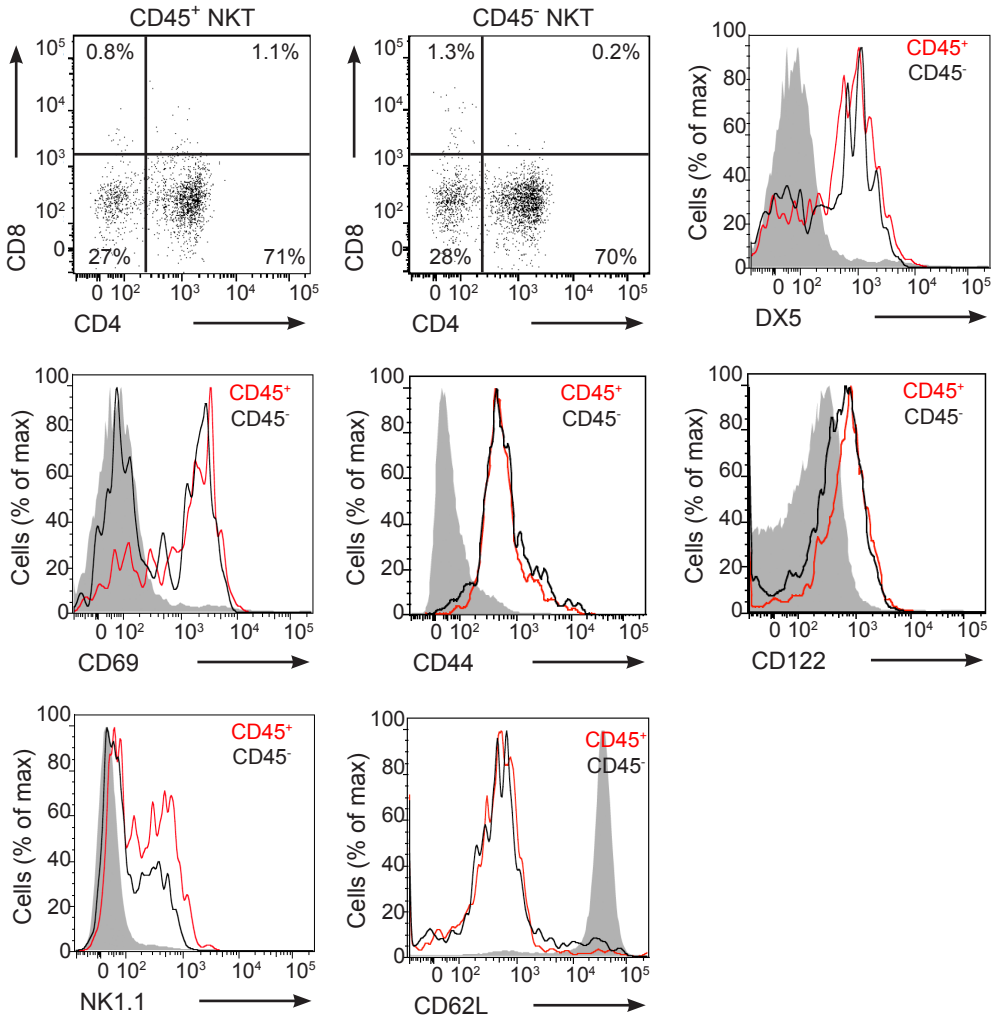
Supplementary Figure 1



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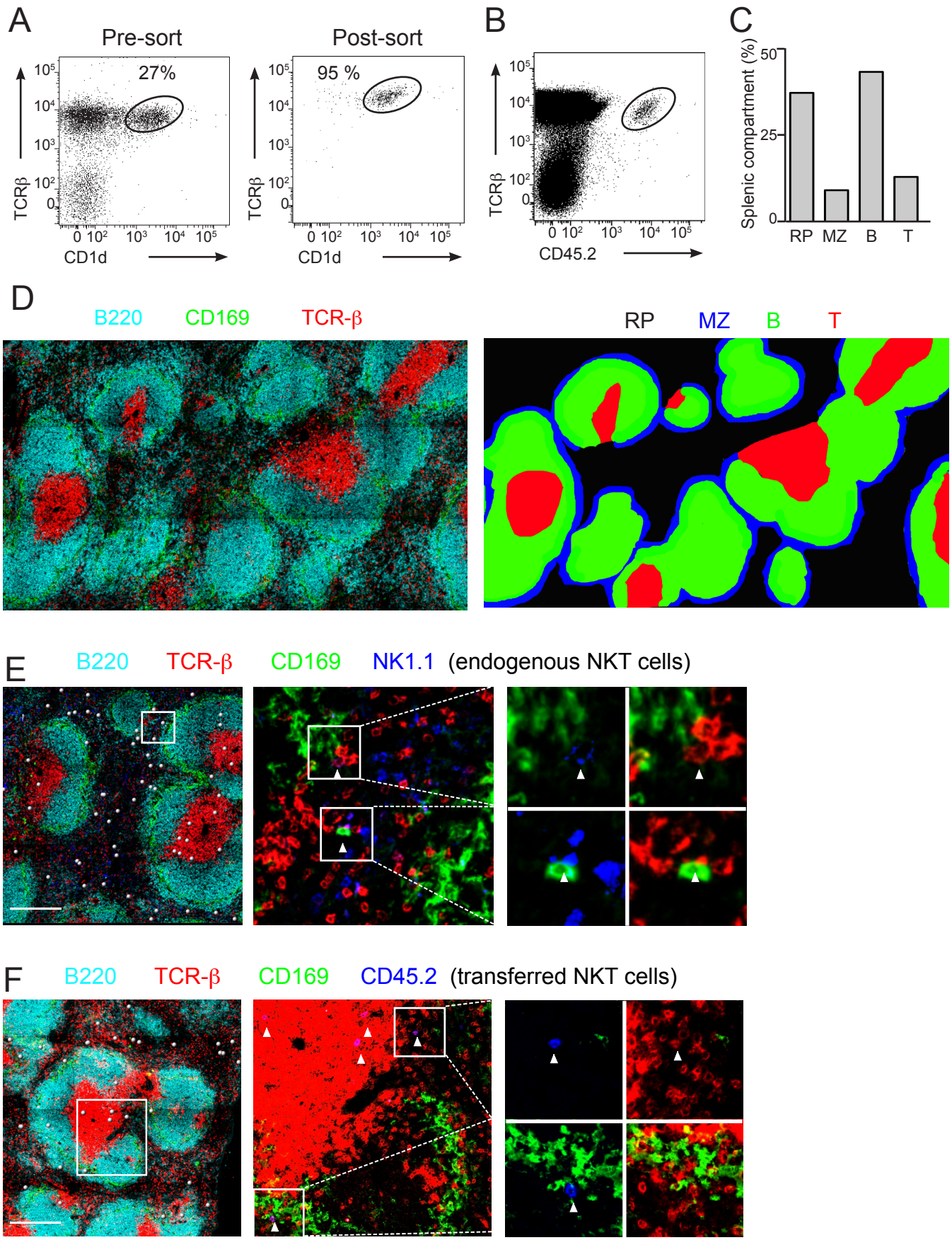
Supplementary Figure 2



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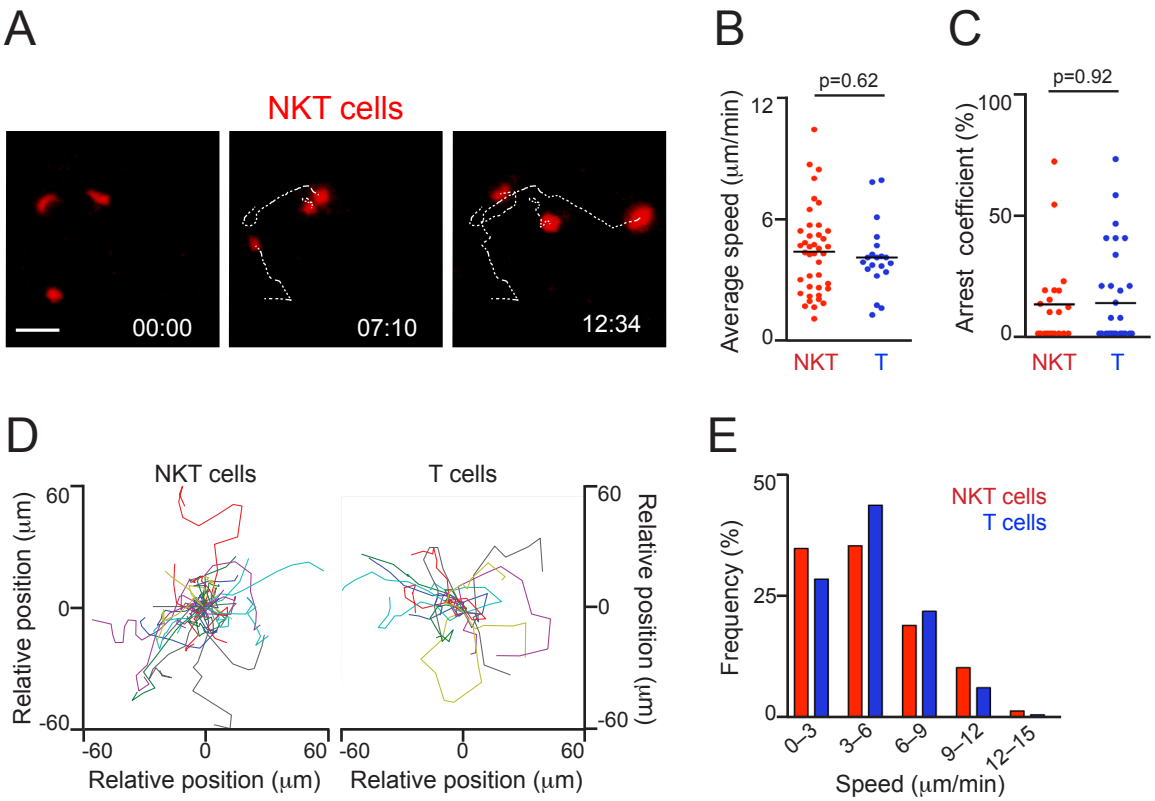
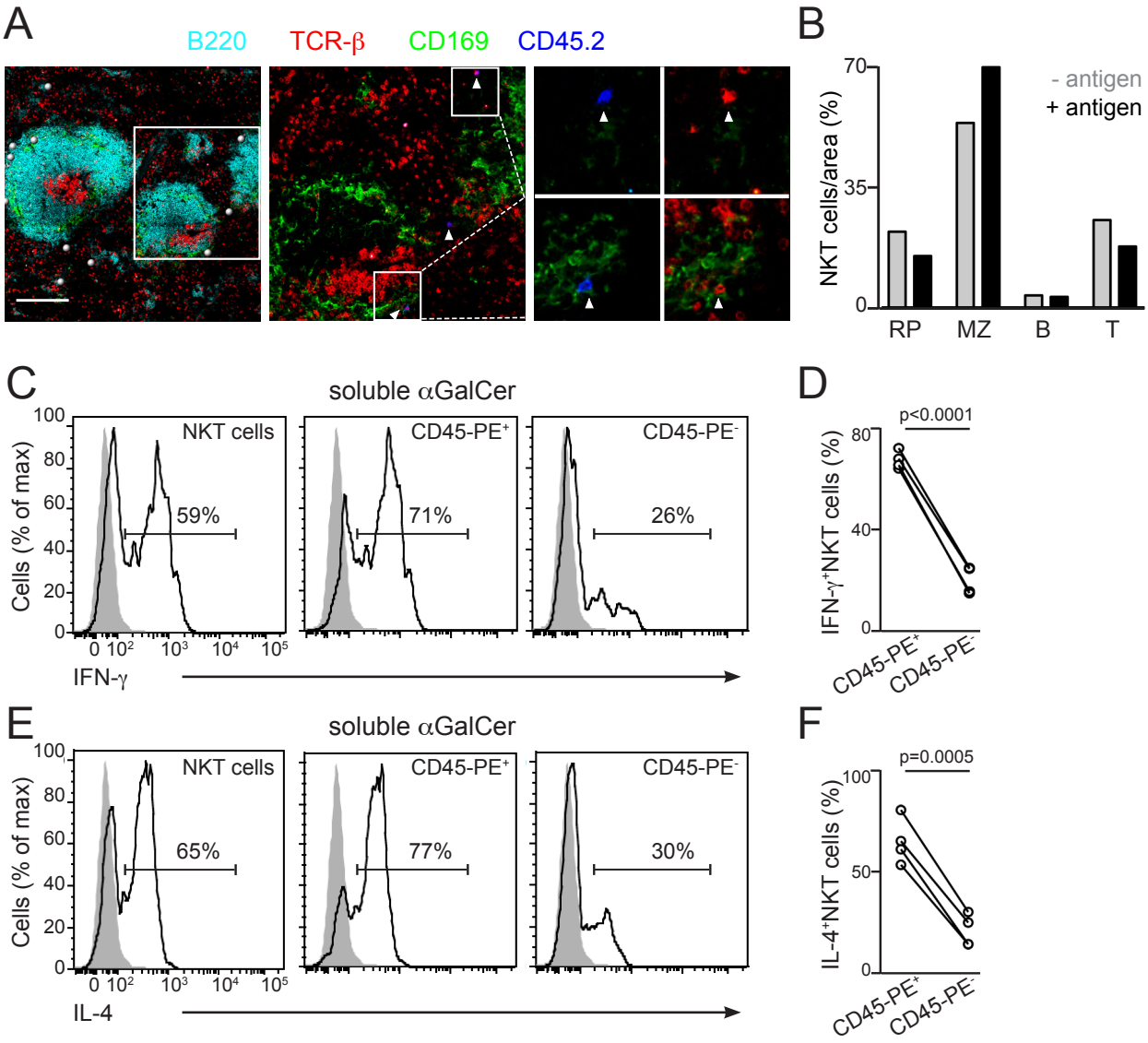
Supplementary Figure 3



Supplementary Figure 4



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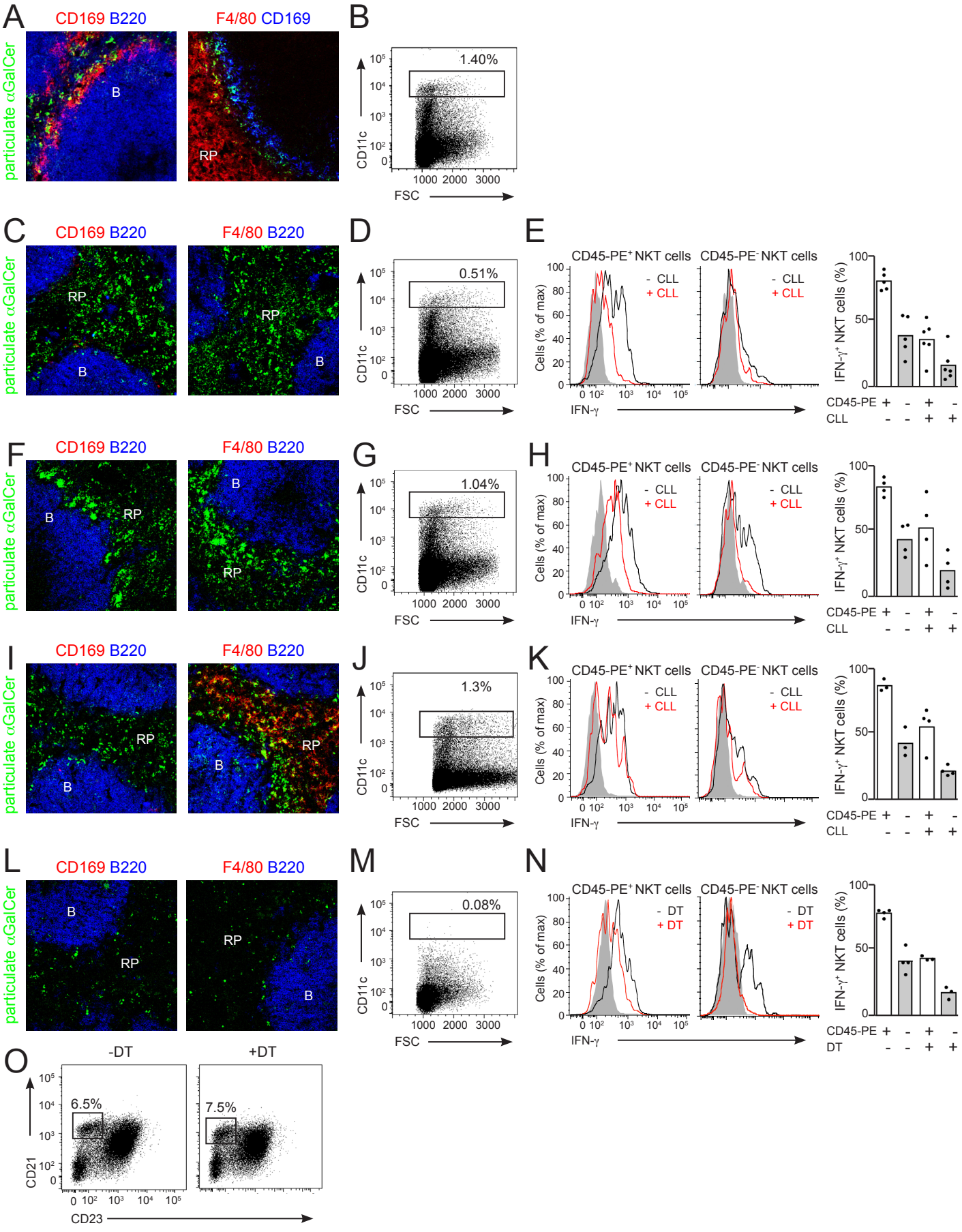
Supplementary Figure 5



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SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. *In vivo* antibody labelling of splenic NKT cells. Expression of CD4 and CD8, NK1.1, DX5, CD69, CD44, CD122 and CD62L in NKT cells that are highly ($CD45^+$) or poorly ($CD45^-$) labelled at 3 min after *in vivo* injection of CD45 antibody. Grey profiles show the indicated staining for conventional T cells. Data represent 5 independent experiments with 2 mice per experiment

Supplementary Figure 2. Distribution of splenic NKT cells. (**A,B**) Adoptive transfer of NKT cells. (**A**) Single cell suspensions were prepared from spleens of Vα14 transgenic mice and depleted of $B220^+$ cells (left). Sorted cells were >95% CD1d-Tet^+ cells (right). (**B**) 3 x 10^6 purified NKT cells were adoptively transferred into CD45.1 congenic recipient mice. 14-16 h later transferred NKT cells were detected as TCR-β^+-$CD45.2^+$ cells in the spleen of recipient animals. (**C,D**) Identification of splenic compartments in cryosections. Spleen cryosections were stained with B220 (cyan), TCR-β (red) and CD169 (green). Splenic compartments were identified as follows: MZ was limited by CD169 macrophages and identified by the B220 staining of MZ B cells. PALS were defined by TCR-β staining and B cell follicles were defined by B220 staining. The remaining area was classified as the RP. Processed images (**D,** right) were analysed in ImageJ to determine the area occupied by each specific compartment (**C**, and previously described (Aoshi et al, 2008)). (**E,F**) Confocal microscopy identification of endogenous (**E**) and adoptively transferred (**F**) NKT cells in spleen sections stained with B220 (cyan), CD169 (green), TCR-β (red) and NK1.1 (blue, **E**) or CD45.2 (blue, **F**). Data were pooled from 2 independent experiments with 2 mice each.

Supplementary Figure 3. Dynamics of splenic NKT cells. Splenic NKT cells were imaged by multi-photon microscopy after slice-opening of the spleen (**A**) Snapshot images showing NKT cells (red) at the indicated time points. Individual cell tracks are coloured in pink. Stamp, min:sec. Bars, 20 μm. (**B-E**) Average speed (**B**), arrest coefficient (**C**), migratory tracks (**D**) and speed distribution (**E**) for NKT cells and T cells. Each dot represents an individual cell. Data were pooled from 2 independent experiments with 2 mice each. *p*, unpaired two-tailed t-test

Supplementary Figure 4. NKT cell activation occurs in the MZ. (**A-B**) Confocal microscopy identification of adoptively transferred NKT cells 2h after antigen injection. (**A**) Spleen sections were stained with B220 (cyan), CD169 (green), TCR-β (red) and CD45.2 (blue). (**B**) Quantification of distribution of NKT cells in the RP, MZ, B cell follicles (B) and PALS (T). Bars, 200 μm. (**C-F**) Mice were injected with soluble αGalCer (0.5 μg) and 2 h later they received CD45-PE 3 min before analyses. Flow cytometry profiles and quantification of intracellular IFN-γ (**C, D**) and IL-4 (**E, F**) for total (left) and highly (middle, CD45-PE^{+}) or poorly (right, CD45-PE^{-}) CD45-PE labelled NKT cells (TCR-β^{+}αGalCer-CD1d tetramer^{+}B220^{-}). *p*, paired t-test. Data represent 2 independent experiments with at least 3 mice each.

Supplementary Figure 5. Effect of MZ disruption in NKT cell activation. Confocal microscopy images (**A,C,F,I,L**) and flow cytometry profiles (**B,D,G,J,M,O**) of spleens of WT mice (**A,B**), WT mice treated with clodronate liposomes (CLL) for 2 days (**C-E**), 6 days (**F-H**) or 16 days (**I-K**) and CD11c-DOG mice treated with DT for 2 days (**L-O**). Mice received an i.v. injection of particulate lipids (green) 2 h before analyses. Splenic sections for immunofluorescence (**A,C,F,I,L**) were stained with B220 (blue), CD169 or F4/80 (red). (**B,D,G,J,M**) Flow cytometry profiles for splenocytes showing the CD11c^{high} population. (**E,H,K,N**) Flow cytometry profiles and

quantifications for IFN-γ secretion in NKT cells from mice injected with αGalCer (for 2h) and CD45-PE antibody 3 min before analyses showing highly (left, CD45-PE$^+$) and poorly (right, CD45-PE$^-$) *in vivo* labeled NKT cells. (**O**) Flow cytometry profile showing MZ B cell population in CD11c-DOG mice untreated (left) or treated (right) with DT. Data represent 2 independent experiments with at least 2 mice per experiment.

SUPPLEMENTARY MOVIES

Supplementary Movie 1. Dynamics of NKT cells in the splenic RP. NKT cells (red) were adoptively transferred into WT recipients and spleens were imaged by multi-photon microscopy. Representative tracks of cell movement are traced (pink). Long ticks represent 20 μm. A movie of the XY projection (left) is shown together with three-dimensional representations of the imaged volume (right).

Supplementary Movie 2. Dynamics NKT cells in the splenic RP. NKT cells (red) and T cells (blue) were adoptively transferred into WT recipients and spleens were imaged by multi-photon microscopy. Representative tracks of cell movement are traced and coloured according to cell type. Long ticks represent 20 μm.

Supplementary Movie 3. Dynamics NKT cells in splenic sections. NKT cells (red) were adoptively transferred into WT recipients and spleens were imaged by multi-photon microscopy after slice-opening of the tissue. Representative tracks of cell movement are traced. Long ticks represent 20 μm.

Supplementary Movie 4. NKT cells arrest after antigen administration. NKT cells (red) and T cells (blue) were adoptively transferred into WT recipients that were

injected with particulate αGalCer (green) 2 h before imaging. Representative tracks of cell movement are traced and coloured according to cell type. Long ticks represent 10 μm.

Supplementary Movie 5. NKT cells arrest after antigen administration. NKT cells (red) and T cells (blue) were adoptively transferred into WT recipients that were injected with particulate αGalCer (green) 2 h before imaging. Representative tracks of cell movement are traced and coloured according to cell type. Long ticks represent 10 μm.