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Supplemental Information

Differential Intrasplenic Migration of Dendritic Cell Subsets Tailors Adaptive Immunity

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Supplemental Experimental Procedures

Diphtheria toxin treatment. Diphtheria toxin (DT) was purchased from Sigma-Aldrich and titrated in Zbtb46-DTR bone marrow chimeric mice (due to variability between different lots). For transient DC depletion in Zbtb46-DTR bone marrow chimeric mice, 60ng of DT/gram of body weight was injected i.p. on day 0 followed by a second dose of 40ng of DT/gram on day two. For alloantibody experiments, HOD RBCs were transfused on day three.

Flow cytometry Analysis and Antibodies. Single cell suspensions of spleen were acquired either with LSR II (BD), or MACSQuant (Miltenyi) flow cytometers and analyzed using FlowJo software (Tree Star). The following antibodies were used for staining different cell subsets (from BioLegend if not specified): TCRβ (H57-597), B220 (RA3-6B2), MHC II (M5/114.15.2), CD11c (N418), 33D1 (33D1), XCR1 (ZET), Va2 (B20.1), CD4 (GK1.5), Ly6C (HK1.4), Ly6G (1A8), F4/80 (CI:A3-1), CD11b (M1/70) and goat polyclonal anti-mouse Ig (BD Pharmingen).

GFP-RBC uptake. GFP^+ RBCs were collected from transgenic mice expressing enhanced Green Fluorescent Protein under the direction of the human ubiquitin C promoter, leukoreduced, and stored as described for HOD^+ RBCs for 12 days. Half an hour after RBC transfusion, the spleen was harvested and processed to obtain a single cell suspension. The uptake of GFP^+RBCs was analyzed by flow cytometry in TCRβ- B220-MHC II+CD11c+) DC subsets.

In vitro T cell proliferation assay. Splenic dendritic cells from WT or $Batf3^{-/-}$ mice were enriched by MACS isolation (Miltenyi Biotec) according to manufacturer suggested protocols. Purified DCs were plated at different numbers in a 96-well round bottom tissue culture treated plate. After pulsing with 100ug/ml OVA for 1 hour at 37°C, free OVA was washed away. Then the DCs were co-cultured with CFSE labeled purified OT-I and OT-II T cells at different DC/T ratios for 72 hours. OT-I and OT-II proliferation was determined by CFSE dilution after gating on CD45.1+ CD8+ or CD45.1+CD4+ cells, respectively, by flow cytometry. Numbers on the histograms indicate the percentage of proliferating cells in the indicated gates.

In vivo T cell proliferation assay. One million transgenic $CD45.1^+$ $CD4^+$ or $CD8^+$ T cells, isolated from OT-II or OT-I mice were purified with the $CD4^+$ or $CD8^+$ negative isolation kit (Miltenyi Biotec or StemCell), CFSE labeled and adoptively transferred i.v. into recipient mice. Twenty-four hours later, either 5ug of OVA (plus 1ug of LPS) or one unit of OVA-expressing HOD RBCs were injected. Three days after immunization spleens were collected and T cell proliferation was assayed by determining CFSE dilution of transgenic T cells by flow cytometry.

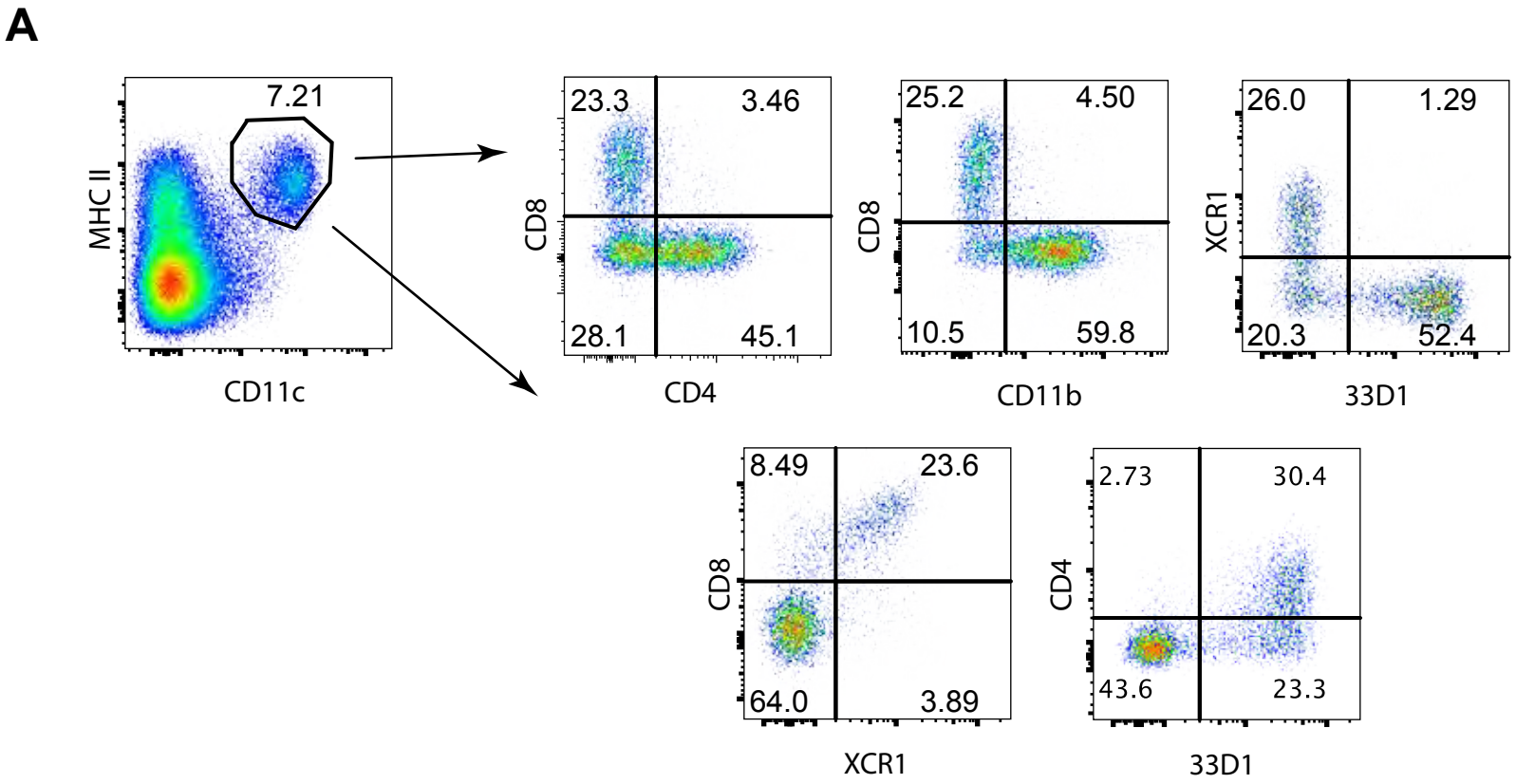
Image analysis. Imaris software was used to determine the distance of DC subsets from the WP border as defined by MOMA-1 staining. The contour of the WP boundary was defined manually using the inner surface of $MOMA^+$ cells. The centroids of DC subsets were determined using the Imaris spots algorithm based on the fluorescent intensity of 33D1 or XCR1. The distance of each centroid within the while pulp to the nearest point of the WP boundary was determined using the Imaris XT "distance transformation" algorithm. Histograms and 3D surface plots of T cell staining were made in image J.

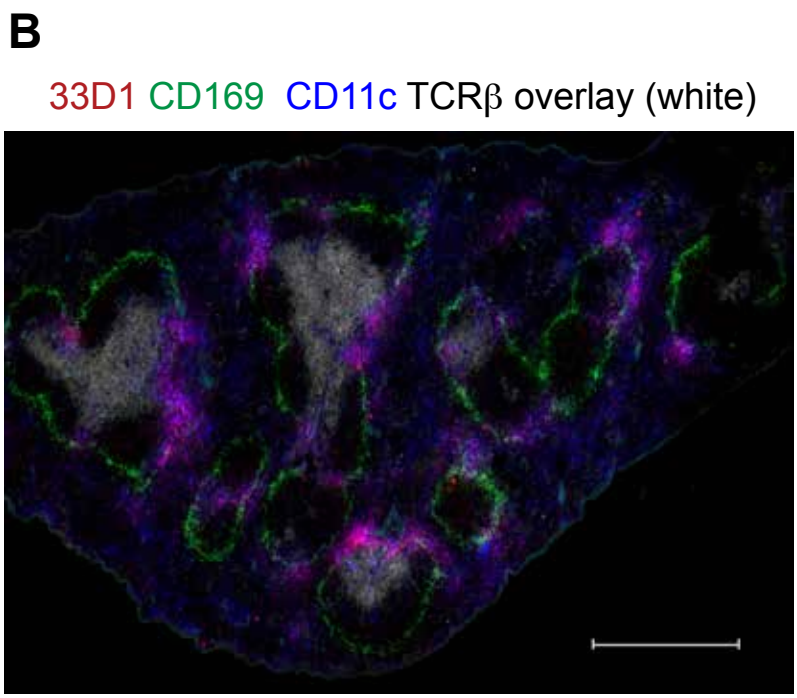
Serum alloantibody analysis. Serum from transfused mice was collected three weeks after RBC transfusion. To identify the presence of alloantibodies, sera were added to 96 well plates coated either with 5uL of HOD^{+} RBCs or with 5uL of HOD^{-} RBCs (negative control). After 30 minutes of incubation plates were washed and stained with anti-Ig conjugated with APC for 30 minutes. The stained samples were washed and the total Igs were analyzed by flow cytometry (Hendrickson et al., 2011).

Transplantation. Wild type mice were irradiated with 2 doses of 650 rad three hours apart. Two hours after the second treatment, 10^6 bone marrow cells from Zbtb46-DTR mice were adoptively transferred by i.v. injection into wild type recipient mice. All experiments with bone marrow chimeric mice were performed 7-10 weeks after bone marrow transplant.

Figure S1: DC subset markers and distribution in spleens from naïve wildtype mice.
Related to Figure 1

(A) Representative flow cytometry images of splenic DC markers. Gated on TCRβ-CD19- cells that are MHCII+CD11c+ showing the distribution of the two dominant DC subsets with a variety of markers. Also shown is the overlap of CD8 with XCR1 and CD4 with 33D1 on splenic DCs. (B-C) The same spleen as in Figure 1B and Figure 1C with the T cell zone indicated by an overlay of TCR (white). (B) 33D1 (red); CD169 (green); CD11c (blue); TCR (white). Scale bar = 500um. (C) XCR1 (red); CD169 (green); CD11c (blue); TCR (white). Scale bar = 500um. One representative spleen of 3 from 6 independent experiments.





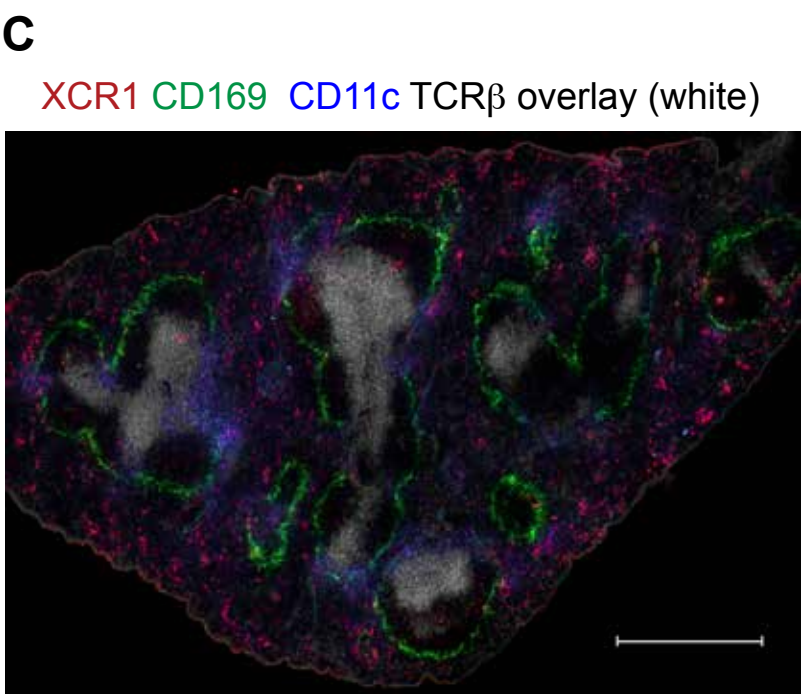
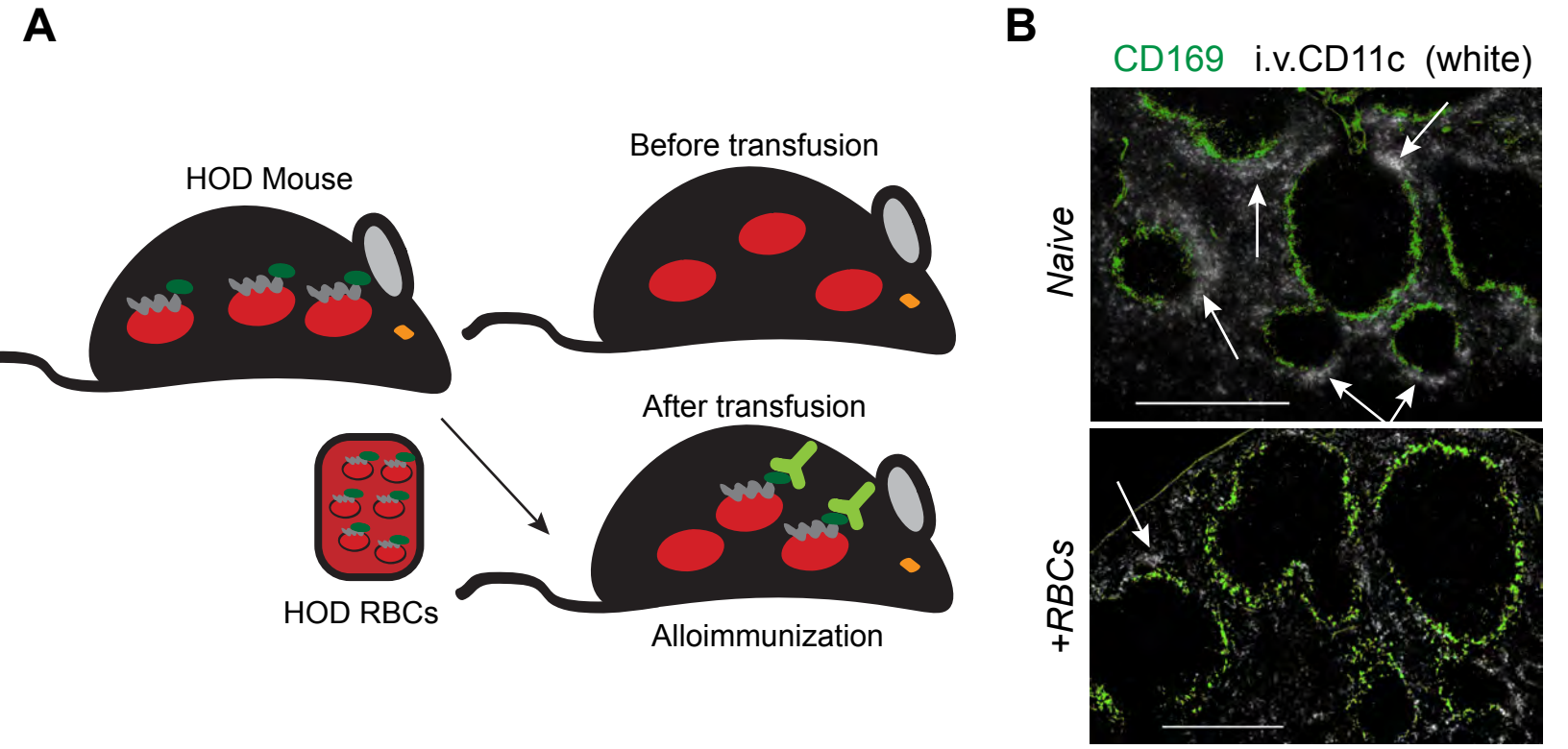
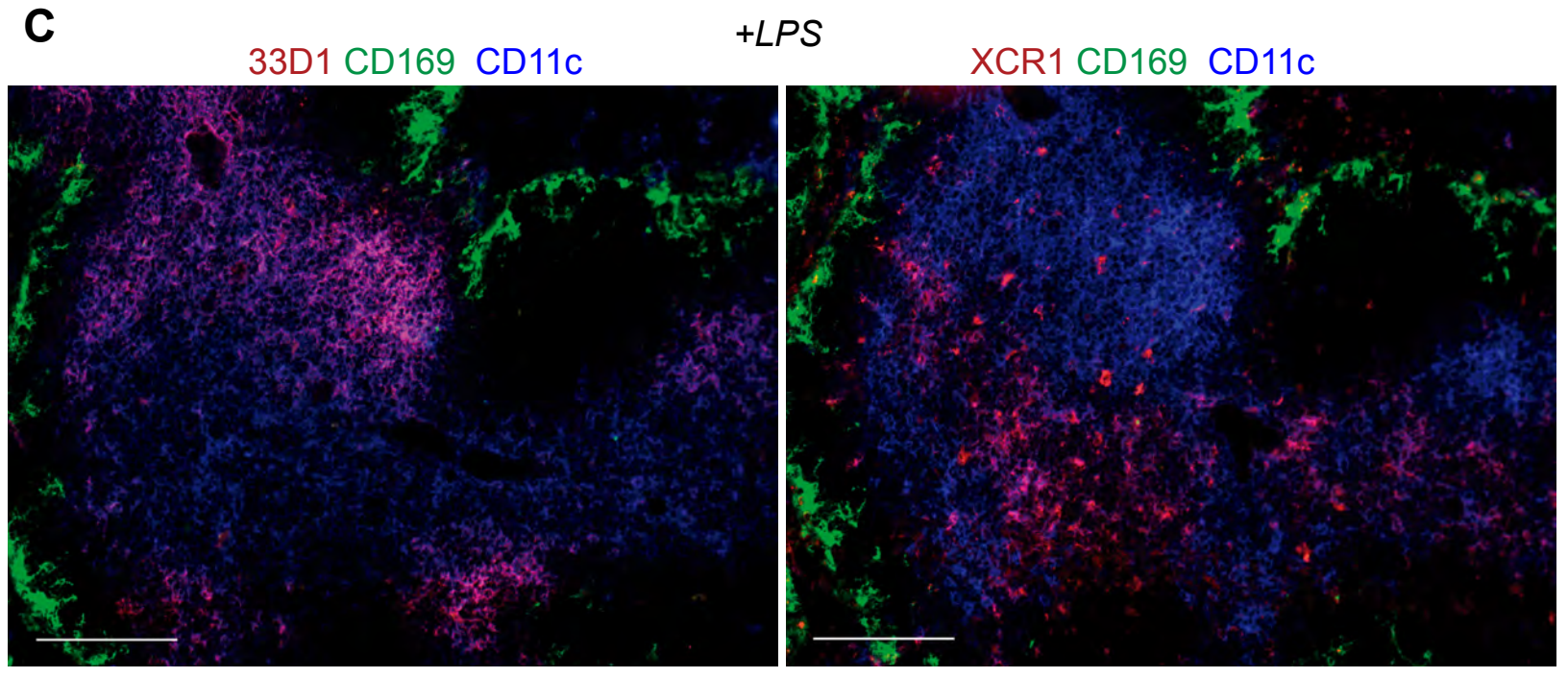
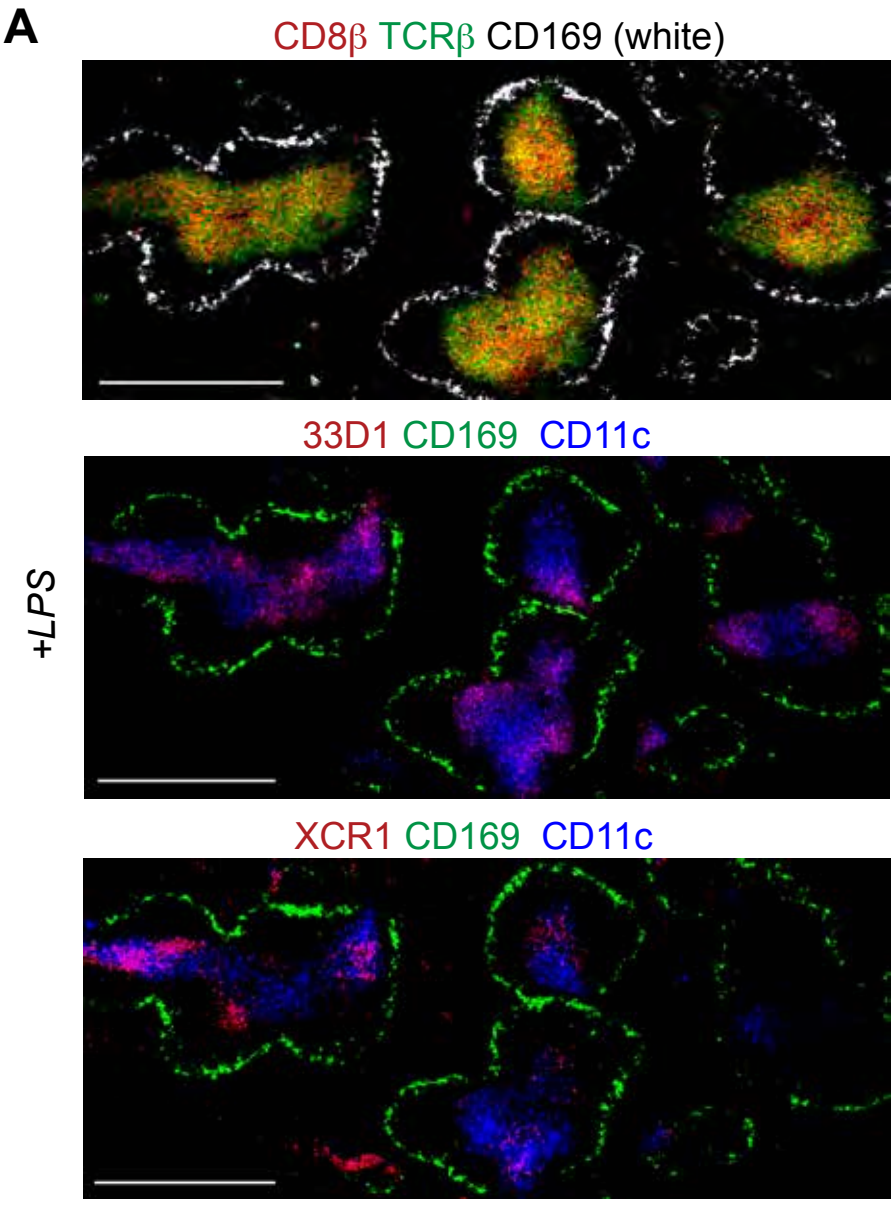


Figure S2: RBC alloimmunization model and DC subset segregation in the white pulp with multiple stimuli. Related to Figure 2

(A) In vivo red blood cell (RBC) alloimmunization model. RBCs were collected from HOD transgenic mice, processed and stored in a similar manner as human RBCs are in the clinical setting and then transfused into recipient mice.
(B) Fluorescence image of a spleen from a naïve mouse (top) or a mouse transfused (bottom) with RBCs 6-8 hours prior to in vivo DC labeling with anti-CD11c (white). CD169 (green) staining performed after sacrifice. Arrows indicate area of DCs stained outside of the WP by anti-CD11c in vivo labeling (white). Scale bar = 100um. n = 2-5 mice/group. Representative of three independent experiments. (C) Fluorescence image of a single white pulp from a wildtype mouse transfused with 15µg of lipopolysaccharide (LPS) 4 hours prior, demonstrating differential location of 33D1+ (left) and XCR1+ (right) DCs (red). CD169 (green); CD11c (blue). Scale bar=100µm. Images are representative of five independent experiments with 2-3 mice/experiment.







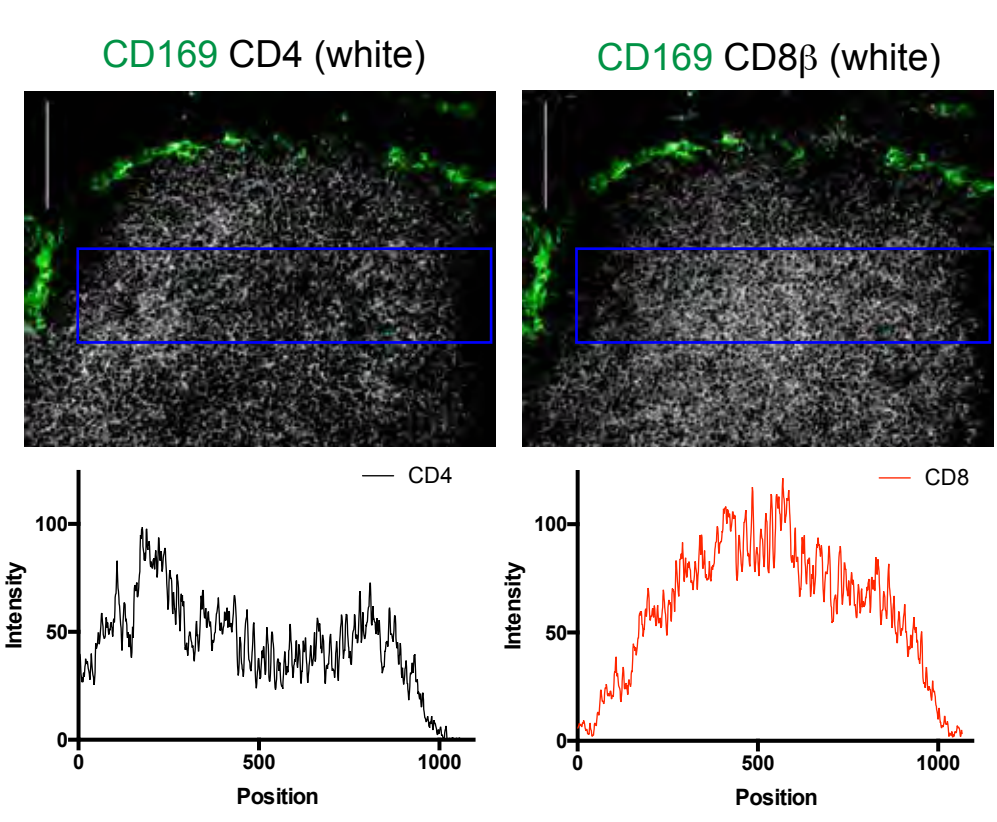


Figure S3: CD4+ and CD8+ T cells occupy preferential niches in the T cell zone at steady state and after immunization.
Related to Figure 3

(A) Fluorescence image of a spleen from a wildtype mouse 6-8 hours after i.v. injection of LPS. Upper panel shows CD8β (red), TCRβ (green) and CD169 (white); Middle panel shows 33D1 (red), CD169 (green) and CD11c (blue); Bottom panel shows XCR1 (red), CD169 (green) and CD11c (blue). Scale bar = 500um. Images are representative of three spleens tested across four independent experiments. (B) (Upper panel): Fluorescence staining shows CD4 (white, left) and CD8β (white, right) together with CD169 (green) of the same follicle, respectively. (Bottom panel): Histogram below the images showing relative intensity of a cross-section (blue box) of the CD4 (left) and CD8β staining (right).

Figure S4: Subdomain organization of T cells, DCs and each DC subset is disrupted in the spleen of immunized *Ccr7*-deficient mice. Related to Figure 4

(A) Individual intensity images of TCRβ (left) or CD11c (right) in wildtype and *Ccr7*-/- mice 6-8 hours after RBC transfusion (from Fig. 4A). A clear loss of appropriate T cell and DC localization is observed in *Ccr7*-deficient mice, although significant overlap of the two disorganized populations exist. (B) Fluorescence image of a spleen from *Ccr7*-/- mouse without transfusion (naive, top) or 6-8 hours after RBC transfusion (+RBC, bottom). 33D1 or XCR1 (red); CD169 (green); CD11c (blue). Scale bar = 500um. n = 2-3 mice/group. Representative of two independent experiments.

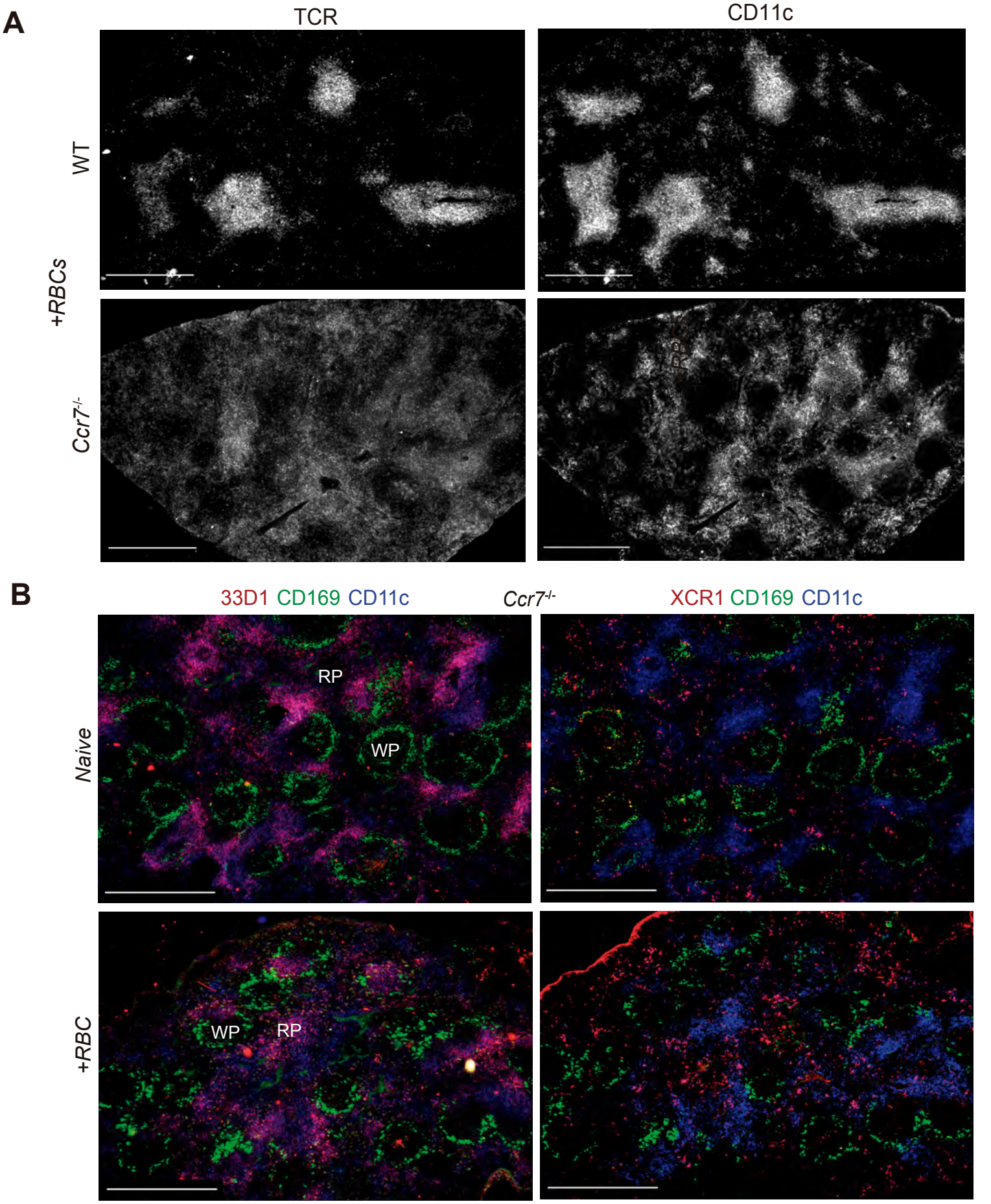


Figure S5: Intrasplenic 33D1+ DC migration is critical for allogeneic responses to transfused RBCs.
Related to Figures 6 - 7

(A) Fluorescence image of the *Dock8*$^{-/-}$ spleen from Fig. 6b with the T cell zone demonstrated by an overlay of TCR staining (white). 33D1 or XCR1 (red); CD169 (green); CD11c (blue). Scale bar = 500um. N = 2-3 mice/group. (B) Anti-RBC antibody in the sera of mice 21 days following HOD RBC transfusion in *Dock8*$^{+/-}$, *Dock8*$^{-/-}$ and naïve mice. **P<0.002. n= 2-8 mice/group. Representative of three independent experiments. (C-D) Bone marrow chimera mice were generated using *Zbtb46*-DTR (CD45.1.2) and *Dock8*$^{-/-}$ (CD45.2) bone marrow in a 50:50 ratio. Eight weeks after reconstitution, mice were (+DT) or were not (-DT) administered DT twice over a three-day period. Seven days after the final DT treatment, the presence of marginal zone B cells (C) or one day after the final DT treatment, the presence of DC subsets (D) was assessed by flow cytometry. No evidence of marginal zone B cell loss was observed (C). DT treatment eliminated all WT CD45.1.2+ (Zbtb46-DTR) DCs while leaving only CD45.2+ *Dock8*-deficient DCs in the spleen of mice receiving DT (D).