Experimental Procedures:

Mice

C57BL/6J were purchased at Janvier Labs. CD11c YFP (Lindquist et al., 2004), OT-I (Hogquist et al., 1994), OT-II (Barnden et al., 1998), IFNγ-YFP (Reinhardt et al., 2009), Kbm1 (Schulze et al., 1983), MHCII (Madsen et al., 1999), tdTomato (Kastenmuller et al., 2013), UbGFP (Schaefer et al., 2001), XCR1-DTR-Venus (Yamazaki et al., 2013) and various inter-crosses thereof were maintained at in-house facilities. zDC mice (Satpathy et al., 2012) were kindly provided by Lino Teichmann, Clec9a$^{+/cre}$ x Rosa$^{+/tdTomato}$ mice (Schraml et al., 2013) were kindly provided by Caetano Reis e Sousa.

Treatment of mice

For depletion of CD4^{+} T cells in vivo, mice received 500 µg anti-CD4 (GK1.5; BioXcell) or isotype control (LTF-2; BioXcell) i.p. on d-3 and d-1. For depletion of XCR1^{+} DC, transgenic mice and control littermates were treated with 0.5 µg DTX (Merck Millipore) on d-1 and d0 i.p.. To block entry of naïve T cells mice received 100 µg anti-CD62L (Mel 14; BioXcell) i.p..

Virus purification and generation

Highly purified viruses have been used for this study. Adenovirus (Ad-GFP) (Stabenow et al., 2010) was purified using a Cesium Chloride density gradient and was kindly provided by Percy Knolle (Technical University, Munich,

Germany). MVA and Vaccinia Viruses were purified using two consecutive sucrose cushions. MVA-GP (Frenz et al., 2010) was kindly provided by Gerd Sutter (LMU, Munich, Germany). *MVA-WT, MVA-OVA, MVA-OVA-GFP,* MVA-NP-SIINFEKL-GFP *and MVA-OVA-tdTomato* have been previously described (Kastenmuller et al., 2013). MVA–GP-Venus and *MVA-OVA-Kb* were generated based on standard methods (Staib et al., 2004).

Bacterial infection

Recombinant *L.m.*-B8R was a gift from Ross Kedl (University of Colorado, Denver). The strain was maintained as a –80°C stock in brain-heart infusion/50% glycerol (BHI/50% glycerol). Before each experiment, recombinant *L.m.*-B8R was grown overnight at 37°C with aeration.

Flow Cytometry

Cells were surface stained with anti-CD8 (5H10; Caltag Laboratories), anti-CD44 (IM7, BD Biosciences), CD3 (145-2C11; BD), NK1.1 (PK136; BD), CD4 (GK1.5; BD), CD11b (M1/70; eBioscience), CD8 (2G9; Caltag), MHCII (M5/114.15.2; BD), 25D1-biotinylated (in house) (Porgador et al., 1997), CD69 (H1.2F3; BD), CD25 (3C7; BD), CD127 (A7R34; DB), KLRG1 (2F1; DB) CD11c (N4.18; BD). Intracellular cytokine staining was performed with anti-IFNγ (XMG1.2; BD), TNFα (MP6-XT22; BD), IL2 (JES6-5H4; BD) using the Cytofix/Cytoperm kit (BD Biosciences). Flow cytometric data were collected on an LSRII or Canto II (BD Biosciences) and analyzed with FlowJo software (TreeStar).

Immunofluorescence Staining

LN and spleens were harvested and fixed using PLP buffer (0.05 M phosphate buffer containing 0.1 M L-lysine (pH 7.4), 2 mg/ml $NaIO_4$ and 10 mg/ml paraformaldehyde) for 12h, then dehydrated in 30 % sucrose prior to embedding in OCT freezing media (Sakura Finetek). 30 µm sections were cut on a CM3050S cryostat (Leica), adhered to Superfrost Plus slides (VWR), stained, mounted with Fluormount G (Southern Biotech) and acquired on a 710 confocal microscope (Carl Zeiss Microimaging). Frozen sections were permeabilized and blocked in 0.1 M Tris (AppliChem) containing 0.3 % Triton X-100 (GERBU Biotechnik), 1 % FCS (Biochrom AG), 1 % GCWFS (Sigma Aldrich) and 1% normal mouse serum (Life Technologies). Serial lymph node sections were prepared, each section was visually inspected using epifluorescent light microscopy, and several representative sections from different LN areas were acquired using confocal microscopy for detailed analysis. The following antibodies were used for staining: anti-CD8 (2G9; Caltag), ERTR-7 (Santa Cruz), anti-B220 (RA3-6B2; BD), CD69 (polyclonal R&D Systems), GFP (polyclonal, Invitrogen), CD4 (GK1.5, BD), NK1.1 (PK136, BD). Unconjugated primary antibodies were stained with AF-conjugated secondary antibodies (Invitrogen).

Quantitative analysis of imaging data

Semi-automated analysis was performed using the Imaris software tools. *Distance quantification:* Both infected cells and the various T cell populations were localized using Imaris spot function and relative distance was calculated

using Excel software calculating the minimal distance in 3D. To calculate the distance to the LN capsule spots outlining the capsule were placed manually using ERTR7 staining as guidance. *Co-localization and cluster quantification:* T cell clusters were defined semi-automated using a surface generation tool (Imaris) and incorporated at least 3 cells. Co-localization with other cell populations were counted manually.

Automated image analysis of cluster formation

Cluster formation of pure OT-I clusters and mixed clusters of OT-I, OT-II and polyclonal $CD4^+$ T cells was evaluated quantitatively by automated image analysis based on a previously developed method (Klauschen et al., 2009). Subsequent to image segmentation, cells and cell clusters including composition of clusters were computed using a connected component algorithm. Because of the large field of view and therefore limited image resolution on the level of single cells, individual cells cannot be identified robustly within clusters. We therefore determined the average cell size based on the size distribution of single cells as 13 voxels and used this information to estimate cluster composition (the results were robust to cell size estimate variations, data not shown). The computations were performed with the GNU Octave scientific computing framework on standard hardware.

Intravital two-photon imaging

Mice were anesthetized with isoflurane (Baxter; 2.5% for induction, 1~1.5% for maintenance, vaporized in an 80:20 mixture of O_2 and air), popliteal LN were exposed and intravital microscopy was performed. The imaging system was composed of a Chameleon laser (Coherent) tuned to 820nm or 930nm and a Zeiss 780 upright microscope equipped with a 20× water immersion lens (NA 1.0, Zeiss) and ZEN acquisition control software. The microscope was enclosed in an environmental chamber in which anesthetized mice were warmed by heated air and the surgically-exposed LN was kept at 36-37°C with warmed PBS. For dynamic imaging we typically used a z-stack of 57 μm and 3μm step size and acquired every 40sec. Raw imaging data were processed and analyzed with Imaris (Bitplane).

Clodronate Liposome

For *in vivo* depletion of LN macrophages, mice were injected in the footpad with 20μl of clodronate containing liposomes or empty liposomes as control (Encapsula) 7 days before infection.

Surgical lymph vessel disruption

For surgical lymph vessel disruption, mice were anesthetized with isoflurane (Baxter; 2.5% for induction, 1~1.5% for maintenance, vaporized in an 80:20 mixture of O2 and air) and popliteal LNs were exposed. Afferent lymphatic vessels were surgically disconnected and the wound was closed using tissue

adhesive (3M VetBond). Successful lymph vessel disruption was tested using trpyan blue injection in the footpad.

Cell labeling

Isolated lymphocytes were labeled with 1 μM Cell Tracker Green, 100 μM Cell Tracker Blue or 10μM Cell Tracker Orange (Invitrogen), as previously described (Kastenmuller et al., 2013).

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