D-tryptophan from probiotic bacteria influences the gut microbiome and allergic airway disease

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Background: Chronic immune diseases, such as asthma, are highly prevalent. Currently available pharmaceuticals improve symptoms but cannot cure the disease. This prompted demands for alternatives to pharmaceuticals, such as probiotics, for the prevention of allergic disease. However, clinical trials have produced inconsistent results. This is at least partly explained by the highly complex crosstalk among probiotic bacteria, the host's microbiota, and immune cells. The identification of a bioactive substance from probiotic bacteria could circumvent this difficulty.

Objective: We sought to identify and characterize a bioactive probiotic metabolite for potential prevention of allergic airway disease.

Methods: Probiotic supernatants were screened for their ability to concordantly decrease the constitutive CCL17 secretion of a human Hodgkin lymphoma cell line and prevent upregulation of costimulatory molecules of LPS-stimulated human dendritic cells.

Results: Supernatants from 13 of 37 tested probiotic strains showed immunoactivity. Bioassay-guided chromatographic fractionation of 2 supernatants according to polarity, followed by total ion chromatography and mass spectrometry, yielded $C_{11}H_{12}N_2O_2$ as the molecular formula of a bioactive substance. Proton nuclear magnetic resonance and enantiomeric separation identified D-tryptophan. In contrast, L-tryptophan and 11 other D-amino acids were inactive. Feeding D-tryptophan to mice before experimental asthma induction increased numbers of lung and gut regulatory T cells, decreased lung T_H2 responses, and ameliorated allergic airway inflammation and hyperresponsiveness. Allergic airway inflammation reduced gut microbial diversity, which was increased by D-tryptophan.

Conclusions: D-tryptophan is a newly identified product from probiotic bacteria. Our findings support the concept that defined bacterial products can be exploited in novel preventative strategies for chronic immune diseases. (J Allergy Clin Immunol 2017;139:1525-35.)

***Key words:** D-tryptophan, probiotic bacteria, bacterial substance, screening, immune modulation, allergic airway disease, gut microbiota*

Chronic immune diseases, such as allergies, inflammatory bowel disease, or diabetes, are highly prevalent in industrialized countries, and a further increase of burden caused by noncommunicable diseases is expected for the next decades.[1] Currently available pharmaceuticals improve symptoms but cannot cure the disease. Accordingly, there is an increasing demand for proved alternatives to pharmaceutical products from both health care professionals and consumers.[2]

Probiotic bacteria have been shown to modify immune responses *in vitro*[3-5] and in animals[6,7] and are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host." Accordingly, they have been proposed as an alternative to classical therapies for the treatment of immune diseases.[8] However, apart from acute infectious diarrhea,[9] clinical trials for different indications, such as primary prevention of allergic diseases[10-22] or treatment of chronic inflammatory bowel disease,[23] were highly inconsistent. Accordingly, a consensus paper[24] and the European Food Safety Authority[25] stated that a role for probiotic microbes for prevention of allergic manifestations is not established.

One important reason for the conflicting results is most likely the complexity of the reciprocal crosstalk between probiotic bacteria and the host's microbiota and immune cells. Even in healthy subjects, the gut microbiome differs remarkably among individual patients.[26,27] In addition, both the microbiome and immunity can be substantially altered under disease conditions.[28] Thus it is hard to predict the precise functionality of a probiotic strain in individual patients. In addition, there is a lack of

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Abbreviations used

AAI:	Allergic airway disease
CDM:	Chemically defined medium
DC:	Dendritic cell
Foxp3:	Forkhead box p3
IDO:	Indoleamine 2,3-dioxygenase
LGG:	*Lactobacillus rhamnosus* GG
OTU:	Operational taxonomic unit
SLC6A14:	Solute carrier family 6 amino acid transporter member 14
Treg:	Regulatory T

mechanistic understanding that is important to establish biological plausibility for any claimed health effect.

The use of specified substances derived from probiotic microbes could provide an attractive alternative to overcome these problems. Other than living bacteria with complex fates and response patterns in the host, they should have definable properties with a provable mode of action. Thus far, only very few candidate structures or substances have been demonstrated as bioactive agents and even less with preclinical evidence for therapeutic effects.[29]

Therefore the aim of the present study was (1) to establish a screening tool for the detection of T_H2-decreasing immune activity in probiotic supernatants, (2) to identify a soluble bacterial molecule that mediates this activity, (3) to test the putative substance in a mouse model of allergic airway disease (AAI), and (4) to obtain insight into potential underlying mechanisms.

METHODS

For detailed information on reagents, culture conditions of bacteria and human cells, generation of human monocyte-derived dendritic cells (DCs), structural elucidation of D-tryptophan (Sigma-Aldrich, St Louis, Mo), cytokine/chemokine quantification, murine T-cell differentiation, flow cytometry, quantitative RT-PCR, microbiota analysis, isolation of intestinal lamina propria cells, and animal experiments (induction of experimental asthma and lung function analyses), see the Methods section in this article's Online Repository at www.jacionline.org.

Bacterial strains

Bifidobacteria, lactobacilli, lactococci, *Escherichia coli* Nissle 1917, *Enterococcus faecium*, and *Streptococcus thermophilus* were obtained from different providers (see Table E1 in this article's Online Repository at www.jacionline.org). All strains were grown until stationary phase and a minimum cell number of 10^8 colony-forming units/mL. Cell-free supernatants were obtained by means of centrifugation (at 6000 rpm for 5 minutes at 20°C), followed by filtration through a 0.22-μm pore size surface-modified Polyethersulfone Membrane (Millipore, Darmstadt, Germany). No bacterial growth was observed when aliquots from supernatants were cultured in bacterial growth medium. Otherwise, supernatants were stored immediately after collection in aliquots at −80°C until further use.

Bioassays for screening for immunomodulatory activity in probiotic supernatants

Two biological assays based on downmodulation of costimulatory molecules on human DCs and of CCL17 secretion by a human Hodgkin lymphoma T-cell line (KM-H2) were set up. Human immature DCs were matured with 0.1 μg/mL LPS from *E coli* (Sigma-Aldrich) in the presence or absence of 200 μL of bacteria-free supernatants for 24 hours, followed by flow cytometric analysis of costimulatory molecules.

Similarly, 200-μL supernatants were added to 3 to 5 × 10^6 KM-H2 cells for 24 hours. Supernatants were collected from KM-H2 cells by means of centrifugation and stored at −80°C until quantification of CCL17. The corresponding amount of blank MRS medium was added to control for the dilution of KM-H2 culture medium with different volumes of bacterial supernatants. Blank bacterial growth medium and supernatants from *Lactobacillus rhamnosus* DSM 20021, which has no probiotic activity, were used as negative controls in both screening assays.

Animals and oral supplementation with D-tryptophan

All animal experiments were conducted under the Federal Guidelines for the Use and Care of Laboratory Animals (Az 55.2-1-54-2532-137-13) and approved by the Government of the District of Upper Bavaria and Schleswig-Holstein (V244-13313/2016 [7-1/10]). Six- to 8-week-old female BALB/c mice were obtained from Charles River (Sulzfeld, Germany) and housed in individually ventilated cages, with 2 mice each maintained in specific pathogen-free conditions. A standard extruded pellet diet and sterile filtered drinking water were provided *ad libitum*. For quantification of D-Tryptophan in mouse sera, D-Tryptophan was dissolved in drinking water at concentrations of 1.8 or 18 mg/dL (approximately 0.09 and 0.9 mg/d per mouse). Control animals received pure water ($n = 8$ per group). No changes in behavior or body weight were noted in the supplemented animals compared with control animals. Animals were killed after 14 days, and sera were immediately stored at −80°C until analysis.

For testing prevention of AAI, mice received 50 mmol/L D-Tryptophan starting at least 3 days before the first sensitization until death on day 25. For microbiome analyses, the cecum was cut off and immediately stored at −80°C until further processing.

Statistical analyses

Bioassays and animal experiments. Results of bioassays and animal experiments are presented as means with SDs. The Student *t* test with the Dunn multiple comparison test or 2-way ANOVA with the Bonferroni posttest was used, where appropriate. Tests applied are presented in the respective figure legends. *P* values of less than .05 were considered significant (version 5.0; GraphPad Prism Software, La Jolla, Calif).

Microbial diversity. Bacterial diversity was assessed by means of molecular barcoding of 16S rRNA genes in cecum samples of 6 animals per group. To this end, DNA was directly extracted from the cecum by using a kit-based protocol (PowerSoil DNA Isolation Kit; MO BIO Laboratories, Carlsbad, Calif). Fragments of 315 bp were amplified within the variable regions V5 and V6 of the 16S rRNA gene by using S-D-Bact-0785-a-S-18 (5′-GGMTTAGATACCCBDGTA-3′) and S-*-Univ-1100-a-A-15 (5′-GGGTYKCGCTCGTTR-3′) as primers.[30] Sequencing of amplicons was performed on the Illumina MiSeq platform (Illumina, San Diego, Calif) by using paired-end technology (see the Methods section in this article's Online Repository for details). Sequences were deposited in National Center for Biotechnology Information accession no. PRJNA304109.

Reads were analyzed with the software package QIIME (http://qiime.org). Operational taxonomic units (OTUs) were picked within the 13_8 version of the Greengenes reference database[31] at a similarity level of 95% sequence identity. Sequences were subsampled to 15,000 reads per sample, which reflects the number of reads obtained in the sample with the lowest number of reads after quality control. This number was still sufficient to reach a plateau when collectors' curves were calculated based on of OTU_{95}. The taxonomy assignment was done with the RDP classifier 2.2.[32] Principal coordinate analysis was generated on the unweighted UniFrac distance matrix by using the ape package within the R software environment (http://www.r-project.org), and statistical significance was determined with the Student *t* test. The α-diversity of each sample was measured by using the Chao1 metric[33] and compared between treatments by using the nonparametric 2-sample *t* test (ie, with Monte Carlo permutations for significance testing). β-Diversity was calculated by using the phylogenetic method UniFrac.[34] The nonparametric analysis of similarity was performed to examine the β-diversity distance matrix for significant

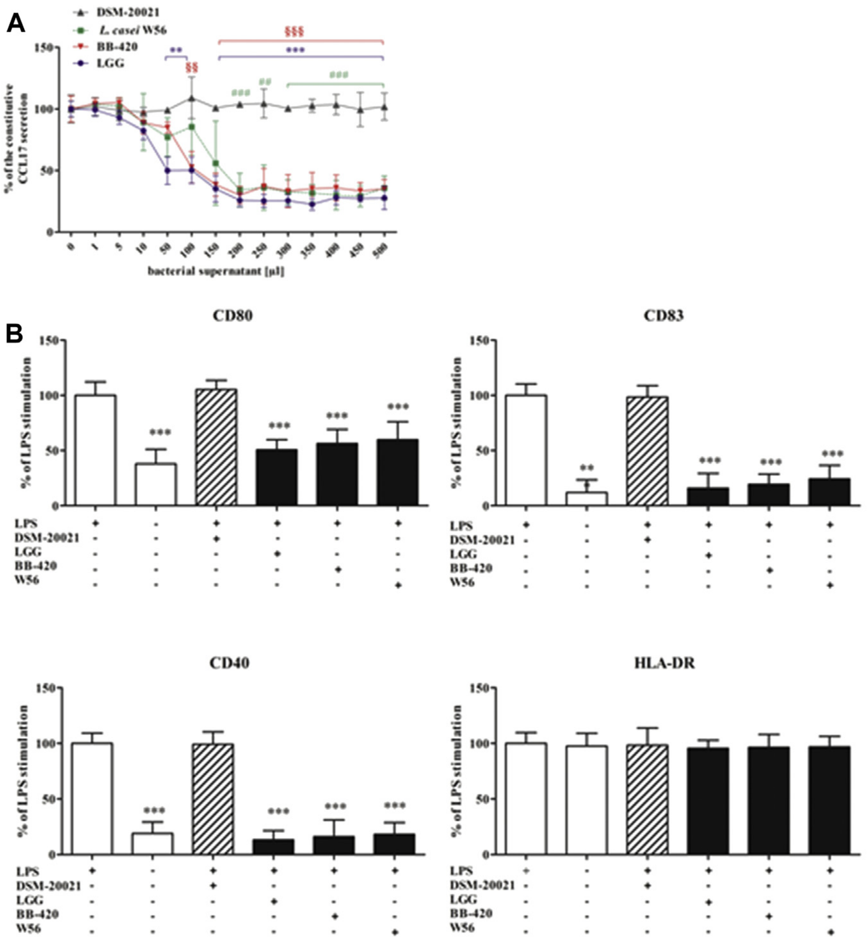

FIG 1. Screening of supernatants from different probiotic strains for immune activity on human cells. **A,** Dose-dependent capacity of bacterial supernatants from LGG *(fx1)*, *Bifidobacterium* BB-420 *(fx2)*, and *Lactobacillus casei* W56 *(fx3)* to lower CCL17 secretion of human Hodgkin lymphoma KM-H2 cells. The negative control was nonprobiotic *Lactobacillus rhamnosus* DSM-20021 *(fx4)*. Three independent experiments in duplicates are shown (mean ± SD percentages relative to CCL17 secretion of untreated KM-H2 cells). LGG: ***P* < .005 and ****P* < .0005, *L casei* W56: ##*P* < .005, ###*P* < .0005, BB-420: §§*P* < .005, §§§*P* < .0005, Student *t* test. **B,** Capacity of supernatants from LGG, *Bifidobacterium* BB-420, *Lactobacillus casei* W56, or nonprobiotic *Lactobacillus rhamnosus* DSM-20021 to prevent full upregulation of costimulatory molecules and HLA-DR on LPS-stimulated human monocyte-derived DCs. +/-, With/without bacterial supernatant. Five independent experiments are shown (mean ± SD percentages relative to LPS alone). ***P* < .01 and ****P* < .001, Dunn multiple comparison test.

differences between groups of samples; differences in OTU abundance between groups were tested for significance by means of nonparametric ANOVA.

RESULTS

Identification and characterization of a bioactive probiotic substance

Screening of crude probiotic supernatants for downregulation of CCL17. To develop a high-throughput screening system for the detection of T_H2-downregulatory activity in supernatants from probiotic bacteria, we made use of high constitutive secretion of the T_H2-associated CCL17 by the human Hodgkin lymphoma T-cell line KM-H2.

KM-H2 cells were incubated with increasing volumes of supernatants from *Lactobacillus rhamnosus* GG (LGG), *Bifidobacterium* BB-420, and *Lactobacillus casei* W56 to identify the threshold for downregulation of CCL17. Supernatants from all 3 probiotic strains led to a significant dose- and time-dependent reduction of CCL17 concentrations to approximately 30% relative to supernatant from the nonprobiotic *Lactobacillus rhamnosus* DSM-20021 (Fig 1, *A*). The minimum volume (200 µL) leading to that reduction was used in all subsequent experiments.

Because the numerous ingredients of the bacterial culture medium interfered with the detection of specific signals in mass spectrometry, the bacteria were cultivated in less complex, chemically defined medium (CDM1). The potency of supernatants from probiotic strains cultivated in CDM1 versus standard medium to decrease CCL17 concentrations was comparable (see Fig E1 in this article's Online Repository at www.jacionline.org). Subsequent testing of supernatants from 37 probiotic strains revealed that 7 of 21 *Lactobacillus* species strains, 5 of 10 *Bifidobacterium* species strains, and 1 of 3 *Lactococcus* species strains decreased CCL17 secretion without affecting cell viability (see Fig E2 in this article's Online Repository at

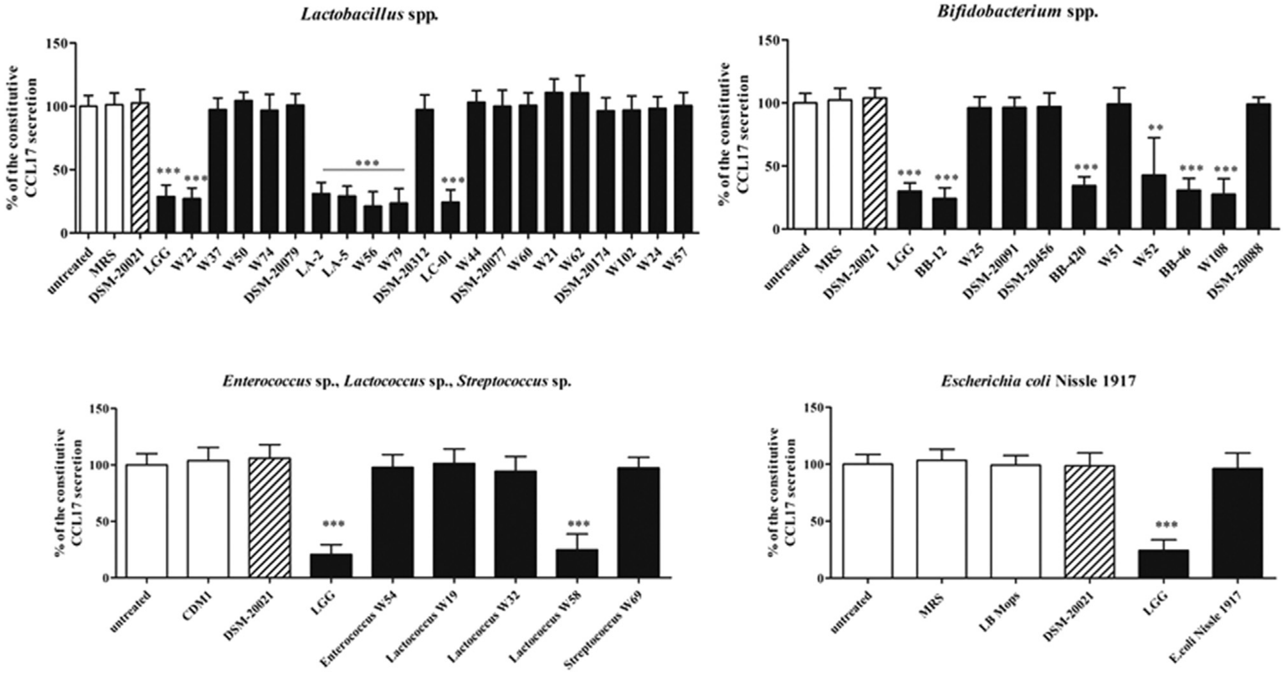

FIG 2. Overview on the ability of bacterial supernatants from all 37 strains to decrease CCL17 secretion of KM-H2 cells. *Shaded bars*, Nonprobiotic *Lactobacillus rhamnosus* DSM-20021 (negative control); LGG was included as a positive control in all experiments with strains other than lactobacilli. *Open bars*, Untreated KM-H2 cells and medium control cells. Three independent experiments in duplicates are shown (mean + SD percentages relative to CCL17 secretion of untreated KM-H2 cells). $^{**}P < .005$ and $^{***}P < .0005$, Student *t* test.

www.jacionline.org). In contrast, none of the *Streptococcus thermophilus*, *Enterococcus faecium*, or *E coli* Nissle 1917 strains influenced CCL17 levels (Fig 2 and see Table E1).

Verification of results from CCL17-based screening assays. To confirm the observed immunomodulatory activity, we evaluated the efficacy of probiotic supernatants to decrease the expression of costimulatory molecules on human monocyte-derived DCs. On recognition of antigen, naive DCs undergo a complex maturation process.[35] Although fully activated DCs induce adaptive immune responses, incomplete activation leads to tolerance.[36] Therefore we screened for reduced expression of costimulatory molecules in the presence of probiotic supernatants. All 13 supernatants that had already been preidentified as "immunomodulatory" in the CCL17-based screen also significantly decreased the percentages of LPS-induced CD83-, CD80-, CD86-, and CD40-expressing mature DCs, whereas the remaining supernatants were inactive on DCs (Fig 1, *B*). None of the supernatants affected the viability of DCs (see Fig E2). Thus both bioassays produced 100% concordant results. For a complete overview of the bioactivity of all strains, see Table E1.

Fractionation of selected probiotic supernatants yields 3 bioactive fractions of different polarity. LGG has been most frequently used in clinical studies.[37] Therefore we selected supernatants from LGG and further supernatants of *L casei* W56 for further enrichment and stepwise chemical characterization of the putative metabolite. During this procedure, each subfraction was retested for bioactivity in both the KM-H2 and DC bioassays.

Bacterial supernatants were subjected to semipreparative chromatography, yielding 11 MeOH/H_2O extracts. The highest immunomodulatory activity was found in the 20% fraction, along with slightly lower activities in the 40% and 50% MeOH fractions (Fig 3). Therefore we chose this fraction for further purification.

Isolation and identification of the bioactive substance in 20% MeOH/H_2O extracts. Chromatographic subfractionation of the 20% MeOH/H_2O fraction yielded 10 subfractions (see Fig E3 in this article's Online Repository at www.jacionline.org), 3 of which showed activity in bioassays. These subfractions and their closest neighbors were re-evaluated by means of reverse-phase, ultraperformance liquid chromatography, high-resolution time-of-flight mass spectrometry to generate total ion chromatograms. By identifying similarities in the chromatograms, we identified a substance that, according to peak retention time and molecular mass information, was only present in the bioactive subfractions, being highest in subfraction 7 from *L casei* W56 and subfraction 6 from LGG (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org). The extracted mass spectrum strongly suggested that this substance was composed of the tryptophan ions $(2M+H)^+$ and $(M+H)^+$ and its fragment, $(M+H\text{-}NH_3)^+$ (see Fig E4, *B*).

After careful enrichment of the bioactive substance by repeated chromatography runs, the isolated candidate substance of both strains showed bioactivity in both screening assays. High-resolution mass spectrometric analyses by using Fourier transform ion cyclotron resonance mass spectrometry confirmed $C_{11}H_{12}N_2O_2$ as the molecular formula of these ions (see Fig E4, *C* and *D*). Further analyses by using proton nuclear magnetic resonance provided detailed information on the functional group distribution and molecular structure: the doublets and triplets (δ7.8-7.0) showed the occurrence of an indole ring. Resonance signals at the region of δ3.9-3.8 and δ3.2-3.1 could also be

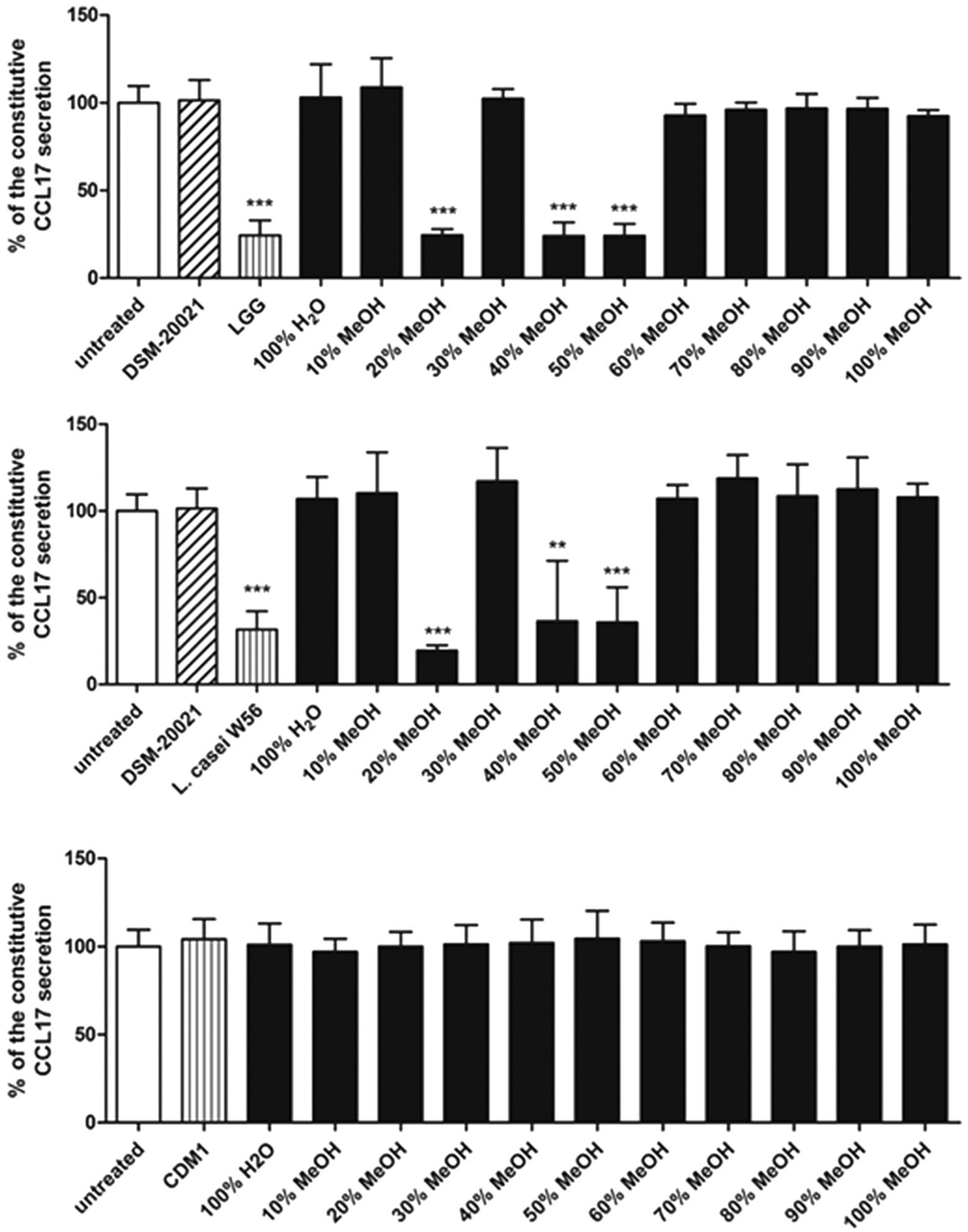

FIG 3. Capacity of subfractions of probiotic supernatants to decrease CCL17 secretion in KM-H2 cells. Subfractions with different polarity (MeOH/H_2O gradient chromatography) from supernatants of LGG *(top)* or *Lactobacillus casei* W56 *(middle)*. Negative controls were nonprobiotic DSM-20021 and blank CDM1 medium *(bottom)*. Three independent experiments in duplicates are shown (mean ± SD percentages relative to constitutive CCL17 secretion of untreated KM-H2 cells). $^{**}P < .005$ and $^{***}P < .0005$, Student *t* test.

assigned to β-CH and α-CH protons, respectively (see Fig E5 in this article's Online Repository at www.jacionline.org). Thus there was a close agreement between standard tryptophan and our bioactive subfraction.

Because L-tryptophan is a standard component of the bacterial growth medium, we hypothesized that the bioactivity is related to the D-form of this amino acid. Indeed, enantiomeric separation of the purified subfraction confirmed the presence of D- and L-tryptophan (see Fig E6, *A*, in this article's Online Repository at www.jacionline.org), whereas the corresponding subfraction of blank medium contained only L-tryptophan (see Fig E6, *B*).

Immunomodulatory activity in probiotic supernatants is restricted to the D-form of tryptophan. To verify whether bioactivity was indeed restricted to the D-isomer of tryptophan, we tested different concentrations of synthetic L- and D-tryptophan in the CCL17 bioassay. Only D-tryptophan showed dose-dependent immune activity (Fig 4). Moreover, none of 12 other polar and nonpolar neutral D-amino acids tested showed any bioactivity (Table I).

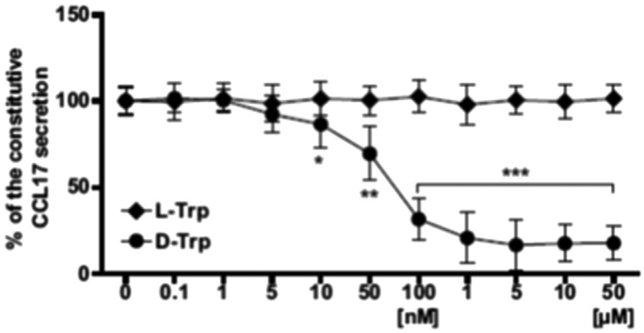

FIG 4. Effect of tryptophan L- and D-isomers on CCL17 secretion by KM-H2 cells. KM-H2 cells were stimulated with different concentrations of synthetic L- and D-isomers of tryptophan followed by CCL17 quantification in KM-H2 culture medium after 24 hours. *Circles*, D-tryptophan; *diamonds*, L-tryptophan. Three independent experiments in duplicates are shown (mean ± SD percentages relative to constitutive CCL17 secretion of untreated KM-H2 cells). *$P < .05$, **$P < .005$, and ***$P < .0005$, Student *t* test.

Bacterial supernatants and D-tryptophan modulate cytokine profiles of enriched human DCs. To obtain a first insight into mechanisms underlying this bioactivity, we quantified the cytokines secreted by highly enriched DCs (see Fig E2, *D*) after treatment with the bacterial supernatants or synthetic D-tryptophan. All probiotic supernatants and D-tryptophan strongly induced IL-10 and decreased LPS-induced IFN-g, IL-12, and IL-5 in these cultures. In contrast, cytokine patterns were unaffected by the control supernatants and amino acids (Table II). Overall, this resulted in increased IL-10/IL-12 ratios and, with the exception of *BB-46*, in decreased IL-5/IFN-g ratios.

Preclinical effects of oral D-tryptophan supplementation

D-tryptophan influences allergic airway inflammation and T_H2 immune responses. If it is to be used as an oral intervention in patients with allergic diseases, D-tryptophan needs to be absorbed from the gut. Oral supplementation of mice with 0.9 mg/d D-tryptophan increased D-tryptophan serum levels significantly (Fig 5, *A*), indicating enteric uptake and systemic distribution. Pretreatment of mice with D-tryptophan for 3 days and throughout experimental "asthma" induction decreased numbers of total bronchoalveolar lavage fluid cells, which was mainly caused by a reduction in eosinophil numbers (Fig 5, *B* and *C*). Furthermore, this supplementation improved airway hyperreactivity to methacholine (Fig 5, *D*). Because this suggested an involvement of T_H2 responses, we analyzed lung T cells: D-tryptophan reduced Il-4–producing T cells and Il-4 levels in bronchoalveolar lavage fluid (trend, Fig 5, *E* and *F*, and see Fig E7 in this article's Online Repository at www.jacionline.org for splenic cells) but not Ifn-g-producing T_H1 cells. Furthermore, D-tryptophan treatment significantly increased Helios-positive regulatory T (Treg) cell numbers, whereas total forkhead box p3 (Foxp3)$^+$ cell numbers remained unchanged (Fig 5, *G*).

To further substantiate these *in vivo* findings, we performed T-cell differentiation assays *in vitro*. In line with the *in vivo* observations, D-tryptophan reduced T_H2 cell differentiation, whereas T_H1 differentiation remained unaffected (Fig 6, *A* and *B*, and see Fig E8, *A*, in this article's Online Repository at www.jacionline.org). Consequently, *Il4* and *Gata3* expression and Il-13 secretion were reduced, whereas *Ifng* expression remained unaffected. However, Treg cells showed increased *Foxp3* expression on mRNA and protein levels (Fig 6, *C*, and see Fig E8, *B*).

D-tryptophan induces gut Treg cells and increases intestinal microbial diversity in allergic airway inflammation. In addition to the observed pulmonary immune response, the frequency of Foxp3$^+$ T cells was locally increased in the colons of supplemented mice with AAI compared with nonsupplemented mice with AAI (Fig 7, *A*). Altered gut immunity might be driven directly by D-tryptophan and/or indirectly through altered gut microbiota.

A diversity analysis of bacteria by 16S rRNA–based barcoding demonstrated a strongly reduced community richness and diversity at the level of OTU_{95} in mice with AAI (Fig 7, *B*). Supplementation with D-tryptophan increased the bacterial diversity of AAI mice, resulting in comparable α-diversity patterns compared with those of healthy animals. Although the original diversity was not completely restored after D-tryptophan application, its effect on microbial community composition was significant (see Fig E9, *A*, in this article's Online Repository at www.jacionline.org).

Independent of the health status of the animals' D-tryptophan supplementation, all samples were dominated by the phyla Bacteroidetes and Firmicutes (19.4% to 27.7% and 65.9% to 78.4% of the total sequences). As expected, the phylum Firmicutes mainly consisted of members of the order Clostridiales. Other phyla, including Actinobacteria and Proteobacteria, were also present, although at significantly lower abundance. At the family level, Lachnospiraceae, Odoribacteraceae, Rikenellaceae, Ruminococcaceae, S24-7, and an unclassified bacterial family belonging to the Clostridiales (see Fig E9, *B*) dominated. The latter was mainly present in mice with AAI, forming 58.6% of the total community. However, Lachnospiraceae were less abundant in animals with AAI (5.5%) compared with control animals (13.7%), D-tryptophan–treated mice with AAI (20.6%), or D-tryptophan–treated mice without AAI (27.5%). Odoribacteraceae were strongly affected by D-tryptophan because their relative abundance tripled in both groups of supplemented animals (3.9% vs approximately 1.1%). In contrast, Rikenellaceae showed a decreased abundance in the D-tryptophan groups (1.1% to 2.0%) compared with the control groups (4.6% to 7.7%). Interestingly, Ruminococcaceae, which were strongly reduced in the control mice affected with AAI (3.7%) recovered through application of D-tryptophan (8.9%): this was comparable with abundance in the control group of mice without AAI. Members of the S24-7 family were affected by neither AAI nor application of D-tryptophan. Overall, D-tryptophan supplementation increased intestinal bacterial diversity in D-tryptophan–treated mice with AAI, such that the bacterial diversity pattern was more comparable with healthy control mice (PBS/PBS; Fig 7, *B*). Thus our results suggested that D-tryptophan treatment re-establishes a healthy microbial community genotype in mice with AAI.

DISCUSSION

In the present work, for the first time, we identified D-tryptophan as a bacterial substance produced by the probiotic strains LGG and *L casei* W56. We demonstrate that D-tryptophan decreases the production of T_H2 cytokines and chemokines in

TABLE I. Percentage of surface marker–expressing mature DCs treated with synthetic D-amino acids*

	D-alanine	D-histidine	D-isoleucine	D-leucine	D-methionine	D-phenylalanine
CD83	97.7 ± 2.3	103.1 ± 0.3	100.5 ± 1.1	97.1 ± 2.4	102.4 ± 2.2	99.6 ± 2.0
CD86	99.2 ± 2.1	102.5 ± 0.4	99.8 ± 1.2	101.9 ± 1.3	102.2 ± 2.5	99.2 ± 3.3
CD80	98.3 ± 2.6	102.0 ± 0.9	98.2 ± 1.5	100.3 ± 1.6	100.4 ± 0.2	92.4 ± 3.5
CD40	102.3 ± 3.4	101.4 ± 3.2	100.4 ± 2.4	100.4 ± 1.7	102.7 ± 0.6	100.4 ± 2.7
HLA-DR	98.1 ± 1.1	99.9 ± 0.9	100.1 ± 0.3	98.0 ± 10.0	98.9 ± 2.0	98.0 ± 3.2

	D-proline	D-serine	D-threonine	D-tryptophan	D-tyrosine	D-valine
CD83	100.9 ± 0.4	100.8 ± 0.3	102.6 ± 0.5	7.6 ± 3.3	101.6 ± 0.6	102.1 ± 1.2
CD86	101.2 ± 1.9	101.1 ± 2.8	102.1 ± 0.7	24.1 ± 2.7	102.2 ± 0.9	101.8 ± 0.9
CD80	100.3 ± 0.1	100.1 ± 2.8	100.8 ± 0.4	12.1 ± 1.7	101.6 ± 0.4	99.6 ± 2.0
CD40	99.2 ± 1.3	100.8 ± 1.1	100.6 ± 1.5	15.2 ± 6.5	100.4 ± 1.3	101.4 ± 2.4
HLA-DR	98.4 ± 2.6	98.6 ± 0.6	97.1 ± 3.9	88.9 ± 3.0	98.4 ± 2.4	100.0 ± 1.0

Three independent experiments are shown (mean ± SD percentages relative to LPS-induced expression).

*DCs were stimulated with LPS (0.1 μg/mL) in the presence of the indicated D-amino acids (10 μmol/L). Percentages of CD83-, CD86-, CD80-, or CD40-expressing DCs were assessed.

TABLE II. Cytokine regulation by probiotic supernatants or D/L-tryptophan in human LPS-treated DCs*

	IL-10 (pg/mL)		IL-5 (pg/mL)		IFN-g (pg/mL)		IL-12 (pg/mL)		Ratios			
									IL-10/IL-12		IL-5/IFN-g	
	-	LPS	-	LPS	-	LPS	-	LPS	-	LPS	-	LPS
Medium	3.20	2.90	14.70	68.30	112.50	2238.80	102.10	2092.80	0.031	0.001	0.131	0.031
DSM-20021	6.80	4.80	33.60	55.90	330.00	2520.50	447.80	2217.30	0.015	0.002	0.102	0.022
LGG	432.90	787.90	9.10	5.40	372.70	105.70	79.20	106.90	5.466	7.370	0.024	0.051
LA-2	107.30	591.70	8.00	10.30	111.60	437.70	89.30	238.00	1.202	2.486	0.072	0.024
LA-5	81.30	305.70	7.60	8.00	113.30	531.80	87.50	331.10	0.929	0.923	0.067	0.015
LC-01	452.40	924.50	7.90	2.40	109.30	211.30	76.90	67.80	5.883	13.636	0.072	0.011
BB-12	234.90	735.70	11.00	10.90	75.40	437.00	91.50	228.20	2.567	3.224	0.146	0.025
BB-46	813.50	1230.70	14.00	13.60	13.50	637.90	95.10	202.30	8.554	6.084	1.037	0.021
BB-420	450.40	915.40	8.80	8.40	81.50	783.70	102.50	356.90	4.394	2.565	0.108	0.011
L-Tryptophan	5.70	4.90	12.00	61.40	45.00	2031.50	88.30	1993.00	0.065	0.002	0.267	0.030
D-Tryptophan	56.90	202.50	10.30	20.60	21.90	1129.50	82.50	871.90	0.690	0.232	0.470	0.018
L-Proline	†	6.00	14.80	57.70	88.90	2133.90	99.80	1938.00	†	0.003	0.166	0.027
D-Proline	5.90	4.00	15.80	69.10	92.60	2295.40	90.60	1911.90	0.065	0.002	0.171	0.030

*DCs were stimulated in the presence or absence of LPS (0.1 μg/mL) with supernatants from 200 μL of bacterial cell-free supernatants or tryptophan enantiomers (10 μmol/L) for 14 hours. Nonprobiotic DSM-20021 and blank medium (CDM1) were used as negative controls. D/L-proline and L-Tryptophan were used as controls for D-tryptophan.

†Less than the detection limit.

human peripheral and murine immune cells and, more importantly, prevents full development of AAI when fed to mice. Aside from immune modulation, this can occur also through maintenance of a diverse gut microbiota, which was otherwise lost in animals with experimental asthma.

Probiotic bacteria have been shown to modify immune responses *in vitro*[3,4] and in animal studies,[5,6] but clear evidence for clinical efficacy in the treatment of chronic inflammatory disorders is largely lacking. Because the reciprocal interaction of probiotic bacteria with the host's microbiota and immune system is extremely complex, use of defined small substances with a predictable mode of action might provide an interesting alternative for prevention of allergic disease in subjects at risk.

D-amino acids are nonproteinogenic enantiomers of L-amino acids. Until the discovery of free D-aspartate and D-serine in the mammalian brain as neurotransmitters in the late 1980s, D-amino acids were considered to play no role in higher organisms. Thus far, research on D-amino acids in mammals has been mainly restricted to the nervous system because of the relative abundance of D-aspartate and D-serine in the brain[38] and the difficulty of detecting D-amino acids at trace levels.[39] Thus very little is known on D-tryptophan uptake[40] and metabolism in human subjects,[41] and it has been assumed that higher organisms use D-tryptophan poorly.[42] By developing highly sensitive assays, we demonstrated systemic distribution of D-tryptophan in mice after oral uptake.

In contrast to higher organisms, numerous bacteria, including probiotic bacteria, produce D-amino acids, such as D-glutamate and D-alanine, by using them mainly for cross-linking glycan chains in the peptidoglycan wall.[43,44]

The regulation of bacterial L-tryptophan biosynthesis and degradation is well known.[45] A role for D-tryptophan in bacterial communication was only recently discovered by demonstrating its requirement for disassembly of biofilms in *Bacillus subtilis*.[46] Other soluble substances produced by probiotic bacteria are less investigated thus far.[4,47]

Human subjects are potentially exposed to microbially generated D-amino acids[48] because body surfaces and the environment harbor an abundant and high diversity of microbes.[49] Similar to what has already been shown for

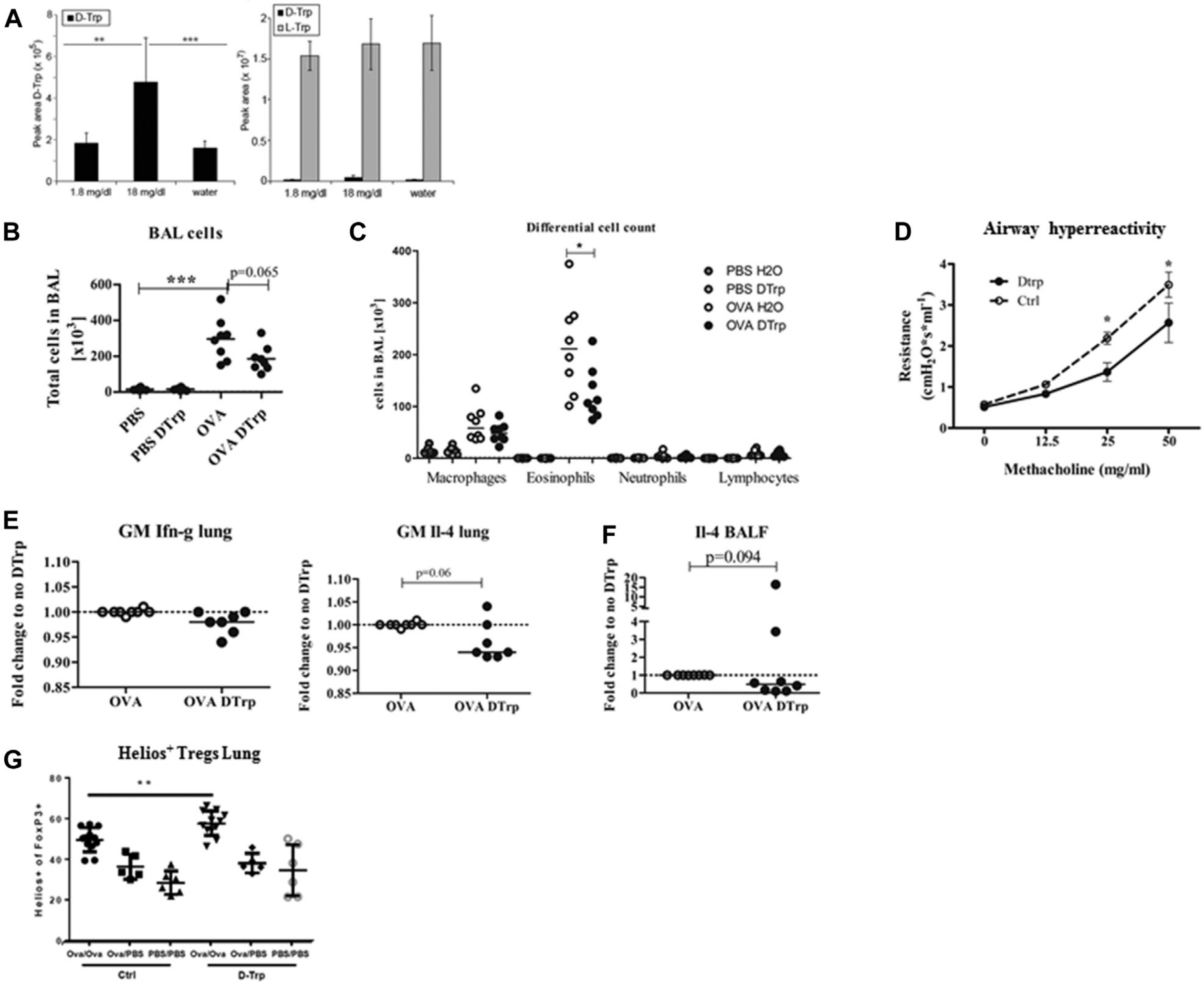

FIG 5. Oral D-tryptophan reduces allergic airway inflammation. **A,** Serum D-tryptophan in mice receiving D-tryptophan (50 mmol/L) in drinking water or water only (ultraperformance liquid chromatography mass spectrometry peak areas). Note the different scales for D-tryptophan *(solid bars)* and L-tryptophan *(shaded bars)*. $**P = .006$ and $***P = .004$, Welch Test, mean ± SD. **B,** Total number of cells in bronchoalveolar lavage fluid *(BAL)*. **C,** Differential cell count. **D,** Measurement of airway resistance to increasing doses of methacholine (2-way ANOVA with the Bonferroni posttest). **E,** Geometric mean (fold change) of Ifn-g and Il-4 in lung-derived $CD3^+CD4^+$ lymphocytes. **F,** Il-4 levels in bronchoalveolar lavage of mice, as assessed by using a Cytometric Bead Array. **G,** Helios-positive Treg cells of lung-derived $CD3^+CD4^+Foxp3^+$ lymphocytes. Student *t* test: $*P < .05$, $**P < .01$, and $***P < .001$. Fig 5, *B, C, E* and *F,* n = 8 mice per group, Mann-Whitney *U* test, median ± SD, $*P < .05$, $***P < .001$, and Fig 5, *D* and *G,* n = 6 to 12 mice per group.

acyl-homoserine lactones from gram-negative bacteria,[50-53] means to recognize and interact with bacterial D-amino acids, including D-tryptophan, could have evolved.

This hypothesis is supported by several observations. First, human cells used in our bioassays responded to D-tryptophan but to neither L-Tryptophan nor any other tested D-amino acid.

Second, at least 2 surface receptors for D-tryptophan exist in human subjects: the G protein–coupled receptor GPR109B[54] is expressed on macrophages, monocytes, adipose tissue, and lung[55] and mediates attraction of neutrophils on binding of D-tryptophan or its metabolite, D-Kynurenine. Of note, when we extracted and analyzed published transcriptomic data,[56] GPR109B was significantly decreased in airway epithelial cells and T cells from patients with asthma as opposed to control subjects, indicating a potential role for this receptor in allergic disease (see Table E2 in this article's Online Repository at www.jacionline.org).

The second receptor, solute carrier family 6 amino acid transporter member 14 (SLC6A14, alias $ATB^{0,+}$), transports D-tryptophan and 4 other D-amino acids across epithelial cells.[57] Because the receptor is expressed in the intestine, SLC6A14 is exposed to high microbial load and diversity. SLC6A14 is further expressed at exceptionally high levels in the fetal lung (based on our own data [see Fig E10 in this article's Online Repository at www.jacionline.org] and those of Su et al[58]). The physiologic role of SLC6A14 in fetal life is unknown thus far. However, it is tempting to speculate a mechanistic link for prenatal intervention trials using probiotic bacteria.

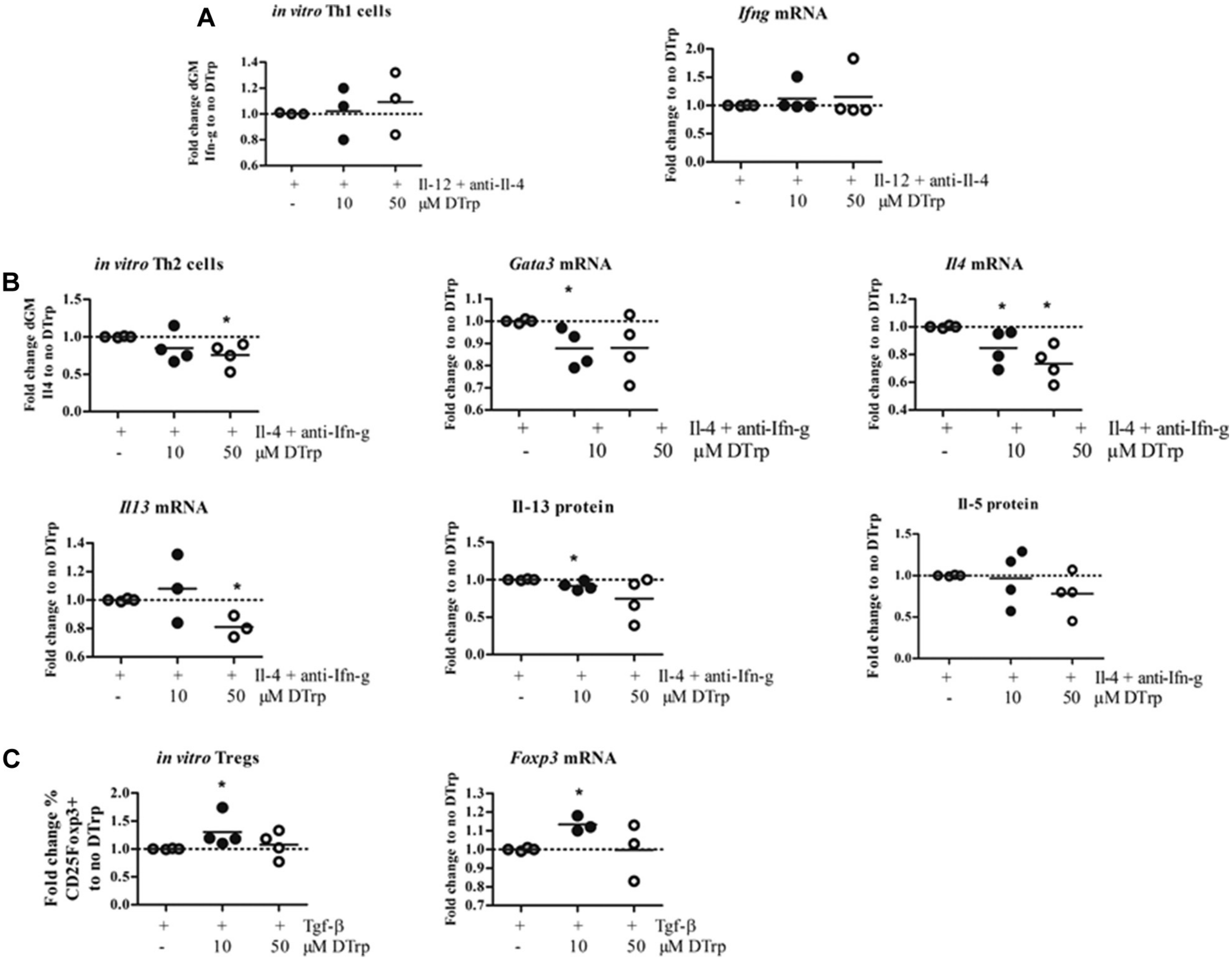

FIG 6. D-tryptophan *(DTrp)* influences *in vitro* primary T-cell differentiation. Primary murine splenocytes were differentiated toward $\mathrm{T_H}1$ **(A)**, $\mathrm{T_H}2$ **(B)**, and Treg **(C)** cells with respective cytokine mixes in the presence of 0, 10, or 50 µmol/L D-tryptophan (dissolved in water). Differentiation was assessed by means of flow cytometry, quantitative RT-PCR, and the Cytometric Bead Array for Il-13 and Il-5 protein levels from culture supernatants. Graphs depict fold changes to differentiated cells not treated with D-tryptophan. $^*P < .05$, n = 3 to 4 independent experiments, Mann-Whitney *U* test.

Three enzymes, tryptophan 2,3-dioxygenase (TDO), indoleamine 2,3-dioxygenase (IDO) 1, and the more recently discovered IDO2, can metabolize tryptophan. Although tryptophan 2,3-dioxygenase is specific for L-tryptophan, IDO1 channels both D- and L-tryptophan into the kynurenine pathway. IDO activation leads to tryptophan depletion and thereby promotes peripheral tolerance,[59] which contrasts our findings. However, IDO1 seems not to be important for the induction of immune tolerance in the airways but instead promotes $\mathrm{T_H}2$ responses through effects on lung DCs,[60] which we suggest could be counteracted by D-tryptophan. In addition, IDO2, which is also expressed on DCs[61] and has a slightly different substrate specificity, could further modulate D-tryptophan metabolism.[62]

Thus far, we concentrated on the 20% MeOH fraction for identification of the putative substance because this was the subfraction with the highest immunomodulatory activity and polarity. Bioactivity was further detected in the 40% and 50% MeOH fractions, holding the potential for the discovery of further small immunoactive substances. Our bioassays were designed to detect substances that induce a tolerogenic profile in DCs and decrease levels of the allergy-related chemokine CCL17. Therefore it is possible that further immunoregulatory substances not related to allergic disease were overlooked.

D-tryptophan could influence immune homeostasis either directly, as shown in our screening assays, or indirectly by shifting the structure of the microbiome of the host. Apart from the observed immunomodulatory properties of D-tryptophan, we do not have direct mechanistic links explaining the altered gut microbiota or protection from AAI. However, in line with our own findings, Trompette et al[63] demonstrated that a change in the gut microbiota caused by dietary fermentable fibers induces production of metabolites involved in protection from AAI. These metabolites have further been associated with increased frequencies of Foxp3$^+$ Treg cells.[64] The lung microbiota and a population of Foxp3$^+$ Treg cells have further been shown to protect neonatal mice from exaggerated type 2 immune responses in a murine model of house dust mice–induced AAI,[65] which supports a role of both immune parameters also in adult mice.

In summary, for the first time, we identified that D-tryptophan acts as an immunomodulatory substance produced by probiotic

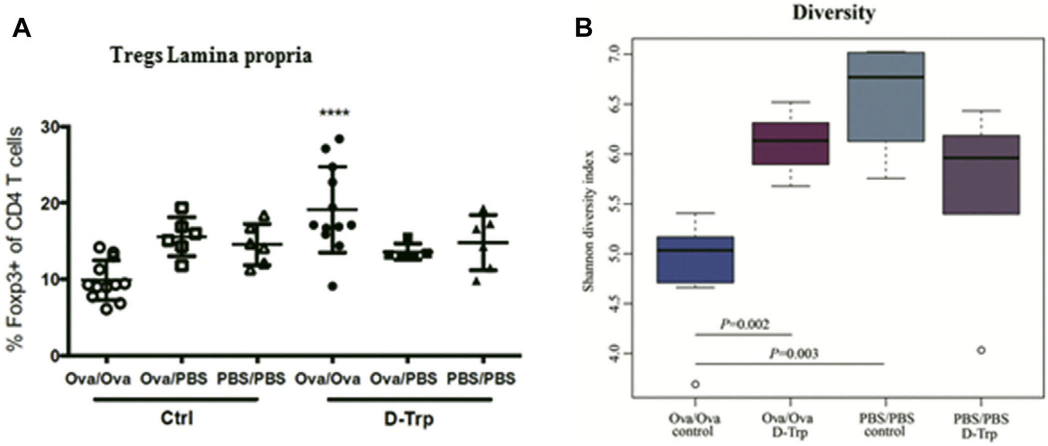

FIG 7. Oral D-tryptophan *(DTrp)* supplementation increased gut Treg cell numbers and the intestinal bacterial community in mice with AAI. **A,** Percentage of Foxp3$^+$ cells within CD3$^+$CD4$^+$ T cells in the lamina propria of the colon. *****P* < .0001, n = 6 to 12 mice per group, Student *t* test. **B,** α-Diversity of bacterial communities. Shannon diversity index was used to estimate bacterial diversity for each treatment (Wilcoxon rank sum test).

strains. Our results suggest that tryptophan can potentially influence both immune responses and the constituents of intestinal microbiota and can conceivably reduce the degree of hyperactivity severity of AAI. In addition to immune modulation, this can occur through the maintenance of a diverse gut microbiota, which was otherwise lost in animals with AAI.

We conclude that bacteria-derived D-tryptophan can play a wider role in human health than previously thought. Overall, our findings support the concept that defined bacterial products can provide the basis for future development of preventive strategies for chronic inflammatory disorders.

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Key messages

- **D-tryptophan is a newly identified immunomodulatory probiotic substance.**
- **When fed to mice, D-tryptophan increased the gut microbial diversity and ameliorated AAI.**
- **Although the biology of live probiotic bacteria is very complex, D-tryptophan has a provable mode of action that might be exploited for prevention or treatment of allergic diseases.**

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