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**Activation of adenosine receptor A2a by gut symbionts promotes
immune tolerance**

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Abstract

The gut microbiota is critical to immune homeostasis^{1–4}, but our understanding of the underlying molecular mechanisms is very limited. Here, we demonstrate a division of labor among members of the eight-membered “model microbiome” altered Schaedler flora⁵ in promoting distinct immunophenotypes. We report that *Parabacteroides goldsteinii* ASF519 induces immune tolerance by promoting interleukin (IL)-10 production in a variety of myeloid-derived immune cells. The IL-10 induction is dependent on the activation of adenosine receptor A2a by microbial enzymes of the methionine cycle. ASF519 colonization in mice increased the level of adenosine in ceca and induced IL-10 secreting dendritic cells in colonic lamina propria. These immunophenotypes were pharmacologically reversed by A2a blockage. In mouse models of human autoimmune diseases, ASF519 supplementation significantly ameliorated insulinitis in type 1 diabetes and collagen-induced arthritis. This study unveils a novel paradigm of gut microbiota-adenosine receptor interactions in immune tolerance and potentially provides a new therapeutic strategy for immune disorders.

Introduction

Previous studies have demonstrated reciprocal interactions between the immune system and the gut microbiota^{1,6,7}. The importance of the gut microbiota to immune function has been demonstrated by a wide range of immune functional and structural deficiencies in germ-free (GF) or antibiotic-treated mice²⁻⁴ that can be rescued by microbial recolonization in the gut. We now appreciate that gut microbial dysbiosis caused by genetic susceptibility, antibiotic use, or Westernized diet, for example, can influence intestinal diseases, immune disorders, cancer, and metabolic syndromes⁷. However, it is necessary to move from microbiome-wide association studies to causal microbe identification, and ultimately to the underlying molecular mechanisms to fully exploit the microbiome for therapeutic intervention. Currently, our knowledge of microbial immunomodulation is mainly mechanistically limited to activation of Toll-like receptors (e.g., TLR2/4/6), members of G protein-coupled receptors (GPCR 41/43/109a, P2XR, and TGR5), and nuclear receptors (AhR, PXR, FXR), or inhibition of histone deacetylase in immune cells, by microbially derived polysaccharides, short-chain fatty acids, ATP, and bile acids, and amino acid derivatives⁸.

Synthetic microbial consortia consisting of known members retain a lower complexity of inter-microbial and microbe-host interactions than natural gut microbiota but still show tremendous immunological and ecological impacts in the gut⁹. Examples include the 17-member Treg inducing Clostridia consortium¹⁰, the 11-member IFN γ + CD8+ T cell inducing bacterial assembly¹¹, and the multi-functional 8-member altered Schaedler flora (ASF)⁵. The immunomodulatory effects of gut microbes appear independent of microbial phylogeny¹². The ASF is genetically competent in terms of gene richness¹³ and its wide phylogenetic distribution (**Extended Data Fig. 1a**), and more importantly, it can resolve immunological, neurological, and metabolic defects in germ-free mice¹⁴⁻²². It increases IL-10 production and promotes Treg proliferation in the colon¹⁶ and is able to mimic the specific pathogen-free microbiota in reducing insulinitis in *MyD88*^{-/-} NOD mice²³. These results indicate a pronounced immune tolerance-eliciting function of this defined community, but the identity of the responsible microbes and molecules is yet undetermined. Here, we dissected the role of each individual ASF member in a panel of immune regulation assays and elucidated a novel microbial immunomodulatory

mechanism involving the adenosine receptor A2a. These functional microbiome studies have the potential to open novel therapeutic avenues for the prevention and treatment of immune diseases.

Results

Division of labor among murine intestinal microbes in *in vitro* immune regulation

Dendritic cells (DCs) are professional antigen-presenting cells that play a critical role in inducing immunity and maintaining immune tolerance. Whether they are tolerogenic or immunogenic mainly depends on their cytokine production profiles and impact on T cells. We interrogated the immunoregulatory potential of each ASF member by stimulating bone marrow-derived dendritic cells (BMDCs) with filter-sterilized spent medium (SSM) collected during bacterial growth (**Extended Data Fig. 1b**). We found that SSM from *Parabacteroides goldsteinii* ASF519 dramatically induced the production of the anti-inflammatory cytokine IL-10 in a bacterial growth-dependent manner (**Fig. 1a; Extended Data Fig. 1c**). In contrast, *Clostridium spp.* ASF356 only induced IL-10 at the exponential stage, while a weak IL-10 induction was observed with *Eubacterium plexicaudatum* ASF492 and *Pseudoflavonifractor spp.* ASF500. Measurement of another anti-inflammatory cytokine, TGF- β , revealed that *Lactobacillus intestinalis* ASF360 and *Lactobacillus murinis* ASF361 induced BMDCs to release this cytokine (**Fig. 1a**), which positively correlated with bacterial growth (**Extended Data Fig. 1c**); ASF519 had a mild effect on TGF- β . For their immunogenic potential, although the uninoculated bacterial culture medium induced a high level of pro-inflammatory TNF- α , this effect was diminished by the monoculture of 7 ASF members (**Fig. 1a**), with the exception of *E. plexicaudatum* ASF492 that displayed a recurring TNF- α release when the stationary-phase SSM was used. These measurements revealed distinct cytokine inducing profiles of ASF members coupled with growth phase-specific microbial physiology (**Fig. 1a**). Notably, ASF519 is the only member that induced two anti-inflammatory cytokines, IL-10 and TGF- β (**Fig. 1b**).

We then tested the impact of exposed BMDCs on Treg proliferation via *in vitro* coculture with splenocytes from conventional mice (**Extended Data Fig. 1b**). Interestingly, BMDCs exposed to ASF519 SSM, especially collected from late

exponential and stationary phases, strongly promoted Foxp3⁺ Treg proliferation (80±6% by ASF519 SSM versus 20±9% by uninoculated bacterial culture medium) (**Fig. 1a, Extended Data Fig. 1d**). The Foxp3⁺ Treg subset increased 1.8-fold when cocultured with the ASF519 SSM-stimulated BMDCs (**Fig. 1c**). A weak stimulatory effect on Treg proliferation was observed with three other members (ASF356, ASF361, and ASF502) but the effect peaked at distinct bacterial growth stages. Their impacts on both cytokine profiles and Treg proliferation demonstrate a “division of labor” among ASF members in regulating immunophenotypes of BMDCs (**Fig. 1b**). In particular, ASF519 promotes tolerogenic phenotypes of BMDCs via inducing anti-inflammatory cytokines and Treg proliferation. Considering the high abundance of ASF519 in the feces of ASF mice⁵ and the relatively high prevalence (~23%) of *P. goldsteinii* in the human gut microbiome according to The Human Microbiome Project (**Extended Data Fig. 2**), we were compelled to further explore its immunoregulatory activities.

Synergistic regulation of myeloid cell-specific IL-10 induction

Because IL-10 is produced by a variety of immune cell types and is involved in both innate and adaptive immunity, we tested the IL-10 inducing specificity of ASF519 on a panel of diverse immune cells. We found that stimulation of isolated DCs, bone marrow-derived macrophages, and isolated splenic monocytes all yielded a high level of IL-10 (**Fig. 1d**); however, neither splenic panB nor panT stimulation, nor stimulation of immune-irrelevant BEND3 endothelial cells, showed a significant impact on IL-10 production. These data suggest that ASF519-mediated IL-10 induction is specific to myeloid-derived antigen-presenting cells. We also observed that IL-10 induction is independent of TNF-α signaling (**Extended Data Fig. 3a**), and that it is a slow response, which ramps up after 24 hrs of monotonous stimulation of BMDCs by ASF519 SSM (**Extended Data Fig. 3b and 3c**).

RNA sequencing for BMDCs revealed dramatic transcriptomic changes caused by stimulation with ASF519 SSM versus broth control (adjusted $p < 0.01$, |fold change| > 3) (**Fig. 1e and 1f, Extended Data Fig. 4a and 4b**). Further stimulation by zymosan induced significant effects in broth-treated rather than ASF519 SSM-treated cells (**Extended Data Fig. 4b-4d**), suggesting that ASF519 SSM may increase the tolerance of BMDCs to immunogenic zymosan. ASF519-induced differentially expressed genes have a wide association with the regulation of immune

functions, cell communication, apoptosis, and other biological processes (**Extended Data Fig. 4e**). Notably, negative regulation of antigen processing and presentation of peptide or polysaccharide antigen via MHC class II, and negative regulation of oligodendrocyte apoptotic process, are enriched most in upregulated and downregulated gene sets, respectively. At the gene level (**Fig. 1g, Extended Data Fig. 4f**), we observed a dramatic induction of tolerogenic *Il10*, lipocalin-2 (*Lcn2*), and indoleamine 2, 3-dioxygenases (*Ido1/2*), as well as a reduction of the co-stimulatory molecule *Cd40* and inflammatory cytokines, including *Il12b*, *Il23a*, and *Il1f9* (or *Il-36* gamma). It is known that IL-36 gamma promotes inflammation and DC maturation; IL-12B and IL-23A together build up the heterodimeric inflammatory IL-23 which is associated with autoimmune diseases and cancer. Their decreased transcript abundance may additively enhance the tolerogenic phenotypes of BMDCs. It is also notable that this step-wise stimulation with ASF519 SSM added before zymosan exerted a synergistic and antagonistic effect on certain transcripts (**Fig. 1g, Extended Data Fig. 4g-4j**). For instance, *Il10*, *Ido2*, and U90926 were synergistically induced; whereas suppression of *Il12b*, *Il1f9*, and *Cd40* by ASF519 SSM was attenuated by subsequent zymosan stimulation. We validated this synergy on *Il10* at the protein level (**Fig. 1h**). Therefore, components of ASF519 SSM can induce multifaceted tolerogenic phenotypes of BMDCs *in vitro*.

Microbial activation of adenosine receptor A2a induces IL-10 production

To further dissect this immunomodulatory mechanism, we sought to identify the responsible molecules using bioanalytical techniques (**Fig. 2a**). The IL-10 inducing function of ASF519 SSM was largely heat-labile (**Extended Data Fig. 5a**) and immobilized proteinase K digestion of ASF519 SSM completely abolished IL-10 induction (**Fig. 2b**). These results, plus a dose-dependent IL-10 induction by concentrated crude proteins (**Fig. 2c**), confirmed the proteinaceous nature of the responsible molecules. We then performed gel filtration to fractionate crude proteins in ASF519 SSM. BMDC assays revealed consecutive fractions of an early elution peak exhibiting strong IL-10 induction (**Fig. 2d**). The elution chromatogram demonstrated that these bioactive fractions were in the excluded volume of the gel filtration column that was designed for separating 20-8,000 kDa globular proteins (**Extended Data Fig. 5b-5c**), suggesting a large structure is formed. Native- and SDS-PAGE analysis of these bioactive fractions confirmed the existence of a

complex architecture that is susceptible to proteinase K digestion (**Extended Data Fig. 5d**). Mass spectrometry analysis of major gel bands identified proteins associated with cysteine and methionine metabolism, including adenosylhomocysteinase (AHCY), O-acetyl-L-serine sulfhydrylase (OASS), methionine adenosyltransferase (MAT), and O-acetylhomoserine sulfhydrylase (METY). Because the integrity of proteinaceous factors is essential for IL-10 induction, we speculated metabolites of these enzymes are the actual effectors, which are naturally heat-labile or dependent on substrate availability. This hypothesis is supported by the known immunosuppressive role of naturally occurring nucleosides in mammals, such as adenosine, inosine, and 5'-methylthioadenosine (MTA), which can also stem from bacterial methionine metabolism and purine metabolism. These nucleosides activate membrane adenosine receptors (A1, A2a, A2b, and A3) on a variety of immune cells to promote tolerogenic or anti-inflammatory phenotypes²⁴.

To test whether ASF519-mediated IL-10 induction is mediated by the activation of adenosine receptors, we applied adenosine receptor-specific antagonists to BMDC assays. Intriguingly, supplementation of the A2a inhibitor ZM241385 drastically dampened IL-10 induction in a dose-dependent manner in response to ASF519 SSM (**Fig. 2e**). By contrast, antagonists of A1, A2b, and A3 at the tested concentrations had no significant impact (**Fig. 2e**). The engagement of A2a in IL-10 induction was pharmacologically reproduced using bioactive gel-filtration fractions as stimuli of BMDCs in inhibition assays (**Extended Data Fig. 5e**) and further confirmed with the genetic knockout of A2a where A2a^{-/-} BMDCs disabled IL-10 induction when exposed to ASF519 SSM (**Fig. 2f**). A2a activation then increased the intracellular level of cyclic AMP (**Extended Data Fig. 5f**), which is known to be involved in the transcriptional regulation of *Il10*. Therefore, activation of adenosine receptor A2a signaling in BMDCs is required for IL-10 induction by microbial proteins.

AHCY can hydrolyze S-adenosylhomocysteine (SAH) to generate adenosine, a natural ligand of adenosine receptors. To validate its role in IL-10 induction, we first applied the AHCY inhibitor DZA to BMDC assays, which revealed that AHCY inhibition decreased IL-10 induction in a dose-dependent manner (**Fig. 2e**). We then heterologously expressed and purified recombinant enzymes (**Extended Data Fig. 6a-6d**), including AHCY, OASS, MAT, METY, and methionine synthase (MS), for *in*

vitro enzymatic and immunological assays. As expected, only purified AHCY demonstrated the enzymatic activity of using SAH as a substrate to produce adenosine (**Fig. 2g**). Using purified AHCY to stimulate BMDCs successfully induced IL-10 in a dose-dependent manner (**Fig. 2h**). Apart from AHCY, two other enzymes of the methionine cycle (MS and MAT) were also able to induce IL-10 at different optimal concentrations (**Fig. 2h**). Because OASS and METY are involved in cysteine metabolism and irrelevant to IL-10 induction, we conclude that enzymatic activities of the ASF519-derived methionine cycle contribute to IL-10 induction in BMDCs (**Fig. 2i**).

ASF519 colonization induces tolerogenic phenotypes

To probe *in vivo* immunoregulatory function, ASF519 was orally introduced to 4-week old germ-free mice for a 2-week long mono-association. Untargeted metabolomics revealed vast changes in the abundance of cecal metabolites caused by ASF519 mono-association (**Extended Data Fig. 7a**). These metabolites were significantly enriched for the metabolism of 13 amino acids, galactose, and amino and nucleotide sugars (**Fig. 3a, extended Data Fig. 7b**). Notably, ASF519 colonization increased cecal adenosine by 15-fold, as well as the adenosine-forming substrate SAH and one of the adenosine-degradation products, adenine, by 9-fold and 73-fold, respectively (**Fig. 3b**). Therefore, ASF519 increases the presence of adenosine and related metabolites in cecal contents of mice.

With qPCR analysis for 34 select genes involved in intestinal integrity, mucin synthesis, and immune functions, we found ASF519 mono-association increased the transcript level of *Il10* in the small intestine and colon and *Il17a* in the colon, and it significantly reduced *Il5* transcripts in the small intestine (**Fig. 3c**). *Il10* up-regulation is consistent with ASF519 SSM-mediated IL-10 induction observed *in vitro*. Other genes involved in mucin synthesis and intestinal integrity were not influenced by ASF519, with the exception of an increase in the cell proliferation promoting *myc* gene in the small intestine (**Extended Data Fig. 8**). Measurement of 18 cytokines in sera revealed a systematic reduction of IL-4 and IL-5 in ASF519 mice (**Fig. 3d and 3e**). IL-5 is mainly secreted by activated T helper 2 cells which originally rely on IL-4 to differentiate from naive T cells, and both IL-4 and IL-5 are involved in the proliferation and function of activated B and T cells. Their concurrent reduction in circulation may imply the effects of ASF519 on systemic immune response.

To validate the role of adenosine receptor A2a in ASF519-mediated immunoregulation *in vivo*, we blocked A2a signaling by i.p. injection of an A2a antagonist, ZM 241485, into antibiotic-pretreated mice that received live or heat-killed ASF519 (**Fig. 3f**). Immunophenotyping of colonic lamina propria cells revealed an increase in IL-10 producing DCs among CD103⁻ CD11c⁺ and CD103⁺ CD11c⁺ populations in live versus killed ASF519 recipients (**Fig. 3g and 3h**). Importantly, these effects were significantly decreased by the A2a antagonist. This IL-10 immunophenotype is consistent with our *in vitro* observation that blockage of A2a rather than other adenosine receptors sufficiently interrupted IL-10 production by BMDCs in response to ASF519 SSM. Other major immune cell types, such as CD11b⁺ F4/80⁺ macrophages, CD4⁺ T cells, and CD19⁺ CD45⁺ B cells, did not show significant changes in their IL-10 secretion (**Extended Data Fig. 9 and 10**). It is also notable that ASF519-treated mice exhibited decreased lamina propria B cells and CD11b⁺ CD45⁺ cells in the colon (**Fig. 3i and 3j**), which was also restored by A2a blockage. Together, these data demonstrate that the immunoregulatory function of ASF519 in colonic lamina propria goes beyond IL-10 induction in DCs to include more general homeostatic activities of leukocytes through A2a activation.

Oral administration of ASF519 ameliorates autoimmune disorders

Type 1 diabetes (T1D) is an autoimmune disease in which insulin-producing beta cells in the pancreas are destroyed by T cells. DCs promote the priming and effector differentiation of autoreactive T cells in the pancreatic lymph nodes²⁵. To test if the immunoregulatory functions of ASF519 can curb the immunopathogenesis in T1D, we introduced ASF519 to T1D-prone non-obese diabetic mice (NOD) by weekly oral gavage from four to thirteen weeks of age (**Fig. 4a**). At 14 weeks, ASF519 recipients had a lower incidence of hyperglycemia (>250 mg/dL, 57%) compared to killed ASF519 recipients (88%) (**Fig. 4b**), indicating an improved pancreatic function as a result of ASF519 supplementation. To measure immunopathogenesis directly in the pancreas, mice were sacrificed at 14 weeks old to perform histological analysis of insulinitis by scoring infiltration of insulin-producing beta cells by pathogenic lymphocytes. In line with improved glucose control, the severity of insulinitis was reduced in ASF519 mice (**Fig. 4c**). Insulin immunostaining on adjacent sections of histological staining also demonstrated that more insulin-producing beta cells were

present in the islets of ASF519 mice. Therefore, ASF519 colonization in NOD mice plays a protective role against T1D progression.

We then asked whether ASF519 could ameliorate other autoimmune diseases. Rheumatoid arthritis is an autoimmune disease that mainly involves the synovial tissues within joints. We introduced ASF519 to DBA mice before the injection of collagen to induce arthritis (**Fig. 4d**). The qPCR analysis confirmed the long-term colonization of ASF519 in the gut of DBA mice as after 3 weeks of the final gavage, the abundance of ASF519 in feces was >11 fold higher in live versus killed ASF519 recipient mice (**Fig. 4e**). Following 30 days post-collagen immunization, mice that received killed ASF519 significantly lost body weight (**Fig. 4f**), whereas ASF519 mice were able to maintain their body weight. In line with that, killed ASF519 recipient mice developed much more severe arthritis than ASF519 mice (**Fig. 4g**). These data demonstrate that ASF519 colonization strongly protected DBA mice from collagen-induced arthritis.

Discussion

Accumulating evidence from mouse and human studies demonstrates that the microbiome is integral to immune regulation, and that alterations to the microbiome may underlie many immune-mediated and metabolic diseases. Here, we leverage the ASF, an important and long-studied “model microbiome” to demonstrate that there exists a division of labor among members of the microbiome in modulating immune regulatory phenotypes. This led us to identify that one specific member, *P. goldsteinii* ASF519, is a potent inducer of IL-10 in myeloid cells in an adenosine receptor A2a-dependent manner both *in vitro* and *in vivo*. By a combination of activity-guided fractionation and genetic methods, we identified that enzymes belonging to the methionine/adenosine cycling pathway were sufficient to induce IL-10 in dendritic cells. Furthermore, gnotobiotic mice mono-colonized with ASF519 exhibited a profound increase in cecal abundance of adenosine, adenine, and S-adenosylhomocysteine. This evidence combined with our data showing that the IL-10 induction is A2a-dependent demonstrates that the IL-10-inducing activity of ASF519 is mediated by adenosine signaling. Finally, we demonstrated that ASF519 supplementation can reduce the severity of arthritis in the CIA model as well as insulinitis resulting from autoimmune diabetes in NOD mice.

303 The novel mechanism we here describe is distinct from known microbially
304 involved IL-10 regulation, including IL-10 induction in DCs, macrophages, or
305 monocytes by bacterial polysaccharide capsules²⁶⁻²⁷, and SCFA-mediated induction
306 through GPCR43 activation in microbiota antigen-specific Th1 cells²⁸. Our study
307 broadens the previously described GPCR interactions with gut microbial
308 metabolites^{29,30}. In regard to the critical role of DCs in both innate and adaptive
309 immunity, the increased IL-10 induction by ASF519 promotes mucosal immune
310 tolerance by reducing antigen presentation to lymphocytes and the immunogenic
311 potency of leukocytes in colonic lamina propria. As A2a signaling is not only involved
312 in immunomodulation, but also metabolism³¹ and cell proliferation³², ASF519
313 mediated A2a activation may modulate multiple biological functions that together
314 manifest the observed benefits in disease models here described.

315 This work serves as a model workflow by which future immune phenotypes
316 can be screened in the ASF system *in vitro* and then interrogated *in vivo* towards the
317 discovery and characterization of novel immunoregulatory functions of the gut
318 microbiome. Furthermore, this workflow may in the future help formulate
319 combinatorial strategies to sharpen the regulatory potency of microbes or their
320 secreted products for therapeutic use. Supplementation with ASF519 or potentially
321 related *Parabacteroides* species may represent a novel strategy to prevent and/or
322 treat autoimmune and inflammatory diseases.

METHODS

Animal models

C57BL/6, 129S1/J, *Il10^{-/-}*, *A2a^{-/-}*, DBA/1J, NOD mice were purchased from Jackson Laboratories and maintained in specific pathogen-free conditions. The *A2a^{-/-}* mice were on a mixed genetic background of BALB/c and 129S. Unless otherwise indicated, 8-12-week-old mice of both genders were used for *in vitro* experiments. Only male DBA and female NOD mice were used for disease studies. This study complied with all ethical regulations involving experiments with mice, and all experimental manipulations were performed in accordance with protocols approved by the Animal Care and Use Committee of Joslin Diabetes Center.

To study the *in vivo* function of *Parabacteroides goldsteinii* ASF519 in mice, we introduced this strain into 4-wk old both male and female germ-free (GF) mice by oral gavage of 200 µl bacterial cells with 1×10^9 CFU in PBS. The control GF group was orally administered with 200 µl PBS. The SPF mice were developed by introducing fecal microbiota of conventional mice to GF mice. After 2 weeks of colonization, we sacrificed mice to collect sera, intestinal tissues, and cecal contents. Shotgun metagenomic sequencing for fecal and cecal samples confirmed mono-colonization. Approximately, 1 cm of middle colon and ileum (5 cm upstream of the cecum) were preserved in 0.5 ml TRIZOL buffer for storage at -80°C.

We tested the engagement of adenosine receptor A2a in ASF519-mediated immune regulation through pharmacological blockade in mice. 9-11-wk old C57BL/6J mice were treated by an antibiotic cocktail (0.5 g/L vancomycin, 1.36 g/L neomycin, plus 3.75 g/L sweetener aspartame) for 48 hrs. Then, the antibiotic water was exchanged with a 10%(w/w) PEG4000 solution in water, and the mice were fasted for 16 hrs. After that, we administered these prepared mice twice at the interval of 3 days by gavaging 200 µl of live or heat-killed *P. goldsteinii* in PBS at 1×10^9 CFU. Both live and killed *P. goldsteinii* recipient groups were further divided with a daily intraperitoneal injection of 100 µl A2a antagonist ZM 241385 (1.25 mg/ml) or vehicle (7.4% DMSO + 7.4% Cremophor + 85.2% PBS) for 17 days. We collected stool samples at 5 timepoints: before-antibiotic treatment, post-antibiotic/pre-10% PEG4000 treatment, post-gavage day 1, day 3, and day 7. On post-gavage day 14, we harvested colon tissues and cecal contents for immunophenotyping and metabolomic analysis, respectively.

To test whether *P. goldsteinii* influences the development of autoimmune diabetes, we introduced this bacterium into non-obese diabetic mice (NOD) which are prone to develop insulinitis and diabetes³³. 4-wk old female NOD mice received 200 µl of live or heat-killed ASF519 in PBS at approximately 1×10^9 CFU by weekly oral administration until 13-wk old. Blood glucose was measured in tail tip blood at 14 weeks. The blood glucose at >250 mg/dL defines hyperglycemia³⁴. We collected the pancreas for histological studies, including Hematoxylin and Eosin staining and insulin immunostaining.

We also tested the protective role of *P. goldsteinii* in the collagen-induced arthritis mouse model³⁵, which is the most commonly studied autoimmune model of rheumatoid arthritis. 10-wk old male DBA/1J mice were orally administered with 200 µl of live or heat-killed ASF519 in PBS at 1×10^9 CFU for twice a week in a course of two weeks. After microbial colonization, mice were immunized by 50 µl of 1 mg/ml collagen emulsion in Complete Freund's Adjuvant (CFA, Sigma-Aldrich #F5506-10ML) that consists of 1 mg/ml of heat-killed *Mycobacterium tuberculosis* (Invitrogen #tlrl-hkmt-1) by subcutaneous injection at about 1.5 cm distal from the base of the tail. This formulation in CFA has been demonstrated to be essential to induce arthritis in mice from the autoimmune response that results in arthritis of major joints within 4-6 weeks of injection. We monitored mice twice weekly for arthritis and bodyweight, assessed arthritis severity by the established qualitative scoring system through observing and measuring the severity of paw inflammation³⁵. Each paw was evaluated and scored individually on a scale of 0-4, with 4 indicating the most severe inflammation. The maximal combined score from 4 paws per mouse is 16.

Collection of bacterial spent media

ASF isolates were provided by Dr. Michael Wannemuehler at Iowa State University. We grew each ASF member in the brain heart infusion broth (BHI) under the anaerobic condition at 37°C. The BHI medium consists of 37 g/L BHI (Sigma), 5 g/L yeast extract, 0.5 g/L L-cysteine, 10 mg/L resazurin, and 5 mg/L hemin, dissolved in 1/2 (v/v) miniQ water and 1/2 (v/v) tap water. After autoclaving, 1 L of BHI was supplemented with 0.2 ml 1% vitamin K, 50 ml newborn calf serum, and 50 ml horse serum, and 10 ml of 20% (m/v) filter-sterilized yeast extract. The medium was placed in the anaerobic chamber overnight to reduce oxygen before bacterial inoculation. The spent media were harvested at indicated time points during growth and then

filter-sterilized twice with 0.2 μ M filters before storage at -80°C. Bacterial cell biomass was estimated by monitoring OD600.

The defined medium used to grow ASF519 contains salts, carbon sources, and supplements. The salts and carbon sources include 13.6 g/L KH_2PO_4 , 0.875 g/L NaCl, 1.125 g/L $(\text{NH}_4)_2\text{SO}_4$, 5.0 g/L glucose, and 0.25 g/L L-cysteine, pH7.2. 1 L of the salts/carbon sources was then supplemented with 0.5 ml of 10 mg/ml hemin, 200 μ l of 1% vitamin K, 1.0 ml of 0.1 M MgCl_2 , 1.0 ml of 0.4 mg/ml $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 ml of 0.8% CaCl_2 , 10 ml ATCC Vitamin complex, and 10 ml ATCC mineral complex. The medium was sterilized with 0.2 μ m filters and oxygen-reduced in an anaerobic chamber before use.

Cell isolation and cultivation

We developed bone marrow-derived dendritic cells (BMDCs) from mouse bone marrow. Mice at 8-12 wk old were euthanized and sacrificed to collect hind legs. After removing skin, flesh, and muscles from the legs, bone marrow was flushed out from bones and collected for red blood cell lysis using the RBC lysis buffer (Invitrogen #00-4333-57) by following the manufacturer's instruction. After centrifugation at 500g for 8 min at 4°C, we resuspended hematopoietic cells in complete DMEM (cDMEM) consisting of 10% fetal calf serum, 1x penicillin-streptomycin solution (100x, Caisson Labs #PSL01), 10 mM HEPES, and 5 μ M 2-mercaptoethanol in Gibco DMEM medium (Thermo fisher #10-566-024). To develop BMDCs, hematopoietic cells were cultured in cDMEM with 20 ng/ml GM-CSF (2 x 10^6 cells in 10 ml per untreated petri dish 100 mm x 15 mm) at 37°C, 5% CO_2 . The supplemented medium was refreshed on days 3 and 6. BMDCs were collected on day 9 and resuspended in the CryoStor CS10 buffer (STEMCELL #07930) for storage at -80°C.

To develop BM-DMs³⁶, hematopoietic cells from bones were transferred into culture-treated dishes (on average 1 leg per plate) for incubation at 37°C for 4 hrs to allow resident macrophage to adhere to the plastic. Cells in the supernatants were collected and cultured in cDMEM with 10 ng/ml M-CSF (2 x 10^6 cells in 10 ml per culture treated dish 100 mm x 15 mm) at 37°C, 5% CO_2 , and the medium was refreshed every 3 days. BM-DMs were collected on day 10 for downstream experiments.

The BEND3 endothelial cell line was cultured in cDMEM without using GM-CSF or M-CSF (5×10^5 cells in 10 ml per culture treated dish 100 mm x 15 mm) at 37°C, 5% CO₂. After 3 days, the medium was removed; cells were treated with 0.25% trypsin (3 ml per petri dish) at 37°C for 5 min to allow cell dissociation. Then, the cells were gently scraped from the plastic and resuspended cells in fresh cDMEM for further assays.

PanB, panT, panDC, and monocytes were isolated from splenocytes of 8-12 wk B6 mice using EasySep Mouse B cell isolation kit (STEMCELL #19854), EasySep Mouse T cell isolation kit (STEMCELL #19848), EasySep Mouse Pan-DC enrichment kit (STEMCELL #19763), and EasySep Mouse Monocyte isolation kit (STEMCELL #19861), respectively.

***In vitro* BMDC stimulation**

1×10^5 cultured or isolated immune cells in 140 µl cDMEM were placed in a 96-well non-tissue culture treated microplate (Corning #351172), mixed with 60 µl of filter-sterilized spent media (SSMs) for 48 hrs incubation at 37°C, 5% CO₂, then supplemented with zymosan at 4 µg/ml for additional 16 hrs incubation under the same condition. Finally, stimulated cultures were centrifuged at 500g for 8 min to harvest supernatants for the measurement of cytokines. To test the effect of ASF519 SSMs on TGF-β production, no zymosan was added during the entire course (64 hrs) of stimulation of BMDCs. IL-10, TGF-β, and TNF-α were quantified by corresponding ELISA kits (Invitrogen).

The impact on Treg proliferation was tested by co-culturing stimulated BMDCs with splenocytes. We placed 1×10^6 BMDCs in 1 ml cDMEM into a 24-well non-tissue culture treated plate (Corning #351147), stimulated cells with 0.5 ml of bacterial SSMs or BHI broth for 2 days at 37°C in the CO₂ chamber, then further stimulated cells with 4 µg/ml zymosan for additional 16 hrs under the same condition. Fresh splenocytes from 129S1 mice were diluted to 1×10^6 cells/ml in phosphate-buffered saline (PBS), stained with Celltrace violet (Thermo fisher #C34571) at 10 µM, 37°C for 20 min, washed once with five volumes of Gibco RPMI1640 medium (Thermo fisher #61870127), finally resuspended in RPMI1640 at 2×10^6 cell/ml. To coculture BMDCs and splenocytes, we removed the supernatants from stimulated BMDCs by centrifugation at 500g for 8 min, resuspended cell pellets in 0.5 ml RPMI1640, then added 0.5 ml of stained splenocytes for 4 days incubation in the

CO₂ incubator at 37°C. Treg proliferation was analyzed by flow cytometry for co-cultured cells stained by mouse CD4 and Foxp3 antibodies.

Flow cytometry

We generated single-cell suspensions from mouse colonic lamina propria tissue using the Lamina Propria dissociation kit (Miltenyi Biotec #130-097-410) by following manufacturers' instruction. The program 37°C_m_LPDK_1 on the gentleMACS Octo dissociator with heaters was used for tissue dissociation and cell extraction. Spleens were harvested and gently grinded with the rubber head of syringe plunge through 70 µm cell strainers to obtain single cell suspension; red blood cells were then lysed with the RBC lysis buffer (Invitrogen #00-4333-57). Cells were preserved in the CryoStor CS10 buffer (STEMCELL #07930) for storage at -80°C.

Single-cell suspensions were stained and analyzed with BD LSRFortessa. The panel included fluorescence conjugated antibodies against CD45, CD4, CD19, CD11b, CD11c, CD103, F4/80, Foxp3, and IL-10. Cells were firstly stained by the Zombi Violet Fixable Viability Kit, incubated with the FcR blocking reagent (anti-CD16/CD32, BioLegend #101302) in the cell staining buffer (BioLegend #420201) at room temperature for 10 min, and then stained for surface markers, including CD45, CD4, CD19, CD11b, CD11c, CD103, and F4/80. Stained cells were then fixed and permeabilized by using the Foxp3 Fix/Perm buffer kit (BioLegend #421403). Permeabilized cells were finally stained for intracellular and nucleus markers. Cells were acquired with a BD LSRFortessa, and analysis was performed with the FlowJo software. Frequencies of each cell type were averaged for each experimental condition.

For flow cytometry analysis of co-cultured cells, the panel included fluorescence conjugated antibodies against CD4, CD8, and Foxp3. Cells were stained with the APC eFluo 780 live/dead dye and then corresponding antibodies by following the same staining protocol used for colonic lamina propria cells.

Low input RNA-Seq

1,000 BMDC cells were collected directly into 5 µl TCL lysis buffer (Qiagen) with 1% 2-mercaptoethanol. Lysates were frozen after 5 min. Smart-seq2 libraries were prepared as previously described³⁷ and sequenced using the Broad Genomics Platform. Reads were aligned to the mouse genome (GENCODE GRCm38/mm10

primary assembly and gene annotation version M16;
https://www.gencodegenes.org/mouse/release_M16). The ribosomal RNA gene annotations were removed from the general transfer format (GTF) file. The gene-level quantification was calculated by featureCounts (<http://subread.sourceforge.net/>). Raw read count tables were normalized by the median of ratios method with the DESeq2 package from Bioconductor and then converted to GenePattern GCT and CLS format. Samples with less than 3 million uniquely mapped reads were automatically excluded from normalization to mitigate the effect of samples with poor quality on normalized counts. Normalized read counts were filtered for robust expression (>10) to avoid confounders from low-level noise and processed in the Multiplot suite and Morpheus (<https://software.broadinstitute.org/morpheus/>). PCA was done using the prcomp function in R on all genes.

Metagenomic data analysis

The genomic DNA was extracted from feces or cecal contents using the ZymoBIOMICS DNA kit (Zymo #D6300). Shotgun metagenomic sequencing was performed using Illumina HiSeq2000 NextGen Sequencer. Data was cleaned by removing mouse genome contamination. Microbial abundance was analyzed by MetaPhlAn2³⁸. The prevalence of *Parabacteroides goldsteinii* in the human gut microbiome studies was analyzed by the curatedMetagenomicData package³⁹ in R.

Bioassays for treated or fractionated samples

To perform acetone precipitation, we mixed 4 times the sample volume of cold (-20°C) acetone to ASF519 SSMs or medium broth in an acetone-compatible bottle and then incubated the mix for at least 60 min at -20°C. The precipitate was collected by centrifugation at 13,000g for 10 min, then air-dried at room temperature for 30 min before adding 1 x PBS buffer to dissolve the protein pellet on ice. Protein concentration was measured by the Pierce BCA protein assay kit (Thermo Fisher #23227). We then evaluated the IL-10 inducing capability of acetone precipitates at varying working concentrations in BMDC assays. To verify the role of proteinaceous components in IL-10 induction and Treg proliferation, we treated SSMs of ASF519 grown in either the defined medium or supplemented BHI with immobilized proteinase K to destroy proteins. In detail, we added 2 ml of 33 mM Tris-HCl pH7.5

to reconstitute 10 U protease-agarose power (1U/200 µl, Sigma #P9290-10UN), mixed 1 ml protein sample with 200 µl of reconstituted protease, killed-protease (generated by heating at 95°C for 10 min), or 33 mM Tris-HCl pH7.5, for incubation at 37°C for 1 hr, followed by centrifugation to remove protease-agarose beads. By following the *in vitro* BMDC stimulation protocol described above, we evaluated the IL-10 inducing capability of heat- and proteinase K treated samples.

For gel filtration to fractionate proteins by size, we loaded the concentrated crude samples, following filtration using 0.2 µm syringe filters, to the AKTA pure system with a 10 ml sample injection loop and a HiLoad 16/600 Superdex 200 pg column. Proteins were eluted into 1x PBS at 1 ml/min and collected every 2 ml in sterile microtubes. We then ran *in vitro* BMDC assays using 10 µl of each fraction as the stimulus to determine bioactive fractions that can induce IL-10 production. The bioactive fractions were pooled and concentrated for further analysis, including protein quantification, native-/SDS-PAGE analysis, and protein identification by mass spectrometry. The gel filtration standard (Bio-Rad #1511901) was analyzed with the same gel filtration settings to indicate the molecular size.

Pharmacological inhibition

To test whether ASF519 mediated IL-10 induction occurs via any of the adenosine receptors, we employed the AHCY inhibitor 3-deazeadenosine (DZA, Sigma #D8296-5MG) to block the formation of adenosine, the A1 antagonist DPCPX (Tocris #0439), the A2a antagonist ZM 241385 (Thermo fisher #10-361-0) and the A2b antagonist MRS-1754 (Tocris #2752), or the A3 antagonist MRS-1523 (Sigma #M1809-5MG). Stocks of antagonists were prepared in 50% DMSO. We treated 1 x 10⁵ BMDCs in 140 µl with 2 µl of a series of diluted antagonists or vehicle (50% DMSO) at 37°C for 30 min, then added 60 µl of broth control, ASF519 SSMs, or protein extracts from SSMs to stimulate BMDCs for 48 hrs in the CO₂ chamber, followed by further stimulation with zymosan at 4 µg/ml for 16 hrs at 37°C in the CO₂ chamber. Finally, we centrifuged the cell culture to collect supernatants for IL-10 ELISA assays.

The impact of TNF signaling on IL-10 induction of BMDCs was tested by blocking the TNF signaling pathway with Etanercept⁴⁰ (Sigma-Aldrich #Y0001969) in the BMDC assay using ASF519 SSMs and zymosan for step-wise stimulation. The

levels of IL-10 and TNF- α in the BMDC supernatants were measured by ELISA kits (Invitrogen).

Expression of His-tagged proteins

The coding genes of methionine adenosyltransferase (MAT), adenosyl homocysteinase (AHCY), O-acetylhomoserine sulfhydrylase (METY), methionine synthase (MS), O-acetylserine sulfhydrylase (OASS, also named cysteine synthase) predicted in the ASF519 genome were cloned in the pET21(+) plasmid. Constructs verified by Sanger sequencing were introduced into NEB Lemo21 DE3 for inducible expression of His-tagged proteins. Bacterial cells were grown at 37°C in LB with 30 μ g/ml chloramphenicol, 50 μ g/ml carbenicillin, and 0.5-1.5 mM L-rhamnose to OD₆₀₀ 0.8, then 0.4 mM IPTG was added to induce protein expression for 20 hrs at 18°C. The recombinant enzymes were purified sequentially with a nickel affinity column and a HiLoad 16/600 superdex 200pg column, in order to remove endotoxin and get high-purity proteins. The purity of fractions in PBS was determined by SDS-PAGE. High-purity fractions were pooled and concentrated with Amicon Ultra-15 10K centrifugal filters, then mixed with an equal volume of 50% glycerol before protein quantification with the BCA kit and storage at -20°C. To verify the adenosine forming enzymatic activity, 1 μ M of all pure enzymes were pre-incubated with 1 mM β -NAD⁺ cofactor at 37°C for 30 min, then supplemented with the substrate SAH at 1.67 mM for incubation at 37°C for 10 min. The level of adenosine was then quantified by the adenosine fluorometric kit (Cell Biolabs #MET-5090). Reactions with PBS replacing pure enzymes served as the control. Each condition was performed in 3 replicates. The impact of pure enzymes on IL-10 induction was evaluated by adding pure enzymes into BMDC assays with 1×10^5 cells in 200 μ l in a 96-well microplate by following the *in vitro* step-wise stimulation protocol. The level of IL-10 in cell culture supernatants was determined by the IL-10 ELISA kit.

Histological analyses

We performed hematoxylin and Eosin (H&E) staining to examine infiltration in pancreatic islets. The slides were deparaffinized in two washes of xylenes at 5 min and then rehydrated in series of decreasing Ethanol concentration, 100%, 95%, 70%, and 50% and distilled water. The slides were stained with hematoxylin (Gill-3, Fisher #3537-32) for 2 min and raised in running distilled water. The slides were

destained (3-4 dips) with acid water and raised with distilled water then blued with ammonia water. After washing with distilled water and a quick 95% ethanol wash, the slides were stained with eosin (Thermo Scientific #6766007) and dehydrated with two washes of 95% ethanol, two washes of 100% ethanol, and two washes of xylene. The slides were mounted with permount (Fisher # SP15-500), cover with a coverslip, and left to dry overnight. Images were taken with EVOS® FL Auto Imaging System (Life Technologies) at 20x in brightfield. The severity of insulitis in islets was assessed by blind pathological scoring.

To confirm the insulin producing function of islets, we conducted immunohistochemical staining of insulin in pancreas. The slides adjacent to those used in H&E staining were deparaffinized in xylenes at 14 min and then rehydrated in series of decreasing ethanol concentration, 100% for 8 min, 95% for 6 minutes, and 70% for 10 min then in distilled water for 5 min. The slides were washed with PBS w/ 0.2% lamb serum for 5 min and then outlined with a PAP pen. The slides were blocked with Avidin (Vector Laboratories, #SP-2001) for 30 minutes, washed with PBS w/ 0.2% lamb serum for 5 min, and then blocked with Biotin (Vector Laboratories, #SP-2001) for 30 min and washed again with PBS w/ 0.2% lamb serum. The slides were blocked with goat serum (Vector Laboratories, #PK-4007) for 1 hr and incubated overnight at 4°C with an anti-Insulin antibody (ab195956, 1:1000). The slide was warmed to room temperature for 1 hr and washed with PBS w/ 0.2% lamb serum for 5 min and then incubated with a biotinylated secondary antibody (Vector Laboratories, #PK-4007) for one hour and washed PBS w/ 0.2% lamb serum for 5 min. Then the slides were incubated with the ABC reagent (Vector Laboratories, # PK-4007) for 1 hr and washed with PBS. The slides were incubated in a peroxidase substrate solution (Vector Laboratories, #SK-4105) for 2 min and wash with distilled water and counterstained with hematoxylin (Gill-3, Fisher #3537-32) for 30 sec, and raised in running distilled water. The slides were then blued with ammonia water for 3 min and raised in running distilled waters. The slides were dehydrated with a series of washes with 70% ethanol for 5 minutes, 95% ethanol for 2 min, 100% ethanol for 2 min, and xylene for 4 min. The slides were mounted with permount (Fisher #SP15-500), covered with a coverslip, and left to dry overnight. Images were taken with EVOS® FL Auto Imaging System (Life Technologies) at 20x in brightfield. We verified that the islets without insulitis still had functional beta cells by immunostaining for insulin.

Mouse Magnetic Luminex assays

We performed a magnetic bead-based multiplex assay (R&D systems #LXSAMSM-18) for the measurement of 18 analytes in sera, including BAFF/BLyS, IFN-gamma, IL-1beta, IL-3, IL-5, IL-10, IL-13, IL-17E, IL-33, GM-CSF, IL-1alpha, IL-2, IL-4, IL-6, IL-12p70, IL-17A, IL-27, and TNF-alpha. Serum samples were analyzed with two replicates in a 96-w plate by following the manufacturer's instructions. The plate was read with Luminex 200.

Metabolomics

We extracted metabolites from approximately 50 mg cecal contents or a single fecal pellet for the analysis of untargeted metabolomics. In detail, we resuspended cecal/fecal samples in 2 ml methanol in 8 ml glass vials (VWR #66011-085) and added 4 ml cold chloroform for vortex mixing and homogenization in an ultrasound water bath for 5 min. We then added 2 ml LC-MS grade water consisting of 100 nM pentanoic acid as the internal standard. After vortex mixing, vials were placed in centrifuge tubes for centrifugation at 3,000 rpm for 10 min. The aqueous phase was transferred to new glass vials and dried with nitrogen. Metabolites were resuspended in a volume of acetonitrile:water (1:1) based on the sample weight (200 µl/100 mg).

Samples were run on a Thermo Fisher Orbitrap IDX mass spectrometer. 5 µl of the extracted sample was injected onto a 5 µm Zic-pHILIC column (150 x 2.1 mm). Solvent A contained 20 mM ammonium carbonate and 0.1% ammonium hydroxide in Water; solvent B contained 97% acetonitrile in water. A linear gradient was used to separate the analytes: 100 to 40% solvent B (0 to 19 min), 40 to 0% solvent B (19 to 28 min), 0% solvent B (28 to 33 min), 0 to 100% solvent B (33 to 36 min), and re-equilibration of the column with 100% solvent B (36 to 45 min) at a flow rate of 0.2 ml/min and a column temperature of 40°C.

The mass spectrometry parameters were as follows: HESI + and -; for MS1: switching polarity, 120,000 resolution, range 65-1000, 40% RF lens, AGC 1.e5, maximum IT 50 ms, using internal calibration; for MS2: threshold at 2e4, dynamic exclusion, AcquirX targeted exclusion, AcquirX targeted inclusion - ddMS2 with HCD stepped 15, 25, 45, detected in orbitrap at 30,000 resolution, cycle of 0.6 sec with MS2 of other if time, same parameters as the inclusion. A pooled sample was created from all samples and used for MS-MS runs using AcquirX. For each AcquirX run, a blank and the pool was run in MS1, then four runs of MS2 ID were performed.

AquirX was run in pos and neg separately. Functional analysis of untargeted metabolome data was performed with the online tool MetaboAnalyst 5.0⁴¹ (<https://www.metaboanalyst.ca/>), using the Mummichog algorithm (positive mode; 3 ppm; p-value cutoff=0.001) and selecting the *Mus musculus* KEGG pathways containing at least 3 entries.

qPCR analysis

We homogenized intestinal tissues in 0.5 ml of TRIZOL buffer using the Retsch Mixer Mill MM400 with two zirconia beads at 25 frequency/sec for 10 min. Then, 100 µl chloroform was added for mixing and incubation at room temperature for 3 min. We centrifuged samples at 12,000g, 4°C for 15 min, transferred 300 µl of the aqueous phase to RNase-free tubes in order to mix with 150 µl 100% ethanol, then isolated and eluted the total RNA with the NucleoSpin RNA XS kit. The quality of extracted RNA was assessed by Agilent RNA chips. The RNA concentration was determined by a NanoDrop spectrophotometer. The cDNA was synthesized by iScript Reverse Transcription Supermix (Bio-Rad #1708841) using 1 µg total RNA as the template. We then performed qPCR using PowerUp SYBR Green Master Mix (Thermo fisher #A25742) on the QuantStudio Dx Real-Time PCR Instrument. Three-step amplification was applied for each primer set. There were two technical replicates for each primer set. Data was analyzed with the delta-delta Ct method⁴², using the mouse *gapdh* gene as the internal control.

For the assessment of bacterial abundance, genomic DNA was extracted from mouse feces using the ZymoBIOMICS DNA kit (Fisher Scientific #50-125-1501), then quantified with the Quantifluor ONE dsDNA System (Promega #E4870). DNA was diluted to 10-50 ng/µl and then used as the template for qPCR using the universal 16S rRNA primers or *P. goldsteinii* ASF519 specific primers. Data was analyzed with the delta-delta Ct method⁴², using the universal 16S rRNA as a reference.

Statistical analysis

Mice at similar ages were randomly allocated into experimental groups. No statistical methods were used to predetermine sample size. Prism software (GraphPad, v8.4.3) was used for all statistical analysis, unless otherwise specified. Student t tests, non-parametric Mann-Whitney U tests, and two-way ANOVA with Tukey's multiple

comparison adjustment was performed as indicated in the figure legends. For all statistical test, the following p values were used: ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05 and not significant (ns). All graphs show the mean with sd or sem specified, with each dot representing a biological sample (unless otherwise specified). In the figure legends, n numbers refer to biological replicates, unless otherwise specified.

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Author contributions

T.X. conceptualized, designed and conducted most of the experiments, performed data analysis and prepared the manuscript. M.W. provided bacterial spent media. L.Z., L.P., and M.Z.I. helped with in vivo mouse experiments. M.S.C. helped with RNA sequencing and data analysis. L.P. stained and imaged pancreas sections. C.O.B. and A.D.K. provided discussion, data interpretation and help with statistical analyses. A.D.K. designed the experiments, interpreted the data and prepared the manuscript. All authors helped to write and edit the manuscript.

Competing interests

A.D.K. is a co-founder and scientific advisor to FitBiomics, Inc. The other authors declare no competing interests.

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Fig. 1 *Parabacteroides goldsteinii* ASF519 induces multifaced tolerogenic phenotypes. **a**, Measurement of cytokine production by BMDCs and Foxp3⁺ Treg proliferation in the coculture of BMDCs and splenocytes, using filter sterilized spent media collected during bacterial growth as the stimuli of BMDCs. These 8 bacterial species are members of the altered Schaedler flora (ASF), a model of the murine gut microbiome. Mean values of three biological replicates are shown. **b**, Prominent immunophenotypes evoked by each ASF member irrespective of bacterial growth stages. All blue cells present statistical significance ($p < 0.05$, two-tailed Student t test) with at least a 35% change in cytokine levels or a 2-fold change in Treg proliferation. **c**, Flow cytometry analysis of Foxp3⁺ CD4⁺ T cells in splenocytes after coculture with BMDCs stimulated by BHI broth or ASF519 SSM. Representative data are shown ($n = 3$). **d**, IL-10 production by cultured or enriched cell types after stimulation with either broth or ASF519 SSM ($n = 3-4$). **e**, Experimental design for RNA sequencing of BMDCs that were exposed to bacterial spent medium and zymosan in

a step-wise manner (n=3). **f**, Intersection of differentially expressed genes (DEGs) (adjusted $p < 0.01$ and $|\text{fold change}| > 3$) in two between-group comparisons, ASF519 versus Broth, and zymosan-involved ASF519_Zym versus Broth_Zym. **g**, Heatmap of select DEGs in either comparisons shown in f. The fold change of each gene was relative to the Broth condition. The bar plot indicates fold changes in ASF519_Zym versus ASF519, indicative of synergy (Syn.) and antagonism (Ant.) between ASF519 and zymosan. **h**, IL-10 production by BMDCs that underwent monotonous (bacterial medium) or step-wise stimulation (bacterial medium + zymosan) (n=3). Data (d, e, and h) are represented as mean $\pm$ sd across biological replicates and analyzed by two-tailed Student t test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Fig. 2 Activation of adenosine receptor A2a by microbial enzymes enhances immune tolerance. **a**, Bioanalytical strategy to identify responsible molecules in bacterial spent media. **b**, Effect of immobilized protease K (ProK) treatment of ASF519 SSM on IL-10 induction in BMDC assays (n=3). The heat killed ProK and ProK vehicle were used as controls. **c**, Dose-dependent induction of IL-10 by enriched crude proteins in BMDC assays (n=3). **d**, IL-10 inducing function of each fraction eluted from gel filtration of crude ASF519 SSM. The UV absorbance of proteins (mAU) and IL-10 readouts were plotted against the elution volume (Ev.) and elution fractions, colored by black and red, respectively. Data are representative of reproducible runs. **e**, Effect of inhibition of adenosine forming enzyme AHCY and adenosine receptors (A1, A2a, A2b, A3) by specific antagonists on IL-10 induction of BMDCs in response to ASF519 SSM. **f**, Validation of A2a engagement in IL-10 induction. Wildtype (WT) and A2a^{-/-} BMDCs were stimulated by fractionated ASF519 proteins or PBS (n=3). **g**, Adenosine formation by purified enzymes in *in vitro* assays fed with the substrate S-adenosylhomocysteine (n=4). MAT, S-adenosylmethionine

860 synthase; AHCY, Adenosylhomocysteinase. **h**, IL-10 induction by purified enzymes
861 that are involved in methionine and cysteine metabolism (n=3). MS, methionine
862 synthase; METY, O-acetyl-L-homoserine sulfhydrylase; OASS, O-acetylserine
863 sulfhydrylase. **i**, Model of ASF519-mediated regulation of IL-10 induction in BMDCs.
864 Enzymes of the methionine cycle interact with immune cells to activate the
865 adenosine A2a receptor signaling pathway to induce IL-10 production. Data (b, c, f
866 and g) are represented as mean $\pm$ sd and analyzed by two-tailed Student t test
867 (*p<0.05, **p<0.01;***p<0.001).

868

Fig. 3 *P. goldsteinii* ASF519 in mice alters the cecal metabolome and immune phenotypes. **a**, Effect of ASF519 mono-association (ASF519m) in gnotobiotic mice on metabolic pathways through functional analysis of cecal metabolome data. The metabolism of 13 amino acids (e.g., Cys and Met), galactose (Gal), and amino and nucleotide sugars were significantly influenced by ASF519m versus germ-free mice (Extended Data Fig. 7b). **b**, Signal density of identified cecal metabolites. Data are represented as mean $\pm$ sem (n=5-8) and analyzed by Mann-Whitney U test ($***p < 0.001$). **c**, qPCR assays on the transcript abundance of select genes in small intestine and colon collected from GF, ASF519m, and SPF mice. Data are represented as mean $\pm$ sem (n=5-8) and analyzed by two tailed Student t test ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$). **d** and **e**, measurement of IL-4 (d) and IL-5 (e) in sera of GF, ASF519m, and SPF mice. Data are represented as mean $\pm$ sem (n=4-8) and analyzed by unpaired, nonparametric Mann-Whitney test, $*p < 0.05$. **f**, Experimental design to test the role of adenosine receptor A2a in ASF519-mediated

885 immunomodulation in antibiotic-treated (ABX) mice. Mice that received two doses of
886 live or killed ASF519 were intraperitoneally injected daily with the A2a antagonist,
887 ZM 241385. **g-j**, Flow cytometry analysis of IL-10 producing CD11c+ dendritic cells
888 (g and h), CD19+ CD45+ B cells (i), and CD11b+ CD45+ cells (j). Data are
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890 Tukey's test (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).
891

Fig. 4 *P. goldsteinii* ASF519 supplementation ameliorates disease severity in mouse models of type 1 diabetes (T1D) and collagen-induced arthritis (CIA). **a**, Experimental design to test the effect of orally administered heat-killed (Ctrl) or live ASF519 on T1D in female NOD mice. **b**, The percentage of mice grouped by non-fasting blood glucose levels at 14-wk old (n=7-8). **c**, Histological insulinitis scores of pancreatic islets in Ctrl and ASF519 mice. Each group has at least 120 islets assessed. The insulin producing function of insulinitis free islets was validated by immunohistochemical staining of insulin. **d**, Experimental design to test the role of ASF519 in the collagen-induced arthritis mouse model. **e**, Comparison of ASF519 abundance in stools before gavage and 21 days post gavage by qPCR. Data are represented as mean±sem (n=5-6) and analyzed by Mann-Whitney U test (**p<0.01; ***p<0.001). **f**, Loss of body weight after immunization with collagen (n=5-6). **g**, Arthritis severity developed on four paws of immunized Ctrl and ASF519 mice. Data (f and g) are represented by mean±sem (n=5-6) and the significance was tested by two-way ANOVA (***p<0.001).

Figures

Figure 1

Parabacteroides goldsteinii ASF519 induces multifaced tolerogenic phenotypes. a, Measurement of cytokine production by BMDCs and Foxp3⁺ Treg proliferation in the coculture of BMDCs and splenocytes, using filter sterilized spent media collected during bacterial growth as the stimuli of BMDCs. These 8 bacterial species are members of the altered Schaedler flora (ASF), a model of the murine gut microbiome. Mean values of three biological replicates are shown. b, Prominent immunophenotypes evoked by each ASF member irrespective of bacterial growth stages. All blue cells present statistical significance (p<0.05, two-tailed Student t test) with at least a 35% change in cytokine levels or a 2-fold change in Treg proliferation. c, Flow cytometry analysis of Foxp3⁺ CD4⁺ T cells in splenocytes after coculture with BMDCs stimulated by BHI broth or ASF519 SSM. Representative data are shown (n=3). d,

IL-10 production by cultured or enriched cell types after stimulation with either broth or ASF519 SSM (n=3-4). e, Experimental design for RNA sequencing of BMDCs that were exposed to bacterial spent medium and zymosan in a step-wise manner (n=3). f, Intersection of differentially expressed genes (DEGs) (adjusted $p < 0.01$ and $|\text{fold change}| > 3$) in two between-group comparisons, ASF519 versus Broth, and zymosan-involved ASF519_Zym versus Broth_Zym. g, Heatmap of select DEGs in either comparisons shown in f. The fold change of each gene was relative to the Broth condition. The bar plot indicates fold changes in ASF519_Zym versus ASF519, indicative of synergy (Syn.) and antagonism (Ant.) between ASF519 and zymosan. h, IL-10 production by BMDCs that underwent monotonous (bacterial medium) or step-wise stimulation (bacterial medium + zymosan) (n=3). Data (d, e, and h) are represented as mean+sd across biological replicates and analyzed by two-tailed Student t test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

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