Regulatory T cell-derived TGF-β1 Controls Multiple Checkpoints Governing Allergy and Autoimmunity

Jacob A. Turner[1,2*], Emmanuel Stephen-Victor[1,2*], Sen Wang[1,2], Magali Noval Rivas[3], Azza Abdel-Gadir[1,2], Hani Harb[1,2], Ye Cui[1,2], Manoussa Fanny[1,2], Louis-Marie Charbonnier[1,2], Jason Jun Hung Fong[1,2], Mehdi Benamar[1,2], Leighanne Wang[1,2], Oliver T. Burton[4], Kushagra Bansal[5], Lynn Bry[6], Chengsong Zhu[7], Quan-Zhen Li[7], Rachel L. Clement[8], Hans C. Oettgen[1,2], Elena Crestani[1,2], Rima Rachid[1,2], Peter T. Sage[8] and Talal Chatila[1,2,9 †]

[1]Division of Immunology, Boston Children's Hospital, Boston, MA, 02115 USA. [2]Department of Pediatrics, Harvard Medical School, Boston, MA, 02115 USA. [3]Division of Pediatric Infectious Diseases and Immunology, Department of Biomedical Sciences, Infectious and Immunologic Diseases Research Center (IIDRC), Cedars-Sinai Medical Center, Los Angeles, CA, 90048 USA. [4]Laboratory of Lymphocyte Signaling and Development, The Babraham Institute, Cambridgeshire CB22 3AT, UK. [5]Molecular Biology & Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore, 560064, India. [6]Massachusetts Host-Microbiome Center, Department of Pathology, Brigham & Women's Hospital, Harvard Medical School, Boston, MA, 02115 USA. [7]Department of Immunology and Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75390, USA. [8]Transplantation Research Center, Renal Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, 02115 USA

[9]Lead Contact

[*]These authors contributed equally to this work

†**Corresponding Author**. Email: talal.chatila@childrens.harvard.edu **(T.A.C).**

Fig. S1. Treg cell-specific overexpression of a *Tgfb1* transgene rescues FA. Related to Figure 1. (A) RT-PCR analysis of *Tgfb1* transcripts in sorted EGFP$^+$ and EGFP$^-$ CD4$^+$ T cells from MLN of *Foxp3*^{YFPcre}, *Foxp3*^{YFPcre}*Il4ra*^{F709} and *Foxp3*^{YFPcre}*Il4ra*^{F709}*Tgfb1*^{Tg} mice (8-12 weeks old). (B, C) LAP staining in Treg cells sorted from the MLN. (D) Changes in core body temperature in OVA-SEB-sensitized mice after oral OVA challenge. (E) Total and OVA-specific serum IgE and MMCP-1 concentrations before sensitization and after challenge. (F,G) Representative flow plots, frequencies and numbers of IL-4$^+$ and GATA-3$^+$ Treg and Teff cells from the MLN as determined by flow cytometry. Each symbol represents an independent sample. Numbers in flow plots indicate percentages. Error bars indicate SEM. Statistical tests: One-way ANOVA with Dunnett's post hoc analysis (A, E), two-way ANOVA (D); Student's *t*-test (C, G). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Data representative of two independent experiments. n=3-8 mice per group.

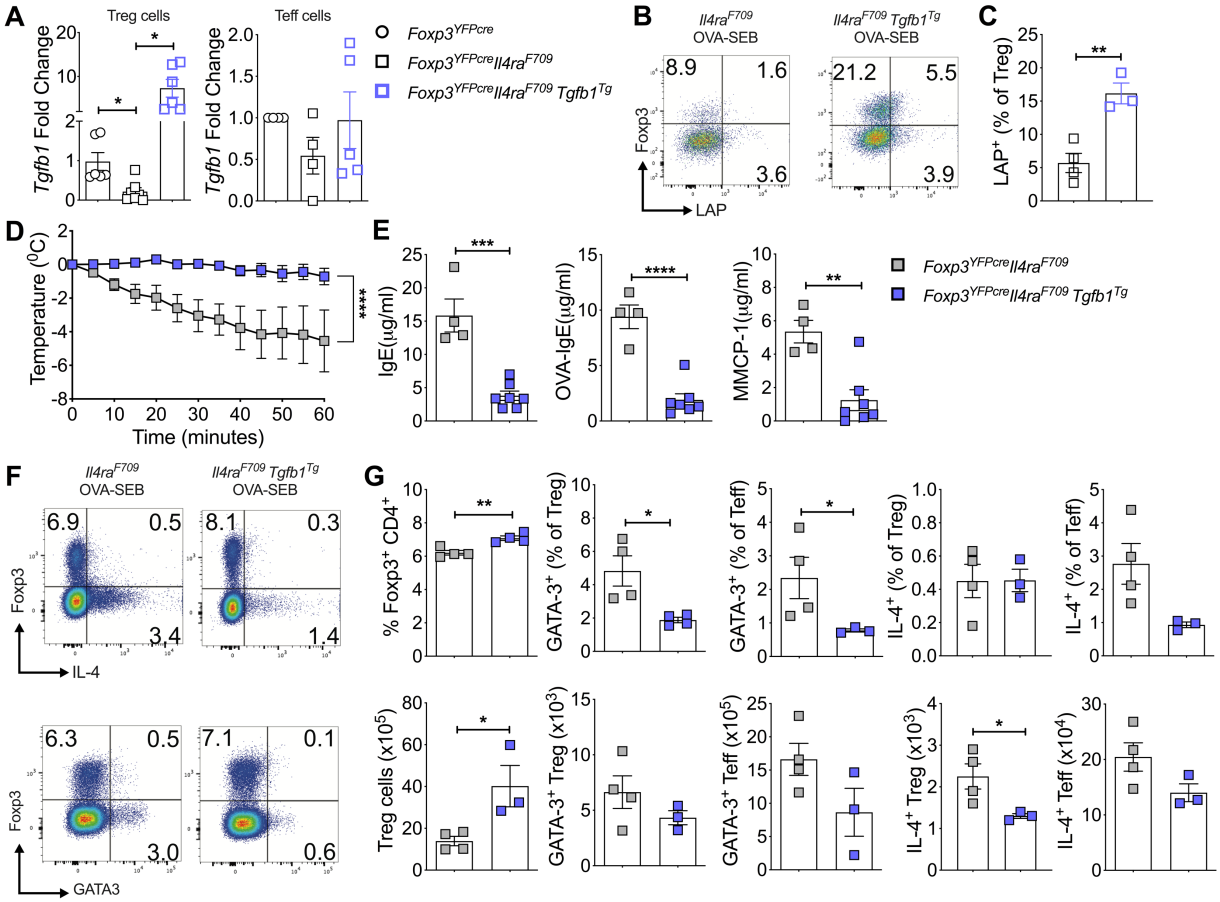
A
Treg cells
Teff cells
Tgfb1 Fold Change
Foxp3YFPcre
Foxp3YFPcreIl4raF709
Foxp3YFPcreIl4raF709 Tgfb1Tg
B
Il4raF709 OVA-SEB
Il4raF709 Tgfb1Tg OVA-SEB
8.9
1.6
3.6
21.2
5.5
3.9
Foxp3
LAP
C
LAP+ (% of Treg)
D
Temperature (0C)
Time (minutes)
E
IgE(μg/ml)
OVA-IgE(μg/ml)
MMCP-1(μg/ml)
F
Il4raF709 OVA-SEB
Il4raF709 Tgfb1Tg OVA-SEB
6.9
0.5
3.4
8.1
0.3
1.4
Foxp3
IL-4
6.3
0.5
3.0
7.1
0.1
0.6
GATA3
G
% Foxp3+ CD4+
GATA-3+ (% of Treg)
GATA-3+ (% of Teff)
IL-4+ (% of Treg)
IL-4+ (% of Teff)
Treg cells (x105)
GATA-3+ Treg (x103)
GATA-3+ Teff (x105)
IL-4+ Treg (x103)
IL-4+ Teff (x104)

Fig. S2. Characterization of TGF-β1 transcript and protein expression in immune cells of *Tgfb1* targeted mice. Related to Figure 6. (A) RT-PCR analysis of *Tgfb1* mRNA expression in Treg and Teff cells of littermate *Foxp3*^{YFPcre}, *Foxp3*^{YFPcre}*Tgfb1*$^{\Delta/+}$ and *Foxp3*^{YFPcre}*Tgfb1*$^{\Delta/\Delta}$ mice. (B and D) Flow cytometric analysis and frequencies of LAP^{+} (B) and GARP^{+} (D) cells from sorted Treg cells that were activated with 1μg/ml anti-CD3 mAb and 100U of IL-2 for 24h. (C) Frequencies of LAP^{+} Teff cells that were activated with 1μg/ml anti-CD3 mAb and 100U of IL-2 for 24h. (E to G) Quantification of TGF-β1 production by ELISA from FACS sorted Treg and Teff cells that were either sham treated or activated with anti-CD3+anti-CD28 mAb-coated dynabeads and 1μg/ml of IL-2 for 48h (E), from FACS sorted B cells that were either sham treated or activated with 10μg/ml αIgM and 1μg/ml recombinant CD40L for 48h (F), and from FACS sorted monocytes that were either sham treated or activated with 1μg/ml LPS for 48h (G). Each symbol represents an independent sample. Numbers in flow plots indicate percentages. Error bars indicate SEM. Statistical tests: One-way ANOVA with Dunnett's post hoc analysis (A to D), and Two-way ANOVA (E to G); **P<0.01, ***P<0.001, ****P<0.0001. Data representative of two independent experiments. n=4-14 mice per group.

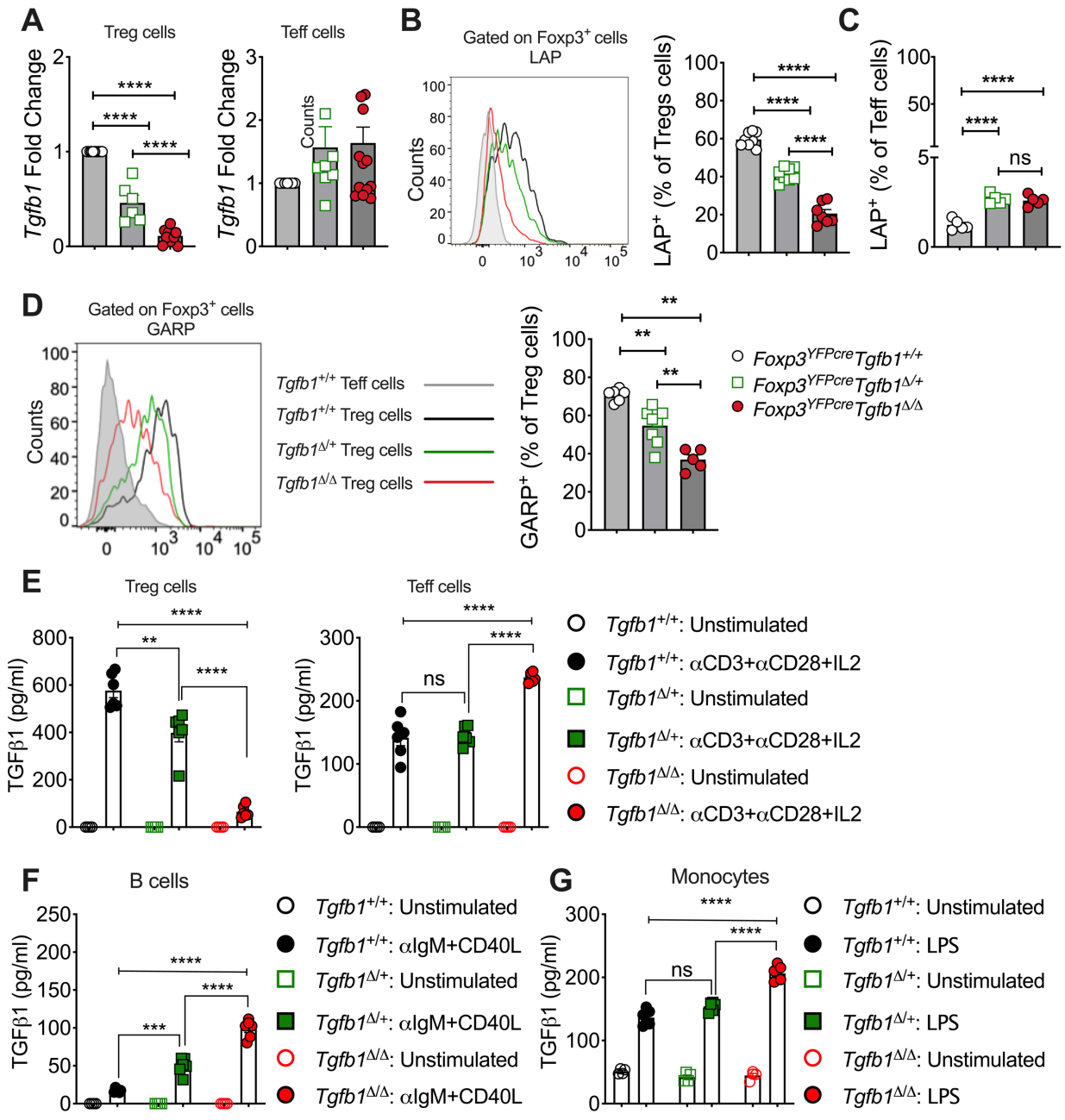
A
Treg cells
Teff cells
Tgfb1 Fold Change
Counts
B
Gated on Foxp3+ cells
LAP
Counts
LAP+ (% of Tregs cells)
C
LAP+ (% of Teff cells)
ns
D
Gated on Foxp3+ cells
GARP
Counts
Tgfb1+/+ Teff cells
Tgfb1+/+ Treg cells
Tgfb1Δ/+ Treg cells
Tgfb1Δ/Δ Treg cells
GARP+ (% of Treg cells)
Foxp3YFPcreTgfb1+/+
Foxp3YFPcreTgfb1Δ/+
Foxp3YFPcreTgfb1Δ/Δ
E
Treg cells
Teff cells
TGFβ1 (pg/ml)
ns
Tgfb1+/+: Unstimulated
Tgfb1+/+: αCD3+αCD28+IL2
Tgfb1Δ/+: Unstimulated
Tgfb1Δ/+: αCD3+αCD28+IL2
Tgfb1Δ/Δ: Unstimulated
Tgfb1Δ/Δ: αCD3+αCD28+IL2
F
B cells
TGFβ1 (pg/ml)
Tgfb1+/+: Unstimulated
Tgfb1+/+: αIgM+CD40L
Tgfb1Δ/+: Unstimulated
Tgfb1Δ/+: αIgM+CD40L
Tgfb1Δ/Δ: Unstimulated
Tgfb1Δ/Δ: αIgM+CD40L
G
Monocytes
TGFβ1 (pg/ml)
ns
Tgfb1+/+: Unstimulated
Tgfb1+/+: LPS
Tgfb1Δ/+: Unstimulated
Tgfb1Δ/+: LPS
Tgfb1Δ/Δ: Unstimulated
Tgfb1Δ/Δ: LPS

Fig. S3. Characterization of *Tgfb1*-deficient Treg cells. Related to Figure 6. (A) Flow cytometric analysis and MFI of CD25, CTLA4, OX40 and ICOS expression in splenic Treg cells. (B) Flow cytometric analysis and frequencies of Ki67 expression in $Foxp3^+$ and $Foxp3^-$ cells among splenic $CD4^+$ T cells. (C) Flow cytometric analysis and frequencies of IL-10 expression in $Foxp3^+$ and $Foxp3^-$ cells among MLN $CD4^+$ T cells. (D) Flow cytometric analysis and MFI of LAP in $CD11c^+MHCII^+$ DCs activated with 1μg/ml LPS for 24h (E) Flow cytometric analysis of an *in vitro* suppression assay with sorted $Foxp3^+$ cells from 8-12 weeks old $Foxp3^{YFPcre}$ (+/+), $Foxp3^{YFPcre}Tgfb1^{\Delta/+}$ (Δ/+), and $Foxp3^{EGFPcre}Tgfb1^{\Delta/\Delta}$ (Δ/Δ) Treg cells and cell-trace violet loaded WT Teff cells. (F) Flow Cytometric analysis of the frequency of YFP^+Foxp3^+ and YFP^-Foxp3^+ Treg cells in the spleen, MLN and LI-LP of female $Foxp3^{+/YFPcre}Tgfb1^{\Delta/\Delta}$. Each symbol represents an independent sample. Numbers in flow plots indicate percentages. Error bars indicate SEM. Statistical tests: Student's *t*-test (A and B), One-way ANOVA with Dunnett's post hoc analysis (C and D), and Two-way ANOVA (E); **P<0.01, ***P<0.001, ****P<0.0001. Data representative of two independent experiments. n=4-9 mice per group.

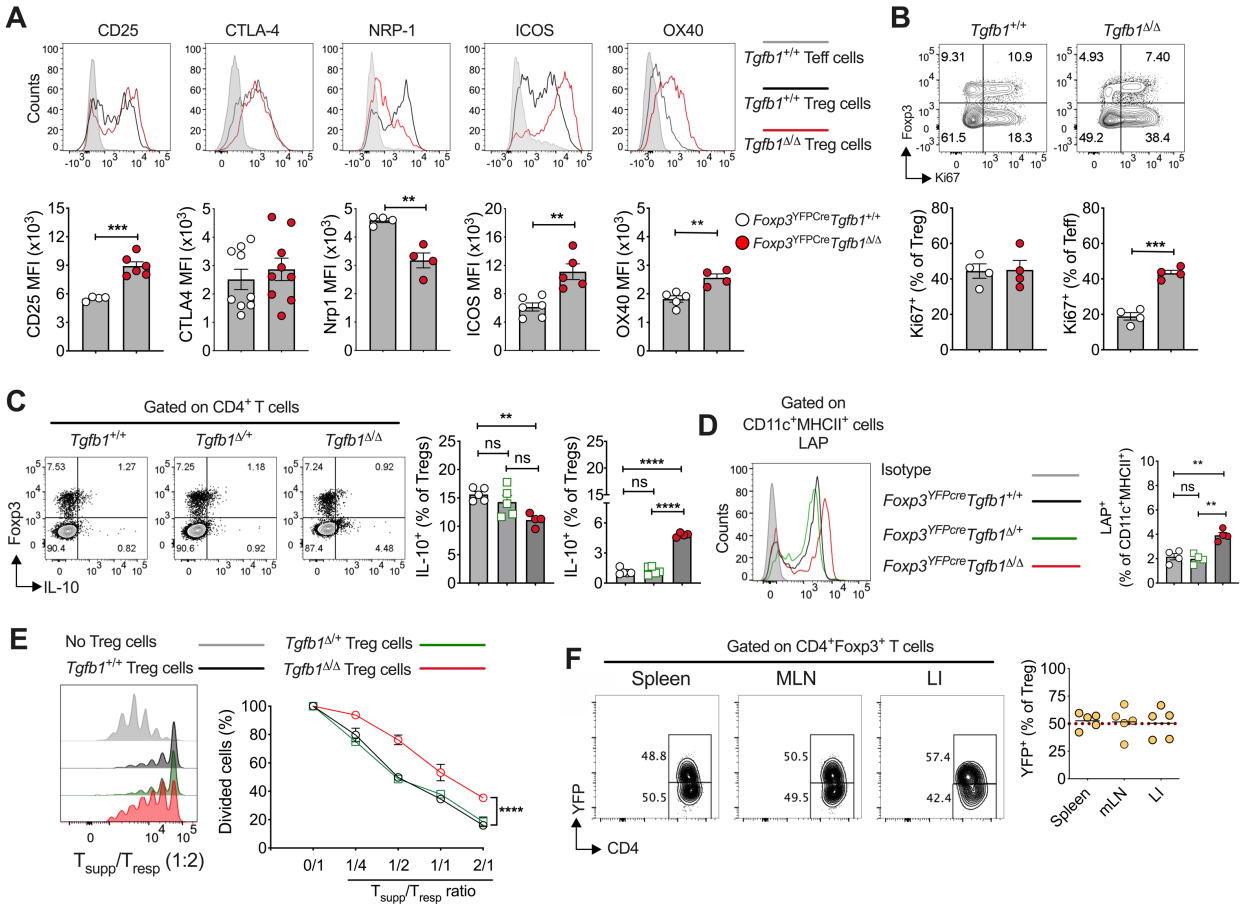
A
CD25
CTLA-4
NRP-1
ICOS
OX40
Counts
Tgfb1+/+ Teff cells
Tgfb1+/+ Treg cells
Tgfb1Δ/Δ Treg cells
CD25 MFI (x10³)
CTLA4 MFI (x10³)
Nrp1 MFI (x10³)
ICOS MFI (x10³)
OX40 MFI (x10³)
Foxp3YFPCreTgfb1+/+
Foxp3YFPCreTgfb1Δ/Δ
B
Tgfb1+/+
Tgfb1Δ/Δ
Foxp3
Ki67
9.31
10.9
61.5
18.3
4.93
7.40
49.2
38.4
Ki67+ (% of Treg)
Ki67+ (% of Teff)
C
Gated on CD4+ T cells
Tgfb1+/+
Tgfb1Δ/+
Tgfb1Δ/Δ
Foxp3
IL-10
7.53
1.27
90.4
0.82
7.25
1.18
90.6
0.92
7.24
0.92
87.4
4.48
IL-10+ (% of Tregs)
ns
D
Gated on CD11c+MHCII+ cells
LAP
Counts
Isotype
Foxp3YFPcreTgfb1+/+
Foxp3YFPcreTgfb1Δ/+
Foxp3YFPcreTgfb1Δ/Δ
LAP+ (% of CD11c+MHCII+)
E
No Treg cells
Tgfb1+/+ Treg cells
Tgfb1Δ/+ Treg cells
Tgfb1Δ/Δ Treg cells
Tsupp/Tresp (1:2)
Divided cells (%)
Tsupp/Tresp ratio
0/1
1/4
1/2
1/1
2/1
F
Gated on CD4+Foxp3+ T cells
Spleen
MLN
LI
48.8
50.5
50.5
49.5
57.4
42.4
YFP
CD4
YFP+ (% of Treg)

Fig. S4. Treg cell-specific *Tgfb1* deletion in *Foxp3*^{EGFPcre} mice is incompletely penetrant due to increased deletion escape. Related to Figure 6. (A) Representative flow cytometry plots acquired from the MLN of 8-12 weeks old *Foxp3*^{EGFcre}*R26*^{YFP} and *Foxp3*^{EGFPcre}*Tgfb1*$^{\Delta/\Delta}$*R26*^{YFP}. (Band C) Flow cytometric analysis and frequencies of LAP and GARP expression from EGFP^{+} and EGFP^{-} cells that were sorted from *Foxp3*^{EGFPcre}*Tgfb1*$^{\Delta/\Delta}$ and activated with 1μg/ml CD3 and 100U of IL2 for 24h. Expression of LAP (B) and GARP (C) in EGFP^{+} and EGFP^{-} cells stained for intracellular Foxp3 post activation. Each symbol represents an independent sample. Numbers in flow plots indicate percentages. Error bars indicate SEM. Statistical tests: Student's *t*-test (A***P<0.001. Data representative of two independent experiments. n=4 mice/group.

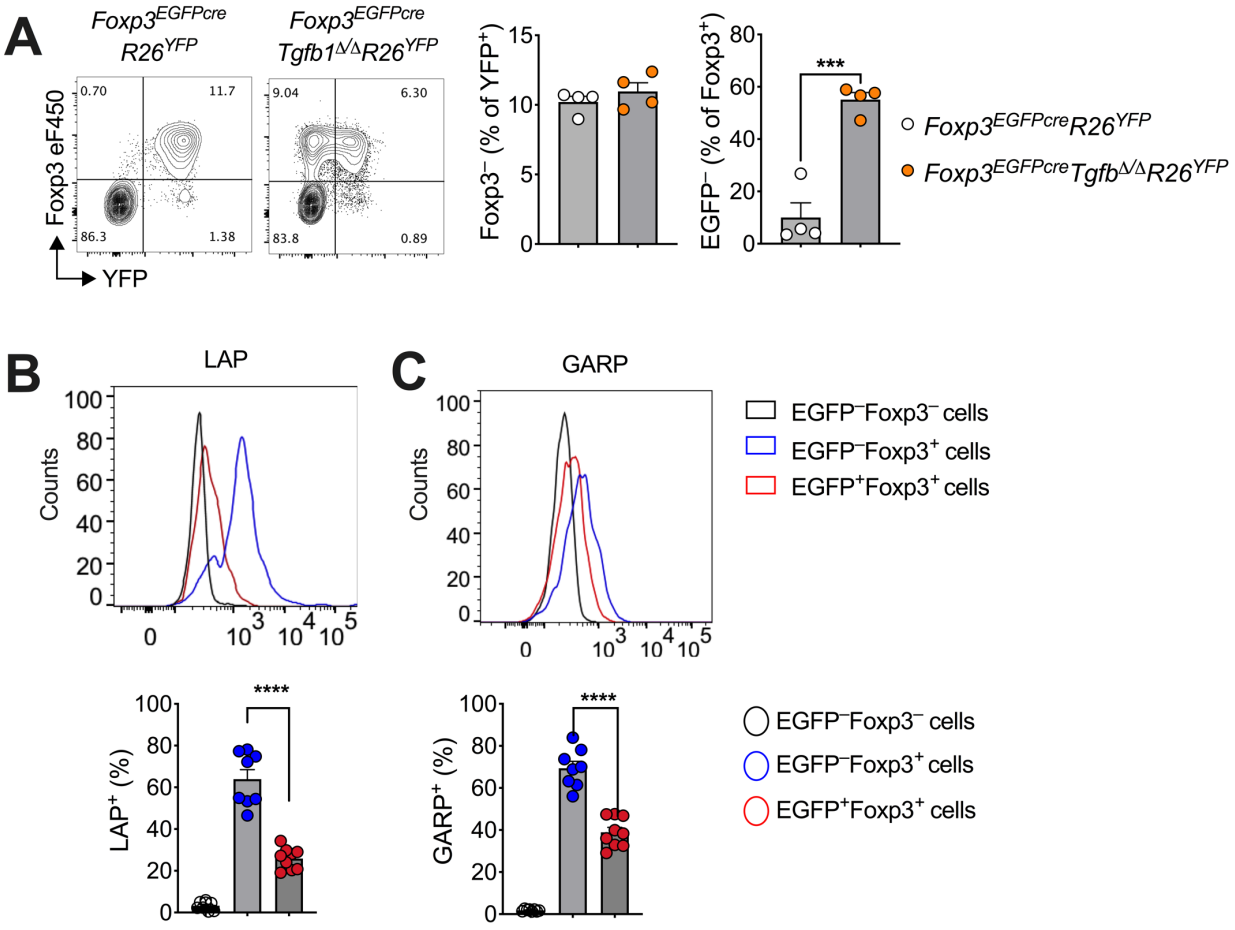

Fig. S5. Treg cell-specific *Tgfb1* deletion in *Foxp3*^{EGFPcre} mice promotes FA. Related to Figure 6. (A) Changes in core body temperature in OVA-SEB-sensitized WT, *Il4ra*^{F709} and *Foxp3*^{EGFPcre}*Tgfb1*$^{\Delta/\Delta}$ mice after 8 weeks of OVA-SEB sensitization followed by oral OVA challenge. (B) Total and OVA-specific serum IgE concentrations and serum MMCP-1 concentrations after anaphylaxis. (C) Representative histological sections from the SI-LP stained with Toluidine Blue. Magnified squares are 600x. (D) quantification the number of mast cells in the sections. (E to G) Flow cytometric analysis and frequencies and numbers of Foxp3$^+$ Treg cells (E), GATA-3$^+$ (F) and IRF-4$^+$ CD4$^+$ Treg cells (G) from the MLN. Each symbol represents an independent sample. Numbers in flow plots indicate percentages. Error bars indicate SEM. Statistical tests: One-way ANOVA with Dunnett's post hoc analysis (A, E), two-way ANOVA (D); Student's *t*-test (C, G). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Data representative of two independent experiments. n=9-18 mice per group.

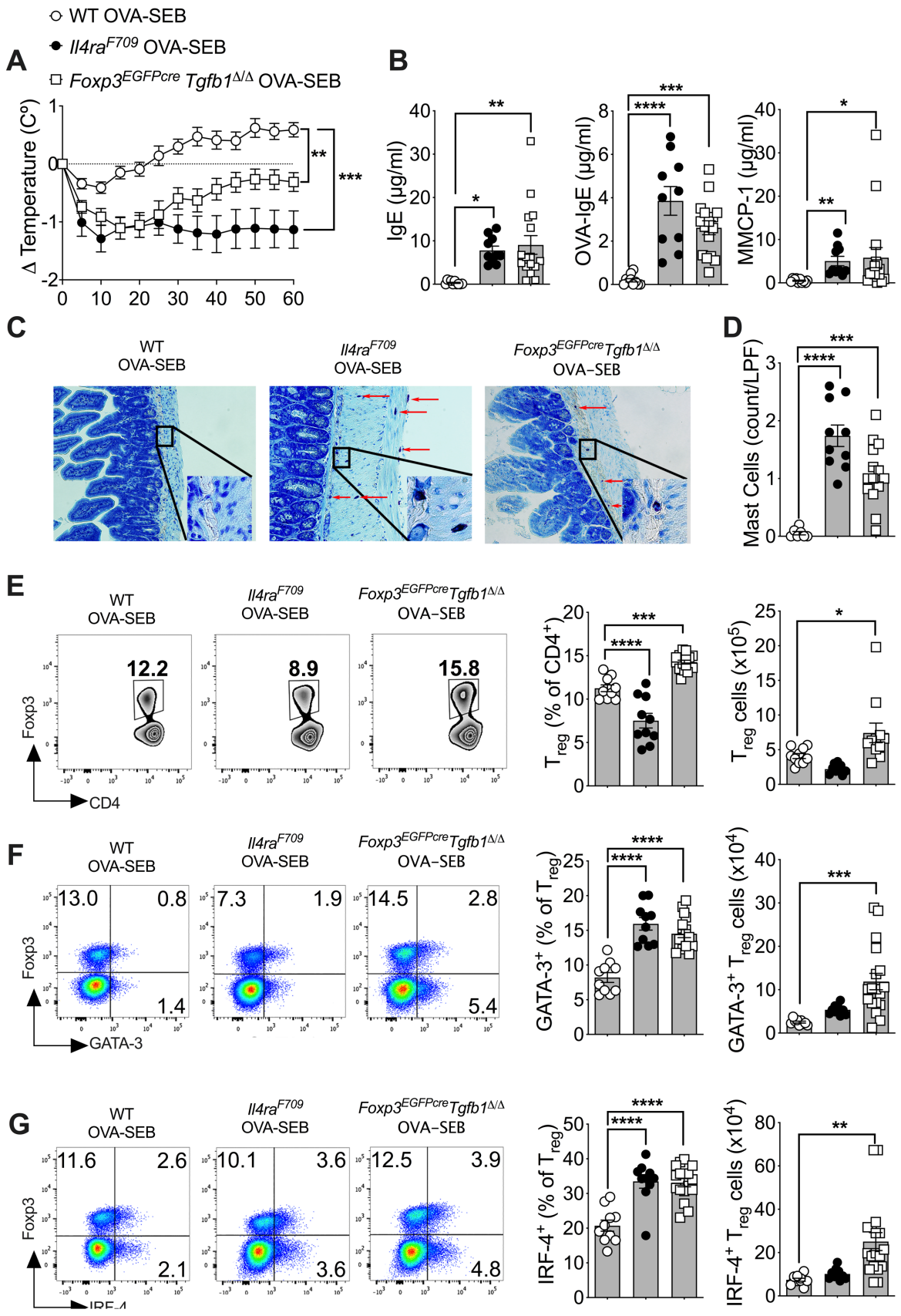
WT OVA-SEB
Il4ra^F709 OVA-SEB
Foxp3^EGFPcre Tgfb1^Δ/Δ OVA-SEB
A
Δ Temperature (C°)
B
IgE (μg/ml)
OVA-IgE (μg/ml)
MMCP-1 (μg/ml)
C
WT OVA-SEB
Il4ra^F709 OVA-SEB
Foxp3^EGFPcre Tgfb1^Δ/Δ OVA-SEB
D
Mast Cells (count/LPF)
E
12.2
8.9
15.8
Foxp3
CD4
T_reg (% of CD4+)
T_reg cells (x10^5)
F
13.0
0.8
1.4
7.3
1.9
14.5
2.8
5.4
GATA-3
GATA-3+ (% of T_reg)
GATA-3+ T_reg cells (x10^4)
G
11.6
2.6
2.1
10.1
3.6
3.6
12.5
3.9
4.8
IRF-4
IRF-4+ (% of T_reg)
IRF-4+ T_reg cells (x10^4)

Fig. S6. Adoptively transferred *Foxp3*^{EGFPcre}*Tgfb1*$^{\Delta/\Delta}$ Treg cells fail to prevent or rescue FA in *Il4ra*^{F709} mice. Related to Figure 6. (A to D) Preventative model. (A) Experimental scheme. CD4$^+$EGFP$^+$ Treg cells sorted from DO11.10$^+$*Foxp3*^{EGFPcre} (*Tgfb1*$^{+/+}$) and DO11.10$^+$*Foxp3*^{EGFPcre}*Tgfb1*$^{\Delta/\Delta}$ (*Tgfb1*$^{\Delta/\Delta}$) mice were transferred into *Il4ra*^{F709} mice, which received 8 weeks of OVA-SEB sensitization and were then orally challenged with OVA. (B) Core body temperature changes after oral OVA challenge. (C) Total and OVA-specific serum IgE and serum MMCP-1 concentrations after anaphylaxis. (D). Frequency of IL-4$^+$ and GATA-3$^+$ CD4$^+$ T cells from the MLN as determined by Flow Cytometry. (E to H) Curative model. (E) Experimental scheme. CD4$^+$EGFP$^+$ Treg cells derived as in (A) were transferred into OVA-SEB-sensitized *Il4ra*^{F709} mice, which were further sensitized for four weeks then challenged with OVA. (F) Core body temperature changes after oral OVA challenge. (G) Total and OVA-specific serum IgE and serum MMCP-1 concentrations. (H). Frequency of IL-4$^+$ and GATA-3$^+$ CD4$^+$ T cells from the MLN. Each symbol represents an independent sample. Numbers in flow plots indicate percentages. Error bars indicate SEM. Statistical tests: two-way ANOVA (B,F); Student's *t*-test (C, D, G, H). *P<0.05, **P<0.01, ****P<0.0001. Data representative of two independent experiments. n=5-8 mice per group.

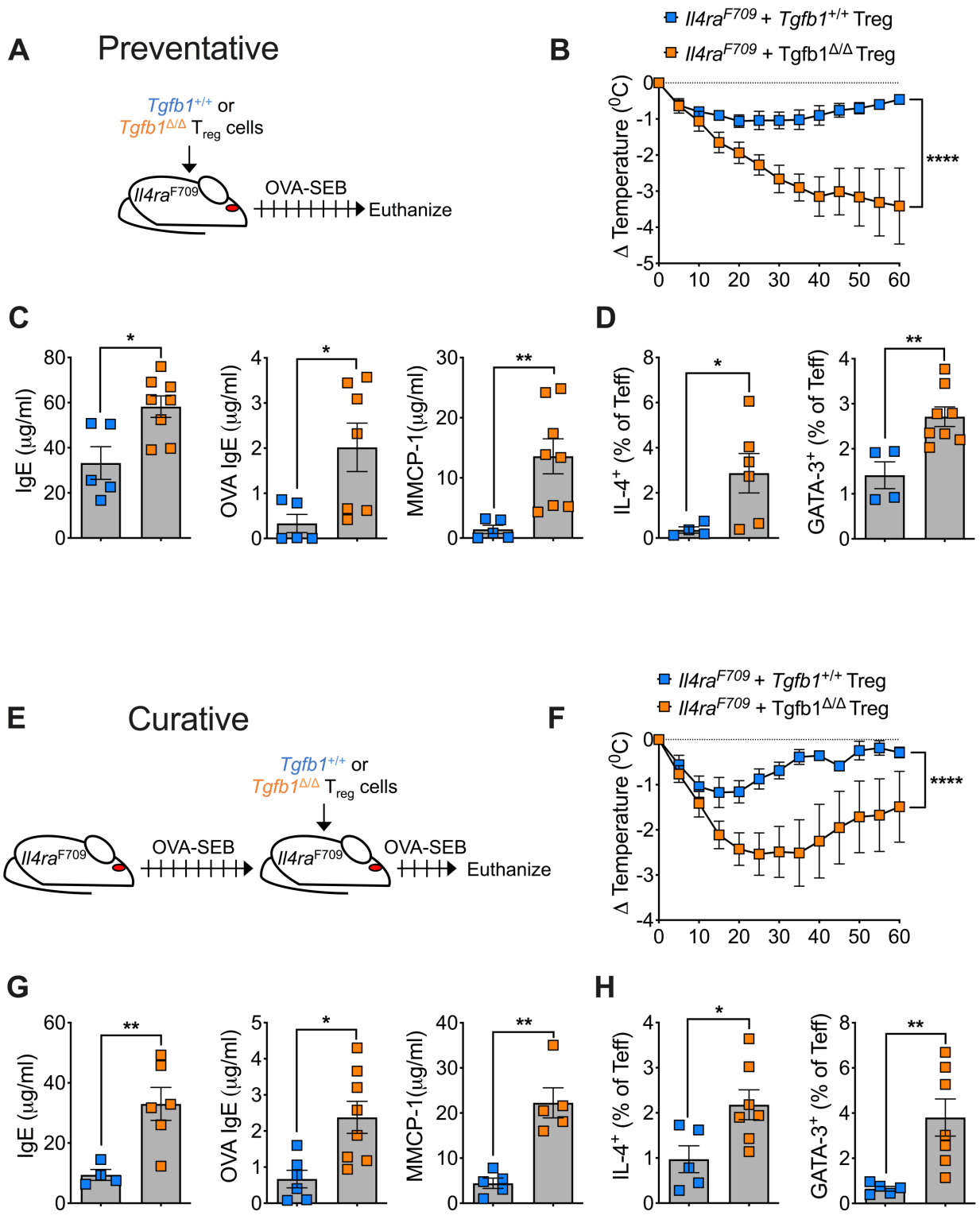
A
Preventative
Tgfb1+/+ or
Tgfb1Δ/Δ Treg cells
Il4raF709
OVA-SEB
Euthanize
B
Il4raF709 + Tgfb1+/+ Treg
Il4raF709 + Tgfb1Δ/Δ Treg
Δ Temperature (0C)
****
C
IgE (μg/ml)
OVA IgE (μg/ml)
MMCP-1(μg/ml)
D
IL-4+ (% of Teff)
GATA-3+ (% of Teff)
E
Curative
F
G
H

Fig. S7. Humoral autoimmunity in *Foxp3*^{YFPCre}*Tgfb1*$^{\Delta/\Delta}$ mice. Related to Figure 7. (A to C) Heat map representation of serum IgM (A), IgA (B) and IgE (C) autoantibodies in *Foxp3*^{YFPcre}, *Foxp3*^{YFPcre}*Tgfb1*$^{\Delta/+}$, and *Foxp3*^{YFPcre}*Tgfb1*$^{\Delta/\Delta}$ littermate mice. Each column number represents an independent mouse. Statistical tests: R package 'limma' and multiple comparisons corrections adjusted to $p<0.05$ (A to C). Data representative of at least two independent experiments with 5 mice per group.

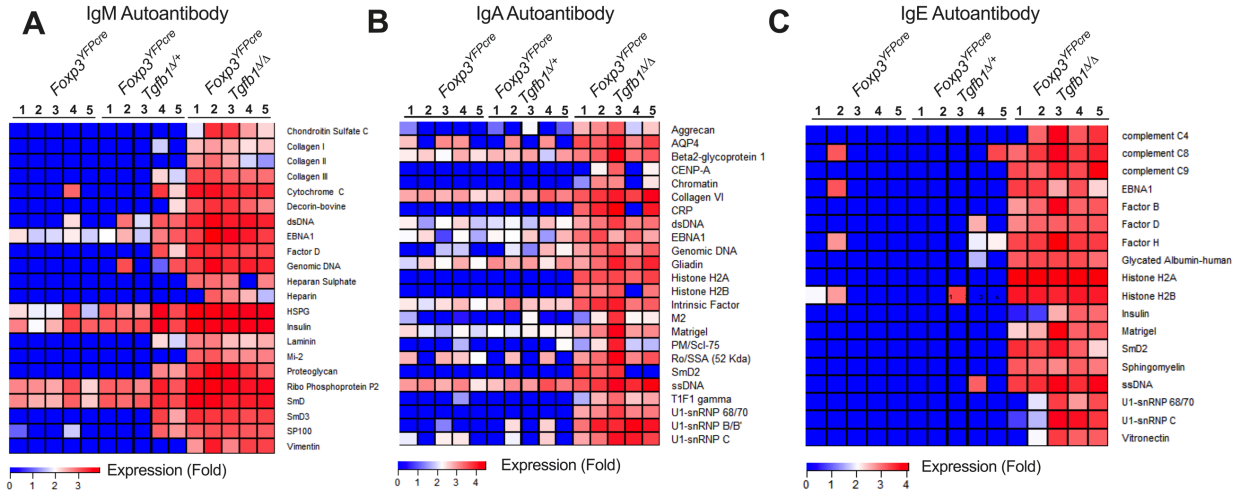

Table S1. Demographic characteristics of Study Subjects. Related to Figure 1.

	Gender	Age (years)	Phenotype
Healthy Controls	F	6	HC
	F	7	HC
	F	8	HC
	M	2	HC
	M	11	HC
	F	6	HC
Atopic Controls	M	4	AD
	M	17	AR
	F	4	AR, AD
	F	1	AD
	M	12	AS, AR
	F	14	AS, AR
	F	19	AR
	M	7	AS, AR
	M	20	AS, AR, AD
	F	8	AR, AD
	M	14	AS, AR
	M	12	AS, AD
	M	14	AS, AD
	M	7	AS
	M	10	AS
	M	11	AR, AD
	M	9	AR
	M	10	AR
	M	12	AS
	F	8	AS, AR, AD
	F	17	AS, AR
	M	14	AS, AR, AD
	M	15	AS, AR
	M	9	AS, AR
Food Allergic Subjects	M	14	FA (peanut, milk, tree nuts, seafood), AS, AR
	M	14	FA (peanut, tree nuts), AS, AR
	M	5	FA (peanut, tree nuts, egg, shellfish), AS, AR, AD
	F	10	FA (peanut, tree nuts, egg, salmon, trout, anchovy)

	F	2	FA (peanut, wheat, barley, rye, oat, almond), AS, AR, AD
	M	1	FA (peanut, milk, soy, egg, wheat, tree nuts, sesame), AD
	M	13	FA (peanut, egg)
	M	14	FA (peanut, tree nuts), AS, AR
	M	1	FA (peanut, tree nuts)
	F	15	FA (peanut, tree nuts, egg, shellfish, lentils, peas, sesame, chickpeas), AS, AR
	F	2	FA (peanut, milk, egg, soy, tree nuts), AD
	F	11	FA (peanut, tree nuts), AR, AD
	F	3	FA (peanut, tree nuts, soy, sesame, sunflower seed, poppy seed, chickpea, lentil, lima bean, fish, shellfish), AR, AD
	F	7	FA (egg, milk, tree nuts)
	M	14	FA (peanut, egg, tree nuts, fish, shellfish), AS, AR
	M	5	FA (egg), AR
	F	17	FA (avocado), AR, AD
	F	3	FA (peanut, tree nuts), AD
	M	7	FA (peanut, tree nuts, egg), AS, AR
	F	10	FA (peanut), AS, AR
	M	11	FA (peanut, tree nuts)

HC: healthy control; FA: food allergy; AS: asthma; AR: allergic rhinitis; AD: atopic dermatitis