Supplemental material

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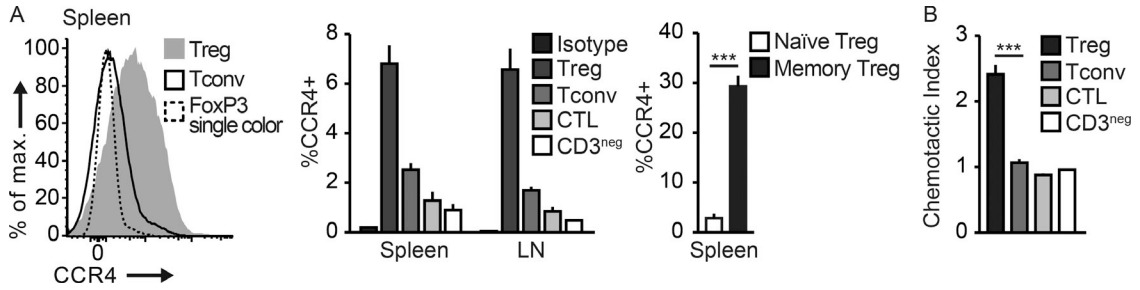


Figure S1. **CCR4 is expressed selectively by T regs. (A)** Spleen and lymph node cells from C57BL/6 mice were analyzed by flow cytometry for CCR4 surface expression on $CD3^+CD4^+Foxp3^+$ (T reg), $CD3^+CD4^+Foxp3^{neg}$ (T conv), $CD3^+CD8^+$ (CTL), $CD3^{neg}$, $CD3^+CD4^+Foxp3^+CD62L^+CD44^{low}$ (naive T reg) and $CD3^+CD4^+Foxp3^+CD62L^{neg}CD44^{high}$ (memory T reg) cells. The relative mean fluorescence intensity to FoxP3 stain alone was calculated (2.97 for T regs and 0.94 for T convs). **(B)** Recombinant murine CCL22 (50 ng/ml) was used to attract splenocytes in a standard in vitro Transwell migration assay. The number of migrated $CD3^+CD4^+Foxp3^+$ (T reg), $CD3^+CD4^+Foxp3^{neg}$ (T conv), $CD3^+CD8^+$ (CTL), and $CD3^{neg}$ cells were determined by flow cytometry, and the chemotactic index was calculated as the ratio of cells migrated to CCL22 and cells migrated to medium. Data are presented as mean ± SEM and are representative for two independent experiments. ***, P < 0.001 (two-sided Student's *t* test).

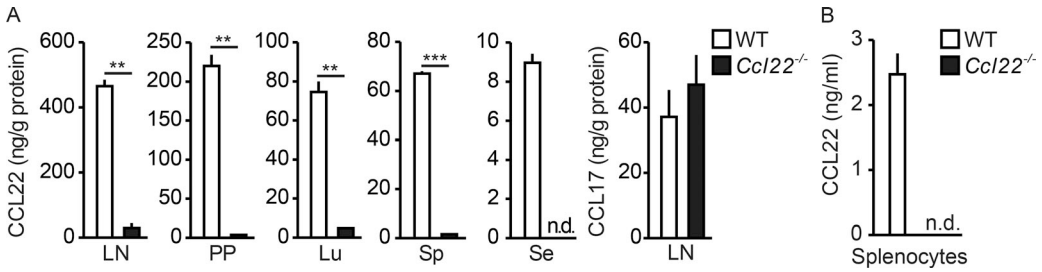


Figure S2. ***Ccl22*$^{-/-}$ mice are deficient for CCL22 but proficient for CCL17 protein. (A)** CCL22 and CCL17 protein levels were measured by ELISA in tissue homogenates of peripheral lymph nodes, Peyer's patches (PP), lung (Lu), spleen (Sp), and serum (Se) of *Ccl22*$^{-/-}$ and C57BL/6 (WT) mice. **(B)** 10^6 splenocytes from *Ccl22*$^{-/-}$ and WT mice were cultured for 96 h, and CCL22 was measured by ELISA in the supernatant. Data are shown as mean ± SEM of four mice per group and are representative of two independent experiments. **, P < 0.01; ***, P < 0.001; n.d., not detectable (two-sided Student's *t* test).

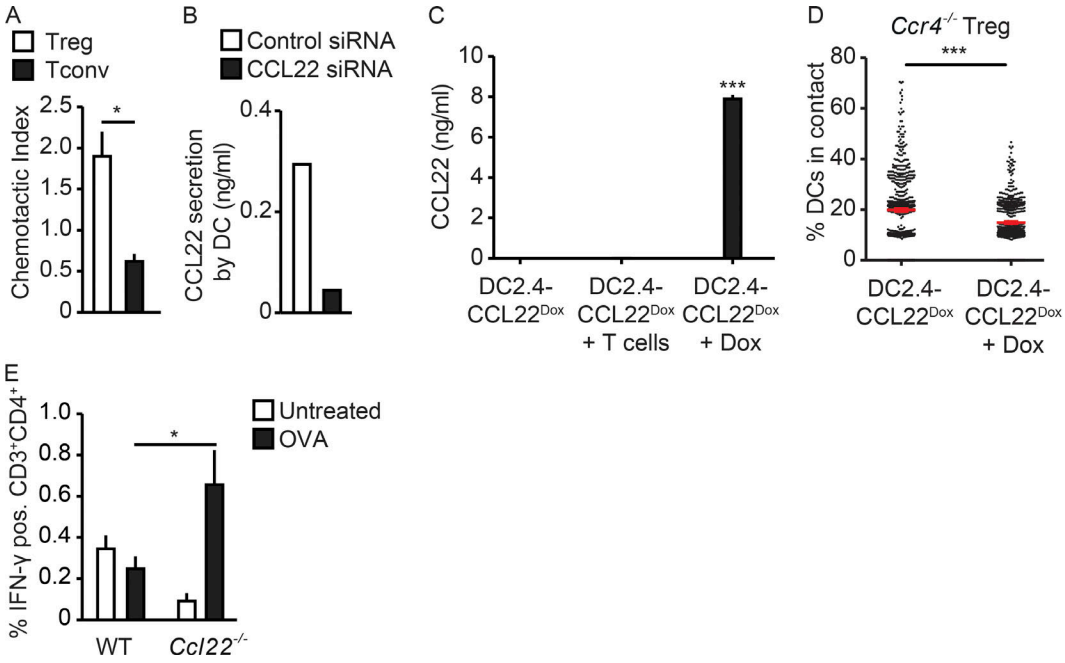


Figure S3. **DC-secreted CCL22 is a strong chemoattractant for T regs, and CCL22-induced DC–T reg contacts depend on CCR4 expression by T regs.** **(A)** Freshly isolated C57BL/6 splenocytes (10^6) were used for a standard in vitro migration assay with supernatant from C57BL/6 and *Ccl22*$^{-/-}$ BMDCs as chemoattractant. The numbers of migrated $CD3^+CD4^+Foxp3^+$ (T reg) and $CD3^+CD4^+Foxp3^{neg}$ (T conv) cells were determined by flow cytometry, and the chemotactic index was calculated as the ratio of cells migrated to the supernatant from C57BL/6 and cells migrated to the supernatant of *Ccl22*$^{-/-}$ BMDCs. **(B)** CCL22 protein expression of control siRNA or CCL22 siRNA-treated DCs was measured in collagenase-dissolved gels by ELISA. Data are representative of two independent experiments. **(C)** 10^5 DC2.4-$CCL22^{dox}$ cells were cultured alone or in the presence of 10^6 CD4-sorted T cells or in medium containing 2 µg/ml doxycycline. CCL22 was determined by ELISA after 3 d of culture. **(D)** 10^6 T regs from *Ccr4*-deficient mice were mixed in a collagen gel with 10^5 DC2.4-$CCL22^{dox}$ without or with doxycycline. DC–T cell interaction was analyzed by confocal microscopy over 8 h. Resulting videos were analyzed for DC–T cell contacts in a standardized manner as described. Each dot represents the percentage of DCs in contact with T cells at one time point. Data are representative of three independent experiments. **(E)** *Ccl22*$^{-/-}$ and WT C57BL/6 mice were injected with OVA protein as described in Fig. 3. The frequency of OVA-specific CD4 T cells ($CD19^{neg}CD3^+CD4^+$) was determined by intracellular IFN-γ staining upon restimulation with $OVA_{323–339}$ peptide (n = 6 mice per group treated with OVA and n = 2 mice per group without treatment). Data are shown as mean ± SEM. *, P < 0.05; ***, P < 0.001 (two-sided Student's t test).

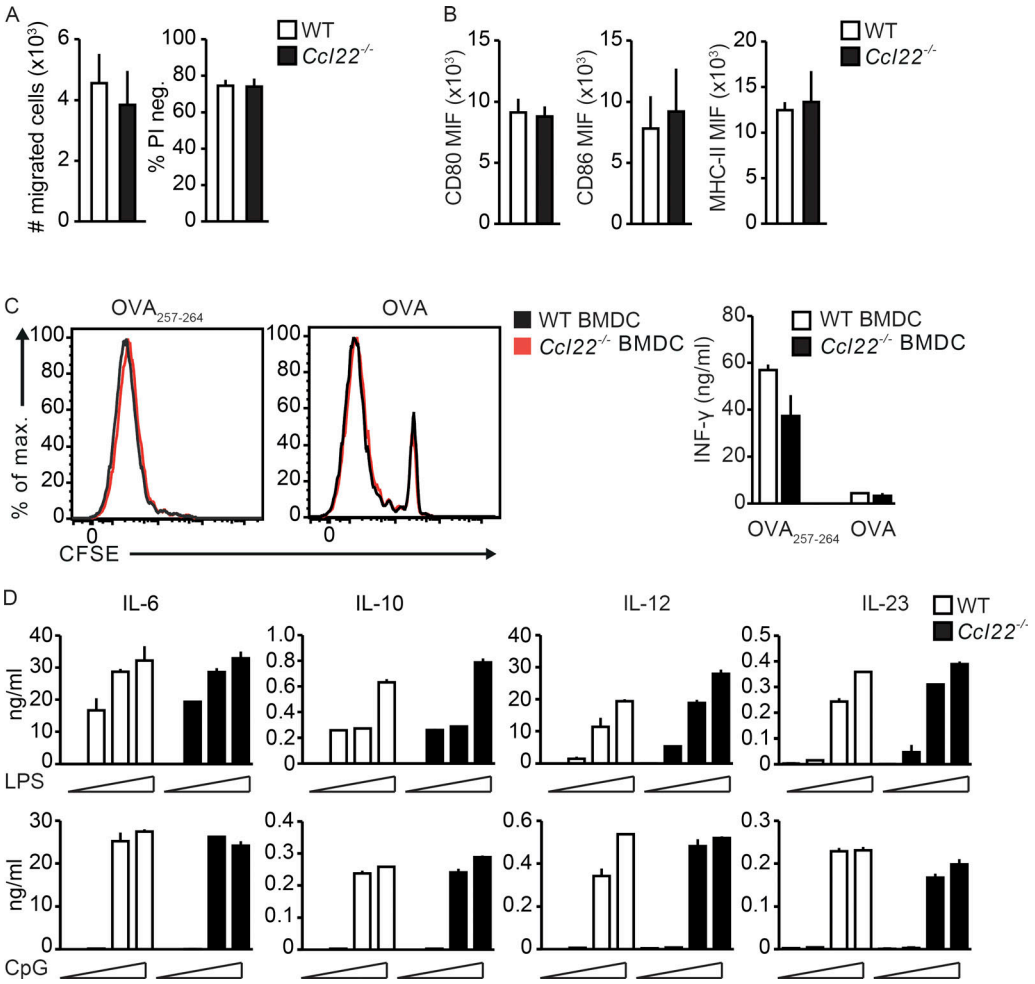


Figure S4. **WT and *Ccl22*$^{-/-}$ DCs do not differ in terms of in vivo migration, survival, costimulatory molecule expression, and in vitro cytokine production. (A)** C57BL/6 mice were subcutaneously injected with CpG-stimulated and eFlour450-stained WT or *Ccl22*$^{-/-}$ BMDCs, and the number and viability (measured by propidium iodide [PI] staining) of DCs recovered from the ipsilateral inguinal lymph node was quantified ex vivo by flow cytometry after 36 h. **(B)** The median intensity of fluorescence (MIF) of CD80, CD86, and MHC class II molecules on freshly generated BMDCs was analyzed by flow cytometry. **(C)** BMDCs were pulsed with $OVA_{257-264}$ peptide SIINFEKL or OVA and activated with CpG 1826 for 5 h, then CFSE-stained OT-1 splenocytes were added for 3 d. To analyze activation, proliferation, and cross-presentation, CFSE levels and IFN-γ in the supernatant were measured by flow cytometry and ELISA, respectively. **(D)** 4 × 10^6 WT or *Ccl22*$^{-/-}$ BMDCs were stimulated with increasing amounts of either LPS (0, 1, 10, and 100 ng/ml) or CpG (0, 0.05, 0.5, and 5 µg/ml) for 6 h. Protein levels of indicated cytokines were analyzed by ELISA in the collected supernatants. Data are shown as mean ± SEM and are representative of two independent experiments.

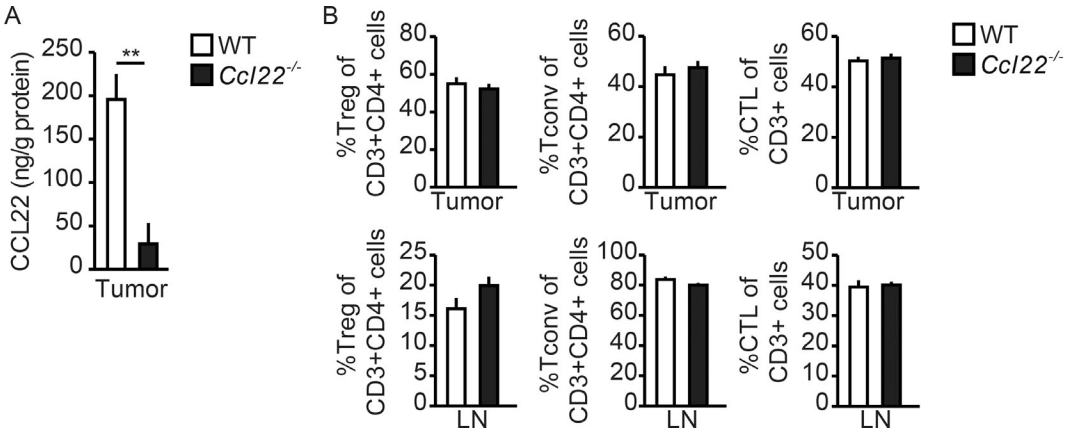
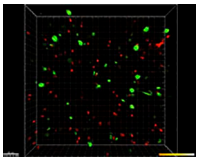
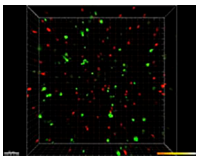


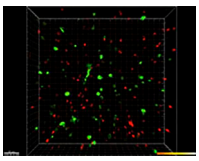
Figure S5. **CCL22 is strongly expressed in the tumor tissue of WT mice but not *Ccl22*$^{-/-}$ mice, and lymphocyte subpopulations in the tumor and lymph nodes are not altered in *Ccl22*$^{-/-}$ mice. (A and B)** *Ccl22*$^{-/-}$ and WT C57BL/6 mice were injected subcutaneously with Panc02-OVA tumors. 25 d after tumor inoculation, CCL22 protein concentration in tumor lysates was determined by ELISA (A), and percentages of $CD3^+CD4^+Foxp3^+$ (T reg), $CD3^+CD4^+Foxp3^{neg}$ (T conv), and $CD3^+CD8^+$ (CTL) in the tumor and in the tumor-draining lymph node were analyzed using flow cytometry (B). Data are shown as mean ± SEM and are representative of two independent experiments (n = 5 mice per group). **, $P < 0.01$ (two-sided Student's t test).



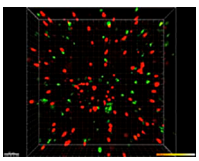
Video 1. **BMDCs (labeled in green with PKH67) were cocultured at 37°C with 5% CO_2 with T reg cells (labeled in red with PKH26) in a 3D collagen gel.**



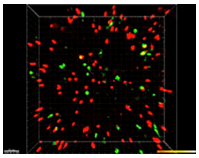
Video 2. **WT BMDCs (labeled in green with PKH67) were cocultured at 37°C with 5% CO_2 with T reg cells (labeled in red with PKH26) in a 3D collagen gel.**



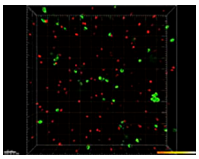
Video 3. ***Ccl22*$^{-/-}$BMDCs (labeled in green with PKH67) were cocultured at 37°C with 5% CO_2 with T reg cells (labeled in red with PKH26) in a 3D collagen gel.**



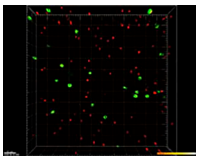
Video 4. **WT BMDCs (labeled in green with PKH67) were cocultured at 37°C with 5% CO_2 with T convs (labeled in red with PKH26) in a 3D collagen gel.**



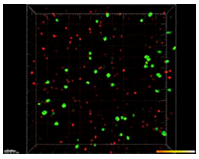
Video 5. ***Ccl22*$^{-/-}$BMDCs (labeled in green with PKH67) were cocultured at 37°C with 5% CO_2 with T convs (labeled in red with PKH26) in a 3D collagen gel.**



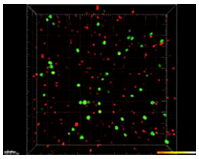
Video 6. **DC2.4 cells without doxycycline preincubation (i.e., without CCL22 expression; labeled in green with PKH67) were cocultured at 37°C with 5% CO_2 with T reg cells (labeled in red with PKH26) in a 3D collagen gel.**



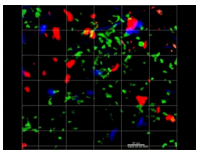
Video 7. **DC2.4 cells with doxycycline preincubation (i.e., with CCL22 expression; labeled in green with PKH67) were cocultured at 37°C with 5% CO_2 with T regs (labeled in red with PKH26) in a 3D collagen gel.**



Video 8. **DC2.4 cells without doxycycline preincubation (i.e., without CCL22 expression; labeled in green with PKH67) were cocultured at 37°C with 5% CO_2 with T convs (labeled in red with PKH26) in a 3D collagen gel.**



Video 9. **DC2.4 cells preincubated with doxycycline (i.e., with CCL22 expression; labeled in green with PKH67) were cocultured at 37°C with 5% CO_2 with T convs (labeled in red with PKH26) in a 3D collagen gel.**



Video 10. **Differently labeled $OVA_{323–339}$-pulsed BMDCs were pretreated with control or CCL22 siRNA 18 h before injection into the footpad of OT-II-Foxp3-GFP mice.** The footpad-draining lymph node was imaged by two-photon microscopy (blue, control DCs; red, CCL22-deficient DCs; green, $FoxP3^+$ cells).