SUPPLEMENTAL MATERIAL

for

Production of chemokines by T regulatory cells is required for therapeutic attenuation of autoimmunity and allograft rejection

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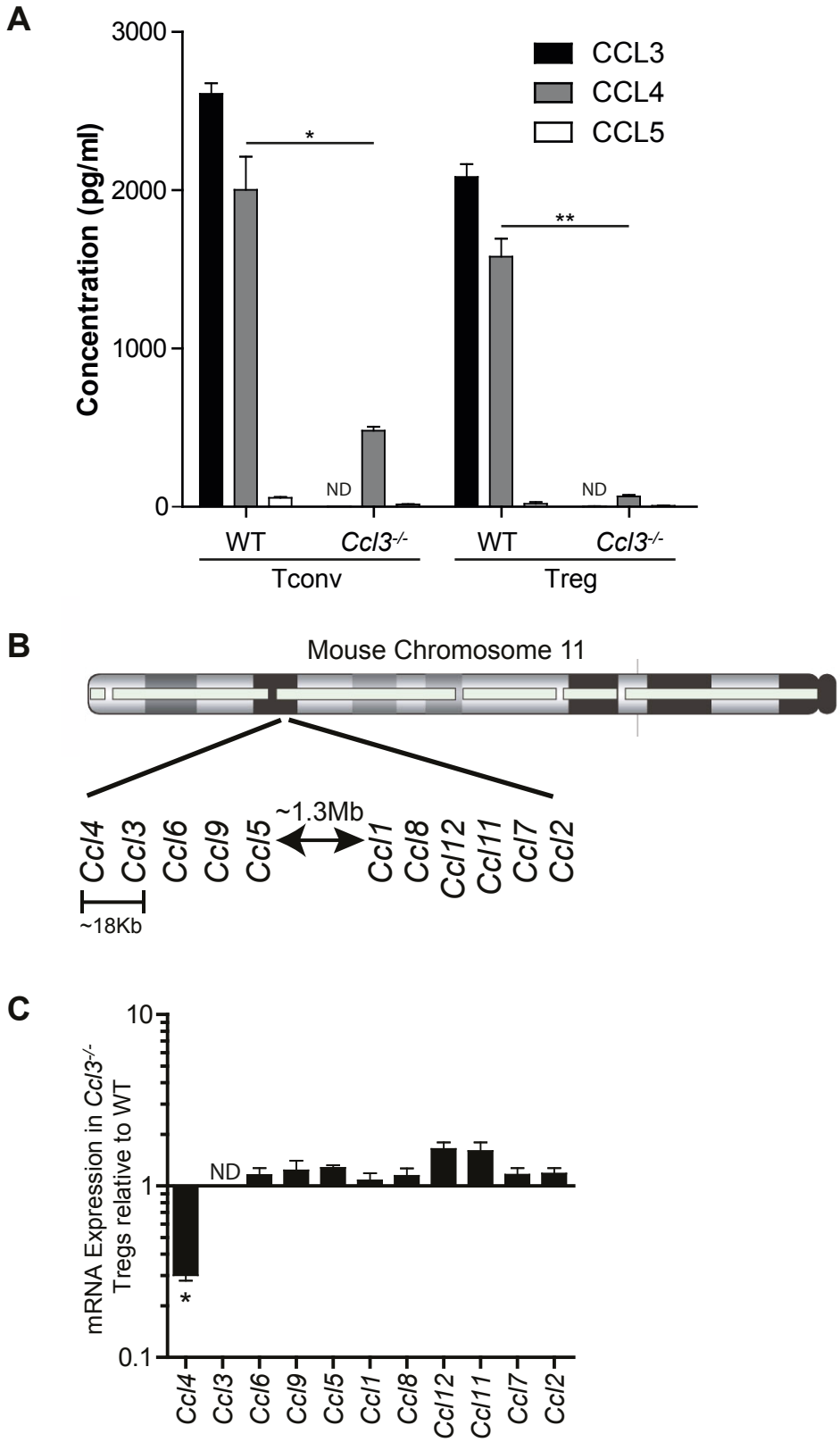
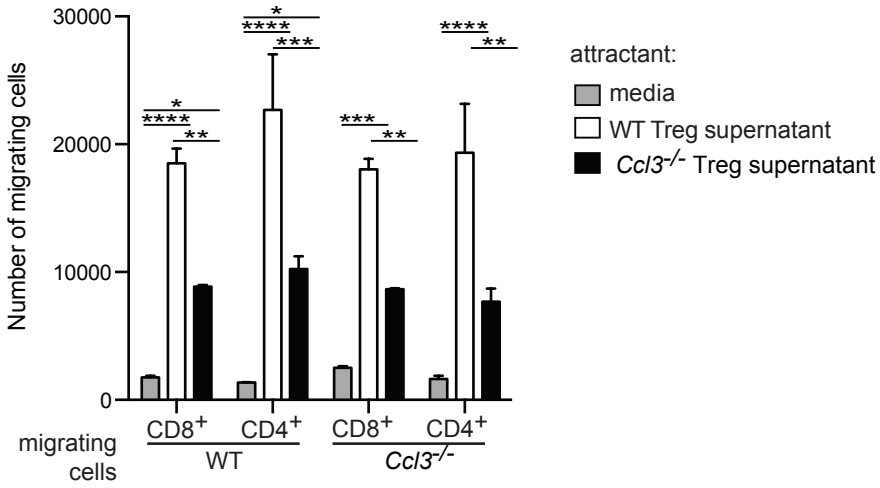
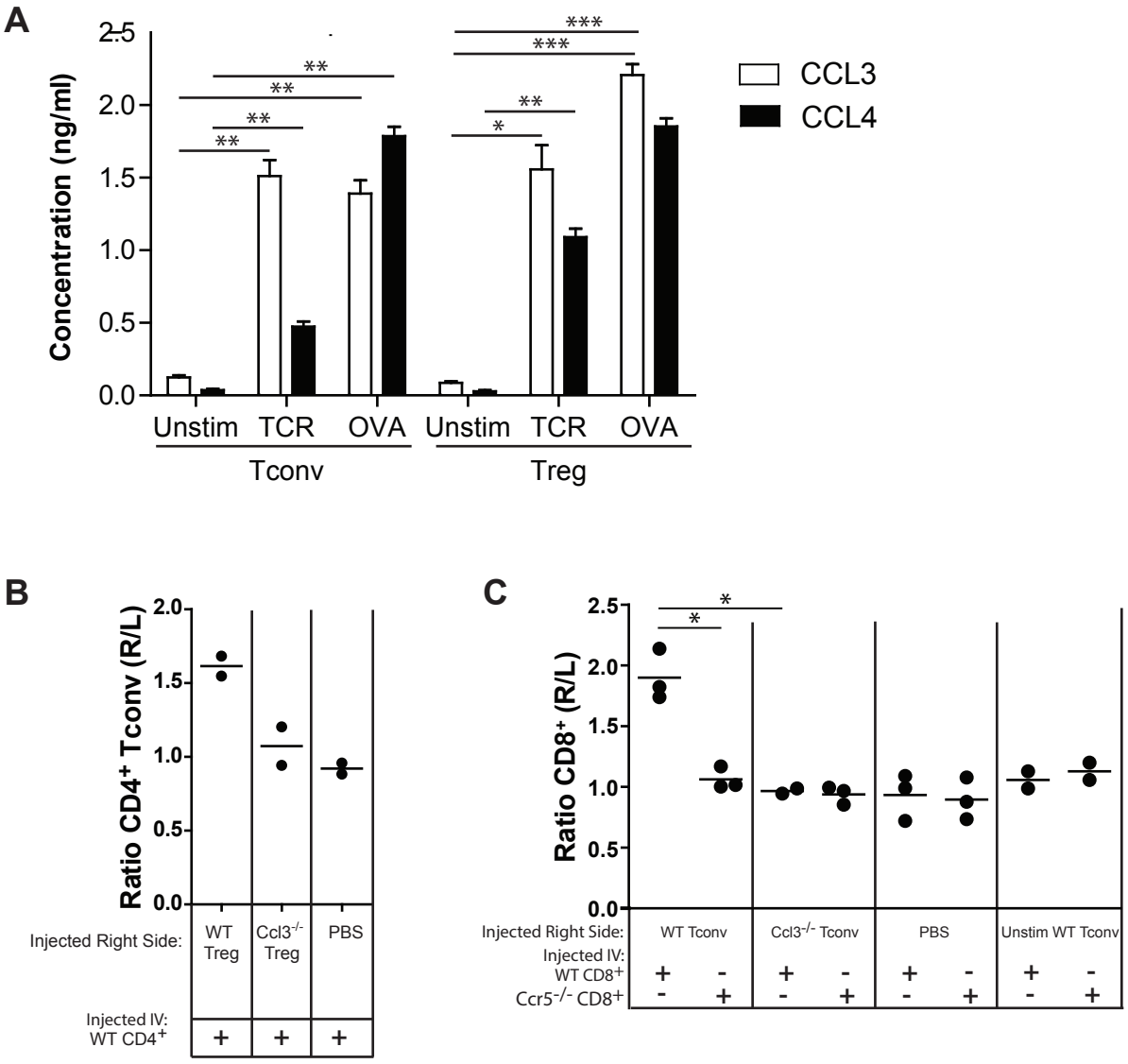


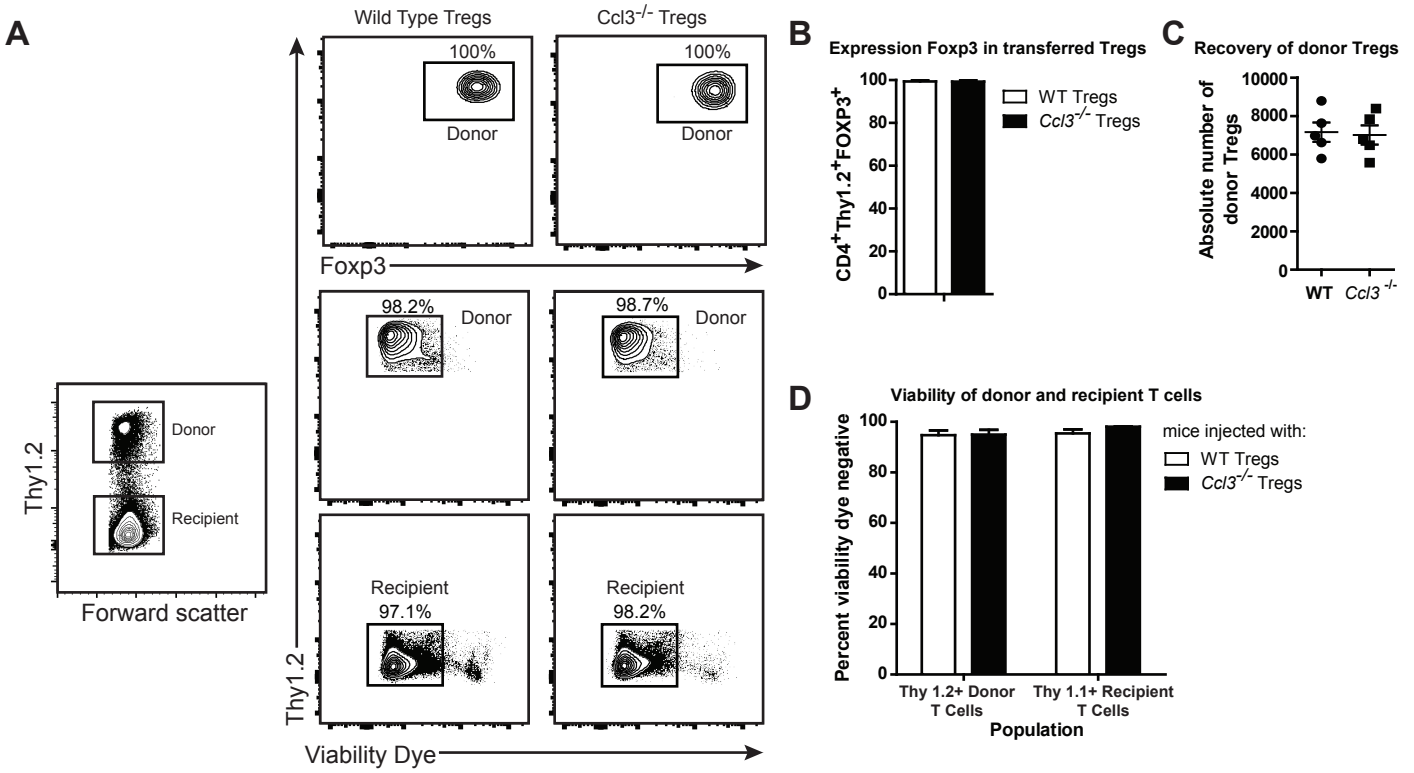
Supplementary Fig. S1: *Ccl3-/- mice are hypomorphic for CCL4.* (**A**) Tregs and Tconv cells were sorted from WT C57Bl/6 and *Ccl3-/-* mice, and stimulated for 48h with plate bound α-CD3, soluble α-CD28, and IL-2. Amounts of CCL3, CCL4 and CCL5 in supernatants were determined by Cytometric Bead Array (n=3) (*P < 0.05, **P < 0.01). (**B**) Depicts the genomic architecture of the CC chemokine cluster on mouse chromosome 11. (**C**) Cells isolated from (A) were analyzed for mRNA levels of the chemokines surrounding the *Ccl3* locus. Graph depicts the change in mRNA for the indicated chemokines in *Ccl3-/-* Tregs relative to WT Tregs (*P < 0.05; ND, not detectable).



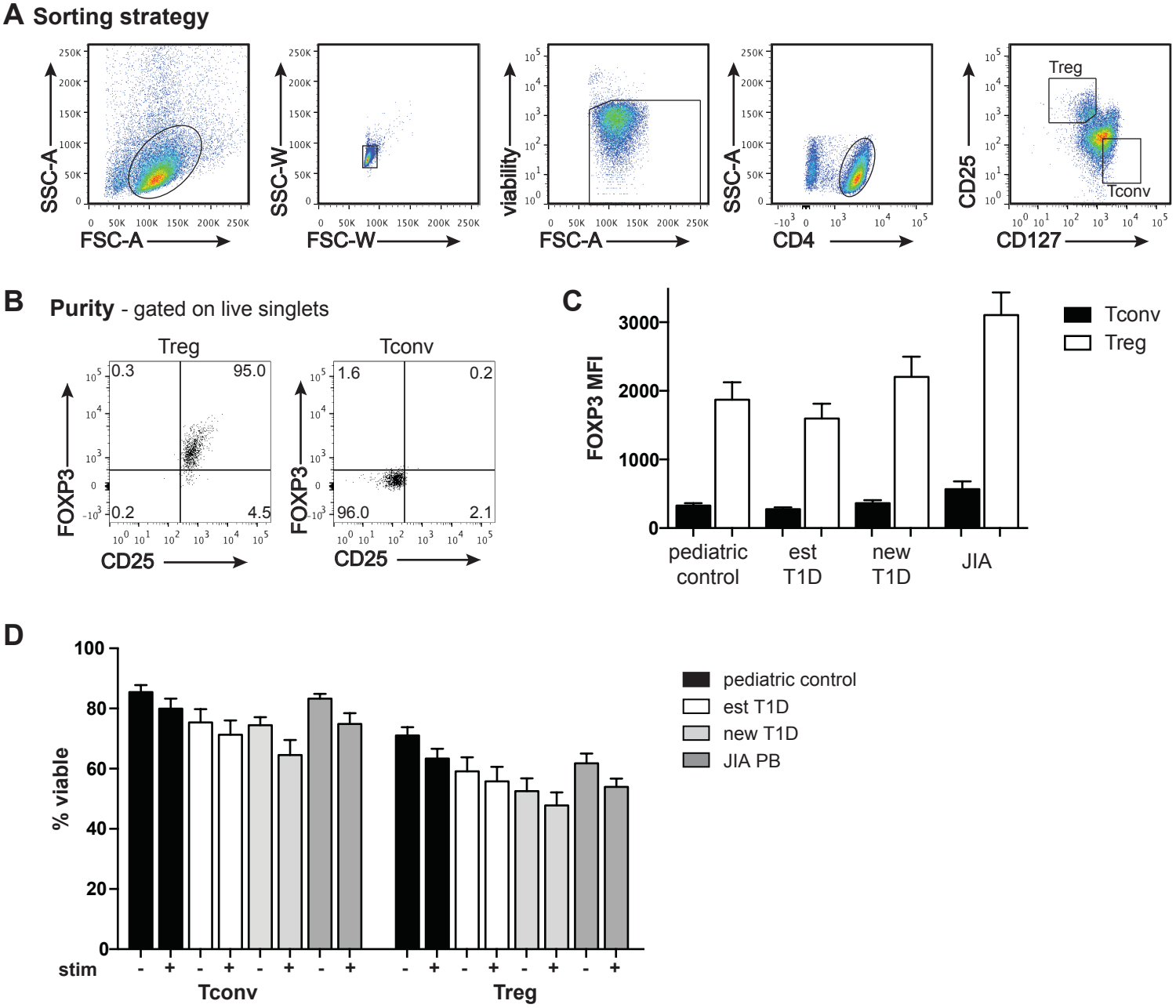
Supplementary Fig. S2: *WT Treg suppernatants attract CD8+ and CD4+ T cells regardles of the capacity of migrating cells to produce chemokine.* Tregs and CD4+ or CD8+ T cells were sorted from WT or *Ccl3-/-* mice.Supernatants from TCR-stimulated Tregs were placed in the bottom of a transwell chamber and 300'000 stimulated CD4+ or CD8+ T cells from WT or Ccl3-/- mice were placed on top. After 3 hours the number of migrated cells in the bottom chamber were counted by flow cytometry. Data are the average ± SEM of technical duplicates, two-way ANOVA with Bonferroni's multiple comparisons test (*P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.0001), no statistical difference was detected between migrating cells (WT or *Ccl3-/-*) with the same attractant condition.



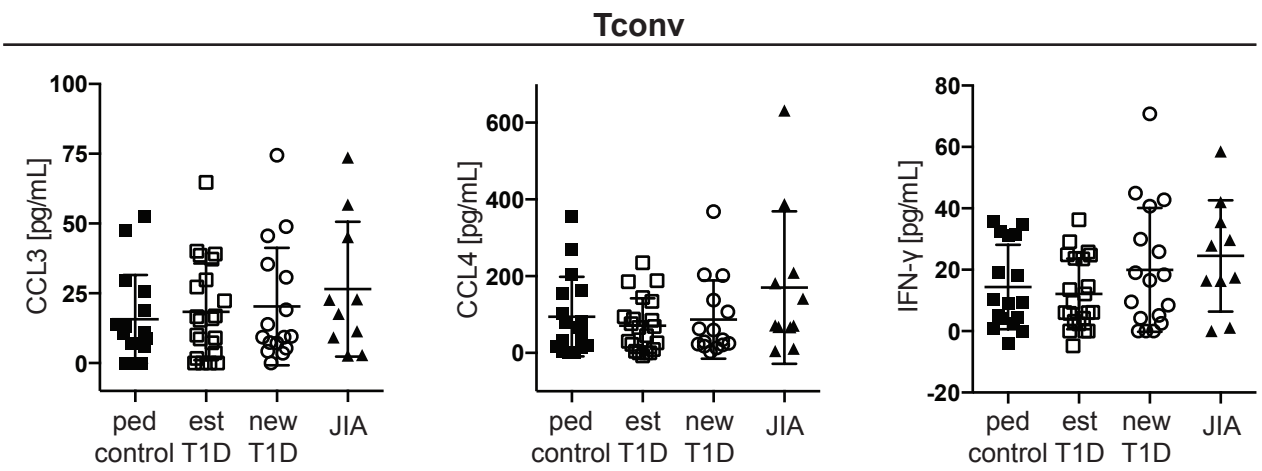
Supplementary Fig. S3: *Ag-induced chemokine production in OTII Tregs and Tconv cells and in vivo migration of CD4+ or CD8+ Tconv cells*. (**A**) OTII Tregs and Tconv cells were stimulated for 48h in the presence or absence of simulation with α-CD3/28 (TCR), or with irradiated BM-DCs that had been pulsed with $OVA_{323-339}$ 12 h prior to T cell stimulation. Amounts of CCL3 and CCL4 in supernatants were determined by Cytometric Bead Array, data shown are the average and SEM (n = 3) (*P < 0.05, **P < 0.01, ***P < 0.005). (**B**-**C**) Mice were injected subcutaneously in the dorsum of the right foot with $1x10^6$ WT or *Ccl3*$^{-/-}$ Tregs (B) or Tconvs (C) similar as described in Figure 5C. (**B**) WT CD4$^+$ T cells were injected intravenously, and after 40 h the ratio of WT CD4$^+$ T cells in the right and left draining popliteal lymph nodes was determined (n = 2, one experiment). (**C**) WT or *Ccr5*$^{-/-}$ CD8$^+$ T cells were injected intravenously, and after 40 h the ratio of WT or *Ccr5*$^{-/-}$ CD8$^+$ T cells in the right and left draining popliteal lymph nodes was determined (n = 2-3, 2 experiments). Each dot represents the R/L ratio from an individual mouse (*P < 0.05).



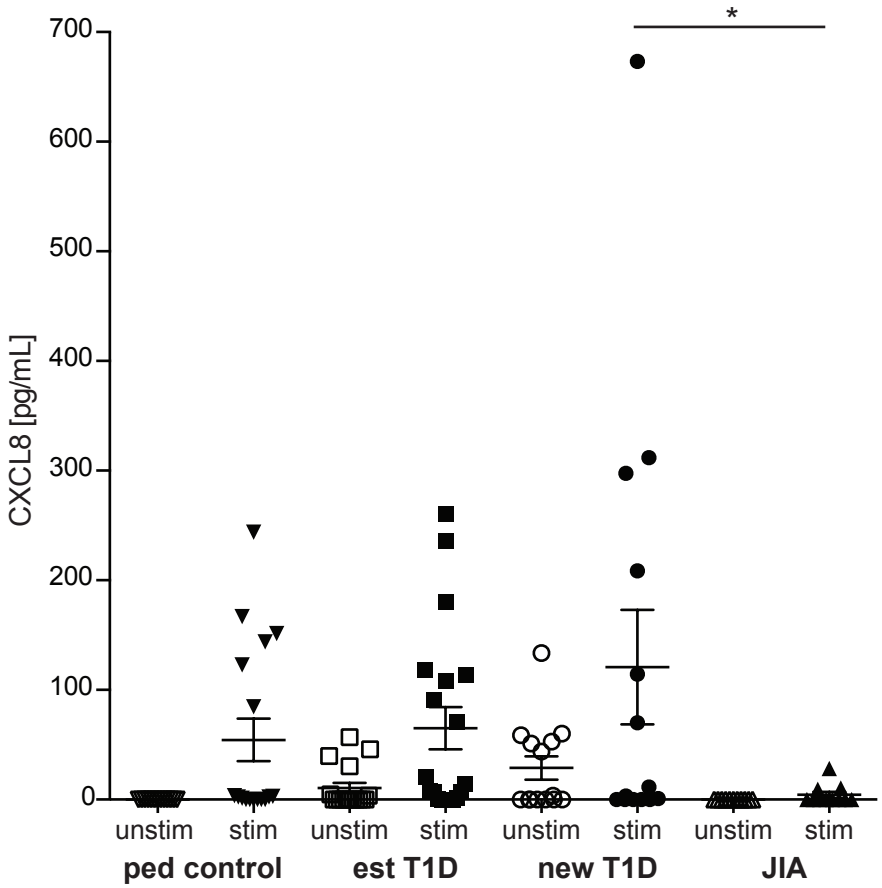
Supplemental Fig. S4: *Ability to produce CCL3 does not affect Treg viability in vivo.* 3-4x10^6 WT or *Ccl3*$^{-/-}$ Thy1.2$^+$ Foxp3^{eGFP} Tregs were injected subcutaneously into the dorsal footpad of Thy1.1$^+$ WT mice. 40hrs post-injection cells were isolated from the draining lymph node and stained with antibodies for CD4, Thy1.2 and viability dye. Foxp3^{eGFP} expression was determined in CD4$^+$Thy1.2$^+$ cells. Shown are representative FACS plots (A), summary graphs for donor cell FOXP3 frequency (B), recovery of donor Tregs in absolute numbers (C) and viability (D), graphs represent the mean and SEM of 3 independent experiments, with C also showing the cell recovery in individual mice. The absolute number of adoptively transferred Tregs reported in (C) compares to an average of 32,428 (6,922 - 66,747, n=4) endogenous Tregs in a single draining lymph node.



Supplementary Fig. S5: *Sorting strategy and purity for human Tregs and Tconv cells.* (**A**) Human Tregs were sorted as live, single, $CD4^{+}CD25^{hi}CD127^{lo}$ cells and Tconv cells as live, single $CD4^{+}CD25^{-}CD127^{hi}$ cells from PBMCs. (**B**) Post-sort purity was assessed by FOXP3 staining. (**C**) Depicts the average mean fluorescence intensity of FOXP3 expression immediately post sorting in Tregs and Tconv cells, shown is the mean and SEM. (**D**) After 48h of culture with or without stimulation with α-CD3/28 and IL-2, supernatants were collected and the viability of the remaining cells was assessed by flow cytometry. Shown is the mean and SEM for unstimulated and stimulated cells. Pediatric controls n=17-18, established T1D n=22, new onset T1D n=17, and JIA n=13).



Supplementary Fig. S6: *Tconv cells from T1D or JIA patients and controls have equal production of CCL3 and CCL4.* FACS-sorted Tconv cells from pediatric controls (n=16-17), established T1D (n=22), new onset T1D (n=16-17) and JIA (n=10) patients were activated with α-CD3/CD28-coated beads in the presence of IL-2 for 48 h at a cell concentration of $1.4x10^5$/mL, supernatants were collected and CCL3, CCL4 and IFN-γ was assessed by CBA assay, and background measurements of the respective unstimulated sample were subtracted and outliers were excluded by the ROUT method (Q=1%).



Supplementary Fig. S7: *Tregs from T1D patients produce CXCL8 at levels similar to controls.* FACS-sorted peripheral blood Tregs from pediatric controls , established T1D, new onset T1D and JIA patients were activated with α-CD3/α-CD28-coated beads in the presence of IL-2 for 48 h at a cell concentration of 1.4 x 105/mL. Supernatants were collected and CXCL8 was assessed by CBA assay, outliers were excluded by the ROUT method (Q=1%) (pediatric controls (n=13-17), established T1D (n=17-19), new onset T1D (n=14) and JIA (n=11); p values: *<0.05 by One-way ANNOVA with Bonferroni post-hoc test).