SUPPLEMENTAL DATA

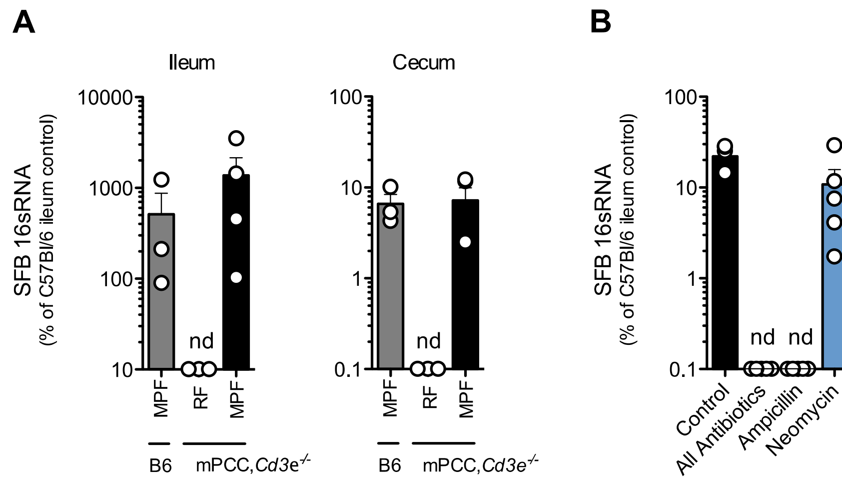

Figure S1 (related to Figure 1**). The MPF commensal flora contains ampicillin-sensitive SFB.**

(A) Amount of SFB 16sRNA in ileum (left) or cecum (right) contents from 6 week old RF- and MPF-housed mPCC,*Cd3e*$^{-/-}$ mice or MPF-housed C57Bl/6 mice (B6) (n=3-4 mice per group, mean±SEM, nd: not detected).

(B) Various antibiotic formulations were given in the drinking water of MPF-housed mPCC,*Cd3e*$^{-/-}$ mice starting 3 weeks prior to naïve 5C.C7 T cell transfer and maintained for the length of the experiment. 9.5 weeks post T cell transfer, cecum contents were harvested and the amount of SFB 16sRNA was quantified. "*All antibiotics*" formulation contains ampicillin, neomycin, vancomycin and metronidazole. (n=5 mice per group, mean±SEM, nd: not detected).

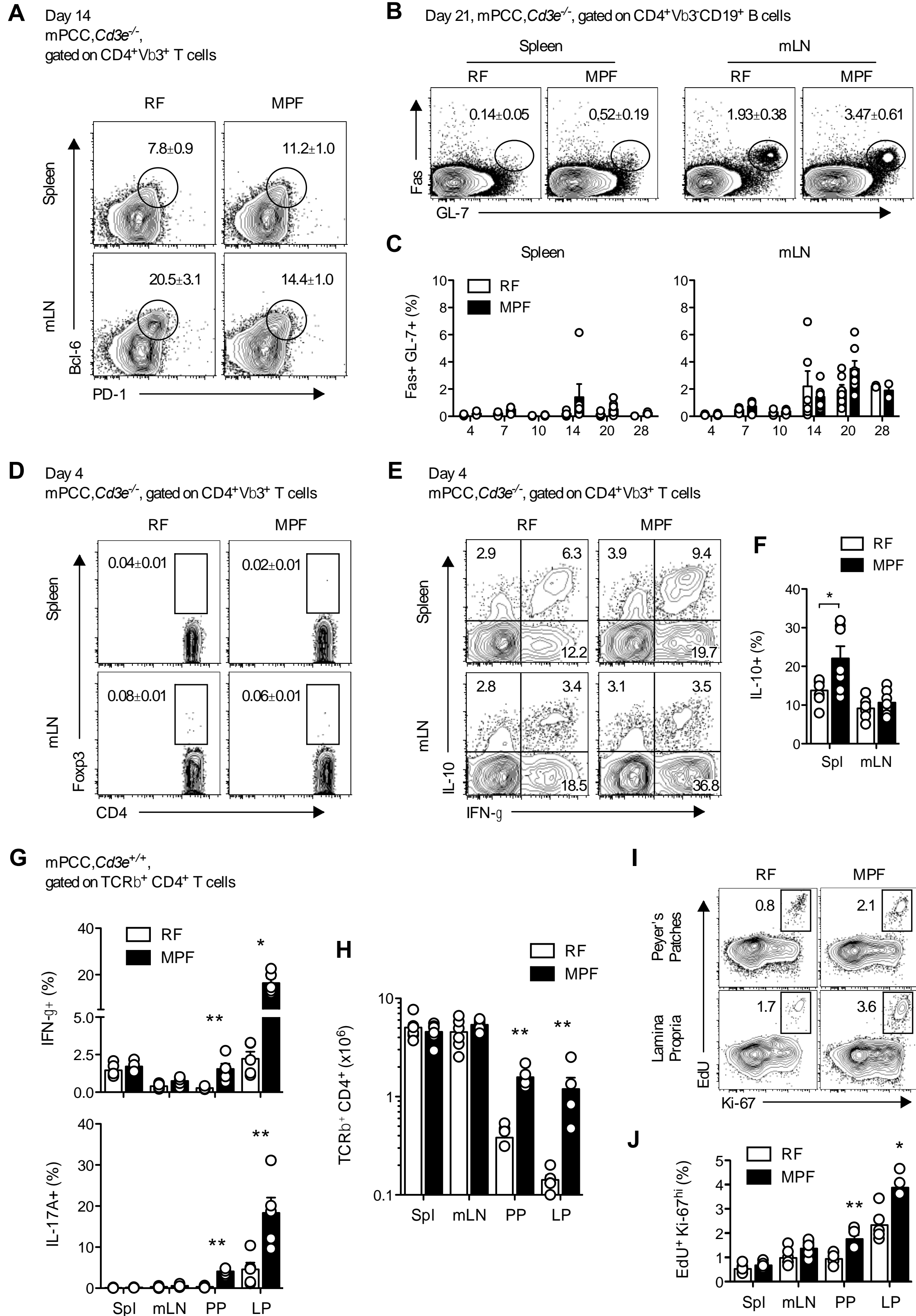

Figure S2 (related to Figure 2).

Figure S2 (related to Figure 2)**. The MPF commensal flora does not specifically oppose classical regulatory T cell or promote Tfh/GC B cell responses, but drives the full maturation of gut-resident polyclonal helper T cell responses.**

(A) Representative PD-1 and Bcl-6 expression profiles gated on live $CD4^{+}V\beta 3^{+}$ T cells in indicated organs of RF- or MPF-housed mPCC,*Cd3e*$^{-/-}$ hosts 14 days post naïve 5C.C7 T cell transfer. Numbers represent the average frequencies ± SEM observed for 3 mice.

(B) Representative Fas and GL-7 expression profiles gated on live $CD4^{-}V\beta 3^{-}CD19^{+}$ B cells in spleen and mLN of RF- or MPF-housed mPCC,*Cd3e*$^{-/-}$ hosts 21 days post T cell transfer. Data represent 7 mice per group pooled from 4 independent experiments. Numbers represent the average frequencies ± SEM observed for 7 mice. (C) Frequency of $Fas^{+}GL\text{-}7^{+}$ cells in live, $CD4^{-}V\beta 3^{-}CD19^{+}$ B cells at indicated time points in spleen and mLN of RF- or MPF-housed mPCC,*Cd3e*$^{-/-}$ hosts. Data (mean±SEM) pooled from 4 independent experiments (n=2-7 mice per group and time points).

(D) Representative Foxp3 expression profiles gated on live $CD4^{+}V\beta 3^{+}$ T cells in spleen and mLN of RF- or MPF-housed mPCC,*Cd3e*$^{-/-}$ hosts 4 days post T cell transfer. Data (mean±SEM) pooled from 3 independent experiments (n=7 mice per group).

(E-F) 4 days post transfer of naïve 5C.C7 T cells into RF- or MPF-housed mPCC,*Cd3e*$^{-/-}$ hosts, the cells were stained for intracellular IL-10 and IFN-γ following a 3h stimulation with PMA and ionomycin. (E) Representative IL-10 and IFN-γ expression profiles and (F) frequency of $IL\text{-}10^{+}$ cells in live, $CD4^{+}V\beta 3^{+}$ T cells in spleen and mLN. Data (mean±SEM) pooled from 3 independent experiments (n=7 mice per group).

(G-J) 5 wk. old RF- or MPF-housed mPCC,*Cd3e*$^{+/+}$ mice were injected with EdU and sacrificed 1 hr. later. (G) Frequency of $IL\text{-}17A^{+}$ and $IFN\text{-}\gamma^{+}$ cells in live, $TCR\beta^{+}CD4^{+}$ T cells isolated from the indicated organs and restimulated for 5.5h with PMA and ionomycin. Data (mean±SEM) pooled from 5 independent experiments (n=5 mice per organ). (H) Absolute number of live, $TCR\beta^{+}CD4^{+}$ T cells recovered from indicated organs. (I) Representative EdU and Ki-67 expression profiles and (J) frequency of $EdU^{+}Ki\text{-}67^{hi}$ cells in live, $TCR\beta^{+}CD4^{+}$ T cells isolated from indicated organs. Data (mean±SEM) pooled from 6 independent experiments (n=5-6 mice per organ).

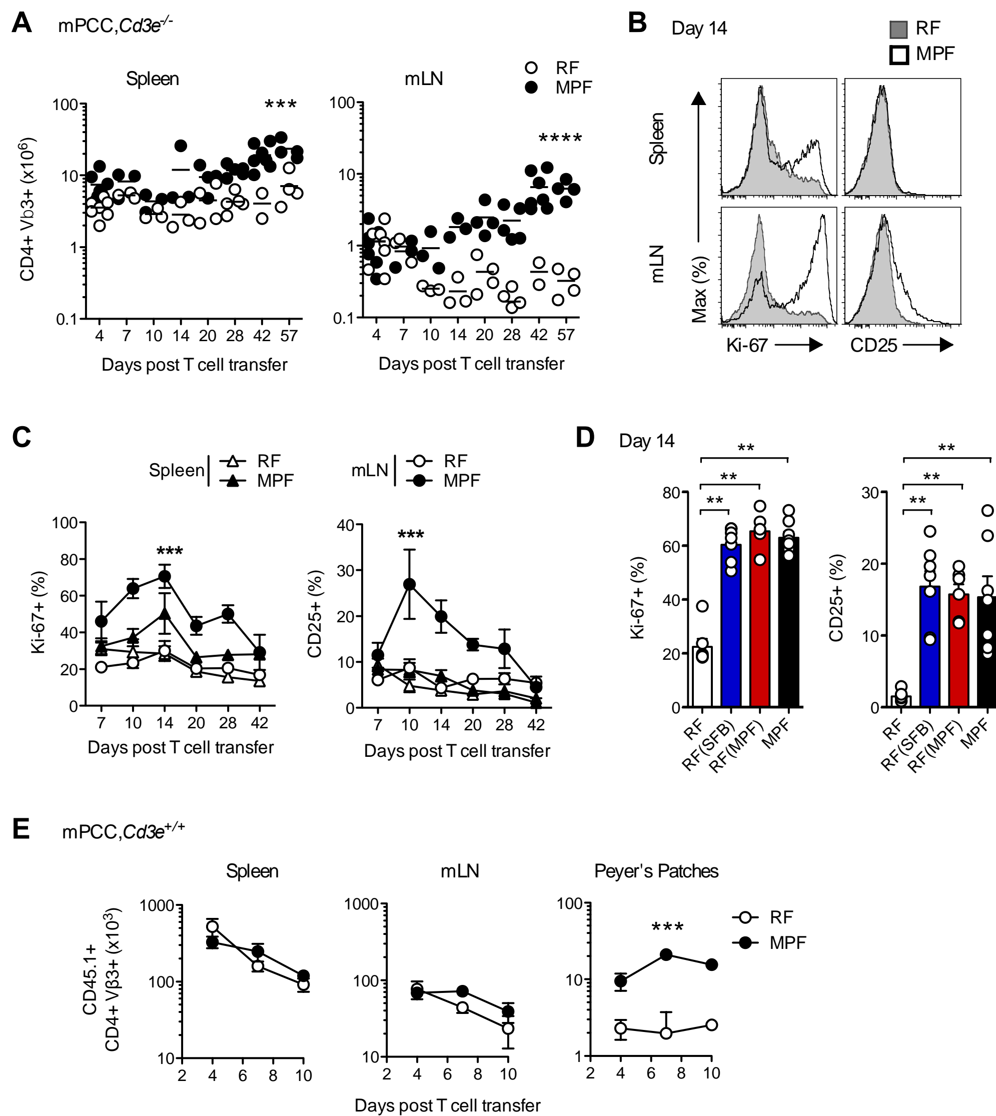

Figure S3 (related to Figure 3**). Full kinetic of the ongoing T cell response in T cell-deficient and T cell-replete hosts.**

(A) Absolute number of donor $CD4^{+}V\beta3^{+}$ T cells in spleen and mLN of RF- or MPF-housed hosts at indicated time points post naïve 5C.C7 T cell transfer. Individual values and average per time point are displayed. Data pooled from 5 independent experiments (n=2-7 mice per time point).

(B) Representative CD25 and Ki-67 expression profile gated on live, $CD4^{+}V\beta3^{+}$ T cells isolated from spleen or mLN of indicated mPCC,*Cd3e*$^{-/-}$ host 14 days post T cell transfer.

(C) Frequencies of Ki-67^{+} and CD25^{+} cells in live, CD4^{+}Vβ3^{+} T cells isolated from spleen or mLN of RF- or MPF-housed mPCC,*Cd3e*$^{-/-}$ hosts at indicated time points post T cell transfer. Data (mean±SEM) pooled from 5 independent experiments (n=2-7 mice per time point).

(D) Frequencies of Ki-67^{+} and CD25^{+} cells in live, CD4^{+}Vβ3^{+} T cells isolated from mLN of the indicated mPCC,*Cd3e*$^{-/-}$ host 14 days post T cell transfer. Data (mean±SEM) pooled from 3 independent experiments (n=6-7 mice per group).

(E) Absolute number of donor CD45.1^{+}CD4^{+}Vβ3^{+} T cells in spleen, mLN and Peyer's patches of RF- or MPF-housed mPCC,*Cd3e*$^{+/+}$ hosts at indicated time points post naïve CD45.1^{+} 5C.C7 T cell transfer. Data (mean±SEM) pooled from 2 independent experiments (n=2-3 mice per time point).

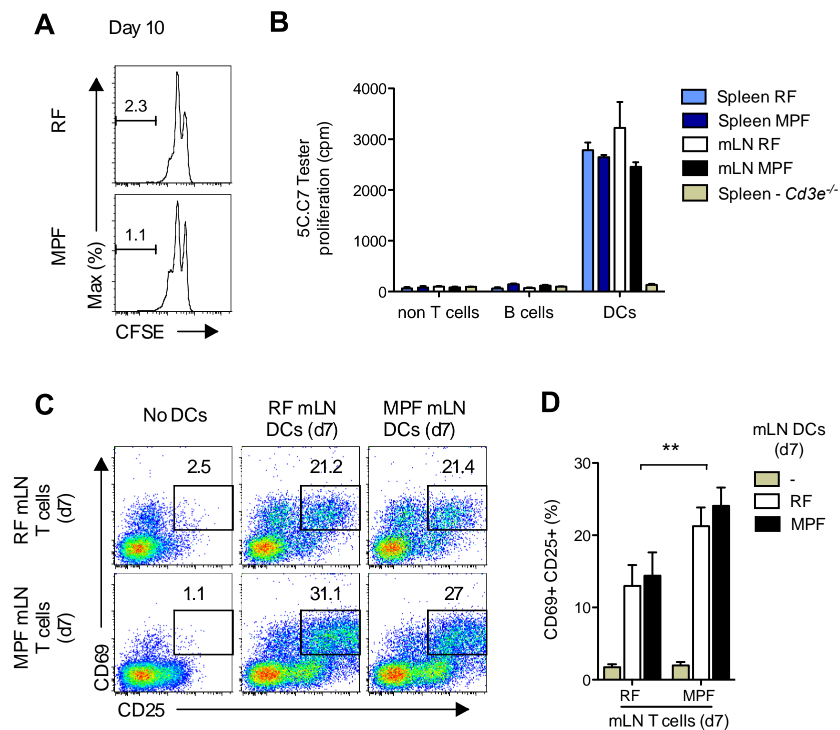

Figure S4, (related to Figure 4)**. The MPF commensal flora does not have a detectable impact on self-antigen presentation *in vivo*.**

(A) 10 days post transfer of naïve carboxyfluorescein succinimidyl ester (CFSE)-labeled 5C.C7 T cells into RF- or MPF-housed *Cd3e*$^{-/-}$ mice, the hosts mLN were harvested. Histograms represent CFSE dilution in gated live, CD4^{+}Vβ3^{+} T cells.

(B-D) 7 days post transfer of naïve 5C.C7 T cells into RF- or MPF-housed mPCC,*Cd3e*$^{-/-}$ mice, the hosts spleen and mLN were harvested and dissociated using collagenase D. Transferred 5C.C7 T cells when then directly purified, as described in methods for T cell purification. Alternatively, cell suspensions were further enriched for Thy1.2 (30-H12)-depleted (non-T), Thy1.2/NK1.1/CD11b/CD11c-depleted (B cells) cell populations, using two rounds of negative selection with dynabeads (Dynal) and the indicated antibodies, or CD11c^{+} dendritic cells (DC) using anti-mouse CD11c microbeads according to the manufacturer's instructions (Miltenyi Biotec; purity ≥95% CD11c^{+} cells).

(B) Isolated antigen-presenting cell populations were further irradiated (3000 rads) and cultured for 68h at a 1:2 ratio with 5C.C7 Tester cells (*in vitro* 3 day pre-activated and rested 5C.C7,*Rag2*$^{-/-}$ T cells), as previously described (Singh and Schwartz, 2003). 3H-Thymidine was added to the culture for the last 24h. 3H-Thymidine incorporation from triplicate wells is displayed (mean±SEM).

(C-D) 3 x 10^4 purified $CD4^+V\beta3^+$ T cells (mLN T cells) were cultured for 16h with 7.5 x 10^3 CD11c-enriched dendritic cells (mLN DCs) from the indicated hosts (4:1 ratio). (C) Representative CD69 and CD25 expression profile and (D) frequency of $CD69^+CD25^+$T cells at 16h from pooled triplicates. Data (mean±SEM) pooled from 5 independent experiments. ** $P<0.01$ in a 2 way ANOVA.

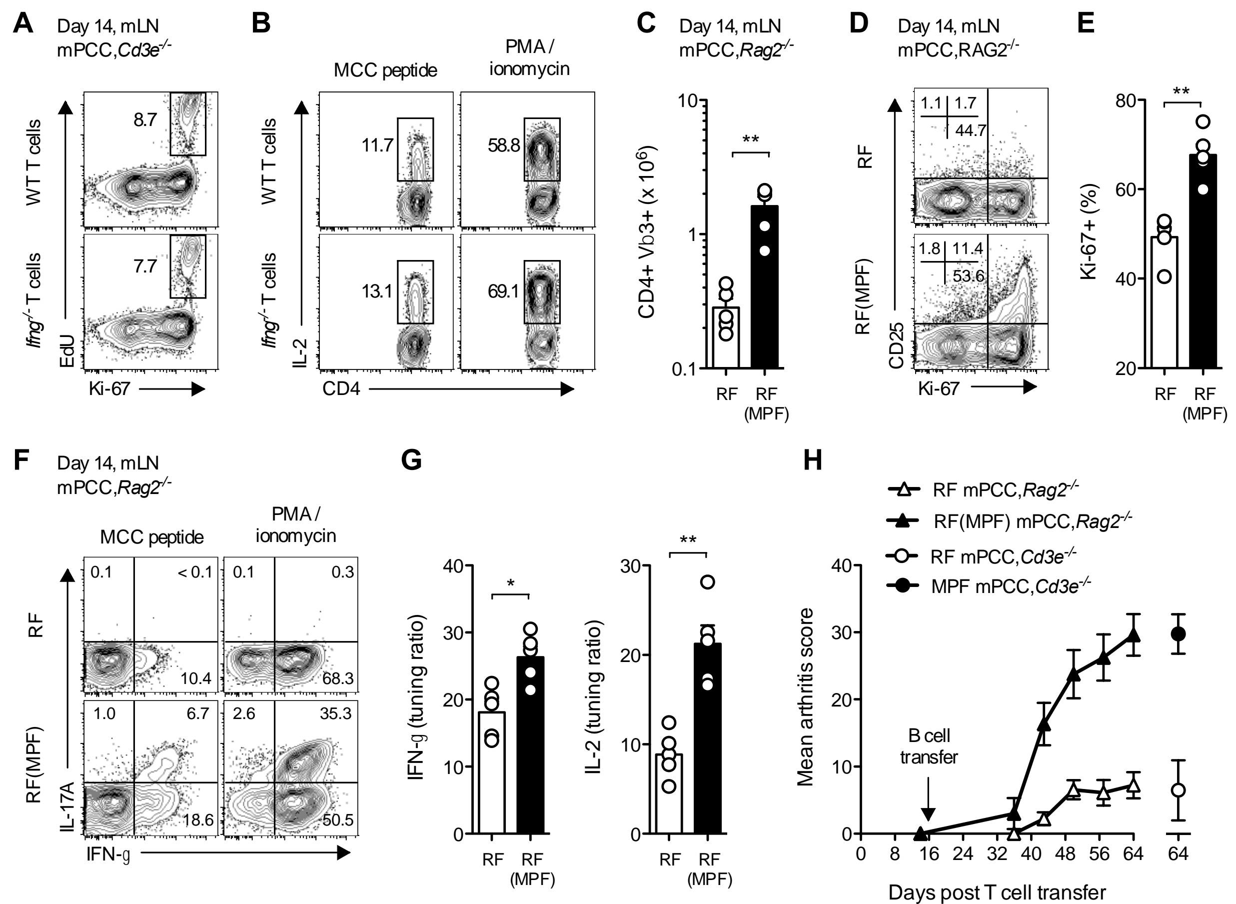

Figure S5 (related to Figure 6)**. The impact of the MPF commensal flora on T cell tuning is independent of T cell-derived IFN-γ or the development of overt autoimmune pathologies.**

(A) Representative EdU and Ki-67 expression profiles and (B) representative IL-2 expression profile, following a 3h restimulation with PMA and ionomycin or 3μM MCC peptide, in live, $CD4^{+}V\beta3^{+}$ T cells isolated from mLN of mPCC,*Cd3e*$^{-/-}$ hosts 14 days post naïve WT or *Ifng*$^{-/-}$ 5C.C7 T cell transfer.

(C) Absolute number of live, $CD4^{+}V\beta3^{+}$ T cells, (D) representative CD25 and Ki-67 expression profiles and (E) frequency of Ki-67^{+} cells in live, $CD4^{+}V\beta3^{+}$ T cells recovered from mLN of mPCC,*Rag2*$^{-/-}$ hosts 14 days post naïve 5C.C7 T cell transfer. Cohoused-RF(MPF) mPCC,*Rag2*$^{-/-}$ mice were cohoused for 3 weeks with MPF-housed

mPCC,*Cd3e*$^{-/-}$ mice prior to T cell transfer. Data (mean±SEM) pooled from 2 independent experiments (n=5 mice per group).

(F-G) 14 days post transfer of naïve 5C.C7 T cell into mPCC,*Rag2*$^{-/-}$ hosts, the cells isolated from mLN were restimulated for 3h with PMA and ionomycin or 3μM MCC peptide. (F) Representative expression profiles for IFN-γ^{+} and IL17A^{+} and (G) tuning ratio, as described in experimental procedures, for IFN-γ and IL-2 in gated live, CD4^{+}Vβ3^{+} T cells. Data (mean±SEM) pooled from 2 independent experiments (n=5 mice per group).

(H) 14 days post naïve 5C.C7 T cell transfer, RF-housed or cohoused-RF(MPF) mPCC,*Rag2*$^{-/-}$ mice were further injected with 25 x 10^{6} naive polyclonal B cells purified from RF-housed mPCC,*Cd3e*$^{-/-}$ mice using a mouse B cell isolation Kit according to the manufacturer's instructions (Miltenyi Biotec; purity ≥95% B220^{+} cells). Bi-weekly arthritis scores for mPCC,*Rag2*$^{-/-}$ hosts and day 64 arthritis scores for control RF- or MPF-housed mPCC,*Cd3e*$^{-/-}$ hosts, injected at day 0 with naive 5C.C7 T cells, are displayed. Data (mean±SEM) pooled from two independent experiments (n=9 mice per group for RAG2$^{-/-}$ hosts and 4-5 mice per group for CD3ε$^{-/-}$ hosts).

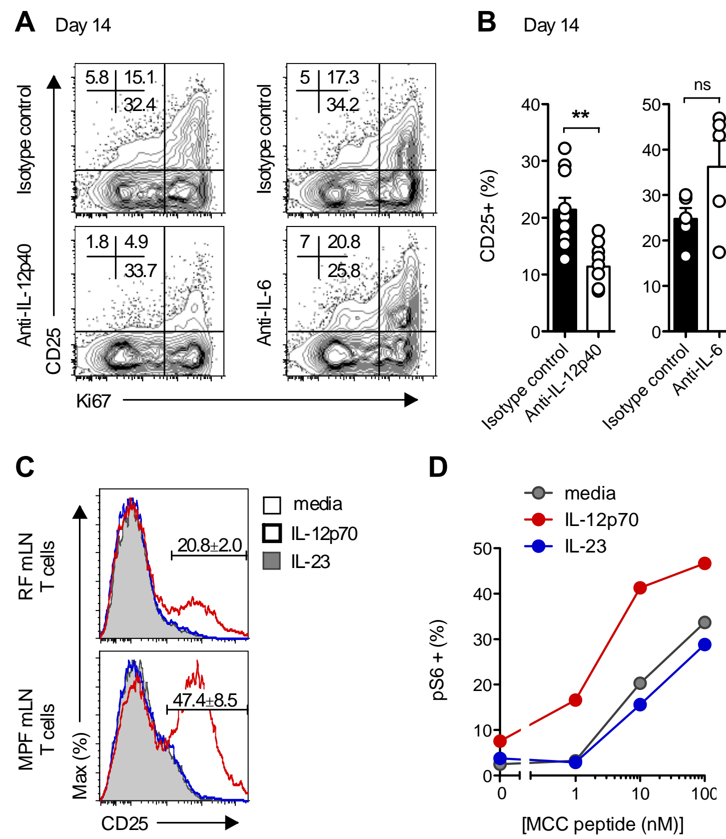

Figure S6 (related to Figure 7)**. IL12p70, but not IL-6 or IL-23 directly regulates CD25 and mTOR activation in tuned T cells.**

(A-B) Starting at day 7 post naïve 5C.C7 T cell transfer, MPF-housed mPCC,*Cd3e*$^{-/-}$ hosts were further injected twice daily i.p. with anti-IL12p40, anti-IL6 or corresponding isotype control mAb. *In vivo* neutralization of IL-6 was achieved by injecting 0.5mg of mAb MP5-20F3 at day 7 and 0.25mg every other day until day 14 and compared to control Rat IgG1 (HRPN) (all BioXCell). (A) Representative CD25 and Ki-67 expression profiles and (B) frequency of CD25^{+} cells in live, CD4^{+}Vβ3^{+} T cells isolated from mLN at day 14. Data (mean±SEM) pooled from 4 or 3 independent experiments respectively (n=9-10 mice per group for anti-IL12p40 and 5 mice per group for anti-IL-6).

(C-D) 7 days post naïve 5C.C7 T cell transfer, CD4^{+}Vβ3^{+} T cells were purified from mLN of RF- or MPF-housed mPCC,*Cd3e*$^{-/-}$hosts and cultured with the indicated cytokines (10ng/ml) for 16h alone (C) or 40h with fresh CD3ε$^{-/-}$ splenocytes and MCC

peptide (D). (C) Representative CD25 expression profiles in live, $CD4^{+}V\beta3^{+}$ T cells in 1 out of 4 independent experiments. Frequencies of $CD25^{+}$ T cells following IL-12p70 and IL-23 stimulation are displayed as mean ± SEM from all 4 experiments. (D) Frequency of $pS6^{+}$ cells in live, $CD4^{+}V\beta3^{+}$ T cells isolated from MPF-mLNs. 1 out of 3 independent experiments is displayed.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Autoantibody titrations.

Autoantibody titers were determined by staining B10.A *Rag2*$^{-/-}$ target cells consisting of pooled lymph nodes, spleen and bone marrow cell suspensions fixed in 2% paraformaldehyde (PFA) (Sigma-Aldrich) and further permeabilized overnight in 90% methanol at -20 °C. Targets were blocked with 100µg/ml donkey Fab_2 (Jackson ImmunoResearch) and further incubated with serial dilution of each individual mouse serum in staining buffer (see below). Targets were then washed and incubated with FITC-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch).

SFB Quantification and monoassociation of GF mice.

Genomic DNA was prepared from ileum or cecum contents using the QiaAmp DNA Stool Mini kit according to the manufacturer's instructions (Qiagen). Quantitative PCR analysis was carried out using the QuantiFast™ SYBR® Green PCR kit (Qiagen) and the primer sequences for SFB and bacterial 16S rDNA described by Barman et al. (Barman et al., 2008). The amount of SFB 16s rDNA is presented as a relative percentage compared to an external control sample consisting of pooled genomic DNA from ileum contents of MPF-housed C57BL/6 mice. Samples less than 3 Ct away from the H_20 control were considered "not detected" (nd).

For association of germ-free mice with SFB (a kind gift from Dr. Yoshinori Umesaki (Umesaki, Okada, Matsumoto, Imaoka, & Setoyama, 1995)), fecal pellets isolated from SFB mono-associated mice were reconstituted in sterile PBS 1x and 200 µl of this suspension was administered to each germ-free mouse by gavage in a sterile isolator.

SFB reconstitution was confirmed by qPCR of fecal 16S rDNA relative to negative GF controls. Mono-associated mice were maintained for 3 weeks in sterile isolators and then cohoused with animals in a specific pathogen-free facility.

Cell isolation and adoptive transfer.

For adoptive transfer experiments involving naïve 5C.C7 T cells, suspensions of pooled peripheral (cervical, axillary, brachial, inguinal) and mesenteric lymph nodes from B10.A 5C.C7 TCR transgenic, *Rag2*$^{-/-}$ mice (> 90% CD4^{+}Vβ3^{+} T cells), were injected i.v. (suborbital route) into indicated hosts.

For isolation of purified tuned 5C.C7 T cells from spleen, mLN or Peyer's patches of RF- or MPF-housed mPCC,*Cd3e*$^{-/-}$ hosts or total cells from mPCC,*Cd3e*$^{+/+}$ hosts, the indicated organs were incubated for 45min at 37 °C in PBS 1x with 2.5mg/ml Collagenase D (Roche) and 3mM $CaCl_2$ prior to dissociation on a gentleMACS™ Dissociator (Miltenyi Biotec).

For isolation of purified tuned 5C.C7 T cells from lamina propria of RF- or MPF-housed hosts, 1 cm pieces of PBS 1x-washed small intestine were incubated for 20 min at 37 °C in PBS 1x. Tissues were then incubated for 45min at 37 °C in E/R complete medium (see below) with 2.5mg/ml Collagenase D (Roche) and 3mM $CaCl_2$ prior to dissociation on a gentleMACS™ Dissociator (Miltenyi Biotec).

T cells were further enriched from cell suspensions using two rounds of negative selection with dynabeads (Dynal) and antibodies to B220 (RA3-6B2), CD11b (M1/70), I-A/I-E (M5/114) and NK1.1 (PK136) (all functional grade purified, eBioscience; cell purity typically ≥80% CD4^{+}Vβ3^{+} T cells).

***In vitro* analysis of T cell responsiveness.**

10,000 purified, tuned or naïve 5C.C7 T cells (see above) were cultured with half-log dilutions of the synthetic moth cytochrome *c* (MCC_{88-103}) peptide (ANERADLIAYLKQATK) in the presence of irradiated (3000 rads) splenocytes from B10.A, *Cd3e*$^{-/-}$ mice in 200µl of a 1:1 mix of RPMI 1640 and Eagle's Hank's Amino Acids (EHAA; Invitrogen), supplemented with 10% fetal bovine serum (FBS), 4mM glutamine, 200µg/ml penicillin, 200µg/ml streptomycin, 25µg/ml gentamicin and 50µM 2-ME (E/R complete medium). CD69 and pS6 expression levels were assayed in pooled triplicates at 16h and 40h, respectively. When indicated, rmIL-12p70 (Peprotech, 10ng/ml) or rmIL-23 (eBioscience, 10ng/ml) was added to the co-culture. For proliferation, 1µCi of tritiated thymidine was added per well at 60h and cells were cultured for an additional 24h. Cells were harvested using a Brandel 96-well harvester and incorporated tritium was counted using a Wallac Trilux 1450 scintillation counter.

***Ex vivo* staining and analysis of cytokine production by T cells.**

Cell suspensions from mPCC,*Cd3e*$^{-/-}$ mice were prepared from transfer recipients by mechanically dispersing spleen or mLN in sterile PBS containing 5% FBS. One million cells per organ were then directly stained or re-stimulated for 3h *in vitro* in the presence of 2.5 x 10^6 splenocytes from B10.A, *Cd3e*$^{-/-}$ mice in 2mL E/R complete medium and 3µM MCC peptide or 100ng/ml PMA and 1µg/ml ionomycin (Sigma). Alternatively, purified CD4$^+$ T cells were re-stimulated for 6h *in vitro* in the presence of 10µg/ml anti-CD3 (145-2C11, BD Bioscience) or 100ng/ml PMA and 1µg/ml ionomycin (Sigma), in the presence of 10µg/ml anti-CD28 (37-51, Bio X Cell).

For experimental tracking of 5C.C7 T cells in T cell replete mPCC,*Cd3e*$^{+/+}$ hosts, total $CD4^{+}$ T cells were enriched prior to staining using the same protocol used for the isolation of purified tuned T cells in T cell deficient hosts, adding antibodies to CD8 (clone 53-6.7, functional grade purified, eBioscience).

Antibodies for flow cytometry are described in the tables below, according to the fixation protocol used. Surface staining was performed in PBS 1x, with 2mM EDTA, 2% BSA and 1% sodium azide (Sigma) (staining buffer). For intracellular staining, cells were fixed using BD Bioscience Cytofix/Cytoperm™ or eBioscience Foxp3 Fixation/Permeabilization solution according to the manufacturers instructions and subsequent staining done in eBioscience Permeabilization Buffer. For pS6 staining, cells were fixed in 2% paraformaldehyde (PFA) (Sigma-Aldrich) and permeabilized overnight in 90% methanol at -20 °C. Staining was then performed in staining buffer. Intranuclear staining for EdU was performed using the Click-iT™ EdU Flow Cytometry kit (Invitrogen) according to the manufacturer's instructions with eBioscience Foxp3 Fixation/Permeabilization solution used as fixative buffer prior to co-staining for EdU and Ki-67 or with BD Bioscience Cytofix/Cytoperm™ solution used as fixative buffer prior to co-staining for EdU and IFN-γ. Data were collected on a FACSCanto II flow cytometer and further analyzed using FlowJo software (Treestar).

Surface staining

Molecule	Clone	Source	Fluorochrome
CD4	RMA-4	Biolegend	Pacific Blue
Vβ3	KJ25	BD Biosciences	Biotinylated
TCRβ	H57-597	BD Biosciences	Biotinylated
CD45.1	A20	Biolegend	APC
CD45.2	104	Biolegend	PE-Cy5.5
CD69	H1-2F3	Biolegend	PE
CD25	PC61	Biolegend	APC
PD-1	J43	BD Biosciences	PE
CD19	eBio1D3	eBiosciences	eFluor® 450
GL-7	GL-7	eBiosciences	Alexa Fluor® 647
Fas/CD95	Jo2	BD Biosciences	PE
LIVE/DEAD® Fixable Near-IR dye		Invitrogen	-

Intracellular staining with BD Bioscience Cytofix/Cytoperm™

Molecule	Clone	Source	Fluorochrome
IL-2	JES6-5H4	Biolegend	PE
IL-17A	TC11-18H10.1	Biolegend	FITC
IFNγ	XMG1.2	Biolegend	APC, PerCP/Cy5.5, PE-Cy7
IL-10	JES5-16E3	Biolegend	APC

Intracellular staining with eBioscience Foxp3 Fixation/Permeabilization solution

Molecule	Clone	Source	Fluorochrome
Foxp3	FJK-16s	eBiosciences	APC
Ki-67	B56	BD Biosciences	PE
Bcl-6	K112-91	BD Biosciences	Alexa Fluor® 647

Intracellular staining with PFA fixation + 90% Methanol permeabilization.

Molecule	Clone	Source	Fluorochrome
pS6 Ribosomal Protein (Ser235/236)	D57.2.2E	Cell Signaling	Alexa Fluor® 488, Alexa Fluor® 647

SUPPLEMENTAL REFERENCES

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