Supplementary Materials for

Gut IgA enhances systemic IgG responses to pneumococcal vaccines through the commensal microbiota

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This document includes:

Supplementary Materials and Methods

Human subjects

The age range of donors from the NYC cohort was from 22 to 61 years (median 34 years) and 66.67% were females. Exclusion criteria for IGAD patients in the NYC cohort included serum IgA > 7 mg/dl, progression to common variable immunodeficiency, recent immunomodulatory treatments such as steroids or biologics targeting cytokines, antibiotics use within six weeks and surgical procedures likely to affect humoral immunity such as splenectomy or intestinal resection. IGAD responders and non-responders to pneumococcal vaccination were defined as IGAD patients with a presence or absence, respectively, of protective serum IgG against more than 4 serotypes of 14 total after administration of Pneumovax23. Two IGAD patients determined to have an inadequate antibody response after receiving Pneumovax23 were immunized with Prevnar13 at a later date. However, neither of these two patients showed an increase in the number of protective IgG titers against pneumococcal serotypes in subsequent blood work. Compared to IGAD vaccine responders, a higher percentage of IGAD vaccine non-responders had a concomitant IgG2 or IgG4 subclasses deficiency (**Table S2**). Of note, IgG2 is the human equivalent of mouse IgG3 and mediates humoral immunity against PPS in humans (*68*, *69*).

Following the conclusion of this study, the European Society for Immunodeficiencies revised the diagnostic criteria for selective IgA deficiency (https://esid.org/Working-Parties/Clinical-Working-Party/Resources/Diagnostic-criteria-for-PID2#Q7). According to these new criteria, a patient with selective IgA deficiency must have normal serum IgG and normal IgG responses to vaccination. While acknowledging these new diagnostic criteria, it may be worth considering that IgA deficient patients with impaired IgG responses to polysaccharides, including

pneumococcal vaccines, remain poorly characterized. These patients, defined in our study as IGAD-NRs, may be part of a large spectrum of biological conditions in which IgA deficiency induces a progressive alteration of the IgG response. Selective IgA deficiency as defined by the new diagnostic criteria could stand at one extreme of this spectrum of IgA disorders, whereas common variable immunodeficiency progressing from selective IgA deficiency would stand at the opposite extreme (*24*). IgA deficiency with impaired IgG responses to pneumococcal vaccines and/or combined IgG subclass deficiency would stand in between these two extremes of such heterogeneous spectrum of IgA disorders (*22–24*, *70*).

Analysis of human stool samples

Approximately 500 mg from each donor was blended into a slurry (40-50 mg/mL) in pre-reduced bacterial LYBHIv4 culture medium (*71*) containing 37 g/l Brain Heart Infusion (BHI) Broth (BD Biosciences), 5 g/l yeast extract (BD Biosciences), 1 g/l D-xylose, 1 g/l D-fructose, 1 g/l D-galactose, 1 g/l cellobiose, 1 g/l maltose, 1 g/l sucrose, 0.5 g/l N-acetylglucosamine, 0.5 g/l L-arabinose, 0.5 g/l L-cysteine, 1g/l malic acid, 2 g/l sodium sulfate, 0.05% Tween 80, 20 mg/mL menadione, 5 mg/l hemin, and 0.1 M MOPS (3-(N-morpholino)propanesulfonic acid) at pH 7.2. The slurries were passed through sterile 100-μm strainers to remove large debris. To store for later gavage of gnotobiotic mice, slurries were diluted 1:20 in LYBHIv4 media with 15% glycerol and stored at -80°C.

Analysis of murine blood and stool samples

Blood was collected from mice in ethylenediamine tetraacetic acid (EDTA) tubes pre-immunization (PI) and post-immunization at days 3, 7, 14, and 21 by submandibular vein puncture. Additional blood was collected in EDTA tubes from an orbital socket after sacrificing the animal. Plasma was isolated by incubating blood in EDTA tubes at room temperature for 20

minutes and spinning for 20 minutes at 15,000 G and collected into sterile microtubes for freezing at -80°C until further use. Murine tissue was collected after isoflurane-induced euthanasia followed by exsanguination and placed in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 2% heat-inactivated FBS (Life Techologies). Single cell suspensions were created from spleen and MLNs through tissue homogenization between frosted glass slides. PPs were incubated in dissociation buffer (HBSS supplemented with 10% FBS, 5 mM EDTA and 15 mM HEPES) 20 minutes at 37°C before meshing with a 70-□m cell-strainer (Fisher) and a syringe plunger. Splenic cell suspensions were treated with red blood cell lysis buffer (KD Medical) before staining for flow cytometry.

Colonization of GF mice

Cecal contents from WT or *Igha*$^{-/-}$ mice were dissolved in 5 ml sterile phosphate buffer solution (PBS) by vortexing. 200 μL of this murine fecal slurry. Colonized mice were handled under aseptic conditions. All immunization experiments in reconstituted GF mice were performed at least 14 days after bacterial colonization.

Isolation and identification of translocated bacteria

Spleen, liver, MLNs, mesenteric adipose tissue and a small piece of small intestine (positive control) from steady state WT and *Igha*$^{-/-}$ mice were aseptically dissected, weighed and collected into sterile PBS containing 0.5 g/ml reduced cysteine. To ensure aseptic collection, instruments were autoclaved, sterile consumables used, buffers prepared in a biosafety cabinet and sterile-filtered, and euthanized mice bathed in freshly prepared Clidox (Pharmacal, 1:3:1 S-Base:water:S-Activator ratio) prior to dissection. New instruments were used after initial incision. Tissues (whole organs for spleen, MLNs, MAT, spleen-sized piece of liver from same lobe, small SI piece without feces) were homogenized in 1 ml reduced PBS with sterile 3.5-mm

beads using a bead beater (Bio-Spec) before clearing by centrifugation (600 × g, 3 minutes). Under strict anaerobic conditions, tissue homogenates were passed through 100 μm filters and 100 μl was inoculated onto reduced BBL™ Chocolate II Agar (Becton Dickinson) and incubated under anaerobic conditions at 37°C for 72 hours. Colonies were counted and representative colonies were picked, expanded in liquid pre-reduced bacterial LYBHIv4 culture medium (*71*) containing 37 g/l Brain Heart Infusion (BHI) Broth (BD Biosciences), 5 g/l yeast extract (BD Biosciences), 1 g/l D-xylose, 1 g/l D-fructose, 1 g/l D-galactose, 1 g/l cellobiose, 1 g/l maltose, 1 g/l sucrose, 0.5 g/l N-acetylglucosamine, 0.5 g/l L-arabinose, 0.5 g/l L-cysteine, 1g/l malic acid, 2 g/l sodium sulfate, 0.05% Tween 80, 20 mg/mL menadione, 5 mg/l hemin, and 0.1 M MOPS (3-(N-morpholino)propanesulfonic acid) at pH 7.2, and identified using MALDI-TOF mass spectrometry against a custom database following acetonitrile extraction (Bruker Biotyper) (*72*).

Culture of bacteria

Streptococcus pneumoniae was streaked out on blood agar plates and incubated overnight at 37°C, after which one colony was picked with a sterile tip and inoculated in 5 ml pre-warmed BBL™ Brain Heart Infusion Broth media (BD) and incubated at 37°C overnight. The overnight culture was then sub-cultured by transferring 500 μl to 50 ml pre-warmed media (as above) and incubated 2-4 hours at 37°C until optical density (OD) 600 nm was approximately 0.3. *Streptococcus pneumoniae* was then made replication-deficient through incubation with 50 μg/ml mitomycin-C (Sigma Aldrich) at 37°C for 1 h, and washed twice with PBS before immunization as described below.

Immunizations

Each mouse was i.v. or i.p. immunized with 2.87 μg Pneumovax23 (Merck), 1.625 μg Prevnar13 (Pfizer), 50 μg TNP-aminoethilcarboxymethyl (AECM)-Ficoll (Biosearch Technologies), 50 μg TNP-LPS (Biosearch Technologies). Each of these immunogens was diluted into 100-200 μL sterile PBS before injection. 50 μg NP-OVA-16 supplemented with 100 μl Imject Alum Adjuvant according to manufacturer's instructions (Thermo Scientific) was injected i.p. Replication-deficient *Streptococcus pneumoniae* prepared as above was i.v. injected at 2×10^7 cells/mouse. For anti-PD-1 experiments, mice were treated with rat IgG2a mAb RMP1-14 to mouse PD-1 (Bio X Cell) or rat IgG2a mAb 2A3 as control (Bio X Cell). Mice received 200 μg i.p. of either anti-PD-1 or control mAb in 200 μl sterile dilution buffer (Bio X Cell) one day prior to Prevnar13 immunization and on days 1, 4, 7, 10, 13, 16, and 19 post-immunization.

Flow cytometry and cell sorting

For extracellular/intracellular IgG3 and IgG1, cells were first blocked with 25% normal rat serum and then stained for surface markers, including anti-IgG3-biotin followed by streptavidin-APC staining. After fixation and permeabilization (BD Cytofix/Cytoperm), cells were blocked again with 25% normal rat serum before addition of intracellular antibodies, including FITC-conjugated IgG3 diluted in BD Perm/Wash (BD Biosciences). For IgA-coated bacterial staining, bacteria isolated from fecal pellets were washed in staining buffer (1% BSA PBS w/v) and blocked for 20 minutes on ice with staining buffer supplemented with 20% normal rat serum (Invitrogen) prior to staining with anti-IgA antibody 1:100 (final 1:200) for 30 minutes (*36*). To quantify serum IgG1 specific to commensals, bacteria were optionally incubated with matched mouse serum diluted 1:50 in staining buffer and incubated on ice for 30 minutes. After washing with staining buffer, samples were stained with a biotinylated anti-IgG1 antibody (1:25) for 30 minutes on ice before another wash and staining with streptavidin-APC. After washing, bacteria

were resuspended in a saline buffer including 0.9% NaCl and 10 mM HEPES and supplemented with 1.25 nM SYTO Green Fluorescent Nucleic Acid Stain (Life Technologies). Cells were acquired with LSR Fortessa or LSR II (BD Biosciences) and data were further analyzed by FlowJo V10 software (TreeStar). For cell sorting, B cells were purified using Miltenyi Pan B cell isolation Kit II (Cat. 130-104-443), followed by FACS staining for sorting. Cells were resuspended in PBS with CD16/32 Fc Block and Live/Dead staining (Invitrogen) before staining with antibodies to surface markers. Live $CD11c^{-}Ly6G^{-}CD3^{-}$ $B220^{+}AA4.1^{-}CD21^{high}CD23^{lo}$ MZ B cells and $CD21^{+}CD23^{+}$ FO B cells were FACS-sorted (Influx BD) with Influx (BD Biosciences). The purity of sorted cells was consistently >95%.

ELISA

For total Ig subclass quantification, Immulon 4 HBX 96-well plates (ThermoFisher Scientific) were coated with a primary antibody (Southern Biotech) diluted in UltraCoat ELISA Coating Buffer (Leinco Technologies) at 50 μl per well overnight at 4°C. Antigen-specific Ig subclass quantification was determined by coating Immulon 4HBX 96-well plates overnight at 4°C with 50 μl/well of the following antigens diluted in PBS at 5 μg/mL: TNP-bovine serum antigen (BSA), CPS9 or CPS14 from *Streptococcus pneumoniae*, *Staphylococcus aureus*-derived lipoteichoic acid, *Salmonella typhimurium*-derived LPS, *E Coli* 0111:B4-derived LPS, or mitomycin-C-inactivated whole *Streptococcus pneumoniae* (4×10^6 bacteria/mL). For detection of pneumococcal polysaccharides (PPS)-specific antibodies following vaccination with Pneumovax23 (Merck) or Prevnar13 (Pfizer), plates were coated with Pneumovax23 in PBS at 20 μg/mL. Pneumovax23 encompasses 23 capsular polysaccharides, including 12/13 of the saccharides of the capsular antigens of *Streptococcus pneumoniae* serotypes in Prevnar13. For detection of high- and low-affinity NP-specific antibodies, plates were coated with NP-BSA ratio

7 (NP7) or NP-BSA ratio 23 (NP23), respectively, (Biosearch Technologies) at 10 μg/mL. Coated plates were then washed with 0.1% polysorbate 20 (Fisher) in PBS and blocked with milk powder or 1% BSA in PBS for 2 hours at room temperature. After washing, serum or fecal supernatant serially diluted in ELISA dilution buffer (1% BSA 0.1% polysorbate 20 PBS) was added to each well and incubated overnight at 4°C. After additional washing, plates were incubated for 2 hours at room temperature with a horseradish peroxidase (HRP)-conjugated secondary antibody (Southern Biotech) diluted in ELISA dilution buffer. After further washing, plates were developed with two-component tetramethylbenzidine HRP substrate (Seracare Life Sciences) and stopped with an equal volume of 1M H_2SO_4. OD values were determined at 450-nm wavelength on Epoch microplate spectrophotometer (BioTek). Human sCD14 was measured through Human CD14 DuoSet ELISA kit following manufacturer's instructions (R&D Systems). For microbiota-specific IgG1 quantification, bacteria pellets from small intestine or colonic fecal content was resuspended in 500 μl PBS and the resulting suspension was frozen in dry-ice and then thawed in a 37°C water bath for five cycles (*73*). The resulting lysates were centrifuged at 8000 g for 10 minutes and supernatants were collected as commensal antigen lysates. For quantification of IgG1 and IgM reactive to surface antigen from whole bacteria, bacterial pellets were frozen in sterile-filtered LB media with 30% glycerol, thawed at room temperature, washed, resuspended in 500 μl PBS and heat-killed at 85°C for 1 hour. Lysate or whole bacteria suspension protein concentration was measured with Nanodrop. Immulon 4 HBX 96-well plates (Thermo Scientific) were coated with 5 μg/mL lysate protein or whole bacteria in PBS and incubated overnight at 4°C. In each mouse, commensal-specific titers were determined as the difference in OD values between commensal-coated and PBS-coated wells. When not expressed in units of optical density (OD), the relative concentration of antigen-specific antibodies in serum

from immunized mice was expressed in arbitrary units (AU) calculated from a standard curve obtained with serum from previously-immunized mice with high titers of specific antibodies run contemporaneously. An appropriate control serum was used as standard to quantify total antibody concentrations in mice (Bethyl Laboratories, Inc.).

RNA-seq analysis

Standard quality control and mapping of sequence reads

The fastq files generated from Illumina RNA-seq were preprocessed and quality controlled using the CLC Genomics Workbench Version 9.0.1. The raw reads were trimmed and filtered to remove possible adapter sequences and low-quality nucleotides at the ends. After trimming, sequence reads shorter than 50 nucleotides were discarded according to the following parameters: phred score > Q30, base error probability score > 0.05, and base ambiguities < 2, read-length < 50 bp. Trimmed reads were aligned to the *Mus musculus* genome, and count data was generated using CLC Genomics Workbench Version 9.0.1.

Differential expression analysis

Statistical analysis was carried out using R-language (R-project.org) and packages available through the Bioconductor project (www.bioconductor.org). Normalization factors to scale RNA-seq library size were calculated using TMM method (*74*, *75*) and converted to counts per million (CPM) reads using the cpm function (edgeR package). Gene transcripts with a CPM >1 in at least one sample were kept for further analysis. The remaining data was then transformed using *voom* (*76*). Gene/transcript-expression profiles of non-Igh genes were compared between B cells from *Igha*$^{-/-}$ and WT mice using linear mixed-effect models with cell-type (MZ vs FO B cells) and mouse strain (WT vs *Igha*$^{-/-}$ mice) as fixed factors. A random intercept was used for each parent mouse the B cells were derived from. Models were fitted using the *limma* package

framework and hypothesis of interest were tested via contrasts using the moderated t-test (*77*). P-values were corrected for multiple hypothesis testing and are presented with false discovery rate (FDR) values. Gene and transcripts annotation was carried out with Biomart (*78, 79*) using the *mmusculus_gene_ensembl* dataset from 01/07/2019.

Gene set and gene pathway analysis

Over-representation analysis (ORA) and gene-set enrichment analysis (GSEA) were performed with the HALLMARK and C2CP collections from the MolSigDB database (http://software.broadinstitute.org/gsea/msigdb/index.jsp) as well as custom gene sets. Custom gene sets representing GC B cell differentiation and PC differentiation were defined using publicly available GSE60927 data (*40*) as genes up-regulated (fold change (FCH) >2, FDR <0.05) when comparing GC B cells vs FO B cells (GC B cell differentiation), and the top-50 down-regulated (FCH >1.5, FDR <0.05) genes when comparing bone marrow PCs vs splenic plasmablasts (this differentially expressed gene set was defined as PC differentiation). A gene-set representing B cell anergy was defined as up-regulated genes (FCH >2, FDR <0.05) when comparing anergic MD4ML5 cells vs naïve non-anergic MD4 cells (control) using data previously published (*43*). Gene-set variation analysis (GSVA) (*80*) was used to calculate gene-sets activity per sample for proliferation (Hallmark_G2M_checkpoint) and custom gene-sets. Statistical analysis of GSVA scores was performed using the same methods as gene-expression (limma package framework).

16S rRNA gene sequencing and analysis

16S rRNA gene data was generated following Earth Microbiome Project protocols for DNA extraction, library prep and sequencing (*81*). Raw sequencing data was demultiplexed, quality filtered and processed using QIIME2 v2020.8.0 (*82*). Amplicon sequence variants (ASVs) were

estimated using DADA2 (*83*) and taxonomy assigned using GreenGenes (*84*). Based on ASV tables, bacterial functions and pathways were predicted using PICRUST2 (*85*). Diversity measures were calculated rarefying at 1,000 sequences per sample; alpha diversity was estimated using Faith's phylogenetic diversity and observed features, and beta diversity with unweighted UniFrac (*86*). Differential abundance analysis was performed using LefSe (*87*) and ANCOM (*88*). Segmented filamentous bacteria was identified based on taxonomic assignment to the string "*Candidatus Arthromitus*" in GreenGenes. Statistical testing and plotting were performed using the *scipy* 1.5.2 and *seaborn* 0.11.1 packages in Python 3.7.13.

Metabolomics

Metabolome analysis was performed using both capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) and liquid chromatography time-of-flight mass spectrometry (LC-TOFMS) in two modes for cationic and anionic metabolites. For CE-TOFMS, each 50-μL sample was mixed with 450 μL of 10 μM methanol-containing internal standards and mixed. Then, 500 μl chloroform and 200 μl Milli-Q water were added, mixed thoroughly and centrifuged at 2,300 × g, 4°C for 5 minutes. The water layer (400 μL × 1) was filtrated through a 5-kDa cut-off ULTRAFREE-MC-PLHCC filter (Human Metabolome Technologies) to remove macromolecules. The filtrate was centrifugally concentrated and resuspended in 50 μL of ultrapure water immediately before the measurement. For LC-TOFMS, each 500 μl (human) or 300 μl (mouse) sample was mixed with either 1,500 μl (human) or 900 μl (mouse) of 1% formic acid in acetonitrile (v/v) containing 6 μM internal standards and centrifuged at 2,300 x g, 4°C for 5 minutes. Then, the supernatant was filtrated by using a Hybrid SPE phospholipid 55261-U column (Supelco) to remove phospholipids. The filtrate was desiccated and resuspended in either

200 µl (human) or 120 µl (mouse) of 50% isopropanol in Milli-Q water (v/v) immediately before the measurement.

Peaks detected in CE-TOFMS or LC-TOFMS analysis were extracted using an automatic MasterHands ver. 2.17.1.11 integration software (Keio University) in order to obtain peak information, including m/z, migration time (MT) in CE, retention time (RT) in LC, and peak area. The peak area was then converted to relative peak area by dividing the Metabolite Peak Area by the Internal Standard Peak Area. The peak detection limit was determined based on signal-noise ratio; S/N = 3. Putative metabolites were then assigned from HMT's standard library and Known-Unknown peak †4 library on the basis of m/z and MT or RT. The tolerance was ± 0.5 minutes in MT/RT and ±10 ppm. If several peaks were assigned the same candidate, the candidate was given the branch number. Absolute quantification was performed in 110 metabolites, including glycolytic and tricarboxylic-acid-cycle-intermediates, amino acids, and nucleic acids. All the metabolite concentrations were calculated by normalizing the peak area of each metabolite with respect to the area of the internal standard and by using standard curves, which were obtained by single-point (100 µM) calibrations. Standardized relative peak area data were used to identify metabolites showing statistically significant differences between defined group phenotypes (Kruskal Wallis with Dunn's correction for human data and Mann-Whitney for mouse data, p value <0.05). Data represent standardized values of relative area in detected peaks where values below the limit of detection were assigned a value of epsilon(2^-52). Heat maps show standardized relative peak areas of significantly differentially abundant metabolites and have been row mean-centered and row-normalized. Heat maps were generated using the *gplots* R-package.

Mouse B cell cultures

A single cell suspension of splenocytes was prepared as above and purified for B cells using either MACs Mouse B cell Isolation Kit (Miltenyi Biotech) or EasySep Mouse B cell Isolation Kit (Stemcell Technologies). Cells were plated at 0.2×10^6 cells/ml in 96-well round bottom plates in 200 μl RPMI medium containing 10% FBS, 1% penicillin-streptomycin (Life Sciences) and 55 μM 2-mercaptoethanol and stimulated with 1 μg/ml anti-CD40 (Biolegend) and 100 ng/ml IL-4 (R&D Systems), or 10 μg/ml LPS (Invivogen) for 6 days. Supernatant was collected on day 6 for ELISA and cells were harvested for flow cytometry.

Human B cell cultures

3×10^5 PBMCs (200 μl/well) were cultured in 96-well round bottom plates and stimulated with 500 ng/ml trimeric CD40L (Enzo Life Sciences) and 100 ng/ml IL-21 (Peprotech) for 7 days. RPMI culture medium containing 10% FBS and 1% penicillin-streptomycin (Life Sciences) was prepared with or without 120 mg/L BCAAs at a 2:5:5 ratio of L-valine (0.17 nmol/L), L-leucine (0.38 nmol/L), and L-isoleucine (0.38 nmol/L). Supernatant was collected at day 7 and analyzed for immunoglobulin titers via ELISA.

Histology and immunofluorescence

Colon and small intestine from WT or *Igha*$^{-/-}$ mice were incubated for 4h in Methanol-Carnoy's fixative at room temperature, then immersed in 100% ethanol at 4°C for 1h prior incubation with xylene solution at room temperature. Tissue was then embedded in paraffin solution and 4-μm sections were cut before staining with hematoxylin and eosin at Mount Sinai's Biorepository and Pathology CoRE service. Slides were analyzed with an Aperio AT2 (Leica) and scored by a pathologist from Mount Sinai Hospital.

Table S1. Inflammation grading of gut tissue samples from WT and *Igha*$^{-/-}$ mice

	Inflammation score
169_WT	0
170_IgAKO	0
172_WT	0
230_IgAKO	0
206_WT	0
207_IgAKO	0
209_WT	0
208_IgAKO	0
All samples show no histological evidence of inflammation; some processing artifacts are noted.	
Dr. Zhu Hongfu, MD, a pathologist from Icahn School of Medicine at Mount Sinai, analyzed these paraffin-embedded mouse tissue sections.	

Table S2. NYC cohort of IGAD patients

Relevant phenotypic traits	**HCs** (n = 27)	**IGAD-Rs** (n = 10)	**IGAD-NRs** (n = 6)
Gender (% female)	66.67%	66.67%	66.67%
Age, years (median [IQR])	33 [10]	33 [13]	40 [3]
IgG-targeted Pneumococcal serovars post-Pneumovax23 (median of 14)	N/A	11 [4]	2 [3]
Serum antibody titers, mg/dL (median [IQR])[a]			
IgA	196 [105]	<7	<7
IgM	110 [51]	84 [51]	72 [116]
IgG	1170 [268]	1111 [455]	812 [322]
IgG1	644 [117]	729 [99]	621 [264]
IgG2[b]	381 [119]	312 [173]	125 [100]
IgG3	39 [39]	61 [40]	34 [120]
IgG4[c]	29 [36]	12 [61]	1 [0]
Recurrent infections, *n* (%)			
Bronchitis	N/A	5 (62.5)	4 (80)
Otitis	N/A	1 (12.5)	1 (20)
Pneumonia	N/A	5 (62.5)	2 (40)
Sinusitis	N/A	7 (87.5)	3 (60)

IQR, interquartile range

[a]Ig classes: HCs, n = 11; IGAD pneumococcal vaccine responder (IGAD-Rs), n = 8; IGAD pneumococcal vaccine non-responders (IGAD-NRs), n = 6.
IgG subclasses: HCs, n = 8; IGAD-Rs, n = 8; IGAD-NRs, n = 5.
[b]Of patients with IgG subclass data, 1 IGAD-R and 3 IGAD-NR had IgG2 titers below the sufficiency cut-off of 124 mg/dL.
[c]Of patients with IgG subclass data, 3 IGAD-R and 4 IGAD-NR had IgG4 titers below the sufficiency cut-off of 124 mg/dL

Inclusions per assay:
Flow cytometry: HCs, n = 17; IGAD-Rs, n = 6; IGAD-NRs, n = 6.
sCD14 quantification: HCs, n = 18; IGAD-Rs, n = 9; IGAD-NRs, n = 6.
Metabolomics: HCs, n = 9; IGAD-Rs, n = 4; IGAD-NRs, n = 3.
GF mouse reconstitution: HCs, n = 4; IGAD-Rs, n = 2; IGAD-NRs, n = 2.
In vitro assays: HCs, n = 10.

Table S3. BCN cohort of IGAD patients

Relevant phenotypic traits	**Pediatric HCs** (n = 11)	**Pediatric IGAD patients** (n = 14)	**Adult IGAD patients** (n = 17)
Gender (% female)	45.45%	42.85%	64.71%
Age, years (median [IQR])	8 [7]	10 [5]	56 [19]
Serum antibody titers, mg/dL (median [IQR])[a]			
IgA	123 [47]	<10	<10
IgM	117 [66]	79 [34]	95 [82]
IgG	1138 [143]	1235 [243]	1278 [555]
IgG1	813 [26]	999 [210]	807 [297]
IgG2	312 [58]	335 [271]	300 [256]
IgG3	74 [2]	43 [14]	44 [41]
IgG4	17 [4]	35 [98]	N/A

IQR, Interquartile range.

[a]Ig classes: pediatric healthy controls, n = 7; pediatric IGAD patients, n = 14; adult IGAD patients, n = 17.

IgG subclasses: pediatric healthy controls, n = 2; pediatric IGAD patients, n = 12; adult IGAD patients, n = 17.

Adult HCs, n = 23; relevant phenotypic traits were not available.

Supplementary Figures

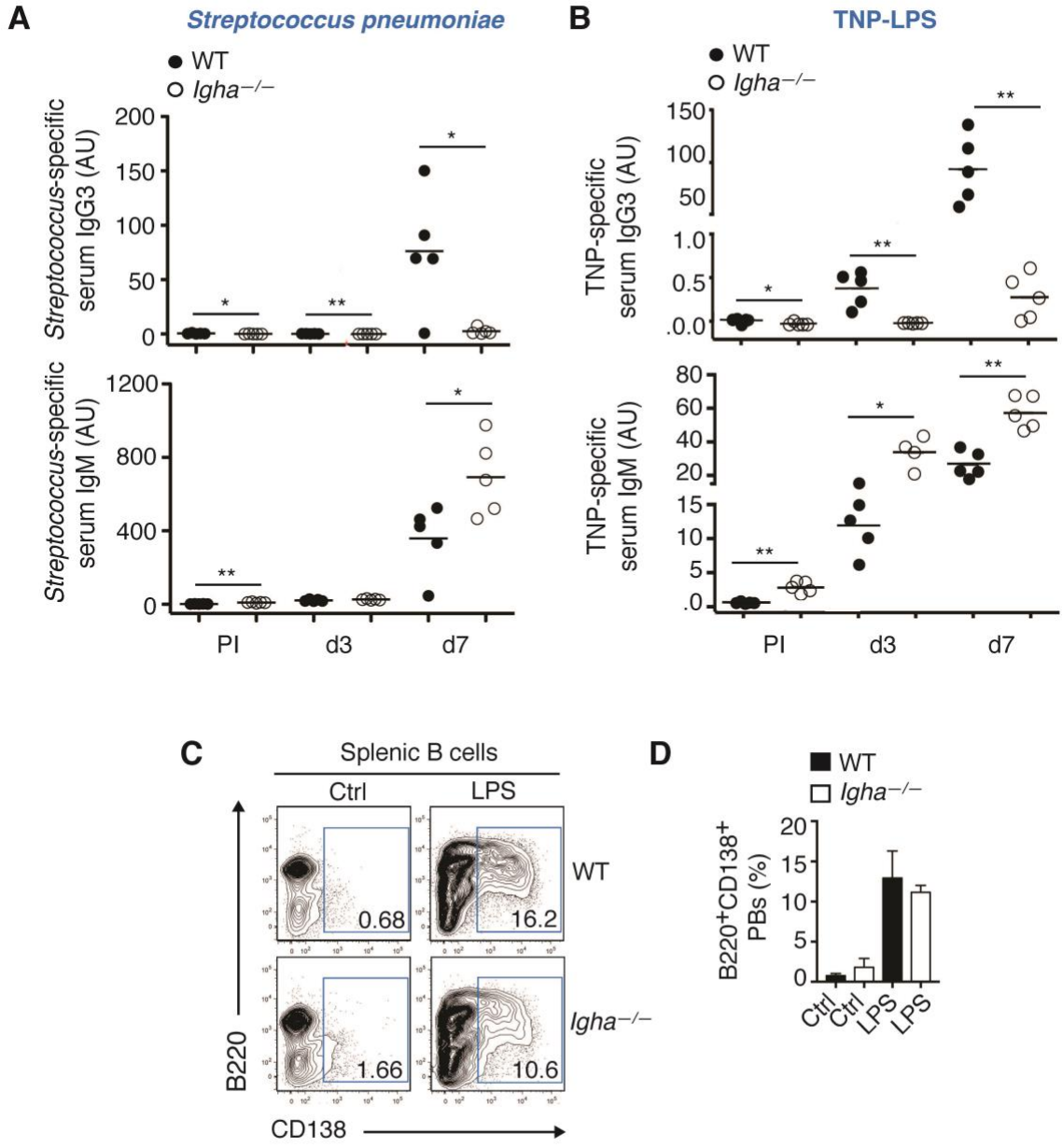

Fig. S1. IgA enhances systemic IgG3 responses to TI antigens. (**A**) ELISA of serum IgM and IgG3 to whole mitomycin C-inactivated *Streptococcus pneumoniae* from 5 WT or *Igha*$^{-/-}$ mice prior to immunization (PI) or 3 and 7 days following i.v. immunization with mitomycin C-treated

non-replicating *Streptococcus pneumoniae*. WT mice in this experiment were purchased from Jackson Laboratories. (**B**) ELISA of serum IgM and IgG3 to TNP from 5 WT and 4-5 *Igha*$^{-/-}$ mice PI or 3 and 7 days after i.v. immunization with TNP-LPS. (**C**) Flow cytometry of B220 and CD138 molecules on purified splenic B cells from representative WT or *Igha*$^{-/-}$ mice following exposure to medium alone (Ctrl) or LPS for 6 days. B220^{+}CD138^{+} cells correspond to PBs. Numbers indicate PB frequency (% of live cells). (**D**) Summary of the frequency (% of live) of B220^{+}CD138^{+} PBs obtained from purified splenic B cells isolated from 6 WT or *Igha*$^{-/-}$ mice and cultured as in (C). Results summarize one (A-B) or three (D) experiments involving 2 WT or *Igha*$^{-/-}$ mice or show representative plots from mice summarized in S1D (C). Data are presented with mean (A-B) or mean ± s.e.m. (D); significance was determined using a two-tailed unpaired Mann-Whitney test; *P < 0.05, **P < 0.01.

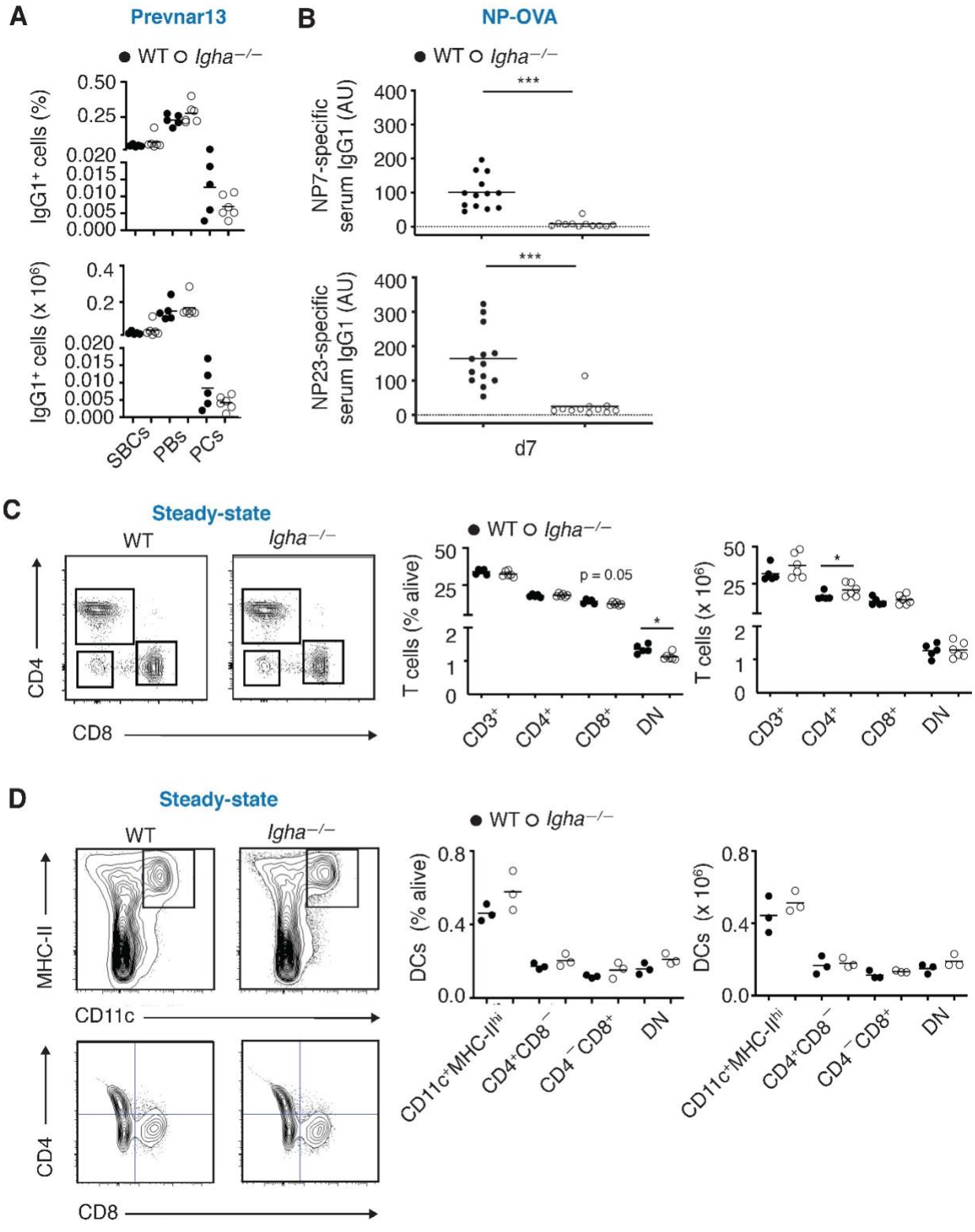

Fig. S2. **IgA enhances systemic IgG1 responses to TD antigens without having major effects on T cells and DCs.** (**A**) Flow cytometry of intracellular IgG1 (icIgG1) and extracellular IgG1

(ecIgG1) expression by splenic class-switched ecIgG1^{+}icIgG1^{-} B cells (SBCs), ecIgG1^{+}icIgG1^{+} PBs, and ecIgG^{lo}icIgG^{+} PCs from 5 WT or 7 *Igha*$^{-/-}$ mice 28 days following i.v. immunization with Prevnar13. Frequency (% of B220^{+} cells, top) and absolute number (bottom) of SBCs, PBs and PCs are indicated. (**B**) ELISA of serum high-affinity IgG1 to NP7-BSA and low-affinity IgG1 to NP23-BSA in 13 WT or 11 *Igha*$^{-/-}$ mice 7 days following i.p. immunization with NP15-OVA and alum. Data from individual mice are identical to d7 data from Figure 2C. (**C**) Flow cytometry of total CD3^{+} T cells, CD4^{+}, CD8^{+} and CD4^{-}CD8^{-} (double negative, DN) T cell subsets from the spleen of 5 WT or 6 *Igha*$^{-/-}$ mice at steady state. Representative flow cytometry contour plot (left) pre-gated on live CD3^{+} T cells, frequency of T cell subsets (% live, middle), and absolute cell numbers (right) are indicated. (**D**) Flow cytometry of total CD11c^{+}MHC-II^{high} dendritic cells (DCs) as well as CD4^{+}CD8^{-}, CD4^{-}CD8^{+} and CD4^{-}CD8^{-} (DN) DC subsets from the spleen of 3 WT or *Igha*$^{-/-}$ mice at steady state. Representative flow cytometry contour plots (left), frequency (% live) and absolute numbers are indicated. DCs were first gated on CD3^{-}B220^{-} cells. WT mice in this experiment were purchased from Jackson Laboratories. Results summarize 1 (C-D, right) or 2 (A-B) experiments or show representative flow cytometry plots (C-D, left). Data are presented with mean; significance was determined using a two-tailed unpaired Mann-Whitney test. *p <0.05, ***p <0.001.

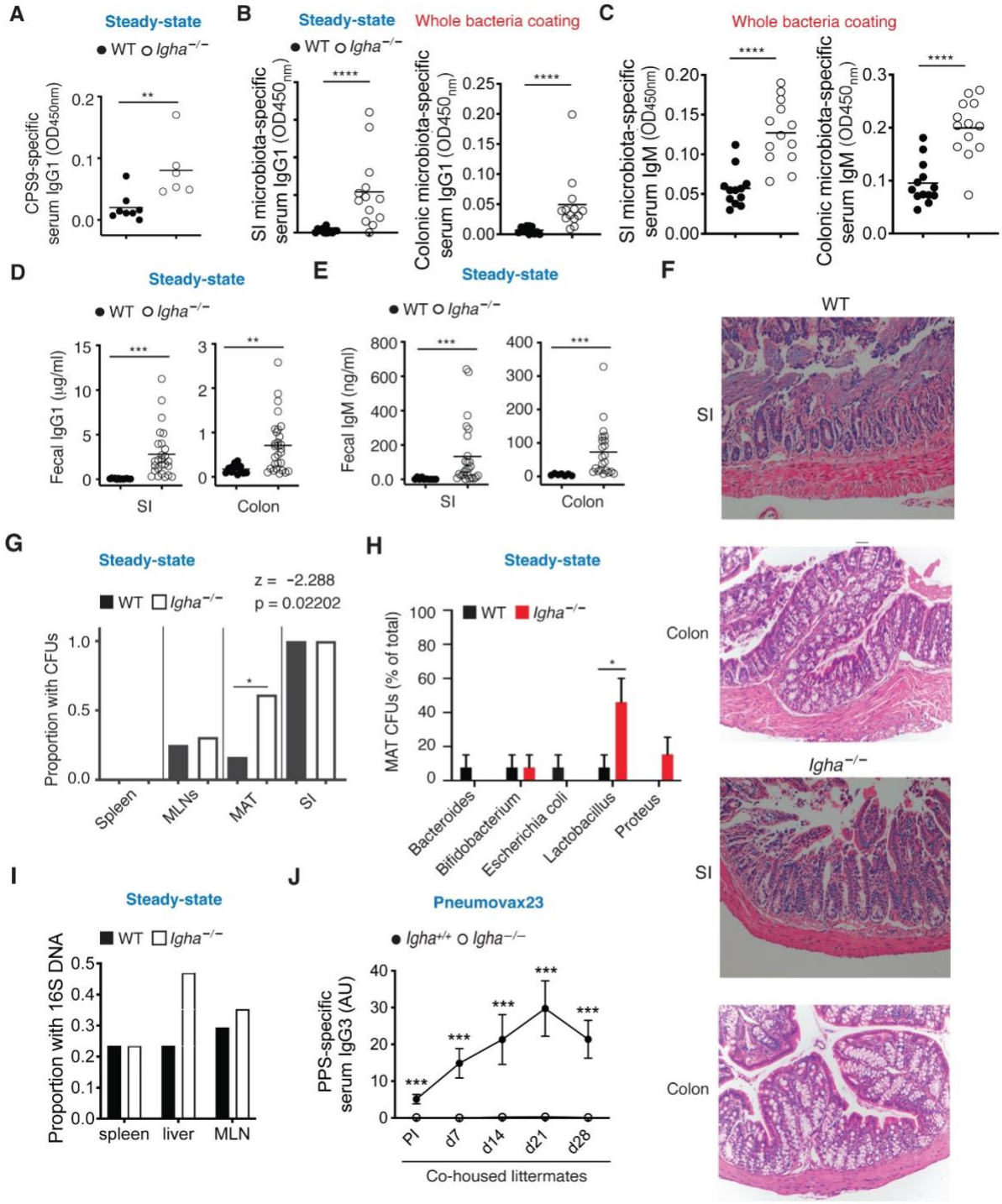

Fig. S3. **IgA restrains systemic IgG and IgM responses to translocated gut antigens.** (**A**) ELISA of serum IgG1 to pneumococcal CPS9 from 8 WT or 6 *Igha*$^{-/-}$ mice. (**B**, **C**) ELISA of

serum IgG1 (B) and IgM (C) reactive to surface antigens from heat-killed whole bacteria inhabiting the small intestine (SI) or colon of 12-13 WT or 13 *Igha*$^{-/-}$ mice. (**D**) ELISA of free total IgG1 from the SI or colon of 12-13 WT or 26-29 *Igha*$^{-/-}$ mice. (**E**) ELISA of free total IgM from the SI or colon of 7-10 WT or 22-25 *Igha*$^{-/-}$ mice. (**F**) Hematoxylin and eosin staining of tissue sections from the SI and colon of representative WT or *Igha*$^{-/-}$ mice (pathological score 0, Table S1). (**G**) Overall percentage of agar plates from each organ homogenate that resulted in the identification of CFUs after incubation in an anaerobic chamber. Results for spleen, MLN, and MAT are from 8-13 WT and 10-13 *Igha*$^{-/-}$ mice, while positive controls from the small intestine are from 8 WT and 10 *Igha*$^{-/-}$ mice. Based on the same data as in Figure 3E. (**H**) Summary of CFUs of anaerobic bacteria identified in homogenates of MAT from 12 WT or 13 *Igha*$^{-/-}$ mice at steady state. The frequency (%) of colony-positive agar plates containing each taxonomically identified genus per experiment is shown. (**I**) Proportion of spleen, liver or MLNs with detectable 16S DNA by qPCR in 17 WT or *Igha*$^{-/-}$ mice at steady state (4/17 livers from WT vs 8/17 livers from *Igha*$^{-/-}$ mice had detectable 16S DNA). (**J**) ELISA of serum IgG1 to PPS from 9 *Igha*$^{+/+}$ or 5 *Igha*$^{-/-}$ co-housed littermate mice from *Igha*+/- parents prior to immunization (PI) and 7, 14, 21, or 28 days following i.p. immunization with Pneumovax23. Data are presented as mean ± s.e.m.; significance was determined using two-tailed unpaired Mann-Whitney test; *p <0.05, ***p <0.001. Data derive from one experiment (A, J), summarize 2 (B-C) 4-5 experiments (G-H), or are representative of 4 WT and 4 *Igha*$^{-/-}$ mice (F). Data are presented with mean (A-E) or mean ± s.e.m. (H, J); significance was determined using two-tailed unpaired Mann-Whitney test (A-E, H, J), unpaired t test (C, right), or a two-tailed two proportions z-test (H). *p < 0.05, **p < 0.01, ***p <0.001

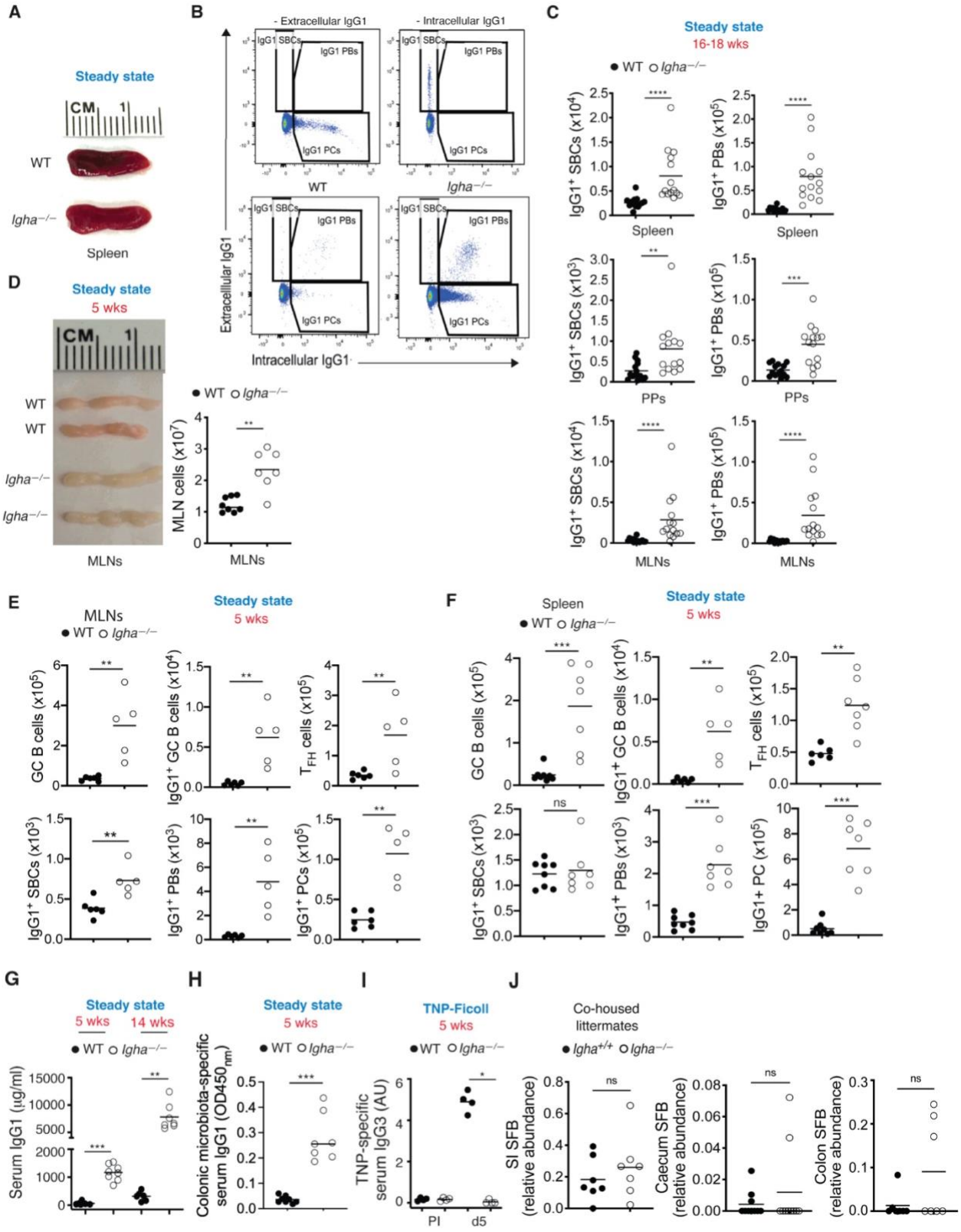
A
Steady state
CM 1
WT
Igha−/−
Spleen
B
- Extracellular IgG1
- Intracellular IgG1
IgG1 SBCs
IgG1 PBs
IgG1 PCs
WT
Igha−/−
Extracellular IgG1
Intracellular IgG1
C
Steady state
16-18 wks
WT Igha−/−
IgG1+ SBCs (x10^4)
IgG1+ PBs (x10^5)
Spleen
IgG1+ SBCs (x10^3)
IgG1+ PBs (x10^5)
PPs
IgG1+ SBCs (x10^4)
IgG1+ PBs (x10^5)
MLNs
D
Steady state
5 wks
CM 1
WT
WT
Igha−/−
Igha−/−
MLNs
WT Igha−/−
MLN cells (x10^7)
MLNs
E
MLNs
Steady state
5 wks
WT Igha−/−
GC B cells (x10^5)
IgG1+ GC B cells (x10^4)
TFH cells (x10^5)
IgG1+ SBCs (x10^3)
IgG1+ PBs (x10^3)
IgG1+ PCs (x10^5)
F
Spleen
Steady state
5 wks
WT Igha−/−
GC B cells (x10^5)
IgG1+ GC B cells (x10^4)
TFH cells (x10^5)
IgG1+ SBCs (x10^3)
IgG1+ PBs (x10^3)
IgG1+ PC (x10^5)
ns
G
Steady state
5 wks
14 wks
WT Igha−/−
Serum IgG1 (µg/ml)
H
Steady state
5 wks
WT Igha−/−
Colonic microbiota-specific serum IgG1 (OD450nm)
I
TNP-Ficoll
5 wks
WT Igha−/−
TNP-specific serum IgG3 (AU)
PI
d5
J
Co-housed littermates
Igha+/+ Igha−/−
SI SFB (relative abundance)
Caecum SFB (relative abundance)
Colon SFB (relative abundance)
ns

Fig. S4. **IgA starts limiting the gut microbiota-driven expansion of systemic IgG1$^+$ B cells and IgG1-secreting plasma cells at an early age.** (A) Images of spleen from a representative adult WT or *Igha*$^{-/-}$ mouse. (**B**) Flow cytometric strategy to identify IgG1$^+$ switched B cells (SBCs), IgG1$^+$ PBs, and IgG1$^+$ PCs based on extracellular (ecIgG1) and intracellular IgG1 (icIgG1) staining. Top left panel, splenic negative control without extracellular anti-IgG1 staining; top right panel, splenic negative control without intracellular anti-IgG1 staining. Bottom left and right panels, splenic SBCs, PBs and PCs from representative WT or *Igha*$^{-/-}$ mice. (**C**) Absolute numbers of IgG1$^+$ SBCs and PBs in spleen, PPs and MLNs from 14 WT or *Igha*$^{-/-}$ mice aged 16-18 weeks. (**D**) Image of representative MLNs from 2 WT or 2 *Igha*$^{-/-}$ mice aged 5 weeks (left) and absolute numbers of total cells in MLNs from 8 WT or 7 *Igha*$^{-/-}$ mice aged 5 weeks. (**E, F**) Absolute numbers of total GC B cells, IgG1$^+$ GC B cells, T_{FH} cells, switched B (SBCs) cells, IgG1$^+$ PBs, and IgG1$^+$ PCs in MLNs (E) or spleen (F) from 6-8 WT or 5-7 *Igha*$^{-/-}$ mice aged 5 weeks. (**G**) Total serum IgG1 from 6-7 WT or 7-8 *Igha*$^{-/-}$ mice aged 5 or 14 weeks. (**H**) ELISA of serum IgG1 to colonic microbiota from 8 WT or 7 *Igha*$^{-/-}$ mice aged 5 weeks. (**I**) ELISA of serum IgG3 to TNP from 4 WT or 4 *Igha*$^{-/-}$ mice aged 5 weeks prior to immunization (PI) and 5 days following i.p. immunization with TNP-Ficoll. (**J**) Relative SFB abundance in the SI (i.e., ileum), caecum, and colon from 7-10 *Igha*$^{-/-}$ mice and *Igha*$^{+/+}$ co-housed littermates calculated by 16S rDNA sequencing. Data are from one representative experiment of 2-3 (A, D), summarize 2 experiments (C. G), or one individual experiment (E-F, H-I). Data are presented with mean; a two-tailed unpaired Student's t-test was performed when data were determined to follow a Gaussian distribution (G), otherwise a Mann-Whitney test was used to determine significance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

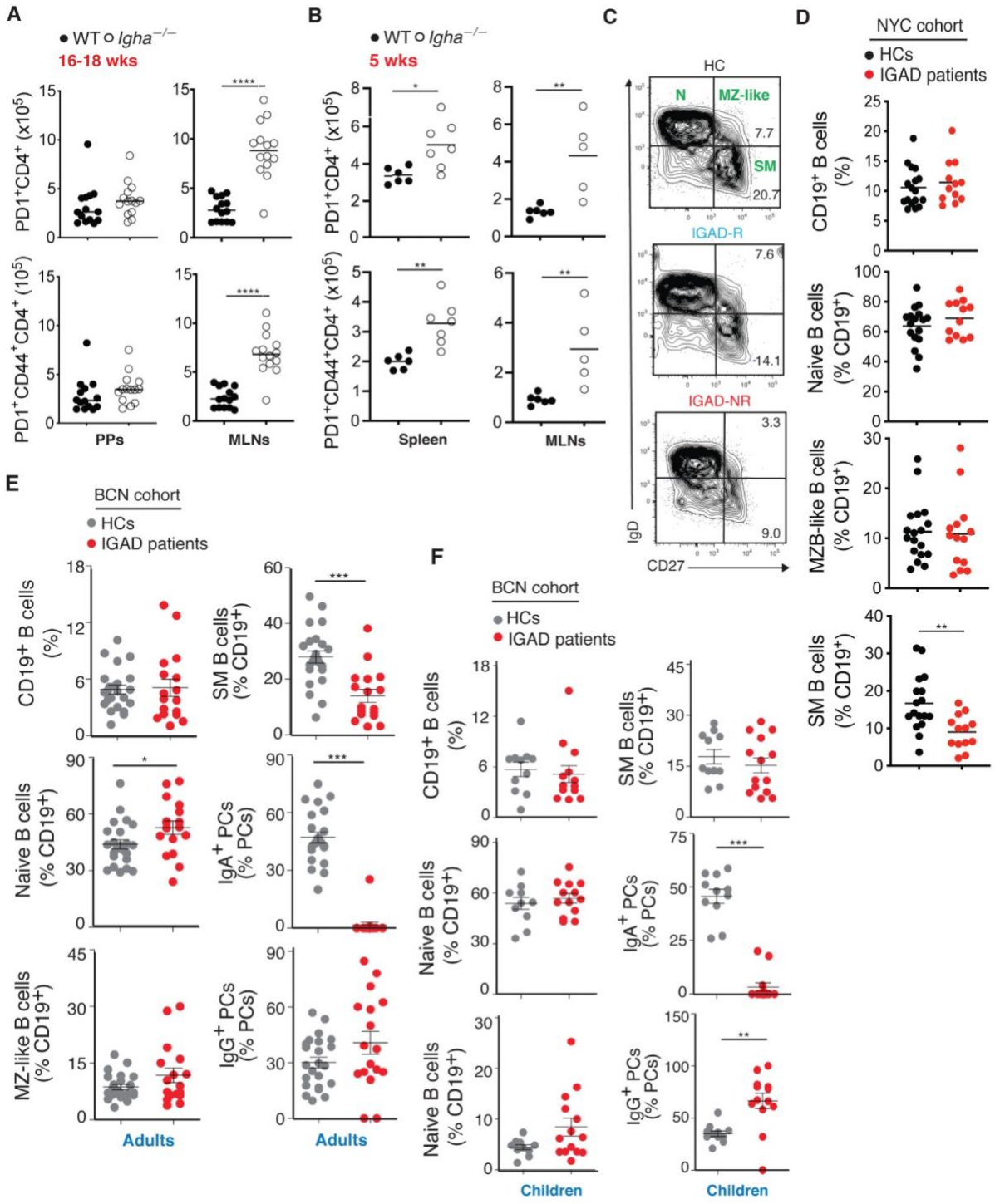

Fig. S5. IgA constrains the gut microbiota-driven expansion of PD-1$^+$CD4$^+$ T cells in mice and IgA loss perturbs the frequency of circulating switched memory B cells and plasma cells in humans. (**A**) Absolute numbers of PD-1$^+$CD4$^+$ and antigen-experienced PD-

$1^+CD44^+CD4^+$ T cells from PPs and MLNs of 16-18 wk-old WT or *Igha*$^{-/-}$ mice at steady state. (**B**) Absolute numbers of PD-1^+CD4^+ and antigen-experienced PD-$1^+CD44^+CD4^+$ T cells from spleen and MLNs of 5 wk-old WT or *Igha*$^{-/-}$ mice at steady state. (**C**) Flow cytometry analysis of IgD and CD27 on circulating naïve (N) IgD^+CD27^-, MZ-like IgD^+CD27^+ and switched memory (SM) IgD^-CD27^+ B cells in a representative adult healthy control (HC) donor and two representative adult IGAD patients from the NYC cohort. Numbers indicate frequency (% of $CD19^+$ cells). (**D**) Frequency of circulating total $CD19^+$ B cells (% of live), naïve, MZ-like and switched memory (SM) B cell subsets (% of $CD19^+$) from adult donors of a NYC cohort comprised of 17 HCs and 12 IGAD patients. (**E**) Frequency of circulating $CD19^+$ total B cells (% of live), $IgM^+IgD^+CD27^-$ naïve B cells, $IgM^{hi}IgD^{int}CD27^+$ MZ-like B cells, $IgD^-IgM^-CD27^+$ SM B cells (% of $CD19^+$), IgA^+ PCs, and IgG^+ (IgM^-IgA^-) PCs (% of $CD19^+CD27^{hi}CD38^{hi}$) from adult donors of a BCN cohort comprised of 23 HCs and 17 IGAD patients. All subsets were first gated on $CD19^+$non-$CD38^{++}CD27^{++}$ cells. (**F**) Frequency of circulating $CD19^+$ total B cells (% of live), $IgM^+IgD^+CD27^-$ naïve B cells, $IgM^{hi}IgD^{int}CD27^+$ MZ-like B cells, $IgD^-IgM^-CD27^+$ SM B cells (% of $CD19^+$), IgA^+ PCs, and IgG^+ PCs (% of $CD19^+CD27^{hi}CD38^{hi}$) from children of a BCN cohort comprised of 11 HCs and 14 IGAD patients, gated as in (E). Data summarize 2 (A) or 1 (B) independent experiments, show a representative flow cytometry contour plot (C) or paired experiments wherein flow cytometry was performed on human peripheral blood mononuclear cells upon reception of IGAD blood samples paired with HC blood samples (D-F). Data are presented with median (A), mean (B, D) or mean ± s.e.m. (E-F); significance was determined using two-tailed unpaired t-test for data were determined to follow a Gaussian distribution (A, right), otherwise a Mann-Whitney test was used. *$p < 0.05$, ***$p < 0.001$.

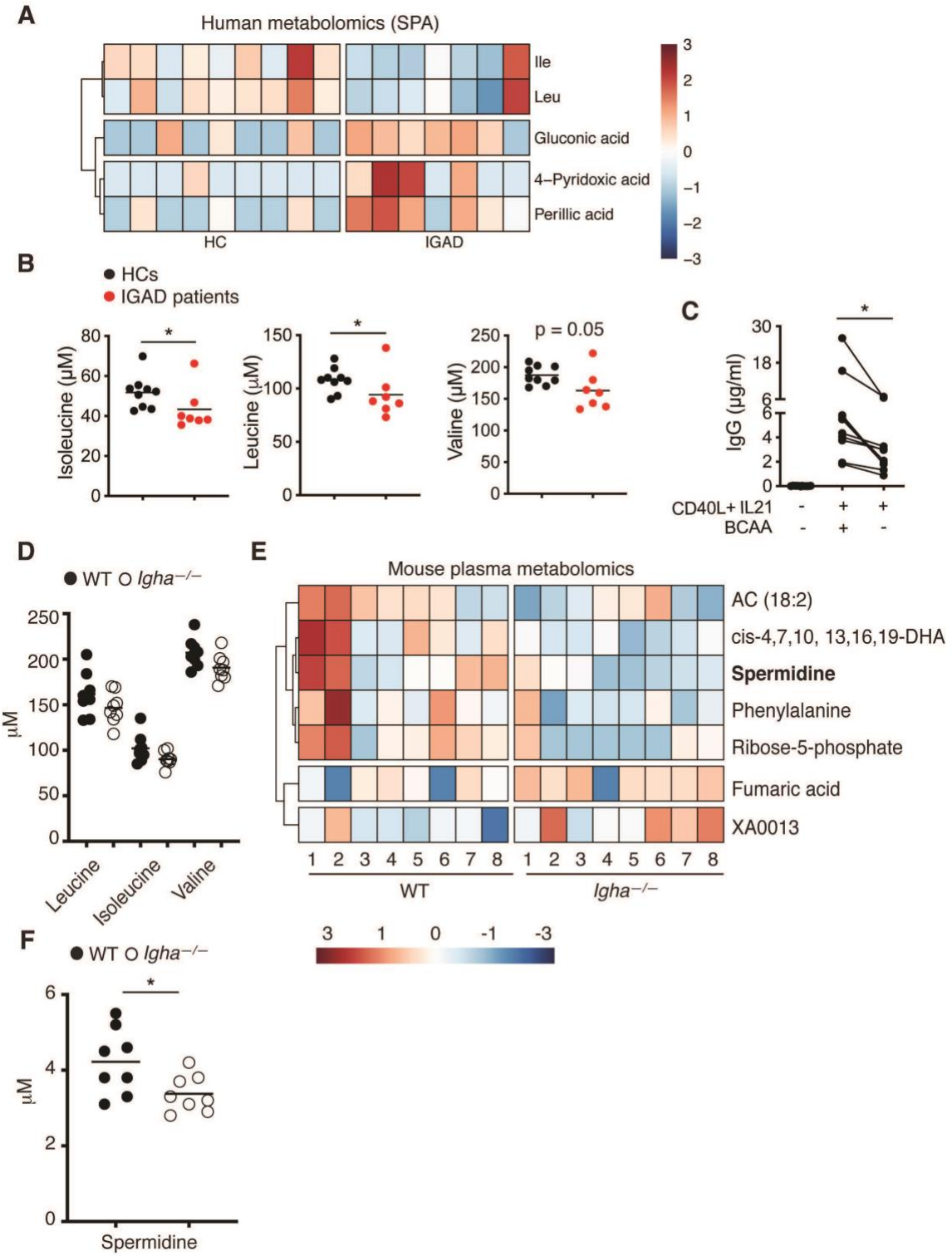

Fig. S6. **IgA optimizes the amount of circulating BCAAs, which show IgG-enhancing potential.** (**A**) Heat map of differentially abundant plasma metabolites from 9 HCs and 7 IGAD patients. Data represent standardized peak areas and have been row mean-centered and row-

normalized. Metabolites highlighted in bold are discussed in the text. SPA, standardized peak area. Leu, Leucine. Ile, Isoleucine. (**B**) Plasma concentration of leucine, isoleucine and valine from 9 HC or 7 IGAD patients. Quantitative estimation (μM) was performed by normalizing relative peak areas and comparing to standard curve. (**C**) ELISA of IgG secreted by B cells from 10 HCs upon exposure of peripheral blood mononuclear cells to medium alone (ctrl) or T cell-associated stimuli CD40L and IL-21 for 6 days in the presence or absence of 120 mg/L BCAAs at a 2:5:5 ratio of L-valine, L-leucine, and L-isoleucine. (**D**) Plasma concentration of leucine, isoleucine and valine from 8 WT or 8 *Igha*$^{-/-}$ mice. Quantitative estimation (μM) was performed by normalizing relative peak areas and comparing to standard curve. (**E**) Heat map of plasma metabolomics from 8 WT or 8 *Igha*$^{-/-}$ mice. Significantly different metabolites are shown and spermidine is highlighted in bold. AC, acylcarnitine; DHA, docosohexahenoic acid. (**F**) Plasma concentration of spermidine from 8 WT or 8 *Igha*$^{-/-}$ mice. Quantitative estimation (μM) was performed by normalizing relative peak areas and comparing to standard curve. Metabolomics (A, B, D-F) was from one experiment including multiple biological replicates. *In vitro* BCAA experiment (C) summarizes two independent experiments involving peripheral blood mononuclear cells from 10 HCs. Data are presented with mean and significance was determined through Kruskal-Wallis test with Dunn's correction for multiple comparisons (A, E), Mann-Whitney (B, F) or paired t test (C). *$p < 0.05$.