Supporting Information

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SI Methods

Study Design. MZ twins were recruited by launching a national televised appeal as well as internet notification in Germany with support from the German Multiple Sclerosis Society (DMSG). Inclusion criteria for study participation were MZ twins with an MS diagnosis according to the revised McDonald criteria or CIS in one twin only. Exclusion criteria were antibiotic, glucocorticosteroidal, or immunosuppressive treatment, gastrointestinal infection, or diet irregularities in the 3 mo before study entry. In total, 34 pairs (Table 1) visited the outpatient department at the Institute of Clinical Neuroimmunology in Munich for a detailed interview on past and present medical, family, and social history, a neurological examination, and a nutrition questionnaire. To confirm the MS diagnosis, medical records including MRI scans were obtained and reviewed. Fecal samples were directly collected in hospital or were taken at home, stored at −20 °C, and transferred to the hospital in cooling bags. Finally, all samples were stored at −80 °C. Buccal swabs for zygosity testing were taken from all participants. The study was approved by the local Ethics Committee of the Ludwig-Maximilians University Munich, and all participants gave written informed consent.

16S rRNA Sequencing and Analysis. The V3–V5 region of the bacterial 16S rRNA gene was amplified using the universal forward (5′-CCGTCAATTCMTTTGAGTTT-3′) and reverse (5′-ACTCCTA CGGGAGGCAGCAG-3′) primers incorporating the FLX Titanium adapters and a unique barcode sequence. PCR products were sequenced on a 454 GS FLX titanium pyrosequencer (Life Sciences) at BGI-Shenzhen. Analysis was performed using QIIME v1.9 as described (35). Essentially, amplicon sequences were quality-filtered and grouped by OTUs using the SortMeRNA method (36) using the Greengenes version 13.8 97% dataset for closed reference. Sequences that did not match reference sequences in the Greengenes database were dropped from the analysis. Taxonomy was assigned to the retained OTUs based on the Greengenes reference sequence, and the Greengenes tree was used for all downstream phylogenetic community comparisons. Samples were filtered to at least 10,000 sequences per sample, and OTUs were filtered to retain only OTUs present in at least 5% of samples, covering at least 0.01% of total reads. After filtering, sequences were rarefied to the lowest number of sequences per sample: 10,975 sequences in human samples and 8,137 sequences in mouse samples. For comparison between human and mouse samples, the human and mouse datasets were combined before OTU filtering and rarefaction. The resulting OTUs were filtered as described above, and samples were rarefied to 6,200 sequences per sample. Alpha diversity was calculated using the phylogenetic diversity index method (37). For analysis of beta diversity, pairwise distance matrices were generated by phylogenetic metric of weighted UniFrac (38) and used for PCoA. For comparison of individual taxa, samples were not rarefied. Instead, OTU abundances were normalized using variance-stabilizing transformation, and taxa distributions were compared using the Wald negative binomial test from the R software package DESeq2 (as described in refs. 38 and 39) with Benjamini–Hochberg correction for multiple comparisons. All statistical analyses of differences between individual bacterial species were performed using QIIME v.1.9 or R (packages DESeq2 and phyloseq).

Metagenomic Analysis of Human and Mouse Samples. We performed metagenomic sequencing of the gut microbiome in 16 pairs of identical twins, each composed of a sibling affected by MS and one unaffected sibling. In addition, stool samples from 25 germ-free mice that were colonized with four twin pair samples also underwent metagenomic sequencing. Each human and mouse fecal sample produced at least 30 million paired-end DNA reads 100 bp in length. Sequence quality, evaluated using FastQC, was high in the majority of sequences across all samples. We used the HMP Unified Metabolic Analysis Network (HUMAnN2) tool to calculate the relative abundance of specific microbes, gene families, and metabolic pathways. This software pipeline uses MetaPhlAn2 to obtain a list of abundant organisms by aligning sequences to genes unique to known bacterial species. DNA sequences are subsequently aligned to genomes of the identified organisms using the Bowtie 2 aligner and an annotated pan-genome database, ChocoPhlAn. Unmapped DNA reads undergo translated alignment to the bacterial proteome using the software Diamond and a large protein database, UniRef50. The product of sequence alignment is a quantitative relative abundance of specific protein families. The HUMAnN2 software subsequently uses this information to determine the number complete copies of specific metabolic pathways using MetaCyc, a database mapping metabolic reactions to pathways. After obtaining quantitative measurements of gut bacterial abundance, gene families, and metabolic pathways, we performed association testing to identify pathogenic and protective factors in MS. We calculated the pairwise sum of absolute differences of microbial relative abundance between two individuals to determine if gut bacterial flora are more similar between twins with discordant phenotypes than between pairs of unrelated individuals. We used logistic regression (adjusting for twin pair and number of genome equivalents sequenced per sample) to examine associations between each gut bacterial variable and MS phenotype. We corrected for multiple comparisons and adjusted P values using a false-discovery rate of 5%. We applied the same rigorous statistical approach to mouse samples and also adjusted for the twin pair from which mice were colonized.

Colonization of Germ-Free RR Mice with Human MS Twin-Derived Fecal Samples. For the human-to-mouse fecal transfer experiments we selected a subgroup of five discordant twin pairs, mainly based on pragmatic criteria such as relatively young age (20–40 y), female sex, and either no treatment or treatment only with IFN-β (Table S1). One gram of human fecal material was suspended in 15 mL prereduced PBS (PBS supplemented with 0.1% L-cysteine hydrochloride monohydrate) and was vortexed at room temperature for 5 min. Large insoluble particles were allowed to settle by gravity for 5 min. The supernatant was transferred to an anaerobic crimped tube (Sigma-Aldrich). Prereduced glycerol (containing 0.1% L-cysteine hydrochloride monohydrate) was added to a final concentration of 20%, and tubes were frozen at −80 °C. Tubes were sprayed thoroughly with Virkon (V.P. Produkte) before being transferred to the gnotobiotic isolators. Germ-free RR mice were gavaged with ≈300 μL of fecal bacterial suspension. In addition, mice colonized with healthy twin fecal material were injected with 250 μg anti–IL-10 (JES5-2A5; BioXcell) or isotype control antibodies once every week. All animal procedures were in accordance with the guidelines of the Committee on Animals of the Max Planck Institute of Neurobiology and the Max Planck Institute of Immunobiology and Epigenetics with a license from the Regierung von Oberbayern as well as the Regierungspräsidium Freiburg.

Cell Isolation and Flow Cytometry. Isolation and phenotyping of immune cells by flow cytometry were done as previously described (7). Briefly, single-cell suspensions were prepared from spleens by mechanical disruption via forcing through 40-μm cell strainers (Thermo Fisher Scientific). For the isolation of lymphocytes from the small intestine, the intestine was collected in ice-cold HBSS buffered with 15 mM Hepes. After careful removal of fatty tissue and fecal contents, the intestine was opened longitudinally and cut into small pieces. The intestinal fragments were washed three times for 15 min with stirring in HBSS containing 5 mM EDTA, 15 mM Hepes, and 10% FBS. Next, intestinal pieces were washed once for 5 min with stirring in RPMI medium containing 15 mM Hepes and 10% FBS, followed by an incubation step at 37 °C with stirring in RPMI medium with 15 mM Hepes, 10% FBS, and 100 U/mL collagenase D (Roche Diagnostics). The digested tissue was washed twice in HBSS containing 5 mM EDTA before the lymphocytes of the small intestine were resuspended in 5 mL of 40% Percoll (Sigma-Aldrich) and overlaid on 2.5 mL of 80% Percoll. Percoll gradient separation was performed by centrifugation at 780 × *g* for 20 min at room temperature. Small intestinal lamina propria lymphocytes were harvested from the interphase of the Percoll gradient and washed once in RPMI medium containing 15 mM Hepes and 10% FBS. For detection of cell-surface markers, cells were stained in FACS buffer (PBS containing 1% BSA and 0.1% NaN_3) with fluorochrome-labeled antibodies: PerCP-Cy5.5–conjugated anti-CD4 (RM4-5); eFluor 450-conjugated anti-CD45 (30-F11); PerCP-Cy5.5–conjugated anti-B220 (RA3-6B2); FITC-conjugated anti-CD11c (HL3), and PE-Cyanine 7–conjugated anti-CD11b (M1/70). For intracellular cytokine staining, 2×10^6 cells/mL were stimulated for 16 h with anti-CD3 antibody (1 μg/mL) (BD Pharmingen). Brefeldin A (5 μg/mL) (Sigma-Aldrich) was added for the last 5 h. After surface staining, cells were fixed and permeabilized using the Transcription Factor Staining Buffer Set (eBioscience) and stained intracellularly using the following antibodies: PE-conjugated anti-IL17 (TC11-18H10), FITC-conjugated anti–IFN-γ (XMG1.2), and APC-conjugated anti-FoxP3 (FJK-16s). All antibodies were purchased from BD Pharmingen, eBioscience, or BioLegend. Cells were acquired on a FACSVerse flow cytometer (BD Biosciences), and analysis was performed using FlowJo (TreeStar) software.

ELISA. For the measurement of cytokine production by T cells, 2×10^5 splenocytes were cultured in the presence of 1 μg/mL anti-CD3 antibody (BD Pharmingen) or 1 μg/mL anti-CD3 and 0.5 μg/mL anti-CD28 antibody (BD Pharmingen). Cytokines in cell-culture supernatants were measured by ELISA with antibody pairs for IFN-γ (BD Biosciences), IL-17 (eBioscience), and IL-10 (R&D Systems). Serum titers of anti-MOG antibodies were quantified as previously described (6).

Cytokine Profiling of Human $CD4^+$ T Cells. From a subgroup of eight twin pairs, including three of the donors of fecal samples, we used available frozen PBMC specimens for in vitro cytokine production analyses. Frozen PBMC specimens from these pairs were thawed, and $CD4^+$ T cells were isolated using the RosetteSep Kit (Stemcell Technologies). For assessment of IFN-γ, IL-4, and IL-17A secretion, 1×10^5 $CD4^+$ T cells per well in triplicate were stimulated for 48 h with 5 μg/mL PHA. Levels of IFN-γ, IL-4, and IL-17A in the supernatants were measured by Luminex Bead-based Multiplex Assay (R&D systems) using a Bio-Plex MAGPIX Multiplex Reader (Bio-Rad). For assessment of IL-10 secretion, 1×10^5 $CD4^+$ T cells per well in triplicate were stimulated for 96 h with either 5 μg/mL PHA or 1 μg/mL anti-CD3 (clone OKT3; BioLegend) and anti-CD28 (clone: CD28.2; eBioscience) antibodies, respectively. Levels of IL-10 in the supernatants were measured by ELISA according to the manufacturer's instructions (eBioscience).

Real-Time qPCR. RNA was prepared from ileum and colon of gnotobiotic mice using the Qiagen RNeasy mini kit (Qiagen) followed by conversion to cDNA using a verso cDNA synthesis kit (Thermo Scientific). Triplicate wells of PCR with gene-specific primers were performed using the Absolute QPCR SYBR Green Mix or Absolute QPCR mix with ROX (Thermo Fisher Scientific) following the manufacturer's instructions and were measured using a 7900HT Real-Time PCR System (Applied Biosystems).

Statistical Analysis. GraphPad Prism 6 (GraphPad Software, Inc.) was used for all statistical analysis. P values < 0.05 were considered to be significant.

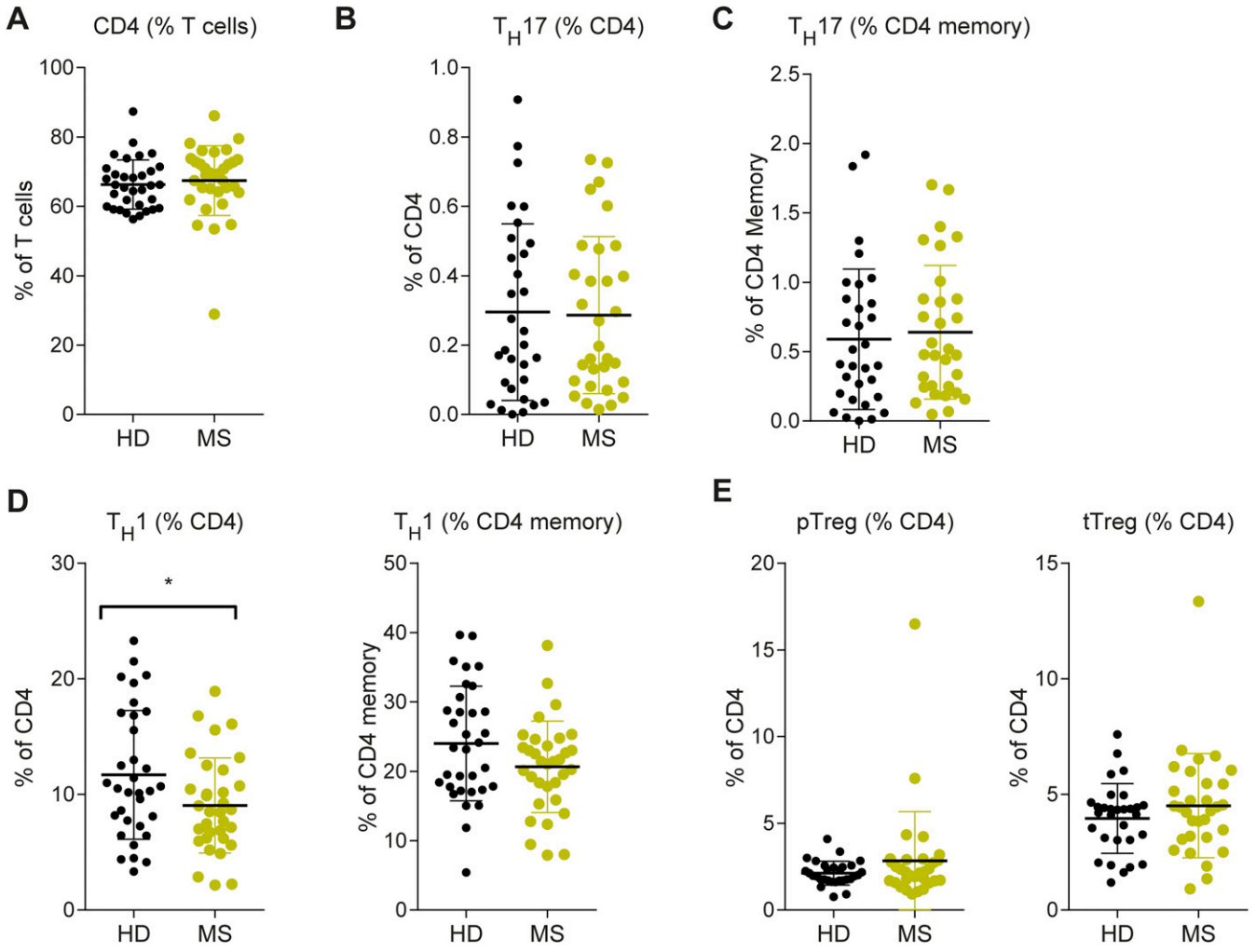

Fig. S1. Immune-phenotyping of PBMCs from twin pairs did not reveal profound changes in T cell subset composition. Frozen PBMC specimens from all twin pairs ($n = 34$) were thawed and immediately subjected to 10-color flow cytometry for quantification of T cell subsets including CD4 T cells, Th17 cells (defined by simultaneous expression of $CCR4^+$, $CCR6^+$, $CD161^+$, $CD146^+$), Th17 memory cells, Th1 cells (defined as $CCR4^-$, $CCR6^-$, $CD183^+$), Th1 memory cells, peripheral regulatory T cells (defined as $FoxP3^+$ $Helios^-$), and thymic regulatory T cells (defined as $FoxP3^+$ $Helios^+$). (*A*) CD4 T cells are depicted as percentage of T cells. (*B–E*) All other populations are depicted as percentage of CD4 T cells. Each dot represents an individual measurement, and horizontal lines and whiskers indicate mean ± SEM. *$P < 0.05$ (Mann–Whitney *u* test).

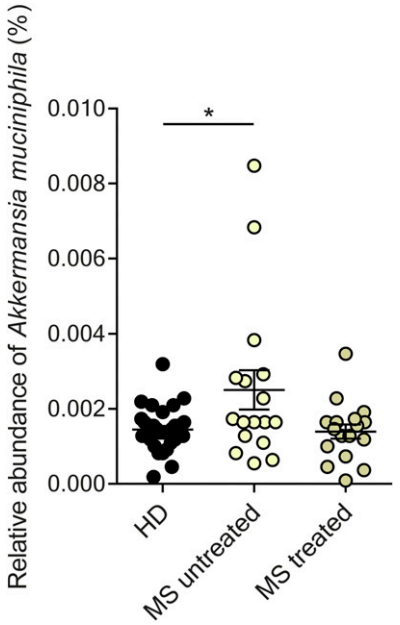

Fig. S2. Differential abundance of *Akkermansia muciniphila* in fecal samples of healthy twins (HD; $n = 34$) compared with co-twins with untreated MS (MS untreated; $n = 17$) or treated MS (MS treated; $n = 17$). Each dot represents an individual stool sample, and horizontal lines and whiskers indicate mean ± SEM. *$P < 0.05$ (Mann–Whitney u test).

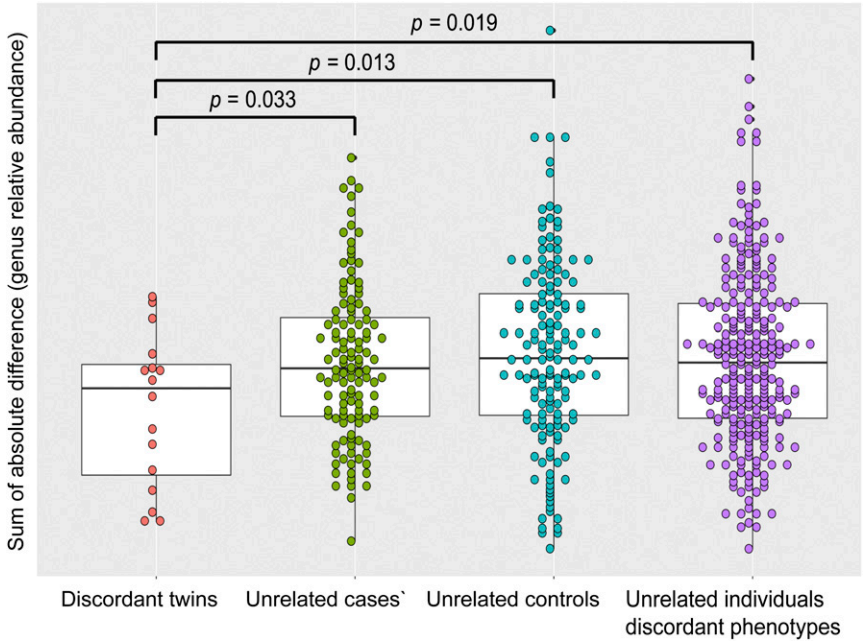

Fig. S3. Comparison of the metagenomic profiles of healthy and MS twins revealed a high similarity of the gut microbiomes between discordant twins. Pairwise analysis of gut microbial composition (sum of absolute differences in genus relative abundance between two individuals) shows that gut bacterial flora are more similar within twin pairs discordant for MS (discordant twins, $n = 32$) than within pairs of unrelated individuals with MS (unrelated cases; $n = 16$), unrelated healthy individuals (unrelated controls; $n = 18$) or unrelated healthy and MS individuals (unrelated individuals discordant phenotypes; $n = 34$).

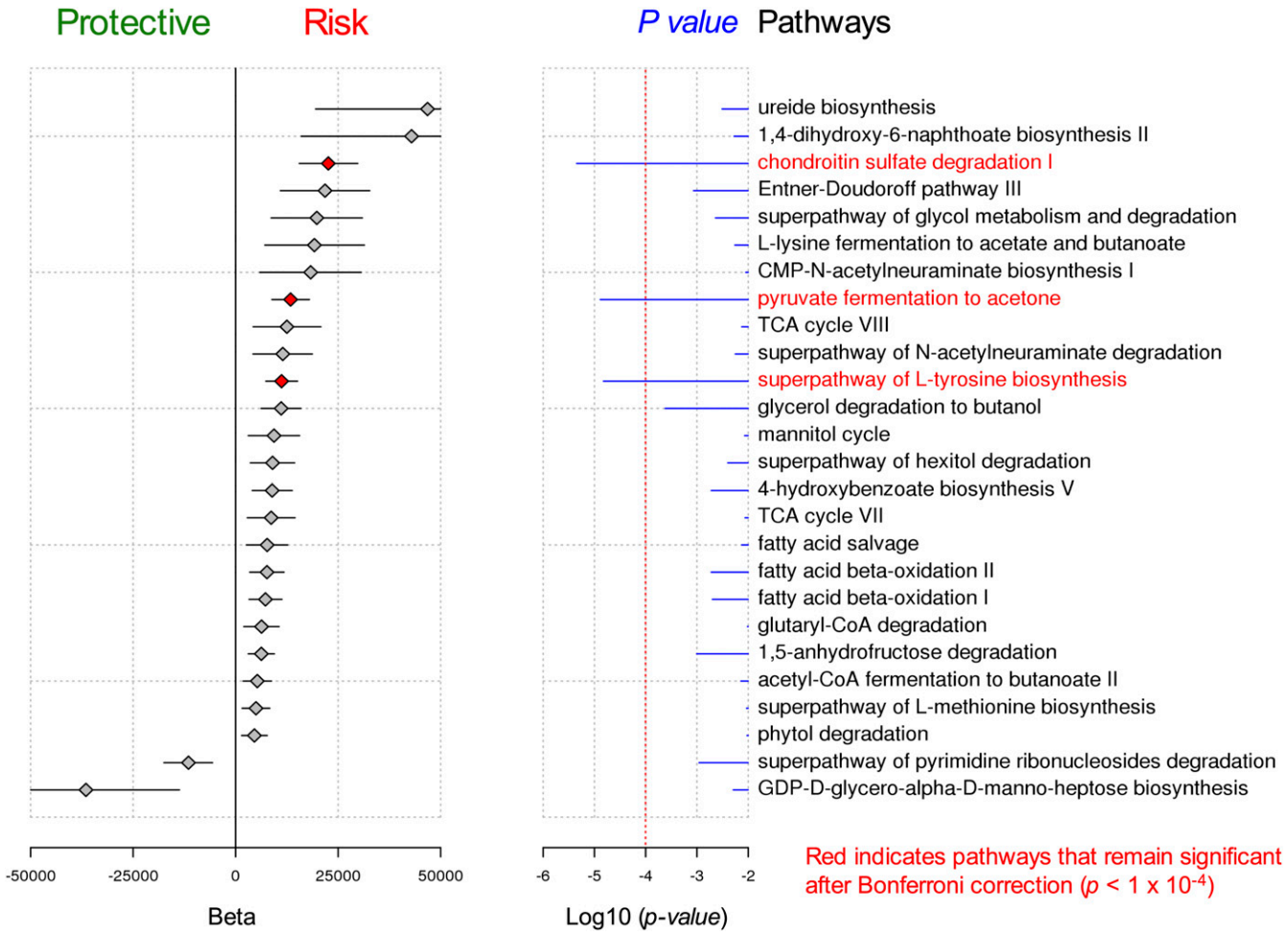

Fig. S4. Shotgun metagenomic sequencing of mouse fecal pellets uncovered several metabolic pathways associated with MS. Examination of gut microbiome metabolic pathways identified by metagenomic sequencing in 25 germ-free mice colonized by stool from four twins discordant for MS. Pathways that remained significantly associated with MS after multiple testing correction included chondroitin sulfate degradation (beta = 22,600, $P = 4.5 \times 10^{-6}$), pyruvate fermentation to acetone (beta = 13,400, $P = 1.3 \times 10^{-5}$), and superpathway of L-tyrosine biosynthesis (beta = 11,200, $P = 1.5 \times 10^{-5}$).

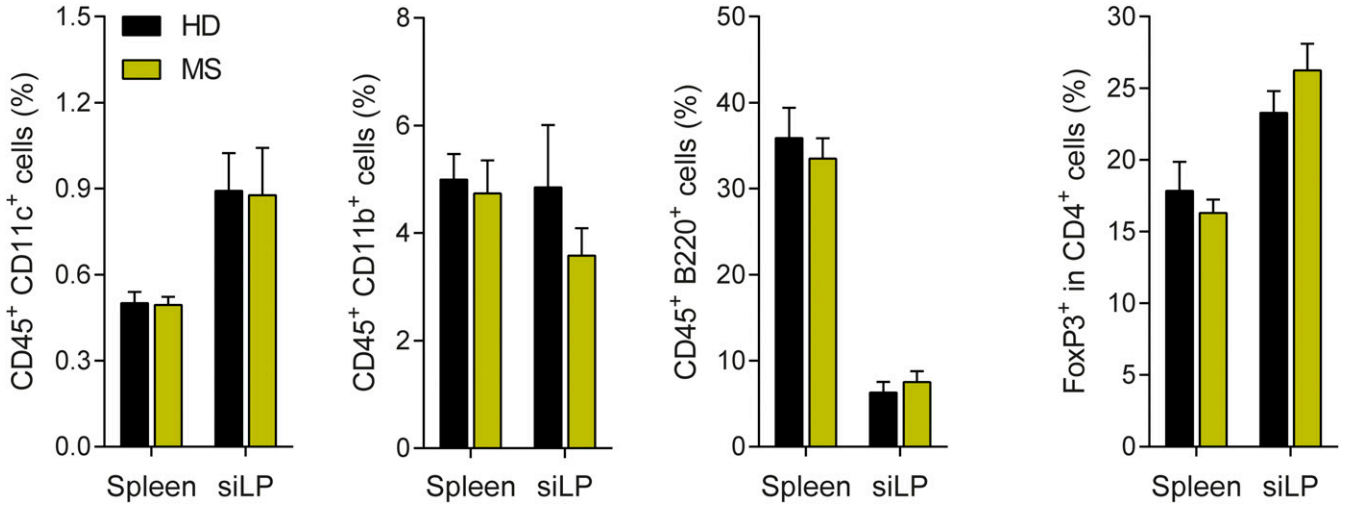

Fig. S5. No differences were seen in immune cell populations of mice receiving control or MS patient fecal material. Bar graphs display frequencies (± SEM) of dendritic cells ($CD45^+CD11c^+$), macrophages ($CD45^+CD11b^+$), B cells ($CD45^+B220^+$), as well as regulatory T cells ($CD4^+FoxP3^+$) in spleen and small intestinal lamina propria (siLP) of germ-free SJL/J mice receiving fecal material from healthy twins (HD) or MS patients (MS). $n = 10–13$ mice per group. Data were pooled from three independent experiments.

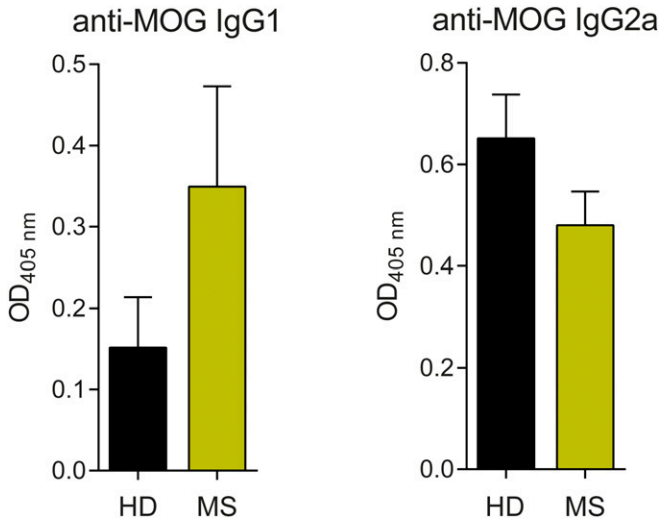

Fig. S6. No differences were seen in serum autoantibody titers in recipients of healthy twin (HD; $n = 16$) or MS twin (MS; $n = 19$) microbiota. Serum MOG-specific IgG1 or IgG2a antibodies were measured by sandwich ELISA. Values shown are the absorbances at 405 nm. Bars depict mean ± SEM.

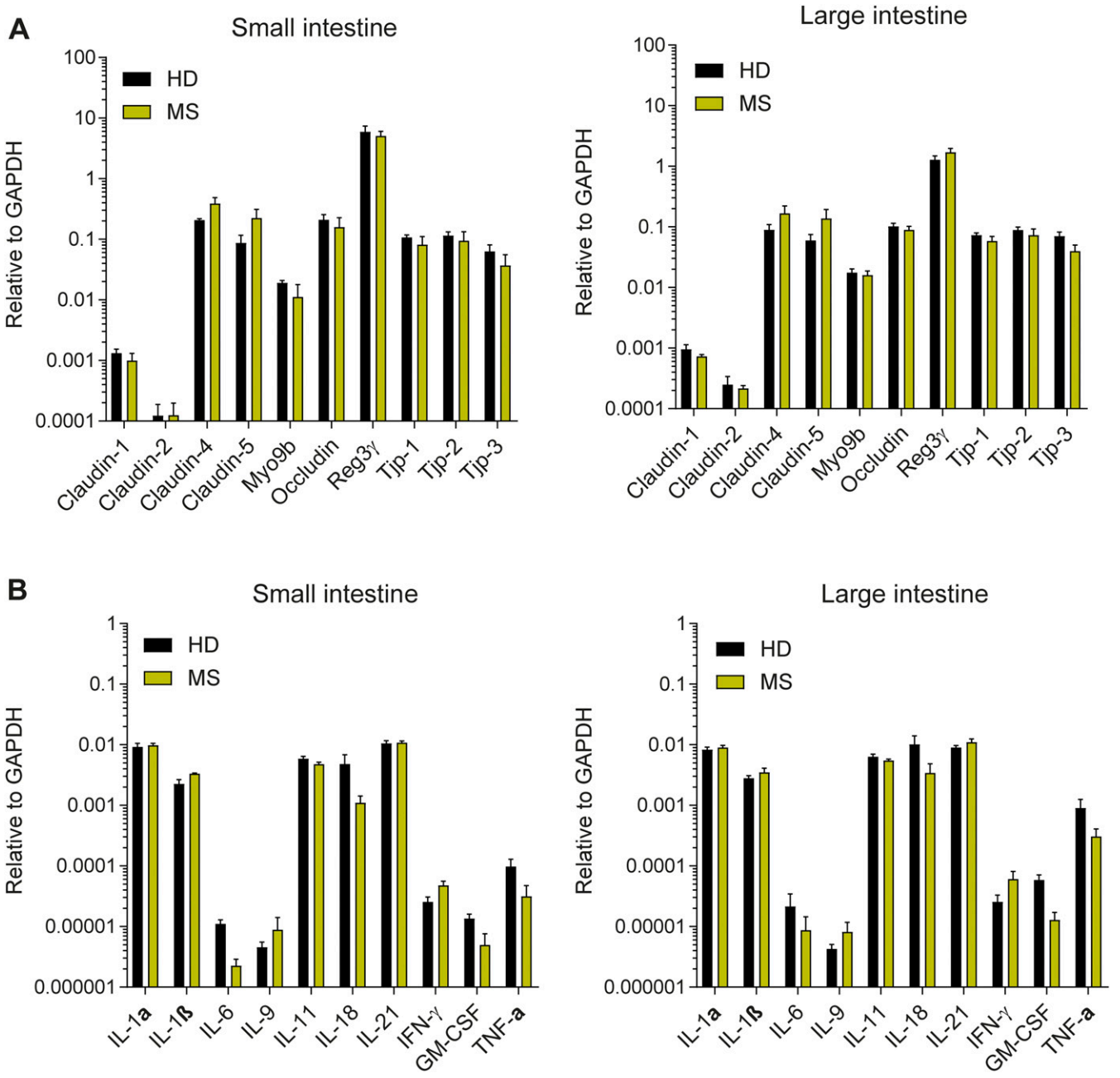

Fig. S7. No differences were seen in the expression of cytokines and tight junction proteins in recipients of healthy (HD) or MS twin microbiota. Expression levels of tight junction proteins (*A*) and cytokines (*B*) were measured by real-time qPCR. (*Left*) Expression levels in small intestine (ileum). (*Right*) Expression levels in colon. $n = 3$ mice per group. Bars depict mean ± SEM.

Table S1. Clinical characteristics of the five MZ twin pairs discordant for MS used for colonization experiments

Twin pair	Age in years	Gender	Disease duration in years	Disease course	EDSS	DMD
1	40	Female	14	RR-MS	2.0	None in 3 mo prior
	40	Female	N.a.	N.a.	N.a.	N.a.
2	20	Female	6	RR-MS	2.0	IFN-β
	20	Female	N.a.	N.a.	N.a.	N.a.
3	21	Female	1	RR-MS	2.0	IFN-β
	21	Female	N.a.	N.a.	N.a.	N.a.
4	29	Female	15	RR-MS	4.5	None in 3 mo prior
	29	Female	N.a.	N.a.	N.a.	N.a.
5	40	Female	2	RR-MS	1.5	IFN-β
	40	Female	N.a.	N.a.	N.a.	N.a.

DMD, disease-modifying drugs; EDSS, Expanded Disability Status Scale; N.a., not applicable; RR-MS, relapsing-remitting MS.

Table S2. Efficiency of transfer of human microbiota to mouse recipients

Donor	Transferred genera, *n* (%)	Nontransferred genera *n* (%)
HD	122.5 (90.725)	12.5 (9.275)
MS	118.5 (89.95)	13.25 (10.05)

HD, healthy twins; MS, MS twins.