Table S1. Cytokine production by *ex vivo*-stimulated splenocytes from CCR9$^{-/-}$ and *wt* mice.

Mice	*Oral*	*IFN-γ*	*IL-2*	*IL-4*	*IL-10*
Wt	OVA	1102.8 ± 177 ***	70.1 ± 10.1 **	313 ± 51 *	1689.5 ± 429.5 *
	PBS	2506.9 ± 356	174.2 ± 24.3	149.1 ± 24.6	372.4 ± 78.3
CCR9$^{-/-}$	OVA	1756.9 ± 365.8	167.8 ± 53.2	156.1 ± 33.7	319.3 ± 27.4 *
	PBS	1626.4 ± 275.6	185.6 ± 37.6	124.2 ± 53.7	529.4 ± 193.4

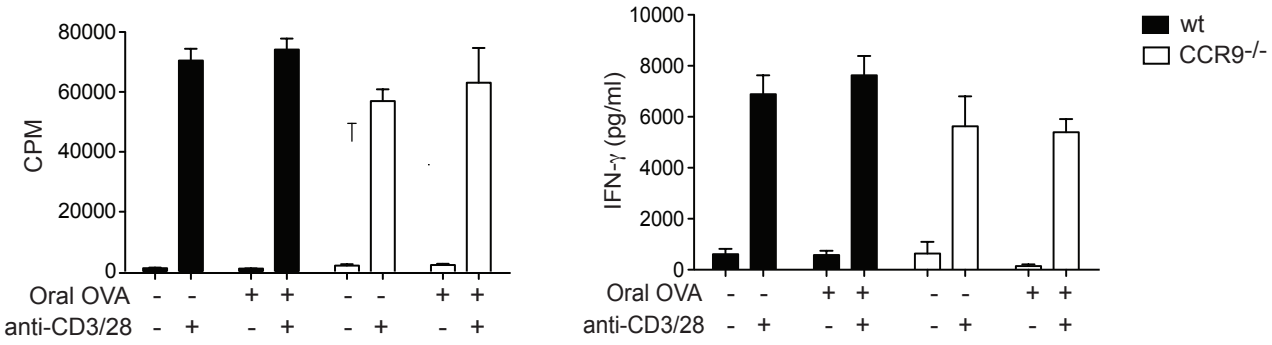
Cytokine concentrations as pg/ml. Mean ± SEM (n= 2-3) *p<0.05, **p<0.01, *** p<0.001

Table S2. Cytokine production by *ex vivo*-stimulated splenocytes from VAD and control mice.

Mice	*Oral*	*IFN-γ*	*IL-2*	*IL-4*	*IL-10*
Control	OVA	702.1 ± 91 **	98.1 ± 16.1 ***	243.8 ± 21.3 ***	1714.8 ± 130.7 ***
	PBS	1846.6 ± 230.2	334.6 ± 23.4	40.7 ± 16.9	240.9 ± 46.5
VAD	OVA	3032.6 ± 505	228 ± 31.4	301.6 ± 72.4	201.3 ± 63.8
	PBS	2984.1 ± 627.9	324.6 ± 32.8	314.4 ± 57.9	169.1 ± 59.5

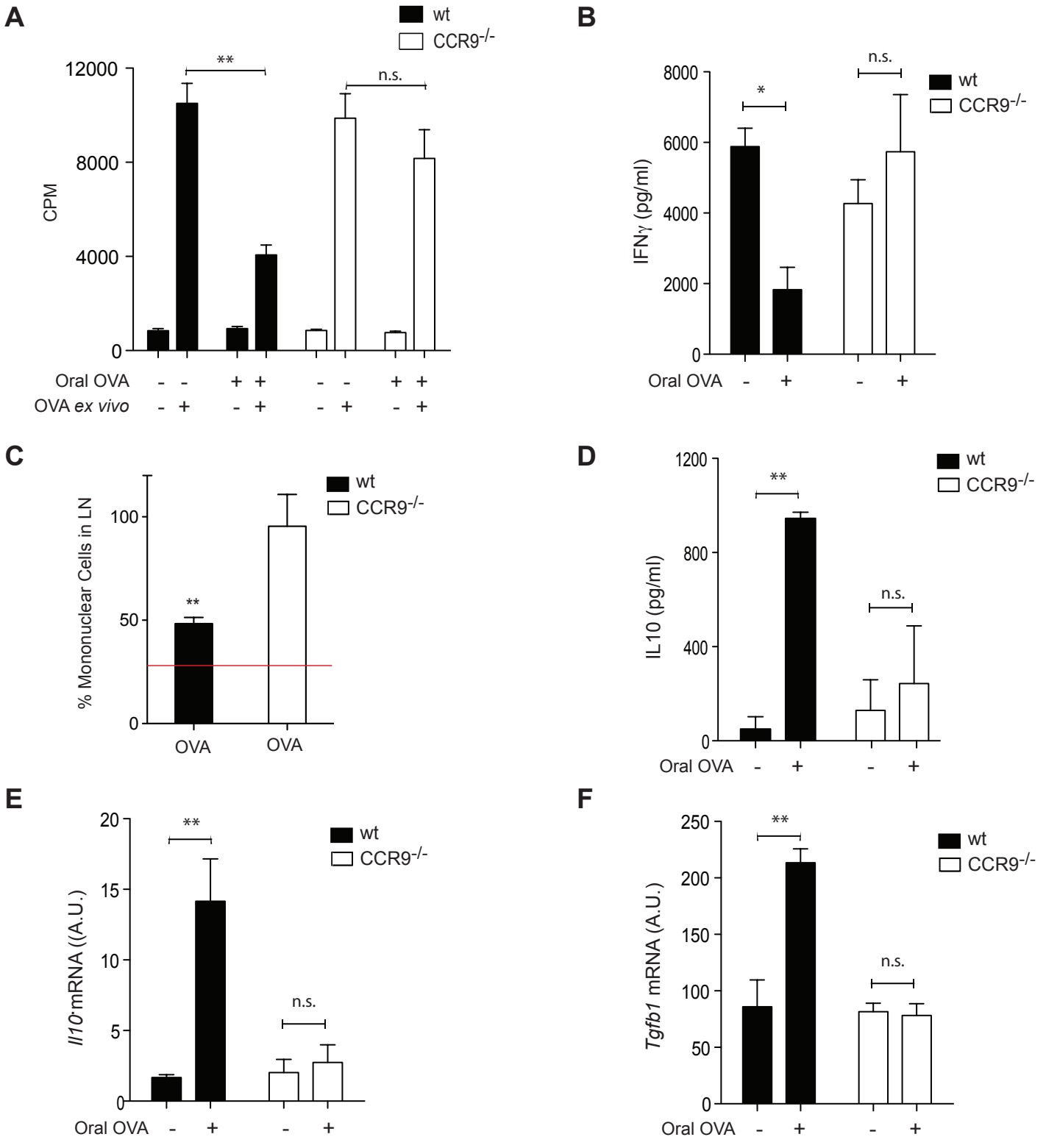
Cytokine concentrations as pg/ml. Mean ± SEM (n= 2-3) **p<0.01, *** p<0.001

Suppl. Figure 1



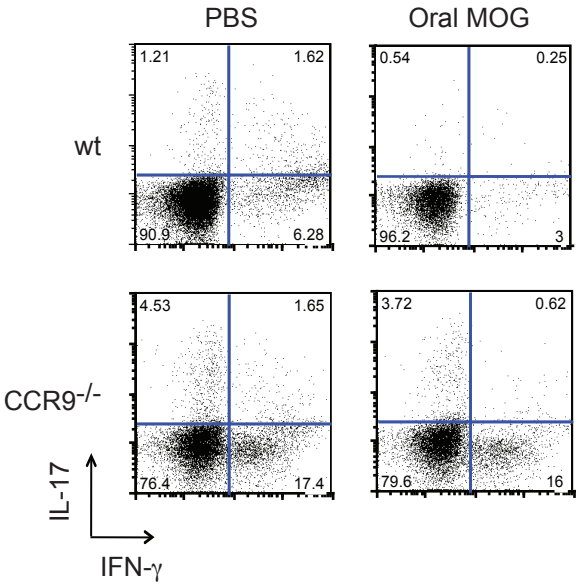
Suppl. Figure 1. *Ex vivo* polyclonal T cell activation in *wt* and CCR9-/- mice. *Wt* and CCR9$^{-/-}$ mice were supplemented daily with oral PBS or 2.5 mg OVA for 5 days, and then they were immunized s.c. with OVA plus CFA. After 7 days the mice were challenged with OVA in the left footpad and PBS in the right footpad. 24 hours later the mice were sacrificed. Total splenocytes were polyclonally stimulated *ex vivo* with plate-bound anti-CD3 (1µg/ml) plus anti-CD28 (10µg/ml). T cell proliferation was measured after 72 hours. IFN-γ was measured in the culture supernatant after 48 hours. Mean ± SEM.

Suppl. Figure 2



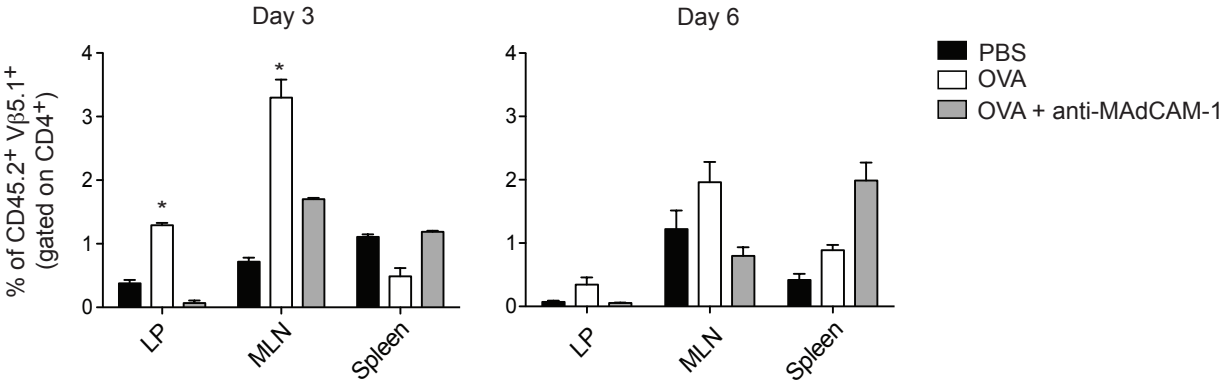
Suppl. Figure 2. CCR9 is required for OT induction. *Wt* and CCR9$^{-/-}$ mice were supplemented daily with oral PBS or 2.5 mg OVA for 5 days, and then they were immunized s.c. with OVA plus CFA. After 7 days the mice were challenged with OVA in the left footpad and PBS in the right footpad. 24 hours later the mice were sacrificed. Inguinal lymph node CD4$^+$ T cells were cultured with 1µg/ml OVA in the presence of mytomicin-c-treated APC. (**A**) T cell proliferation was assessed after 72 hours. (**B**) IFN-γ was measured in the culture supernatant after 48 hours. (**C**) Cellularity in popliteal draining lymph node (LN). The number of mononuclear cells in LN from OVA-fed mice was normalized with respect to the number of cells in PBS-fed mice (n=7-9 mice/group). The solid red line indicates the average percentage of mononuclear cells in non-draining LN of PBS-fed mice as reference. (**D**) IL-10 was measured in the culture supernatant after 48 hours. (**E, F**) *Il10* and *Tgfb1* mRNA expression in OVA-stimulated CD4$^+$ T cells. Mean ± SEM, **p<0.01

Suppl.Figure 3



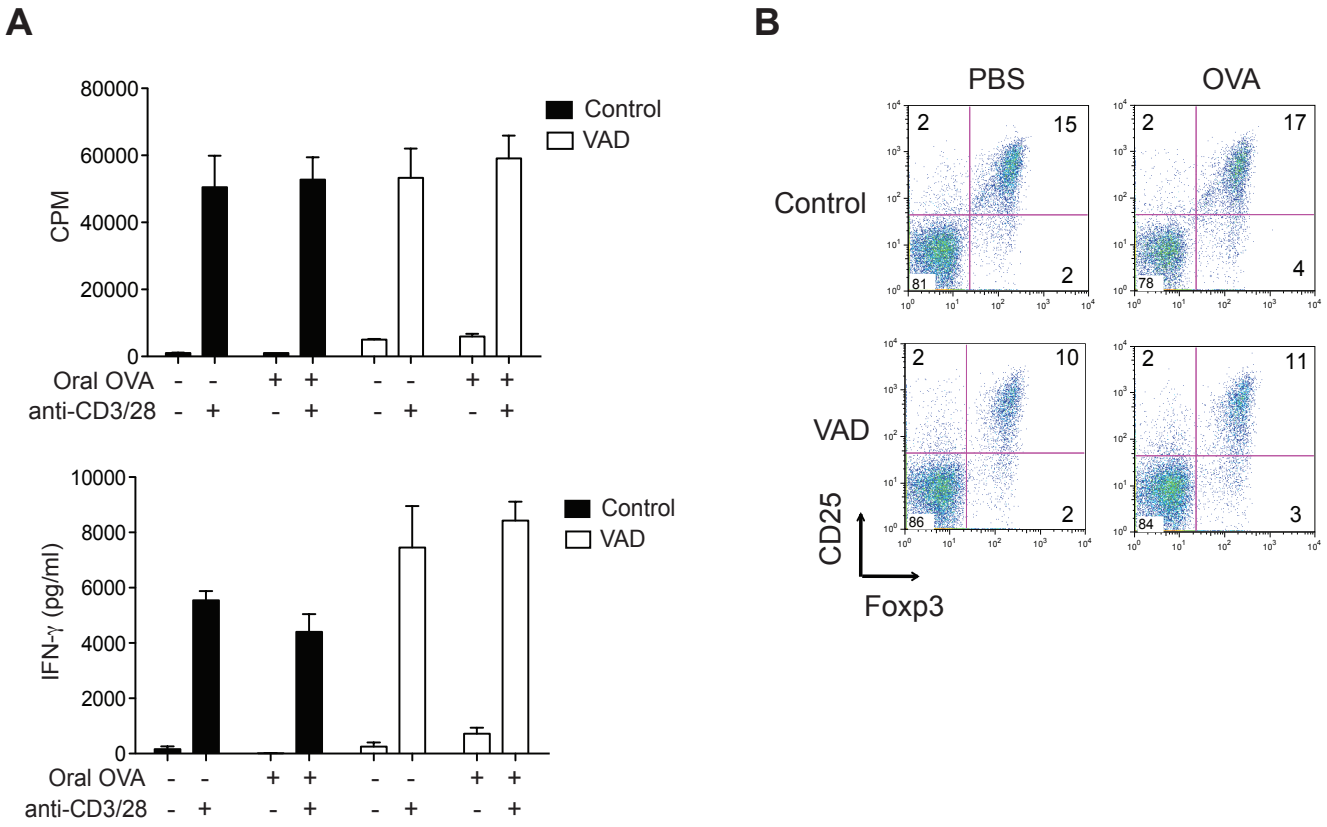
Suppl. Figure 3. Th1 and Th17 responses in *wt* and CCR9$^{-/-}$ mice. *Wt* and CCR9$^{-/-}$ mice were supplemented daily with oral PBS or 0.25 mg MOG_{35-55} for 5 days, and then they were immunized s.c. with MOG_{35-55} plus CFA. At day 16, the mice were sacrificed and total splenocytes were stimulated ex vivo with 20 μg/ml MOG_{35-55} in the presence of GolgiPlug for 5 hours and then analyzed for their intracellular expression IL-17 and IFN-γ. Numbers indicate the percentages of IL-17 or IFN-γ producing cells (FACS plots were gated on $CD4^+$ cells).

Suppl. Figure 4



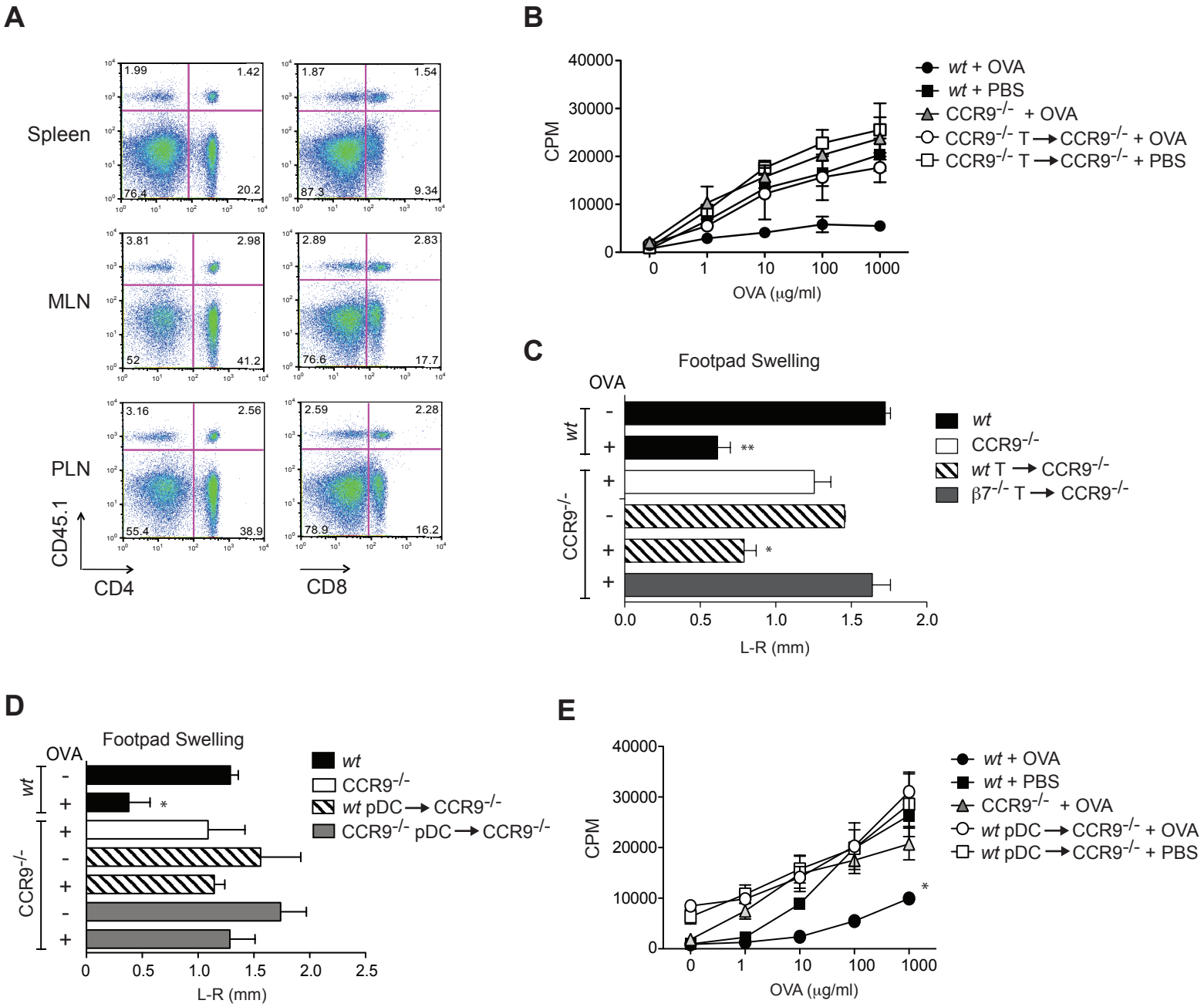
Suppl. Figure 4. Generation of gut-homing OVA-specific CD4 T cells during OT induction. 5x10^6 CD45.2$^+$ OT-II CD4$^+$ T cells were adoptively transferred into congenic CD45.1$^+$ *wt* recipients. 24 h later the mice were daily supplemented with 2.5mg OVA via oral gavage for 5 days. One group of mice was simultaneously treated with anti-MAdCAM-1 mAb. The frequencies of exogenous CD45.2$^+$ Vβ5.1$^+$ OT-II CD4$^+$ T cells infiltrating the LP, MLN and spleen were analyzed at days 3 and 7 after starting feeding OVA (n=3 mice/group). Mean ± SEM. *p<0.05

Supplementary Figure 5



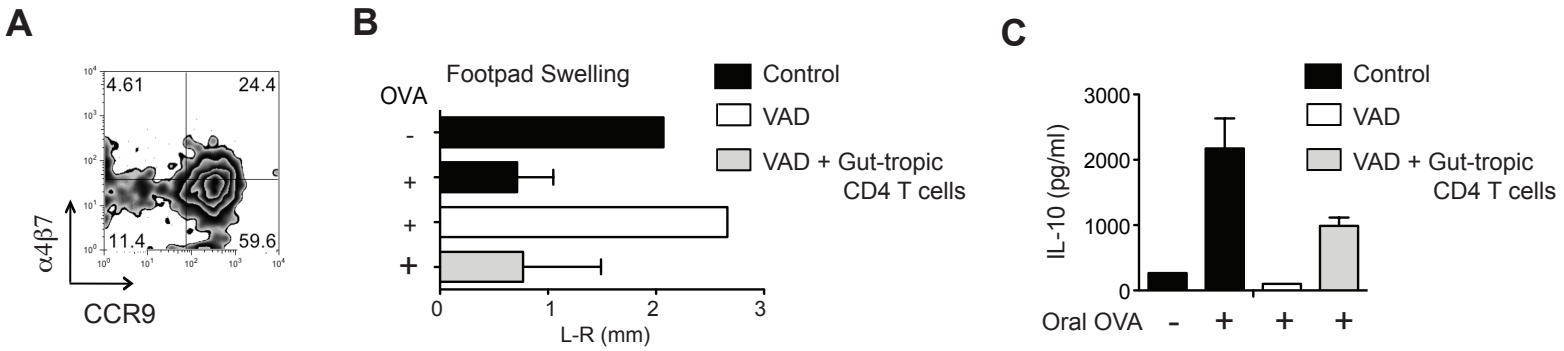
Suppl. Figure 5. *Ex vivo* polyclonal T cell activation and T_{REG} frequencies in control and vitamin A-depleted (VAD) mice. Mice on a control or VAD diet were supplemented daily with oral PBS or 2.5 mg OVA for5 days, and then they were immunized s.c. with OVA plus CFA. Seven days later the mice were challenged with OVA in the left footpad and PBS in the right footpad. (**A**) Total splenocytes were polyclonally stimulated *ex vivo* with plate-bound anti-CD3 (1μg/ml) plus anti-CD28 (10μg/ml). T cell proliferation was measured after 72 hours. IFN-γ was measured in the culture supernatant after 48 hours. (**B**) Frequency of T_{REG} in MLN from mice on a control or a VAD diet. Mean ± SEM

Suppl. Figure 6



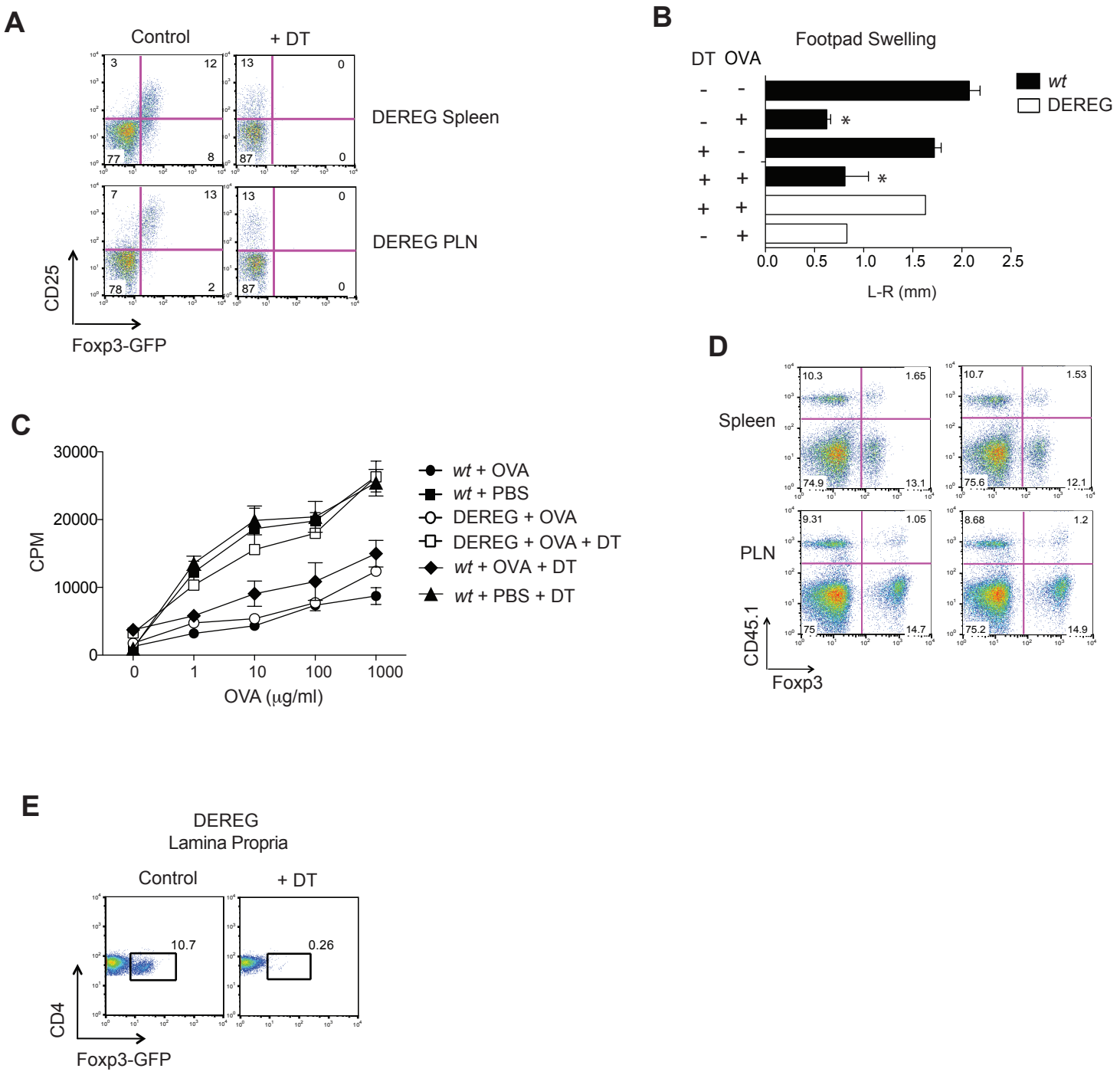
Suppl. Figure 6. Adoptive transfer of CCR9$^{-/-}$ T cells, β7$^{-/-}$ T cells or *wt* pDC does not rescue OT in CCR9$^{-/-}$ mice. *Wt* T cells, CCR9$^{-/-}$ T cells, β7$^{-/-}$ T cells or *wt* pDC were adoptively transferred into CCR9$^{-/-}$ recipients. After that the mice were supplemented daily with oral PBS or 2.5 mg OVA for 5 days and then they were immunized s.c. with OVA plus CFA. Seven days later the mice were challenged with OVA in the left footpad and PBS in the right footpad. (**A**) FACS plots show the proportion of *wt* T cells (CD45.1^{+}) in different lymphoid compartments 16 days post-transfer into CCR9$^{-/-}$ (CD45.2^{+}) mice. (**B**) Total splenocytes were stimulated with 1 mg/ml OVA and T cell proliferation was measured after 72 hours (n=3-4 mice/group). (**C, D**) DTH responses were measured after 24 hours (n=3-4 mice/group). (**E**) Total splenocytes were stimulated with 1 mg/ml OVA and T cell proliferation was measured after 72 hours (n=3-4 mice/group). Mean ± SEM. *P<0.05, **P<0.01

Suppl. Figure 7



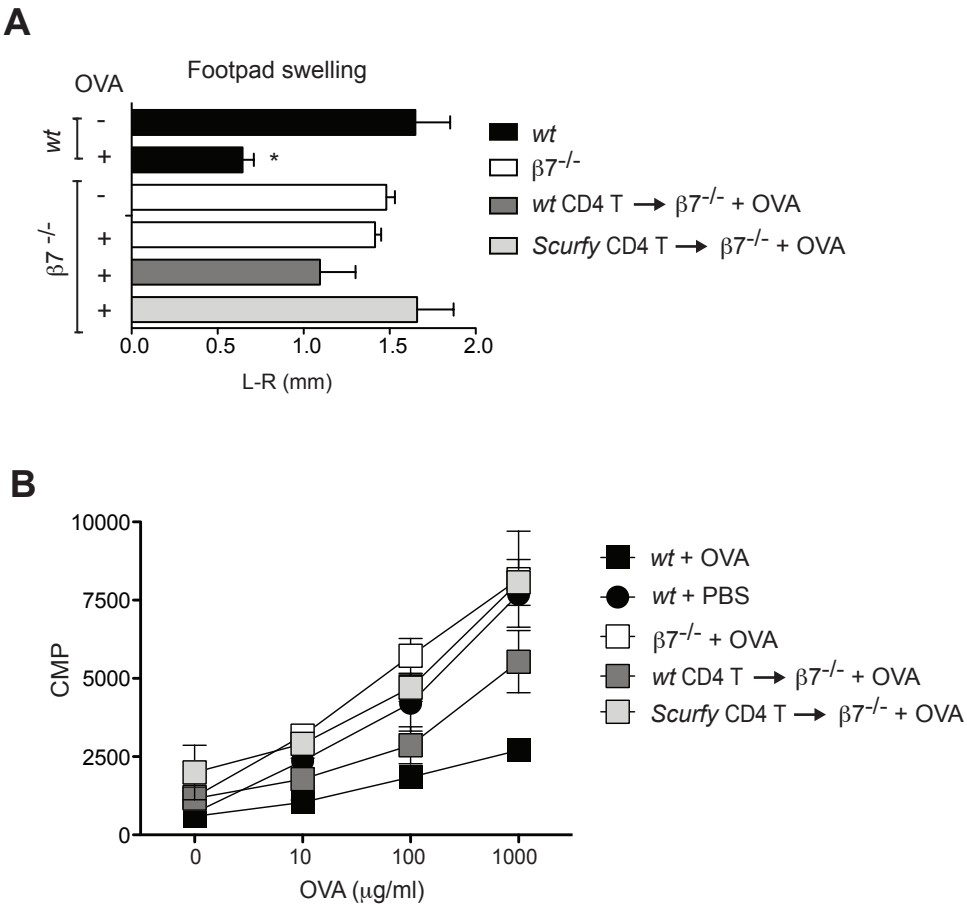
Suppl. Figure 7. Adoptive transfer of antigen-specific gut-tropic CD4 T cells rescues OT in VAD mice. Ex vivo-generated gut tropic OT-II CD4 T cells (**A**) were transferred into VAD mice. After that the mice were supplemented daily with oral PBS or 2.5 mg OVA for 5 days and then they were immunized s.c. with OVA plus CFA. Seven days later the mice were challenged with OVA in the left footpad and PBS in the right footpad. (**B**) DTH responses were measured following OVA challenge into the footpad after 24h. (**C**) IL-10 was measured in the culture supernatant after 48 hours stimulation with 1 mg/ml OVA. n=1-2 mice/group; Mean ± SEM

Suppl. Figure 8



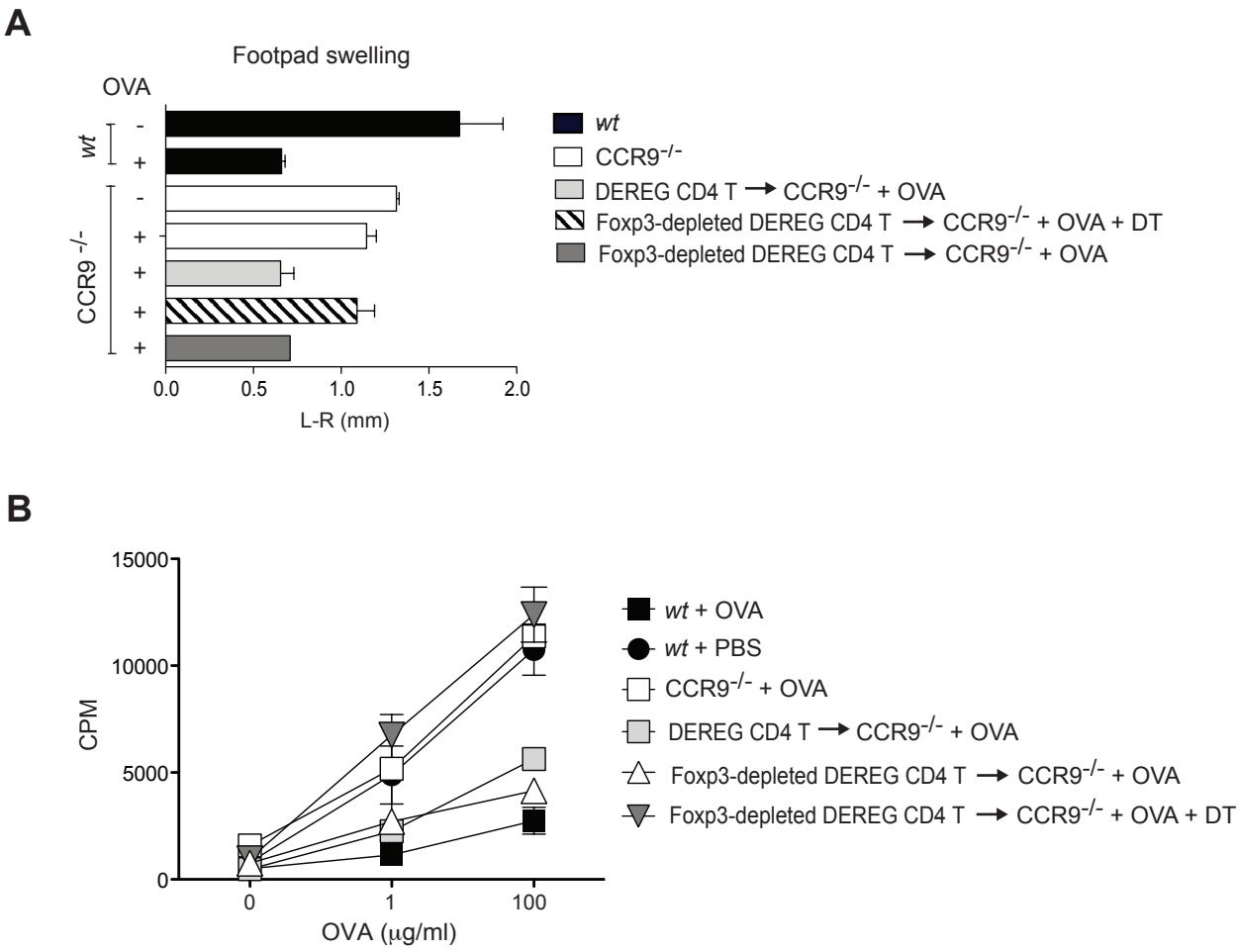
Suppl. Figure 8. T_{REG} depletion abrogates OT. *Wt* and DEREG mice were supplemented daily with oral PBS or 2.5 mg OVA for 5 days, and then they were immunized s.c. with OVA plus CFA. Seven days later the mice were challenged with OVA in the left footpad and PBS in the right footpad. Mice were treated or not with diphtheria toxin (DT) to deplete T_{REG}. (**A**) CD4 T cell expression of Foxp3-GFP and CD25 in spleen and PLN of untreated or DT-treated DEREG mice. FACS plots were gated on viable CD4+ T cells. (**B**) DTH responses were measured after 24 hours (**C**) Total splenocytes were stimulated with the indicated OVA concentrations and proliferation was measured after 72 hours. (**D**) FACS plots show the proportion of $CD4^+Foxp3^+$ T cells ($CD45.1^+$) in different lymphoid compartments 16 days post-transfer into $CCR9^{-/-}$ ($CD45.2^+$) mice. (**E**) Foxp3-GFP expression in CD4 T cells from small intestine lamina propria of untreated or DT-treated DEREG mice. FACS plots were gated on viable cells. n=1-2 mice/condition; Mean ± range

Suppl. Figure 9



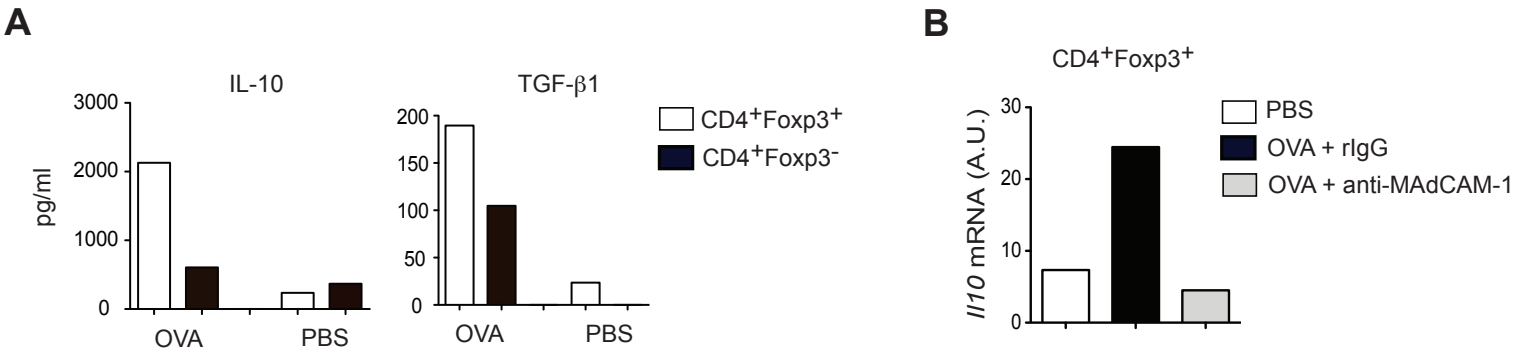
Suppl. Figure 9. Adoptive transfer of Foxp3-deficient CD4 T cells from *Scurfy* mice does not rescue OT in CCR9$^{-/-}$ mice. Naïve CD4 T cells from *wt* or *Scurfy* mice were adoptively transferred into CCR9$^{-/-}$ recipients. After that the mice were supplemented daily with oral PBS or 2.5 mg OVA for 5 days, and then they were immunized s.c. with OVA plus CFA. Seven days later the mice were challenged with OVA in the left footpad and PBS in the right footpad. (**A**) DTH responses were measured after 24 hours. (**B**) Total splenocytes were stimulated with the indicated OVA concentrations. Proliferation was assessed after 72 hours. n=2 mice/group; Mean ± range

Suppl. Figure 10



Suppl. Figure 10. *Wt* iT_{REG}, but not nT_{REG}, are required to rescue OT in CCR9$^{-/-}$ mice. CD4+ T cells from DEREG mice either treated with DT (Foxp3-depleted DEREG) or not (DEREG) were adoptively transferred into CCR9$^{-/-}$ mice and then they were orally supplemented with OVA for 5 days. One group of CCR9$^{-/-}$ mice receiving Foxp3-depleted DEREG CD4 T cells was treated daily with DT during OVA feeding. Mice were then immunized s.c. with OVA plus CFA and, seven days later, the mice were challenged with OVA in the left footpad and PBS in the right footpad. (**A**) DTH responses were measured after 24 hours. (**B**) Total splenocytes were stimulated with the indicated OVA concentrations and proliferation was measured after 72 hours. n=1-2 mice/group; Mean ± range

Suppl. Figure 11



Suppl. Figure 11. Gut-tropic Foxp3$^+$ T_{REG} are the major source of IL-10 in OT. DEREG mice were supplemented daily with oral PBS or 2.5 mg OVA for 5 days and treated i.p. with anti-MAdCAM-1 or control rIgG. After that the mice were immunized s.c. with OVA plus CFA. Seven days later the mice were challenged with OVA in the left footpad and PBS in the right footpad. The next day the mice were euthanized and Foxp3$^+$ (GFP$^+$) CD4$^+$ T cells and Foxp3$^-$ (GFP$^-$) CD4$^+$ T cells were FACS sorted from pooled spleens (2/3 mice/group) and stimulated ex vivo with 100 µg/ml OVA in the presence of spleen-DC. (**A**) IL-10 and TGF-β1 production was analyzed in the culture supernatants at 60 h. (**B**) Il10 mRNA expression was assessed in sorted Foxp3$^+$ CD4$^+$ T_{REG}.

Supplementary Methods

EAE. Mice were injected s.c. in the flank with 100 μg MOG_{35-55} in 0.15 ml of PBS emulsified in a equal volume of complete Freund's adjuvant (CFA) containing 4 mg of *Mycobacterium tuberculosis* H37 RA per ml (Difco™ , Detroit Michigan). Pertussis toxin (200 ng/mouse/injection) (List Biological Laboratories, Campbell, CA) was administered i.p. at the time of immunization and 48 hours later. Animals were scored for EAE as follows: 0, no disease; 1, tail paralysis; 2, hind limb weakness; 3, hind limb paralysis; 4, hind limb plus forelimb paralysis; 5, moribund.

Mouse treatments. DT was administered intraperitoneally (i.p.) (8 μg/kg) every day during the OT protocol. Anti-MAdCAM-1 (MECA-367; BD Pharmingen, San Diego, CA) was administered i.p. (2mg/kg) every day from days -1 to 6 and every other day from days 8 to 14 of the OT protocol.

Cell isolation and adoptive cell transfer. Single-cell suspensions were prepared from spleens harvested from naïve (non-tolerized) *wt*, $CCR9^{-/-}$ or $\beta7^{-/-}$ mice. Total T cells were negatively selected using the Pan T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions (purity > 95%). $CD4^+$ T cells were isolated from spleens and peripheral lymph nodes (PLN) of non-tolerized *wt* or DEREG mice using the mouse CD4 isolation kit (Miltenyi Biotec). pDC were isolated by negative selection (Miltenyi Biotec) from the bone marrow of B16-FLT3L treated *wt* and $CCR9^{-/-}$ mice, according to the manufacturer's instructions (purity > 92%). Total T cells ($25x10^6$), $CD4^+$ T cells ($15x10^6$) or pDC ($4x10^6$) were injected i.v. into *wt*, $CCR9^{-/-}$, or $\beta7^{-/-}$recipients.

Cell proliferation and cytokine measurement. Single-cell suspensions were prepared from the

spleens of *wt* and CCR9$^{-/-}$ or from control and VAD mice that were orally treated with either PBS or OVA and then used for DTH induction. Cells ($3x10^5$ cells/well) were cultured in 200 μl/well of X-vivo 15 media (Cambrex Bio Whittaker, East Rutherford, NJ) at 37°C and 5% CO2 in 96-well flat-bottom culture plates and in the presence of graded concentrations of antigen (0 - 1,000 μg/ml) or 1 μg/ml anti-CD3ε plus 10 μg/ml anti-CD28 mAbs (BD Pharmingen, San Diego, CA). In some experiments CD4$^+$ T cells were isolated from PLN by magnetic sorting and stimulated with antigen in the presence of mytomicin-c-treated CD11c$^+$ APC. Seventy-two hours later the cells were pulsed for 16 hours with 1 μCi/well [^{3}H]thymidine (Perkin Elmer, Waltham, MA), then harvested and counted in a scintillation counter. For cytokine determination, cultured supernatants were collected after 24 hours (IL-2) or 48 hours (other cytokines), and cytokine concentrations were determined by capture ELISA, according to the manufacturer's instructions (BD Biosciences, San Jose, CA). Intracellular cytokine staining (IL-17 and IFN-γ) was performed in cells stimulated for 6 hours with MOG peptide (20μg/ml) in the presence of GolgiStop (BD Biosciences). Cytokine mRNA for *Il10*, *Tgfb1* and *Actb* (encoding β-actin) were assessed in PLN CD4$^+$ T cells stimulated with OVA (1μg/ml) for 48h using "Assays-on-Demand" real-time PCR kits (Applied Biosystems).

***Ex vivo* generation of gut-tropic OT-II CD4 T cells**. Naïve Thy1.1$^+$ OT-II CD4$^+$ T cells ($1.5x10^6$) were activated with spleen-DC ($0.4x10^6$) plus 100 nM $OVA_{323-339}$ in the presence of 100 nM of all-*trans* retinoic acid (Sigma-Aldrich, St Louis, MO), as described [1]. Cells were cultured in X-vivo 15 media (Cambrex Bio Whittaker, East Rutherford, NJ) in 24-well plates (1 ml/well). Four days later the expression of CCR9 and α4β7 was evaluated by flow cytometry. Gut-tropic OT-II CD4$^+$ T cells ($5x10^6$) were injected i.v. into VAD mice.

Immunophenotype. The following mAbs were purchased from BD Pharmingen: anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CCR9 (ebioCW-1.2), anti-α4β7 (DATK32), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD25 (PC61.5), anti-Foxp3 (FJK-16s), anti-CD44 (IM-7), anti-CD62L (MEL-14).

References.

1. Villablanca EJ, Mora JR. Competitive homing assays to study gut-tropic t cell migration. Journal of visualized experiments : JoVE 2011.