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

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Abstract (250/250 words)

Background Chronic immune diseases (CIDs), such as asthma, are highly prevalent. Currently available pharmaceuticals improve symptoms, but cannot address the underlying pathologies. This prompted demands for alternatives to pharmaceuticals such as probiotics for prevention of allergic disease. However, clinical trials have given 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. 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 lower 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 two supernatants according to polarity, followed by total ion chromatograms and mass spectrometry, yielded $C_{11}H_{12}N_2O_2$ as molecular formula of a bioactive substance. Proton nuclear magnetic resonance and enantiomeric separation identified D-tryptophan. In contrast, L-tryptophan and eleven other D-amino acids were inactive.

Feeding D-tryptophan to mice prior to experimental asthma induction, increased numbers of gut foxp3+ T cells, lowered systemic Th2 responses, and ameliorated allergic airway inflammation and hyperresponsiveness. Allergic airway inflammation reduced the gut microbial diversity, which was restored by D-tryptophan.

Conclusions. D-tryptophan is a newly identified product from probiotic bacteria. Our findings support the concept that defined bacterial products might be exploited in novel preventative strategies for CIDs.

Key messages

- D-tryptophan is a newly identified immune modulatory probiotic substance
- When fed to mice, D-tryptophan restores the gut microbial diversity and ameliorates allergic airway disease
- While 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.

Capsule summary

The complexity of the host-bacterial crosstalk questions therapeutic approaches with live bacteria. By showing that the probiotic substance D-tryptophan improves airway inflammation, we provide proof-of-principle that defined microbial products might be exploited for therapeutic purposes.

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

List of abbreviations

AAI	Allergic airway disease
BALF	Bronchoalveolar lavage fluid
CCL17	Chemokine ligand 17 (TARC: thymus and activation regulated chemokine)
FT-ICR	Fourier transform ion cyclotron resonance
LGG	*Lactobacillus rhamnosus GG*
MS	Mass spectrometry
PFP	Pentafluorophenyl
TIC	Total Ion Chromatograms

98 UPLC Ultra Performance Liquid Chromatography

99

Introduction

Chronic immune diseases such as allergies, inflammatory bowel disease or diabetes are highly prevalent in industrialized countries and a further increase of burden due to non-communicable diseases is expected for the next decades (1). Although symptoms of chronic immune diseases are treatable, the underlying pathologies remain incurable. Accordingly, there is an increasing demand for proven alternatives to pharmaceutical products from both healthcare 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 diarrhoea (9), clinical trials for different indications such as primary prevention of allergic diseases (e.g. (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 individuals, the gut microbiome differs remarkably among individuals (26,27). In addition, the microbiome as well as the immunity can be substantially altered in 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 mechanistic understanding which is important to establish biological plausibility for any claimed health effect.

To overcome these problems, the utilization of specified substances derived from probiotic microbes could provide an attractive alternative. Other than living bacteria with complex fates and response patterns in the host, they should have definable properties with provable mode

126 of action. So far, only very few candidate structures or substances have been demonstrated as
127 bioactive agents and even less with preclinical evidence for therapeutic effects (29).
128 Therefore, the aim of the present study was 1) to establish a screening tool for the detection of
129 Th2-lowering immune activity in probiotic supernatants, 2) to identify a soluble bacterial
130 molecule that mediates this activity, 3) to test the putative substance in a mouse model of
131 allergic airway disease (AAI) and 4) to obtain insight into potential underlying mechanisms.

Materials and 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, cyto/chemokine quantification, flow cytometry, microbiota analysis, isolation of intestinal lamina propria cells and animal experiments (induction of experimental asthma, lung function analyses) see Methods section in this article's Online Repository at www.jacionline.org.

Bacterial strains

Bifidobacteriae, Lactobacilli, Lactococci, E. Coli Nissle 1917, Enterococcus faecium, and *Streptococcus thermophilus* were obtained from different providers (Table E1) All strains were grown until stationary phase and a minimum cell number of 10^8 CFU/ml. Cell free supernatants were obtained by centrifugation (6000 rpm; 5 min; 20° C) followed by filtration through 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 immune modulatory activity in probiotic supernatants

Two biological assays based on down-modulation 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 0.1µg/mL lipopolysaccharide (LPS) from *E. coli* (Sigma-Aldrich, St. Louis, USA) in the presence or absence of 200 µL bacteria free supernatants for 24 h followed by flow cytometric analysis of costimulatory molecules. Similarly, 200 µL supernatants were added to 3-5 x 10^6 KM-H2 cells for 24 h. Supernatants were collected from KM-H2 by centrifugation and stored at -80 °C until quantification of CCL17. To control for the dilution of KM-H2 culture media with different volumes of

159 bacterial supernatants, the corresponding amount of blank MRS medium was added. Blank
160 bacterial growth medium and supernatants from *Lactobacillus rhamnosus* DSM 20021, which
161 has no probiotic activity, were used as negative controls in both screening assays.
162
163 **Animals and oral supplementation with D-tryptophan**
164 All animal experiments were conducted under the Federal Guidelines for the Use and Care of
165 Laboratory Animals (Az 55.2-1-54-2532-137-13) and was approved by the Government of the
166 District of Upper Bavaria. Female Balb/c mice from 6-8 weeks old were obtained from
167 Charles River (Sulzfeld, Germany) and housed in individually ventilated cages with two mice
168 each in specific pathogen free conditions. A standard extruded pellet diet and sterile filtered
169 drinking water were provided *ad libitum*. For quantification of D-tryptophan in mouse sera,
170 D-tryptophan (Sigma-Aldrich, St. Louis, USA) was dissolved in the drinking water at
171 concentrations of 1.8 mg/dL or 18 mg/dl (approximately 0.09 and 0.9 mg/day per mouse).
172 Control animals received pure water (n=8 per group). No changes in behavior or body weight
173 were noted in the supplemented animals compared to controls. Animals were sacrificed after
174 14 days and sera were immediately stored at -80 °C until analysis.
175 For testing prevention of AAI, mice received 50 mM D-tryptophan starting at least three days
176 before the first sensitization until sacrificing on day 25. For microbiome analyses, the caecum
177 was cut off and immediately stored at -80 °C until further processing.
178
179 **Statistical analyses**
180 ***Bioassays and animal experiments***
181 Results of bioassays and animal experiments are given as means with standard deviation.
182 Student´s t test with Dunn´s Multiple Comparison Test or Two-way ANOVA with Bonferroni
183 post test were used where appropriate. Tests applied are given in the respective figure legends.

184 P values ≤ 0.05 were considered significant. (GraphPad Prism Software version 5.0, Inc. La
185 Jolla, CA 92037 USA).
186
187 ***Microbial diversity***
188 Electropherogram evaluation was performed using Peak Scanner Software v1.0 (Applied
189 Biosystems). T-RFLP data were imported into T-REX (30) and a data matrix based on peak
190 height was generated. Peak sizes smaller than 50bp were excluded from analysis; alignments
191 were done using a clustering threshold of 2 bp. Fragments with relative abundance of less
192 than 1% were considered as background noise. The resulting data matrix was exported for
193 principal component analysis (PCA) within the R software environment (www.R-project.org),
194 using the function PCA of the R-package vegan. Data analysis was repeated with binarized T-
195 RFLP data (presence/absence of peaks).

196 **Results**

197 **I. Identification and characterization of a bioactive probiotic substance**

198 **Screening of crude probiotic supernatants for downregulation of CCL17**

199 To develop a high-throughput screening system for the detection of Th2-downregulatory
200 activity in supernatants from probiotic bacteria, we made use of the high constitutive secretion
201 of the Th2-associated CCL17 by the human Hodgkin Lymphoma T cell line KM-H2.

202 To identify the threshold for downregulation of CCL17, KM-H2 cells were incubated with
203 increasing volumes of supernatants from *Lactobacilus rhamnosus GG (L. rhamnosus GG),*
204 *Bifidobacterium* BB-420 and *Lactobacillus casei* W56. Supernatants from all three probiotic
205 strains led to a significant dose- and time dependent reduction of CCL17 concentrations to
206 ~30% relative to supernatant from the non-probiotic *Lactobacillus rhamnosus* DSM-20021
207 (**Fig 1*A***). The minimum volume (200µl) leading to that reduction was used in all subsequent
208 experiments.

209 As the numerous ingredients of the bacterial culture medium interfered with the detection of
210 specific signals in mass spectrometry, the bacteria were cultivated in less complex medium
211 (CDM1). The potency of supernatants from probiotic strains cultivated in CDM1 versus
212 standard medium to lower CCL17 concentrations was comparable (**Fig E1*A*, *B***). Subsequent
213 testing of supernatants from 37 probiotic strains revealed that 7 of 21 *Lactobacillus* sp.
214 strains, 5 of 10 *Bifidobacterium* sp. strains, and 1 of 3 *Lactococcus* sp. strains lowered CCL17
215 secretion. In contrast, none of the *Streptococcus thermophilus*, *Enterococcus faecium* or *E.*
216 *coli Nissle 1917* strains influenced CCL17 levels (**Fig 2**; **Table E1**).

217

218 **Verification of results from CCL17-based screening assays**

219 To confirm the observed immune modulatory activity, we evaluated the efficacy of the
220 probiotic supernatants to lower the expression of co-stimulatory molecules on human
221 monocyte derived dendritic cells (DC). Upon recognition of antigen, naïve DCs undergo a

222 complex maturation process (31). While fully activated DCs induce adaptive immune
223 responses, incomplete activation leads to tolerance (32). Therefore, we screened for reduced
224 expression of co-stimulatory molecules in the presence of probiotic supernatants. All 13
225 supernatants that had already been pre-identified as "immune modulatory" in the CCL17-
226 based screen also significantly decreased the percentages of LPS-induced CD83, CD80, CD86
227 and CD40 expressing mature DCs, whereas the remaining supernatants were inactive on DCs.
228 Thus, both bioassays gave 100% concordant results. (**Fig 1*B***). For a complete overview of the
229 bioactivity of all strains see table E1.
230
231 **Fractionation of selected probiotic supernatants yields three bioactive fractions of**
232 **different polarity**
233 *L. rhamnosus GG* has been most frequently used in clinical studies (33). Therefore, we
234 selected supernatants from *L. rhamnosus GG* and further of *L. casei* W56 for further
235 enrichment and stepwise chemical characterization of the putative metabolite. During this
236 procedure, each sub-fraction was retested for bioactivity in both the KMH2 and DC bioassays.
237 Bacterial supernatants were subjected to semi-preparative chromatography yielding 11
238 MeOH/H_20 extracts. The highest immune modulatory activity was found in the 20% fraction
239 along with slightly lower activities in the 40% and 50% MeOH fractions (**Fig 3**). Therefore,
240 we chose this fraction for further purification. Chromatographic sub-fractionation yielded ten
241 sub-fractions, three of which showed activity in the bioassays.
242
243 **Isolation and identification of the bioactive substance in 20% MeOH/H_20 extracts**
244 Chromatographic sub-fractionation of the 20% MeOH/H_20 fraction yielded ten sub-fractions,
245 three of which showed activity in the bioassays (**Fig E2*A, B***). These sub-fractions and their
246 closest neighbours were re-evaluated via reversed phase UPLC-High Resolution TOF MS to
247 generate Total Ion Chromatograms. By identifying similarities in the chromatograms, we

248 identified a substance that, according to peak retention time and molecular mass information,
249 was only present in the bioactive sub-fractions, being highest in sub-fraction 7 from *L. casei*
250 W56 and sub-fraction 6 from *L. rhamnosus GG* (**Fig E3*A***). The extracted mass spectrum
251 strongly suggested that this substance was composed of the tryptophan ions $[2M+H]^+$,
252 $[M+H]^+$ and its fragment $[M+H-NH_3]^+$ (**Fig E3*B***).
253 After careful enrichment of the bioactive substance by repeated chromatography runs, the
254 isolated candidate substance of both strains showed bioactivity in both screening assays. High
255 resolution mass spectrometry analyses by FT-ICR-MS confirmed $C_{11}H_{12}N_2O_2$ as the
256 molecular formula of these ions (**Fig E3*C, D***). Further analyses by proton NMR provided
257 detailed information on the functional group distribution and molecular structure: The
258 doublets and triplets (δ7.8-7.0) showed the occurrence of an idole ring. Resonance signals at
259 the region of δ3.9-3.8 and δ3.2-3.1 could also be assigned to β-CH and α-CH protons,
260 respectively (**Fig E4**). Thus, there was a close agreement between standard tryptophan and
261 our bioactive sub-fraction.
262 Since L-tryptophan is a standard component of the bacterial growth medium, we hypothesized
263 that the bioactivity is related to the D-form of this amino acid. Indeed, enantiomeric
264 separation of the purified sub-fraction çonfirmed the presence of D- and L-tryptophan (**Fig**
265 **E5*A***), while the corresponding sub-fraction of blank medium contained solely the L-form
266 (**Fig E5*B***).
267

268 **The immune modulatory activity in probiotic supernatants is restricted to the D-form of**
269 **tryptophan**

270 To verify if the bioactivity was indeed restricted to the D-isomer of tryptophan, we tested
271 different concentrations of synthetic L- and D-tryptophan in the CCL17 bioassay. Only D-
272 tryptophan showed a dose dependent immune activity (**Fig 4**). Moreover, none of twelve other
273 polar and non-polar neutral D-amino acids tested showed any bioactivity (**Table I**).

274

275 **Bacterial supernatants and D-tryptophan modulate cytokine profiles of human DC**

276 To obtain a first insight into mechanisms underlying this bioactivity, we quantified the
277 cytokines secreted by DCs after treatment with the bacterial supernatants or synthetic D-
278 tryptophan. All probiotic supernatants and D-tryptophan strongly induced IL-10 and lowered
279 LPS-induced IFN-γ, IL-12 and IL-5. In contrast, cytokine patterns were unaffected by the
280 control supernatants, and amino acids (**Table II**). Overall this resulted in increased IL-10/IL-
281 12 ratios and – with the exception of *BB-46* - in decreased IL-5/IFN-γ ratios.

282

283 **II. Preclinical effects of oral D-tryptophan supplementation**

284 **D-tryptophan influences allergic airway inflammation and Th2 immune responses**

285 If it is to be used as an oral intervention in allergic diseases, D-tryptophan needs to be
286 absorbed from the gut. Oral supplementation of mice with 0.9 mg/day D-tryptophan increased
287 D-tryptophan serum levels significantly (**Fig 5*A***) indicating enteric uptake and systemic
288 distribution. Pre-treatment of mice with D-tryptophan for 3 days and throughout experimental
289 "asthma" induction lowered total BALF cells, which was mainly caused by a reduction of
290 eosinophils (**Fig 5*B, C***). Furthermore, the supplementation improved airway hyperreactivity
291 to methacholine (**Fig 5*D***). As this suggested an involvement ofTh2 responses, we analysed
292 splenic T cells: D-tryptophan significantly reduced splenic Il-13 with a numerical reduction of
293 Il-4 producing $CD3^+CD4^+$T cells, (**Fig 5*E***) while IFNγ, $Foxp3^+$ and $Il\text{-}10^+$ T cells remained
294 unchanged. In addition, percentages of $CD40^+$ and $CD80^+$ DCs were reduced by half (**Fig 5*F*,**
295 all experimental groups in Fig E6). The frequency of $Foxp3^+$ T cells in the colon was
296 increased in supplemented AAI mice compared to non-supplemented AAI mice, indicating a
297 local immune response.

298

D-tryptophan induces gut Tregs and restores intestinal microbial diversity in allergic airway inflammation

In addition to the observed systemic immune response, the frequency of $Foxp3^+$ T cells was locally increased in in the colon of supplemented AAI mice compared to non-supplemented AAI mice (**Fig 6*A***). Altered gut immunity might be driven directly by D-tryptophan and/or indirectly via altered gut microbiota.

A diversity analysis of bacteria by 16S rRNA gene fingerprinting, demonstrated a significantly reduced bacterial diversity in mice with AAI compared to mice without AAI. Overall, D-tryptophan supplementation increased intestinal bacterial diversity in AAI, D-tryptophan treated mice, such that the bacterial diversity pattern was comparable to 'healthy' control mice (PBS/PBS). (**Fig 6*B***). Thus, our results suggested that D-tryptophan treatment reestablishes a "healthy" microbial community genotype in AAI mice.

Discussion

In the present work we identified for the first time D-tryptophan as bacterial substance that is produced by the probiotic strains *L. rhamnosus GG* and *L. casei W56*. We demonstrate that D-tryptophan lowers the production of Th2 cyto- and chemokines in human peripheral and murine immune cells and more importantly prevents full development of AAI when fed to mice. Beneath immune modulation, this may 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* (e.g.(3,4)) and in animal studies (e.g. (5,6)), but clear evidence for clinical efficacy in treatment of chronic inflammatory disorders is largely lacking. As the reciprocal interaction of probiotic bacteria with the host's microbiota and immune system is extremely complex, utilization of defined small substances with predictable mode of action might provide an interesting alternative for prevention of allergic disease in individuals at risk.

D-amino acids are non-proteinogenic enantiomers of L-amino acids. Until the discovery of free D-aspartate and D-serine in mammalian brain as neurotransmitters in the late 1980s, D-amino acids were considered to play no role in higher organisms. So far, research on D-amino acids in mammals has been mainly restricted to the nervous system, due to the relative abundance of D-aspartate and D-serine in the brain (34) and the difficulty to detect D-amino acids at trace levels (35). Thus, very little is known on D-tryptophan uptake (36) and metabolism in humans (37) and it has been assumed in the past that higher organisms utilize D-tryptophan poorly (38). 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, using them mainly for cross-linking glycan chains in the peptidoglycan wall (39,40).

The regulation of bacterial L-tryptophan biosynthesis and degradation is well known (41). A role for D-tryptophan in bacterial communication was only recently discovered, by demonstrating by demonstrating its requirement for disassembly of biofilms in *B. subtilis* (42). Other soluble substances produced by probiotic bacteria are less investigated so far (4,43).

Humans are potentially exposed to microbial-generated D-amino acids (44) since body surfaces and the environment harbor an abundant and high diversity of microbes (45). Similar to what has already been shown for acyl-homoserine lactones from gram-negative bacteria (e.g.(46–49)), 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 neither to L-tryptophan or any other tested D-amino acid; second, at least two surface receptors for D-tryptophan exist in humans: The G protein-coupled receptor GPR109B (50) is expressed on macrophages, monocytes, adipose tissue and lung (51), and mediates attraction of neutrophils upon binding of D-tryptophan or its metabolite D-kynurenine. Of note, when we extracted and analysed published transcriptomic data (52), GPR109B was significantly decreased in airway epithelial cells and T cells from patients with asthma as opposed to controls, indicating a potential role for this receptor in allergic disease. (Table E2).

The second receptor, SCL6A14 (solute carrier family 6 amino acid transporter member 14, alias $ATB^{0,+}$) transports D-tryptophan and four other D-amino acids across epithelial cells (53). As the receptor is expressed in the intestine, SCL6A14 is exposed to high microbial load and diversity. SCL6A14 is further expressed at exceptionally high levels in the fetal lung (own data Fig E7 and (54). The physiological role of SLC6A14 in fetal life is unknown so far. But it is tempting to speculate a mechanistic link for prenatal intervention trials using probiotic bacteria.

So far, we concentrated on the 20% MeOH fraction for identification of the putative substance as this was the sub-fraction with the highest immune modulatory activity and polarity. Bioactivity was further detected in the 40% and 50% MeOH fractions holding the potential for the discovery of further small immuno-active substances. Our bioassays were designed to detect substances that induce a tolerogenic profile in DCs and decrease the allergy-related chemokine CCL17. Therefore, it is possible that further immune regulatory 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 immune modulatory properties of D-tryptophan, we do not have direct mechanistic links explaining the altered gut microbiota or the protection from AAI. However, in line with our own findings, Trompette and colleagues demonstrated that a change in the gut microbiota due to dietary fermentable fibers induces the production of metabolites involved in protection from AAI (55). These metabolites have further been associated with increased frequencies of $Foxp3^+$ regulatory T cells (56). The lung microbiota and a population of $Foxp3^+$ regulatory T 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 (57) which supports a role of both immune parameters also in adult mice.

In summary, we identified for the first time that D-tryptophan acts as immune modulatory substance that is produced by probiotic strains. Our results suggest that D-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 may occur through the maintenance of a diverse gut microbiota, which was otherwise lost in animals with AAI.

We conclude that bacteria-derived D-tryptophan may play a wider role in human health than previously thought. Overall, our findings support the concept that defined bacterial products

387 might provide the basis for future development of preventive strategies of chronic
388 inflammatory disorders.
389

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569

570

571 **Table I. Percentage of surface marker expressing mature DCs treated with synthetic D-**

572 **amino acids***

	D-Ala	D-His	D-Isoleu	D-Leu	D-Met	D-Phe
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-Pro	D-Ser	D-Thr	D-Trp	D-Tyr	D-Val
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

573 *DCs were stimulated with LPS (0.1μg/ml) in the presence of the indicated D-amino acids

574 (10μM). Percentages of CD83, CD86, CD80 or CD40 expressing DCs were assessed.

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

576

577 **Table II. Cytokine regulation by probiotic supernatants or D/L-tryptophan in human**

578 **LPS treated DCs***

	IL-10 [pg/ml]		IL-5 [pg/ml]		IFN-□ [pg/ml]		IL-12 [pg/ml]	
	-	LPS	-	LPS	-	LPS	-	LPS
Medium	3.2	2.9	14.7	68.3	112.5	2238.8	102.1	2092.8
DSM-20021	6.8	4.8	33.6	55.9	330.0	2520.5	447.8	2217.3
LGG	432.9	787.9	9.1	5.4	372.7	105.7	79.2	106.9
LA-2	107.3	591.7	8.0	10.3	111.6	437.7	89.3	238.0
LA-5	81.3	305.7	7.6	8.0	113.3	531.8	87.5	331.1
LC-01	452.4	924.5	7.9	2.4	109.3	211.3	76.9	67.8
BB-12	234.9	735.7	11.0	10.9	75.4	437.0	91.5	228.2
BB-46	813.5	1230.7	14.0	13.6	13.5	637.9	95.1	202.3
BB-420	450.4	915.4	8.8	8.4	81.5	783.7	102.5	356.9
L-Trp	5.7	4.9	12.0	61.4	45.0	2031.5	88.3	1993.0
D-Trp	56.9	202.5	10.3	20.6	21.9	1129.5	82.5	871.9
L-Pro	**	6.0	14.8	57.7	88.9	2133.9	99.8	1938.0
D-Pro	5.9	4.0	15.8	69.1	92.6	2295.4	90.6	1911.9

579 * DCs were stimulated in the presence or absence of LPS (0.1μg/ml) with supernatants from
580 200μl bacterial cell free supernatants or tryptophan enantiomers (10μM) for 14h. Non-
581 probiotic DSM-20021 and blank medium (CDM1) were used as negative control. D/L-prolin
582 and L-tryptophan were used as controls for D-tryptophan. ** below detection limit
583

584 **Figure legends**

585 **Figure 1. Screening of supernatants from different of probiotic strains for immune**
586 **activity on human cells**

587 **A,** Dose-dependent capacity of bacterial supernatants from *Lactobacillus rhamnosus* GG (-●-),
588 *Bifidobacterium* BB-420 (-▼-) and *Lactobacillus casei* W56 (-■-) to lower CCL17 secretion of
589 human Hodgkin lymphoma KM-H2 cells. Negative control: non-probiotic Lactobacillus
590 DSM-20021(-▲-).Three independent experiments in duplicates (mean percentages ± SD,
591 relative to CCL17 secretion of untreated KM-H2 cells). Student´s t test; **p ≤ 0.005, ***p ≤
592 0.0005. **B,** Capacity of supernatants from *Lactobacillus rhamnosus* GG, B*ifidobacterium* BB-
593 420, *Lactobacillus casei* W56 or non-probiotic *Lactobacillus* DSM-20021 to prevent full
594 upregulation of costimulatory molecules and HLA-DR on LPS stimulated human monocyte
595 derived dendritic cells. +/- with/without bacterial supernatant.
596 Five independent experiments (mean percentages ± SD relative to LPS alone). Dunn´s
597 Multiple Comparison Test; **p < 0.01, ***p < 0.001.
598

599 **Figure 2. Overview on the ability of bacterial supernatants from all 37 strains to lower**
600 **CCL17 secretion of KM-H2 cells.**
601 Shaded bars: non-probiotic *Lactobacillus* DSM-20021 (negative control); *L. rhamnosus GG*
602 was included as positive control in all experiments with strains other than Lactobacilli. White
603 bars: untreated KMH2 and medium controls
604 Three independent experiments in duplicates (mean percentages ± SD, relative to CCL17
605 secretion of untreated KM-H2). Student´s t test; **p < 0.005 and ***p < 0.0005.
606

607 **Figure 3. Capacity of subfractions of probiotic supernatants to lower CCL17 secretion in**
608 **KM-H2 cells.**

609 Subfractions with different polarity (MeOH/H2O gradient chromatography) from
610 supernatants of *Lactobacillus rhamnosus* GG (top), *Lactobacillus casei* W56 (middle),
611 Negative controls: Non-probiotic DSM-20021 and blank CDM1 medium (bottom). Three
612 independent experiments in duplicate (mean percentages ± SD, relative to constitutive
613 CCL17 secretion of untreated KM-H2 cells). Student's t test; *p<0.05, **p<0.005,
614 ***p<0.0005.
615

616 **Figure 4. Effect of tryptophan L- and D-isomers on CCL17 secretion by KM-H2 cells.**
617 KM-H2 cells were stimulated with different concentrations of synthetic L-and D-isomers of
618 tryptophan followed by CCL17 quantification in KM-H2 culture media after 24h. (●) D-
619 tryptophan, (♦) L-tryptophan.
620 Three independent experiments in duplicates (mean percentages ± SD, relative to constitutive
621 CCL1 7 secretion of untreated KM-H2 cells). Student's t test; *p < 0.05, **p < 0.005, ***p <
622 0.0005.
623

624 **Figure 5. Oral D-tryptophan reduces allergic airway inflammation**
625 **A,** Serum D-tryptophan in mice receiving D-tryptophan (50 mM) in drinking water or water
626 only (UPLC-MS peak areas). Note the different scales for D-tryptophan (black bars) and L-
627 tryptophan (grey bars). Welch Test, mean ± SD, ** p= 0.006, ***p= 0.004. **B,** Total number of
628 cells in BALF. **C,** Differential cell count in BALF. Mac, macrophages; Neut, neutrophils; Eos,
629 eosinophils; Lymph, lymphocytes. **D,** Measurement of airway resistance to increasing doses
630 of methacholine. **E,** Percent Il-4^+ and Il-13^+ cells within spleen $CD3^+CD4^+$ T cells.
631 **F,** $CD40^+$ and $CD80^+$ on spleen $CD11b^{high}$DCs D-tryptophan (black bars), water (white bars).
632 **A)** n=8 **B)** n=3-4, **C-F)** n=7-8 mice per group. Box and whisker plots: Maximum and
633 minimum values (whiskers), the upper and lower quartiles (boxes) and median (horizontal
634 line). Student's T-test, **C, D)** Two-way ANOVA with Bonferroni post-test. *$P < 0.05$, ***$P <$
635 0.001.

636

637 **Figure 6. Oral D-tryptophan supplementation increased gut Treg and restored the**

638 **intestinal bacterial community in mice with AAI**

639 **A)** Percentage of Foxp3^{+} cells within gated CD3^{+}CD4^{+} T cells. Cells were isolated from the

640 lamina propria of the colon. . Each symbol represents an individual mouse. Student's *t*

641 test.***P<0.0001.

642 **B)** Unweighted UniFrac distance matrix based on OTU counts was used to perform Principal

643 Coordinate Analysis. The generated scatterplot indicates dissimilarities between individual

644 samples

645

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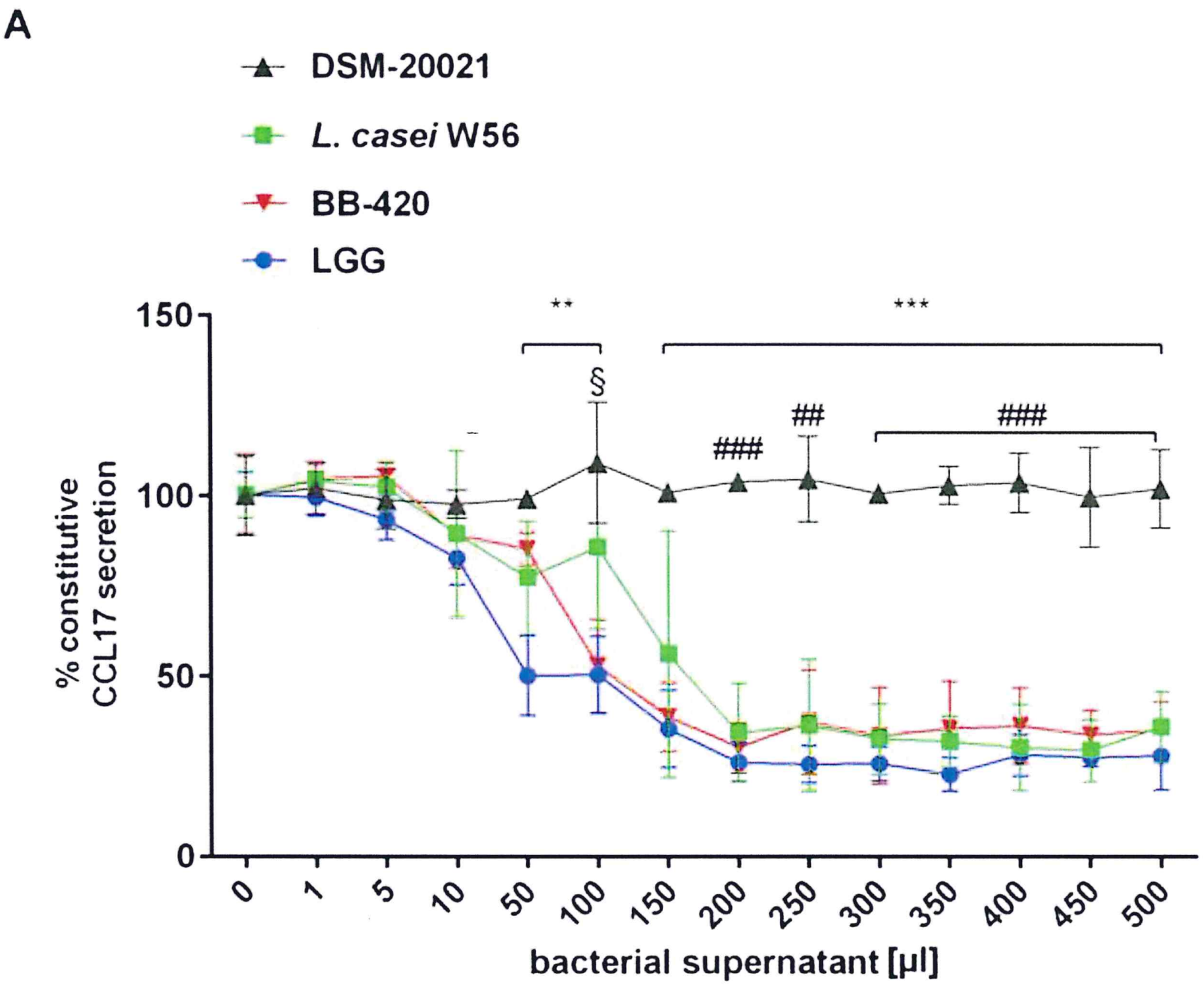

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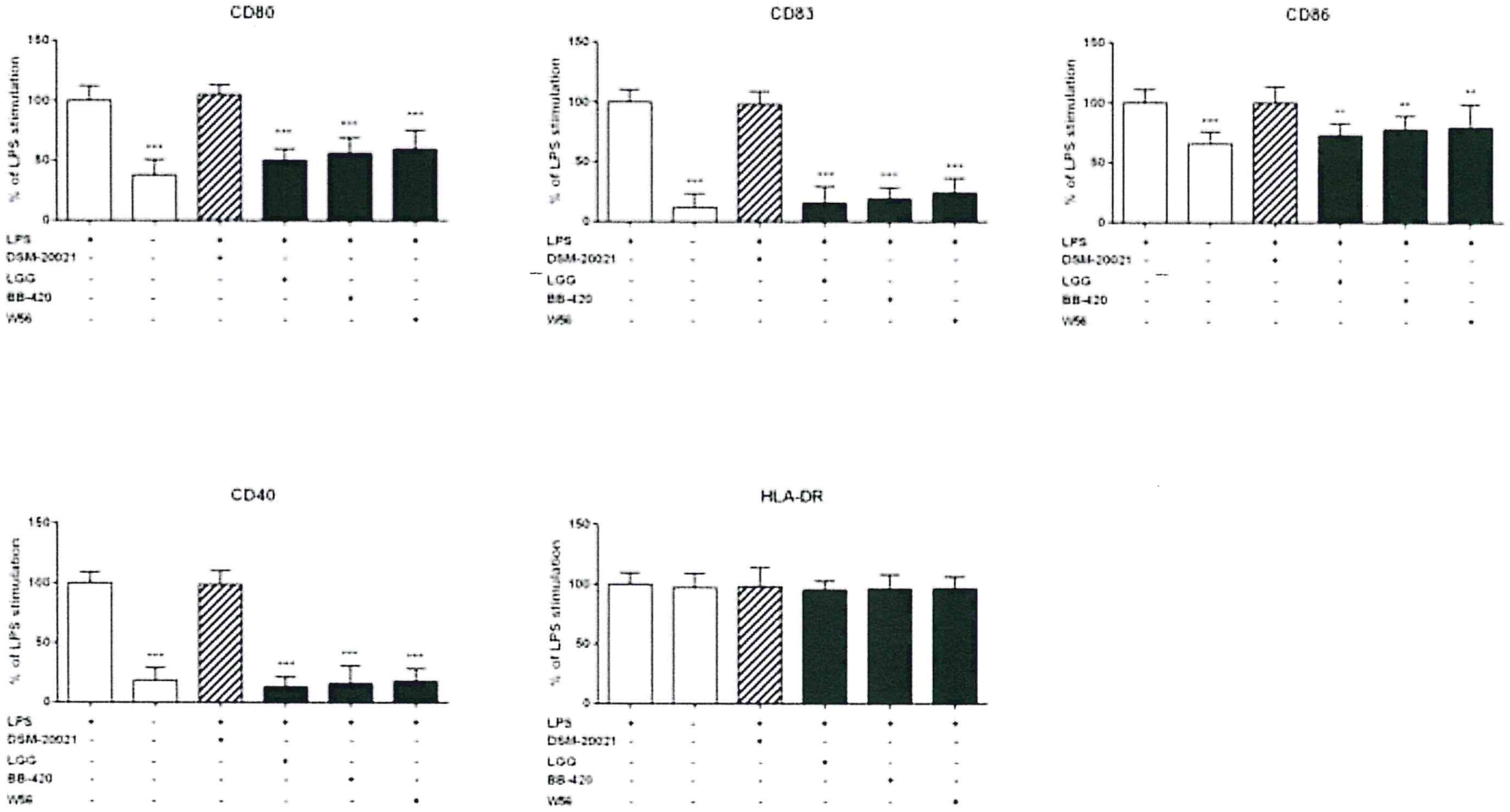

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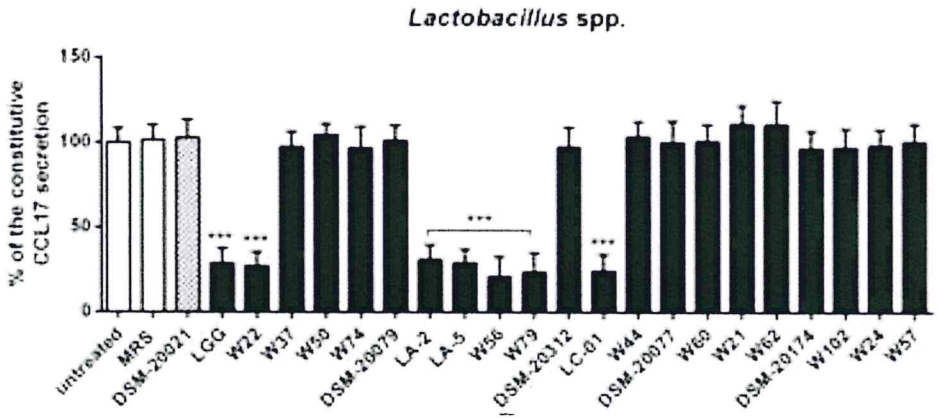

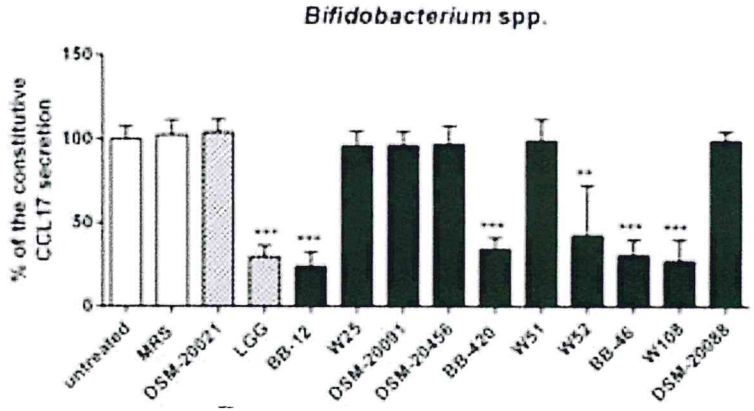

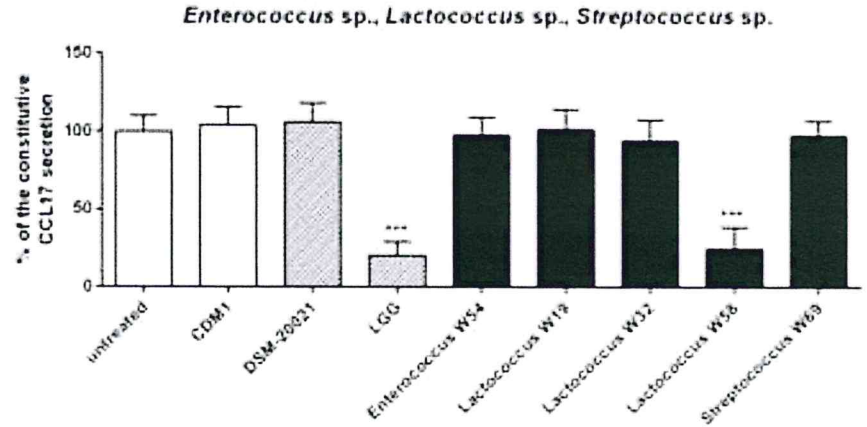

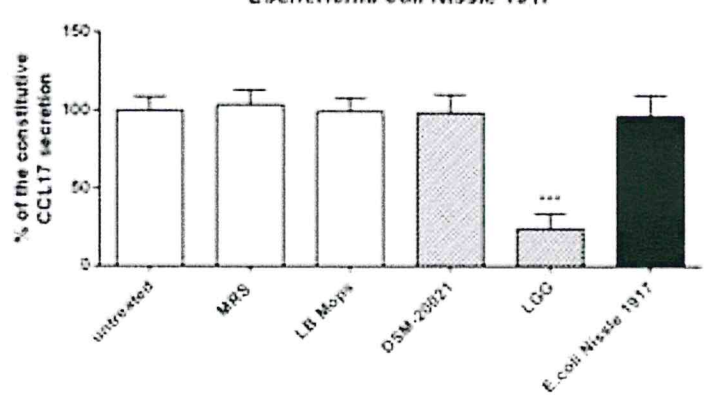

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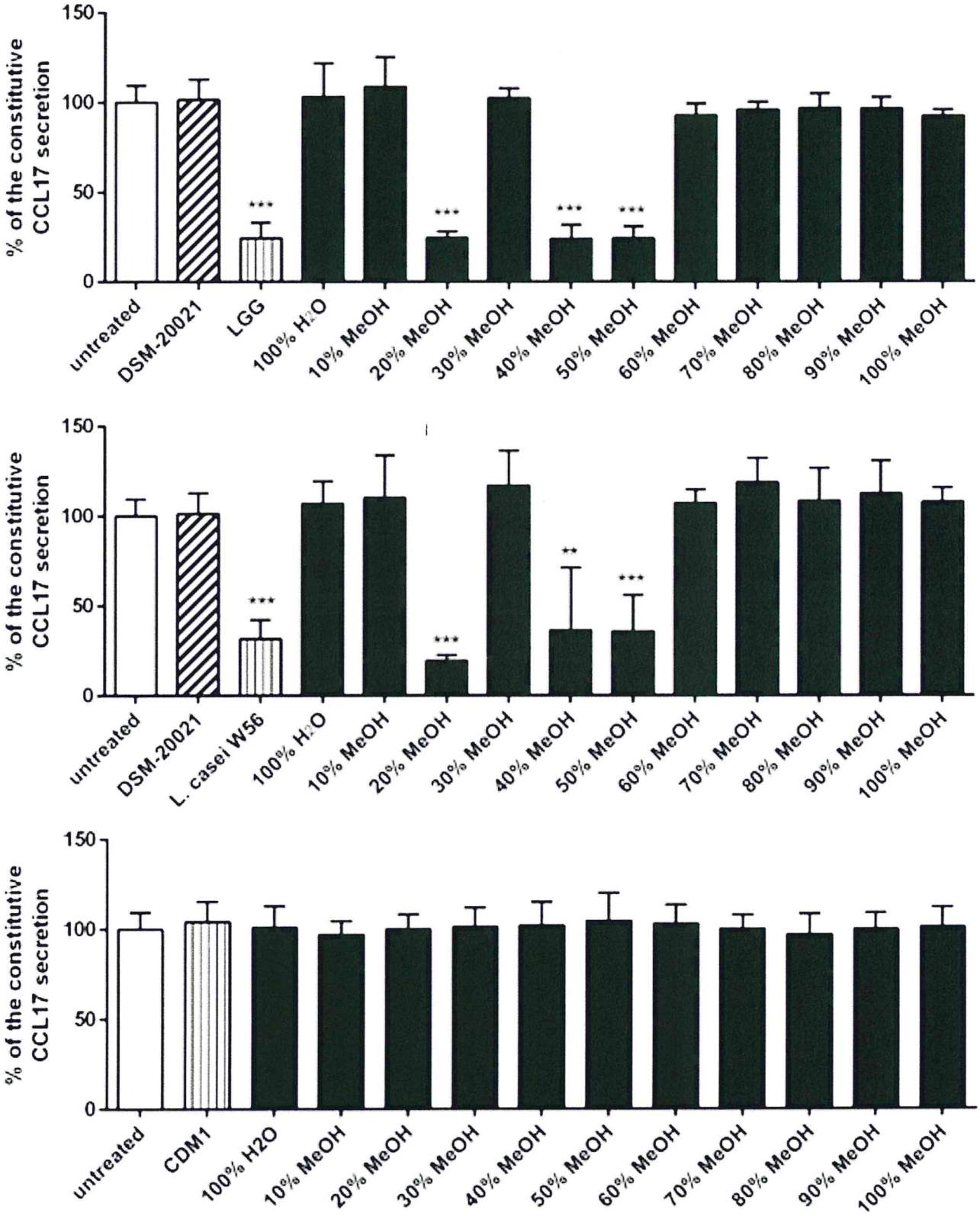

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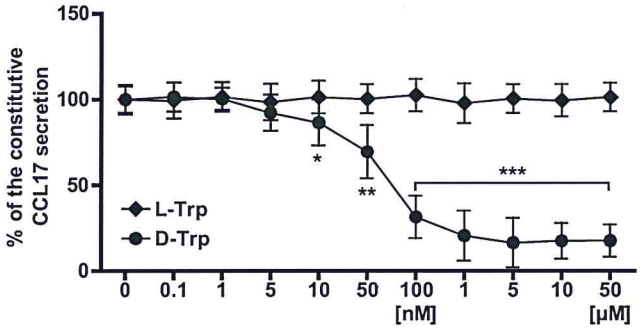

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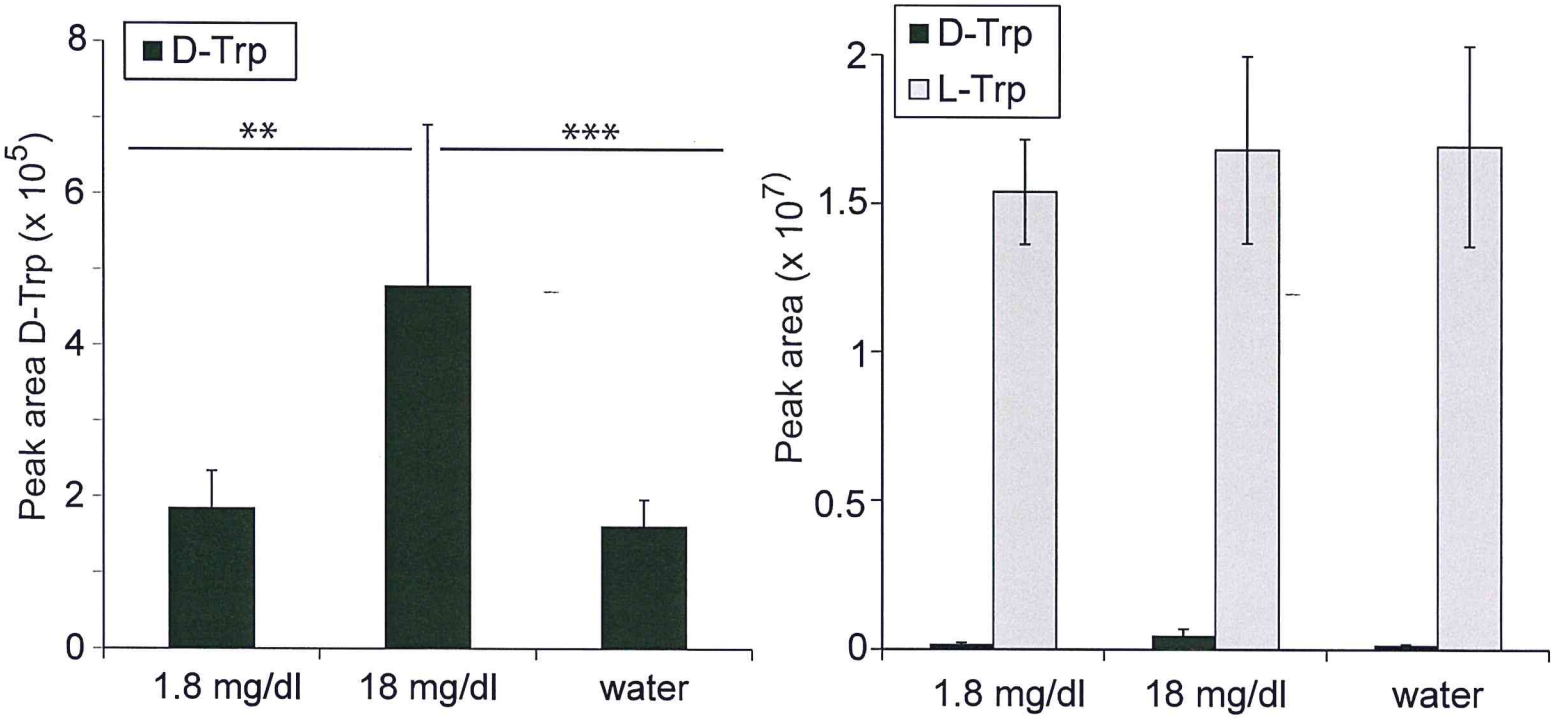

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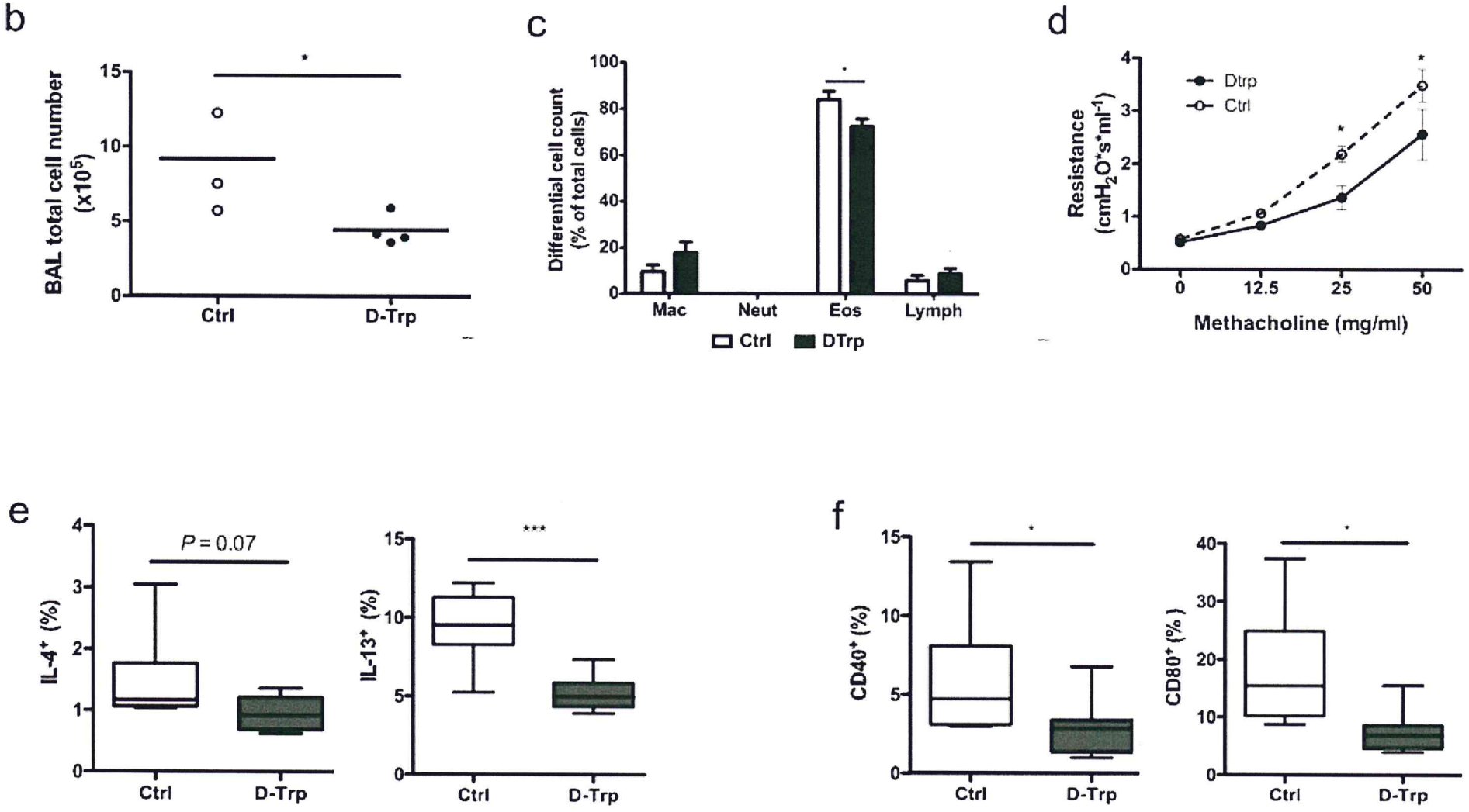

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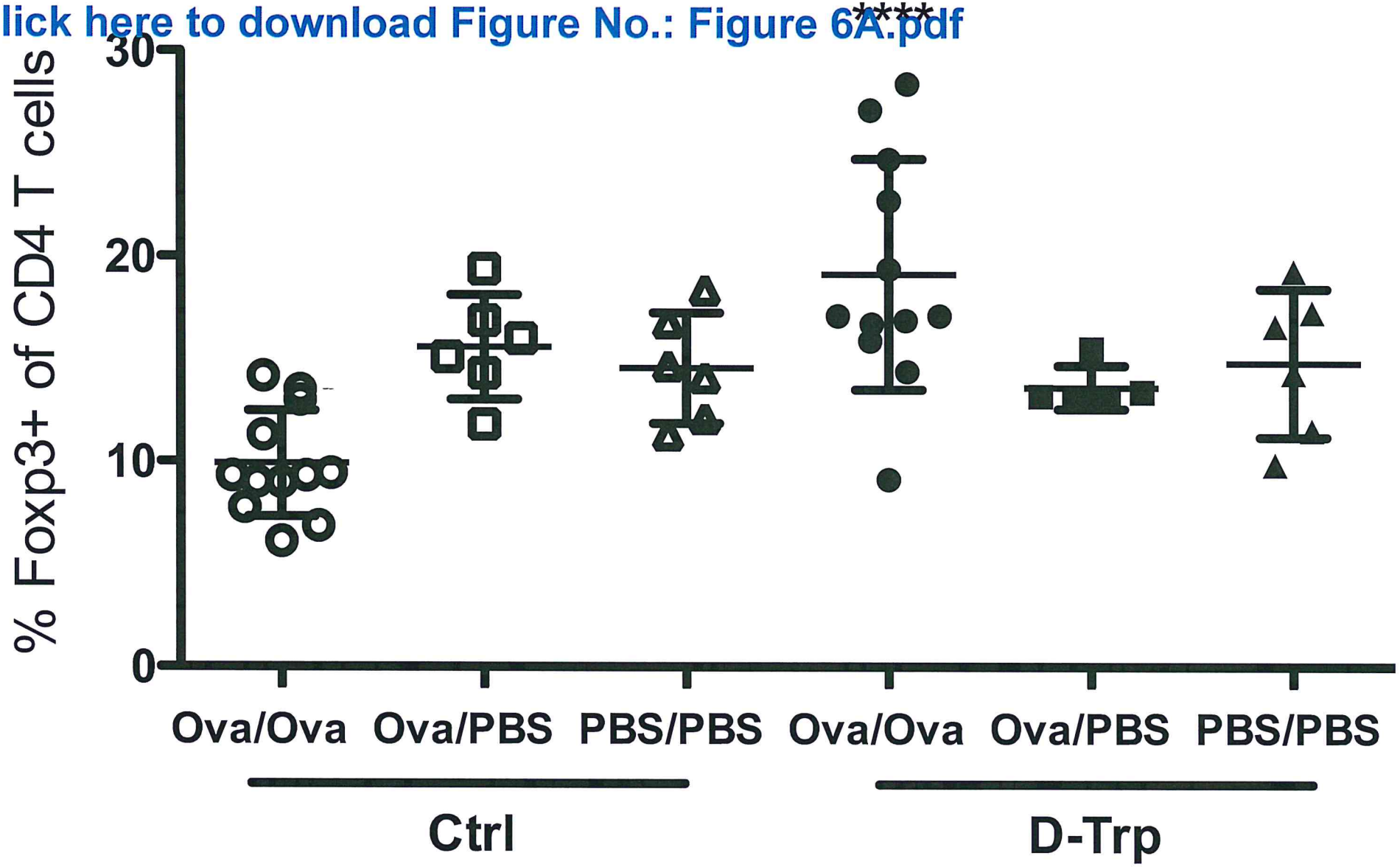

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