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Supplementary Materials for

Divergent T follicular helper cell requirement for IgA and IgE production to peanut during allergic sensitization

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

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Methods

Serum collection. Collected blood was incubated at RT for 1 hour and the resulting clot removed. Samples were centrifuged for 10 minutes at 1500 *g* and the serum were collected and stored at -80 °C prior to analyses.

Enzyme-linked immunospot (ELISpot) assay. 96 well Elispot multiscreen HTS plates (Merck Millipore) were coated with 20 µg/mL crude PN extract (Greer Laboratories) in PBS at 4 °C overnight. The plates were washed in sterile PBS, blocked with complete media (RPMI with 10% fetal bovine serum, 2% penicillin/streptomycin, 2mM L-glutamine, 25mM Hepes buffer and 55µM β-mercaptoethanol) for 2h at 37 °C. 1 x 10^6 cells from MLN or PP of mice 8 to 10 days after 2^{nd} intragastric immunizations with peanut and cholera toxin were added in duplicates and diluted 1:2 in three serial dilutions and cultured at 37 ° C for 20-22h. AP-conjugated goat anti-mouse IgA antibodies (Southern Biotech) were added and incubated for 2h at 37 ° C. Antibody-forming cells were visualized using blue AP substrate kit (Vector Labs) and quantified using an ImmunoSpot analyzer (Cellular Technology Limited).

Immunofluorescence Analysis. Mesenteric lymph nodes from T-*Bcl6*$^{+/+}$ mice and from T-*Bcl6*$^{-/-}$ littermate controls harvested 8 days after PN+CT immunization were mounted in a cryomold with O.C.T. Compound (Tissue-Tek, Sakura), and stored at -80°C prior to sectioning (6µm) and staining. Briefly, frozen sections were blocked with 10% normal rat serum in 1% BSA, 0.1% Tween 20 PBS and stained with the following antibodies from Biolegend unless otherwise indicated: B220 (RA3-6B2),TCRβ (H57-597), GL7, CD45.2 (104), IgD (11-26c.2a), IgA (mA-6E1, eBioscience), CD35 (8C12, BD Pharmingen). The images were acquired immediately after staining with the Nikon eclipse Ti microscope using 10x objectives.

Cellular analyses by flow cytometry. For T cells and germinal center B cell analyses, mice were sacrificed 8 days after a single oral dose of PN and cholera toxin. For plasmablast analyses, mice were sacrificed 10 days after two oral dose of PN and cholera toxin. PP and MLN were harvested and homogenized and the cells were washed and resuspended in 2% Fetal Bovine Serum in Phosphate Buffered Saline before being incubated with fluorochrome-conjugated antibody cocktail for 30 min on ice. Anti-mouse F4/80 (BM8), B220 (RA3-6B2), TCRβ (H57-597), CD4 (RM4-5), GL7 (GL7), CD19 (6D5), IgD (11-26c.2a), CXCR5 (L138D7), PD-1 (RMP1-30), CD44 (IM7), IL-17 (TC11-18H10.1) antibodies were purchased from Biolegend. Anti-mouse CD95 (Jo2), IgG1 (A85-1) and CD16/32 (2.4G2) antibodies were purchased from BD Biosciences. Biotinylated peanut agglutinin was purchased from Vector Laboratories. Anti-mouse FOXP3 (FJK-16s), RORγT (AFKJS-9) and IgA (mA-6E1) antibodies and LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit were purchased from Thermofisher scientific. All flow cytometry samples were scanned on BD FACS LSRII (BD Biosciences) or MACSQuant (Miltenyi) flow cytometers and analyzed by FlowJo software (Version 9.3.2, TreeStar).

Intracellular cytokine staining. For intracellular cytokine staining, cells were stimulated with PMA (50ng/mL) and ionomycin (1μg/mL) for 90 mins in complete RPMI (10% FBS, 25mM Hepes, 2-Mercaptoethanol and 1% Penicillin and Streptomycin) at 37^{o}C before incubating with Golgistop (BD Biosciences) for another 3 hours. After surface staining, cells were fixed and permeabilized according to manufacturer's instruction using BD Fixation/Permeabilization Solution Kit (BD Biosciences). Intracellular staining of cytokines was performed before analysis by flow cytometry as described above.

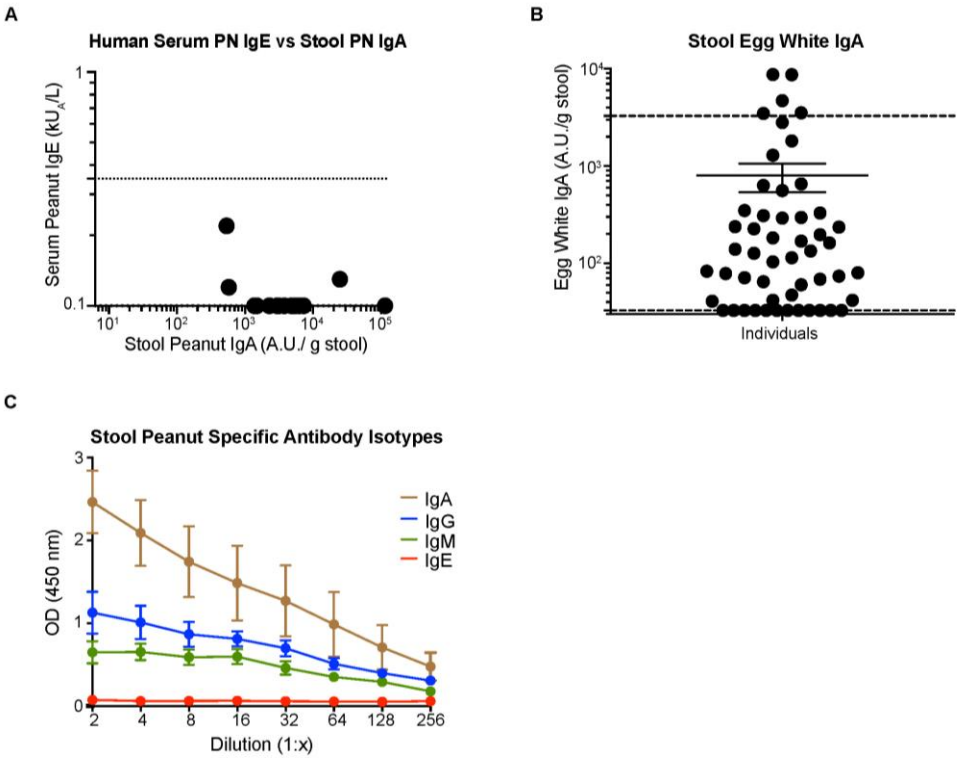

Figure S1. Healthy humans make stool IgA but not serum IgE against food antigens.

A) ELISA quantification of stool peanut-specific IgA (PN IgA) levels in correlation with serum PN IgE concentration detected by ImmunoCAP in healthy human (n=27) stool samples normalized by weight. Dotted line denote serum PN IgE levels cutoff for allergic patients (0.35 kU$_A$/liter). (B) ELISA quantification of stool egg white-specific IgA levels in correlation in healthy human (n=27) stool samples normalized by weight. Dotted lines denote interdecile range. Detection limit of 6.25 arbitrary units (A.U.) relative to a standard of pooled stools with (A) high PN IgA and (B) egg white IgA is the intersection point of X and Y axes. (C) ELISA dilution curves of mean stool PN Ig isotypes in healthy human (n=27) stool samples normalized by weight. All four isotypes from a single sample were assessed on the same plate for comparison of optical density (OD). Error

bars indicate SD.

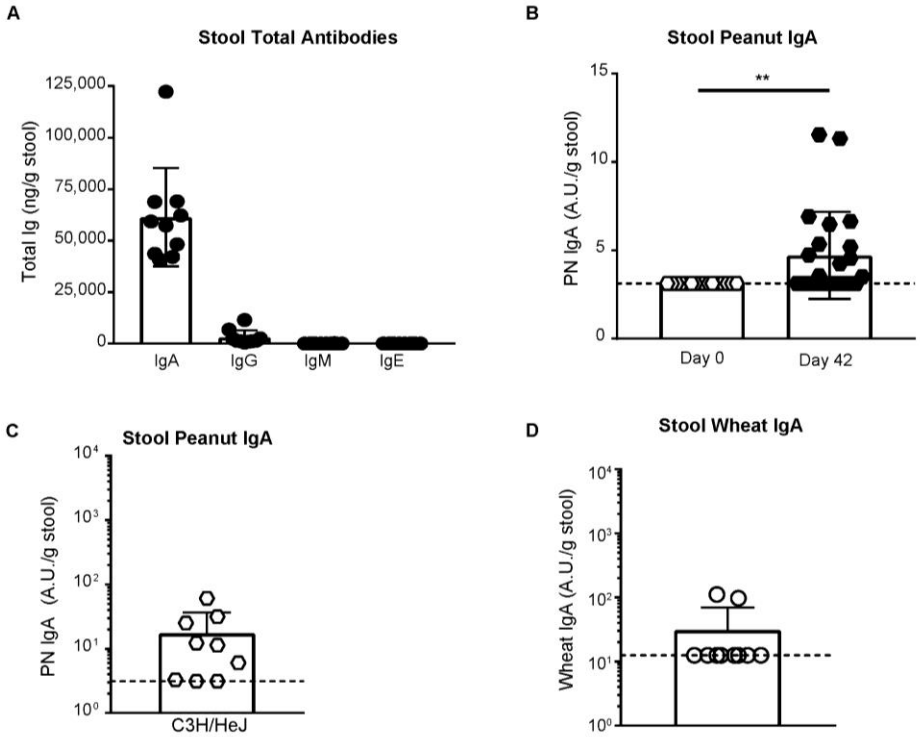

Fig. S2. Daily exposure to food induces minimal food-antigen reactive IgA.

(A) ELISA quantification of total stool antibodies by isotype in wildtype C57BL/6 (WT) mice plotted in linear scale. (B) ELISA quantification of peanut-specific IgA (PN IgA) in stool of 8-week-old adult WT mice exposed to PN for 6 weeks versus naïve mice plotted on linear scale. Dotted line indicates detection limit of assay at 3.12 arbitrary units (A.U.) relative to standards. (C) ELISA quantification of PN IgA in stool of 8-week old adult C3H/HeJ mice exposed to PN for 6 weeks. Dotted line indicates baseline level of PN IgA prior to peanut exposure and detection limit of assay at 3.12 arbitrary units relative to standards. (D) ELISA quantification of wheat-specific IgA in stool of 8-week-old adult WT mice. Dotted line indicates detection limit of assay at 12.5 A.U. relative to standards. Medians at each time point were compared with Mann-Whitney U test, whereby (**) indicates significant differences with p-value <0.01. Error bars indicate SD. Pooled data from 2-3 independent experiments with minimum of 4 mice each.

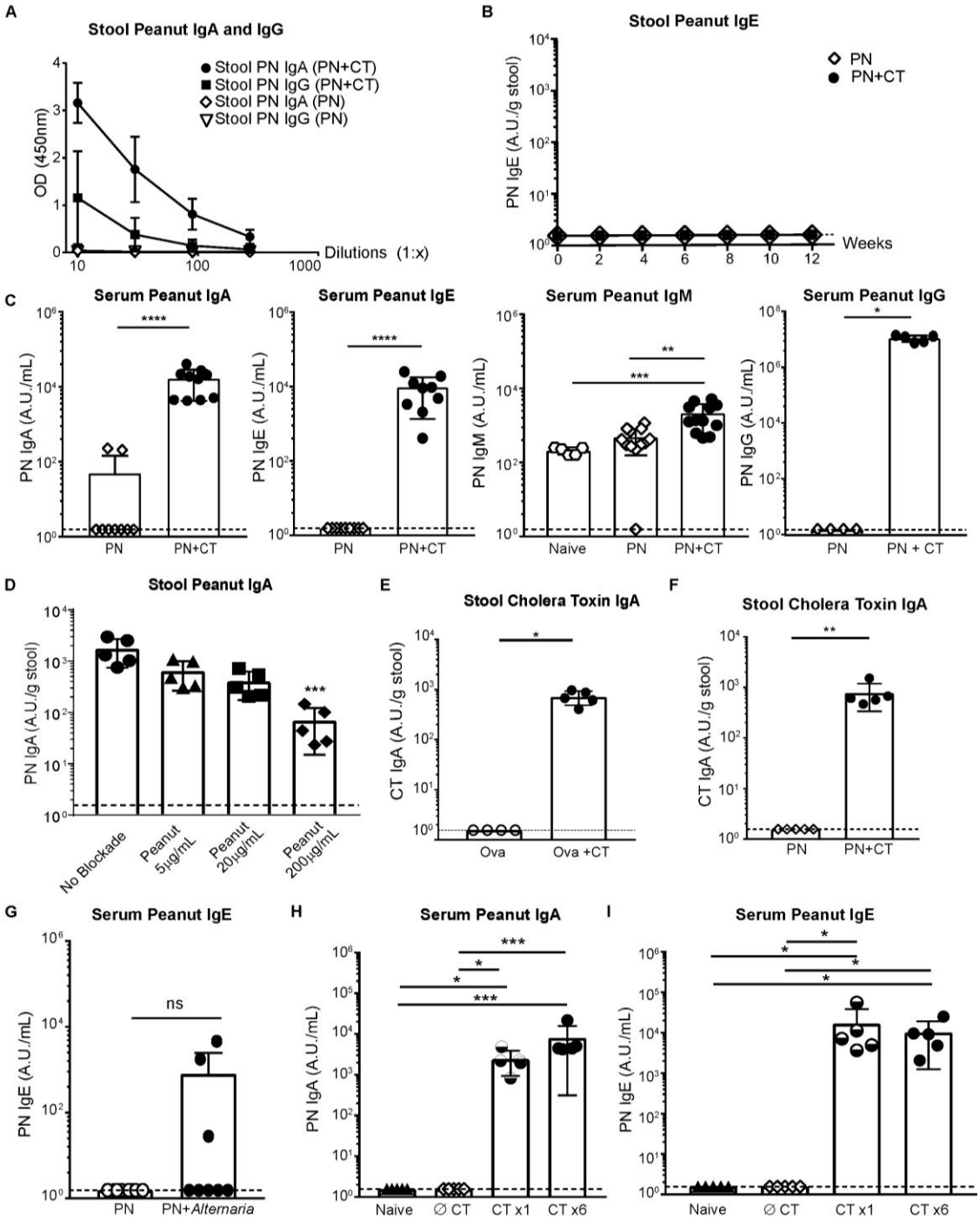
A
Stool Peanut IgA and IgG
Stool PN IgA (PN+CT)
Stool PN IgG (PN+CT)
Stool PN IgA (PN)
Stool PN IgG (PN)
OD (450nm)
Dilutions (1:x)
B
Stool Peanut IgE
PN
PN+CT
PN IgE (A.U./g stool)
Weeks
C
Serum Peanut IgA
Serum Peanut IgE
Serum Peanut IgM
Serum Peanut IgG
PN IgA (A.U./mL)
PN IgE (A.U./mL)
PN IgM (A.U./mL)
PN IgG (A.U./mL)
PN
PN+CT
Naive
PN + CT
D
Stool Peanut IgA
PN IgA (A.U./g stool)
No Blockade
Peanut 5µg/mL
Peanut 20µg/mL
Peanut 200µg/mL
E
Stool Cholera Toxin IgA
CT IgA (A.U./g stool)
Ova
Ova +CT
F
Stool Cholera Toxin IgA
CT IgA (A.U./g stool)
PN
PN+CT
G
Serum Peanut IgE
PN IgE (A.U./mL)
ns
PN
PN+Alternaria
H
Serum Peanut IgA
PN IgA (A.U./mL)
Naive
∅ CT
CT x1
CT x6
I
Serum Peanut IgE
PN IgE (A.U./mL)
Naive
∅ CT
CT x1
CT x6

Fig. S3. Specific IgA, IgG, IgM and IgE to peanut are present in the serum of mice exposed to peanut and CT.

(A) ELISA dilution curves of stool peanut-specific IgA (PN IgA) and PN IgG, measured on the same plate, from wildtype (WT) mice immunized with peanut alone (PN) or peanut with cholera toxin (PN+CT) intragastrically. (B) ELISA quantification of peanut-specific IgE (PN IgE) in stool or (C) PN-specific antibodies in serum by isotypes in WT mice immunized with PN or PN+CT. Median of each group were compared using Mann-whitney U test or Kruskal-Wallis analysis (> 2 groups) and Dunn's post-hoc test. (D) Quantification of PN-specific IgA with competitive ELISA showing peanut dose-dependent blockade of stool PN-IgA after incubation with 0, 5, 20 or 200 µg/mL of PN extract. Median of each group was compared against median of control sample (no inhibition) using Kruskal-Wallis analysis and Dunn's post-hoc test. ELISA quantification of cholera toxin (CT) -specific IgA in stool of WT mice immunized with (E) OVA or OVA+CT or (F) PN or PN+CT. (G) ELISA quantification of serum PN IgE in WT mice immunized with PN or PN+*Alternaria* 1 week after 6^{th} immunization. ELISA quantification of (H) PN IgA or (I) PN IgE in serum of naive, ØCT, CTx1 or CTx6 immunized mice 1 week after 6^{th} immunization. Median of each group (> 2 groups) were compared using Kruskal-Wallis analysis and Dunn's post-hoc test. Dotted lines indicate the detection limits of the assays at 1.56 arbitrary units (A.U.) relative to the standard. Data are representative or pooled from 3 independent experiments with 3-5 mice per group. Mice in each group were cohoused littermates. Median of each group was compared between groups with Mann-Whitney U test, unless otherwise stated, whereby (*), (**),(***) or (****) indicates significant differences with p-value <0.05, <0.01, <0.001 or <0.0001, respectively. Ns denotes not significant while error bars indicate SD.

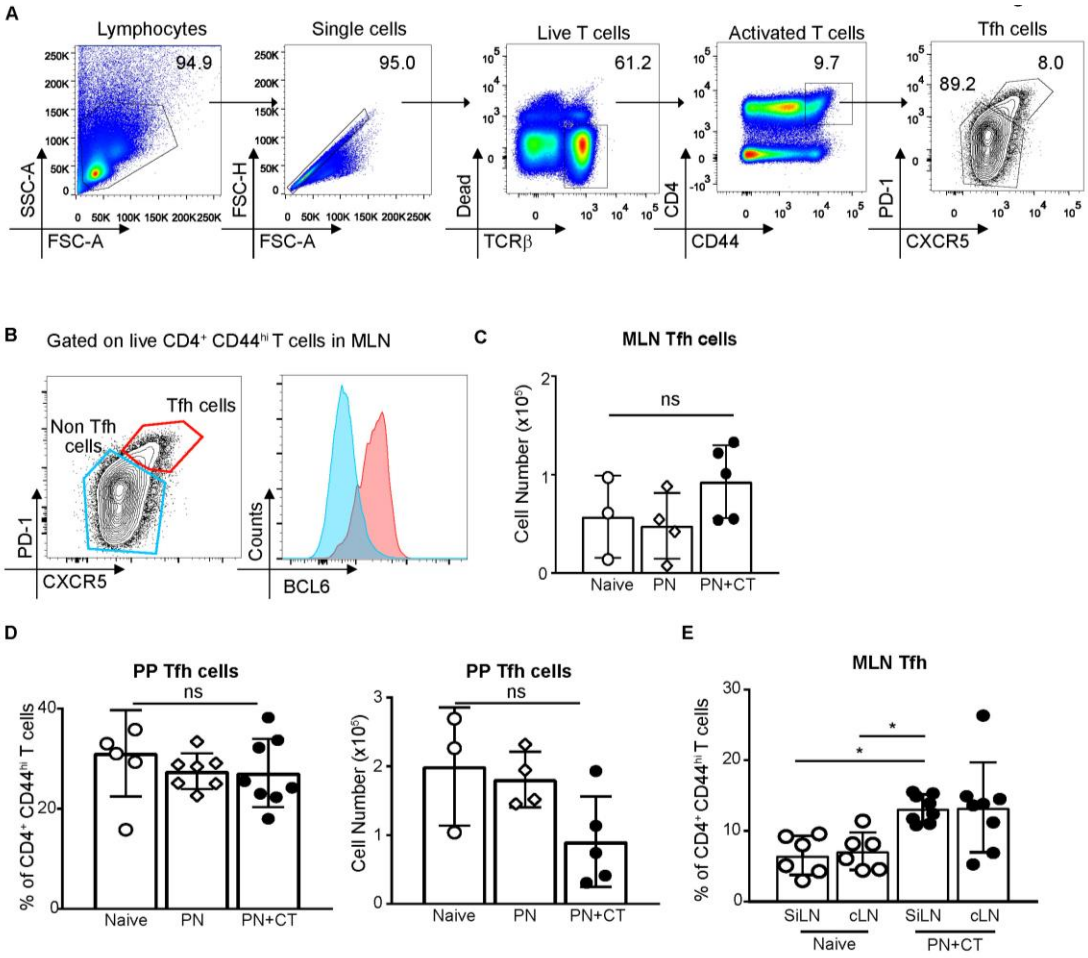

Fig. S4. Exposure of mice to peanut and cholera toxin does not increase T_{FH} cell numbers in mesenteric lymph nodes and Peyer's patches. (A) Gating strategy of T follicular helper (T_{FH}) cells and (B) Histogram showing BCL6 expression in T_{FH} ($CXCR5^{hi}$ $PD1^{hi}$) and Non-T_{FH} population ($CXCR5^{neg\text{-}lo}$ $PD1^{neg\text{-}lo}$) in mesenteric lymph nodes (MLN). (C) Numbers of T_{FH} cells in the MLN. (D) Frequencies and numbers of T_{FH} cells in the Peyer's patches (PP). (E) Frequencies of T_{FH} cells in small intestinal MLN (SiLN) or colonic MLN (cMLN) isolated from WT mice 8 days after immunization with 1 dose of PN or peanut and cholera toxin (PN+CT). Representative data from 3 independent experiments with minimum of 3 mice per group. Median of each group (> 2 groups) was compared using Kruskal-Wallis analysis and Dunn's post-hoc test whereby (*) indicates

significant differences with p-value < 0.05 and ns denotes not significant while error bars indicate SD.

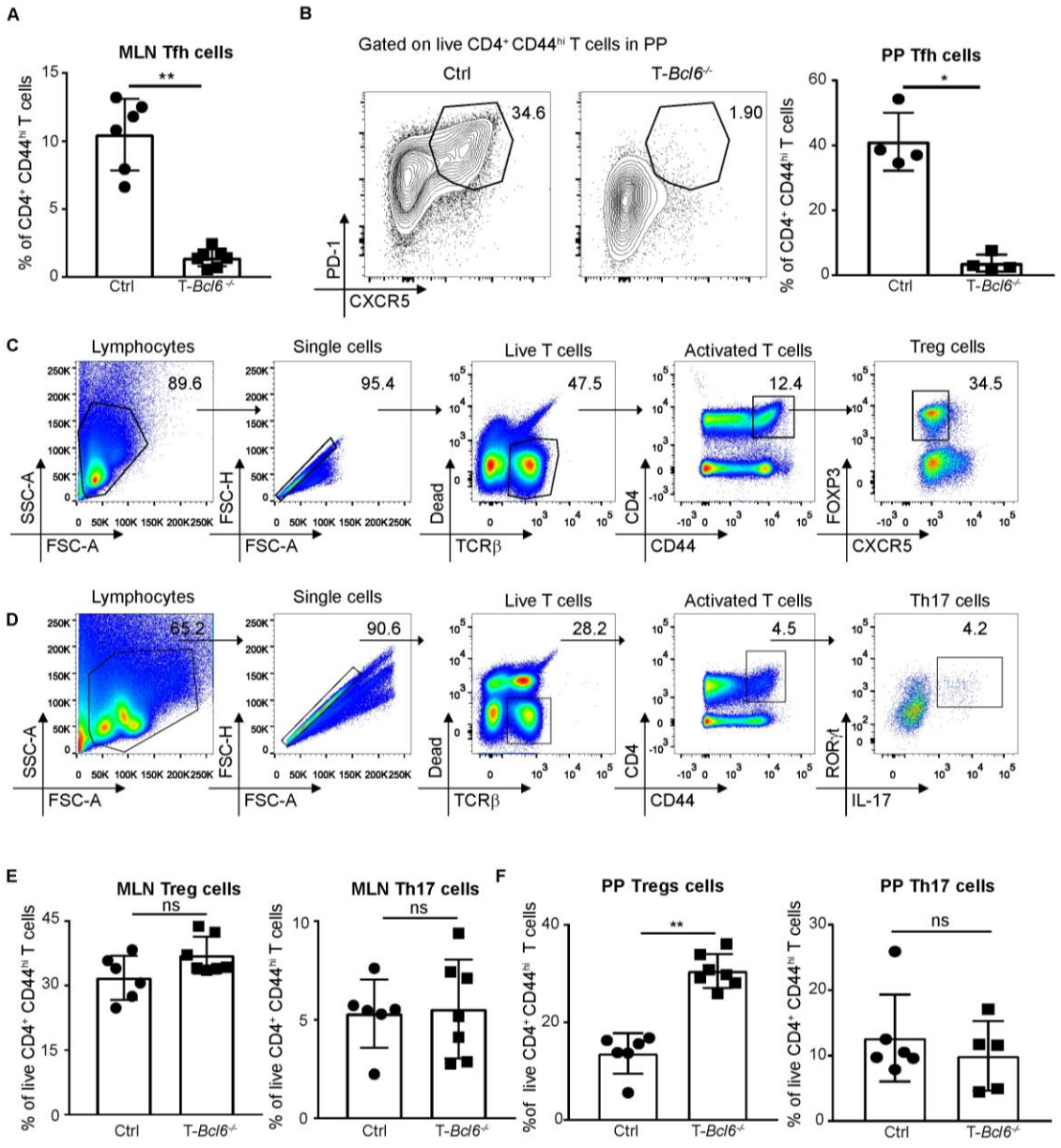

Fig. S5. T_{FH} but not T_{reg} or T_H17 cells were decreased in MLNs and PPs of T-*Bcl6*$^{-/-}$ mice. (A) Frequencies of T follicular helper (T_{FH}) cells in mesenteric lymph nodes (MLN) or (B) Peyer's patches (PP) of control (*Bcl6*$^{flox/flox}$) (Ctrl) or T-*Bcl6*$^{-/-}$ (*CD4*^{cre}*Bcl6*$^{flox/flox}$) cohoused littermates 8 days after 1 peanut with cholera toxin (PN+CT) immunization. T_{FH} cells were gated on CD4$^+$ CD44^{hi} live activated T cells (TCRβ$^+$). Gating strategy of (C) T_{regs} or (D) T_H17 cells in MLN of control mice. Frequencies of T_{regs} and T_H17 cells in (E) MLN and (F) PP of T-*Bcl6*$^{-/-}$ (*CD4*^{cre}*Bcl6*$^{flox/flox}$) cohoused littermates 8 days after 1

peanut with cholera toxin (PN+CT) immunization. Representative data from 3 independent experiments with at least 3 mice per group. Median of each group was compared using Mann Whitney-U test whereby (*) or (**) indicates significant differences with p-value < 0.05 or <0.01 respectively. Ns denotes not significant while error bars indicate SD.

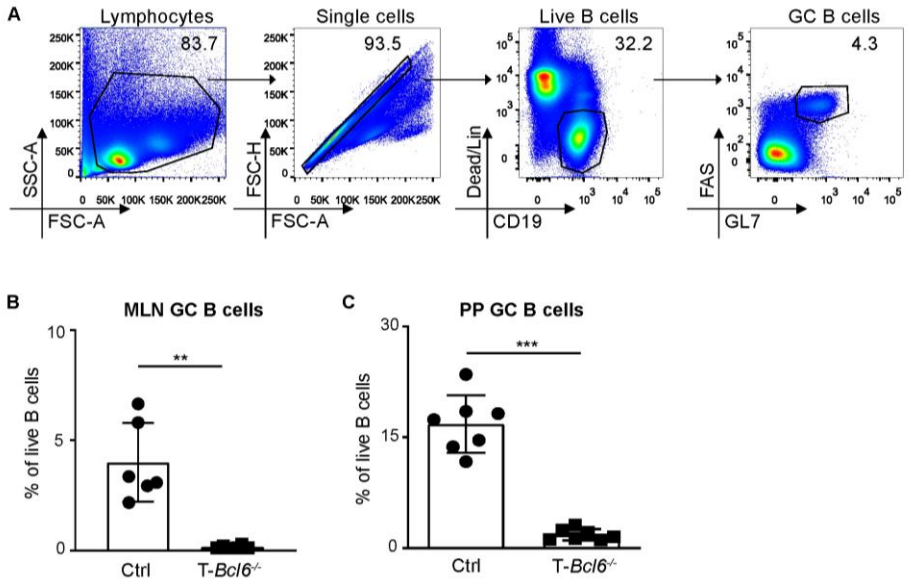

Fig. S6. GC B cells are almost absent in T-*Bcl6*$^{-/-}$ mice. (A) Gating strategy of germinal center (GC B cells) in mesenteric lymph nodes (MLN) of control *Bcl6*$^{flox/flox}$ (Ctrl) mice. Lin is defined as TCRβ^{+}, NK1.1^{+}, CD14^{+}, CD4^{+}. Frequencies of GC B cells in (B) MLN and (C) PP of Ctrl or T-*Bcl6*$^{-/-}$ (*CD4*^{cre}*Bcl6*$^{flox/flox}$) cohoused littermates 8 days after 1 peanut with cholera toxin (PN+CT) immunization. Representative data from 3 independent experiments with at least 3 mice per group. Median of each group was compared using Mann Whitney-U test whereby (**) or (***) indicates significant differences with p-value <0.01 or <0.001 respectively. Ns denotes not significant while error bars indicate SD.

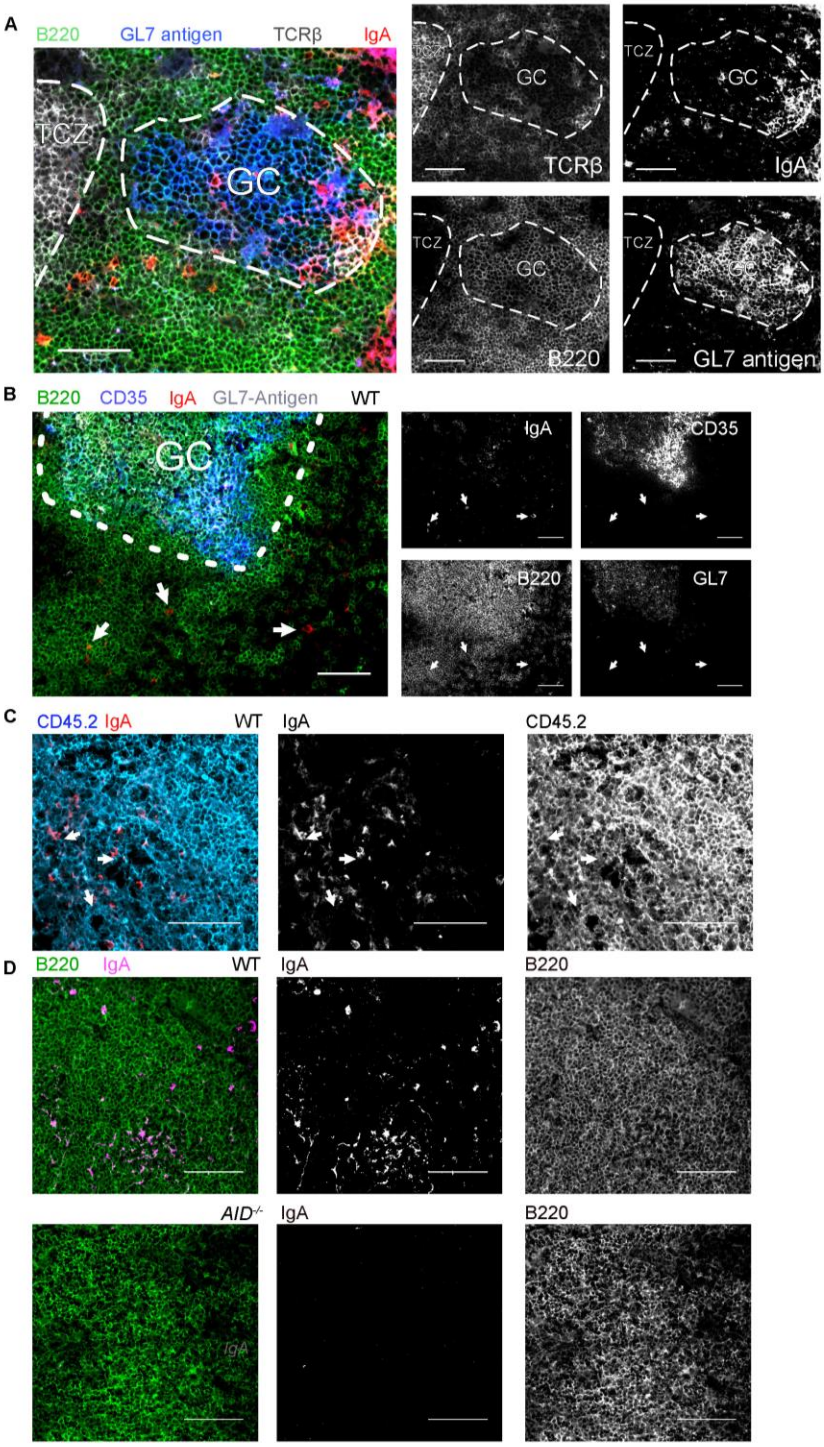

Fig. S7. IgA$^+$ B cells can be found colocalizing with T cells outside GCs. (A) Immunofluorescence images of IgA$^+$ B cells (B220$^+$ IgA$^+$) and colocalization with T cells (TCRβ) outside germinal centers (GCs) (B220$^+$ GL7$^+$) in MLN of wildtype (WT) mice. GC and T cell zones (TCZ) are demarcated with dotted lines. (B) Immunofluorescence

images of IgA^+ B cells ($B220^+$ IgA^+) outside germinal center (GC) ($B220^+$ $GL7^+$) with stromal cell marker (CD35) in MLN of WT mice. GC is demarcated with dotted line while arrows highlight IgA^+ B cells outside GC. (C) Immunofluorescence images of IgA^+ cells and lymphocyte marker (CD45.2) in MLN of WT mice. (D) Immunofluorescence images of IgA^+ B cells ($B220^+$) in MLN of WT and Activation-induced cytidine deaminase knockout (*AID*$^{-/-}$) mice. Representative images of 3 independent experiments. Scale bars represent 100µm.

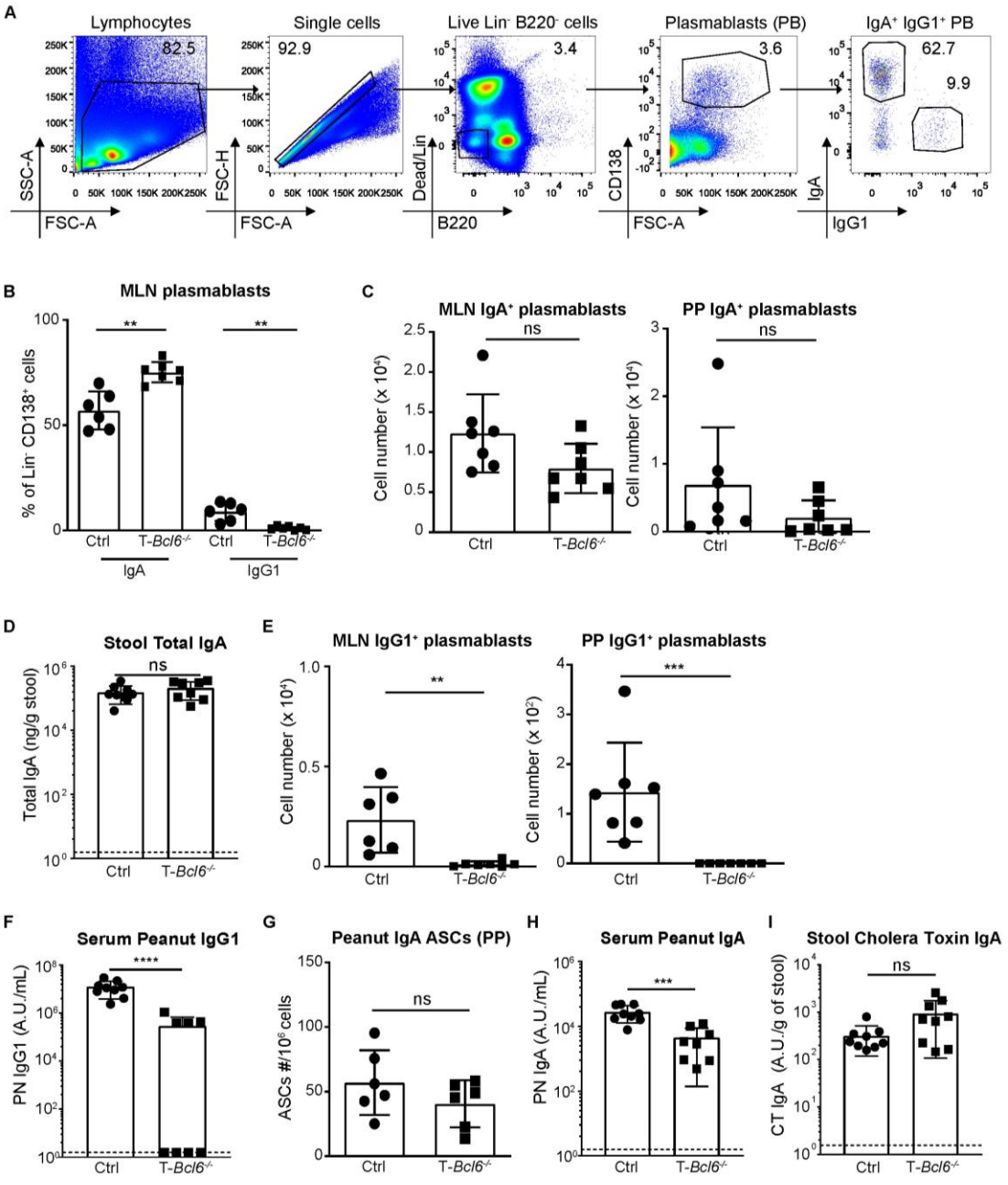

Fig. S8. IgG1$^+$ but not IgA$^+$ plasmablasts are reduced in T-*Bcl6*$^{-/-}$ mice. (A) Gating strategy of IgA and IgG1 plasmablasts in the mesenteric lymph nodes (MLN) of control *Bcl6*$^{flox/flox}$ (Ctrl) mice. Lin is defined as TCRβ$^+$, NK1.1$^+$, CD14$^+$, CD4$^+$. (B) Frequencies of IgG1 and IgA plasmablasts and (C) numbers of IgA plasmablasts in the MLN and Peyer's patches (PP) of Ctrl or T-*Bcl6*$^{-/-}$ (*CD4*^{cre}*Bcl6*$^{flox/flox}$) mice 8 days after 2 weekly immunizations with peanut and cholera toxin (PN+CT). (D) ELISA quantification of total

IgA in stool of Ctrl and T-*Bcl6*$^{-/-}$ cohoused littermates. Dotted line denote detection limit of assay with reference to IgA standard. (E) Numbers of IgG1 plasmablasts in the MLN and PP of Ctrl or T-*Bcl6*$^{-/-}$ mice 8 days after 2 weekly immunizations with peanut and cholera toxin (PN+CT). (F) ELISA quantification of serum PN IgG1 in Ctrl or T-*Bcl6*$^{-/-}$ cohoused littermates 1 week after 6th immunization with PN+CT. (G) Enumeration of peanut-specific IgA (PN IgA) antibody-secreting cells (ASCs) in PP of Ctrl or T-*Bcl6*$^{-/-}$ cohoused littermates by ELISPOT 8 days after 2 weekly immunizations with peanut and cholera toxin (PN+CT). ELISA quantification of (H) Serum PN IgA and (I) Stool cholera toxin-specific IgA (CT IgA) in Ctrl or T-*Bcl6*$^{-/-}$ cohoused littermates 1 week after 6th immunization with PN+CT. Representative (A) or pooled data (B-I) from 2-3 experiments with at least 3 mice. Dotted lines indicate the detection limits of the assays at 1.56 arbitrary units (A.U.) relative to the standard. Median of each group were compared using Mann-whitney U test whereby (**),(***) or (****) indicates significant differences with p-value <0.01, <0.001 or <0.0001, respectively. Ns denotes not significant while error bars indicate SD.

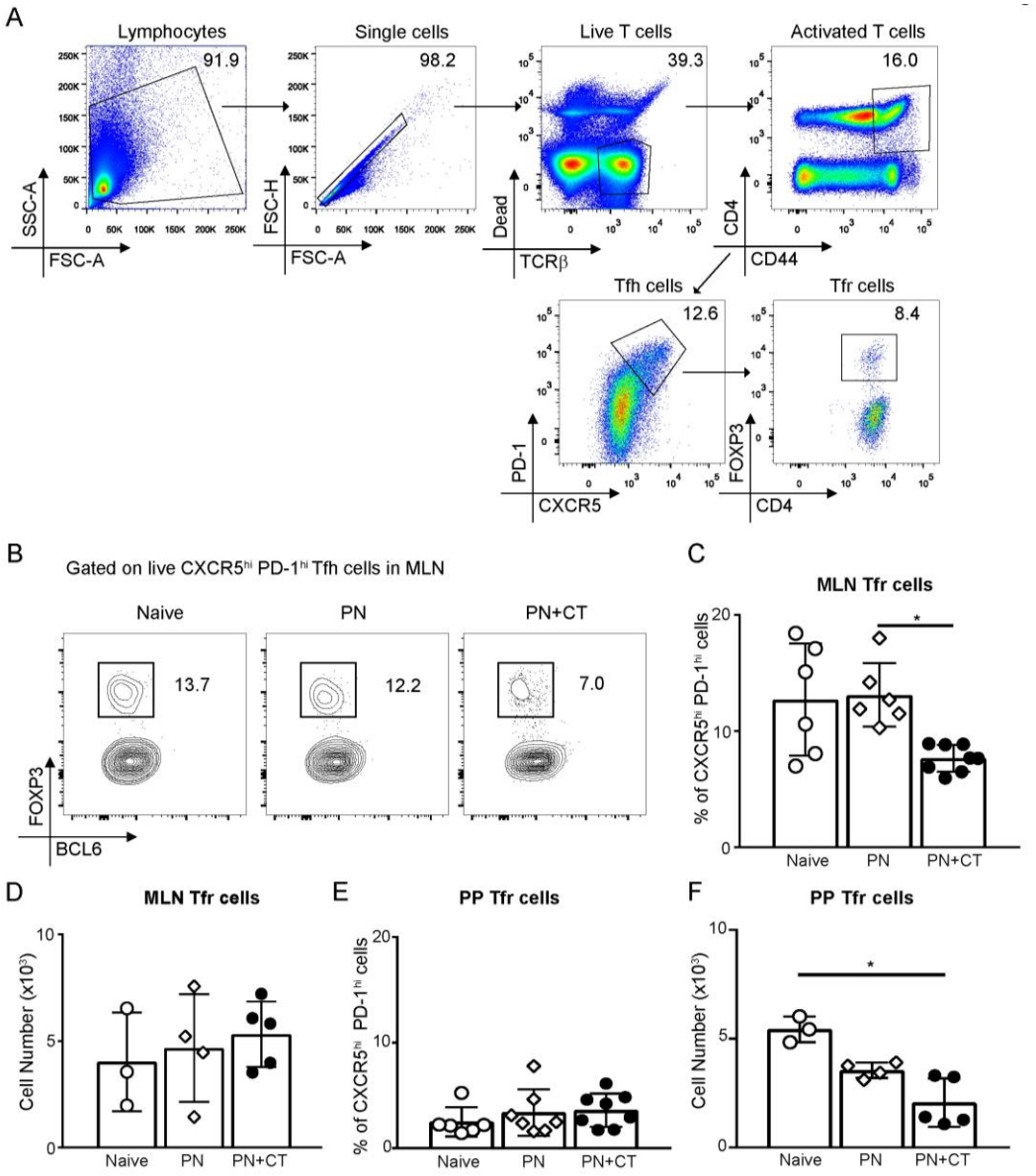

Fig. S9. T_{FR} cell numbers are reduced in PP but not MLN after peanut and cholera toxin exposure. (A) Gating strategy of T follicular regulatory (T_{FR}) cells and T follicular helper (T_{FH}) cells in mesenteric lymph nodes (MLN) of wildtype (WT) mice 1 week after 1 PN+CT immunization. (B) Representative flow plot and frequencies (C, E) and cell numbers (D, F) of Tfr cells in the MLN and Peyer's patches (PP) of WT mice 8 days after immunization with PN+CT. Representative or pooled data (C and E) from 2-3 independent experiments with at least 3 mice in each group. Median of each group was compared using Kruskal-Wallis analysis and Dunn's post-hoc test (*) indicates significant

differences with p-value <0.05 while ns denotes not significant and error bars indicate SD.

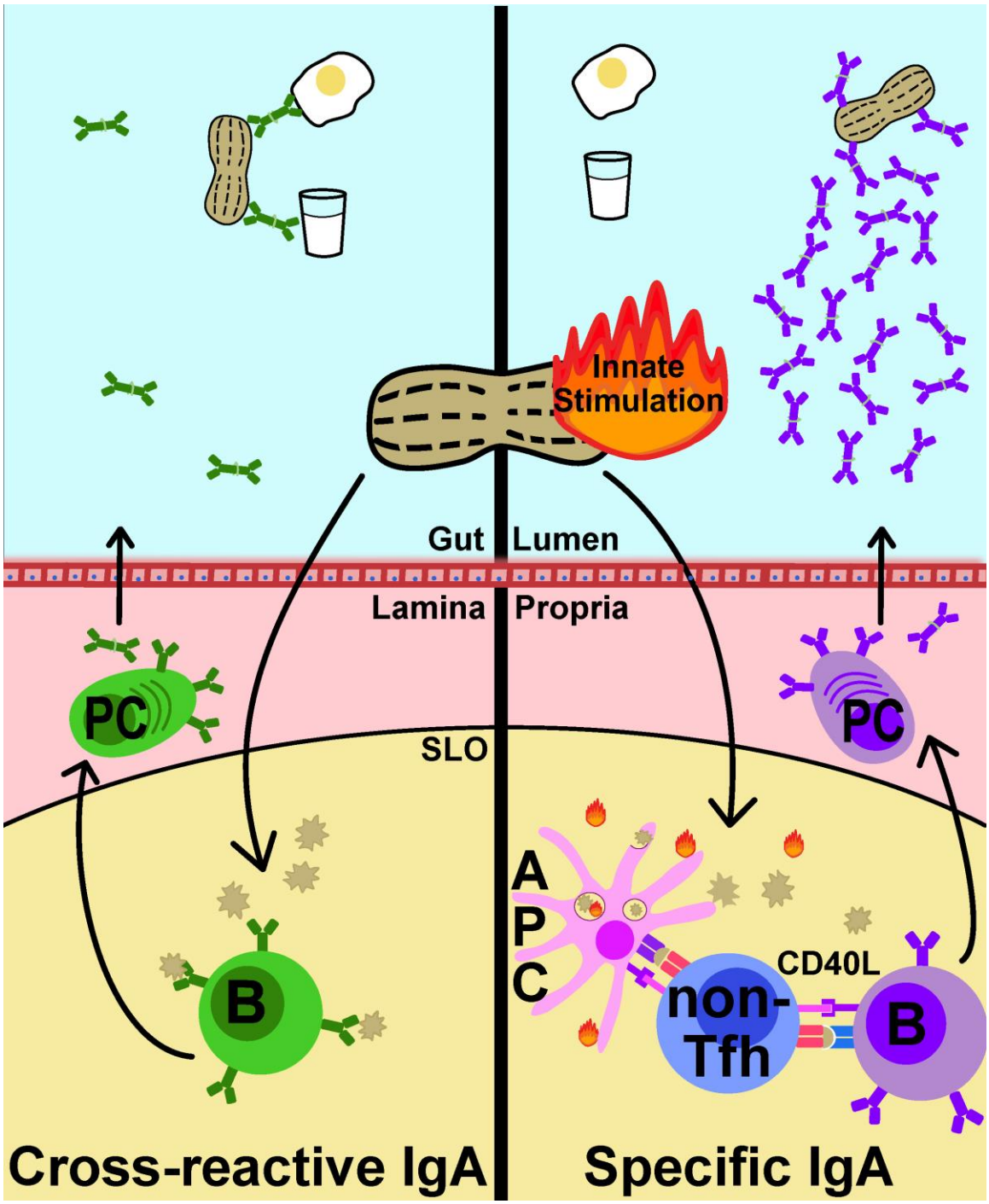

Fig. S10/Graphical Abstract. Model of cross-reactive and specific gut IgA induction to peanut. At steady state, peanut antigens that cross the gut barrier induce cross-reactive IgA (left) in a T cell-independent manner. For illustration purposes, the green antibodies bind to peanut but also egg and milk. In the presence of an innate inflammatory stimulus (right) such as a mucosal adjuvant (depicted as fire), specific IgA

to peanut is made. This requires antigen presentation to T cells. $CD4^+$ T cells and CD40L are required for B cell production of peanut-specific IgA, which does not cross-react with other antigens. However, T_{FH} cells are not required for production of this highly specific IgA. Primed B cells through either path can become IgA plasma cells that reside in the lamina propria and produce gut IgA, in this case binding to subsequent ingestion of peanut. SLO, Secondary lymphoid organs; APC, Antigen presenting cells; non-T_{FH}, $CD4^+$ T cell that is not a T follicular helper cell; B, B cells; PC, plasma cells