Immunity
Supplemental Experimental Procedures

Regulatory T cell Reprogramming Toward a Th2-Cell-like Lineage Impairs Oral Tolerance and Promotes Food Allergy.

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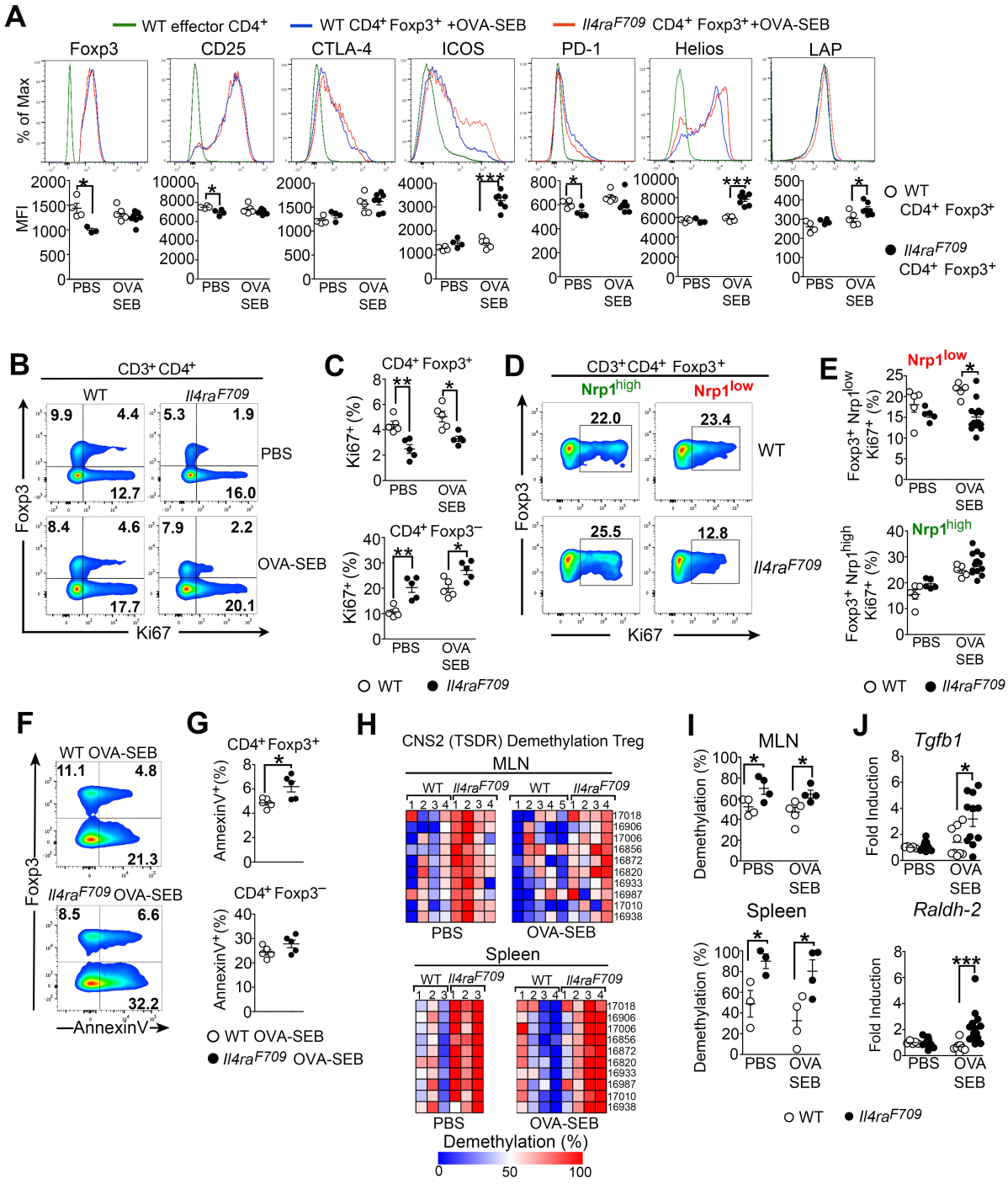

A
WT effector CD4+
WT CD4+ Foxp3+ +OVA-SEB
Il4raF709 CD4+ Foxp3+ +OVA-SEB
Foxp3
CD25
CTLA-4
ICOS
PD-1
Helios
LAP
% of Max
MFI
PBS
OVA SEB
WT CD4+ Foxp3+
Il4raF709 CD4+ Foxp3+
B
CD3+ CD4+
WT
Il4raF709
9.9
4.4
12.7
5.3
1.9
16.0
PBS
8.4
4.6
17.7
7.9
2.2
20.1
OVA-SEB
Foxp3
Ki67
C
CD4+ Foxp3+
Ki67+ (%)
CD4+ Foxp3−
WT
Il4raF709
D
CD3+CD4+ Foxp3+
Nrp1high
Nrp1low
22.0
23.4
WT
25.5
12.8
Il4raF709
Foxp3
Ki67
E
Nrp1low
Foxp3+ Nrp1low Ki67+ (%)
Nrp1high
Foxp3+ Nrp1high Ki67+ (%)
WT
Il4raF709
F
WT OVA-SEB
11.1
4.8
21.3
Il4raF709 OVA-SEB
8.5
6.6
32.2
Foxp3
AnnexinV
G
CD4+ Foxp3+
AnnexinV+(%)
CD4+ Foxp3−
WT OVA-SEB
Il4raF709 OVA-SEB
H
CNS2 (TSDR) Demethylation Treg
MLN
WT
Il4raF709
17018
16906
17006
16856
16872
16820
16933
16987
17010
16938
PBS
OVA-SEB
Spleen
Demethylation (%)
0
50
100
I
MLN
Demethylation (%)
Spleen
J
Tgfb1
Fold Induction
Raldh-2
WT
Il4raF709

Figure S1. Characterization of Treg cells of food allergic *Il4ra*^{F709} mice. (A) Flow cytometric analysis and mean fluorescence intensity (MFI) of Foxp3, CD25, CTLA-4, ICOS, PD-1, Helios and LAP expression in MLN Treg cell isolated from PBS and OVA-SEB WT and *Il4ra*^{F709} mice. **(B, C)** Flow cytometric analysis **(B)** and frequencies (**C**) of Ki67^{+} cells in CD4^{+}Foxp3^{-} Tconv and CD4^{+}Foxp3^{+} Treg cells in the MLN of the respective mouse groups in (A). **(D, E)** Flow cytometric analysis **(D)** and frequencies (**E**) of Ki67^{+} cells in Nrp1^{low} and Nrp1^{high} CD4^{+}Foxp3^{+} Treg cells from the MLN of the respective mouse groups in (A). **(F, G)** Flow cytometric analysis **(F)** and frequencies **(F)** of apoptotic (Annexin V^{+}) CD4^{+}Foxp3^{-} Tconv and CD4^{+} Foxp3^{+} Treg cells isolated from the MLN of OVA-SEB-sensitized WT and *Il4ra*^{F709} mice. **(H)** Heat map representation of the demethylation status of *Foxp3* CNS2 region CpG motifs in Foxp3^{EGFP+} Treg cells from the MLN or spleens of PBS and OVA-SEB-sensitized WT and *Il4ra*^{F709} mice. Individual CpG motifs are numbered with reference to the transcription initiation site of *Foxp3*. **(I)** Average percent demethylation of *Foxp3* CNS2 CpG motifs in Treg cells isolated from the respective mouse groups in (H). **(J)** Real time PCR analysis of *Tgfb1* and *RALDH-2* mRNA expression in the SI of PBS and OVA-SEB-sensitized WT and *Il4ra*^{F709} mice. N=3-14 mice/group, representative of 2 independent experiments (Panels **A-G**) or one experiment (panels **H-J**). *p<0.05, **p<0.01 and ***p<0.001 by 1-way ANOVA with post-test analysis.

(Related to Figure 1)

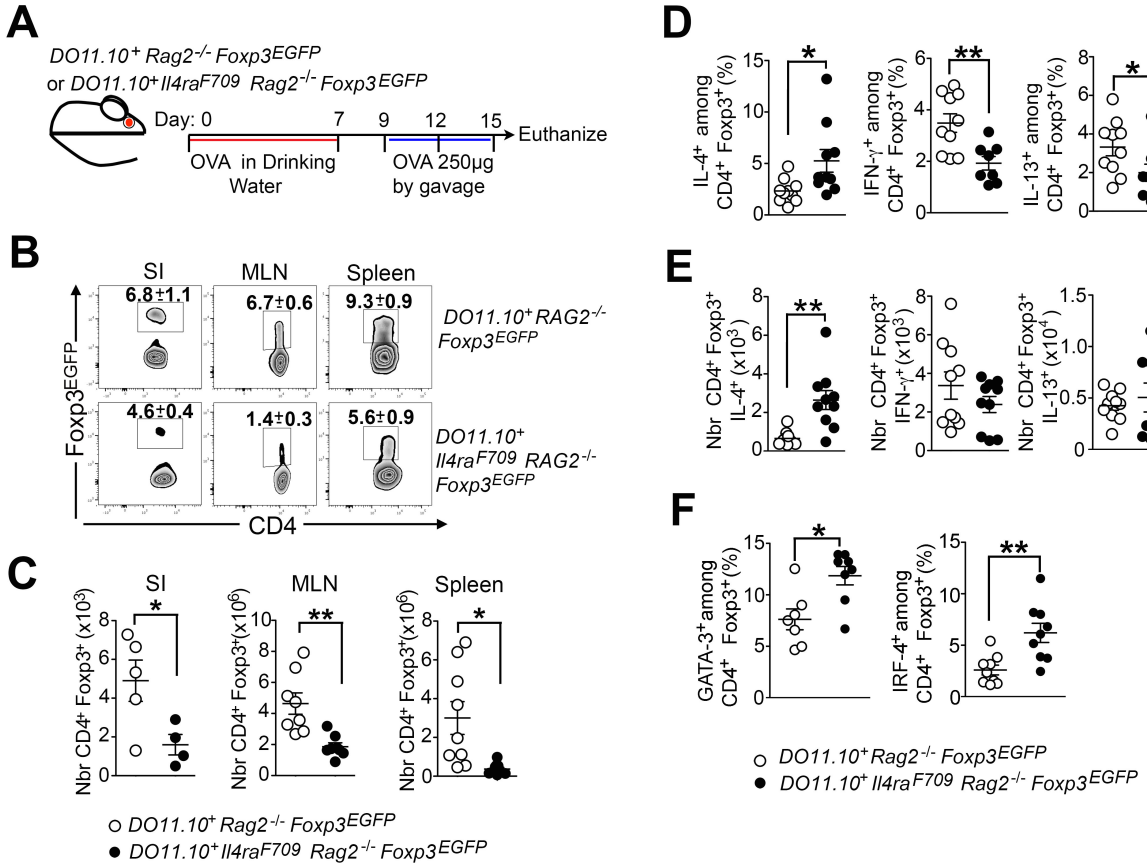


Figure S2. Defective formation of OVA-specific iTreg cells in *DO11.10^{+}Il4ra^{F709}Rag2$^{-/-}$Foxp3^{EGFP}* mice. (A) Schematic representation of the experimental design. *DO11.10^{+}Il4ra^{F709}Rag2$^{-/-}$Foxp3^{EGFP}* and *DO11.10^{+}Rag2$^{-/-}$ Foxp3^{EGFP}* mice were fed OVA at 1% in drinking water for 7 days, then 250µg OVA by oral gavage at days 9, 12 and 15. At day 17, the mice analyzed for iTreg cells induction as detected by EGFP expression. **(B, C)** Flow cytometric analysis **(B)** and numbers **(C)** of iTreg cells induced in the SI, MLN and spleen of the mouse groups shown in (A). At baseline, there is no expression of Foxp3^{EGFP+} iTreg cells (data not shown). **(D, E)** Frequencies **(D)** and numbers **(E)** of IL-4, IFN-γ and IL-13 secreting cells among CD4^{+}Foxp3^{+} iTreg cells from the MLN of the respective mouse strains shown in (A). **(F)** Percentages of GATA-3^{+} and IRF-4^{+} iTreg cells isolated from the MLN of *DO11.10^{+}Il4ra^{F709}Rag2$^{-/-}$Foxp3^{EGFP}* and *DO11.10^{+}Rag2$^{-/-}$Foxp3^{EGFP}* following OVA treatment. N=4-12 mice/group, representative of 2 independent experiments. *P<0.05, **p<0.01 and ***p<0.001 by Student's unpaired two tailed *t* test.

(Related to Figure 2)

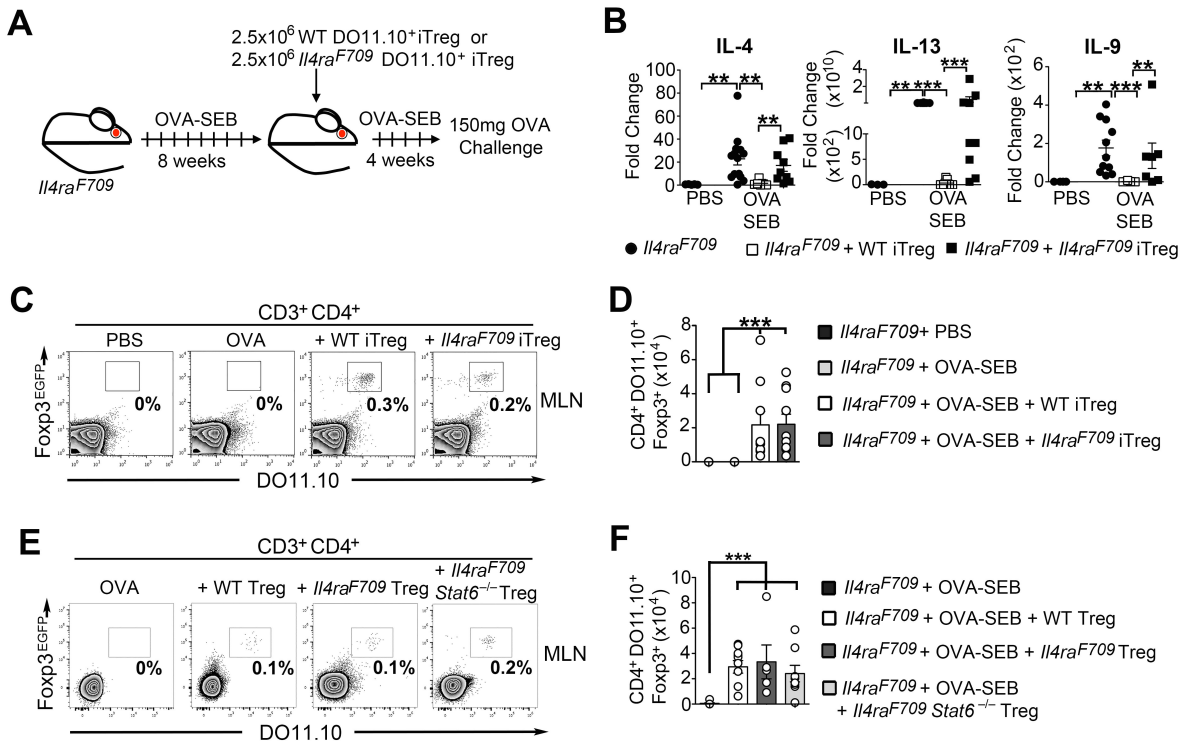


Figure S3. OVA-specific *Il4ra*^{F709} Treg cells fail to induce oral tolerance. (A) Schematic representation of the experimental design. WT and *Il4ra*^{F709} D011.10^{+}Foxp3^{EGFP+} iTreg cells were given to OVA-SEB-sensitized *Il4ra*^{F709} recipients, which were then sensitized for 4 more weeks then challenged with OVA. Comparison groups of PBS or OVA-SEB-sensitized *Il4ra*^{F709} mice that did not receive iTreg cells were included. **(B)** *IL-4*, *IL-13* and *IL-9* mRNA levels in the SI of OVA-SEB-sensitized *Il4ra*^{F709} recipients of transferred iTreg cells. **(C, D)** Flow cytometric analysis and numbers **(D)** of transferred WT and *Il4ra*^{F709} D011.10^{+}Foxp3^{EGFP+} iTreg cells retrieved in the MLN of recipient *Il4ra*^{F709} mice. **(E, F)** Flow cytometric detection (**E**) and enumeration (**F**) of transferred WT DO11.10^{+}Foxp3^{EGFP+} or *Il4ra*^{F709}DO11.10^{+}Foxp3^{EGFP+} STAT6-sufficient or deficient Treg cells in the MLN of *Il4ra*^{F709} recipients retrieved following OVA challenge. N=4-15 mice per group; representative of two independent experiments**.** *p<0.05; **p<0.01; ***p<0.001. 1-and 2-way ANOVA with post-test analysis.

(Related Figure 3)

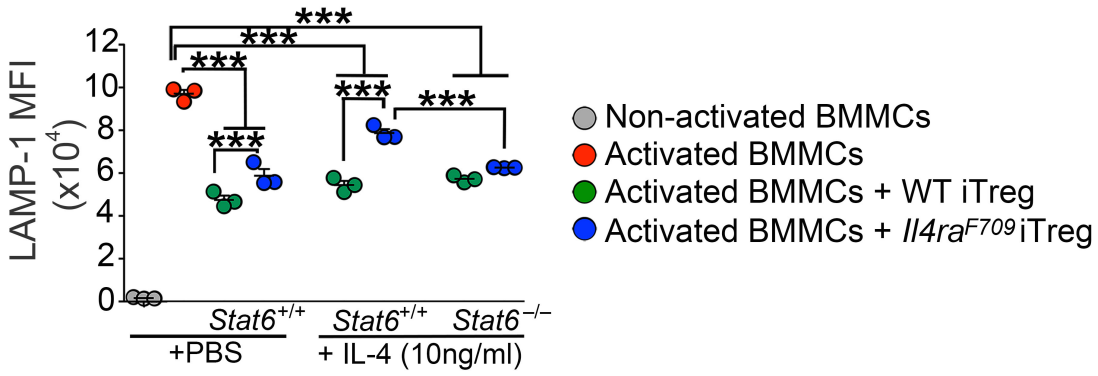


Figure S4. *Il4ra*^{F709} Treg cells functionality is impaired by excessive STAT6 signaling. LAMP-1 expression by IgE-activated BMMCs co-cultured with STAT6-sufficient or -deficient WT and *Il4ra*^{F709} iTreg cells in the absence or presence of IL-4. Results are representative of 3 independent experiments. N=3-7 mice per group**.** *p<0.05; **p<0.01; ***p<0.001. 1-and 2-way ANOVA with post-test analysis.

(Related to Figure 3)

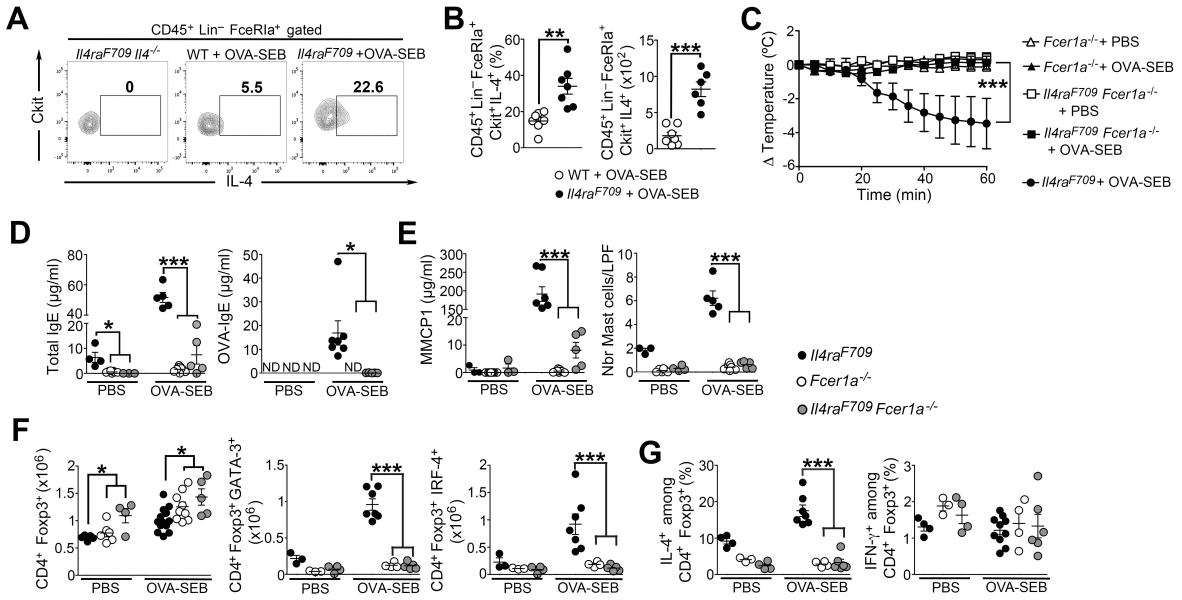


Figure S5. **Dysregulated FcεRIα signaling drives the deficiency and Th2 reprogramming of Treg cells in food allergic *Il4ra*^{F709} mice**. **(A)** Flow cytometric analysis of IL-4 production by mast cells isolated from the SI lamina propia of OVA-SEB-sensitized WT and *Il4ra*^{F709} *mice.* Mast cells isolated from *Il4ra*^{F709}*Il4*$^{-/-}$ double mutant mice that lack IL-4 were used as IL-4 staining controls. **(B)** Percentages and numbers of IL-4 secreting mast cells secreting in the lamina propia of OVA-SEB-sensitized WT and *IL4ra*^{F709} mice. **(C)** Core body temperature changes in PBS and OVA-SEB-sensitized *Fcer1a*$^{-/-}$, *Il4ra*^{F709} and *Il4ra*^{F709}*Fcer1a*$^{-/-}$ mice following oral challenge with OVA. **(D)** Total and OVA-specific serum IgE concentrations in the respective group post analphylaxis. **(E)** Serum MMCP-1 concentrations (left panel) and SI mast cell number (right panel) in the respective mouse group post anaphylaxis. **(F)** Numbers of CD4$^+$Foxp3$^+$ Treg, GATA-3$^+$ and IRF-4$^+$ Treg cells in MLN of PBS and OVA-SEB-sensitized mouse groups. **(H)** Percentages of Treg cell secreting IL-4 and IFN-γ in the MLN of mice the respective groups. N=3-10 mice per group, representative of two independent experiments**.** *p<0.05; **p<0.01; ***p<0.001 by 1 and 2-way ANOVA with post-test analysis.

(Related to Figure 5)

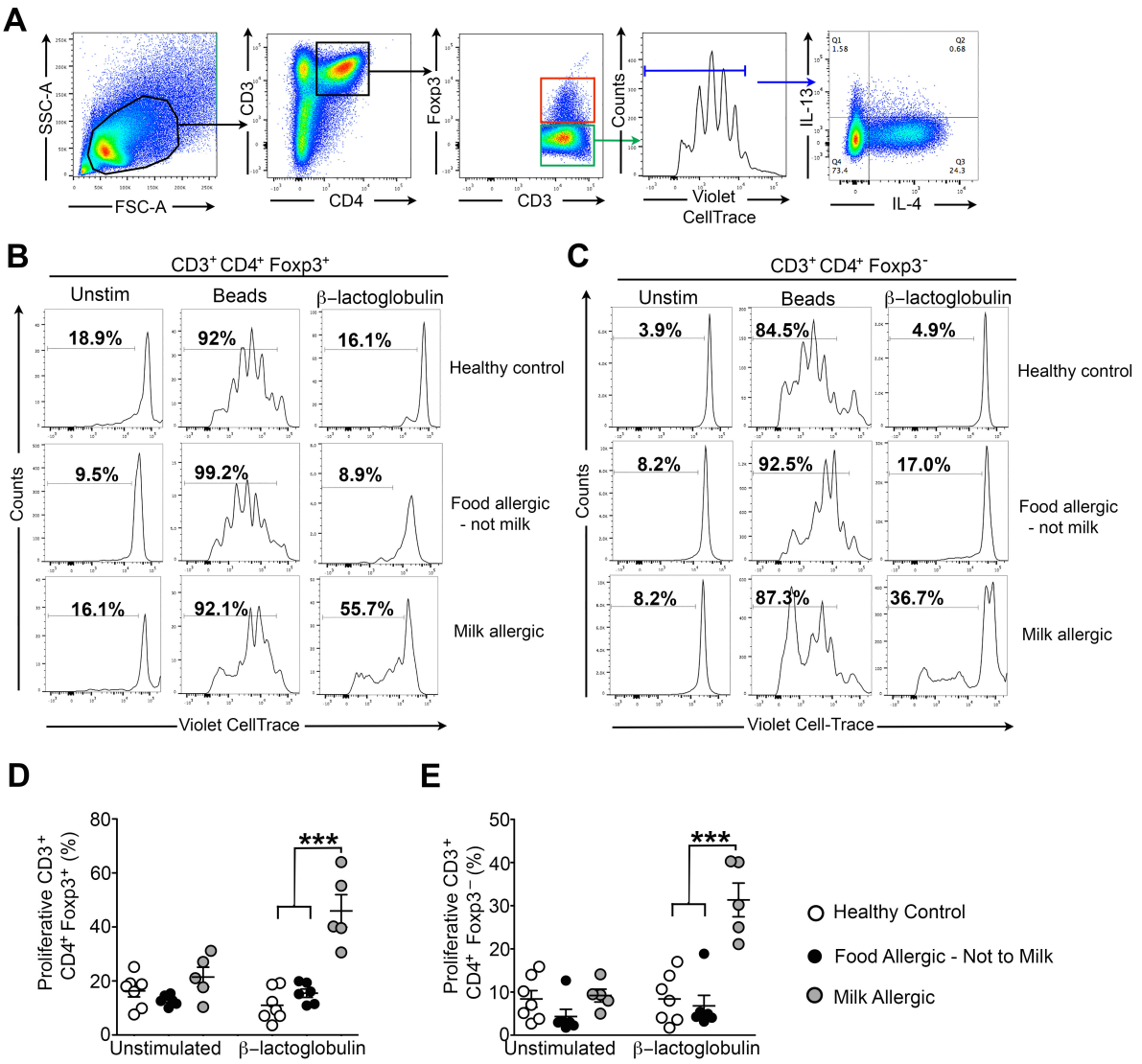
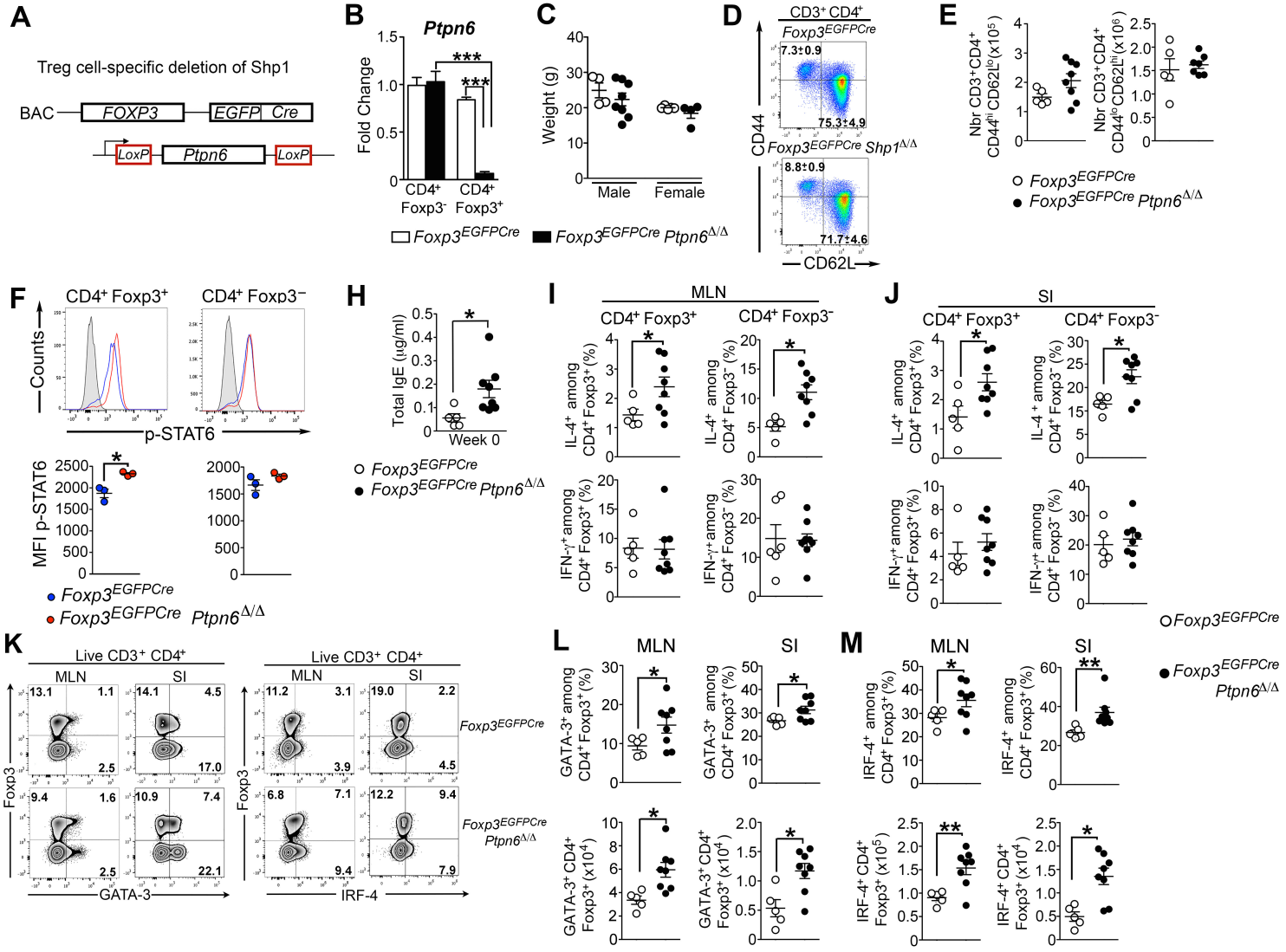


Figure S6. Proliferation of Treg and Tconv cells of milk allergic subjects to β-lactoglobulin stimulation. (A) Flow cytometric gating strategy used to analyze proliferation and cytokine production of β-lactoglobulin-specific $CD4^+$ and $Foxp3^+$ Treg cells. PBMCs were labeled with the Violet CellTrace dye and cultured for 5 days in the absence or presence of anti-CD3/CD28 activation beads or with β-lactoglobulin. **(B, C)** Flow cytometric analysis of proliferating $CD4^+Foxp3^-$ Tconv (**B**) or $CD4^+Foxp3^+$ Treg cells (**C**) isolated from age matched healthy controls, food allergic (but not to milk) or milk allergic children after stimulation with anti-CD3/CD28 beads or β-lactoglobulin. **(D, E)** Percentages of proliferating $CD4^+Foxp3^+$ Treg cells (**D**) and $CD4^+Foxp3^-$ Tconv cells (**E**) of the groups shown in (B, C). N=5-7 human subjects; *p<0.05, **p<0.01 and ***p<0.001 by 1 -way ANOVA with post-test analysis.

(Related to Figure 6)


A
Treg cell-specific deletion of Shp1
BAC
FOXP3
EGFP Cre
LoxP
Ptpn6
LoxP
B
Ptpn6
Fold Change
***
***
CD4+ Foxp3-
CD4+ Foxp3+
Foxp3EGFPCre
Foxp3EGFPCre Ptpn6Δ/Δ
C
Weight (g)
Male
Female
D
CD3+ CD4+
Foxp3EGFPCre
7.3±0.9
75.3±4.9
Foxp3EGFPCre Shp1Δ/Δ
8.8±0.9
71.7±4.6
CD44
CD62L
E
Nbr CD3+CD4+ CD44hi CD62Lo(x10^5)
Nbr CD3+CD4+ CD44lo CD62Lhi(x10^6)
Foxp3EGFPCre
Foxp3EGFPCre Ptpn6Δ/Δ
F
CD4+ Foxp3+
CD4+ Foxp3−
Counts
p-STAT6
MFI p-STAT6
Foxp3EGFPCre
Foxp3EGFPCre Ptpn6Δ/Δ
H
Total IgE (µg/ml)
Week 0
Foxp3EGFPCre
Foxp3EGFPCre Ptpn6Δ/Δ
I
MLN
CD4+ Foxp3+
CD4+ Foxp3-
IL-4+ among CD4+ Foxp3+(%)
IL-4+ among CD4+ Foxp3-(%)
IFN-γ+ among CD4+ Foxp3+(%)
IFN-γ+ among CD4+ Foxp3-(%)
J
SI
CD4+ Foxp3+
CD4+ Foxp3-
IL-4+ among CD4+ Foxp3+(%)
IL-4+ among CD4+ Foxp3-(%)
IFN-γ+ among CD4+ Foxp3+(%)
IFN-γ+ among CD4+ Foxp3-(%)
Foxp3EGFPCre
Foxp3EGFPCre Ptpn6Δ/Δ
K
Live CD3+ CD4+
MLN
SI
Live CD3+ CD4+
MLN
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13.1
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17.0
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22.1
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2.2
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6.8
7.1
9.4
12.2
9.4
7.9
Foxp3EGFPCre
Foxp3EGFPCre Ptpn6Δ/Δ
Foxp3
GATA-3
Foxp3
IRF-4
L
MLN
SI
GATA-3+ among CD4+ Foxp3+(%)
GATA-3+ among CD4+ Foxp3+(%)
GATA-3+ CD4+ Foxp3+(x10^4)
GATA-3+ CD4+ Foxp3+(x10^4)
M
MLN
SI
IRF-4+ among CD4+ Foxp3+(%)
IRF-4+ among CD4+ Foxp3+(%)
IRF-4+ CD4+ Foxp3+(x10^5)
IRF-4+ CD4+ Foxp3+(x10^4)
Foxp3EGFPCre
Foxp3EGFPCre Ptpn6Δ/Δ

Figure S7. Th2 reprogramming of Treg cells of food allergic *Foxp3*^{EGFPCre}*Ptpn6*$^{\Delta/\Delta}$ mice. (A) Scheme for the generation of *Foxp3*^{EGFPCre}*Ptpn6*$^{\Delta/\Delta}$ mice. **(B)** Real time PCR analysis of *Ptpn6* mRNA transcripts in Tconv (CD4^{+}Foxp3^{-}) and Treg (CD4^{+}Foxp3^{+}) cells of *Foxp3*^{EGFPCre} and *Foxp3*^{EGFPCre}*Ptpn6*$^{\Delta/\Delta}$ mice. **(C)** Body weights of 8 weeks old male and female *Foxp3*^{EGFPCre} and *Foxp3*^{EGFPCre}*Ptpn6*$^{\Delta/\Delta}$ mice. **(D)** Flow cytometric analysis of CD3^{+}CD4^{+} Tconv cells isolated from the MLN from of *Foxp3*^{EGFPCre} and *Foxp3*^{EGFPCre}*Ptpn6*$^{\Delta/\Delta}$ mice and stained for CD44 and CD62L. Numbers indicate the percentage of cells ± S.E.M. in the respective quadrant. **(E)** Numbers of naïve (CD44^{low} CD62L^{high}) and effector memory (CD44^{high} CD62L^{low}) CD4^{+} T cells in the MLN of *Foxp3*^{EGFPCre} and *Foxp3*^{EGFPCre}*Ptpn6*$^{\Delta/\Delta}$ mice**. (F)** p-STAT6 expression in CD4^{+}Foxp3^{+} Treg cells or CD4^{+}Foxp3^{-} Tconv cells from *Foxp3*^{EGFPcre} or *Foxp3*^{EGFPcre}*Ptpn6* $^{\Delta/\Delta}$ mice following treatment with IL-4. **(H)** Serum IgE concentrations in 8 weeks old *Foxp3*^{EGFPCre} and *Foxp3*^{EGFPCre}*Ptpn6*$^{\Delta/\Delta}$ mice. **(I, J)** Frequencies of IL-4^{+} and IFN-γ^{+} CD4^{+}Foxp3^{+} Treg and CD4^{+}Foxp3^{-} Tconv cells isolated from the MLN **(I)** and SI **(J)** of OVA-SEB-sensitized *Foxp3*^{EGFPCre} and *Foxp3*^{EGFPCre}*Ptpn6*$^{\Delta/\Delta}$ mice. **(K)** Flow cytometric analysis of GATA-3 and IRF-4 expression in CD4^{+}Foxp3^{+}Treg cells isolated from the MLN and SI of OVA-SEB-sensitized *Foxp3*^{EGFPCre} and *Foxp3*^{EGFPCre}*Ptpn6*$^{\Delta/\Delta}$ mice. **(L, M)** Frequencies and numbers of CD4^{+}Foxp3^{+} GATA-3^{+} **(L)** and IRF-4^{+} **(M)** Treg cells isolated from the MLN and SI of OVA-SEB-sensitized *Foxp3*^{EGFPCre} and *Foxp3*^{EGFPCre}*Ptpn6*$^{\Delta/\Delta}$ mice. N=3-8 mice per groups, representative of two independent experiments**.** *p<0.05; **p<0.01 by unpaired Student *t* test.

(Related to Figure 7)

Table S1: Clinical characteristics of enrolled subjects

Subject	Age (Y)	Sex	Type of food allergy	Total IgE (kU_A/L)	Allergen-specific IgE (kU_A/L)	Skin Test Wheal (mm)
1 HC	3	M	None	N/A	N/A	N/A
2 HC	5	M	None	N/A	N/A	N/A
3 HC	2	M	None	N/A	N/A	N/A
4 HC	5	M	None	N/A	N/A	N/A
5 HC	3	F	None	N/A	N/A	N/A
6 HC	3	F	None	N/A	N/A	N/A
7 HC	5	F	None	N/A	N/A	N/A
8 HC	2	M	None	N/A	N/A	N/A
9 HC	3	F	None	N/A	N/A	N/A
10 HC	13	F	None	N/A	N/A	N/A
11 HC	2	M	None	N/A	N/A	N/A
12 HC	8	F	None	N/A	N/A	N/A
13	7	F	Peanut	384	15	20
14	4	M	Peanut	57	0.38	25
15	4	M	Peanut	233	15	20
16	8	F	Peanut	196	76	Not done
17	4	F	Peanut	363	96	Not done
18	4	F	Peanut	366	>100	Not done
19	11	M	Peanut	2337	>100	Not done
20	5	F	Walnut	221	29.8	Not done
21	<1	M	Milk	280	8.22	8
22	5	F	Milk	56	8	15
23	<1	F	Milk	138	9.75	Not done
24	5	M	Milk	3110	>100	Not done
25	2	F	Milk	52	1.7	10
26	6	F	Milk	1858	>100	Not done
27	9	M	Milk	2484	>100	Not done
28	3	M	Milk	764	95	30

HC: Healthy Control; F: Female; M: Male; N/A: Not Applicable

(Related to Figure 6)

Supplementary Methods

Flow cytometry. The following murine antibodies were used: IL-4 (11B11, BD Biosciences), Thy1.1 (HiS51), Thy1.2 (30-H12), Foxp3 (FJK-16S), DO11.10 (KJ126), IFN-γ (XMG1.2), IL-13 (eBio13a), GATA-3 (TWAJ), IRF-4 (3E4), ICOS (C398.4A), Helios (22F6), Ki67 (SolA15), CD25 (PC61.5), CTLA4 (UC10-4B9) (eBiosciences), CD4 (RM4-5), CD3 (145-2C11), AnnexinV, PD-1 (29F.1A12), IL-17 (TC11-18H10.1), IL-9 (RM9A4), c-kit (2B8), LAP (TW7-16B4), Fcεr1α (MAR-1) (Biolegend) and Nrp1 (R&D Systems). AnnexinV staining kit (Biolegend) was used according to the manufacturer's instructions in order to stain early apoptic cells. Intracellular cytokine staining was performed as previously described (Rivas et al., 2012). For the *in vitro* assessment of the OVA-specific T_R cells proliferation, MLN cells from OVA-SEB-sensitized mice were labeled with Violet CellTrace (Invitrogen) and co-cultured with OVA peptide-pulsed WT bone marrow derived dendritic cells during 4 days. Dead cells were routinely excluded from the analysis based on the staining of eFluor 780 Fixable viability dye (eBioscience). Stained cells were analyzed on an LSRII Fortessa or a FACSCalibur cytometer (BD Biosciences) and data were processed using Flowjo (Tree Star Inc.).

Cell purification. Cell suspensions were enriched in $CD4^+$ T cells by MACS negative selection (Miltenyi) and further sorted using a BD FACSAria II based on CD4 and EGFP (Foxp3) expression. For lamina propia lymphocyte isolation, jejunum section of the small intestine were harvested and the tissue was prepared as previously described (Rivas et al., 2012).

Methylation analysis. The methylation status of the TSDR of Foxp3 (CNS2) in Foxp3^{+} Treg cells were analyzed as described previously (Floess et al., 2007; Schmitt et al., 2012).

ELISAs. Murine mast cell protease 1 (MMCP-1), total and OVA-specific IgE concentrations were measured in the sera of treated mice by ELISAs, as described previously (Noval Rivas et al., 2013).

Quantitative real-time PCR analysis. Jejunum sections were snap-frozen in liquid nitrogen and homogenized in Trizol (Life Technologies). RNA was extracted from the homogenized tissues and from the cell-sorted CD4^{+}Foxp3^{-} Tconv and CD4^{+} Foxp3^{+} Treg cells by using the RNeasy kit (Qiagen). Reverse transcription was performed using Superscript III and oligo dT (Invitrogen). TaQman gene probes were used with TaQman Universal Fast Master Mix (Applied Biosystems) and ran on a Step-One-Plus machine (Applied Biosystems). GAPDH was used as an endogenous control (Applied Biosystems) and WT mouse RNA as the exogenous control. Samples were run in triplicates and the relative expression was calculated using the ΔΔCt method.

iT$_{R}$ – mast cell co-culture. WT bone marrow-derived mast cells (BMMCs) were generated as previously described (Burton et al., 2013). WT *Foxp3^{EGFP}*, WT *Foxp3^{EGFP}Stat6$^{-/-}$*, *Il4ra^{F709}Foxp3^{EGFP}* or *Il4ra^{F709}Foxp3^{EGFP}Stat6$^{-/-}$* iTreg cells were generated *in vitro* as described above. For the *in vitro* LAMP-1 release assay, 6 to 12 weeks old BMMCs were incubated with iTreg cells (ratio 1:1) overnight at 37ºC in the presence or absence of IL-4 (10ng/ml), recombinant IL-3 (5ng/ml, Peprotech), stem cell factor (5ng/ml, Peprotech) and anti-DNP-IgE (1µg/ml, Sigma Aldrich). The cells were washed and incubated with a mix of DNP-Albumin (1µg/ml, Sigma-Aldrich), anti-LAMP-1 (CD107a; Biolegend), anti-Fcer1a, anti-c-kit and anti-CD4 (Biolegend). After 10 minutes of incubation at 37ºC, the cells were washed and analyzed on a BD

LSR II Fortessa.

Histological analysis. Intestinal mast cells were enumerated by microscopic examination of jejunal sections fixed in 10% formaldehyde and stored in ethanol 70% before toluidine blue staining by the Harvard Rodent Histopathology Facility. Jejunal sections were also stained with chloroacetate esterase as previously described (Burton et al., 2013).

Human Subject Selection. Demographics and allergen reactivity of study subjects are detailed in Table S1. Children older than 2 years were considered milk allergic if, in addition to milk-related clinical symptoms, a skin-prick test (SPT) reaction with a wheal diameter ≥ 8mm was observed and/or allergen-specific IgE ≥ 15kU/L were detected. Children younger than 2 years were considered milk allergic if an SPT reaction with a wheal diameter ≥ 6mm was observed and/or allergen-specific IgE ≥ 5kU/L were detected (2010). Control subjects were age group-matched with negative history of food allergy. Non-milk food allergic subjects manifested reactivity to peanut or tree nuts by SPT or allergen-specific IgE together with allergen-related clinical symptoms. Exclusion criteria included subjects who are immunodeficient, with autoimmune diseases, are on chronic immunosuppressive therapy, with allergic colitis to milk, eosinophilic or esophagitis, gastroenteritis or protein induced enterocolitis. After obtaining written consent from the patients and/or their guardians, 10ml of blood were collected in heparinized tubes and the samples were processed as detailed below.

Human sample analysis. For the analysis of the human peripheral Treg cells, fresh blood was stained with the following antibodies: CD3 (HIT3a), CD4 (OKT4), CD25 (BC96), IL-4 (8D4-8), IL-13 (JES10-5A2) (Biolegend), Foxp3 (236A/E7), GATA-3 (TWAJ), IRF-4 (3E4) (eBioscience). The intracellular staining was performed with the

eBioscience Foxp3 kit and the stained cells were analyzed on an LSRII Fortessa (BD Biosciences, CA). Ficoll-Paque (GE Healthcare) purified human PBMC were labeled with the Violet CellTrace dye (Invitrogen) and cultured during 5 days either in complete medium, or with T cell activation/expansion beads (Miltenyi), or in the presence of β-lactoglobulin (1mg/ml, Sigma Aldrich). After 5 days, cells were stimulated during 4 hours with PMA (50 ng/ml; Sigma- Aldrich) and ionomycin (500 ng/ml; Sigma-Aldrich) in the presence of Golgi Plug (BD Biosciences), stained and subsequently analyzed on LSRII Fortessa to assess their cytokine production and their proliferation.

pSTAT6 detection. *Foxp3*^{EGFPcre} and *Foxp3*^{EGFPCre}*Ptnp6*$^{\Delta/\Delta}$ splenic CD4^{+} T cells were isolated by MACS (Miltenyi Biotech). Cells were stimulated with murine IL-4 (Peprotech) for pSTAT6 analysis. After 30 minutes, the cells were washed 3 times then fixed for 20 minutes on ice using a 2% paraformaldehyde solution, permeabilized with 90% methanol solution for 30 minutes and stained in PBS with anti-CD4 (Biolegend) and pSTAT-6 (pY641, BD Bioscience). The analysis was performed on a LSRII Fortessa cytometer and the data processed using Flowjo (Tree Star Inc).

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