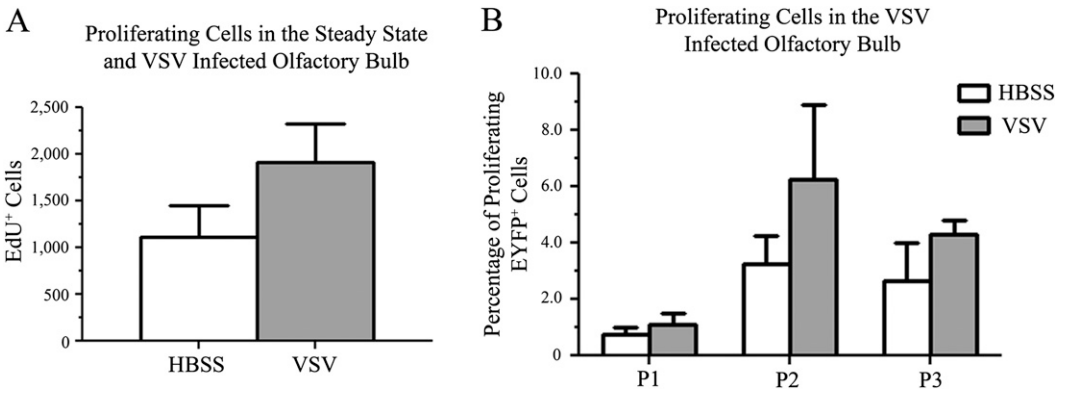

Fig. S7. VSV-induced bDC accumulation is not because of proliferation of EYFP$^+$ cells. (*A*) EdU was used to test for VSV-induced bDC proliferation. VSV infection resulted in a significant increase in EdU incorporation compared with vehicle treated controls. (*B*) Despite increased EdU levels, EdU was absent in all three EYFP$^+$ CD45$^+$ CD11b$^+$ populations, and only present in EYFP^{neg} cells (not shown). Data are representative of five replicates from three experiments. Error bars represent SEM.

Table S1. Phenotyping data associated with EYFP$^+$ populations, microglia (MG), and conventional DC (cDC) represented as geometric mean fluorescence intensities

Marker	cDC	P1	P2	P3	MG
CD4	148	0	0	0	0
CD8a	198*	0	0	31*	0
CD11c	1,498*	933*	2,677*	4,167*	199*
CD40	149	175*	254*	128	124
CD45R	328*	23	0	714*	4
CD49b	8	0	0	539*	37
CD80	394*	267*	572*	183*	115*
CD86	341*	478*	2,475*	454*	246*
CD103	102	1,155*	1,183*	180*	852*
CD115	0	618*	172	8	456
CD122	7	0	0	170*	0
DEC205	95	0	0	128*	0
F4/80	176*	1,330*	1,274*	184*	475*
Gr-1	29*	0	1,565*	37*	0
Ly-6C	358*	69*	38,626*	1,838*	42*
MHC I	6,439*	12,505*	18,737*	4,537*	5,518*
MHC II	5,173*	0	1,310*	1,543*	0
NK1.1	11	1,490*	0	462*	438*
Siglec H	7	56	49	0	1

*P value < 0.05 compared with isotype controls.