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Supplemental Information

Intestinal Microbial Diversity during Early-Life Colonization Shapes Long-Term IgE Levels

Julia Cahenzli, Yasmin Köller, Madeleine Wyss, Markus B. Geuking, and Kathy D. McCoy

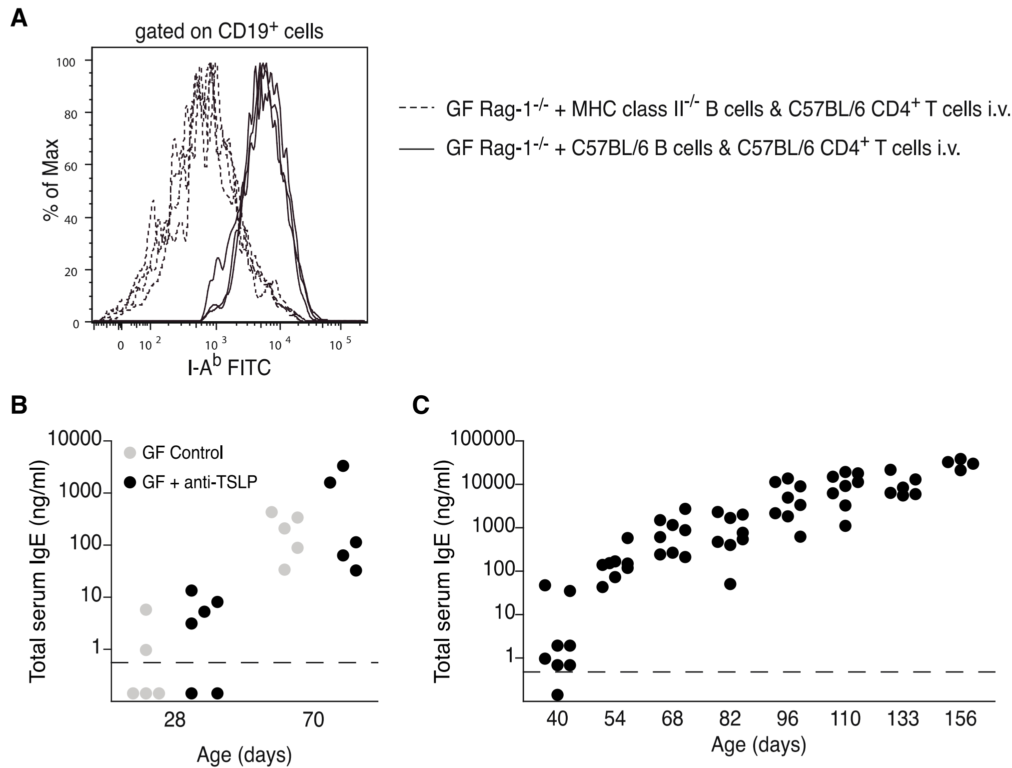

Figure S1. IgE induction in germ-free mice is independent of TSLP, related to Figure 5. (A) Flow cytometric analysis of I-A^{b} expression on B cells from wild-type or I-A$^{b-/-}$ C57BL/6 B cells that were transferred into germ-free *Rag-1*$^{-/-}$ mice in combination with wild-type C57BL/6 CD4^{+} T cells (n=3-4 mice per group). This data excludes cross-contamination with wild-type MHC class II competent B cells from the co-transferred wild-type CD4^{+} T cell preparation. **(B)** Total serum IgE levels were measured longitudinally in germ-free mice left untreated (n=5; grey circles) or administered a neutralizing anti-TSLP monoclonal antibody by intraperitoneal injection two times per week from the time of weaning (n=5-6; black circles) until day 70. **(C)** Total serum IgE levels were longitudinally measured in germ-free TSLP-R$^{-/-}$ mice (n=4-8 per time point). In **(B)** and **(C)** each point represents an individual mouse and the horizontal dotted line delineates the lower limit of detection of the IgE ELISA assay (0.8 ng/ml). Data in panel B are representative of two independent experiments.

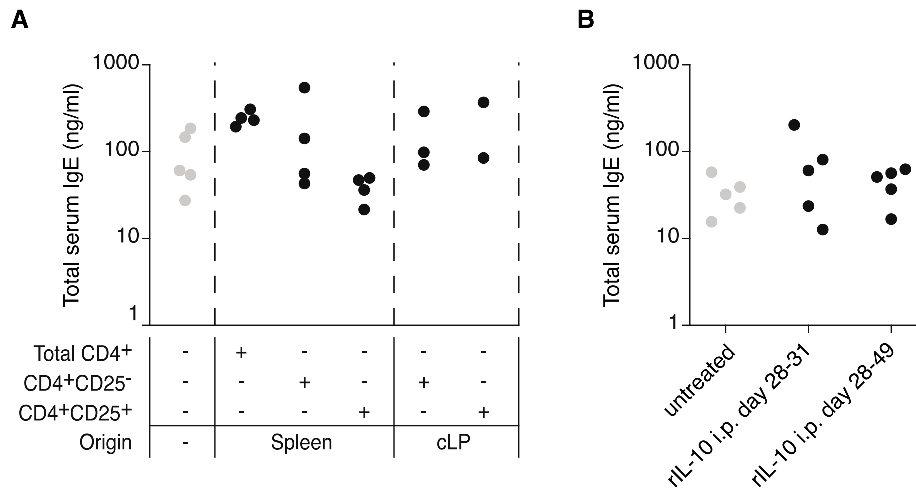

Figure S2. Adoptive transfer of $CD4^+CD25^+$ regulatory T cells or administration of recombinant IL-10 does not inhibit elevated serum IgE levels in germ-free mice, related to Figure 6. (A) Germ-free mice were left untreated (n=5; grey circles) or adoptively transferred at the time of weaning (day 28) with enriched $CD4^+$, $CD4^+CD25^-$ or $CD4^+CD25^+$ (black circles) T cells from spleen (n=4) or colon lamina propria (cLP, n=2-3) of SPF donor mice, as indicated. Total serum IgE levels were measured 70 days post transfer. **(B)** Germ-free mice were left untreated (n=5; grey circles), or administered rIL-10 (n=5; black circles) daily until day 31, or daily until day 31 followed by twice a week until day 49 (n=5; black circles), as indicated. Total serum IgE levels were measured 49 days post-treatment. In (A) and (B) each point represents an individual mouse and the horizontal dotted line delineates the lower limit of detection of the IgE ELISA assay (0.8 ng/ml). Data in **(A)** are representative of two independent experiments.

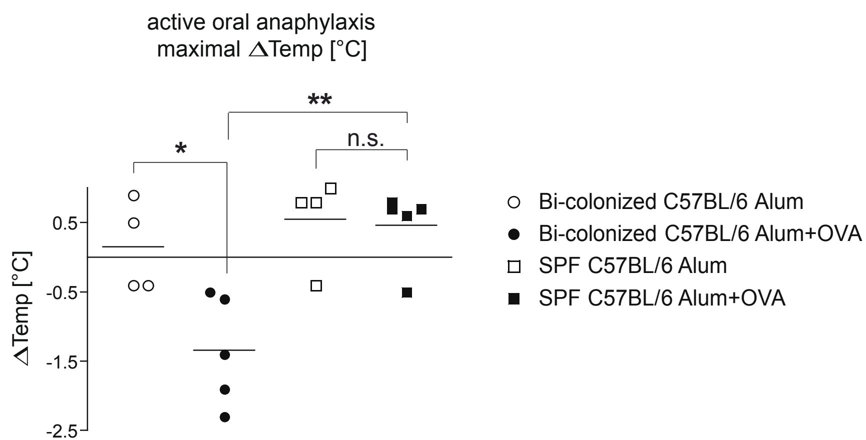

Figure S3. Bi-colonized C57BL/6 mice are more prone to active oral anaphylaxis than SPF C57BL/6 mice, related to Figure 7. Bi-colonized (circles, n=4-5 per group) and SPF (squares, n=4-5 per group) C57BL/6 mice were subcutaneously sensitized with 2mg Alum alone (open symbols) or 50µg OVA in 2mg Alum (closed symbols) and two weeks later orally challenged with 50mg OVA (active oral anaphylaxis). Rectal temperature was measured every 5-10min for 60 min after the oral challenge and the maximal ∆Temperature [°C] is plotted. **P≤0.01, *P≤0.5, and n.s = not significant (P>0.05), unpaired t test.

Table S1. Primer sets used for detection of cytokines, related to Figure 5.

Gene	Primer sequences (5'-3')
Il4	Fwd: TCATCGGCATTTTGAACGAG Rev: CGTTTGGCACATCCATCTCC
Il13	Fwd: GAGCAACATCACACAAGACCAGA Rev: GGCCAGGTCCACACTCCATA
Il10	Fwd: TTTGAATTCCCTGGGTGAGAA Rev: GGAGAAATCGATGACAGCGC
Gapdh	Fwd: CATCAAGAAGGTGGTGAAGC Rev: CCTGTTGCTGTAGCCGTATT

Table S2. Primer sets used for detection of ASF, related to Figure 6.

Species	Primer sequences (5'-3')
ASF 356 *Clostridium sp.*	Fwd: GATGCCTCCTAAGAACCGTATGC Rev: GCGGACGGGTGAGTAACGT
ASF 360 *Lactobacillus acidophilus*	Fwd: AAGTCGAGCGAGCTGAACCA Rev: ATCCCAGACTTTAGGGCAGGTTA
ASF 361 *Lactobacillus murinus*	Fwd: AGGTGGCTATGCTACCGCTTT Rev: CTCAGTTCGGCTACGCATCAT
ASF 457 *Mucispirillum schaedleri*	Fwd: CGTTTGCAAGAATGAAACTCAAA Rev: CACAGCATTATCTCTAACGCCTT
ASF 492 *Eubacterium plexicaudatum*	Fwd: GGGACGACGTCAAATCATCAT Rev: GGCTTCGCTTCCCTCTGTCT
ASF 500 Fusiform-shaped bacterium	Fwd: TCGGACAACTGAAGGGAATCC Rev: CCAGTGCCATGCGACACA
ASF 502 *Clostridium sp.*	Fwd: GAGGGAAGAGAGGGTGACTTAGC Rev: TCTTATGCGGTATTAGCAATCATTTC
ASF 519 *Parabacteroides distasonis*	Fwd: TTGCCGTTGAAACTGGTTGA Rev: GGAGTTCTGCGTGATATCTATGCA

Supplemental Experimental Procedures

Maintenance of gnotobiotic mice

Germ-free mice were maintained at the Farncombe Axenic and Gnotobiotic Unit, McMaster University, Canada or in the Clean Mouse Facility, University of Bern, Switzerland. The absence of bacteria in germ-free mice was routinely confirmed by aerobic and anaerobic culture, Gram-stain, and Sytox DNA stain of cecal contents. The absence of parasites, bacteria, and viruses was independently confirmed every three months by shipping sentinel germ-free mice to Charles River Research Animal Diagnostic Services (MA, USA) or Biolytix AG (Witterswil, CH).

The ASF colony was maintained within a flexible-film isolator to avoid flora diversification. Absence of other bacterial species was confirmed using aerobic and anaerobic culture followed by 16S sequencing of individual colonies as previously described (Geuking et al., 2011).

Isotype-specific ELISA for the detection of serum antibodies

ELISA plates (Nunc) were coated overnight with isotype-specific rat anti-mouse IgE (2.5 μg/ml, clone 6HD5) or 1 μg/ml goat anti-mouse IgA, IgM, IgG1, IgG2a, IgG2c or IgG3 (Southern Biotechnologies) capture antibodies at 4°C in 0.1 M $NaHCO_3$. Plates were washed with 0.05% Tween 20 in PBS and blocked for 2 h at room temperature with 5% bovine serum albumin (for IgE ELISA) or 2.5% bovine serum albumin (for all other isotype-specific ELISAs) in PBS. Blocking buffer was flicked off and serially diluted serum samples or standards (IgE standard clone TIB-141 or standards for IgA, IgM, IgG1, IgG2a, IgG2c and IgG3 from Zymed), were added and incubated for 2 h at room temperature. Captured IgE was detected with biotinylated anti-mouse IgE (clone RIE-4) at 2.5 μg/ml followed by horseradish peroxidase-conjugated streptavidin (Biolegend) at 1 μg/ml. Captured IgA, IgM, IgG1, IgG2a, IgG2c and IgG3 were detected by horseradish peroxidase-conjugated anti-mouse IgA, IgM or IgG (Sigma). ABTS peroxidase solution (0.1 mg/ml 2,2'-azino-bis-[3-ethylbenzthiazidine-6-sulfonic acid], 0.05% H_2O_2 in 0.1 M NaH_2PO_4) was used for the colorimetric reaction. ELISA plates were analyzed at OD 405 nm with a microplate reader (Bio-Rad) and standard curves and antibody concentrations were calculated using Microplate Manager III software (Bio-Rad).

Axenic *in vivo* administration of IL-10.

For IL-10 treatment germ-free C57BL/6 mice (28-days old) were administered recombinant IL-10 (500 ng i.p, R&D) daily on four consecutive days (rIL-10 day 28-31) or daily on four consecutive days followed by twice a week until day 49 of age (rIL-10 day 28-49). For all procedures the germ-free status was confirmed at the end of each experiment.

Axenic adoptive cell transfer

For transfer of $CD4^+$ T cells into 28-day-old germ-free mice, $CD4^+$ (5×10^6), $CD4^+CD25^+$ ($1\text{-}5\times10^6$) or $CD4^+CD25^-$ ($0.2\text{-}1\times10^6$) T cells from spleen or cLP were enriched using CD4 microbeads or the regulatory T cell isolation kit (Miltenyi Biotec). Germ-free status was confirmed at the end of the experiment.

Quantitative real time PCR

DNA impurities were removed from all RNA samples using a DNA-free kit (Ambion). RNA quality and concentration were assessed with RNA 6000 Nano kit (Agilent) on a Bioanalyzer 2100 (Agilent). Synthesis of cDNA was performed with a maximum of 1 μg of RNA (RIN>7), random hexamers and Superscript III RT (Invitrogen Life Technologies) according to the manufacturer's instructions. Gene expression was calculated relative to *Gapdh* (ΔΔCt) with the positive control of the assay set to 1 (for εGLT: B cells stimulated with anti-CD40 and IL-4 for 24-36h; for *Il4*, *Il10* and *Il13*: *in vitro* Th2 polarized $CD4^+$ T cells). Primer sequences (5'- 3') for detection of εGLT: Fwd GCCTGCACAGGGGGCAGAAG; Rev ATGACCCTGGGCTGCCTGGT.

Flow cytometry antibodies

Antibodies and corresponding clones used for flow cytometry were as follows: CD4-PerCP (RM4-5), CD4-FITC (GK1.5), IFN-γ-FITC (XMG1.2), IL-17A-PE (TC11-18H10), IgE-FITC or -biotin (R35-72) conjugated to streptavidin-PE, B220-APC-Cy7 (RA3-6B2), CD25-PE (7D4), and I-A^b-FITC (25-9-17) were purchased from BD PharMingen. CD90.2-APC (30-H12), IgM-PE (R6-60.2), CD19-APC (6D5), CD45RB-PacificBlue (C363-16A), CD3-AlexaFluor700 (17A2) and CD117 (2BA)-APCeFluor780 were purchased from Biolegend.