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Acsbg1 maintains intestinal immune homeostasis and controls inflammation by regulating ST2$^+$ Tregs

Martina Palatella[1], Friederike Kruse[1], Honglei Ji[2,3], Alina K. Loriani Fard[4], Maike Becker[5,6], Carolin Daniel[5,6], Maria Rohm[2,3], Jochen Huehn[1,6]

[1]Department Experimental Immunology, Helmholtz Centre for Infection Research, Braunschweig, Germany

[2]Institute for Diabetes and Cancer (IDC), Helmholtz Center Munich, Neuherberg, Germany

[3]German Center for Diabetes Research (DZD), Munich, Germany

[4]Mouse Pathology Platform, Helmholtz Centre for Infection Research, Braunschweig, Germany

[5]Institute for Diabetes Research, Research Group Immune Tolerance in Diabetes, Helmholtz Center Munich, Neuherberg, Germany

[6]Division of Clinical Pharmacology, Department of Medicine IV, Ludwig-Maximilians-University, Munich, Germany

[7]Cluster of Excellence RESIST (EXC2155), Hannover Medical School, Hannover, Germany

**Corresponding author:*
Jochen Huehn, Department Experimental Immunology, Helmholtz Centre for Infection Research, Inhoffenstrasse 7, 38124 Braunschweig, Germany
Phone: +49 531 6181 3310
Email: jochen.huehn@helmholtz-hzi.de

Abstract

The immune balance in mucosal tissues depends on a delicate interplay between inflammatory T helper 17 (Th17) cells and immunosuppressive regulatory T cells (Tregs). But what happens when this balance is disturbed? In this study, we uncovered a critical role for acyl-CoA synthetase bubblegum family member 1 (*Acsbg1*) in shaping Th17and Treg dynamics. Using *Acsbg1*-deficient mice, we show that while its absence does not disrupt homeostasis under steady-state conditions, it significantly alters Treg populations, particularly in gut-associated tissues. Under high-fat diet-induced metabolic stress, *Acsbg1*-deficient mice display mild metabolic changes but maintain systemic immune and metabolic function, indicating that Acsbg1 is dispensable for metabolic adaptation *in vivo*. However, upon infection with *Citrobacter rodentium*, these mice exhibit excessive Th1/Th17-driven inflammation and impaired resolution, accompanied by a strong reduction in IL-10-producing and $ST2^+$ Treg subsets. The impact is even more striking in an adoptive transfer colitis model, where *Acsbg1*-deficient Tregs fail to control inflammation, resulting in severe colitis and tissue damage. Our findings identify Acsbg1 as a key regulator of $ST2^+$ Treg function and a central player in mucosal immune homeostasis, highlighting its potential as a therapeutic target for inflammatory bowel disease and colorectal cancer.

Keywords

Acsbg1, Tregs, Th17 cell, colitis, mucosal immunology, fatty acid metabolism

Abbreviations

ACS: acyl-coenzyme A synthetases · **Acsbg1**: acyl-CoA synthetase bubblegum family member 1 · **BAT**: brown adipose tissue · **CD:** chow diet · **CFU:** colony forming unit · **CTV:** CellTrace Violet · **d.p.i.:** days post-infection · **EchoMRI:** echo magnetic resonance imaging · **FACS:** fluorescence-activated cell sorting · **FAs**: fatty acid · **FAS**: fatty acid synthesis · **FAO**: fatty acid oxidation · **GTT**: glucose tolerance test · **H&E:** haematoxylin and eosin · **HDL:** high-density lipoproteins · **HFD:** High-fat diet · **i.p.:** intraperitoneal injections · **iTreg**: *in vitro*-induced regulatory T cell · **ITT**: insulin tolerance test · **LDL:** low-density lipoproteins · **LCN-2**: lipocalin-2 · **Lin:** lineage · **mLNs**: mesenteric lymph nodes · **NEFA:** non-esterified fatty acids · **OD:** optical density· **OXPHOS**: oxidative phosphorylation · **PI:** proliferation index · **pLNs**: peripheral lymph nodes · **PPs**: Peyer's patches · **RT-qPCR:** real time quantitative PCR · **SAT**: subcutaneous adipose tissue · **SI**: small intestine · **Tconv:** conventional T cell · **Th**: T helper cell · **Tnaïve:** naïve T cells · **Treg**: regulatory T cell· **VAT**: visceral adipose tissue.

Introduction

The immune system is finely tuned to maintain homeostasis while effectively responding to threats. Central to this balance are $CD4^+$ T cell subsets, in particular regulatory T cells (Tregs) and T helper 17 (Th17) cells, which have opposing but complementary functions. Tregs suppress inflammation, promote immune tolerance, and aid in tissue repair [1-4], whereas Th17 cells defend mucosal barriers against pathogens but, when dysregulated, can drive autoimmunity and chronic inflammation [5]. Maintaining the balance between these subsets is critical, as perturbations contribute to immune-mediated diseases, including inflammatory disorders and cancer [6, 7]. Despite their importance, the molecular mechanisms that control this balance remain incompletely understood.

Emerging evidence suggests that cellular metabolism is a fundamental regulator of T cell fate, influencing differentiation, function, and stability [8]. Upon activation, naïve T cells (Tnaïve) undergo metabolic reprogramming, shifting from catabolic to anabolic pathways, such as aerobic glycolysis, to sustain their bioenergetic requirements [9]. Notably, Tregs and T_{H17} cells rely on different metabolic programs: Th17 cells depend on aerobic glycolysis and acetyl-CoA carboxylase 1-mediated *de novo* fatty acid synthesis (FAS), whereas Tregs rely primarily on oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) [10, 11]. These metabolic differences highlight the critical role of metabolism in shaping immune cell identity, yet many of the underlying molecular mechanisms remain elusive.

In addition to metabolism, epigenetic regulation also plays a pivotal role in determining T cell fate [12, 13]. Our group previously identified a unique epigenetic signature in *in vivo* generated Th17 cells characterized by selective demethylation of key genes, including *Il17a*, *Zfp362*, *Ccr6*, *Acsbg1*, *Rora*, *Dpp4*, and *Dclk1* [14]. Among these, *acyl-CoA synthase bubblegum family member 1* (*Acsbg1*), an enzyme within the acyl-coenzyme A synthetases (ACS) family, stands out for its potential link between metabolism and immunity [15, 16]. ACS enzymes activate fatty acids (FAs) by converting them to acyl-CoA molecules, thereby integrating them into metabolic pathways essential for cellular function [17].

Our recent *in vitro* studies have shown that *Acsbg1* is selectively expressed not only in Th17 cells, but also in *in vitro* induced Tregs (iTregs), where it plays a critical role in their differentiation [18]. Furthermore, in an adoptive transfer colitis model, *Acsbg1*-deficient Tnaïve cells exacerbated colitis and promoted pathogenic immune responses in immunodeficient recipient mice, suggesting an essential role for Acsbg1 in maintaining immune balance [18]. Recent evidence has further identified Acsbg1 as a metabolic checkpoint essential for mitochondrial fitness in Tregs, particularly in maintaining tissue-protective $ST2^+$ Treg subsets critical for resolution of lung inflammation [19]. However, beyond the lung, the tissue-specific role of Acsbg1 in immune regulation remains largely unexplored. Given the unique immunological environment of the intestine, where Tregs and Th17 cells respond to dietary antigens, commensal microbiota and pathogens, perturbations in their balance can lead to inflammatory bowel disease and other gut-related disorders. This underscores the importance of investigating the role of Acsbg1 in this context.

In this study, we investigate the role of *Acsbg1* in regulating Treg and Th17 cell homeostasis *in vivo*, with a particular focus on the gut. Using *Acsbg1*-deficient mice, we show that Acsbg1 is highly expressed in tissue-protective $ST2^+$ Tregs across gut-related organs and is essential for their maintenance during both homeostatic and inflammatory conditions. The absence of *Acsbg1* leads to increased intestinal inflammation and impaired generation of tissue-protective $ST2^+$ Tregs. These findings highlight Acsbg1 as a critical regulator of the mucosal immune balance and a potential player in immune-mediated diseases.

Material and methods

Mice

Acsbg1 knockout mice (B6-Acsbg1$^{tm1a(Eu\text{-}comm)Hmgu}$ mice) were generated as previously described [18] and maintained on a C57BL/6J background. All mice used in this study, including B6-Acsbg1$^{tm1a(Eu\text{-}comm)Hmgu}$ (*Acsbg1*$^{-/-}$), B6;hybrid-Rag2^{tm1Cgn} (*Rag2*$^{-/-}$), B6-Rorc(gt)-GFP^{EBE}-Foxp3^{tm1Flv} (Foxp3^{RFP} x RORγt^{GFP}) and B6-Foxp3$^{tm1(hCD2/CD52)Shori}$-Ptprc^{a} (Foxp3^{hCD2}) mice, were bred and maintained at the Helmholtz Centre for Infection Research (Braunschweig, Germany), under specific opportunistic and pathogen-free conditions. RORγt × Foxp3 mice were rederived to have a complex microbiota, including segmented filamentous bacteria, to increase the frequency of Th17 cells. *Acsbg1*$^{+/+}$ and *Acsbg1*$^{-/-}$ littermate controls were generated by crossing heterozygous *Acsbg1*$^{+/-}$ breeders. Importantly, all littermates were co-housed from birth, avoiding post-weaning regrouping to minimize microbiota-related confounders. All mice were housed in isolated, ventilated cages and handled by appropriately trained personnel and dedicated animal care staff to ensure the highest possible standards of hygiene and animal welfare as approved by institutional authorities (see Ethics). Genotyping was performed via PCR using published primers [18].

Cell isolation from organs

To prepare single-cell suspensions from lymphoid and non-lymphoid organs, mice were sacrificed by CO_2 asphyxiation. Lymphoid organs, such as spleen, PPs, pLNs and mLNs were mechanically disrupted using a plunger and PBS (Gibco) containing 0.2% BSA (Merck). Cell suspensions were filtered through a nylon mesh (100 µm for spleen; 30 µm for lymph nodes). Spleens and colon samples were processed as recently described [18]. Cells were isolated by 40%/80% Percoll (GE Healthcare) gradient centrifugation (780 g, room temperature, 20 min, acceleration and brake off). Cells from the interphase layer were collected, washed and resuspended in PBS 0.2% BSA. Single-cell suspensions from lung, liver and visceral adipose tissue (VAT) were digested in the same medium as the colon. Small intestine (SI) lamina propria cells were isolated using a dissociation kit (Miltenyi Biotec) in conjunction with the gentleMACS™ Octo Dissociator with heaters according to the manufacturer's instructions.

Flow cytometry and cell sorting

Flow cytometric analysis was performed as recently described [18, 20]. Cells were stained for viability, surface, and intracellular markers using standard protocols. Stained cells were analyzed using a FACSymphony A5 SE flow cytometer (BD Biosciences). Live cell counts were quantified on a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec).

For fluorescence-activated cell sorting (FACS), single-cell suspensions from the indicated organs were stained with specific antibodies and sorted using a BD FACSAria-II SORP, BD FACSAria-Fusion, or Symphony S6 SE (all from BD Biosciences). Unless otherwise specified, lineage negative (Lin^{-}) cells were identified using the following markers: CD8α, CD45R, CD19, CD11c, and F4/80. Antibodies used in this study are listed in **Supplementary Table 1**. Flow cytometric data were analyzed using FlowJo® 10.10.0 (Tree Star, Ashland, OR).

Real time quantitative PCR

RNA was isolated from sorted Tnaïve, Th17, RORγt$^-$ Tregs, RORγt$^+$ Tregs, Tconv, ST2$^-$ Tregs, and ST2$^+$ Tregs using the RNeasy Plus Mini or Micro Kit (Qiagen). cDNA was synthetized with the Transcriptor First Strand cDNA Synthesis Kit (Roche). RT-qPCR was performed using SYBR Green (Roche) on a LightCycler®480 system (Roche). Relative mRNA expression levels were normalized to the ribosomal protein S9 (*Rps9*). The following primers were used: *Rps9* (F: 5'-CTGGACGAGGGCAAGATGAAGC; R: 5'-TGACTGTGGCGGATGAGCACA); *Acsbg1* (F: 5'-CCAAAGAGTCTCCAAGTCACG; R: 5'- GAGTACAGAAAGGTTCCAGGC).

High-fat diet challenge

Male *Acsbg1*$^{+/+}$ and *Acsbg1*$^{-/-}$ mice were fed standard chow diet (CD) or switched to a HFD for 16 weeks. Body weight was recorded weekly. Body composition, including fat and lean mass, was determined at baseline (week 0) and 8 weeks, using EchoMRI. Glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed after 6 h fasting. For GTT, mice received 1.5 g D-glucose per kg of body weight. For ITT, mice were injected i.p. with 0.8 U insulin per kg of body weight. Tail vein blood glucose level was measured at the indicated times.

To assess metabolic flexibility, mice were fasted overnight, weighed, and refed for 2 h. Body weight and tail blood were collected pre and post refeeding. Plasma levels of glucose, lactate, cholesterol, triglycerides, NEFA, LDL and HDL were measured using a Beckman Coulter AU480 Chemistry Analyzer (Beckman Coulter). Mice were sacrificed at 16 weeks, and VAT, subcutaneous adipose tissue (SAT), and brown adipose tissue (BAT) were collected and weighed. Moreover, VAT samples were processed for flow cytometry analysis.

Infection with *Citrobacter rodentium*

The bioluminescent Kanamycin- and NaI-resistant *Citrobacter rodentium* ICC180 strain [21] was used for all the experiments. 8- to 10-week-old *Acsbg1*$^{+/+}$ and *Acsbg1*$^{-/-}$ mice were orally gavaged with 10^{10} CFUs of *C. rodentium* in a total volume of 100 µl PBS. *Acsbg1*$^{+/+}$ and *Acsbg1*$^{-/-}$ control mice received PBS only. Fecal bacterial burden was assessed at 7- and 14-days post-infection (d.p.i.) by plating serial dilutions of homogenized feces on Kanamycin$^+$ MacConkey agar (Roth) and normalized to fecal weight. At 14 d.p.i., mice were sacrificed, and colons and SI were processed for histological analysis. Single-cell suspensions from colon, SI, mLNs, liver, pLNs and spleen were analyzed by flow cytometry. For spleen bacterial burden, homogenized tissue was plated on Kanamycin$^+$ MacConkey agar and CFUs were quantified after overnight incubation at 37°C.

***In vitro* Treg suppression assay**

Single-cell suspensions were prepared from spleens and pLNs of *Acsbg1*$^{+/+}$ and *Acsbg1*$^{-/-}$ mice as described above. CD4$^+$ Tnaïve (Lin$^-$CD4$^+$CD25$^-$CD62L^{high} cells) and Tregs (Lin$^-$CD4$^+$CD25$^+$ cells) were sorted by FACS. Tregs were stimulated overnight with plate-bound anti-CD3, anti-CD28 and soluble IL-2 (50 ng ml^{-1}). Stimulated Tregs were then co-cultured with freshly sorted CellTrace Violet Cell Proliferation Kit (CTV, Invitrogen)-labeled Tnaïve from *Acsbg1*$^{+/+}$ mice at ratios from 1:1 to 1:32 (Treg:Tnaïve), with 0:1 as control, in 96-well plates (Sarstedt) in technical triplicates. Each culture contained 1×10^5 Tnaïve and 5×10^4 Dynabeads™ Mouse T-Activator CD3/CD28

(Thermo Fisher Scientific) in RPMI containing 10% fetal calf serum, 1 mM sodium pyruvate, 50 U ml^{-1} penicillin and streptomycin, 25 mM HEPES, 50 µM β-mercaptoethanol, and non-essential amino acids (Thermo Fisher Scientific). After five days, the cells were analyzed by flow cytometry. Suppression was calculated based on the proliferation index (PI) of CTV-labeled $CD4^+$ responder cells using FlowJo software. The percentage of suppression was determined using the following formula, where $PI_{Treg}{}^{+}$ is the proliferation index in the presence of Tregs and $PI_{Treg}{}^{-}$ is the index in their absence:

$$\%\ of\ suppression = \left[\frac{\left(PI_{Treg}{}^{-} - PI_{Treg}{}^{+}\right)}{PI_{Treg}{}^{-}}\right] x\ 100$$

Adoptive transfer colitis

Tregs ($Lin^-CD4^+CD25^{high}$) were sorted from pooled spleens and pLNs of 6- to 8-week-old *Acsbg1*$^{-/-}$ or *Acsbg1*$^{+/+}$ mice (CD45.2) as described above. Colitogenic Tnaïve ($Lin^-CD4^+hCD2^-CD25^-CD62L^{high}$) were sorted from pooled spleens and pLNs of 6- to 11-week-old $Foxp3^{hCD2}$ mice (CD45.1). After washing with PBS, 0.75×10^5 *Acsbg1*$^{-/-}$ or *Acsbg1*$^{+/+}$ $CD45.2^+$ Tregs were mixed with 3×10^5 Tnaïve from CD45.1 congenic mice in a 1:4 ratio in PBS. 3×10^5 cells were adoptively transferred into sex- and age-matched *Rag2*$^{-/-}$ mice (6- to 8-week-old) by i.p. injection. Controls received the same amount of Tnaïve or PBS. Recipient mice were monitored for body weight and health status over the course of 8 weeks. At 8 weeks after transfer, mice were sacrificed and the colons were analyzed by histology or flow cytometry.

Lipocalin-2 quantification

LCN-2 levels in fecal and serum samples were measured using the Mouse Lipocalin-2/NGAL DuoSet ELISA kit (R&D Systems), following the manufacturer's instructions. Fecal pellets were collected in sterile 1.5 mL Eppendorf tubes, homogenized in PBS and centrifuged twice at maximum speed for 10 min. Serum was obtained by centrifugation of blood collected directly from the heart. The ELISA assay was performed on appropriately diluted samples. Absorbance was measured at 450 nm and 570 nm. Background correction was performed by subtracting OD_{570} from OD_{450}. LCN-2 concentrations were normalized for dilution factors and for fecal samples also for stool weight.

Histological analyses

Colons and SI were measured and the weight/length ratio (mg/cm) calculated. Colon samples were fixed as Swiss rolls in 4% neutral buffered formaldehyde, and embedded in paraffin. Approximately 3 µm thickness were stained with haematoxylin and eosin (H&E) and analyzed by light microscopy. The evaluation was performed in a randomized and blinded manner. The severity of inflammation was assessed using a histological scoring system adapted from the Jackson Laboratory score [22].

Statistics

GraphPad Prism v10.4.1 (GrapPad Software) was used for statistical analyses and graphs. Unless stated otherwise, data are presented as mean±SEM. Comparisons between groups were made using two-way ANOVA with Šidák's or Tukey's multiple comparison tests, or where appropriate, the Mann-Whitney U test. A p-value below 0.05 was considered significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Specific details for each analysis are given in the figure legends.

Ethics

In accordance with the German Animal Welfare Act (§4, section 4), the sacrifice of animals solely for the removal of organs for scientific purposes is reported to the competent authority. All animal experiments were approved by the Lower Saxony and Upper Bavaria Committee for the Ethical Conduct of Animal Experiments and by the Lower Saxony State Office of Consumer Protection and Food Safety under the approval numbers 33.19-42502-04-20/3540, 33.19-42502-04-23-000465, and ROB-55.2-2532.Vet_02-21-133. This study was conducted in accordance with the principles of the Basel Declaration and the recommendations of FELASA and GV-SOLAS.

Results

Acsbg1 is preferentially expressed in *ex vivo* tissue-resident ST2$^+$ Tregs

We previously identified *Acsbg1* as part of an epigenetic signature distinguishing *ex vivo* Th17 cells from Tnaïve, Th1 cells and Tregs [14]. In a subsequent study, we have shown that Acsbg1 is also expressed in *in vitro* differentiated Th17 cells and iTregs, and that its deficiency impairs their differentiation and exacerbates colitis in an adoptive transfer colitis model [18]. Recently, Kanno et al. identified *Acsbg1* as a metabolic checkpoint essential for mitochondrial fitness in lung Tregs [19]. These findings suggest that *Acsbg1* plays a fundamental role in shaping Th17 cells and Tregs, but its precise *in vivo* role in other tissues remains unclear.

To investigate this, we first assessed *Acsbg1* expression in *ex vivo* CD4$^+$ T cell subsets using real time quantitative PCR (RT-qPCR). Tnaïve, Th17 cells, RORγt$^-$ Tregs and RORγt$^+$ Tregs were isolated from spleen, mesenteric lymph nodes (mLNs), lung, visceral adipose tissue (VAT), liver, colon and small intestine (SI) of Foxp3^{RFP} x RORγt^{GFP} mice. Low levels of *Acsbg1* expression were observed not only in spleen-derived Tnaïve, but also in Th17 cells from all organs examined. In contrast, both RORγt$^-$ and RORγt$^+$ Tregs showed significantly higher levels of *Acsbg1* expression when compared to Th17 cells (**Fig. 1A**). Notably, Tregs from non-lymphoid organs, such as lung, VAT, liver, colon and SI showed between 4- and 22-fold increase in *Acsbg1* expression when compared to Tregs from lymphoid organs. Interestingly, RORγt$^-$ and RORγt$^+$ Tregs from non-lymphoid organs showed comparable levels of *Acsbg1* expression (**Fig. 1A**).

When examining *Acsbg1* expression in tissue-protective ST2$^+$ Tregs from lung, colon and SI, we observed significantly increased expression levels when compared to ST2$^-$ Tregs, which showed low expression levels comparable to conventional T cells (Tconv) (**Fig. 1B**). In conclusion, our data show that *Acsbg1* is expressed at low levels in *ex vivo* Th17 cells, whereas Tregs, and tissue-resident ST2$^+$ Tregs, show significantly higher *Acsbg1* expression, suggesting its potentially critical role in this specialized subset.

Acsbg1-deficient mice harbor fewer effector Tregs

To investigate the impact of *Acsbg1* deficiency on the immune system, with a particular focus on Th17 cells and Tregs, we analyzed the T cell subset composition in multiple organs, including peripheral lymph nodes (pLNs), spleen, mLNs, SI, Peyer's patches (PPs), colon, VAT, lung and liver of 7- to 10-week-old *Acsbg1*$^{-/-}$ mice and *Acsbg1*$^{+/+}$ littermate controls using flow cytometry (**Supplementary Fig. 1A**). Frequencies of total CD4$^+$ T cells, naïve, effector, Tconv, and Th1 cells were comparable between genotypes (**Supplementary Fig. 1B-F**), and no differences were observed in the frequencies or absolute numbers of Th17 and total Tregs (**Fig. 2A-B**). In contrast, *Acsbg1*$^{-/-}$ mice showed a significant reduction in colonic Foxp3$^+$CD25$^+$ Tregs in CD44$^+$ST2$^+$ Tregs in multiple organs including pLNs, mLNs, colon, and VAT of *Acsbg1*$^{-/-}$ mice compared to *Acsbg1*$^{+/+}$ littermate controls (**Fig 2C-D**). However, absolute numbers of these subsets were unchanged (**Fig 2C-D**), indicating that *Acsbg1* deficiency alters subset composition rather than total Treg abundance. While T-bet$^+$ Tregs were unaffected by *Acsbg1* deficiency in all organs, the frequency of RORγt$^+$ Tregs was significantly reduced in the colon of adult *Acsbg1*$^{-/-}$ mice compared to *Acsbg1*$^{+/+}$ littermate controls (**Supplementary Fig. 1G-H**).

To assess whether these changes persist with age, we also profiled >80-week-old *Acsbg1*$^{-/-}$ mice and *Acsbg1*$^{-/-}$ littermate controls. Similar to the younger adult mice, all major CD4$^+$ T cell subsets remained unaffected by *Acsbg1* deficiency (**Supplementary Fig. 2A-F**). However, aged *Acsbg1*$^{-/-}$

mice exhibited a significant reduction in T-bet$^+$ Tregs in the SI compared to *Acsbg1*$^{+/+}$ littermates (**Supplementary Fig. 2G**), whereas the reduced frequency of RORγt$^+$ Tregs observed in the colon of younger adult mice (**Supplementary Fig. 1H**) was not observed in aged mice (**Supplementary Fig. 2H**). Notably, Foxp3$^+$CD25$^+$ Tregs were significantly reduced not only in the colon, but also in other gut-associated tissues, including mLNs, SI, and PPs of aged *Acsbg1*$^{-/-}$ mice compared to *Acsbg1*$^{+/+}$ littermate controls (**Supplementary Fig. 2I**). In addition, CD44$^+$ST2$^+$ Tregs were significantly decreased in VAT, lung and liver of old *Acsbg1*$^{-/-}$ mice (**Supplementary Fig. 2J**). These persistent changes indicate a long-term impairment of effector Treg populations, particularly in gut-associated tissues.

Mild metabolic changes in *Acsbg1*-deficient mice under high-fat diet feeding

High-fat diet (HFD) feeding is known to promote metabolic inflammation, enhance Th17 responses, and reduce Treg frequencies in VAT, contributing to insulin resistance and tissue dysfunction [23-25]. Given the established role of Acsbg1 in lipid metabolism, we investigated whether its deficiency affects systemic metabolic and immunological responses under dietary stress. 8- to 11-week-old male *Acsbg1*$^{+/+}$ and *Acsbg1*$^{-/-}$ mice were fed a HFD for 16 weeks (**Fig. 3A**), while control groups remained on standard chow diet (CD). Although absolute body weights were similar between genotypes (**Supplementary Fig. 3A**), *Acsbg1*$^{-/-}$ mice on HFD showed a significantly increased relative body weight gain over time compared to *Acsbg1*$^{+/+}$ controls (**Fig. 3B**), suggesting a subtle susceptibility to diet-induced weight gain, despite similar baseline weights.

Echo magnetic resonance imaging (EchoMRI) at baseline and after 8 weeks showed no differences in lean or fat mass between genotypes (**Fig. 3C-D**). Furthermore, glucose and insulin tolerance tests (GTT and ITT, respectively) revealed comparable glucose clearance and insulin sensitivity (**Fig. 3E-G**). Metabolic flexibility, assessed via fasting and refeeding, showed similar changes in blood glucose, lactate, cholesterol, non-esterified fatty acids (NEFA), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels in both genotypes, along with comparable fasting-induced weight (**Fig. 3H-I**). Notably, triglyceride levels were elevated in *Acsbg1*$^{-/-}$ mice after refeeding with HFD compared to *Acsbg1*$^{+/+}$ controls.

Among adipose tissues, the weight of subcutaneous adipose tissue (SAT) was modestly increased in *Acsbg1*$^{-/-}$ mice on HFD, while VAT and brown adipose tissue (BAT) were unaffected (**Supplementary Fig. 3B**). VAT immunophenotyping revealed that Tconv frequencies increased in both genotypes in response to HFD (**Supplementary Fig. 4A-B**). Under standard CD, Treg frequencies were slightly lower in *Acsbg1*$^{-/-}$ mice compared to *Acsbg1*$^{+/+}$ controls, matching levels seen in both groups after HFD (**Supplementary Fig. 4C**). Under standard CD, CD44$^+$ST2$^+$ Tregs were significantly reduced in *Acsbg1*$^{-/-}$ mice compared to *Acsbg1*$^{+/+}$ controls, and HFD strongly reduced frequencies of CD44$^+$ST2$^+$ Tregs in both genotypes (**Supplementary Fig. 4D**). Instead, frequencies of Th17 cells and RORγt$^+$ Tregs were slightly and significantly increased, respectively, upon HFD feeding, with no differences being observed between the genotypes (**Supplementary Fig. 4E-F**).

In summary, Acsbg1 is dispensable for maintaining metabolic homeostasis *in vivo*, as its deficiency leads to only mild metabolic alterations under HFD-induced stress conditions, without affecting immune homeostasis or overall systemic metabolic function.

***Acsbg1* deficiency aggravates *C. rodentium* infection by disrupting T cell balance**

Under homeostatic conditions, *Acsbg1*$^{-/-}$ mice showed normal Th17 and Treg frequencies compared to *Acsbg1*$^{+/+}$ controls (**Fig. 2A-B**). However, their reduced frequency of tissue-protective CD44^{+}ST2^{+} Tregs suggest a potential vulnerability to immune dysregulation during inflammation. To test this, we challenged mice with *Citrobacter rodentium*, a pathogen known to induce potent intestinal Th17 responses [26, 27].

Acsbg1$^{+/+}$ and *Acsbg1*$^{-/-}$ mice were orally infected with 10^{10} colony forming units (CFUs) of *C. rodentium*, while uninfected controls received PBS (**Fig. 4A**). Body weight was monitored daily until 14 d.p.i. (**Fig. 4A**). The uninfected control groups gained weight equally. While both infected groups followed similar weight trajectories until 10 d.p.i., *Acsbg1*$^{-/-}$ mice exhibited significantly greater weight loss thereafter, indicating more severe colitis (**Fig. 4A**). At 7 d.p.i., infected *Acsbg1*$^{+/+}$ and *Acsbg1*$^{-/-}$ mice showed similarly elevated fecal and splenic CFUs (**Supplementary Fig. 5A**). However, by 14 d.p.i. *Acsbg1*$^{-/-}$ mice had elevated fecal and serum lipocalin-2 (LCN-2) levels and higher fecal CFUs compared to infected *Acsbg1*$^{+/+}$ littermates (**Fig. 4B-C**). Pathogen clearance was incomplete in the spleen of *Acsbg1*$^{-/-}$ mice, whereas infected *Acsbg1*$^{+/+}$ littermate controls successfully eradicated the bacteria (**Fig. 4C**). In addition, the weight/length ratio of the colon was significantly increased in *Acsbg1*$^{-/-}$ mice (**Fig. 4D**), though histology showed no obvious morphological differences (**Fig. 4E-F, Supplementary Fig. 5B**).

Flow cytometric analysis of colonic lamina propria T cells at 14 d.p.i. revealed increased frequencies and absolute numbers of Th1 and Th17 cells in *Acsbg1*$^{-/-}$ mice compared to *Acsbg1*$^{+/+}$ littermate controls (**Supplementary Fig. 6; Supplementary Fig. 7A-B**). Frequencies of IFN-γ^{+} and IL-17A^{+} single- and also IL-17A^{+}IFN-γ^{+} double-producing CD4^{+} T cell were also elevated (**Fig. 4G**), although their absolute numbers remained unchanged (**Supplementary Fig. 7C-E**). While the frequencies and absolute numbers of Tconv and total Tregs did not differ significantly between infected *Acsbg1*$^{-/-}$ mice and *Acsbg1*$^{+/+}$ littermate controls (**Supplementary Fig. 7F-G**), *Acsbg1*$^{-/-}$ mice showed significantly reduced frequencies of CD44^{+}ST2^{+} Tregs (**Fig. 4H**) and IL-10^{+} Tregs (**Supplementary Fig. 7H**). Interestingly, RORγt^{+} Treg frequencies were unchanged between genotypes, but their absolute numbers were elevated in *Acsbg1*$^{-/-}$ mice (**Supplementary Fig. 7I**), possibly reflecting compensatory expansion triggered by heightened inflammation or the loss of ST2^{+} Tregs. Notably, IL-10 production by RORγt^{+} Tregs was comparable between groups (**Supplementary Fig. 7J**). Altogether, these results demonstrate that *Acsbg1* deficiency disrupts the CD4^{+} T cell balance, characterized by enhanced Th1 and Th17 cell responses and impaired effector Treg subsets, which correlates with more severe disease outcome following *C. rodentium* infection.

Impaired generation of colonic ST2^{+} Tregs in the absence of *Acsbg1* results in more severe colitis

Our data generated so far using whole-body *Acsbg1*$^{-/-}$ mice suggest that Acsbg1 supports the induction of effector Tregs, particularly ST2^{+} Tregs in the gut. To assess whether this role is Treg-intrinsic, we first performed an *in vitro* suppression assay with Tregs from *Acsbg1*$^{-/-}$ and *Acsbg1*$^{+/+}$ mice. Here, no significant differences were observed at most Tregs:Tnaïve ratios, although *Acsbg1*$^{-/-}$ Tregs showed a modestly reduced suppressive activity at the 1:1 ratio, indicating a subtle impairment of the suppressive function (**Supplementary Fig. 8**).

Next, we assessed the Treg-intrinsic effects of Acsbg1 *in vivo* using an adoptive transfer colitis model, in which *Acsbg1*$^{-/-}$ or *Acsbg1*$^{+/+}$ Tregs (CD45.2) were co-transferred with Tnaïve cells (CD45.1) into *Rag2*$^{-/-}$ mice. While *Acsbg1*$^{+/+}$ Tregs protected against colitis, *Acsbg1*$^{-/-}$ Tregs provided only partial protection, with recipients showing significantly greater weight loss (**Fig. 5A**) and higher colon weight/length ratios (**Fig. 5B**). Histological analysis confirmed more severe inflammation in recipients of *Acsbg1*$^{-/-}$ Tregs (**Fig. 5C-D; Supplementary Fig. 9**). While serum LCN-2 levels were

comparable between *Acsbg1*$^{-/-}$ and *Acsbg1*$^{+/+}$ Treg recipients, fecal LCN-2 levels were significantly elevated in *Acsbg1*$^{-/-}$ compared to *Acsbg1*$^{+/+}$ Treg recipients (**Fig. 5E**).

Flow cytometric analysis 8 weeks after adoptive transfer revealed no differences in Treg-to-effector T cell ratios between *Acsbg1*$^{-/-}$ and *Acsbg1*$^{+/+}$ Treg recipients (**Supplementary Fig. 10, Fig. 5F**). Quantification of the total number of cells isolated from the colonic lamina propria revealed a comparable suppression of the lymphopenia-driven homeostatic expansion by *Acsbg1*$^{-/-}$ and *Acsbg1*$^{+/+}$ Tregs (**Fig. 5G**), although *Acsbg1*$^{-/-}$ Treg recipients showed an increased proliferation of both CD45.1^{+} effector T cells and CD45.2^{+} Tregs compared to *Acsbg1*$^{+/+}$ Treg recipients (**Supplementary Fig. 11A-B**). Among the CD45.1^{+} effector T cells, frequencies of Th1 and Th17 cells were unchanged (**Supplementary Fig. 11C-D**). However, pro-inflammatory cytokine production (IFN-γ^{+}, IL-17A^{+}, IFN-γ^{+}IL-17A^{+} and IL-22^{+}) was significantly higher in *Acsbg1*$^{-/-}$ Treg recipients (**Supplementary Fig. 11E-H**).

Strikingly, ST2^{+} Tregs were significantly reduced in both frequency and absolute number in *Acsbg1*$^{-/-}$ Treg recipients compared to *Acsbg1*$^{+/+}$ controls **(Fig. 5H)**, with the numerical loss more pronounced due to reduced total Treg numbers (**Supplementary Fig. 11I**) and loss of ST2^{+} cells among *Acsbg1*$^{-/-}$ Tregs. In contrast, RORγt^{+} Tregs were increased in frequency but not in number (**Supplementary Fig. 11K**), suggesting a relative enrichment rather than expansion. Interestingly, IL-10^{+} Tregs were significantly elevated in both frequency and absolute number among *Acsbg1*$^{-/-}$ Treg recipients, including a marked increase in IL-10^{+}RORγt^{+} Tregs (**Supplementary Fig. 11J, L**). This suggests that while ST2^{+} Tregs are selectively impaired, other effector subsets may expand or upregulate IL-10 expression in a compensatory manner.

Together, these findings demonstrate that Acsbg1 is essential for the generation and/or maintenance of ST2^{+} Tregs during colitis. Its absence compromises the ST2^{+} Treg accumulation, resulting in poorly controlled Th1/Th17-driven inflammatory immune responses and exacerbated tissue damage, underscoring its critical role in mucosal immune regulation.

Discussion

This study reveals that *Acsbg1* is essential for maintaining the balance between pro-inflammatory T cells and tissue-protective ST2$^+$ Tregs in gut-associated tissues. We show that *Acsbg1* deficiency leads to a selective loss of ST2$^+$ Tregs across multiple tissues, resulting in impaired resolution of inflammation and heightened Th1/Th17 responses during intestinal challenge. These findings establish Acsbg1 as a key metabolic regulator of tissue-protective ST2$^+$ Tregs, particularly under inflammatory stress.

While a previous study revealed that *Acsbg1* supports mitochondrial fitness and induction of ST2$^+$ Tregs in the lung, especially during inflammation [19], its role in the intestinal immune compartment remained unclear. Our data extends these findings by demonstrating that Acsbg1 is most highly expressed in ST2$^+$ Tregs from gut-related tissues and required for their maintenance in both steady-state and inflammatory conditions. This positions Acsbg1 as a critical regulator of mucosal immune homeostasis.

Although *Acsbg1* expression is detectable in Th17 cells, it is significantly lower than in Tregs, especially in the intestine, where Th17 cells are highly abundant. Notably, *Acsbg1* expression is significantly higher in Tregs, particularly those from the lung, VAT, colon and SI. Within the Treg compartment, ST2$^+$ Tregs consistently exhibit the highest *Acsbg1* levels, highlighting a subset-specific requirement, and extending previous findings from Kanno et al. [19] in the lung, to additional tissues such as colon and SI.

Despite being clinically and metabolically healthy under steady-state conditions, *Acsbg1*$^{-/-}$ mice exhibited a notable reduction in CD44$^+$ST2$^+$ Tregs in multiple tissues, especially in the colon and other gut-related tissues. These cells are known to promote tissue repair and immune tolerance [28-33], and their loss likely contributes to increased susceptibility to inflammation. Interestingly, ST2$^+$ Tregs in the SI, while expressing *Acsbg1*, were not significantly reduced. One explanation may lie in tissue-specific metabolic thresholds: although *Acsbg1* is elevated in SI-resident ST2$^+$ Tregs, it does not reach the levels observed in colonic or pulmonary counterparts, potentially resulting in a lower dependency on *Acsbg1* for survival or function. Furthermore, CD44$^+$ST2$^+$ Tregs represent a smaller fraction of SI-resident compared to colonic Tregs, where this subset is more abundant and critical for barrier protection and epithelial repair [31]. This may explain why the loss of *Acsbg1* has a more pronounced impact on the colonic Treg compartment. We also compared ST2$^+$ Tregs and RORγt$^+$ Tregs, a subset known to be induced by the gut microbiota and specialized in maintaining mucosal tolerance [34, 35], across steady-state and different inflammatory contexts. ST2$^+$ Tregs were consistently reduced across all tissues and models examined, whereas RORγt$^+$ Treg changes were variable and context-dependent. This reinforces the idea that Acsbg1 selectively supports ST2$^+$ Treg maintenance rather than broadly affecting all Treg subsets.

Given Acsbg1's role in lipid metabolism [36], we tested whether its deficiency impairs systemic responses to HFD. *Acsbg1*$^{-/-}$ mice showed mildly increased weight gain and plasma triglycerides, suggesting a subtle impairment in lipid handling likely due to impaired hepatic triglyceride clearance. Immune profiling of VAT showed minimal differences, except for a pre-existing reduction in ST2$^+$ Tregs, which were strongly reduced by HFD in both genotypes. These results suggest that Acsbg1 is dispensable for metabolic adaptation under dietary stress but remains critical for immune homeostasis under baseline conditions.

In contrast, immune challenge with *C. rodentium* unmasked a clear phenotype. At 14 d.p.i., *Acsbg1*$^{-/-}$ mice displayed heightened inflammation, impaired bacterial clearance, and an imbalance between effector and regulatory T cells. Elevated IFN-γ$^+$ and IL-17A$^+$ T cell responses were coupled with a reduction in ST2$^+$ and IL-10$^+$ Tregs. Interestingly, while the frequency of RORγt$^+$ Tregs remained

unchanged, their absolute numbers were significantly increased in *Acsbg1*$^{-/-}$ mice, possibly reflecting a compensatory expansion in response to heightened inflammation or to the loss of ST2^{+} tissue-repairing Tregs. Despite robust effector responses, *Acsbg1*$^{-/-}$ mice failed to clear *C. rodentium* efficiently, highlighting the critical role of regulatory restraint in resolving inflammation [21, 26]. We propose that an imbalance between effector and Treg cells, marked by insufficient ST2^{+} Tregs may exacerbates tissue damage, delays repair, and impairs barrier function. While bacterial loads were initially similar, the elevated burdens in *Acsbg1*$^{-/-}$ mice by 14 d.p.i. suggest that persistent inflammation, rather than defective immune activation, underlies delayed clearance.

Given the global deletion of Acsbg1, contributions from other immune or non-immune compartments cannot be excluded. Indeed, the increased Th1/Th17 responses could reflect the result of persistent bacterial burden and chronic immune stimulation.

To test more directly whether Acsbg1 plays a Treg-intrinsic role in immune control, we employed an adoptive transfer colitis model. In lymphopenic mice, the introduction of Tnaïve cells leads to autoimmune colitis, a pathology that can be averted by simultaneous transfer of Tregs [37]. Co-transfer of Tnaïve with *Acsbg1*$^{-/-}$ Tregs into *Rag2*$^{-/-}$ mice resulted in more severe colitis compared to recipients of *Acsbg1*$^{+/+}$ Tregs, characterized by greater weight loss, higher inflammation scores and increased Th1/Th17 responses. Interestingly, *Acsbg1*$^{-/-}$ Tregs did not show an impaired ability to suppress effector T cell expansion, as indicated by similar Treg-to-effector T cell ratios between groups. However, we observed that ST2^{+} Tregs were markedly reduced in both frequency and absolute number in mice receiving *Acsbg1*$^{-/-}$ Tregs. While the frequency reduction appeared modest, the absolute loss was more pronounced, likely reflecting a combined effect of slightly lower total Treg recovery and a reduced proportion of ST2^{+} cells within the *Acsbg1*$^{-/-}$ Treg compartment. By contrast, RORγt^{+} Tregs were modestly increased in frequency, but their absolute numbers remained unchanged, suggesting a relative enrichment rather than true expansion. Importantly, this increase did not mitigate disease severity, reinforcing the non-redundant, tissue-protective role of ST2^{+} Tregs during intestinal inflammation. These findings further support the conclusion that Acsbg1 is selectively required for the generation and/or maintenance of ST2^{+} Tregs, rather than affecting the broader Treg pool. However, these findings do not prove causality between ST2^{+} Treg loss and the outcomes observed in the *C. rodentium* infection model. Rather, they suggest that under defined inflammatory conditions, ST2^{+} Tregs are preferentially affected by *Acsbg1* deficiency and may contribute to impaired resolution of inflammation. Other cell-intrinsic or systemic mechanisms likely contribute to the phenotype in the infection model, and future studies using cell type–specific knockouts will be required to dissect these effects more precisely.

Taken together, these findings demonstrate that Acsbg1 is essential for regulating inflammatory responses in the gut by promoting the generation of ST2^{+} Tregs. Its deficiency disrupts the balance between pro-inflammatory T cells and Tregs, resulting in enhanced Th1/Th17 responses, tissue inflammation, and increased risk of autoimmunity. This is consistent with previous work showing that Acsbg1 supports Treg homeostasis by maintaining mitochondrial fitness and facilitating metabolic reprogramming [19]. In the lung, Acsbg1-mediated acyl-CoA synthesis has been shown to be critical for lipid metabolism, enabling ST2^{+} Tregs to fulfil their tissue-protective functions [19]. Our findings extend this role to gut-related organs, where Acsbg1 appears to be equally important for resolving inflammation and maintaining immune tolerance.

T cell activation and differentiation are influenced by T cell receptor signaling, co-stimulation, and environmental cues [33, 38, 39], but metabolism plays an equally fundamental role in shaping T cell fate. Effector T cells, such as Th17 cells, rely primarily on aerobic glycolysis and *de novo* FAS, whereas Tregs rely on OXPHOS and FAO [10, 11]. Tregs, particularly those in the VAT and intestinal mucosa, show an increased dependence on lipid metabolism for survival and function [40, 41]. This may explain why VAT and intestinal Treg populations are most affected by *Acsbg1* deficiency. Kanno

et al. showed that $ST2^+$ Tregs are particularly dependent on Acsbg1-mediated lipid metabolism and are therefore more vulnerable to its loss [19]. Inhibition or deletion of *Acsbg1* impaired Treg proliferation, survival and metabolism, affecting lipid and cholesterol pathways, aerobic glycolysis and the Krebs cycle [19]. While *Acsbg1*$^{-/-}$ mice remained healthy under steady-state conditions, their inability to regulate inflammation became apparent under inflammatory stress, suggesting that Acsbg1-mediated acyl-CoA synthesis is critical during immune challenge when Tregs must rapidly proliferate to resolve inflammation. In addition, Kanno et al. also showed that the IL-33-ST2 axis induces FA uptake in lung Tregs, suggesting a similar mechanism in intestinal Tregs under inflammatory conditions [19].

Our findings have important therapeutic implications. Enhancing Acsbg1 activity may enhance tissue-protective Treg responses in chronic inflammatory diseases, such as inflammatory bowel disease, where dysregulation of the IL-33/ST2 axis undermines effective immune control [42]. Conversely, targeting Acsbg1 pathways may provide a strategy to limit Treg-mediated immunosuppression in cancer. For example, in colorectal cancer, IL-33 drives $ST2^+$ Treg accumulation in the tumor microenvironment, promoting immune evasion [43]. Thus, inhibition of Acsbg1 could modulate immune infiltrates and enhance anti-tumor immunity.

In conclusion, Acsbg1 is essential for meeting the metabolic requirements of $ST2^+$ Tregs, particularly during inflammatory responses in gut-related organs. Its absence disrupts the Th17/Treg balance, leading to chronic inflammation and tissue damage. These findings establish Acsbg1 as a critical regulator of immune homeostasis and a promising therapeutic target for a range of immune-mediated diseases, from chronic inflammation to cancer.

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Authors contributions

MP: Conceptualization, Methodology, Formal analysis, Data curation, Visualization, Writing - original draft; FK: Formal analysis; HJ: Methodology, Formal analysis, Data curation; ALF: Methodology, Formal analysis, Data curation; MB: Methodology, Formal analysis, Data curation; CD: Data curation, Funding acquisition, Supervision; MR: Methodology, Data curation, Funding acquisition, Supervision; JH: Conceptualization, Funding acquisition, Supervision, Writing – original draft; Writing - review & editing

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used DeepL in order to improve readability and language of the written manuscript. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Figure legends

Figure 1. *Acsbg1* is preferentially expressed in ST2$^+$ Tregs. (A) qRT-PCR was performed to assess *Acsbg1* expression in Tnaïve (Lin$^-$CD4$^+$GFP$^-$RFP$^-$CD25$^-$CD62L^{high}), Th17 cells (Lin$^-$CD4$^+$GFP$^+$RFP$^-$), RORγt$^-$ Tregs (Lin$^-$CD4$^+$GFP$^-$RFP$^+$) and RORγt$^+$ Tregs (Lin$^-$CD4$^+$GFP$^+$RFP$^+$) isolated from spleen, mLNs, lung, VAT, liver, colon and SI of Foxp3^{RFP} x RORγt^{GFP} double reporter mice. **(B)** qRT-PCR was performed to assess *Acsbg1* expression in Tconv (Lin$^-$CD4$^+$CD25$^-$), ST2$^-$ Tregs (Lin$^-$CD4$^+$CD25^{high}ST2$^-$), and ST2$^+$ Tregs (Lin$^-$CD4$^+$CD25^{high}ST2$^+$) isolated from lung, colon and SI of *Acsbg1*$^{+/+}$ mice. Each symbol represents the mean of technical duplicates normalized to *Rsp9* expression. Data are presented as mean ± SEM. Results were pooled from two independent experiments (n=4-8 biological replicates). Statistical significance was assessed independently for each tissue using one-way ANOVA with Tukey's multiple comparison test (mean ± 95% CI; **p < 0.01 and ***p < 0.001).

Figure 2. Adult *Acsbg1*-deficient mice have reduced Treg frequencies in gut-related organs. Summary graphs of immunophenotyping performed in pLNs, spleen, mLNs, SI, PPs, colon, VAT, lung and liver of adult 7–10-week-old *Acsbg1*$^{-/-}$ mice and *Acsbg1*$^{+/+}$ littermate controls. **(A-C)** Frequencies and absolute numbers of **(A)** Foxp3$^-$RORγt$^+$ Th17 cells, **(B)** Foxp3$^+$ Tregs, and **(C)** CD25$^+$Foxp3$^+$ Tregs among CD8$^-$CD4$^+$ T cells. **(D)** Frequencies and absolute numbers of CD44$^+$ST2$^+$ cells among Tregs. Each symbol represents a single mouse. Data are presented as mean ± SEM. Results were pooled from 6 independent experiments with 4-10 mice per experimental group. Significance of differences between means was assessed by ordinary two-way ANOVA with Šídák's multiple comparison test (mean ± 95% CI; *P<0.05, **P<0.01, ***P<0.001).

Figure 3. *Acsbg1*-deficient mice have a healthy clinical and metabolic status. (A) Experimental layout of the HFD challenge. Male *Acsbg1*$^{+/+}$ and *Acsbg1*$^{-/-}$ mice were fed a standard CD until 8- to 11-weeks of age. Then, they were challenged with an HFD for 16 weeks, while control groups remained on standard CD. **(B)** Percentage of body weight variation was monitored for 16 weeks. **(C-D)** EchoMRI was performed at **(C)** 0 and **(D)** 8 weeks of monitoring to assess lean and fat mass. **(E-I)** Mice were challenged with 6h of fasting, followed by **(E)** GTT and **(F-G)** ITT. Area under the curve was calculated to compare glucose clearance and insulin sensitivity between groups. **(H)** Clinical blood parameters assessed after overnight fasting and 2h after refeeding. **(I)** Body weight loss measured after overnight fasting. Data are presented as mean ± SEM. Data are pooled from 2 independent experiments with 5-14 mice per experimental group. Significance of differences between means was assessed by **(B, D, H-I)** ordinary two-way ANOVA with Tukey's multiple comparison test or **(C)** non-parametric Mann-Whitney test (mean ± 95% CI; *P<0.05, **P<0.01, ***P<0.001). In **(B)**, the significance of the difference between *Acsbg1*$^{+/+}$ HFD and *Acsbg1*$^{-/-}$ HFD means at individual time points is shown.

Figure 4. *Acsbg1* deficiency exacerbates *C. rodentium* infection by affecting the balance between Th1 cells, Th17 cells and Tregs. *Acsbg1*$^{-/-}$ mice and *Acsbg1*$^{+/+}$ littermate controls were orally infected with 10^{10} CFU of *C. rodentium*, or injected with PBS as controls, and clinically monitored until 14 d.p.i. **(A)** Percentage change in body weight over the course of the experiment. **(B)** At 14 d.p.i., mice were sacrificed, and fecal and serum samples were collected. Quantification of fecal (left) and serum (right) LCN-2. **(C)** CFU of bacteria recovered from feces (left) and spleen (right). **(D)** Length and weight of the colon were assessed and shown as weight/length ratio. **(E-F)** At

14 d.p.i., mice were sacrificed, and colon samples were collected for histological analysis or flow cytometry. **(E)** Examples of H&E staining of colon samples collected from infected or PBS-treated *Acsbg1*$^{+/+}$ and *Acsbg1*$^{-/-}$ mice. Scale bars = 50 μm. * inflammatory cell invasion, E edema, P peritonitis, I ingesta, [cryptepithelial hyperplasia. **(F)** Scatter plot with bars representing the total inflammation score. **(G)** Representative dot plots and frequencies of IFN-γ^{+}IL-17A^{-}, IFN-γ^{-}IL-17A^{+}, and IFN-γ^{+}IL-17A^{+} cells among CD3^{+}CD4^{+} T cells. **(H)** Representative dot plots and frequencies of CD44^{+}ST2^{+} cells among Tregs. Each symbol represents a single mouse. Data are presented as mean ± SEM. Results were pooled from **(A-F)** 9 independent experiments (n = 4-13 biological replicates per group in total) or **(G-H)** 4 independent experiments with 6-12 mice per experimental group. Significance of differences between means was assessed by **(A-D, F)** ordinary two-way ANOVA with Tukey's multiple comparison test or **(G-H)** non-parametric Mann-Whitney test (mean ± 95% CI; *P<0.05, **P<0.01, ***P<0.001). In **(A)**, the significance of the difference between *Acsbg1*$^{+/+}$ infected and *Acsbg1*$^{-/-}$ infected means at individual time points post infection is also shown.

Figure 5. *Acsbg1* deficiency in Tregs impairs the induction of ST2^{+} Tregs and leads to more severe colitis. Tnaïve (Lin^{-}CD4^{+}hCD2^{-}CD25^{-}CD62L^{high}) were sorted from Foxp3^{hCD2} mice (CD45.1), while Tregs (Lin^{-}CD4^{+} CD25^{high}) were sorted from CD45.2 *Acsbg1*$^{+/+}$ or *Acsbg1*$^{-/-}$ mice. Tregs and Tnaïve were adoptively co-transferred in a 1:4 ratio into sex- and age-matched *Rag2*$^{-/-}$ mice by intraperitoneal injection (i.p). injection. Control groups received the same amount of Tnaïve alone or PBS. Mice were monitored for 8 weeks after transfer. **(A)** Percentage change in body weight over the course of the experiment. **(B-I)** Eight weeks after adoptive transfer, mice were sacrificed and **(B)** the length and weight of the colon was assessed and presented as the weight/length ratio. **(C-D)** Colon inflammation was assessed by histology. **(C)** Examples of H&E staining of colons from mice receiving PBS, Tnaïve, Tnaïve plus Tregs from *Acsbg1*$^{+/+}$ mice and Tnaïve plus Tregs from *Acsbg1*$^{-/-}$ mice. Scale bars= 50 μm. * Inflammatory cell invasion, P peritonitis, I ingesta, [crypt epithelial hyperplasia. **(D)** Summary graph of the total histopathological scores for the indicated experimental groups. **(E)** Quantification of serum (left) and fecal (right) LCN-2. **(F)** Ratio of CD45.2^{+} Tregs to CD45.1^{+} colitogenic T cells and **(G)** total number of lymphocytes isolated from the colonic lamina propria (cLP). **(H)** Representative flow cytometry plots, frequencies and absolute cell numbers of ST2^{+} cells among CD45.2^{+}CD4^{+}Foxp3^{+} Tregs. Each symbol represents a single mouse. Data are presented as mean ± SEM. Results were pooled from **(A-E)** 4 independent experiments or **(F-H)** 2 independent experiments with 3-9 mice per experimental group. Significance of differences between means was assessed by **(A-B, D-E, G)** ordinary two-way ANOVA with Tukey's multiple comparison test or **(F, H)** non-parametric Mann-Whitney test (mean ± 95% CI; *P<0.05, **P<0.01, ***P<0.001). In **(A)**, the significance of the difference between Tnaïve plus *Acsbg1*$^{+/+}$ Tregs and Tnaïve plus *Acsbg1*$^{-/-}$ Tregs means at individual time points after adoptive transfer is also shown.