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Supplemental Information

S1P-Dependent Trafficking of Intracellular *Yersinia pestis* through Lymph Nodes Establishes Bubos and Systemic Infection

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Supplemental Figures:

Figure S1: Necrotic lesions in Y. pestis-infected LNs. Progressive necrosis within *Y. pestis*-infected LNs, revealed by staining with propidium iodide, compared to Saline-injected controls. Tissue sections were stained with a 1:10000 dilution of propidium iodide for 1 minute, followed by washing with PBS and mounting.

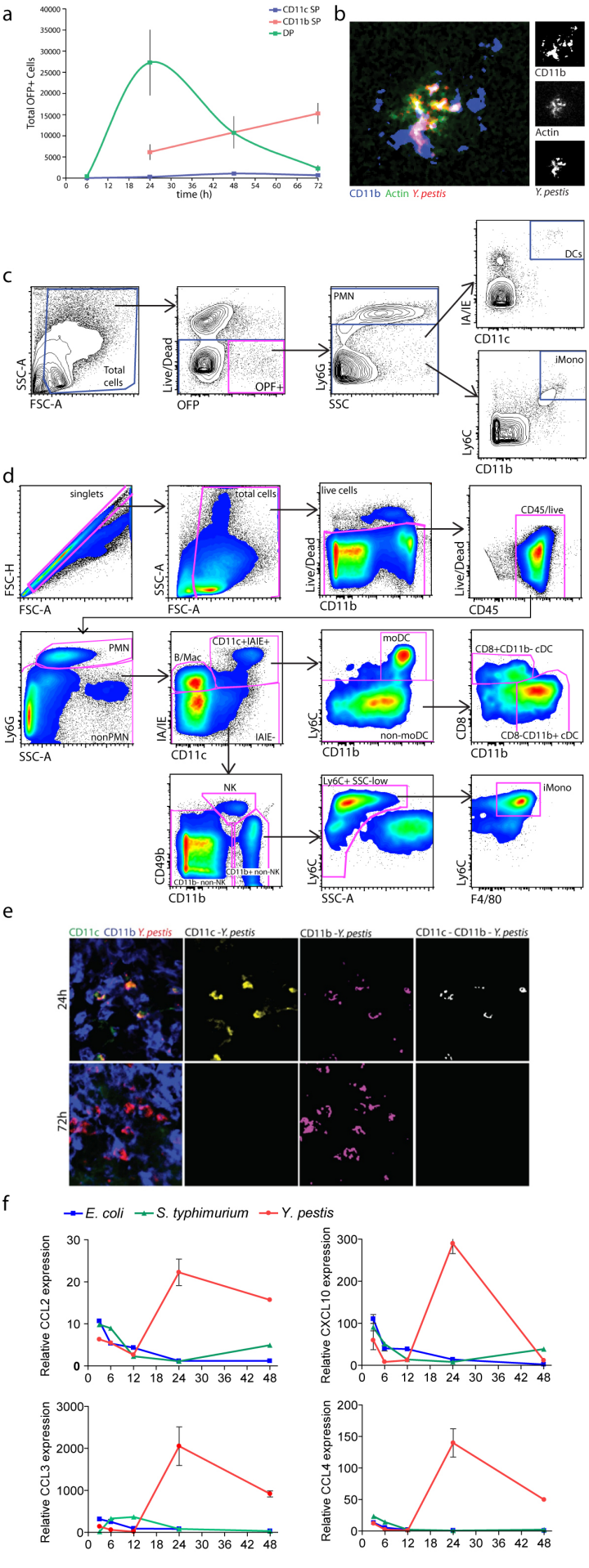

Figure S2: Phenotyping of Y. pestis-infected cells (**a**) Graph represents the total numbers of cells within DLNs determined by flow cytometry to be single positive (SP) and double positive (DP) for markers CD11b and CD11c, after gating on cells staining positive for YP-OFP. For each group, n≥3. (**b**) *Y. pestis* expressing OFP were conjugated to magnetic particles following an established protocol[1] and used to infect the footpads of mice by injection of $1x10^5$ CFU subcutaneously. After 24h, DLNs were isolated, processed to form single cell suspensions, and a magnet was used extract the bacteria within the LN. After washing, the fraction remaining was spun onto slides, fixed, and stained for actin (green) and the surface monocyte/macrophage marker, CD11b (blue). Many intracellular bacteria could be observed within cells that had surface staining for CD11b, and surrounded by actin. (**c**) Gating strategy used to phenotype cells in Figure 2b. (**d**) Gating strategy used to phenotype cells in Figure 2c-d. (**e**) Co-localization images were generated using the ImageJ software program to depict the areas with overlapping signals for myeloid markers CD11c and CD11b and for OFP-expressing protein in images included in Figure 2. CD11c and *Y. pestis* colocalization is depicted as yellow; CD11b and *Y. pestis* colocalization is depicted as magenta; triple colocalization is depicted as white. Note that at 24 hours post infection infected cells primarily express CD11b and CD11c, while at 72 hours *Y. pestis* colocalize primarily with the CD11b but not CD11c marker, suggesting that the bacilli are located mostly within non-DC, monocytic type cells. (**f**) Real time PCR was performed to quantify mRNA levels for the chemokines CCL2, CCL3, CCL4 and CXCL10 present in LNs infected with *E. coli, S. typhimurium or Y. pestis* over a time course.

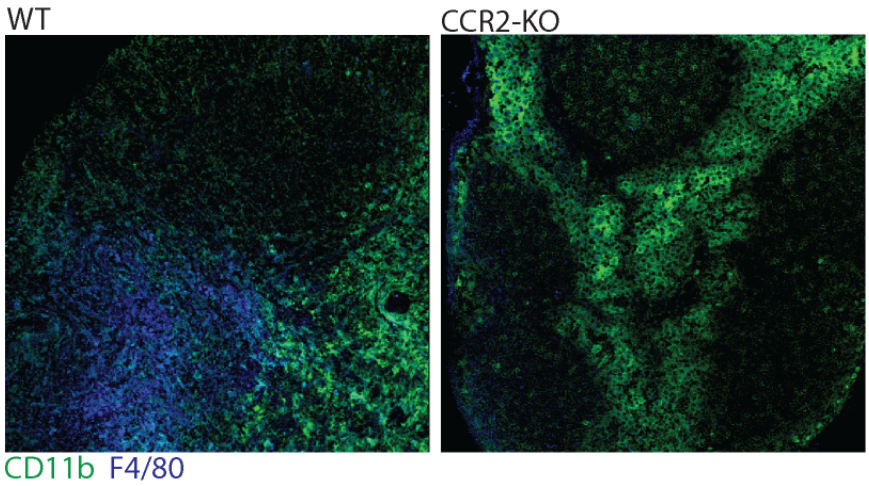

Figure S3: Inflammatory monocytes are absent in DLNs of *Y. pestis*-infected CCR2-KO mice. Images of LNs isolated from mice infected for 24 hours with *Y. pestis*, sectioned and stained for monocyte markers CD11b and F4/80 to demonstrate the differential cellular recruitment into and localization within infected lymph nodes in CCR2-KO mice compared to WT. Images are representative of data acquired using n=2 mice.

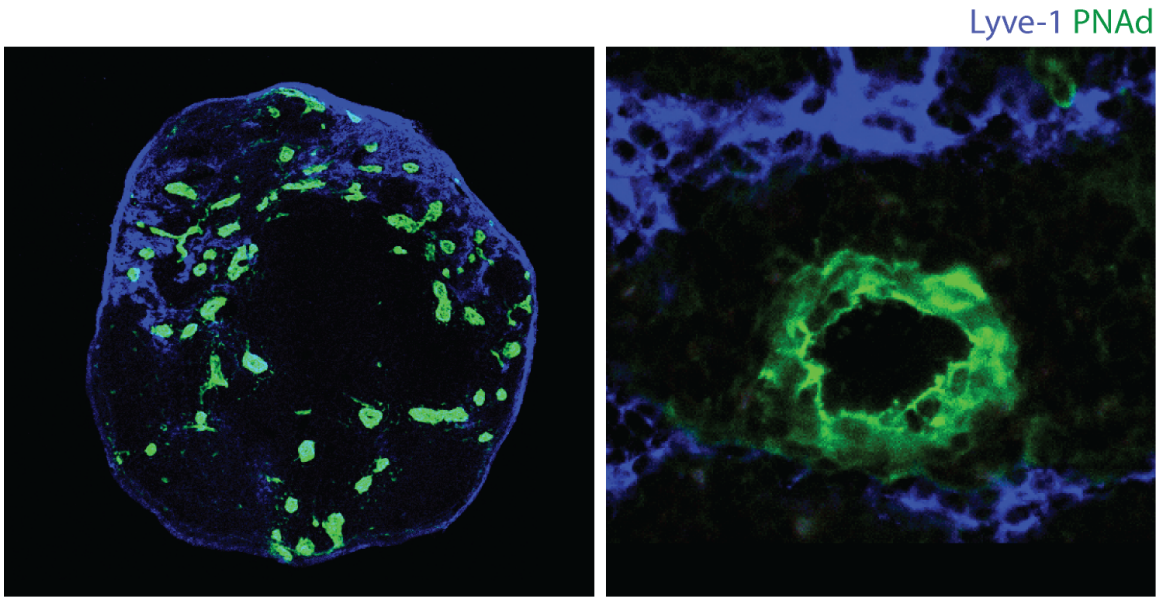

Figure S4: DLN vascular remains intact within buboes. DLNs stained for LN sinuses (Lyve-1, blue) and high endothelial venules (CD31, green) 72 hours after footpad infection with *Y. pestis*. The vasculature appeared intact and without pathology. Images are representative of n=3.

a

		CD11b SP	CD11b$^+$CD11c$^+$
Lymph Node	Resting	89.8% ± 3.2%	77.0% ± 1.8%
	Y. pestis	85.7% ± 2.5%	84.3% ± 1.8%
Footpad	Resting	99.6% ± 0.04%	44.2% ± 11.2%
	Y. pestis	98.0% ± 1.58%	60.4% ± 7.0%

*data do not differ signficantly between resting and infected states

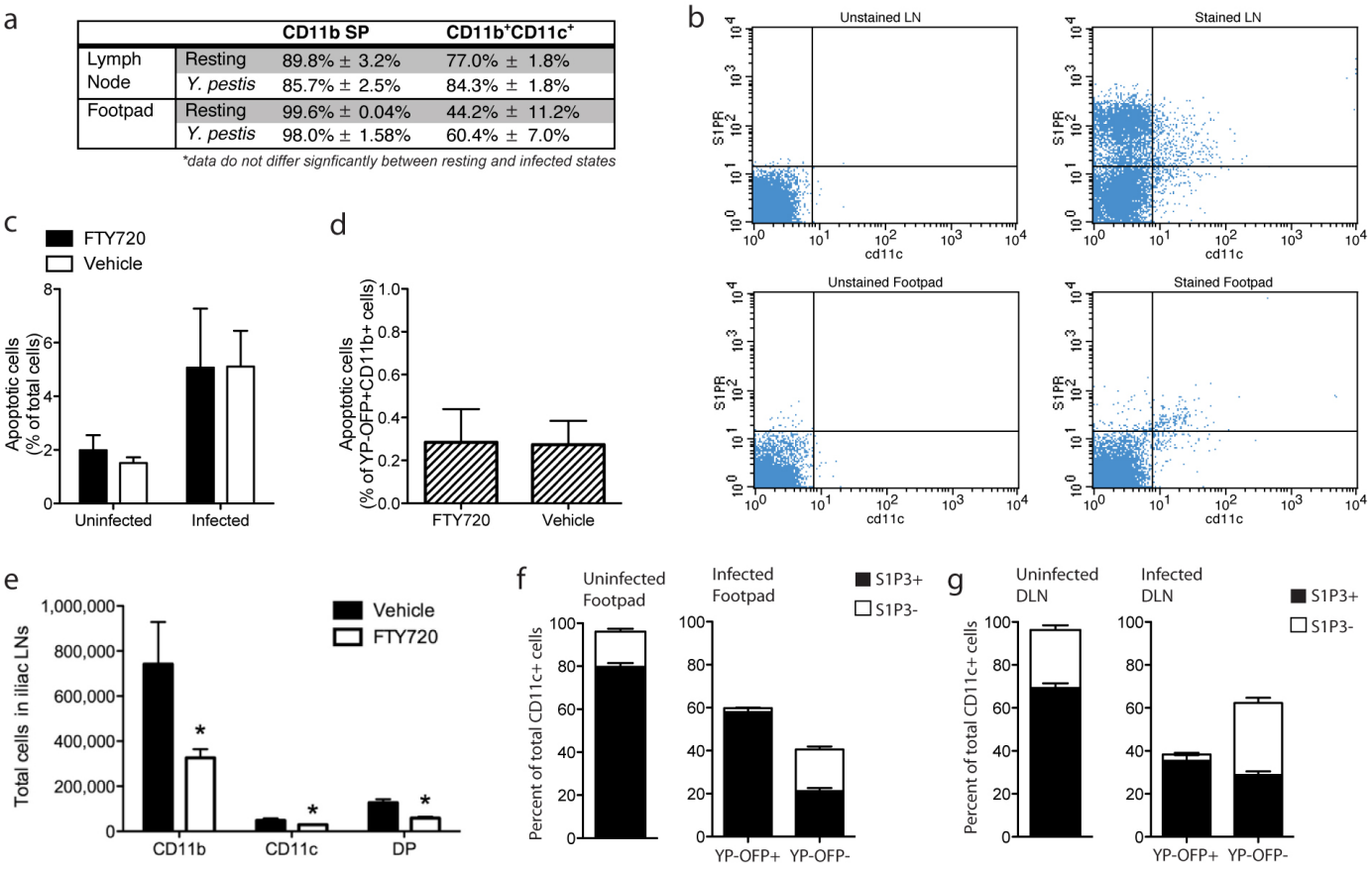

Figure S5: S1P-targeting on DCs and monocytes (**a**) CD11c$^{\pm}$CD11b$^+$ cellular populations express $S1P_1$ in both the LN and the footpad. Significant differences in the numbers of $S1P_1^+$ cells were not observed between the resting state and during infection with *Y. pestis.* (**b**) Representative flow cytometry plots demonstrating effective staining of $S1P_1$ in LNs and footpad tissue are provided. (**c**) FTY720 treatment does not significantly affect the percentage of apoptotic cells at steady state or during infection (p=0.8666). Infection increases the % of apoptotic cells in the lymph node (p=0.0364). (**d**) For infected monocytic cells (CD11b+YP-OFP+) FTY720 treatment also does not increase the percentage of apoptotic cells (p=0.9536) (**e**) Animals were pretreated 24 hours before infection and then administered a daily regimen of S1P-agonist FTY720 or vehicle. Graph represents the total numbers of *Y. pestis*-infected cells (OFP$^+$) staining single and double positive for markers CD11b and CD11c in iliac LNs distal to DLNs 48 hours after infection. FTY720 significantly reduced all CD11b$^+$CD11c$^{\pm}$ infected cell types with p=0.01 by ANOVA. * represents significant differences in numbers for each cell type comparison, determined by Bonferroni's post-test. (**f**) Footpads and (**g**) DLNs were isolated 24h after the injection of saline (for uninfected controls) or *Y. pestis* expressing OFP ($1x10^5$ CFU). Single cell suspensions were stained for CD11c and $S1P_3$ and analyzed by flow cytometry. Graphs represent cell numbers as a percentage of total CD11c$^+$ cells. For infected mice, the proportions of total CD11c cells are separated into infected cells (YP-OFP+) and uninfected cells (YP-OFP-). Most infected cells express $S1P_3$.

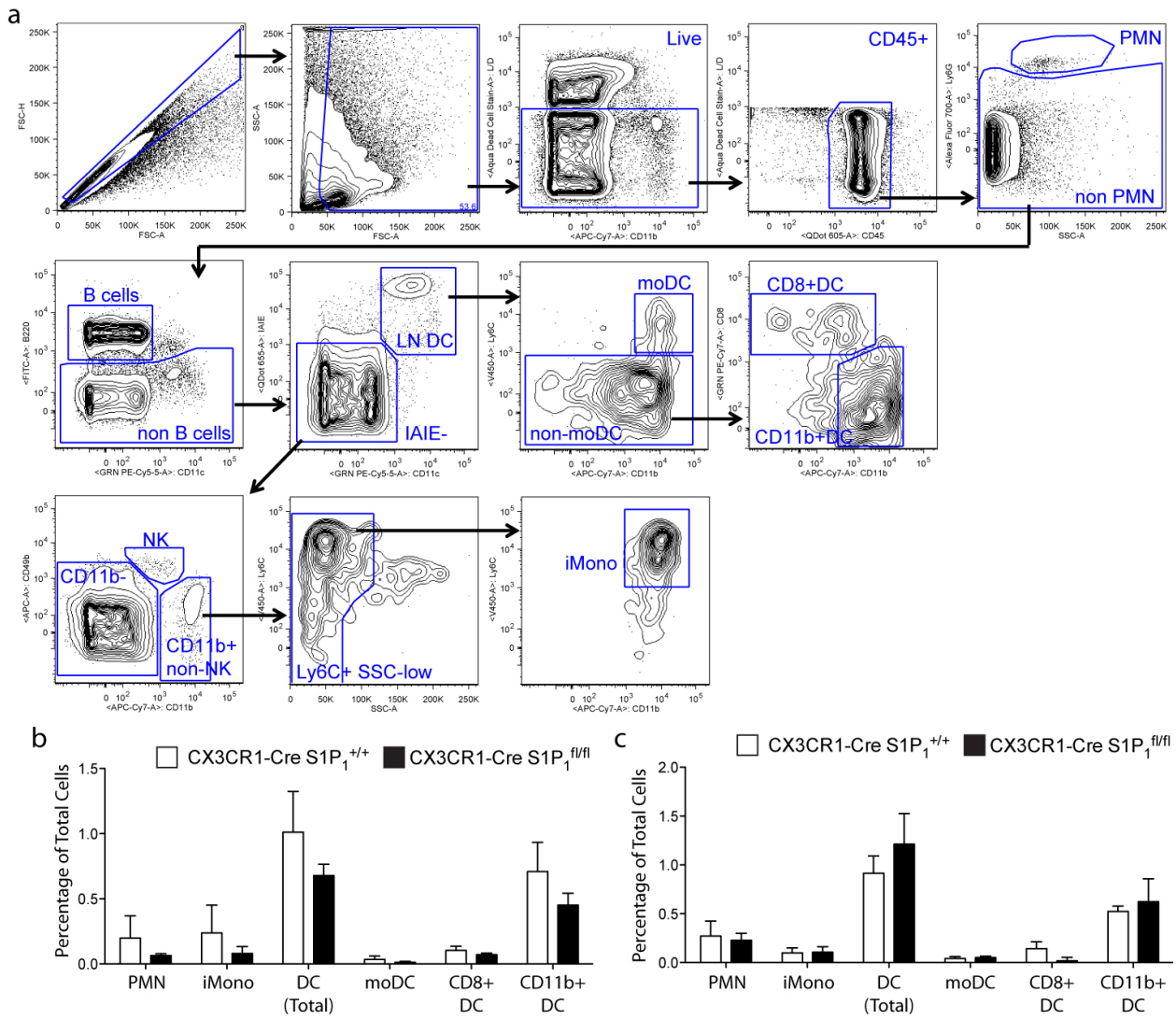

Figure S6: Comparable LN cellularity at baseline in CX3R1-Cre S1P$_1^{fl/fl}$ and control mice. (**a**) Gating strategy used to compare the cellular composition of popliteal and iliac LNs in CX3R1-Cre S1P$_1^{fl/fl}$ and CX3R1-Cre S1P$_1^{+/+}$ mice. No significant differences in cellularity were observed in the (**b**) popliteal or (**c**) iliac LNs.

Supplemental Methods:

Microscopy

For H&E staining, tissues were either frozen sectioned and fixed or prepared by paraffin imbedding, followed by sectioning, deparaffinizing and rehydration in water. Standard H&E protocols were then used, staining first with hematoxilyn, then washing with water, counterstaining eosin, followed by washing, dehydration and mounting.

For frozen sectioning, isolated tissues were flash frozen in OCT (TissueTek), then cryostat sectioned (10 μm) and fixed on slides with acetone for 15 minutes at 4°C. To obtain the image in **Figure 4c**, mice were euthanized and perfused with 4% paraformaldehyde to fix tissues *in situ*. Skin was removed from legs, which were subjected to demineralization according to a published protocol[2]. Briefly, the legs were placed into decalcification buffer (10% EDTA in 0.1M Tris buffer, pH 6.95) and kept in the dark at 4°C for 2 weeks. The buffer was changed every 3-4 days. Demineralized hind legs were then embedded and frozen in OCT for sectioning. For all immuno-staining, tissue sections were rehydrated in PBS containing 1% BSA for 20 minutes, followed by overnight staining with primary antibodies, as indicated in the figure legends: anti-CD11b, anti-CD11c, anti-CD31-biotin (BD Biosciences), anti-F4/80-PE (eBiosciences), anti-Lyve-1. The following secondary antibodies were used: biotinylated anti-hamster (Pharmingen), followed by streptavadin-APC (BD Biosciences) or anti-goat-FITC (Jackson ImmunoResearch).

For TEM, LN tissue was fixed in 4% glutaraldehyde. The samples were treated with 1 % osmium tetroxide (Electron Microscopy Sciences) before dehydration using increasing concentrations of ethanol and embedding in resin. Samples were then sectioned (50–70 nm) and stained with 2 % uranyl acetate/lead citrate (TAAB Laboratories) before viewing using a JM1010 electron microscope (JEOL). Sample processing was performed by the National University of Singapore electron microscopy core facility.

Real time PCR

To quantify chemokine mRNA, we homogenized individual lymph nodes or footpads using ceramic beads for 2 cycles of 20 seconds each by automatic homogenizer, followed by RNA isolation using the

QIAshredder and RNeasy Kits (Qiagen) according to manufacturer's instructions. cDNA was synthesized using the iScript cDNA Synthesis kit (BioRad) and real time PCR was performed using SYBR Green and the iCycler machine (BioRad), with normalization to actin expression. Primers were obtained from IDT-DNA for β-actin: 5' – TGA GAG GGA AAT CGT GCG TGA CAT, 5' – ACC GCT CAT TGA CGA TAG TGA TGA; CCL2: 5' – AGC AGG TGT CCC AAA GAA GCT GTA, 5' – AAA GGT GCT GAA GAC CTT AGG GCA; CCL3: 5' – GCT GAC ACC CCG ACT G, 5' – AGA TCT GCC GGT TTC TCT; CCL4: 5' – CAC CAA TGG GCT CTG AC, 5' – AGG GCT CAC TGG GGT TAG; and CXCL10: 5' – TGC TGC CGT CAT TTT CTG, 5' – TCG CAG GGA TGA TTT CAA.

Flow Cytometry

Tissues were isolated from mice at the time points indicated and minced in RPMI containing 10% FBS and 100U/ml of Collagenase A (Sigma). Single cell suspensions were produced by straining the disrupted lymph nodes through a 70μM cell straining filter (BD Biosciences). Total cells were calculated after counting using a hemacytometer. To stain for DCs and monocytes, we used the following antibodies: anti-CD11b-APC anti-CD11c-FITC (BD Biosciences). Either cells from un-infected controls were individually stained for CD11b or CD11c, or YP-OFP-infected, unstained DLN cells were used as compensation controls. Additional flow cytometry was performed for S1P receptors using antibodies against $S1P_1$ or $S1P_3$ (R&D Systems) followed by staining with anti-rat or anti-rabbit FITC-conjugated secondary antibodies (Jackson ImmunoResearch). Antibodies against CX3CR1 were obtained from Fisher Scientific and used with an AlexFluor647-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch). Additional cell phenotyping of multiple myeloid subsets was performed by staining LN cells in PBS with 3% fetal bovine serum, 10mM EDTA, 5% normal mouse serum, 5% normal rat serum and 1% FC Receptor Block (eBioscience) at 4°C for 30minutes and analyzed by BD LSRII flow cytometer with FlowJo software (Tree Star). FITC-conjugated anti-CD8 (53-6.7), Alexa Fluor® 700- conjugated anti-Ly-6G (1A8), APC-Cy7-conjugated anti-CD11b (M1/70), V450-conjugated anti-Ly-6C (AL-21) are from BD Pharmingen. PE-Cy5.5-conjugated anti-CD11c (N418), PE-Cy7-conjugated anti-F4/80 (BM8), APC-conjugated anti-CD49b (DX5), eFluor® 605NC-conjugated anti-CD45 (30-F11) and eFluor® 650NC-conjugated anti-MHC Class II (I-A/I-E)

(M5/114.15.2) are from eBioscience. LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit is from Molecular Probes. Apoptotic cells were determined using the ApoTag kit (Millipore) according to manufacturer's instructions.

Ex vivo gentamicin protection assay

To quantify the extracellular versus intracellular *Y. pestis* populations within LNs, an *ex vivo* gentamicin protection assay was performed. Gentamicin was chosen as the antibiotic for this experiment because *Y. pestis* is highly susceptible to it[3,4]. DLNs and ILNs were isolated and incubated in antibiotic-free RPMI with 10% FBS and 100µg/mL collagenase at 37°C for 30min. Single cell suspensions were then prepared. This was divided into two equal aliquots, one treated with 2µg/mL gentamicin (pre-determined to be 100% bactericidal) and the other with control (RPMI media) to kill extracellular *Y. pestis* for 30 min at 37°C. After washing with PBS, samples were resuspended in 100µL dH_20 to lyse cells and plated on BHI agar. CFUs were enumerated after 48h growth at room temperature. The fraction of intracellular bacteria was then determined as a percentage of the total by calculation.

For magnetic isolation of *Y. pestis*-containing cells, *Y. pestis* expressing OFP was conjugated to magnetic beads precisely following an established protocol[1]. $1x10^5$ labeled bacteria were injected into the footpads of mice. After 24h, DLNs were harvested and processed to produce a single cell suspension, as previously described. Isolation of bacteria was performed using a magnet, again following the methods provided by Lonnbro et al. Isolated bacteria were resuspended in PBS and applied to slides by cytospinning. Slides were then fixed in acetone, prior to staining for actin (Alexafluor488-conjugated phalloidin, Molecular Probes) and CD11b, followed by visualization by confocal microscopy.

1 Lonnbro, P., Nordenfelt, P., & Tapper, H., Isolation of bacteria-containing phagosomes by magnetic selection. *BMC Cell Biol* 9, 35 (2008).

2 Jonsson, R., Tarkowski, A., & Klareskog, L., A demineralization procedure for immunohistopathological use. EDTA treatment preserves lymphoid cell surface antigens. *J Immunol Methods* 88 (1), 109-114 (1986).

3 Louie, A., Vanscoy, B., Liu, W., Kulawy, R., Brown, D., Heine, H.S., & Drusano, G.L., Comparative efficacies of candidate antibiotics against Yersinia pestis in an in vitro pharmacodynamic model. *Antimicrob Agents Chemother* 55 (6), 2623-2628 (2011).

[4] Hernandez, E., Girardet, M., Ramisse, F., Vidal, D., & Cavallo, J.D., Antibiotic susceptibilities of 94 isolates of Yersinia pestis to 24 antimicrobial agents. *J Antimicrob Chemother* 52 (6), 1029-1031 (2003).