SUPPLEMENTAL INFORMATION

Dynamic interplay among monocyte-derived, dermal and resident lymph node dendritic cells during the generation of vaccine immunity to fungi

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Inventory of supplemental materials

1. Six supplemental figures and legends.

Fig. S1a (supplemental to figure 1). FITC painting to track migrating cells that carry yeast into the lymph nodes of vaccinated mice.

Figure S1b (supplemental to figure 1). Tracking vaccine yeast with various labels.

Figure S2a (supplemental to figure 2). The role of neutrophils in delivery of vaccine yeast into the draining lymph node.

Figure S2b (supplemental to figure 2). The distribution of live and heat-killed yeast among cells in the lymph node.

Figure S2c (supplemental to figure 2). The effect of Pertussis toxin on vaccine yeast delivery into the lymph node.

Figure S7 (supplemental to figure 7). Vaccine priming of T cells following the loss of antigen presenting cell populations.

2. Supplemental methods

Supplemental Figures

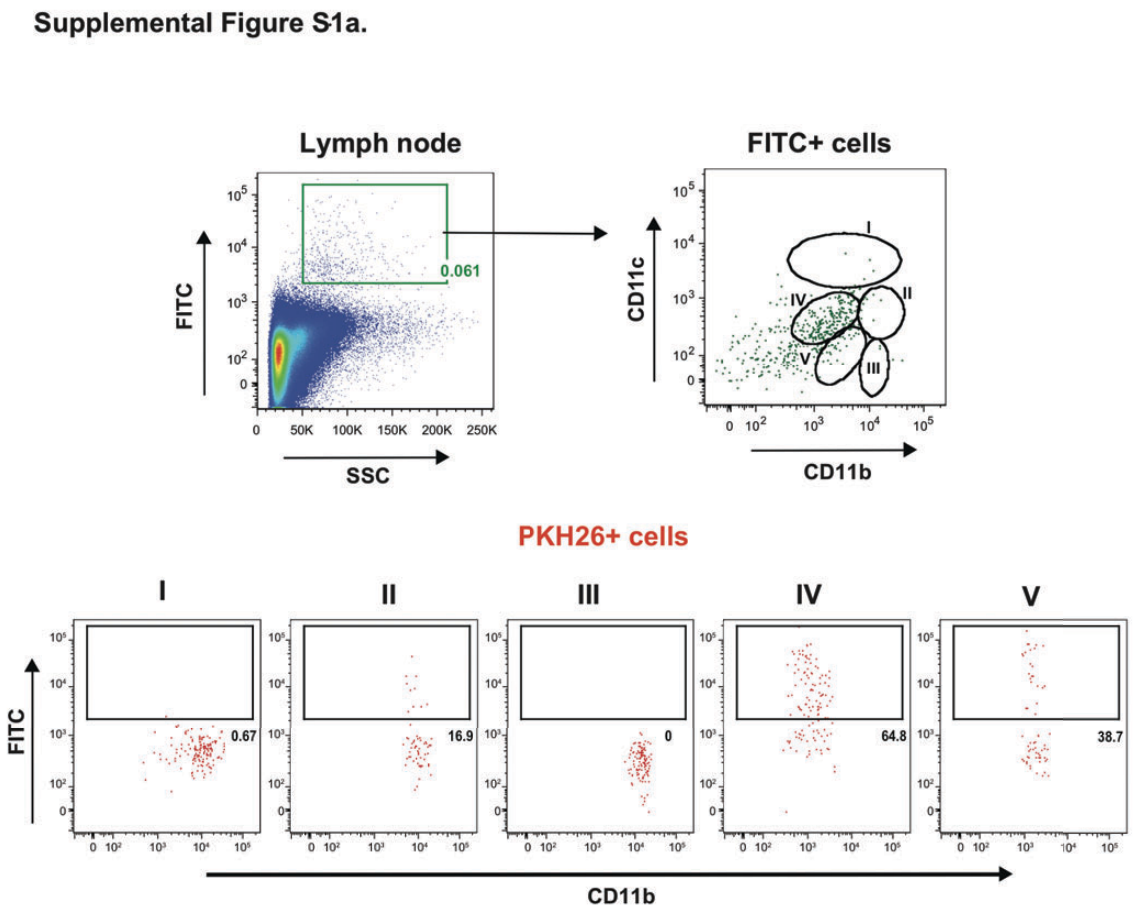


Fig. S1a (supplemental to figure 1). FITC painting to track migrating cells that carry yeast into the lymph nodes of vaccinated mice. Mice were shaved and painted with 2% FITC solution in acetone. Dibutylphthalate (DBP) was added to the FITC solution to induce migration of DCs as a positive control. Mice that were painted were stained for four hours before injecting PKH26^{+} yeast s.c. at the site of painting. Lymph nodes were harvested and analyzed for FITC staining among the five DC populations described in figures 1 and 2, including the cells that harbor PKH26^{+} yeast.

Supplemental Figure S1b.

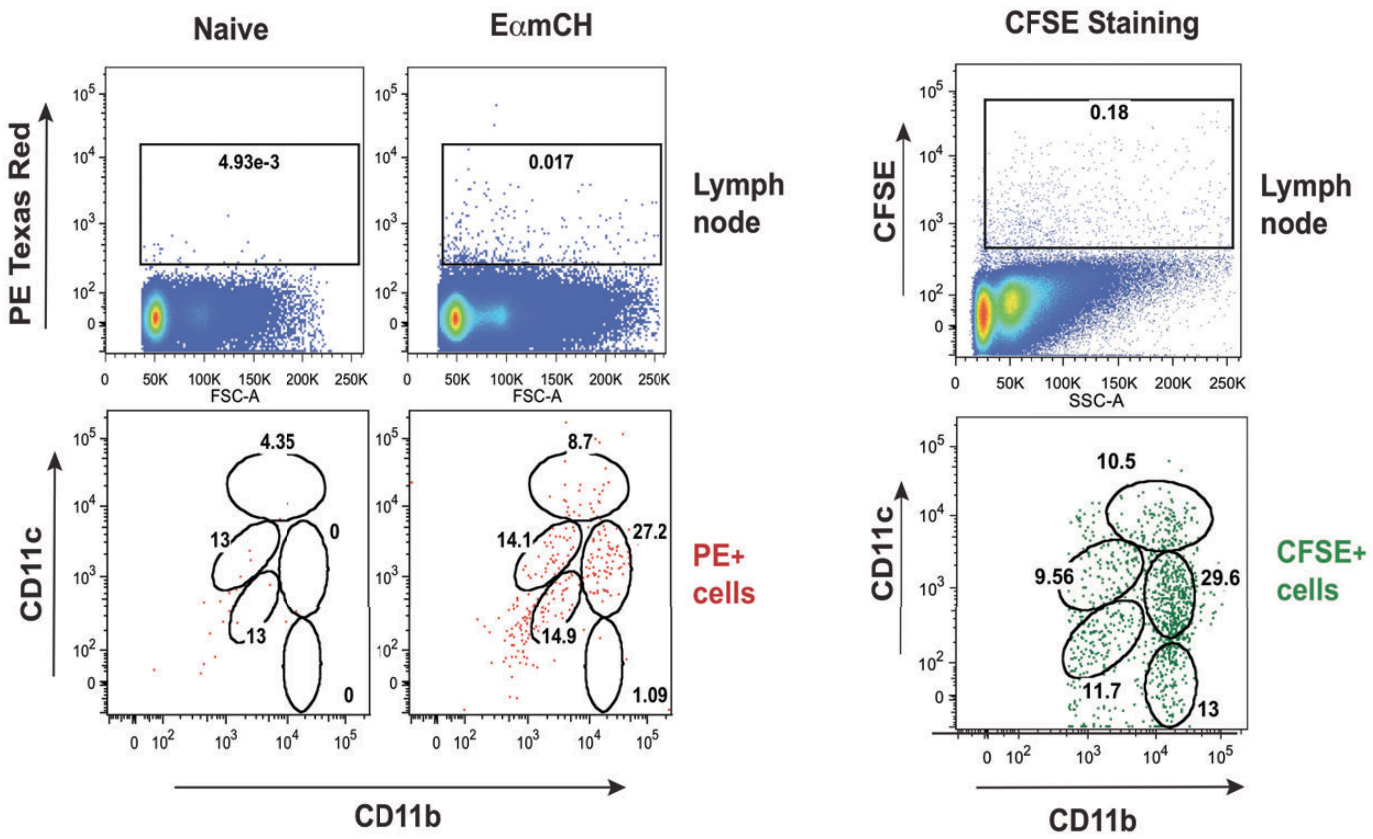


Figure S1b (supplemental to figure 1). Tracking vaccine yeast with various labels. Mice were vaccinated with EαmCherry yeast or yeast labeled with CFSE. The draining lymph nodes were removed 72 hours after mice were vaccinated. Distribution of labeled yeast among lymph node cells was analyzed according to CD11b and CD11c expression. Numbers indicate the percentage of yeast-associated cells in a gate.

Supplemental Figure S2a.

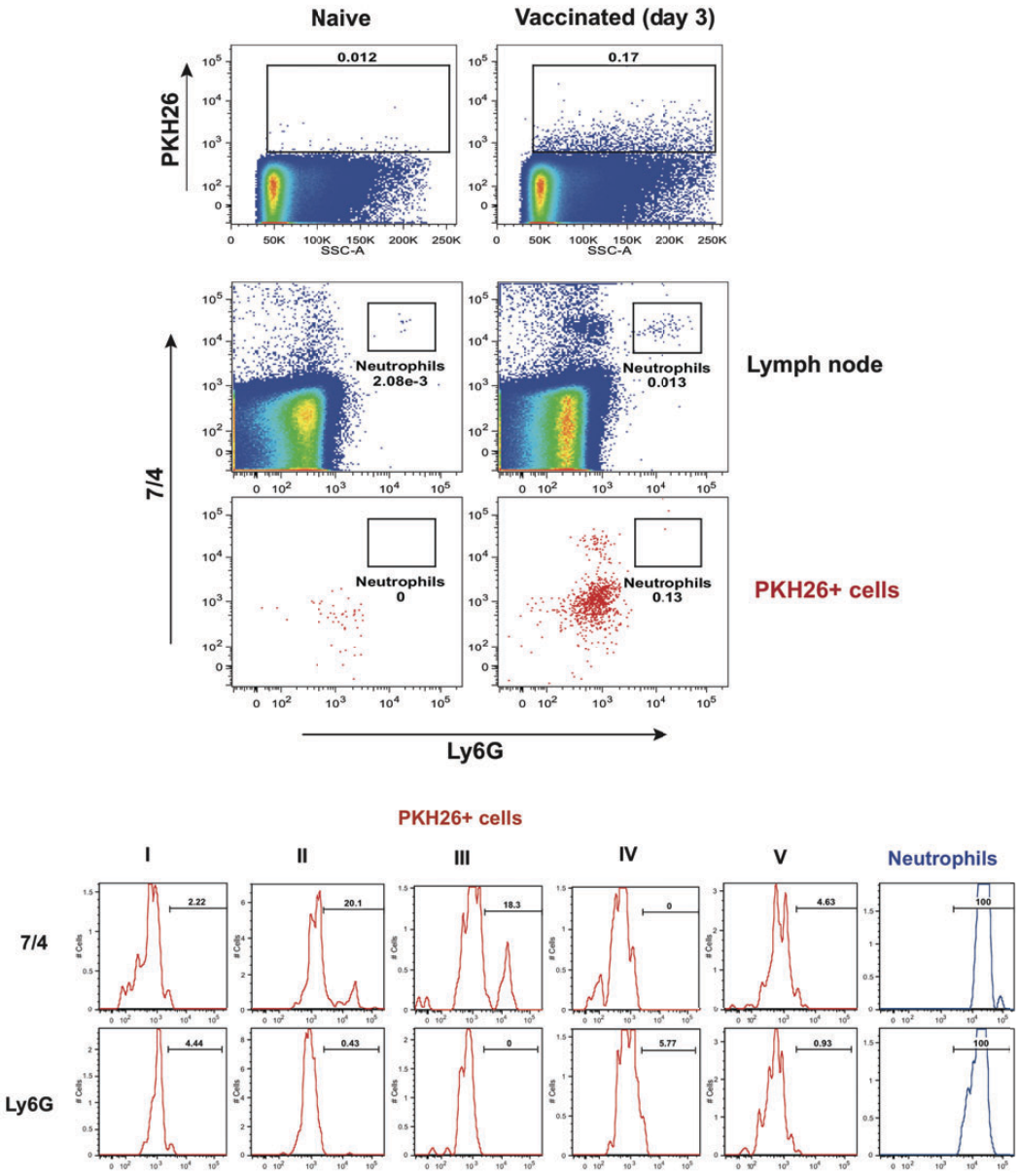


Figure S2a (supplemental to figure 2). The role of neutrophils in delivery of vaccine yeast into the draining lymph node. Mice were vaccinated with PKH26 labeled yeast and draining lymph nodes were removed 72 hours later. In the upper 3 panels, total lymph node cells were stained with Ly6G and 7/4 antibodies to identify and gate on neutrophils (middle), and PKH26^{+} yeast-associated cells in the gate corresponding to neutrophils is shown (bottom). Numbers under the gates correspond to the percent of neutrophils in the node (middle) and the percent of PKH26^{+} cells that are neutrophils (bottom). In the lower panel, the histogram profiles reflect staining with these antibodies on the various monocyte and DC populations and neutrophils.

Supplemental Figure S2b.

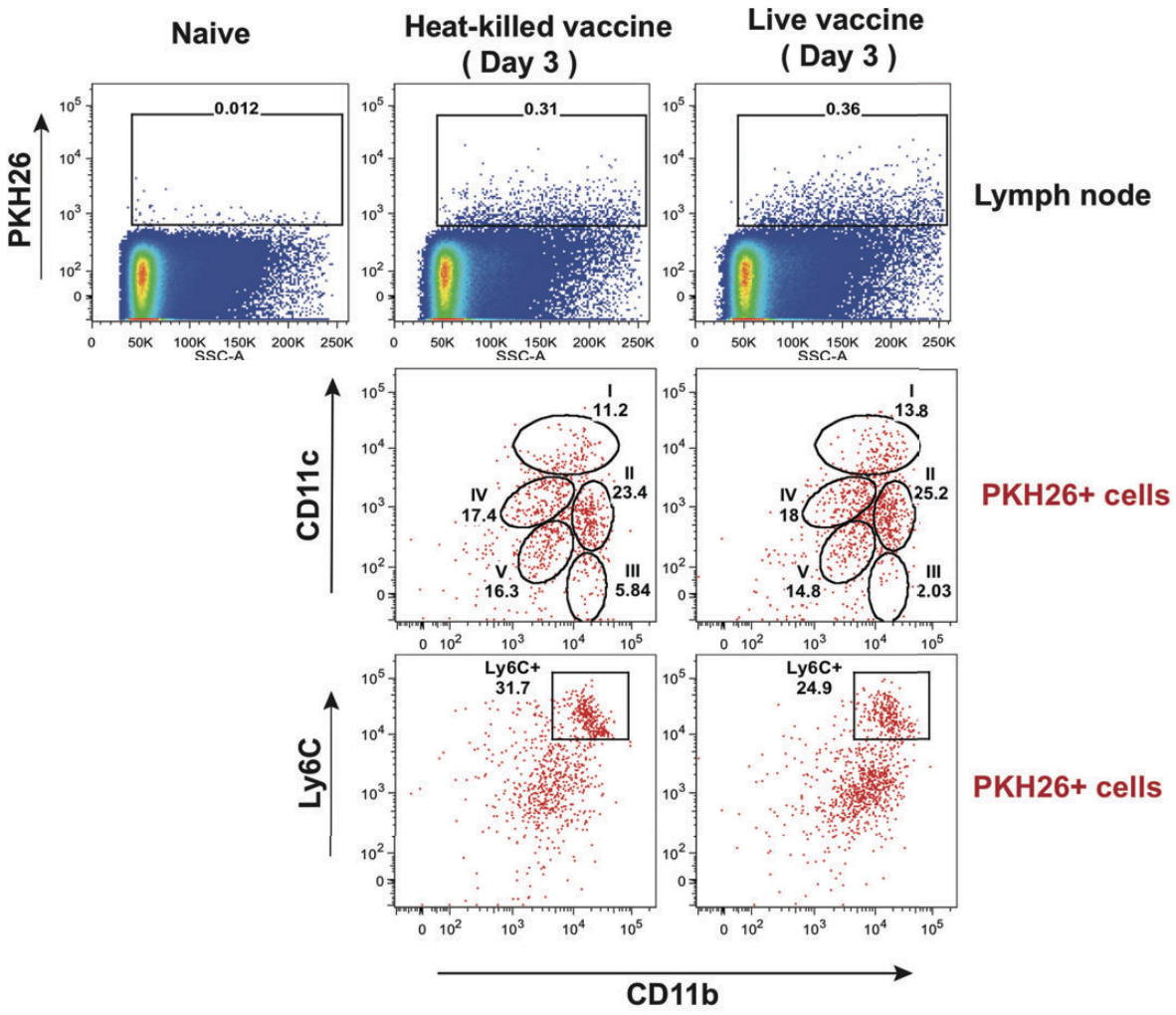


Figure S2b (supplemental to figure 2). The distribution of live and heat-killed yeast among cells in the lymph node. Mice were vaccinated with PKH26^{+} yeast as in Methods and the draining lymph nodes were collected 72 hours after vaccination. The cells were stained with the antibodies shown and gates drawn to identify 5 DC subsets as in Fig. 1. The percentage of PKH26^{+} yeast distributed among the various DC subsets (middle panel) and contained within Ly6c^{+} monocyte-derived DCs (lower panel) are illustrated

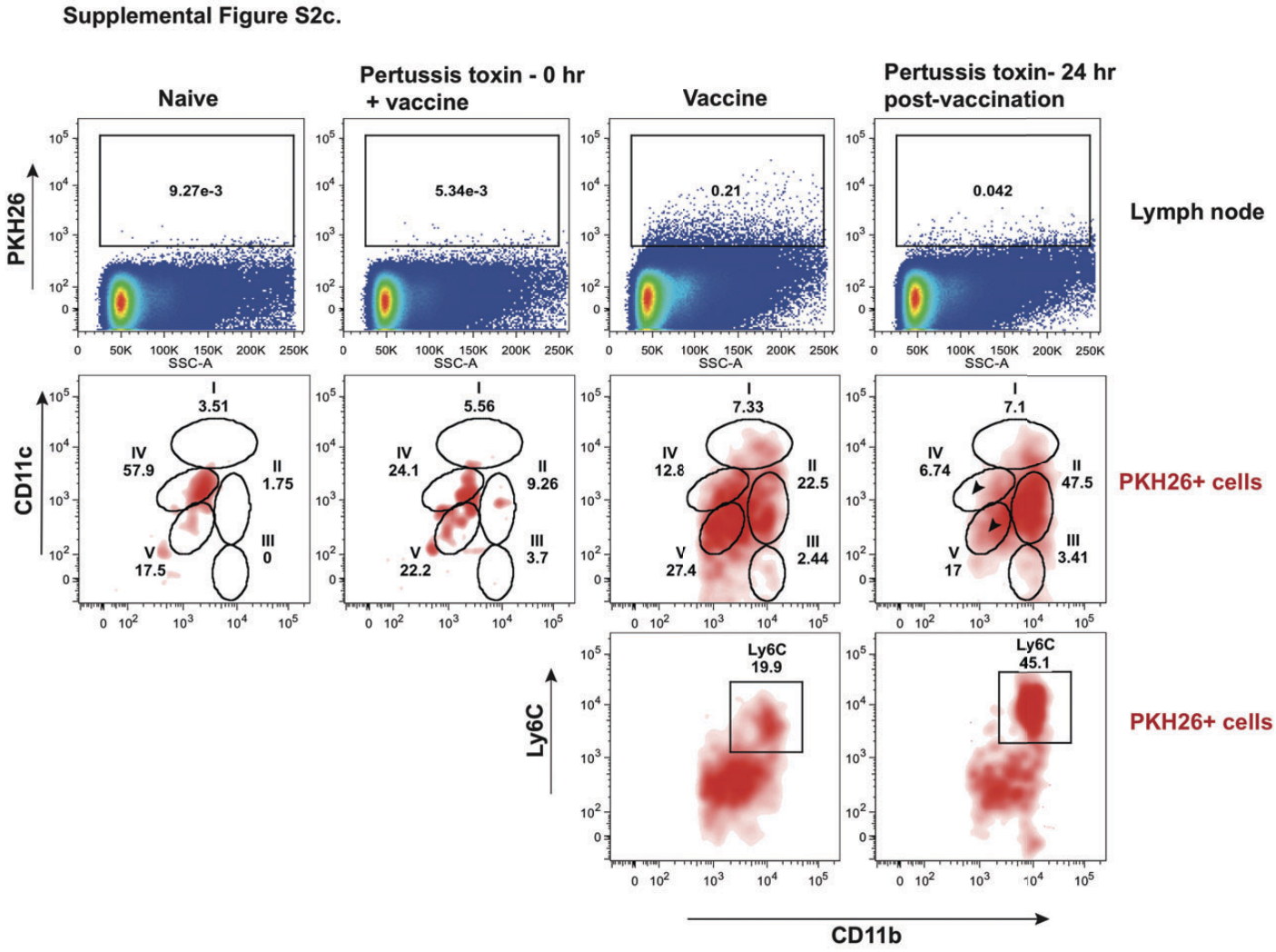
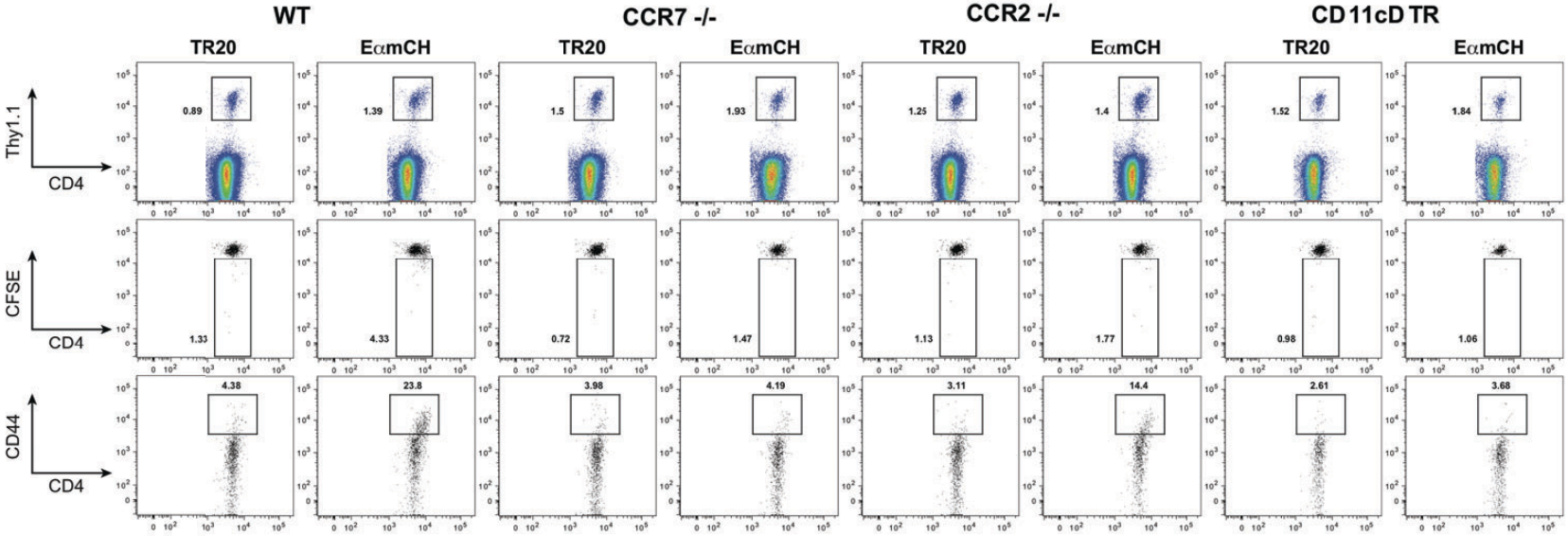


Figure S2c (supplemental to figure 2). **The effect of Pertussis toxin on vaccine yeast delivery into the lymph node.** Mice were vaccinated with heat killed PKH26^{+} yeast and got no toxin or toxin at the time of vaccine injection (0 hour) or 24 hours later as in Methods. Approximately 72 hours after vaccination, the lymph nodes were removed and cells were stained with antibodies shown to identify the 5 DC subsets shown in Fig. 1. The upper panel shows PKH26^{+} cells in the lymph node within the gated region; the middle panel, the distribution of PKH26^{+} cells among the DC subsets; and the lower panel, the proportion of PKH26^{+} cells that are Ly6c^{+} monocyte-derived DC.

Supplemental Figure 7S.



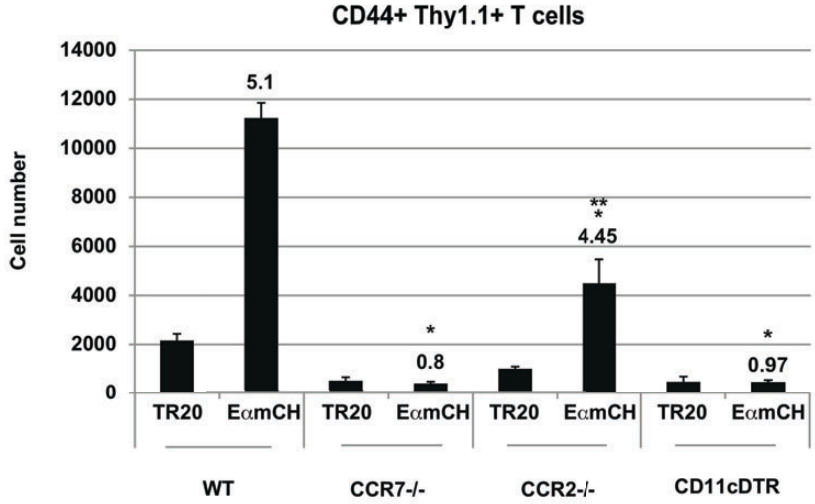


Figure S7 (supplemental to figure 7). **Vaccine priming of T cells following the loss of antigen presenting cell populations.** *In vivo* priming of antigen specific TEa tg cells was analyzed in wild-type, CCR7$^{-/-}$, CCR2$^{-/-}$, and CD11cDTR mice that contain or lack skin-derived DCs, monocyte-derived inflammatory DCs and conventional lymph node resident DCs, respectively. Naïve Thy1.1^{+} TEa T cells ($2x10^5$) were labeled with CFSE and transferred in parallel into wild type or transgenic mice one day before vaccination s.c. with TR20 control or EαmCherry yeast (10^7). Draining nodes were harvested 48 hrs after vaccination. Thy1.1^{+} cells TEa cells were gated (upper row) and analyzed for proliferation (CFSE^{lo}) or activation (CD44^{hi}) *in vivo*. The percent of cells proliferating is displayed in each gate in the 2nd row, and the percent activated, in the 3rd row. Cell numbers and fold-response of Thy1.1^{+} TEa cells vs. TR20 control is enumerated from cell activation and displayed in the bottom panel. *$p<0.001$ for EαmCH vaccine in wild-type vs. other mouse strains. **$p<0.05$ for EαmCH vs. TR20 in CCR2$^{-/-}$ mice.

SUPPLEMENTAL METHODS

EαmCherry vaccine yeast

Briefly, the coding regions for the Eα epitope and red fluroescent protein (mCherry) were fused in frame translationally as a chimera with the truncated BAD1 protein displaying 10 copies of the tandem repeat (the BAD1 truncated strain is named TR20). To streamline the generation of chimeric genes expressing different epitopes, an *Agrobacterium* binary plasmid was constructed that contains unique cloning sites and the *ccdB* gene inserted into the *Nco*I site in the truncated BAD1 gene. Transformants of strain 55 were initially screened for production of the fusion protein using an overlay assay to detect secreted BAD1 (Brandhorst et al., 1999) and subsequently analyzed by Western Blot with αBAD1 antibody on yeast cell extracts to confirm that the fusion protein is the predicted size. Positive yeast lines were also screened for red fluorescence microscopically on an Olympus BX60 fluorescent microscope (Center Valley, PA) and by FACS using the dichroic mirror at 595LP and the PE-Texas red bandpass filter. The strains showing high expression of the transgenic protein were also noted to have visibly pink/red colonies when plated on 7H10 medium, and this characteristic was used to ensure that yeast cells used for *in vivo* and *in vitro* experiments were expressing the Eα-mCherry-TRΔ20 (truncated BAD1) fusion protein at high levels.

Lymph node preparation

Brachial and inguinal LNs were harvested and digested in 1 mg/ml of collagenase D (Roche) in buffer consisting of 50 μg/ml DNase I (Roche), 10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$. Cells were digested in petri dishes for 30 minutes at

room temperature (22-25°C). After digestion, EDTA was added to collagenase buffer to a final concentration of 50 μM. Cells were scrapped and harvested. Lymph node cells were washed once and re-suspended in buffer (0.5% BSA plus 2 mM EDTA in PBS) and washed. Single cell suspensions were analyzed by flow cytometry. For $CD11b^+$ cell enrichment, digested cells were enriched on $CD11b^+$ magnetic beads (IMAG, BD Bioscience) for co-culture. Cell counts were calculated based on flow cytometry percentage of live cell by Live/Dead Violet stain (Molecular probes) and by the number of viable cells determined by trypan blue dye exclusion.

FITC Painting

Mice were shaved and painted at two spots with 50-100 μl of 2% FITC solution in acetone. Dibutylphthalate (DBP) was added to the FITC solution (50:50 mixture of DBP and Acetone) to induce migration of DCs as a positive control (Macatonia et al., 1987). Mice that were painted were stained for four hours before injecting $PKH26^+$ yeast s.c. at the site of painting.

Bone marrow dendritic cells

Bone marrow derived DCs were obtained from the femurs and tibias of C57BL/6 mice. Each bone was flushed with 10mL of 1% FBS in RPMI through a 22G needle. Red blood cells were lysed followed by wash and re-suspension of cells in 10% FBS in RPMI medium. In a petri dish, $2x10^6$ bone marrow cells were plated in 10 mL of RPMI containing 10% FBS plus penicillin-streptomycin (P/S) (HyClone ®), 2-mercaptoethanol and 20 ng/ml of rGM-CSF. The culture media was refreshed every three days. After 10 to 13 days, non-adherent DCs were harvested for use in antigen presentation assays with T cells. Dendritic cells were co-cultured with T cells in RPMI containing 10%FBS and P/S.

Generation and use of YAe antibody

The YAe hybridoma was generously provided by Marc Jenkins (University of Minnesota) (Itano et al., 2003). Monoclonal antibody from ascites was ammonium-sulfate precipitated, purified on protein A/G agarose according to the manufacturer's specifications for the isolation and purification of IgG (product 21001, Pierce Chemical, Rockford, IL), and quantified by measuring OD280. The mAb was biotinylated using the EZ-Link® Sulfo-NHS-LC kit (Thermo Fisher Scientific) according to the manufacturer's protocol. To detect Eα peptide:MHCII display in LN cells, YAe antibody was used at 50-100 μg per sample. YAe staining was performed with staining buffer of PBS containing 1%BSA plus 2mM EDTA.

Flow cytometry

Single cell suspensions of pooled brachial and inguinal LNs were stained to characterize distinct DC cell populations. All samples were blocked with FCγ receptor (CD16/32) (BD Bioscience) for 30 minutes before staining with fluorophore-conjugated antibodies. All events were gated first on FSC/SSC and on live cells based on Violet Fixable Live/Dead stain (Molecular Probes). Fluorophore-conjugated antibodies used were CD11c-APC or -PerCP, CD11b-FITC or PE-Cy7, CD205-PECy7, CD8-PerCP or PECy7, MHCII-FITC, Ly6G-FITC. Ly6C, CD86 and 7/4 antibodies were biotinylated and stained with streptavidin-conjugated to APC, PerCP, or Alexa700. Biotinylated YAe antibody was prepared and used to detect Eα peptide:MHCII display as described above. Samples were always analyzed on the same day. For intracellular Langerin staining, cells were made permeable with Perm/Fix solution (BD Bioscience) for 30 minutes, washed in 1X PermWash buffer (BD Bioscience) and stained with Langerin-FITC (eBioscience). To determine T-cell activation, naïve T-cells co-cultured with DCs were stained

with CD62L-PE, CD25-PECy7, CD69-FITC, CD44-APC, CD4-Alexa700, Thy1.1-PerCP. Antibodies were obtained from BD PharMingen (San Diego, CA) and eBioscience (San Diego, CA). Cell samples were collected for analysis on a LSRII instrument (BD Biosciences, San Jose). Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

FACS Sorting

FACS was conducted at the University of Wisconsin Cancer Center Facility. Yeasts used for vaccination in cell sorting experiments were stained with PKH26 and heat-killed by incubation at 56°C for 25 minutes. Heat-killed yeasts were used for vaccination at 10^7 yeasts/mouse as described (Wüthrich et al., 2005) to comply with facility sorting requirements. Animals were vaccinated with EαRFP as noted above. LNs were harvested 3 days after vaccination, single cell suspensions were labeled with CD11b-FITC, CD11c- APC, B220-Pacific Blue, or DEC205-PECy7, Ly6C-FITC, B220-Pacific Blue, and the cells were re-suspended in RPMI plus 10%FBS and 2mM EDTA. Samples were sorted at $2x10^7$ cells/600μl. Cells were sorted into four, 5-ml tubes of 300 μl of 20% FBS in RPMI, and washed. For co-cultures, sorted cells were placed in 384 well plates with $3x10^5$ CD4 magnetic-bead enriched T cells in a final volume of 120μl. T cells were harvested and analyzed for their activation phenotype 3 days later.

References

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Itano, A.A., McSorley, S.J., Reinhardt, R.L., Ehst, B.D., Ingulli, E., Rudensky, A.Y., and Jenkins, M.K. (2003). Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity. *Immunity 19*, 47-57.

Macatonia, S.E., Knight, S.C., Edwards, A.J., Griffiths, S., and Fryer, P. (1987). Localization of antigen on lymph node dendritic cells after exposure to the contact sensitizer fluorescein isothiocyanate. Functional and morphological studies. *J Exp Med 166*, 1654-1667.

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