SUPPLEMENTAL METHODS

Antibodies and Flow Cytometry. mAbs to Langerin (L31), DEC (NLDC), DCIR2 (33D1), Treml4 (16E5), and a control Ig mAb without receptor affinity (GL117) were produced in-house, purified on protein G columns, and labeled with Alexa Fluor 647 (Life Technologies) per manufacturer's instructions. All flow cytometry stainings were preceded by a 15 min incubation at 4°C with α-CD16/CD32 mAbs (produced in house from 2.4G2 hybridoma, ATCC). Cell suspensions were stained using antibodies from eBioscience or BD Biosciences: Alexa Fluor 700 and PerCP-Cy5.5 α-CD4 (RM4-5), allophycocyanin and PerCP-Cy5.5 α-CD25 (PC61), allophycocyanin α-Foxp3 (FJK-16s), FITC, PerCP-Cy5.5 and PE-Cy7 α-CD45.1 (A20) and α-CD45.2 (104), Alexa Fluor 700 α-CD45 (30-F11), Alexa Fluor 700 and eFluor 450 α-CD3ε (145-2C11), PerCP-Cy5.5 α-CD8α (53-6.7), APC-eFluor 780 and PerCP-Cy5.5 α-CD11b (M1/70), APC-eFluor 780 α-CD11c (N418), PE and PE-Cy7 α-CD11c (HL3), Alexa Fluor 700 α-MHC II (M5/114.15.2), Alexa Fluor 488 α-IL-2 (JES6-5H4), allophycocyanin α-CD62L (MEL-14), FITC and PE α-CD45RB (C363.16A), PE and PerCP-CY5.5 α-CD103 (2E7), PE α-CTLA-4 (UC10-4B9), α-ICOS (7E.17G9), α-Vβ11 (RR3-15), α-PD-1 (J43), and α-SiglecF (E50-2440), PE and PE-Cy7 α-CD44 (IM7), PE-Cy7 α-GITR (DTA-1), α-CD19 (1D3), α-Ter119 (TER-119), α-CD49b (DX5), α-CD5 (53-7.3), α-IFN-γ (XMG1.2), and α-CD127 (A7R34). PE α-PDCA-1 (JF05-1C2.4.1) was from Miltenyi Biotec, and PerCP-Cy5.5 α-CD103 (2E7) and α-IL-17A (TC11-18H10.1) were from Biolegend. Samples were acquired on an LSRII and data were analyzed using FlowJo software (Tree Star).

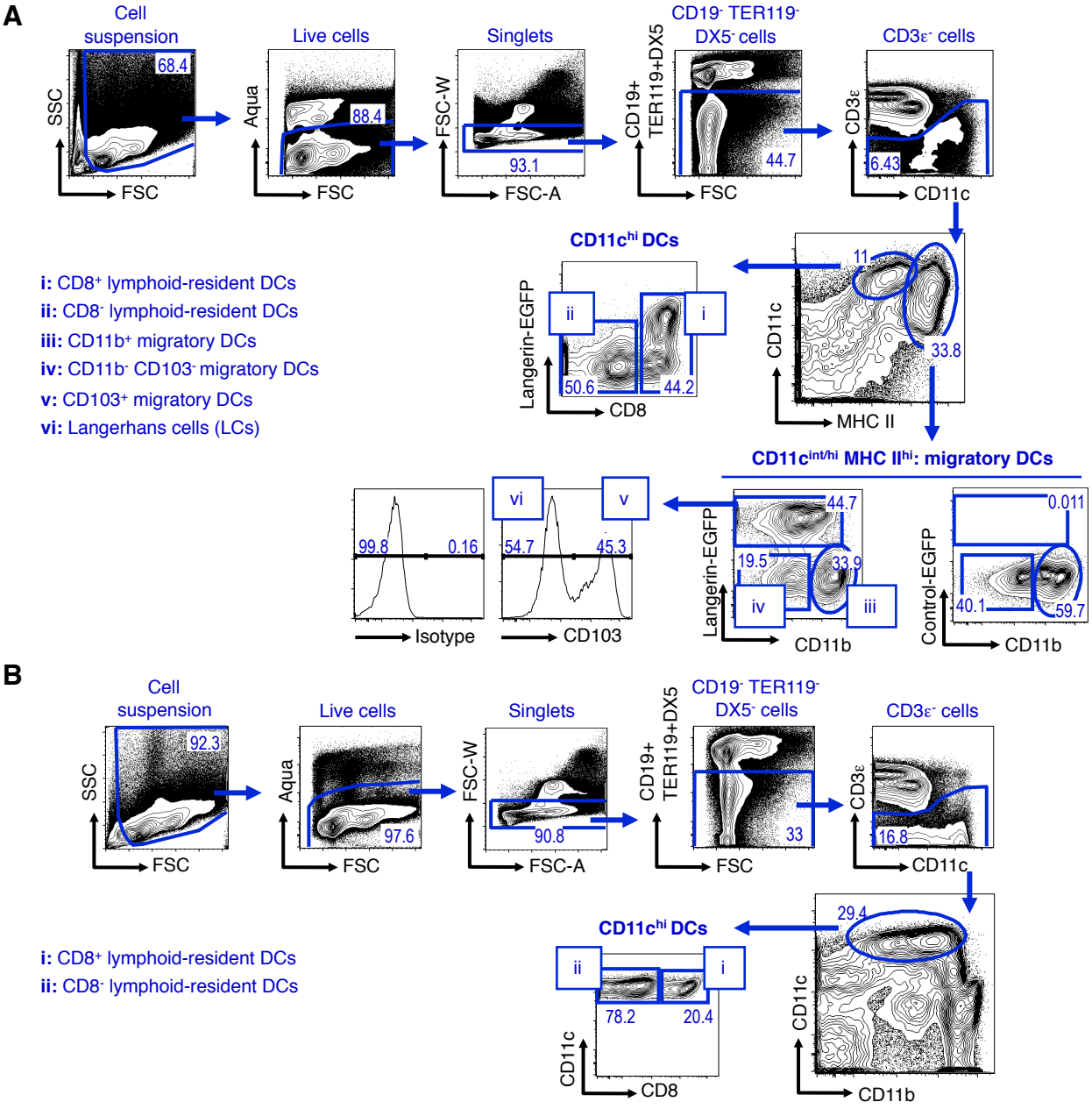
Suppression Assay. B6 mice were transferred with $4x10^6$ 2D2 Foxp3-EGFP CD45.1 $CD4^+$ T cells one day before the s.c. inoculation of 3 μg α-Langerin- or α-DEC- MOGp (day 0). At day 14, Foxp3-$EGFP^+$ or Foxp3-$EGFP^-$ $CD45.1^+$ transferred cells were FACS-sorted from sLN and their function was assessed in an in vitro suppression assay. In brief, $5x10^4$ violet-labeled (1 μM CellTrace violet for 10 min at 37°C) responder MOG-specific $CD45.2^+$ $CD4^+$ T cells/well were cultured in a U-bottom 96-well plate with $1x10^4$ CD11c-bead purified splenic DCs from CD45.1 B6 mice and 12.5 μg/ml MOG35-55p. Sorted Foxp3-$EGFP^+$ $CD45.1^+$ or Foxp3-$EGFP^-$ $CD45.1^+$ T cells were added to the well in decreasing numbers (responder/suppresor ratios = 1:1/3, 1:1/9, 1:1/27, and 1:1/81). Proliferation of responder cells was measured by dilution of the CellTrace violet 4 days later by FACS gating in live $CD4^+$ $CD45.2^+$ $V\beta11^+$ cells. For mixed leukocyte reaction (MLR), $1x10^5$ fresh $CD4^+$ T cells from Balb/c Thy1.1 mice labeled with CellTrace violet (responders) were stimulated with $2x10^4$ CD11c-bead purified splenic DCs from B6 CD45.2 mice. Graded numbers of sorted MOG-specific $CD45.2^+$ Foxp3-$EGFP^+$ cells were added to the cultures in the absence or presence of 12.5 μg/ml MOG35-55p. Proliferation of responder cells was measured by the dilution of the CellTrace violet 4 days later by FACS gating in live $CD4^+$ $Thy1.1^+$ cells. The percentage of suppression was calculated as follow: [1- (% of proliferated T responders in cultures with suppressors / % of proliferated T responders in cultures without suppressors)] x 100.

Isolation of CNS-infiltrating cells. Spinal cords of PBS-perfused mice were mechanically disrupted and digested with Collagenase D (400 U/ml, Roche Diagnostics)

and 50 μg/ml DNase I for 35 min at 37°C, adding 5 μM EDTA for the last 5 min of incubation. Mononuclear cells were enriched by Percoll gradient (67.5% - 30%) centrifugation (800g for 20 min).

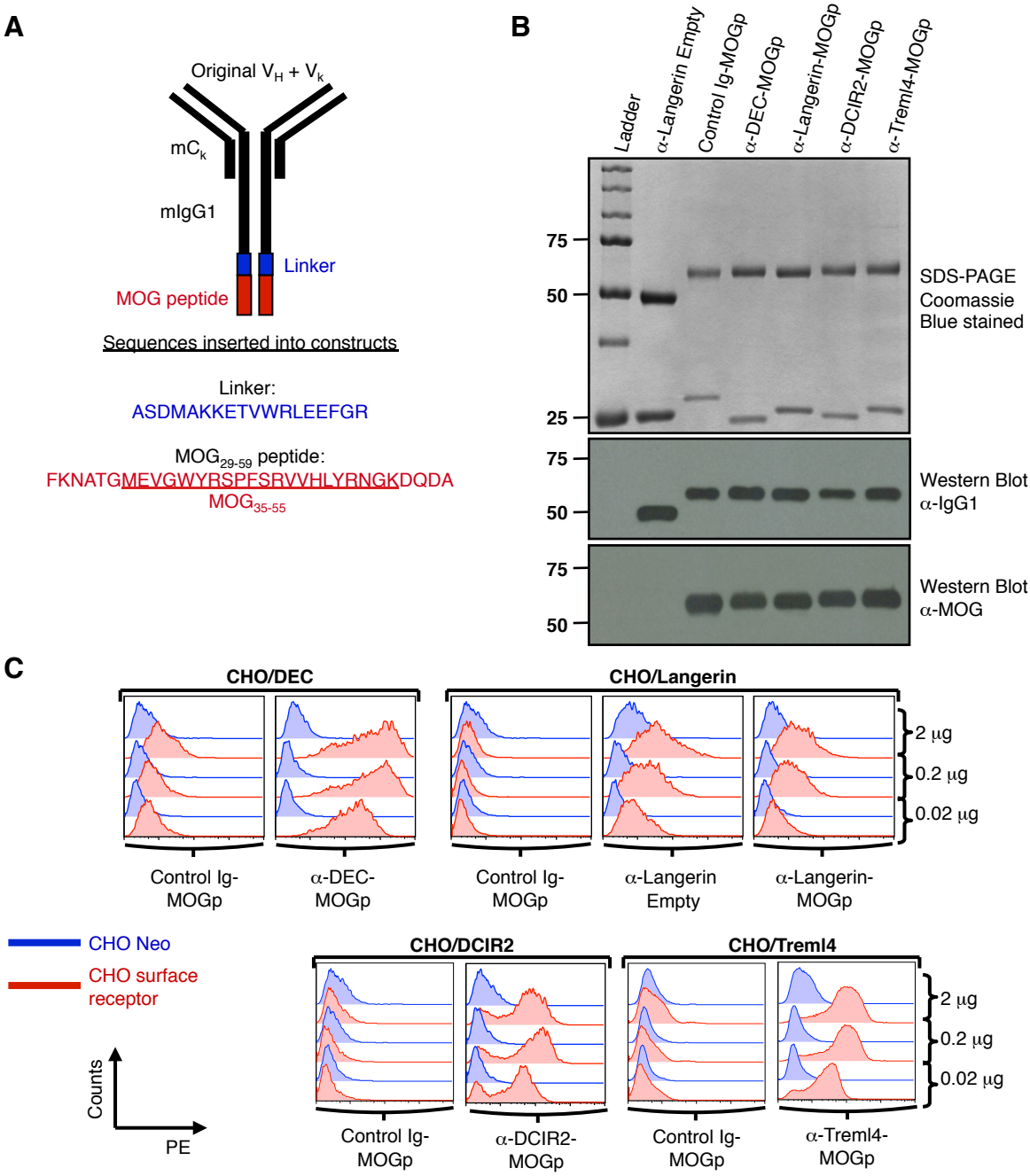
Intracellular cytokine staining. Cells were stimulated with either 12.5 μg/ml MOG35-55p, or 100 ng/ml PMA (Sigma-Aldrich) and 0.5 μg/ml ionomycin (Sigma-Aldrich) at 37°C for 5-6 hrs, adding Brefeldine A (10 μg/ml; Sigma-Alderich) for the last 4-5 hrs to allow accumulation of intracellular cytokines. After staining of surface markers, cells were fixed and permeabilized, followed by staining with α-IL-17A, α-IFN-γ, and IL-2.

Supplemental Figure 1



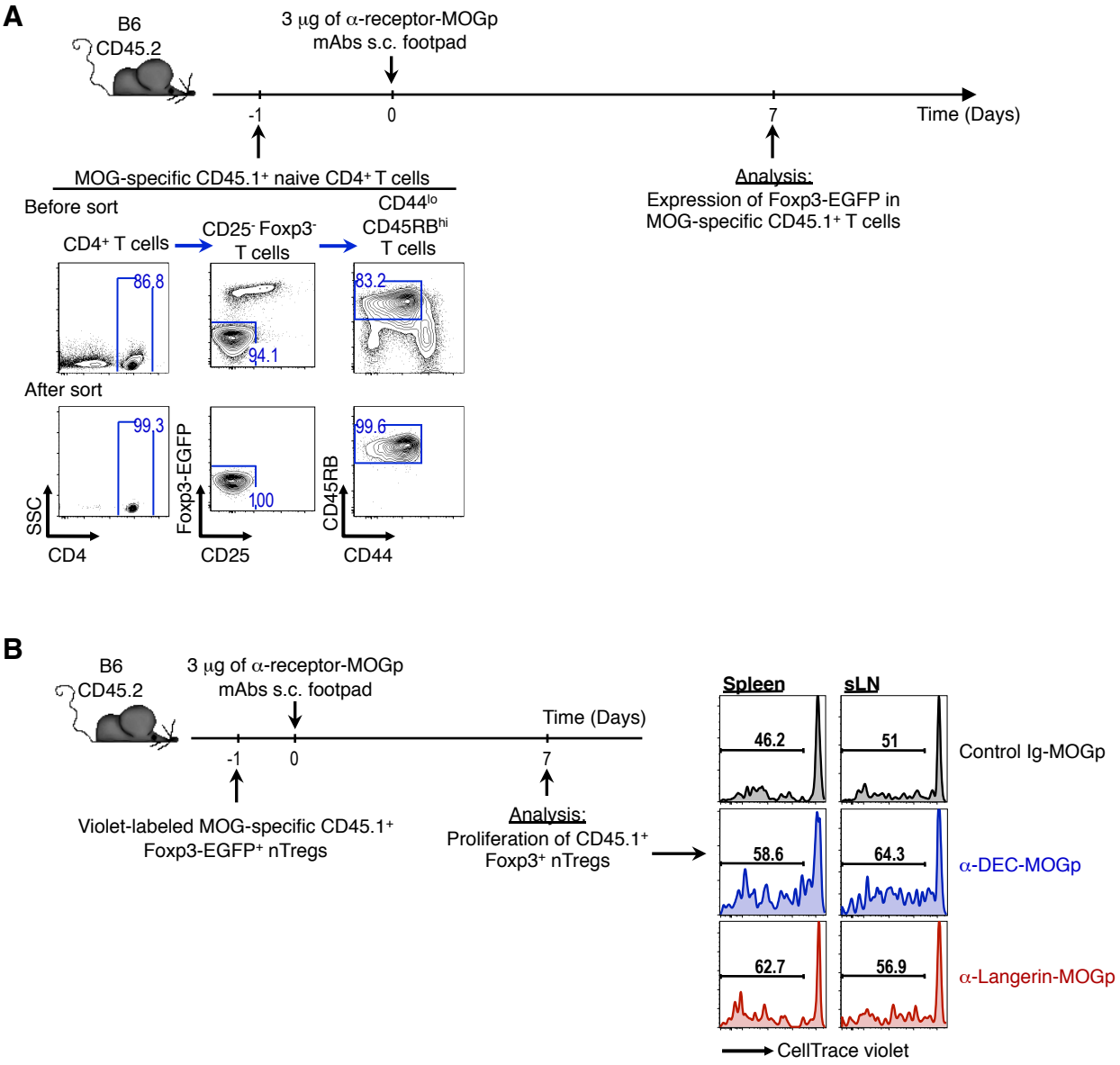
Supplemental Figure 1. Gating strategy to define DCs subsets in the skin draining LN and spleen. sLN *(A)* and spleen *(B)* of Langerin-EGFP mice were harvested and digested using Collagenase D/ DNase I. Whole cell suspensions were stained with a cocktail of antibodies to detect distinct DC subsets. **(A)** Within the single cells suspension, dead cells, doublets, CD19^{+}, TER119^{+}, DX5^{+} and CD3ε^{+} cells were excluded from analysis. The CD11c^{hi} MHC II^{int} population represents lymphoid-resident DCs, and can be further divided into CD8^{+} (gate i) and CD8^{-} DCs (gate ii). CD11c$^{int/hi}$ MHC II^{hi} cells were further analyzed for the expression of Langerin-EGFP and CD11b to define skin migratory DC subsets. Langerin^{-} cells were divided into CD11b^{+} DCs (gate iii) and CD11b^{-} DCs (gate iv). Langerin^{+} cells were further analyzed for the expression of CD103, dividing them into two groups: CD103^{+} DCs (gate v) and CD103^{-} LCs (gate vi). **(B)** Splenic live cells, singlets, CD19^{-}, TER119^{-}, DX5^{-} and CD3ε^{-} cells were selected for further analysis and examined for the expression of CD11c and CD11b. The CD11c^{hi} population represents lymphoid-resident DCs in the spleen and can be further divided into CD8^{+} (gate i) and CD8^{-} DCs (gate ii).

Supplemental Figure 2



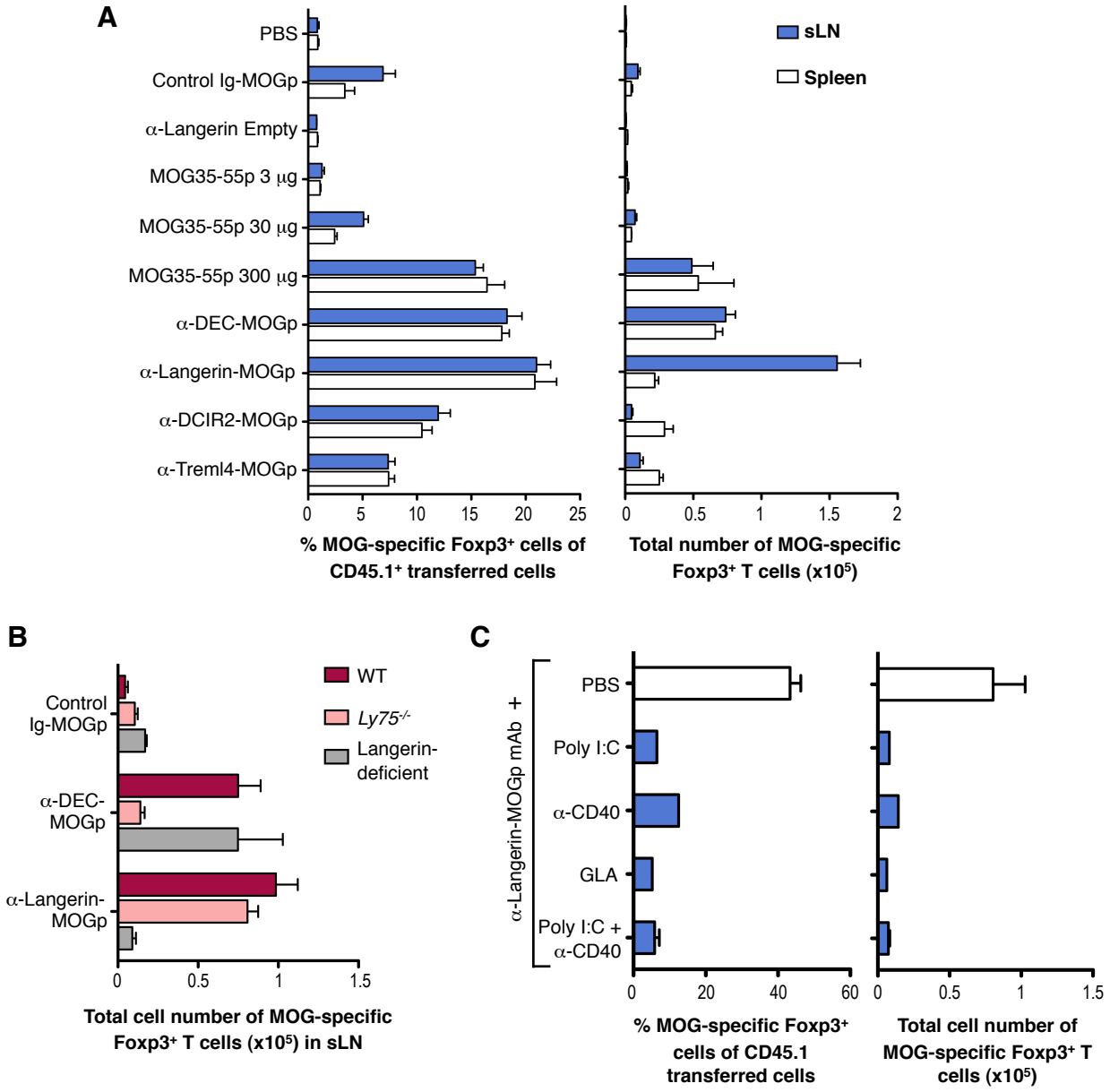
Supplemental Figure 2. Quality control of α-receptor mAbs genetically engineered to express MOGp. (A) Diagrammatic representation of the α-receptor mAb conjugated with MOGp. Constant regions of the original α-receptor mAbs, as well as a control Ig mAb, were replaced by that of the mouse IgG1 and modified to avoid Fc-receptor binding (26). The sequence inserted into the C-terminus domain of the mouse IgG1 heavy chain corresponds to a small linker (blue) and the 29-59 peptide from the myelin oligodendrocyte glycoprotein (MOG; red) containing amino acids 35-55 (underlined). **(B)** Coomassie-stained 10% SDS/PAGE reducing gel comparing fusion mAbs with molecular mass in kDa indicated (upper panel). Western blots of fusion mAbs using HRP-conjugated α-mouse IgG1 (middle panel) or Biotin-conjugated α-MOG followed by HRP-Sva (lower panel). **(C)** FACS analysis of the binding of α-receptor-MOGp mAbs to CHO cells transfected to express different receptors, i.e., DEC, Langerin, DCIR2, Treml4 (red), and control untransfected CHO cells (NEO; blue), using graded doses (0.02-2 μg) of fusion mAbs followed by α-mouse IgG PE.

Supplemental Figure 3



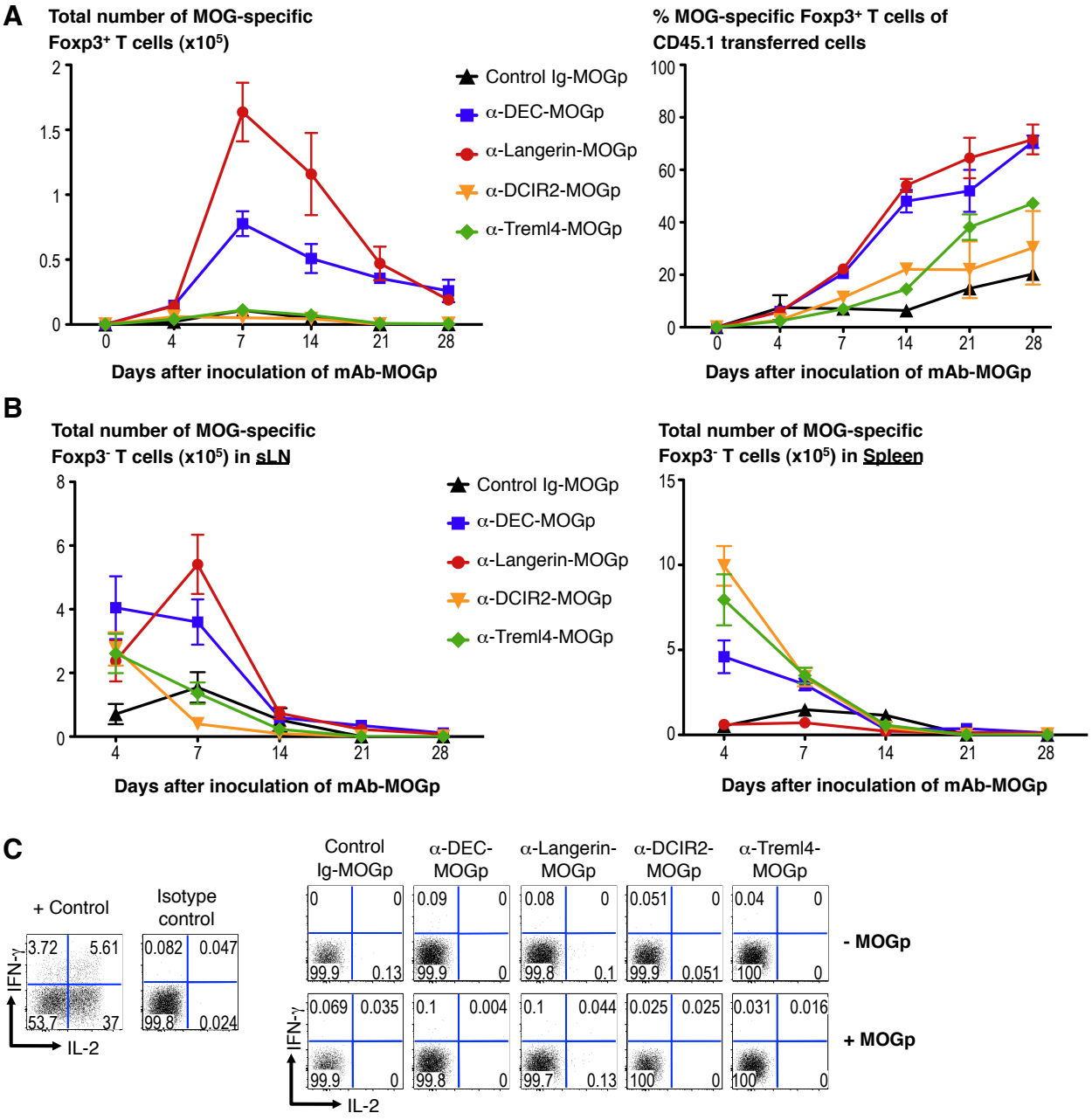
Supplemental Figure 3. Experimental design to evaluate the de novo induction of Foxp3+ T cells, and the expansion of natural Tregs by α-receptor-MOGp mAbs. (A) De novo generation of Foxp3+ T cells from naive CD4+ T cells. 4x10^6 MOG-specific CD45.1+ naive T cells were purified by FACS from MOG-specific Foxp3-EGFP reporter mice (CD25-, Foxp3-EGFP-, CD44lo, CD45RBhi), and were transferred into CD45.2 recipient mice. One day later, mice were inoculated s.c. footpad with 3 μg of α-receptor-MOGp mAbs. The generation of Foxp3-EGFP+ T cells in spleen and sLN was evaluated by FACS 7 days later (shown in Figure 2B). **(B)** Expansion of Foxp3+ nTregs by α-receptor-MOGp mAbs. 1x10^6 CD45.1+ Foxp3+ nTregs, FACS-sorted from MOG-specific Foxp3-EGFP reporter mice, were labeled with CellTrace violet and then transferred into CD45.2 recipient mice. On the next day, mice were injected s.c. with 3 μg of α-Langerin-, α-DEC- or control Ig- MOGp. 7 days after antigen inoculation, the proliferation of Violet-labeled CD45.1+ Foxp3+ nTregs was evaluated by FACS in spleen and sLN. Histograms are gated on CD45.1+ Foxp3-EGFP+ CD4+ T cells. Shown are the frequencies of Foxp3+ nTregs undergoing one or more cell divisions representative of 3 experiments with similar results.

Supplemental Figure 4



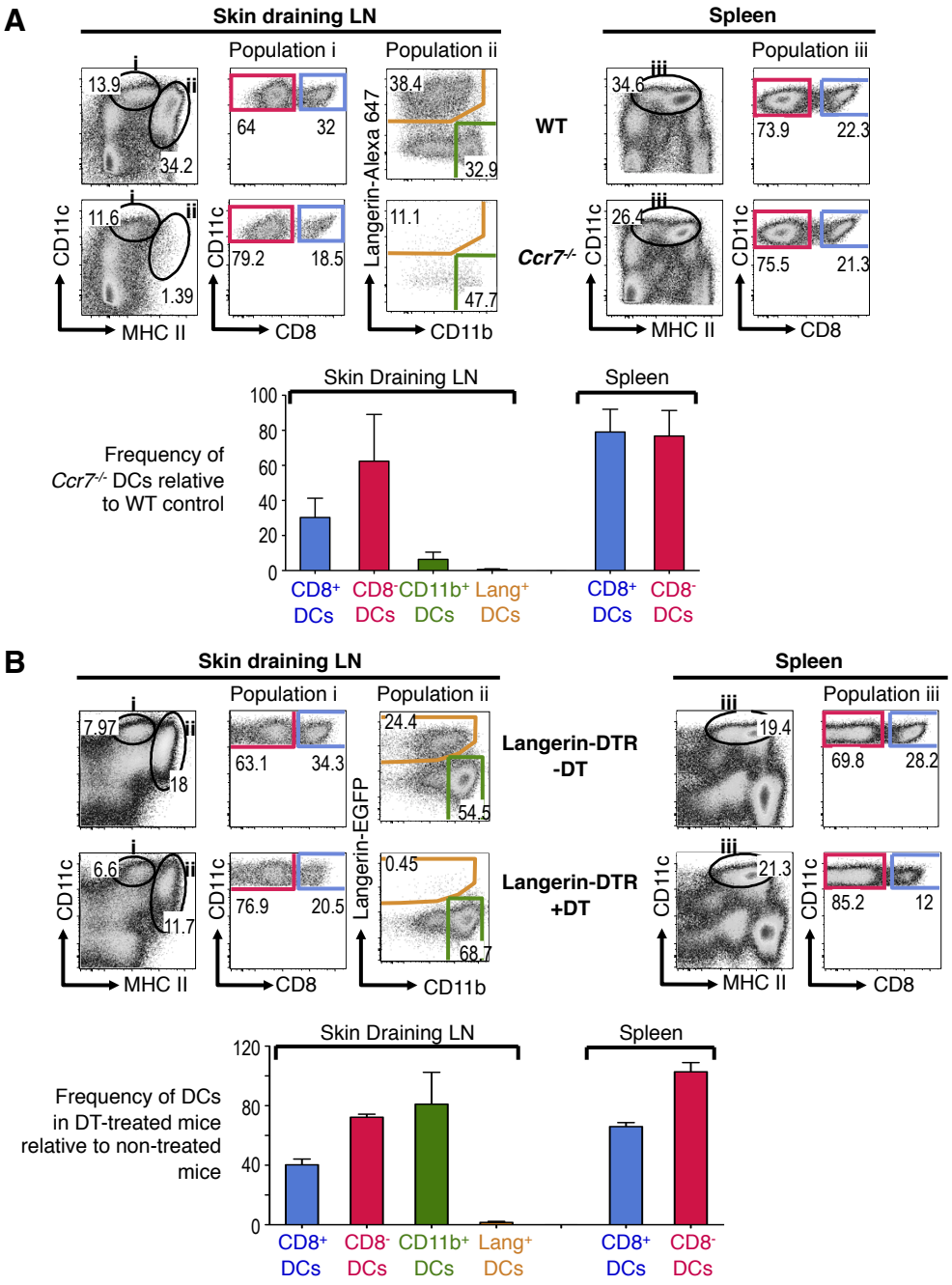
Supplemental Figure 4. Foxp3⁺ T cell generation in vivo using α-DEC and α-Langerin mAbs require steady-state DEC⁺ and Langerin⁺ DCs. (A) B6 CD45.2 mice were transferred with 4x10^6 MOG-specific CD45.1⁺ CD4⁺ T cells. One day later, mice were inoculated with 3 μg of different α-receptor mAbs conjugated with MOGp, control Ig-MOGp mAbs without receptor affinity, un-conjugated α-Langerin mAb, or different doses of soluble untargeted MOG35-55p (3-300 μg). 7 days after antigen inoculation, the percentage (left panel) and total number (right panel) of Foxp3⁺ T cells were evaluated in the sLN and spleen. Bar graphs show the mean ± SEM of 2-10 experiments with a total of 4-20 animals. **(B)** As in *A*, but WT, DEC-deficient (*Ly75*$^{-/-}$) or Langerin-deficient mice were inoculated s.c. with MOGp coupled to α-Langerin, α-DEC or control Ig mAbs. Total number of Foxp3⁺ T cells at day 7 in sLN is shown as the mean ± SEM of two experiments with a total of 4 mice. **(C)** As in *A*, but mice received a s.c. (footpad) inoculation of 3 μg of α-Langerin-MOGp alone (PBS) or in combination with different DC-maturation stimuli, and analysis was performed at day 14 in sLN. Shown is the mean ± SEM of 2 different experiments with 2 animals.

Supplemental Figure 5



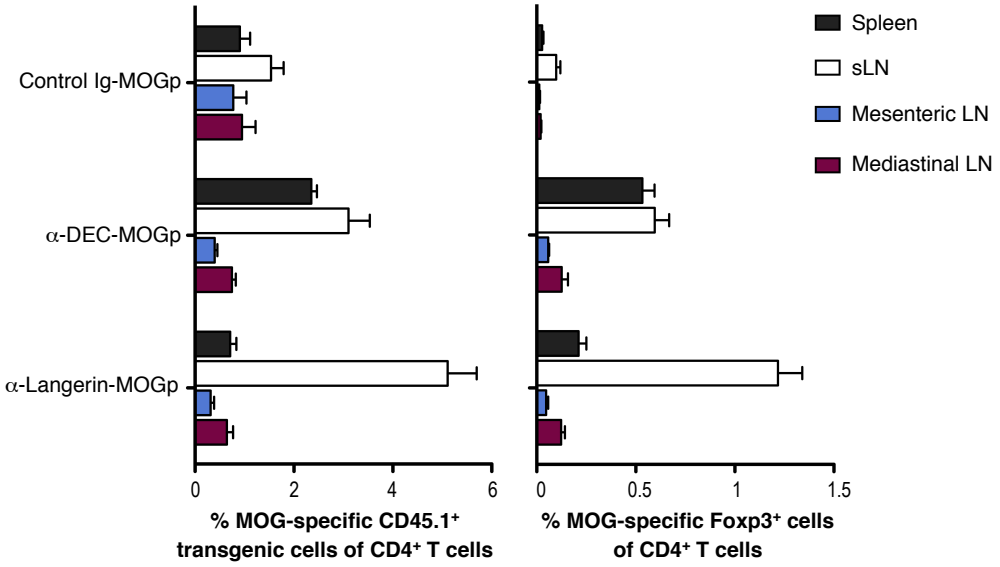
Supplemental Figure 5. Kinetics of Foxp3⁺ T cell generation triggered by α-receptor-MOGp mAbs. (A) B6 CD45.2 mice were transferred with 4x10⁶ MOG-specific CD45.1⁺ CD4⁺ T cells one day before the s.c. inoculation of 3 μg of α-receptor-MOGp mAbs. 4-28 days after antigen inoculation, sLN were analyzed for the total number of Foxp3⁺ cells (left panel) and the % of Foxp3⁺ cells among CD45.1 transferred cells (right panel). The total number of Foxp3⁺ T cells peaked at day 7-14, and gradually declined thereafter, however, significant numbers of MOG-specific Foxp3⁺ T cells were still detected at day 28 after α-DEC and α-Langerin inoculation (~2-3x10⁴ Foxp3⁺ T cells). Data is shown as the mean ± SEM of 2-7 independent experiments with a total of 4-14 animals. **(B)** As in A, but the fate of MOG-specific Foxp3⁻ T cells was followed in the sLN (left panel) and spleen (right panel). **(C)** As in A, but 4 days after α-receptor-MOGp mAbs inoculation, sLN cell suspensions were re-stimulated with 12.5 μg/ml of MOG35-55p in the presence of BFA for 6 hrs followed by intracellular cytokine staining. As positive control, cell suspensions were stimulated with 2 μg/ml of α-CD3 mAb. One representative experiment of 2 is shown.

Supplemental Figure 6



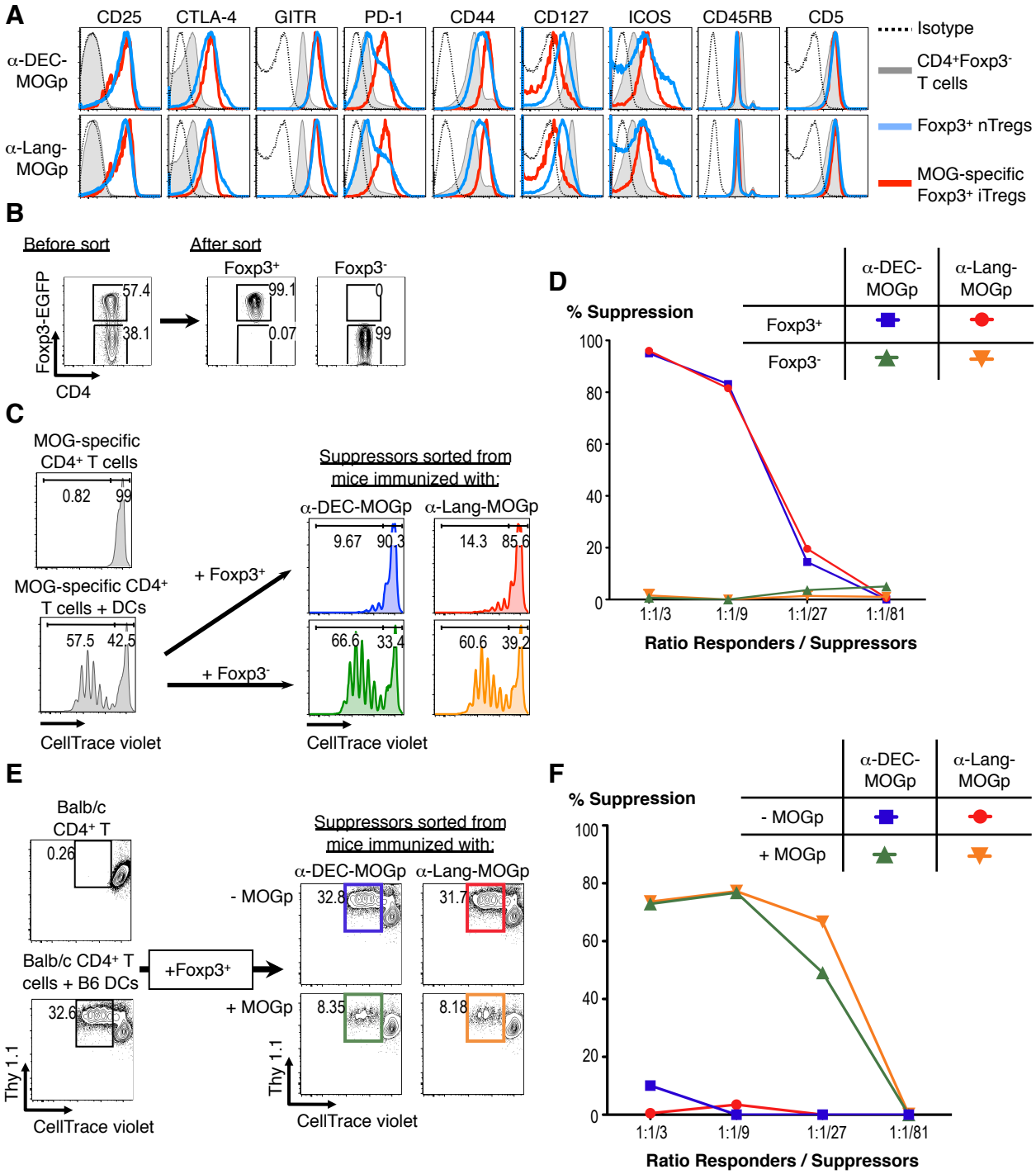
Supplemental Figure 6. *Ccr7*$^{-/-}$ mice lack all subsets of skin migratory DCs, whereas DT-treated Langerin-DTR mice specifically lack CD103^{+} migratory DCs and LCs. (A) sLN and spleen of WT and *Ccr7*$^{-/-}$ mice were analyzed by flow cytometry for the percentage of DC subsets using the gating strategy from Supplemental Figure 1. Upper panels represent FACS plot showing lymphoid-resident CD11c^{hi} DCs (population i and iii) and MHC II^{hi} migratory DCs (population ii) in sLN (left panel) and spleen (right panels). Skin migratory DCs (MHC II^{hi}, population ii) were further divided into Langerin^{+} and CD11b^{+} populations. The bottom bar graph shows the frequency of the indicated population in *Ccr7*$^{-/-}$ relative to WT controls as the mean ± SEM of 4 experiments with 4-7 animals. **(B)** Langerin-DTR/EGFP mice were inoculated i.v. with 500 ng DT at day -1, followed by i.p. inoculations of 250 ng DT at day 1, 3 and 5. At day 7 DT-treated and non-treated mice were analyzed by FACS as in *A*. Mean ± SEM of 2 experiments with a total of 4 mice is shown.

Supplemental Figure 7



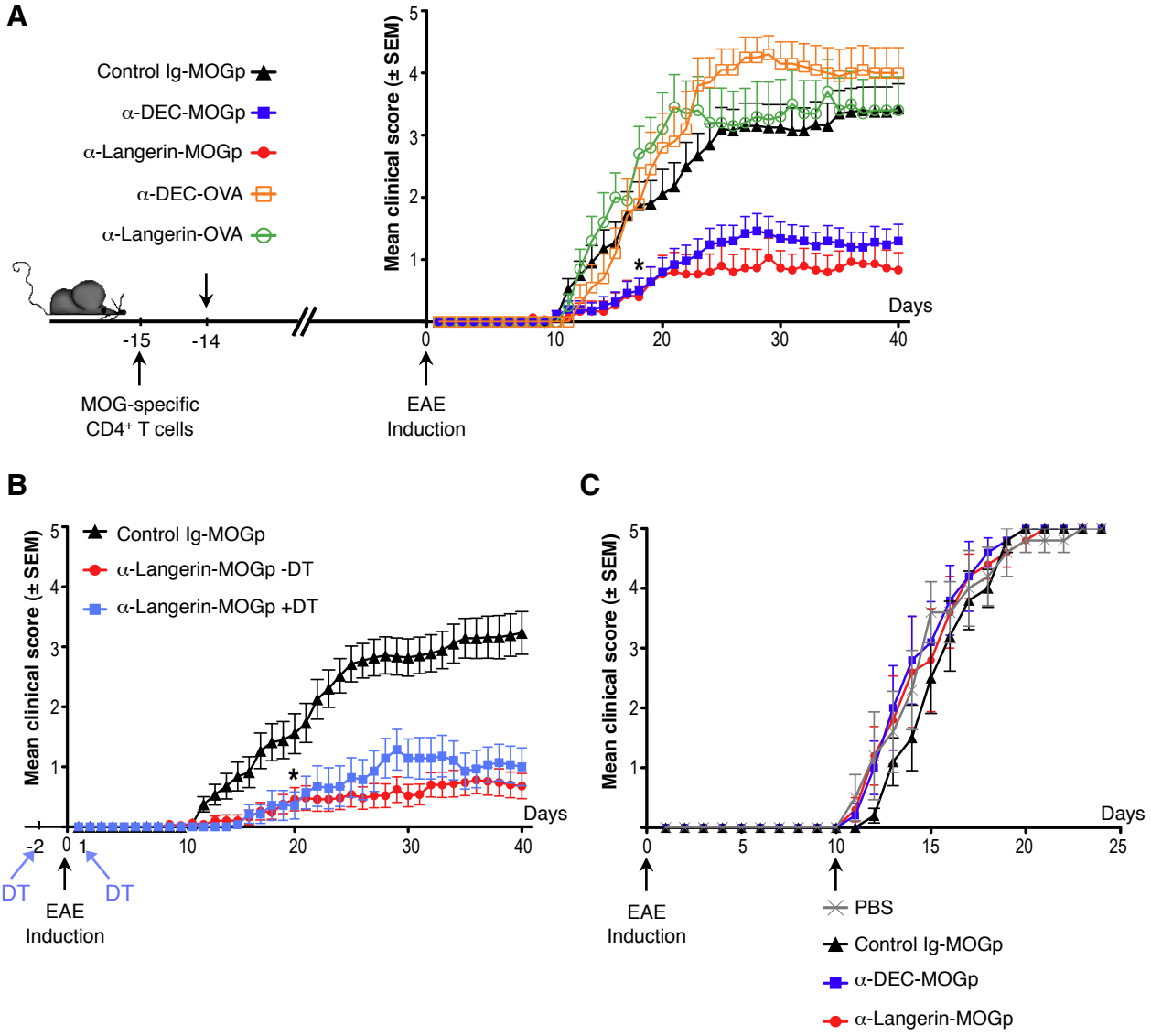
Supplemental Figure 7. MOG-specific Foxp3$^+$ transgenic T cells accumulate preferentially in sLN, but not in LN draining the lung or gut, after inoculation of α-Langerin- and α-DEC- MOGp mAbs. 4x10^6 MOG-specific CD45.1 T cells were transferred into CD45.2 B6 recipient mice one day before the s.c. inoculation of α-DEC-, α-Langerin- or control Ig- MOGp mAbs. Spleen, sLN, mesenteric LN, and mediastinal LN were harvested at day 7 and analyzed for the expression of Foxp3 in MOG-specific transgenic T cells, identified by the leukocyte marker CD45.1 and the T-cell receptor (TCR) Vβ11. The left panel shows the frequency of MOG-specific CD45.1 transgenic T cells. The right panel shows the frequency of MOG-specific CD45.1 transgenic T cells expressing Foxp3. Data is shown as the mean ± SEM of 2-4 experiments with 4-8 animals per group.

Supplemental Figure 8



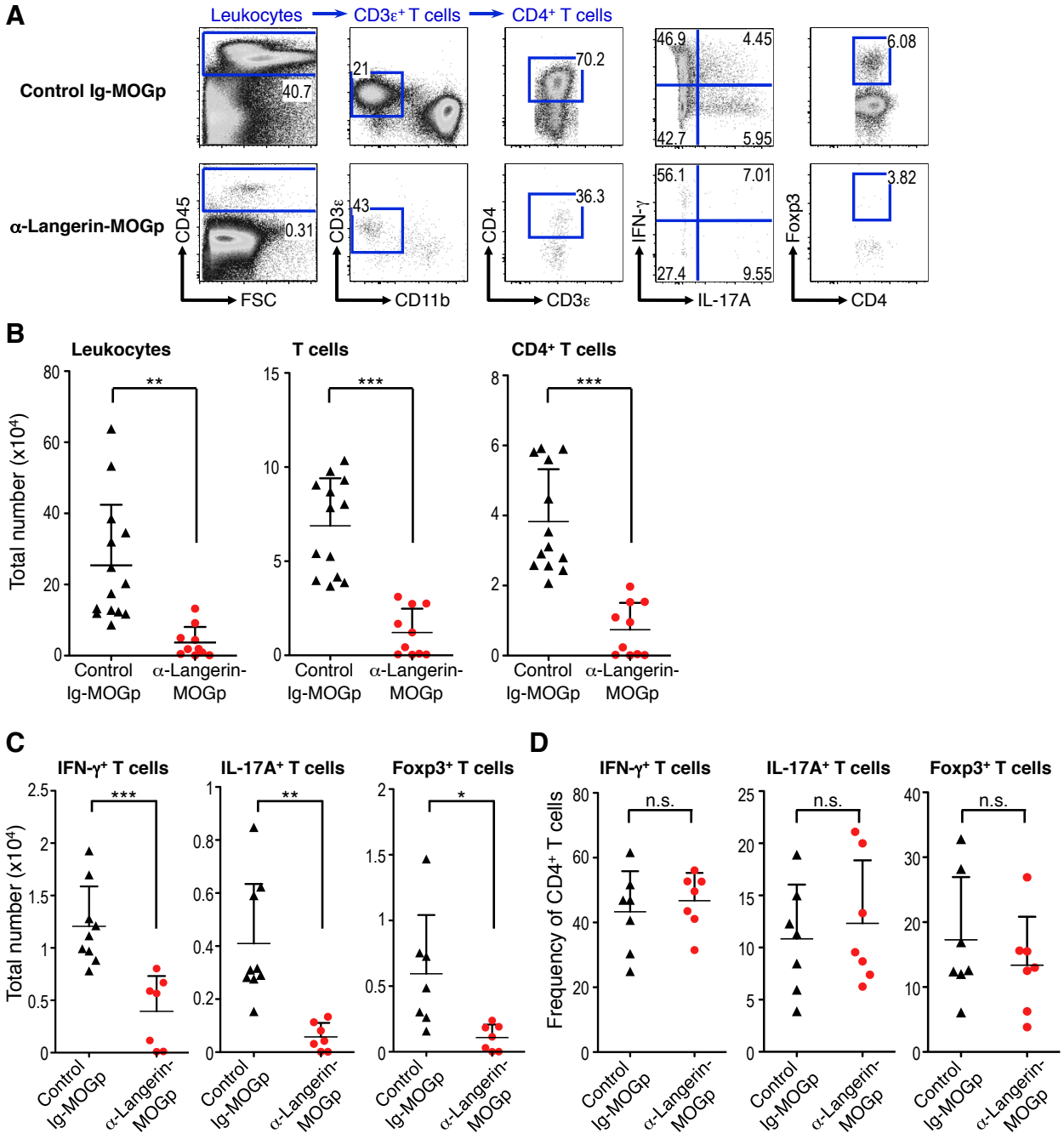
Supplemental Figure 8. α-Langerin and α-DEC-induced Foxp3$^+$ T cells are functional Tregs. B6 mice transferred with 4x10^6 MOG-specific CD4$^+$ T cells, obtained from MOG-specific Foxp3-EGFP mice, were inoculated one day later with 3 μg α-receptor-MOGp mAb s.c., and analysis was performed at day 14 in sLN. **(A)** Phenotype of α-receptor-MOGp mAb induced MOG-specific Foxp3$^+$ T cells (iTregs, red histograms), compared to Foxp3$^+$ nTregs (blue histogram) and Foxp3$^-$ CD4$^+$ T cells (gray filled histogram). One experiment of two analyzed by FACS. **(B)** MOG-specific Foxp3-EGFP$^+$ and Foxp3-EGFP$^-$ T cells before or after FACS-sorting (left and right panel, respectively). **(C)** Violet-labeled MOG-specific CD4$^+$ responder T cells (responders) were co-cultured with CD11c$^+$ DCs (5:1 ratio) plus 12.5 μg/ml MOG35-55p. FACS-sorted Foxp3-EGFP$^+$ or Foxp3-EGFP$^-$ cells were added to the cultures (suppressors; 1:1/9 ratio). Responder cells proliferation was evaluated 4 days later by dilution of the CellTrace violet. **(D)** As in *C*, but the suppressors were added to the culture at the indicated ratios. Depicted is the % of suppression (see methods), as the mean of 2 independent experiments. **(E)** Foxp3-EGFP$^+$ T cells FACS-sorted as in B were tested for suppression in a mixed leukocyte reaction (MLR). Violet-labeled responders CD4$^+$ T cells from Balb/c mice were cultured with CD11c$^+$ DCs from B6 mice (5:1 ratio). MOG-specific Foxp3$^+$ T cells were added to the cultures (suppressors; 1:1/9 ratio) +/- 12.5 μg/ml MOG35-55p. **(F)** As in E, but the suppressors cells were added at different ratios. Shown is the % of suppression as the mean of 2 experiments.

Supplemental Figure 9



Supplemental Figure 9. α-DEC- and α-Langerin conjugated with MOGp, but not OVA, protect from EAE. (A) B6 mice were transferred with 4x10^6 MOG-specific CD4$^+$ T cells one day before the inoculation of 3 μg α-receptor mAbs conjugated with MOGp or OVA. 14 days after, EAE was induced by immunization with MOG35-55p in CFA. The course of EAE is shown as the mean clinical score ± SEM of 10-20 animals in 2-4 experiments. α-DEC- and α-Langerin- MOGp were statistically significant (2-way ANOVA and Bonferroni's post hoc testing) from α-DEC- and α-Langerin- OVA starting at day 18 (*), $p<0.05$. **(B)** As in A, but mice were inoculated i.v. (2 day before EAE induction) and i.p. (1 day after EAE induction) with 250 ng DT. Mean ± SEM of 15-20 animals in 3-4 experiments. No significant differences were found between mice vaccinated with α-Langerin-MOGp +/- DT. On the other hand, α-Langerin-MOGp groups were significantly different from control Ig-MOGp starting at day 20 (* = $p<0.05$; 2-way ANOVA and Bonferroni's post hoc testing). **(C)** As in A, but α-receptor-MOGp mAbs were inoculated 10 days after EAE induction. No significant differences between the groups were found. Shown is one experiment of two with 5 animals per group.

Supplemental Figure 10



Supplemental Figure 10. Flow cytometric analysis of the CNS-infiltrating T cells in α-Langerin-MOGp inoculated mice at the peak of EAE. B6 mice inoculated with 3 μg of α-Langerin- or control Ig-MOGp (given one day after transfer of 4x10^6 MOG-specific CD4$^+$ T cells), were challenged for EAE induction 14 days later. At the peak of the disease (day 19-22), the spinal cord infiltrating cells were analyzed by FACS. **(A)** Shown is a representative FACS plot of CNS-infiltrating cells. **(B)** The total number of CD45$^+$ leukocytes (left panel), CD3ε$^+$ T cells (middle panel), and CD4$^+$ T cells (right panel) is shown as the mean ± SD of 10-14 animals in 2-3 different experiments. **(C)** The total number of CD4$^+$ T cells producing IFN-γ and IL-17A after a short re-stimulation with PMA/ionomycin, or expressing Foxp3 is shown as the mean ± SD of 7 animals in 2 experiments. **(D)** As in C, but the frequency of CD4$^+$ T cells is shown. n.s. is non-significant.

SUPPLEMENTAL TABLE 1. α-Langerin and α-DEC mAbs prevent the development and progression of EAE.

Treatment	Disease Incidence	Day of Onset[A]	Mean Peak Clinical Score[B]	Cumulative disease index[C]
PBS	10/10 (100%)	13.1 ± 1.7	5	126.7 ± 11.4
Control Ig-MOGp	18/20 (90%)	16.3 ± 4.5	4.2 ± 0.92	82.2 ± 30.8
α-DEC-MOGp	12/20 (60%)	18.8 ± 9.3	3 ± 0.6	48.4 ± 22.15
α-Langerin-MOGp	7/15 (46.7%)	19.7 ± 5.2	2.6 ± 0.7	38.4 ± 21
α-DCIR2-MOGp	10/10 (100%)	14.9 ± 1.7	3.65 ± 1	76.65 ± 19.3
α-Treml4-MOGp	9/10 (90%)	14.3 ± 2.8	3.78 ± 0.97	73.6 ± 18.8

[A] Day of onset was calculated as the first day of a clinical score ≥1 for each animal and average within each group.

[B] Mean peak clinical score was measured over the duration of disease per animal and average within each group.

[C] Cumulative disease index was calculated as the sum of daily clinical scores from each animal over the entire observation period, and reported as the average within each group. Mean ± SEM of 2-4 different experiments with a total of 10-20 mice is shown.