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2 **SUPPLEMENTAL INFORMATION**

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5 **Pollutant-induced local alteration of vitamin D metabolism enhances IgE sensitization in**

6 **the gut**

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EXTENDED MATERIALS AND METHODS

Mice. Conventional specific pathogen-free (SPF) C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME) and maintained under specific pathogen-free conditions at the Ohio State University animal care facility. Germ-free C57BL/6 mice were obtained by cesarean derivation and maintained in sterile isolators. The Verigem IgE reporter (IgE GFP) mice were obtained from Dr. Christopher Allen (University of California at San Francisco) and were maintained in our vivarium under SFP condition. All animal experiments were approved by the OSU Animal Care and Use Committee and followed the federal guidelines to avoid unnecessary pain and distress and to minimize animal suffering during the course of the studies.

Cells. The following lines of murine macrophages were obtained from BEI resources (Manassas, VA): J774 cells, wild-type C57BL/6, Myd88 KO C57BL/6 and Trif/Tram double-KO C57BL/6 macrophages. The human intestinal epithelial cell line HT29 clone 19A is a permanently differentiated sub-clone derived from the adenocarcinoma cell line HT29 after sodium butyrate treatment (1) (ATCC, Manassas, VA).

Exposure of mice to subtoxic doses of Cd *in vivo*. Mice aged 8–12 weeks received cadmium chloride ($CdCl_2$, MW = 183.3; Sigma-Aldrich, St. Louis, MO) in drinking water for 4 weeks. The concentration of Cd used in our study (2 ppm or 5 ppm) are approximately 10 times higher than average daily dose people are exposed to in the US. Thus, the average 8-35 μg/day/kg reported by Jarup et al (2)., would equate to 0.16-0.7 μg/day/20g in mice. Since in our study the water consumption per mouse was 2.8 mL/day that would be to equivalent 57-250 ppb [or (0.16-0.7(μg/day)/2.8(mL/day) = 57-250 ng/mL]. Thus, the amount of Cd used in our study could reflect the result of years of chronic exposure of average human. Or shorter exposure by smokers or

47   people living in Cd polluted area (e.g., mining areas). Therefore, Cd was given at the

48   environmentally relevant doses of 10 μM (Cd10) or 25 μM (Cd25) (equivalent to 2 or 5 ppm

49   (μg/L), based on previous studies (3).

50

51   **Allergen sensitization and allergen challenge.** Mice were sensitized by intragastric gavage on

52   days 0 and 7. Briefly, mice were deprived of food for 2 h and given 250 μl of sodium bicarbonate

53   30 min before intragastric gavage of 1 mg of ovalbumin (OVA) and 15 μg cholera toxin in 250 μL

54   of phosphate-buffered saline (PBS). Blood samples were collected on day 14 for analysis of serum

55   IgE and other immunoglobulin isotypes. Nasal antigen challenges were performed on days 15, 16,

56   and 19. For this purpose, mice were anesthetized by intraperitoneal injection of ketamine/xylazine

57   and administered 200 μg of OVA in PBS 100 μL. The body temperature of challenge mice was

58   measured on the skin with a digital thermometer (Heat Spy infrared thermal imaging camera,

59   Wahl, Culver City, CA) to assess the hypothermia associated with allergic responses.

60

61   **Allergen-specific serum IgG and IgA Ab responses.** To determine OVA-specific antibody titers,

62   ELISA was performed with OVA-coated plates as described previously (4, 5). Briefly, microtiter

63   plates were coated with OVA (1 mg/mL). For detection of OVA-specific IgG and IgA Abs, serial

64   dilutions of serum or fecal material extract were added to the plates and the binding antibodies

65   were detected with HRP-conjugated anti-mouse γ- or α-heavy chain-specific antisera (Southern

66   Biotech Associates Inc., Birmingham, AL). Biotin-conjugated rat anti-mouse IgG1, IgG2a/c,

67   IgG2b, or IgG3 monoclonal Abs (mAbs) and HRP-conjugated streptavidin (BD Bioscience, san

68   Jose, CA) were used to measure IgG subclass responses. The Ab titers were determined as the last

69   dilutions of samples that with an absorbance of >0.1 above that of control samples from naïve

70   mice.

71

Analysis of total and allergen-specific serum IgE Ab responses. Total IgE Ab levels were

determined by a BD OptEIA Set Mouse IgE, (BD PharMingen) according to instructions from the

manufacturer. To prevent interference of IgG in the assay, serial dilutions of immune plasma were

previously depleted of IgG by overnight incubation in Reacti-Bind Protein G Coated Plates

(Pierce, Rockford, IL). In order to detect antigen-specific IgE, the microtiter plates were coated

with OVA (1 mg/mL). Serial dilutions of IgG-depleted plasma were then added and IgE were

detected with a biotinylated anti-mouse IgE Ab (BD Biosciences). The IgE titers were determined

as described above for IgG and IgA.

80

Analysis of antigen-specific T helper cell cytokines. Antigen-specific T helper cell cytokine

responses were analyzed by flow cytometry after *in vitro* restimulation and intracellular staining

with cytokine-specific fluorescent antibodies. Briefly, splenocytes were collected on day 14 after

two intraperitoneal sensitizations on day 0 and 7. Cells were restimulated with antigen (i.e., 1 mg/

mL OVA) *in vitro* as previously described (4). After 5 days culture, cells were subjected to

extracellular staining with lineage-specific antibodies [i.e., anti-CD3 (Clone: 17A2), and anti-CD4

(Clone: BL25168) (Biolegend, San Diego, CA)], and after fixation, to intracellular staining using

Th1 (IFNγ (Clone: XMG1.2), TNFα (Clone: MP6-XT22)), Th2 (IL-4 (Clone: 11B11) , IL-5

(Clone: TRFK5), IL-10 (Clone: JES5-16E3), Th17 (IL-17A (Clone: TC11-18H10.1)), and Tfh

(IL-21 (Clone: BL25168)) cytokine-specific antibodies (Biolegend). Labeled cells were analyzed

with an Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA).

92

93   **Assessment of surface body temperature.** Body temperature was assessed by measuring surface

94   body temperature with the aid of infrared thermometers [Heat spy infrared thermal imaging camera

95   (Wahl, Culver City, CA)].

96

97   **Measure of cytokines/chemokines in bronchoalveolar lavages.** Bronchoalveolar lavage fluids

98   were obtained via cannulation of exposed trachea by infusion of 1 mL of sterile PBS through a 22-

99   gauge catheter into the lungs, followed by aspiration of this fluid into a syringe. Aliquots were

100   centrifuged and supernatants were collected and stored at -80°C until analysis. The concentrations

101   of cytokines and chemokines in BALF were evaluated by multiplex assay using the Mouse

102   Cytokine 23-plex Panel (Bio-Rad, Hercules, CA).

103

104   **Analysis of immune cell subset by flow cytometry.** Single-cell suspensions of spleens, lungs,

105   Peyer's patches (PP), or mesenteric lymph nodes (MLN) were stained with fluorescent antibodies

106   directed against the following lineage-specific markers: CD3 (clone: 17A2), CD4 (clone:

107   BL25168), CD8 (clone: 53-6.7), CD19 (clone: 6D5), F4/80 (clone: BM8), c-kit (clone: BL2B8),

108   Gr1 (clone: 1A8), CD11b (clone: M1/70), and CD11c (clone: N418) (Biolegend, San Diego, CA).

109   In selected experiments, cells were also stained with anti-CD38 (clone: 90) or anti-IgA (clone:

110   mA-6E1) (DB Biosciences, San Jose, CA). Labeled cells were analyzed with an Attune NxT flow

111   cytometer (Thermo Fisher Scientific, Waltham, MA).

112

113   **Analysis of lung inflammation and mucus production.** Lung tissues were formalin-fixed and

114   paraffin-embedded. Sections (5 μm thick) were stained with eosin and hematoxylin (H&E) to

115   assess overall structure. Mucus production was visualized by staining with periodic acid-Schiff

116   (PAS) and quantified with the aid of ImageJ software (NIH, Bethesda, MD).

117

Immunohistochemistry analysis of gut tissues. Gut tissues were formalin-fixed and paraffin-embedded. Sections (5 μm thick) were stained with eosin and hematoxylin (H&E) to assess overall structure. Tissues were stained with the following antibodies: anti-NRF-2 (clone: A-10, dilution 1:100) (Santa Cruz Biotech, Dallas, TX), anti-PGE$_2$ (goat polyclonal anti-PGE$_2$, dilution 1:100), or anti-BAFF (goat polyclonal anti-mouse BAFF, dilution 1:100) (Abcam, Cambridge, MA). Nuclei were counterstained with DAPI. Expression of the target molecules was quantified with the aid of ImageJ software (NIH, Bethesda, MD).

125

Evaluation of intestinal permeability. For evaluation of intestinal permeability, mice were deprived of food and water for 2 h and orally administered 200 μL PBS containing 8 mg/20 g of body weight FITC-dextran (4000; FD4, Sigma-Aldrich, Saint Louis, MO). Blood was collected 4 h later, and serum FITC-dextran levels were measured with a Victor V multi-parameter plate reader (Perkin Elmer, Waltham, MA) and quantified using extrapolation against a known-concentration FITC-dextran standard.

132

Real-time RT- PCR. Tissues were collected, snap frozen, and reduced to powder before adding TRIzol (Invitrogen, Carlsbad, CA). Complementary DNA was synthesized using Superscript III (Invitrogen). Real-time PCR was performed as previously described (4) and data were expressed as relative mRNA expression = $2^{-\Delta\Delta Ct}$ where $\Delta Ct = Ct_{unknown} - Ct_{HKG}$, and normalized against two house-keeping genes (HKG): β-actin and HPRT1. The list and sequence of primers are provided in Table S1.

139

140   **Quantification of fecal IgA.** For assessment of IgA levels in the intestinal secretions, freshly

141   emitted fecal pellets from Cd-treated and control mice were dissolved in PBS (1 mL per 0.1 g

142   feces). Concentration of IgA was measured by ELISA using extrapolation against IgA standards

143   and normalized by protein content (4).

144

145   **Culture of gut microbes and 16 rRNA analysis of gut microbiota.** Freshly emitted fecal pellets

146   were collected and normalized by mass (g) of feces. To determine the bacterial load, dilutions of

147   fecal extracts were plated in blood agar and bacteria CFU were counted after 48 h of culture. To

148   determine fungal (*Candida*) load, dilutions of fecal pellet were plated on Sabouraud dextrose agar

149   containing 50 μg/mL of gentamycin and 50 μg/mL of chloramphenicol, and fungal CFU were

150   counted after 24 h of culture. Bacterial tag-encoded FLX amplicon pyrosequencing (Roche,

151   Branford, CT) was used for identification of primary populations of microbes in fecal pellets as

152   previously described(4). Samples were collected twice a day from each individual mouse and

153   pooled to minimize potential daily variation of the microbiota.

154   Bacterial DNA was extracted by conventional methods (Qiagen, Valencia, CA), and 16S

155   rRNA genes were amplified with the modified 16S eubacterial primers 28F, 5'-GAG TTT GAT

156   CNT GGC TCA G-3' and 519R, 5'-GTN TTA CNG CGG CKG CTG-3' for amplifying the 500

157   bp region of 16S rRNA genes. The primer sets used for FLX-Titanium amplicon pyrosequencing

158   were designed with adding linker A and an 8-bp barcode sequence at the 5' end of forward primers

159   as follow: 28F-A, 5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG-barcode-GAG TTT

160   GAT CNT GGC TCA G-3'. The biotin and linker B sequence at the 5' end of reverse primer 519R-

161   B: 5'-Biotin-CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG GTN TTA CNG CGG CKG

162   CTG-3'. HotStarTaq Plus Master Mix kit (QIAGEN, CA, USA) was used for PCR under the

163   following conditions: 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 54°C for 40 s, and

164 72°C for 1 min, and a final elongation step at 72°C for 10 min was also included. The PCR products

165 were cleaned by using Diffinity Rapid Tip (Diffinity Genomics, Inc, West Henrietta, NY), and the

166 small fragments were removed using Agencourt Ampure Beads (Beckman Coulter, CA, USA).

167   Bacterial tag-encoded FLX-Titanium amplicon pyrosequencing (bTEFAP) was performed

168 as described previously (6). In preparation for FLX-Titanium sequencing (Roche, Nutley, New

169 Jersey), DNA fragment sizes and concentration were accurately measured using DNA chips under

170 a Bio-Rad Experion Automated Electrophoresis Station (Bio-Rad Laboratories, CA, USA) and a

171 TBS-380 Fluorometer (Turner Biosystems, CA, USA). A sample of double-stranded DNA ($9.6 \times$

172 10^6 molecules/mL) with an average size of 625 bp were combined with 9.6×10^6 DNA capture

173 beads and then amplified by emulsion PCR. After bead recovery and bead enrichment, the bead

174 attached DNAs were denatured with NaOH, and sequencing primers (Roche) were annealed. A

175 four-region, 454-sequencing run was performed on a GS PicoTiterPlate (PTP) using the Genome

176 Sequencer FLX System (Roche). Forty tags were used on each quarter region of the PTP. All FLX

177 procedures were performed using Genome Sequencer FLX System manufacturer's instructions

178 (Roche). After denoising (USEARCH application) and chimera removal (UCHIIME in *de novo*

179 mode), the sequences ware clustered into operational taxonomic units (OTU) with 96.5% identity

180 (3.5% divergence) using USEARCH, and the seed sequence was put into a FASTA-formatted

181 sequence file. The FASTA files were then queried against a database of high-quality sequences

182 derived from NCBI using a distributed .NET algorithm that utilizes BLASTN+ (KrakenBLAST

183 www.krakenblast.com).

184 The Bray-Curtis index and principal component analysis (PCA) was used to summarize the

185 relationship between microbial communities in the control and Cd-treatment groups. Linear

186 discriminant analysis (LDA) scores were analyzed using the Galaxy software

187 (https://huttenhower.sph.harvard.edu/galaxy/) and the threshold on the logarithmic for

188 discriminative features was set at >2.5.

189

190 **Analysis of metabolites in fecal samples.** Metabolites were analyzed using an adaptation of a

191 previously reported methods (7). Briefly, bacteria-free fecal extracts were mixed with an equal

192 volume of ethyl acetate and centrifuged at 13,000 × g for 15 min. The upper organic layer was

193 diluted by a factor of 10 into H_2O: ACN with 0.1% formic acid and placed into LC vials for HPLC

194 MS analysis (7). Samples (6.4 µL) were injected into the UltiMate 3000 HPLC (Thermo Fisher

195 scientific, Waltham, MA) with an Imtakt Scherzo SM-C18 column (100 mm × 1 mm, 3-µm

196 particle size). Two mobile-phase systems consisting of H_2O with 0.1% formic acid (Solvent A)

197 and 70:30 ACN: H_2O with 0.5% formic acid (Solvent B) were used. A flow rate of 20 µL/min with

198 a column temperature of 55°C at linear gradients from 5% to 90% for 13 min and 90% to 95% for

199 2 min (15 min total) was used before equilibration. Both positive and negative mode runs were run

200 separately with the heated electrospray ionization (HESI) source on the Q Exactive Plus Hybrid

201 Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher scientific). The HESI source was set to

202 a capillary voltage of 3.2 kV with a capillary temperature of 320°C and sheath gas of 7 and

203 auxiliary gas of 1.2. Metabolites that showed significance *(P < 0.05*, t-test) between the control

204 (NoCd) and at least one of the Cd-treated groups were further categorized with the aid of the

205 Human Metabolome Database (HMDB Version 4.0, www.hmdb.ca)

206

207 **Proteomic analysis of fecal samples**. For proteomic analysis, proteins were precipitated by

208 adding 1 volume trichloroacetic acid to 4 volumes of fecal extracts followed by 60 min incubation

209 on ice. The pellets were washed in cold acetone and then resuspended in 50 mM ammonium

210 bicarbonate. Protein concentrations were normalized before sequential addition of 5 mM di-

211   thiothreitol (DTT) and 15 mM iodoacetamide (IAA) to reduce disulfide bonds and alkylate-free

212   cysteine residues. Samples were then trypsin digested (1:30 trypsin:protein) overnight at 37°C.

213   Trifluoroacetic acid (0.5% TFA) was added to stop the digestion. The samples were then

214   centrifuged and the supernatant was removed, then the pellets were dried and resuspended in 50

215   mM acetic acid before analysis.

216   Liquid chromatography-nanospray tandem mass spectrometry (LC/MS/MS) of protein

217   identification was performed on a Thermo Scientific orbitrap Fusion mass spectrometer equipped

218   with an EASY-Spray Sources operated in positive ion mode. Samples were separated on an EASY-

219   Spray nano column (Pepmap^{TM} RSLC, C18 3 μ 100 A, 75 μm × 150 mm, Thermo Scientific) using

220   a 2D RSLC HPLC system from Thermo Scientific. Next, 3 μg of each sample were injected into

221   the μ-Precolumn Cartridge (Thermo Scientific,) and desalted with 0.1% formic acid in water for 5

222   min. The injector port was then switched to inject, and the peptides were eluted off the trap into

223   the column. Mobile phase A was 0.1% formic acid in water, and acetonitrile (with 0.1% formic

224   acid) was used as mobile phase B. Flow rate was set at 300 nL/min. Mobile phase B was increased

225   from 2% to 35% in 80 min, then increased from 35–55% in 10 min, increased again from 55–90%

226   in 5 min, and then kept at 90% for another 4 min before being brought back quickly to 2% in 1

227   min. MS/MS data was acquired with a spray voltage of 1.7 KV and a capillary temperature of

228   275°C. The scan sequence of the mass spectrometer was based on the preview mode data

229   dependent TopSpeed method: the analysis was programmed for a full scan recorded between 400–

230   1,600 m/z and a MS/MS scan to generate product ion spectra to determine amino acid sequences

231   in consecutive scans starting from the most abundant peaks in the spectrum within the next 3 s. To

232   achieve high mass accuracy MS determination, the full scan was performed at Fourier transform

233   (FT) mode and the resolution was set at 120,000. The automatic gain control (AGC) target ion

234   number for FT full scan was set to 2×10^5 ions, maximum ion injection time was set to 50 ms, and

235 the micro scan number was set to 1. MS/MS was performed using ion trap mode to ensure the

236 highest signal intensity of MS/MS spectra using both collision-induced dissociation [(CID) 2+ to

237 4+ charges] and electron-transfer dissociation [(ETD) 4+ to 6+ charges] methods. The AGC target

238 ion number for ion trap MS/MS scan was set to 1,000 ions, maximum ion injection time was set

239 to 100 ms, and the micro scan number was set to 1. The CID fragmentation energy was set to 35%.

240 Dynamic exclusion was enabled with a repeat count of 2 within 40 s and a low mass width and

241 high mass width of 10 ppm.

242   Data analysis was performed using Mascot 2.6.0. The fragmentation spectra were searched

243 against the UniProt *Rattus* protein database with precursor and fragment mass tolerances set to 10

244 ppm and 0.5 Da, respectively, and with up to four missed cleavages. Cysteine

245 carbamidomethylation was set as a fixed modification, whereas methionine oxidation and

246 deamidation of asparagine and glutamine were set as variable modifications for database

247 searching. Data were filtered to enable only proteins for which two unique peptides to be

248 considered, along with high confidence identifications [1% false discovery rate (FDR)]. Search

249 results were compiled in Scaffold using a 1% FDR for proteins and enabling comparison between

250 samples.

251

252 **Fecal material transplant**. Fecal microbiomes were transferred to naïve mice prior to allergic

253 sensitization as previously described (4). Briefly, freshly emitted feces were collected and

254 dissolved in PBS (1 mL/0.1 g feces). Particles were removed by filtration using a 70-μm strainer,

255 and mice received 0.2 mL suspension by gavage.

256

257 ***In vitro* culture with bacteria-free fecal material extracts.** J774 (origin: Balb/c), wild-type

258 C57BL/6, Myd88 KO C57BL/6 or Trif/Tram double-KO C57BL/6 macrophages (BEI resources,

259   Manassas, VA), and HT-29 human epithelial cells were cultured in the presence of 1:50 dilutions

260   of 0.1-μm filtered bacteria-free fecal material extracts from control (NoCd) or Cd-treated mice

261   (Cd10 or Cd25), and then concentration of PGE_2 or mRNA expressions were analyzed by ELISA

262   or RT-PCR. To assess the effect of bacteria-free fecal material extracts on IgE production *in vitro,*

263   spleen or mesenteric lymph node cells ($4x10^5$ cells) from naïve mice were cultured for 4 days in

264   the presence of IL-4 (10 ng/mL), anti-CD40 (1 μg/mL), and bacteria-free fecal material extracts

265   (dilution 1:50) from control or Cd-treated mice. In selected experiments, the COX2-inhibitor

266   celecoxib (Sigma-Aldrich, Saint Louis, MO) was added at a final concentration of 10 μM. Theses

267   stimulated cells were assessed Ig-class switching by flow cytometry or ELISA.

268

269   **Measure of PGE_2.** The concentrations of PGE_2 in fecal materials and culture supernatants were

270   evaluated by competitive ELISA using a Prostaglandin E_2 Parameter Assay kit (R&D Systems,

271   MN).

272

273   ***In vivo* treatment with pharmacological inhibitors of specific pathways.** To address the role of

274   oxidative stress, PGE_2 and Vitamin D_3-metabolizing enzymes, mice received orally by intragastic

275   gavage 250 uL of saline containing 250 mg/kg of N-acetyl L-cysteine (Sigma-Aldritch, Saint

276   Louis, MO), 25 mg/kg of aspirin (Sigma-Aldritch, Saint Louis, MO), or 10 mg/kg of ketoconazole

277   (Sigma-Aldritch, Saint Louis, MO), respectively.

278

279   **Statistical analyses.** Results are expressed as mean ± SD. Statistical significance was determined

280   by one- or two-way ANOVA, followed by the Tukey's multiple range test. All statistical analyses

281   were performed with the StataSE 12.0 software (StataCorp LLC, College Station, TX) and Prism

282   7 software (GraphPad Software, La Jolla, CA).

283   **Table S1.** List of primers used for real-time RT-PCR

Species		Name		Sequence	Size
Mouse	House keeping gene (HKG)	β-actin	F	GCG CAA GTA CTC TGT GTG GA	162
			R	GAA AGG GTG TAA AAC GCA GC	
		Hprt1	F	GAGGAGTCCTGTTGATGTTGCCAG	173
			R	GGCTGGCCTATAGGCTCATAGTGC	
	Dual oxidase	Duox1	F	CCC ACG TTA CCA TTT CCA TCA	204
			R	CAT CTG CAT AGC TGG CTG GA	
		Duox2	F	GGA CAG CAT GCT TCC AAC AAG T	222
			R	GCC TGA TAA ACA CCG TCA GCA	
		Duoxa1	F	CAT CAC CCT CAC AGG CAC C	239
			R	GGA ATG CCA CCC ACA GCA	
		Duoxa2	F	GCC TGG CTT TGC TCA CCA	170
			R	GAG GAG GAG GCT CAG GAT	
	Inflammatory cytokines	Ifnγ	F	ACT GGC AAA AGG ATG GTG AC	237
			R	TGA GCT CAT TGA ATG CTT GG	
		Tnfα	F	CTC TTC AAG GGA CAA GGC TG	253
			R	CGG ACT CCG CAA AGT CTA AG	
		Il-1β	F	TCG CAG CAG CAC ATC AAC AAG	190
			R	CCA GCA GGT TAT CAT CAT CAT CC	
		Caspase-1	F	CCA ATA ATG AAT ACA ACC ACT CG	125
			R	TCC TCC AGC AGC AAC TTC	
		Tgfβ	F	CCC TAT ATT TGG AGC CTG GA	141
			R	CTT GCG ACC CAC GTA GTA GA	
		Il-10	F	GCA CTA CCA AAG CCA CAA AGC AG	88
			R	GTC AGT AAG AGC AGG CAG CAT AGC	
		Kc	F	ACT CAA GAA TGG TCG CGA GG	123
			R	GTG CCA TCA GAG CAG TCT GT	
		Mip2	F	CCT CAA CGG AAG AAC CAA AG	70
			R	ACA TCA GGT ACG ATC CAG GC	
	Ig-Class switching	April	F	GGG GAA GGA GTG TCA GAG TG	148
			R	GCA GGG AGG GTG GGA ATA C	
		Baff	F	AGG CTG GAA GAA GGA GAT GAG	161
			R	CAG AGA GAC GAG GGG AAG GG	
		Ptges	F	AAG CCT AGC CAC ACC ACT TC	100
			R	CCA GCA GGG CTT TCC AGT AA	
	Vitamin D metabolism	Vdr	F	CAC TGC TGC CTC TGA GAA CA	168
			R	ATA ATA CAG ACA GGT GGC AGG AG	
		Cyp24a1	F	ACG TCA CCT CCT TAC CTG GA	165
			R	GAT GCA CCG AGT CGA AGG AG	
		Cyp27b1	F	AAG CTC GCC TCC AGA GTT TTT	88
			R	GGA GAA CCG ACT CAC TGC C	
Human	HKG	β-ACTIN	F	TGG GCA TGG GTC AGA AGG AT	84
			R	GCT CGA TGG GGT ACT TCA GG	
	Ig-Class switching	APRIL	F	AGT CTC CCG GAG CAG AGT TC	103
			R	CGG AGT CAT CTT CTT GTT TTT GGG	
		C4BP	F	GCT TCT CAC TCT TGG GCC AT	216
			R	CAC TGC TGC CTC TGA GAA CA	

284

13

285 Table S2. Metabolites and proteins/peptides most affected by ingestion of Cadmium
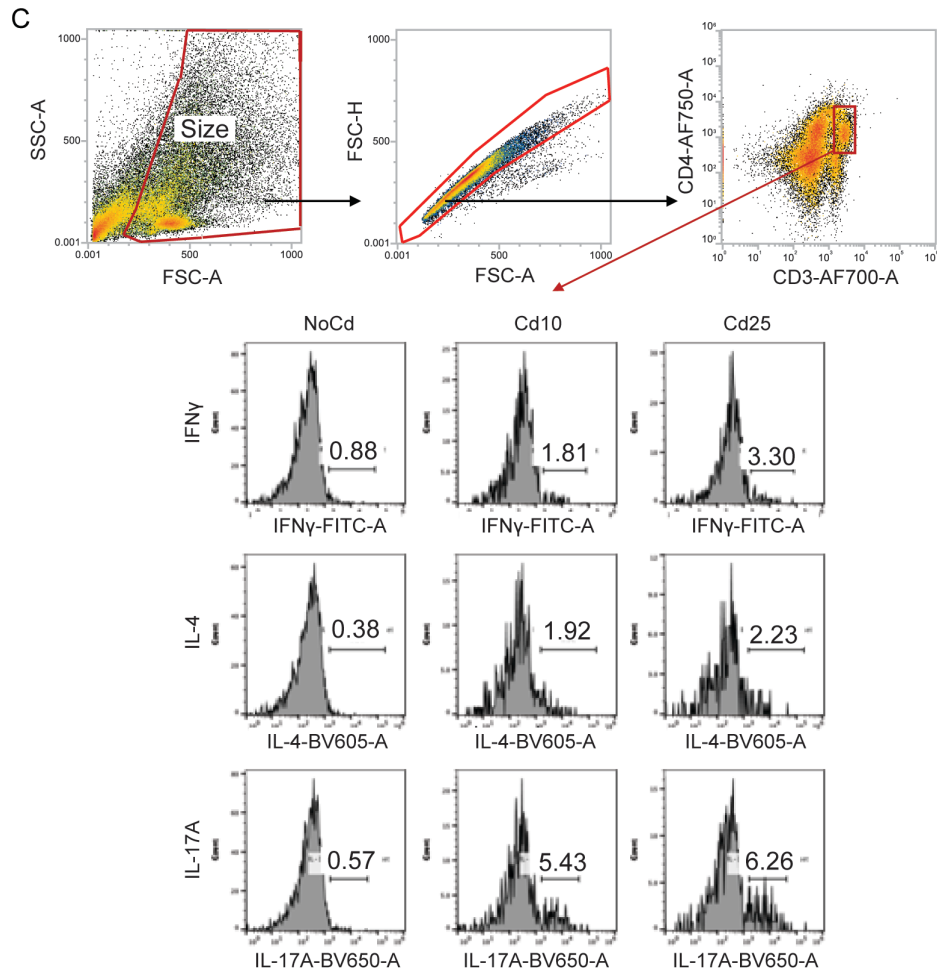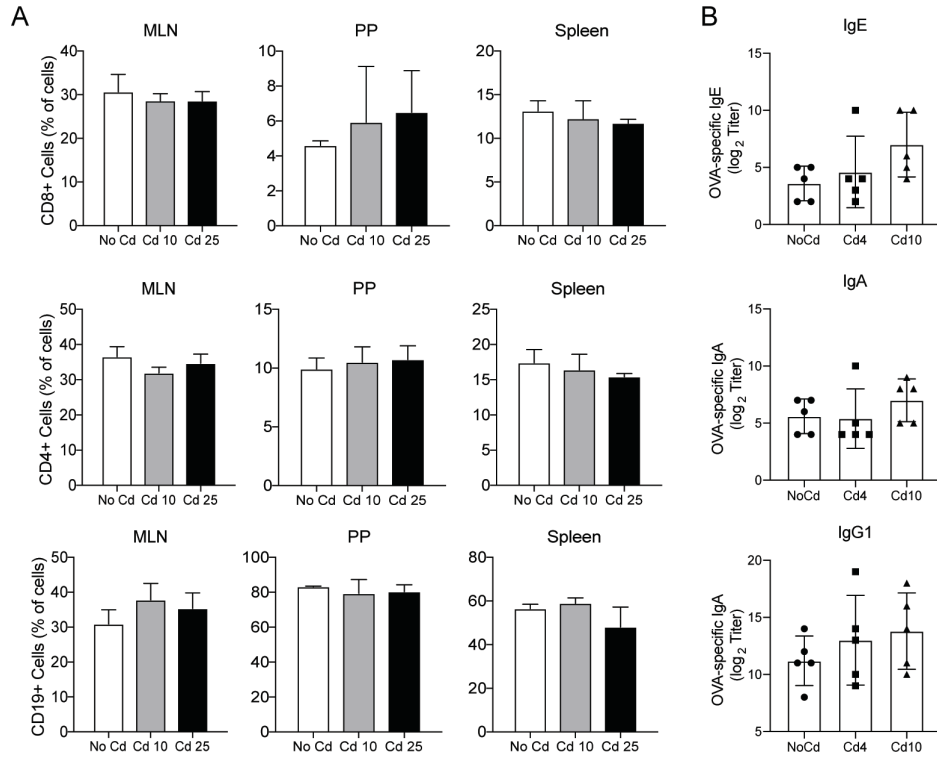
	No Cd	Cd 10
24,25,26,27-Tetranor-23-oxo-hydroxyvitamin D_3	34.725±33.885	2216.896±1325.511
Kallikrein-1	416.5±13.435	238.5±44.548
Mucin-3	3.5±4.950	0±0
Mucin-6	21±2.828	12.5±0.707
Alkaline phosphatase	167±82.024	106±5.657
Aminopeptidase N	685±57.983	580±4.95
Sucrase isomaltase	266±16.971	226±8.485
Ferritin	2±2.828	4.5±0.707
Ferritin heavy chain	5.5±0.707	9.5±3.536
Ig heavy constant alpha	284.5±45.962	266.5±10.607

286
287 Metabolites and proteins/peptides present in fecal extracts were analyzed by HPLC-MS. The
288 amounts of product detected were expressed as mean arbitrary unit (AU) ± one S.D.
289 Note: Fecal materials of mice exposed to Cd 25 contained 1673.890±631.856 AU of 24,25,26,27-
290 Tetranor-23-oxo-hydroxyvitamin D_3.
291

A

MLN — CD8+ Cells (% of cells)
PP
Spleen

MLN — CD4+ Cells (% of cells)
PP
Spleen

MLN — CD19+ Cells (% of cells)
PP
Spleen

B

IgE — OVA-specific IgE ($\log_2$ Titer)

IgA — OVA-specific IgA ($\log_2$ Titer)

IgG1 — OVA-specific IgA ($\log_2$ Titer)

C

SSC-A / FSC-A — Size
FSC-H / FSC-A
CD4-AF750-A / CD3-AF700-A

	NoCd	Cd10	Cd25
IFNγ	0.88	1.81	3.30
IL-4	0.38	1.92	2.23
IL-17A	0.57	5.43	6.26

IFNγ-FITC-A
IL-4-BV605-A
IL-17A-BV650-A

292

293   **Figure S1. Chronic exposure to subtoxic doses of cadmium does not affect the**

294   **frequencies of major immune cell subsets in mucosal and systemic lymphoid tissues**. (A)

295   Mice were provided $CdCl_2$ [10 μM (Cd10) or 25 μM (Cd25)] in drinking water for 28 days. Cells

296   were then isolated from mesenteric lymph nodes (MLN), Peyer's patches (PP) and spleens and

297   analyzed by flow cytometry after staining with lineage-specific fluorescent antibodies. (B) OVA-

298   specific antibody responses from mice exposed $CdCl_2$ [4 μM (Cd4) or 10 μM (Cd10)] in

299   drinking water for 28 days. After exposed to Cd, mice were sensitized with OVA and CT and

300   serum were collected at day 14. (C) Representative flow gating strategy for intracellular

301   cytokines. Spleens were collected on day 14 and restimulated *in vitro* with 1 mg/mL of OVA

302   before flow cytometry analysis of $CD4^+$ T cell cytokine responses. Results are expressed as
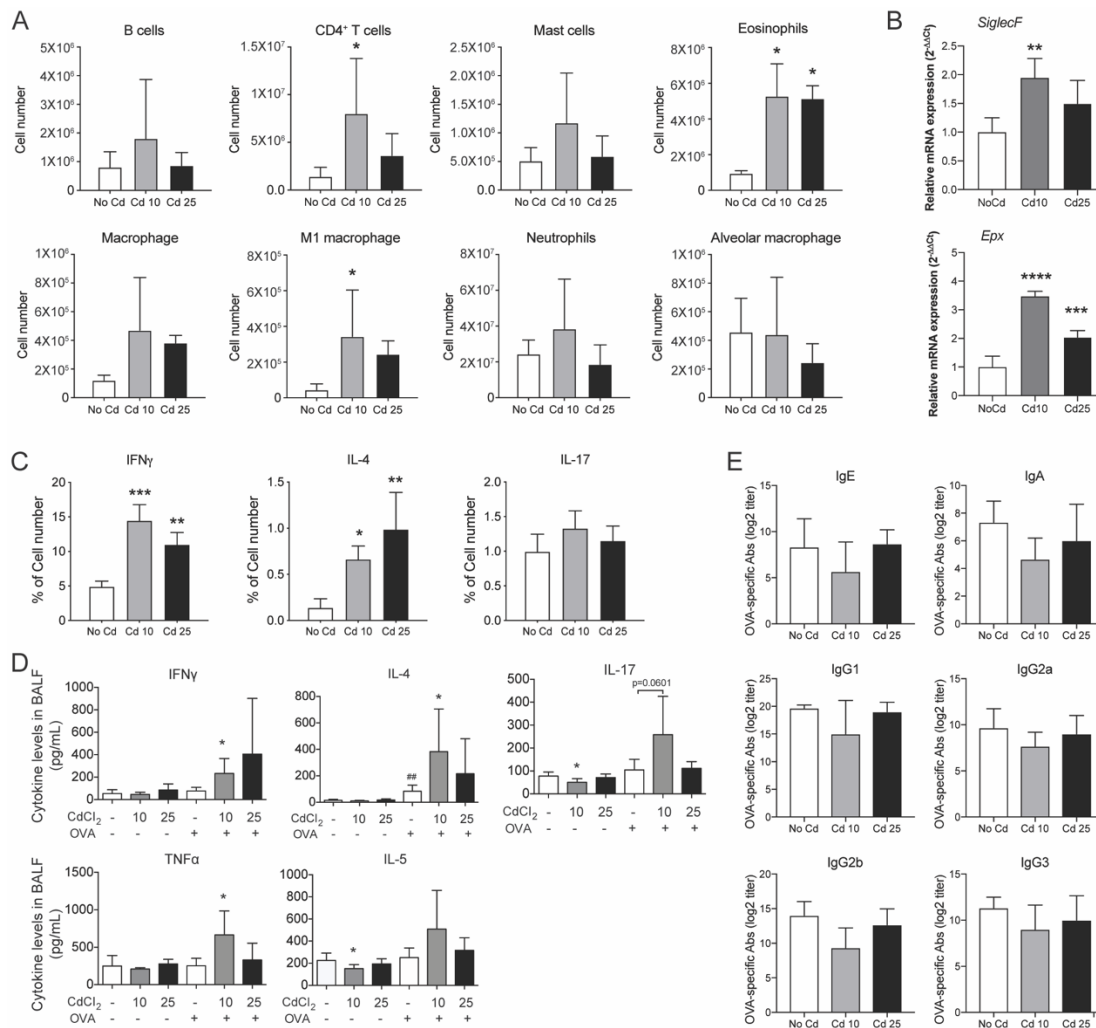
303   mean ± SD. (n = 5 per group).

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Figure S2. Immune cell subset, cytokine and antibody responses in mice chronically exposed to subtoxic doses of cadmium. (A-D) Immune cell subsets, cytokines responses in the lungs after nasal allergen challenges of mice orally sensitized to allergen. (A) Immune cell subsets present in bronchoalveolar lavage fluid (BALF) after allergen challenges were analyzed by flow cytometry. (B) Eosinophils responses in the lungs after allergen challenges were analyzed by expression of *Siglec-F* and *Epx* mRNA. (C) Intracellular cytokines in BAL cells were analyzed by flow cytometry (n = 5). (D) Cytokines responses in BALF were analyzed by multi-plex assay. Data represent one of at least 4 experiments with n = 5 per group. (E) Antibodies (Abs) responses after systemic sensitization of mice exposed to Cd. OVA-specific serum Abs responses on day 14 [after

319 2 i.p. sensitization on days 0 and 7 with OVA allergen (100 μg) with CT (1 μg) as adjuvant] (n=5

320 per group). Data are expressed as mean ± SD. *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$

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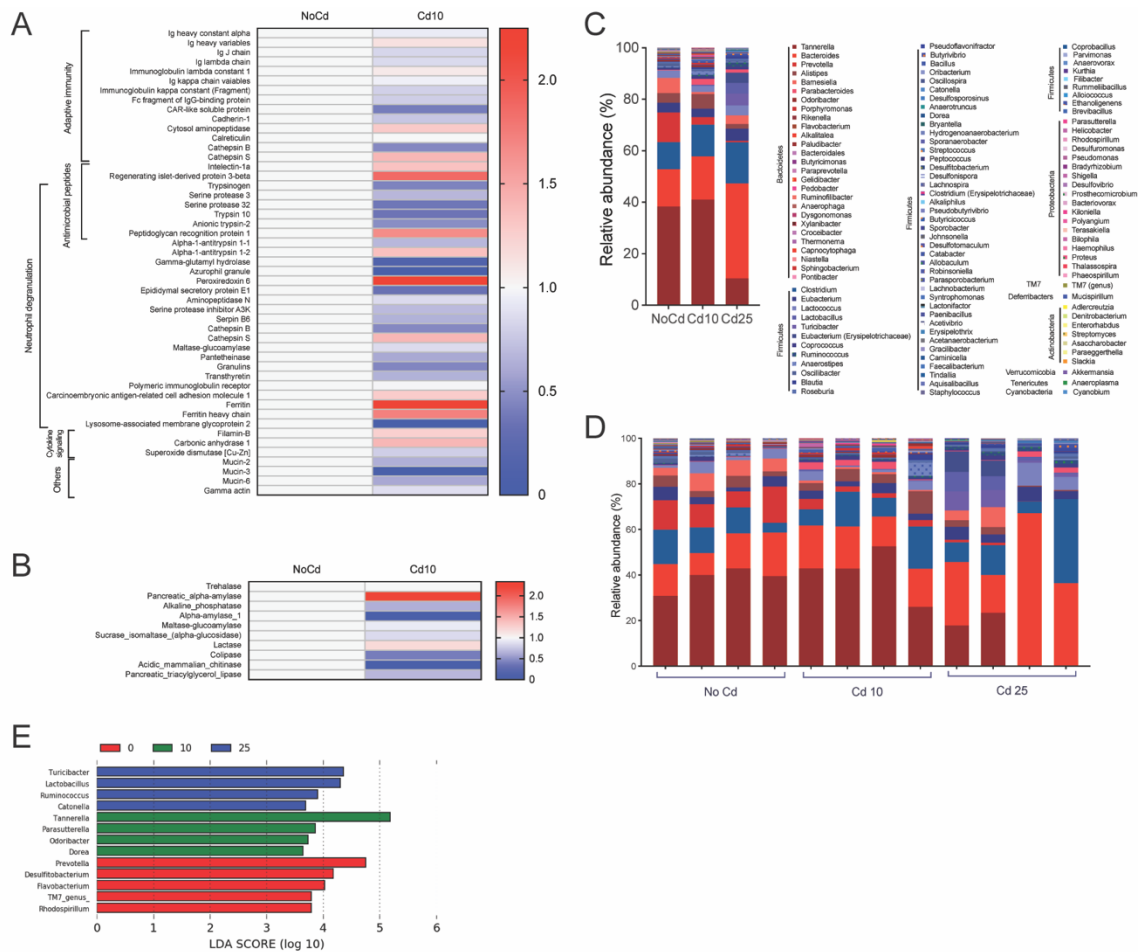
Figure S3. **Chronic ingestion of subtoxic doses of cadmium alters the gut microbiome, and luminal proteomic profiles**

(A and B) Proteomics analysis. Freshly emitted fecal pellets were collected from mice treated with 28 days no Cd (No Cd) or subtoxic doses of Cd (Cd10 and Cd25) and dissolved in PBS (0.1 g feces/ml PBS). Proteomics profiles were analyzed and results expressed as heat-map of mean values showed expression of relative amount of proteins related with (A) immune responses and (B) digestions. (C-E) Composition of microbiome at the genus levels. (C) Mean relative abundance of bacterial genera. (D) Relative abundance of the bacterial genera in individual mice. (E) Linear discriminant analysis (LDA) of bacterial genera. (n = 4 per group). Data are expressed as mean. (n = 4 per group)
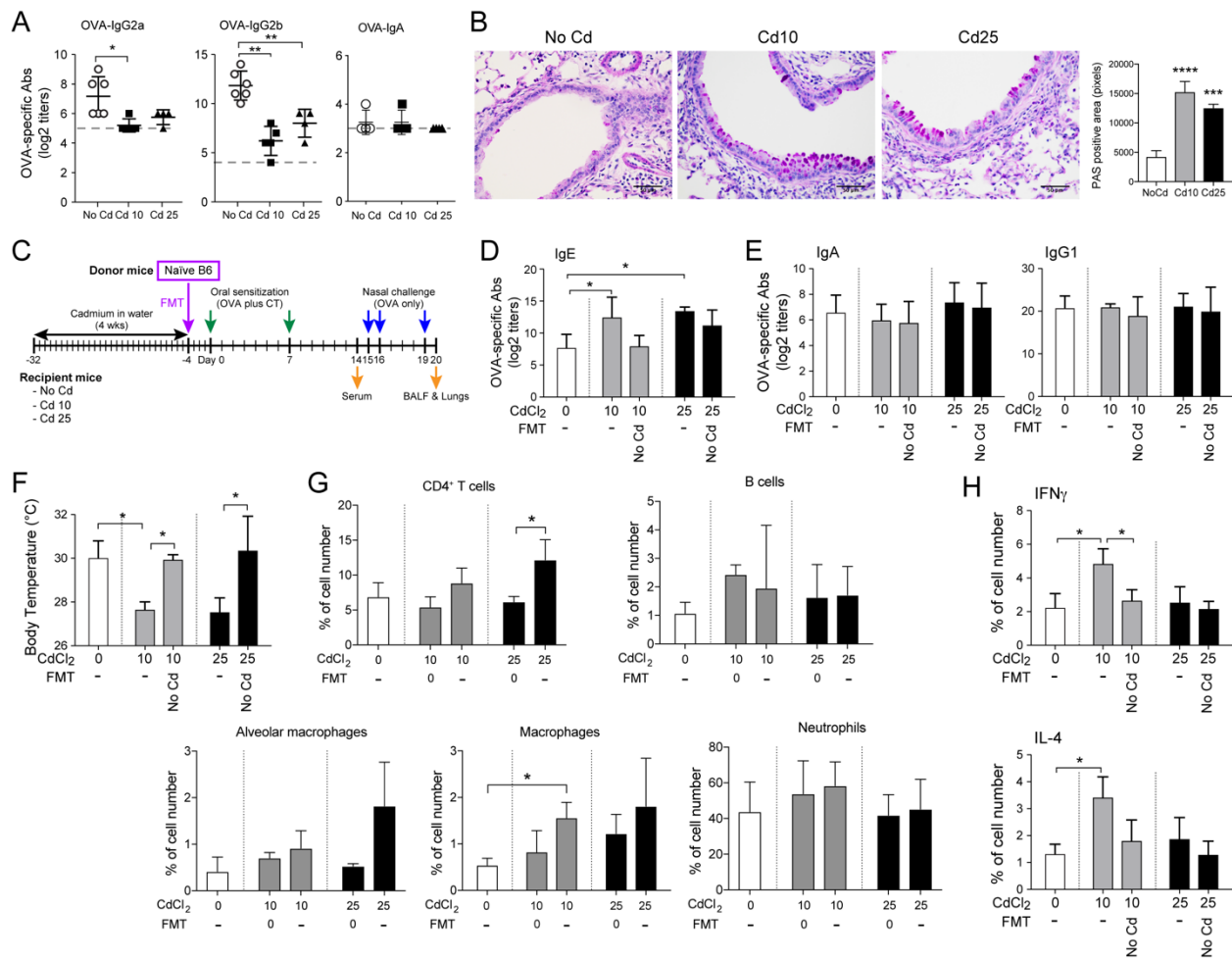
Figure S4. Intestinal content regulates the IgE-promoting effect of Cd.

(A) Allergen-specific serum IgG subclasses and IgA responses after oral sensitization of FMT recipient mice not exposed to Cd (n = 5). (B) Representative lung histology (left panel) and quantification (right panel) of mucus production (PAS staining) after nasal allergen challenge of orally sensitized mice. (C-H) Cd-treated [10 μM (Cd10) or 25 μM (Cd25)] mice were given a single transplantation of fecal material (FMT) from naïve mice before oral sensitization (n = 5). (C) Experimental scheme for transplantation of fecal materials from untreated mice. (D) Allergen-specific serum IgE, (E) IgA and IgG1 responses. (F) Surface body temperature 1 h after nasal allergen challenge. (G) Immune cell subsets present in BALF after nasal allergen challenges were

344   analyzed by flow cytometry. (H) CD4$^+$ T cell cytokine responses in BALF after nasal allergen

345   challenges. Data are expressed as mean ± SD (n = 5). *$p < 0.05$; **$p < 0.01$.

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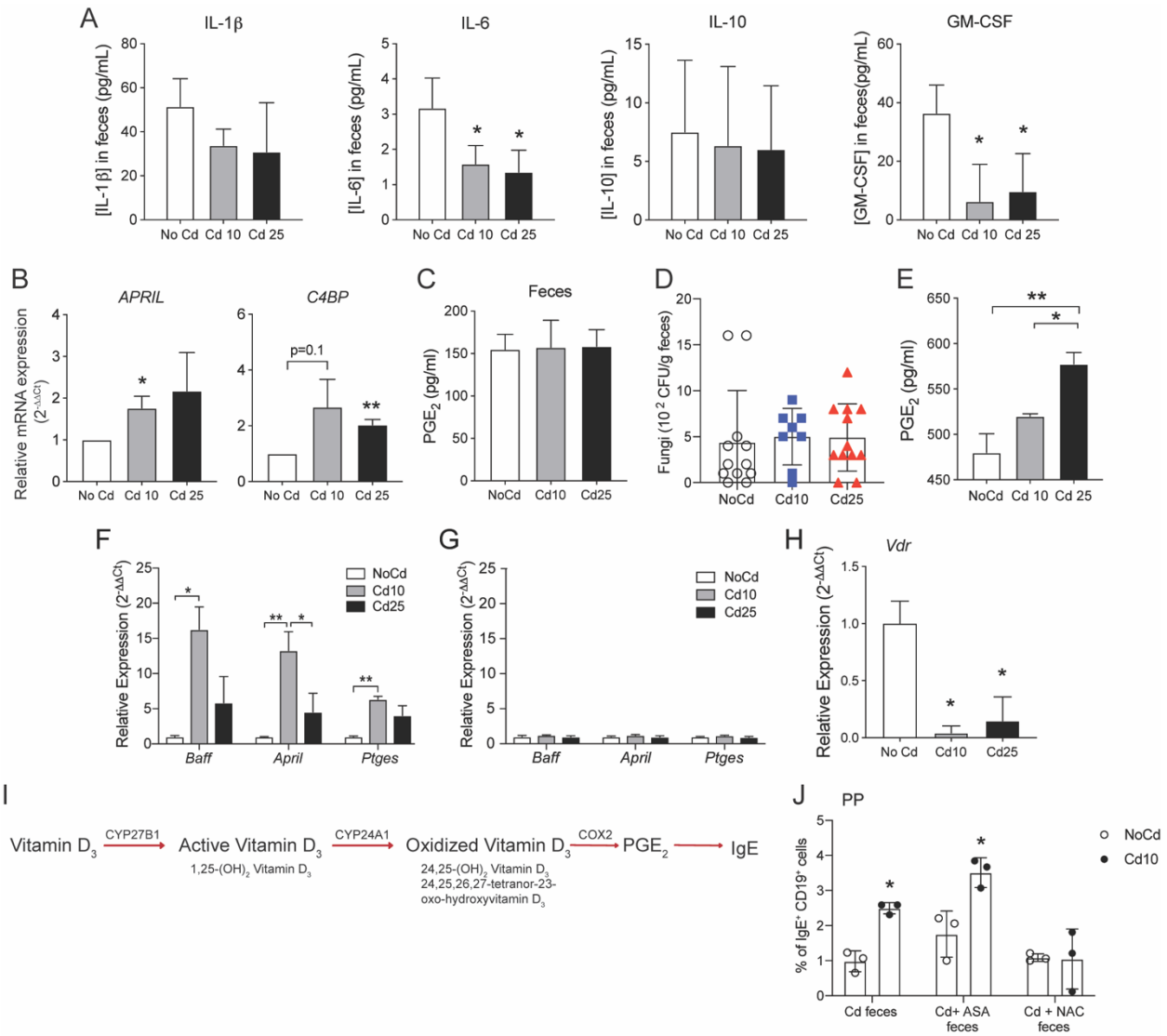
Figure S5. Cytokines and PGE$_2$ levels in fecal extracts of cadmium-treated mice and upstream and downstream signals *in vitro*.

(A)Cytokines levels in bacteria-free fecal extracts were analyzed by multi-plex assay. (B) Expression of *APRIL* and *C4BP* mRNA level from human intestinal epithelial cells (HT-29) were analyzed after cultured with bacteria-free fecal extracts by real-time RT PCR. (C) Level of PGE$_2$ in fecal extracts from Cd-ingested mice. (D) *Candida* load in fecal extracts. Dilution of fecal pellets were normalized by mass (g) of feces, plated on Sabouraud dextrose agar containing 50 μg/mL gentamycin and 50 μg/mL chloramphenicol, and fungal CFU were counted after 24 h culture. (E)

356   PGE$_2$ secretion by *Trif/Tram* double KO murine macrophages cultured 4 h in the presence of

357   bacteria-free fecal extracts. (F and G) Expression of *Baff*, *April* and *Ptges* mRNA responses by

358   splenocytes stimulated for 24 h with anti-CD40, IL-4 in the presence of bacteria-free fecal extracts

359   only (F), or together with the COX2 inhibitor celocoxib (G). (H) Expression of *Vdr* by J774

360   macrophage cell-line cultured 16 h in the presence of bacteria-free fecal extracts. (I) Brief

361   summary of expected mechanism of IgE production through vitamin D$_3$ metabolism. (J) Numbers

362   of IgE$^+$CD19$^+$ cells in culture of payer's patch cells from Verigem IgE mice simulated *in vitro*

363   with anti-CD40 (1 µg/mL) and IL-4 (10 ng/mL)in the presence of bacteria-free fecal extracts from

364   mice exposed to Cd (Cd), mice exposed to Cd and treated with COX2 inhibitor (Cd + ASA), or

365   mice exposed to Cd and treated with antioxidant after exposed to Cd (Cd + NAC) (n = 5). Data are

366   from four independent experiments and are expressed as mean ± SD. *p < 0.05; **p < 0.01;

367   **p<0.001.

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370   References

371   1.   Augeron C, Laboisse CL. Emergence of permanently differentiated cell clones in a human

372   colonic cancer cell line in culture after treatment with sodium butyrate. *Cancer Res.*

373   1984;44(9):3961-9.

374   2.   Jarup L. Hazards of heavy metal contamination. *Br Med Bull*. 2003;68:167-82.

375   3.   Kundu S, Sengupta S, Chatterjee S, Mitra S, Bhattacharyya A. Cadmium induces lung

376   inflammation independent of lung cell proliferation: a molecular approach. *J Inflamm (Lond)*.

377   2009;6:19.

378   4.   Bonnegarde-Bernard A, Jee J, Fial MJ, Aeffner F, Cormet-Boyaka E, Davis IC, et al.

379   IKKbeta in intestinal epithelial cells regulates allergen-specific IgA and allergic inflammation at

380   distant mucosal sites. *Mucosal Immunol.* 2014;7(2):257-67.

381   5.   Kim E, Lembert M, Fallata GM, Rowe JC, Martin TL, Satoskar AR, et al. Intestinal

382   Epithelial Cells Regulate Gut Eotaxin Responses and Severity of Allergy. *Front Immunol.*

383   2018;9:1692.

384   6.   Dowd SE, Wolcott RD, Sun Y, McKeehan T, Smith E, Rhoads D. Polymicrobial nature of

385   chronic diabetic foot ulcer biofilm infections determined using bacterial tag encoded FLX

386   amplicon pyrosequencing (bTEFAP). *PLoS One*. 2008;3(10):e3326.

387   7.   Huang C, Leung RK, Guo M, Tuo L, Guo L, Yew WW, et al. Genome-guided Investigation

388   of Antibiotic Substances produced by Allosalinactinospora lopnorensis CA15-2(T) from Lop Nor

389   region, China. *Sci Rep.* 2016;6:20667.

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