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Supplemental Information

CXCR3 Chemokine Receptor-Ligand Interactions in the Lymph Node Optimize $CD4^+$ T Helper 1 Cell Differentiation

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INVENTORY OF SUPPLEMENTAL INFORMATION

- **Figure S1** (related to **Figure 1**) shows phenotype of DCs transferred and time course of IFN-γ upregulation by OTII cells *in vivo*.
- **Figure S2** (related to **Figure 2**) shows IFN-γ production by WT and *Cxcr3*$^{-/-}$ OTII cells in separate hosts *in vivo* and *in vitro*.
- **Figure S3** (related to **Figure 3**) shows lack of CXCL11 protein production in C57BL/6 mice, upregulation of *Cxcl9* and *Cxcl10* RNA in activated LNs, phenotype of WT, *Cxcl9*$^{-/-}$ and *Cxcl10*$^{-/-}$ DCs, and construction and conformation of the REX3 BAC Tg mice.
- **Figure S4** (related to **Figure 3**) shows quantification of WT and *Cxcr3*$^{-/-}$ OTII movement at 24 hr post T cell transfer.
- **Figure S5** (related to **Figure 6**) identifies LN regions.
- **Movie S1** (related to Figure 3) shows REX3 Tg DCs (blue) interacting with WT (green) and *Cxcr3*$^{-/-}$ (red) OTII cells. Available online.
- **Supplemental Experimental Procedure**
- **Supplemental References**

Figure S1

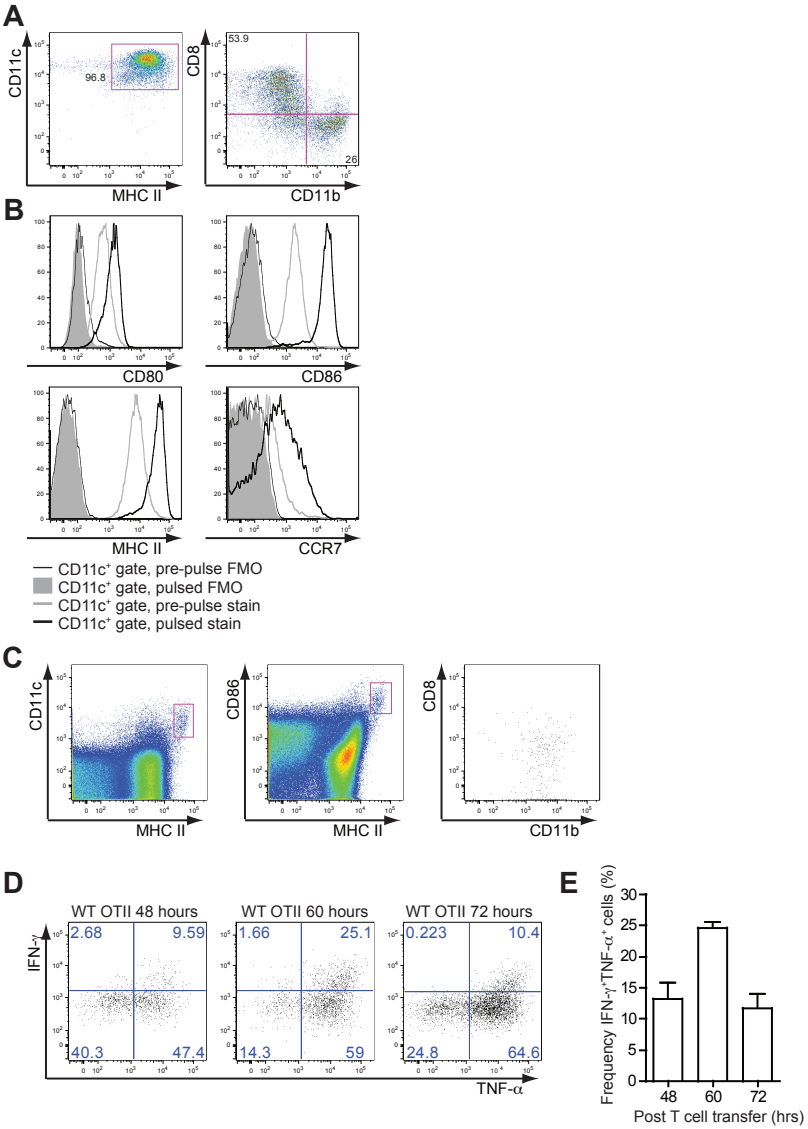

Figure S1. Antigen-pulsed DCs phenotype and time course of IFNγ production in transferred T cells. (**A**) CD11c purified *in vivo* expanded DCs are either $CD8^+$ or $CD11b^+$. (**B**) OVA pulsing and TLR-ligand stimulation induction of DC activation markers CD80, CD86, MHC II, CCR7. Fluorescence minus one (FMO) are indicated for pre- and post-pulse for each marker. (**C**) CMFDA-labeled DCs injected subcutaneously in footpads were tracked to dLNs 24 hr post injection. Only $CD11b^+$ DCs can be found in dLNs (far right panel). (**D**,**E**) IFN-γ production of OTII T cells at times indicated post T cell transfer. Error bars denote SD.

Figure S2

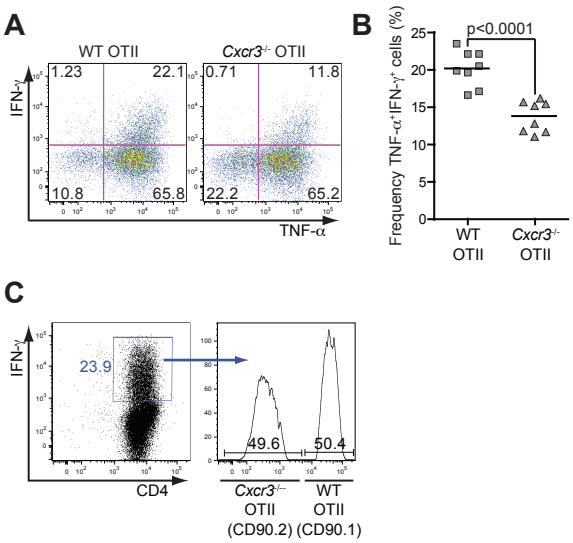

Figure S2. *Cxcr3*$^{-/-}$ OTII cells are defective in IFNγ production *in vivo* but not *in vitro*. Mice received either WT or *Cxcr3*$^{-/-}$ OTII cells following DC transfer. At 60 hr post T cell transfer, dLN T cells were harvested and restimulated to assess cytokine production. (**A**) Representative plots of IFNγ and TNFα production by WT and *Cxcr3*$^{-/-}$ OTII cells transferred into separate hosts. (**B**) Frequency of TNFα^{+}IFNγ^{+} WT (square) and *Cxcr3*$^{-/-}$ (triangle) OTII cells. (**C**) WT (CD90.1) and *Cxcr3*$^{-/-}$ (CD90.2) OTII cells were cultured *in vitro* in the presence of OVA-pulsed DCs. IFN-γ^{+} cells were distributed evenly between CD90.1 and CD90.2 cells after 4 days of culture. Data are representative of three independent experiments (n=3-6).

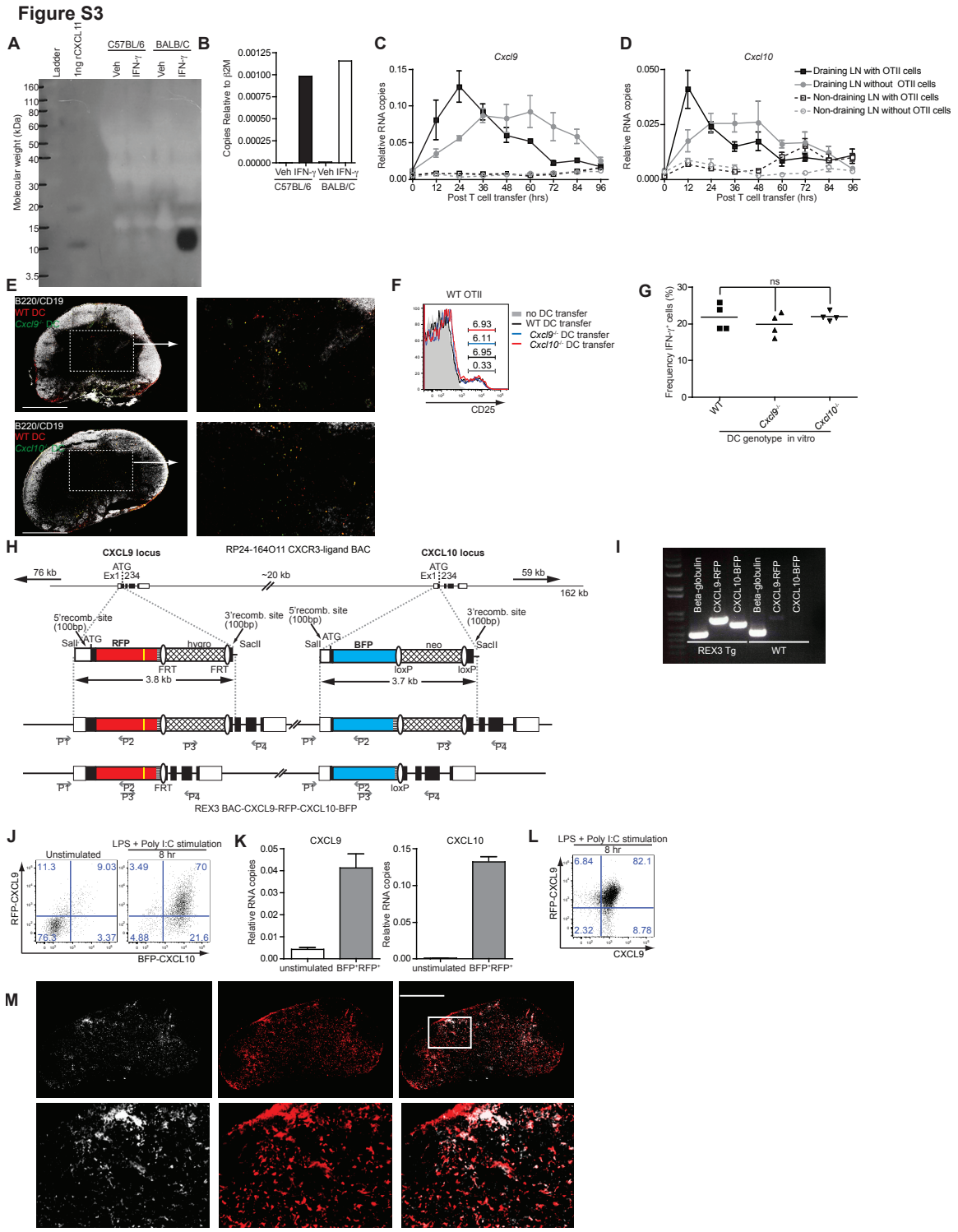

Figure S3. Absence of CXCL11 protein in C57BL/6 mice. CXCL11 (**A**) protein and (**B**) RNA dectected from activated bone marrow-derived macrophages from indicated mice. **Chemokine upregulation in dLN**. Relative RNA levels of (**C**) *Cxcl9* and (**D**) *Cxcl10* in whole draining or non-draining LNs in mice receiving OVA pulsed LPS and PolyI:C activated DC with or without OTII cells, as indicated. **Conformation of *Cxcl10*$^{-/-}$ DC tracking and**

function. (**E**) WT and *Cxcl9*$^{-/-}$ or *Cxcl10*$^{-/-}$ DCs were pulsed, activated, labeled and co-transferred into naïve mice. dLNs were harvested to detect DCs trafficking 24 hr post transfer. (**F**) WT and *Cxcl9*$^{-/-}$ or *Cxcl10*$^{-/-}$ DCs were pulsed, activated and transferred into naïve mice. 24 hr later, naïve WT OTII T cells were given. dLNs were harvested after 3 hr to assess early upregulation of CD25 on transferred T cells. (**G**) WT and *Cxcl9*$^{-/-}$ or *Cxcl10*$^{-/-}$ DCs were assessed for their ability to prime OTII T cells to produce IFNγ after 4 days of *in vitro* culture. Data are representative of 2-3 independent experiments (n=3-6). **Construction of the REX3 transgene and genotyping PCR.** (**H**) RFP and BFP genes were engineered into the ATG start site of the *Cxcl9* and *Cxcl10* genes, respectively, contained in the RP-24-164O11 BAC. Arrows indicate 100 bp regions flanking BAC gene ATG sites, which were used as regions of homology for targeting construct recombineering. SalI and SacII were used to excise targeting constructs from pBluescript prior to recombineering. Antibiotic resistance genes *hygromycin* (*hygro*) and *kanamycin/neomycin* (*neo*) (hatched boxes) were removed by FLP and Cre expressing plasmids for the creation of the completed REX3 BAC Tg. Open box non-coding exons and black box coding exons of *Cxcl9* and *Cxcl10* genes; RFP ORF (red box); BFP ORF (blue box); and SV40 poly A site (stripped box); FRT Flippase Recognition Target and loxP Cre recombinase site. Primers indicate pairs P1->P2 used to screen BAC insertions and Tg lines, amplifying from 5' outside construct insert to inside FP gene and P3->P4 amplfying from inside resistance gene or inside FP gene to 3' outside construction insert. (**I**) Genotyping of REX3 Tg mice by detection of RFP and BFP insertions into the *Cxcl9* and *Cxcl10* locus' respectively, using P1->P2 primers as described in (**H**). *b2-microglobulin* PCR is shown as a DNA positive control. **Confirmation of REX3 Tg reporting of CXCR3 ligands**. (**J,K**) CD11c^{+} DCs were Flt3L-expanded *in vivo* from REX3 Tg mice and either left unstimulated or stimulated for 8 hr with LPS and PolyI:C. (**J**) Representative plots of RFP and BFP upregulation following TLR-ligand stimulation. (**K**) Conformation of expression of *Cxcl9* and *Cxcl10* RNA transcripts from unstimulated and double positive (Cxcl9-RFP^{+}Cxcl10-BFP^{+}) cells obtained by cell sorting. Data normalized to *b2-microglobulin*. (**L-M**) Confirmation of expression of CXCL9 protein with Cxcl9-RFP reporter. (**L**) Representative plot of CXCL9 staining on REX3 Tg DCs stimulated as above. CXCL9$^{-/-}$ cells had no CXCL9 antibody staining (data not shown). (**M**) Conformation of CXCL9 immunohistological staining co-localization with Cxcl9-RFP expression. REX3 Tg dLN taken 36 hr after OTII cell transfer following immunization. Representative images showing co-localization between CXCL9 immunohistological staining and Cxcl9-RFP expression in whole LN (top panels) and high powered of indicated box (lower panels) of CXCL9 staining (left), Cxcl9-RFP (middle), and overlay (right). Bar indicates 500 mm. Error bars denote SEM.

Figure S4

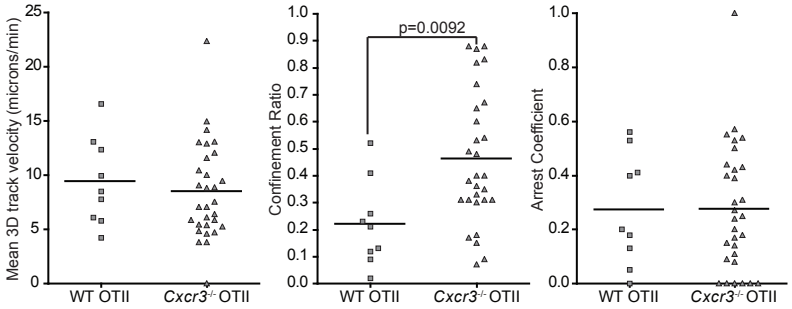

Figure S4. Quantification of intravital multiphoton movies comparing behavior of WT and *Cxcr3*$^{-/-}$ OTII cells at 24-27 hr post T cell transfer. DCs expanded from REX3 Tg mice were prepared as described and subcutaneously injected in WT mice. 24 hr later, WT and *Cxcr3*$^{-/-}$ OTII cells were transferred. Intravital multiphoton imaging of exposed dLN was performed at 24-27 hr post T cell transfer. Mean 3D track velocity, confinement ratio and arrest coefficients are shown for WT and *Cxcr3*$^{-/-}$ OTII cells.

Figure S5

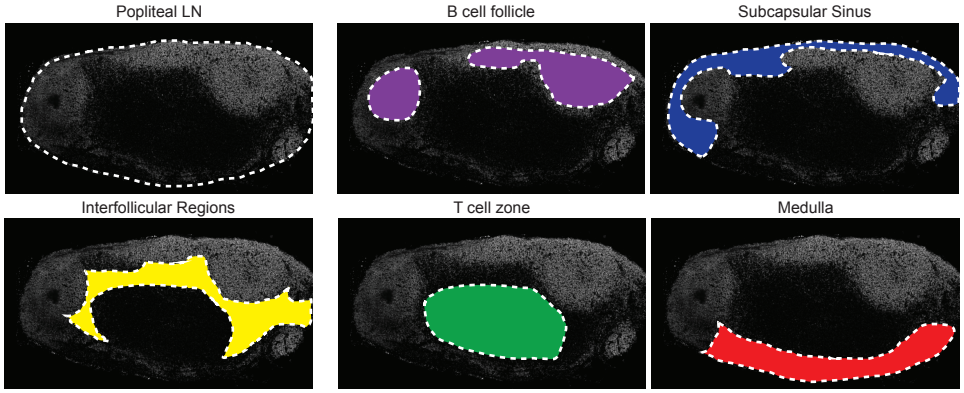

Figure S5. Identification of LN regions. Section of dLN is shown (top left). Color regions indicate areas analyzed as determined by B220 and CD19 staining and LN architecture.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice.

In addition to mice described in Experimental Procedures in the body of the manuscript, C57BL/6-Tg(ACTB-EGFP) (Actin-GFP) mice were provided by A. Wagers (Joslin Diabetes Center, Boston)(Wright et al., 2001), C57BL/6-Tg(GAG-DsRed) (Actin-RFP) mice were purchased from JAX and bred to OTII Tg mice (Vintersten et al., 2004). All mice were bred at Massachusetts General Hospital.

Generation of REX3 Tg mice.

To engineer the *Cxcl9*-RFP targeting construct, a point mutation was inserted (QuikChange Lightning; Agilent Technologies) into the RFP open reading frame (pTagRFP-N vector Evrogen) to increase photostability (Shaner et al., 2008). RFP-SV40 polyA was liberated from the vector and placed upstream of an FRT flanked *hygromycin* resistance gene cloned into pBluescript (Agilent Technologies). This targeting construct was flanked by 100 bp of sequence 5' and 3' of the ATG start codon of *Cxcl9*, so recombineering of this construct led to the insertion of the RFP and *hygromycin* genes at the *Cxcl9* ATG start codon. The *Cxcl10*-BFP targeting construct was engineered with a similar strategy using BFP cDNA (pTagBFP-N vector, Evrogen) and a LoxP flanked *kanamycin* resistance gene. Targeting constructs in pBluescript were excised using SalI and SacII (New England Biolabs) prior to insertion into the *Cxcl9* and *Cxcl10* containing RP24-164O11 BAC (CHORI). The additional LoxP in the pTARBAC1 BAC backbone was removed using recombination (pRed/ET expression, Gene Bridges GmbH) to replace the site with an ampicillin resistance gene. Subsequently, the targeting constructs were recombineered onto the BAC. Correct insertion of targeting vectors was determined using PCR with primers pairs P1 and P2 amplifying from 5' of vector insertion to inside the targeting vector and P3 and P4 amplifying from inside the targeting vector to 3' outside of vector insertion for each *Cxcl9* and *Cxcl10* specific vector (as indicated **Figure S3**). Flp and Cre expression cassettes (Gene Bridges GmbH) were sequentially electroporated into the BAC construct-containing bacteria to remove the antibiotic resistance genes. The 162 kb completed REX3 construct was purified using NucleoBond BAC100 Kit (Clontech), linearized with PI-SceI (NEB) and dialyzed (mini dialysis kit; GE Healthcare) into injection buffer (10 mM Tris pH 7.5, 0.1 mM EDTA, 100 mM, 30 μM spermine (Sigma) 70 μM spermidine (Sigma). A linear REX3 construct injected into C57BL/6xC57BL/6 embryos by the Brigham and Women's Hospital Transgenic Core yielded one founder. University of Michigan Transgenic Animal Model Core also injected a circular REX3 construct into

C57BL/6xC57BL/6 embryos, leading to the generation of 5 additional founders. All 6 founder lines produced transgene positive litters as determined by PCR (**Figure S3I**). All founders were bred to C57BL/6 and litters were screened for similar FP expression pattern by *in vitro* DC culture and *in vivo* immunization. The founder line generated at the Brigham and Women's Hospital Transgenic Core displayed consistently brighter and more reliable reporter expression, and was the primary line used for data generation, although experiments were additionally confirmed with one line generated at Michigan Transgenic Animal Model Core.

Antibodies for Flow Cytometry and Histological staining.

Cells were stained with the viability dye LIVE/DEAD Fixable Blue Cell Stain (Invitrogen) prior to surface staining for detection of CD90.1 (OX-7), CD90.2 (30-H12), CD44 (IM7), CD4 (RM4-5; Invitrogen), CXCR3 (220803; R&D), CD25 (3C7), CD69 (HI.2F3), and CD40L (MI/70). IFNγ (XMG1.2; eBioscience), TNFα (MP6-XT22), IL2 (JES6-5H4) and IL10 (JES5-16E3) were used to stain for intracellular cytokines following Fix&Perm. DCs were labeled for detection of CD11c (N418), MHC II (I-A/I-E, M5/114.15.2), CD8a (53-6.7), CD11b (MI/70), CD86 (GL-1), and CD40 (3/23). All antibodies were purchased from Biolegend unless otherwise indicated. Cell numbers were determined using CountBright beads (Molecular Probes). Sections were stained for detection of CD19 (6D5), B220 (RH3-6B2), CXCL9 (BAF492). Biotin primary antibodies were detected with Cy5-Streptavidin. Stained slides were mounted with Prolong Gold (Molecular Probes), and

Multiphoton Intravital Microscopy and Image Analysis

Mice were anaesthetized and the popliteal LNs microsurgically prepared as previously described {Mempel, 2004 #8} for MP-IVM analysis on a Prairie Ultima IV multiphoton microscope equipped with two MaiTai HP lasers and an Olympus 20x, NA 0.95 lens was used. Imaging depth was typically 80-200 mm below the LN capsule. For four-dimensional recordings of cell migration, stacks of 11 optical sections (512 x 512 pixels) with 4 mm z-spacing were acquired every 15 seconds to provide imaging volumes of 40 mm in depth. Emitted light signals were detected through 455/50 nm, 525/50 nm, 590/50 nm, and 665/65 band-pass filters with non-descanned detectors. Data sets were transformed in Imaris 7.3.1 (Bitplane) to generate maximum intensity projections (MIPs) for export as Quicktime movies and for 3D-tracking of cell migration. Cell tracks were exported for computational analysis of step and track parameters in Matlab (Mathworks). **Movie S1** time-lapse sequences are accelerated 225x over real-time (15 frames per second) for display as Quicktime movie.

RNA Isolation and qPCR.

LNs were dissociated and lysed using gentle MACs dissociator (Miltenyi) with RLT buffer and run over QIAshredder spin columns (Qiagen). RNA was isolated using the RNeasy Mini Kit (Qiagen). Following DNAse I and RNAse out treatment (Invitrogen), cDNA was prepared using Multiscribe RT (Applied Biosystems). qPCR was performed with SYBR Green Master Mix (Applied Biosystems) using a Realplex^2 mastercycler (Eppendorf). Primer sequences for the detection of *Cxcl9* were 5'-AATGCACGATGCTCCTGCA-3' and 5'- AGGTCTTTGAGGGATTTGTAGTGG-3', and for *Cxcl10* were 5'-GCCGTCATTTTCTGCCTCA-3' and 5'-CGTCCTTGCGAGAGGGATC-3'. All samples were normalized using *β2-microglobulin* detected using 5'-CCGAACATACTGAACTGCTACGTAA-3' and 5'-CCCGTTCTTCAGCATTTGGA-3' primers.

Bone Marrow Chimeras.

Mice were irradiated with 800rads in a Cesium-137 irradiator. 4 hr later mice received BM from donor mice i.v. Mice were left to reconstitute for at least 8 weeks prior to experiments. For *Cxcl9*$^{-/-}$ and *Cxcl10*$^{-/-}$ BM recipients chimerism was confirmed by PCR of DNA isolated from splenic DCs.

Detection of CXCL11 protein.

Femurs were removed from 5 C57BL/6 and 5 BALB/c mice and the BM was flushed out and plated in petri dishes with media plus 10 ng/mL M-CSF (Peprotech). After 7 days in culture, BM derived macrophages (BMDMs) were either treated with vehicle or 100 ng/mL (Peprotech) for 5 hr, washed, and then treated with vehicle or 100 ng/mL rIFNγ serum free BMDM media for 48 hr. Supernatant from each mouse strain and treatment was pooled and concentrated 1000-fold using the Amicon Ultra-15 and Ultra-0.5 (Millipore). Concentrated supernatant was run on a 4-12% Bis-Tris gel and blotted on a PVDF membrane (Invitrogen). CXCL11 was detected using a biotinylated CXCL11 antibody (R&D) and peroxidase-conjugated Streptavidin (BD Pharmingen). BMDMs were lysed and RNA and cDNA prepared. RT-PCR was performed with an intron-spanning primer and the data normalized to b-globulin.

SUPPLEMENTAL REFERENCES

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Wright, D.E., Cheshier, S.H., Wagers, A.J., Randall, T.D., Christensen, J.L., and Weissman, I.L. (2001). Cyclophosphamide/granulocyte colony-stimulating factor causes selective mobilization of bone marrow hematopoietic stem cells into the blood after M phase of the cell cycle. Blood *97*, 2278-2285.