Rotavirus susceptibility of antibiotic-treated mice ascribed to diminished expression of interleukin-22

Daniel Schnepf[1*], Pedro Hernandez[2], Tanel Mahlakõiv[1], Stefania Crotta[3], Meagan E. Sullender[4], Stefan T. Peterson[4], Annette Ohnemus[1], Camille Michiels[5], Ian Gentle[6,7], Laure Dumoutier[5], Celso A. Reis[8,9], Andreas Diefenbach[10,11], Andreas Wack[3], Megan T. Baldridge[4], and Peter Staeheli[1,7*]

[1] Institute of Virology, Medical Center University of Freiburg, Freiburg, Germany
[2] Institut Curie, PSL Research University, INSERM U934, CNRS UMR3215, Development and Homeostasis of Mucosal Tissues Group, Paris, France
[3] Immunoregulation Laboratory, The Francis Crick Institute, London, United Kingdom
[4] Department of Medicine, Division of Infectious Diseases, Edison Family Center for Genome Sciences & Systems Biology, Washington University School of Medicine, St. Louis, MO, USA
[5] de Duve Institute, Université catholique de Louvain, Brussels, Belgium
[6] Institute of Medical Microbiology and Hygiene, Medical Center University of Freiburg, Freiburg, Germany
[7] Faculty of Medicine, University of Freiburg, Freiburg, Germany
[8] Instituto de Investigação e Inovação em Saúde, University of Porto, Porto, Portugal
[9] Institute of Molecular Pathology and Immunology, University of Porto, Porto, Portugal
[10] Institute of Microbiology, Infectious Diseases and Immunology, Charité - Universitätsmedizin Berlin, Berlin, Germany
[11] Mucosal and Developmental Immunology, Deutsches Rheuma-Forschungszentrum, an Institute of the Leibniz Gemeinschaft, Berlin, Germany

[*] Corresponding authors:
Daniel Schnepf, Institute of Virology, Medical Center University of Freiburg,
Hermann-Herder-Strasse 11, D-79104 Freiburg, Germany.
Phone: +49-7612036583, E-mail: daniel.schnepf@uniklinik-freiburg.de
and
Peter Staeheli, Institute of Virology, Medical Center University of Freiburg,
Hermann-Herder-Strasse 11, D-79104 Freiburg, Germany.
Phone: +49-7612036579, E-Mail: peter.staeheli@uniklinik-freiburg.de

Short title: Rotavirus susceptibility of antibiotic-treated mice linked to expression of interleukin-22

Key words: rotavirus, antibiotic, germ-free, IL-22

Abstract

The commensal microbiota regulates susceptibility to enteric pathogens by fine-tuning mucosal innate immune responses, but how susceptibility to enteric viruses is shaped by the microbiota remains incompletely understood. Past reports have indicated that commensal bacteria may either promote or repress rotavirus replication in the small intestine of mice. We now report that rotavirus replicated more efficiently in the intestines of germ-free and antibiotic-treated mice compared to animals with an unmodified microbiota. Antibiotic treatment also facilitated rotavirus replication in type I and type III interferon (IFN) receptor-deficient mice, revealing IFN-independent proviral effects. Expression of interleukin-22 (IL-22) was strongly diminished in the intestine of antibiotic-treated mice. Treatment with exogenous IL-22 blocked rotavirus replication in microbiota-depleted wild-type and *Stat1*$^{-/-}$ mice, demonstrating that the antiviral effect of IL-22 in animals with altered microbiome is not dependent on IFN signaling. In antibiotic-treated animals, IL-22-induced a specific set of genes including *Fut2,* encoding fucosyl-transferase 2 that participates in the biosynthesis of fucosylated glycans which can mediate rotavirus binding. Interestingly, IL-22 also blocked rotavirus replication in antibiotic-treated *Fut2*$^{-/-}$ mice. Furthermore, IL-22 inhibited rotavirus replication in antibiotic-treated mice lacking key molecules of the necroptosis or pyroptosis pathways of programmed cell death. Taken together, our results demonstrate that IL-22 determines rotavirus susceptibility of antibiotic-treated mice, yet the IL-22-induced effector molecules conferring rotavirus resistance remain elusive.

54 **INTRODUCTION**

55 The microbiota dramatically alters host susceptibility to multiple viral infections via diverse
56 mechanisms [1-4]. Physical interaction with bacteria enhances viral infectivity for some enteric
57 viruses. Binding to bacterial surface lipopolysaccharides (LPS) enhances the stability of poliovirus
58 virions, resulting in decreased susceptibility of mice treated with an antibiotic cocktail compared to
59 mice with undisturbed microbiota [5, 6]. Binding to bacteria further promotes poliovirus attachment
60 to target cells, thereby facilitating simultaneous infection with multiple virions per cell and
61 promoting viral fitness by enhancing the frequency of genetic recombination between different virus
62 strains [7]. Similar mechanisms may account for the observed reduction of reovirus infectivity in
63 antibiotic-treated mice [5]. Reduced vertical transmission of mouse mammary tumor virus through
64 maternal milk in antibiotic-treated mice has also been observed, attributed to a lack of virus-bound
65 bacterial LPS which stimulate the production of the immunosuppressive cytokine IL-10 [8, 9].

66 For other enteric viruses, both direct and indirect interactions with the microbiota may be at work.
67 Human norovirus infection of B cells was enhanced in the presence of histo-blood group antigen-
68 expressing enteric bacteria [10], and direct binding of human and murine norovirus to commensal
69 bacteria appear to contribute to the proviral effects of the microbiota [11]. Other mechanisms of
70 regulating virus susceptibility rely on modulation of host signaling by the microbiota. Antibiotic
71 treatment decreased replication of persistent murine norovirus in the intestines of wild-type mice
72 but not of mice with defective interferon-λ (IFN-λ) signaling [12], indicating that the bacterial
73 microbiota limits the efficacy of IFN-λ-dependent innate immunity by an unknown mechanism.
74 Interestingly, commensal bacteria were found to facilitate acute murine norovirus infection of distal
75 gut regions while simultaneously inhibiting virus infection of the proximal small intestine [10, 13].
76 Virus inhibition in the proximal gut is secondary to priming of IFN-λ expression by bacteria-
77 biotransformed bile acids [13].

78 The microbiota was further shown to restrict rather than to promote viral growth in several other
79 experimental systems [4, 14, 15]. In these cases, commensal-derived signals provided tonic type I
80 IFN-mediated immune stimulation that lowered the activation threshold of the innate immune
81 system required for optimal antiviral immunity. Accordingly, mice treated with an antibiotic cocktail
82 exhibited impaired epithelial, innate and adaptive antiviral responses resulting in delayed viral
83 clearance after exposure to systemic LCMV or mucosal influenza virus infections [4, 15].
84 Mononuclear phagocytes of microbiome-depleted mice no longer expressed inflammatory response
85 genes so that priming of natural killer cells and antiviral immunity to murine cytometagalovirus was
86 severely compromised [14].

87 How the microbiota modulates rotavirus replication is incompletely understood, despite this being a
88 pathogen of great clinical importance [16]. Antibiotic-mediated microbiome modulation enhanced

89 fecal shedding of a rotavirus live vaccine in adult volunteers [17], and gram-negative probiotics
90 protected against human rotavirus infection of gnotobiotic pigs [18]. However, antibiotic treatment
91 has been reported to suppress rotavirus infection of gut epithelial cells, with an overall reduction in
92 the incidence and duration of diarrhea in suckling mice [19]. Conversely, some mouse colonies have
93 been found to be highly resistant to rotavirus infection, a resistance that can be transferred to
94 susceptible mice via fecal microbial transplantation [20], indicating that a specific component of the
95 microbiome can mediate antiviral effects. Various probiotics including *Bifidobacterium sp.* have been
96 identified as protective against rotavirus infection [21, 22], and particular segmented filamentous
97 bacteria (SFB) were shown to be sufficient to protect mice against rotavirus infection and associated
98 diarrhea [20]. Interestingly, SFB-mediated protection was independent of known rotavirus-impeding
99 factors such as IFN-λ [23-25], interleukin-18 (IL-18) [26] and IL-22 [26, 27], and instead rotavirus
100 resistance correlated with accelerated epithelial cell turnover in this experimental system [20].
101 Bacterial flagellin alone is also sufficient to mediate potent antiviral effects against rotavirus, acting
102 through TLR5 to promote IL-22- and IL-18-dependent antiviral activity [26]. Thus, the relative pro-
103 and antiviral effects of the commensal microbiota on rotavirus infection remain unclear.
104 IFN-λ and IL-22 are both members of the IL-10 cytokine family. Their specific receptor chains (IFN-
105 λR1 and IL-22Rα, respectively) both associate with IL-10Rβ to form functional heterodimeric receptor
106 complexes, and the *Ifnlr1* and *Il22ra1* genes are close relatives located in adjacent positions in the
107 mouse and human genomes [28, 29]. The IFN-λ and the IL-22 receptors are highly expressed in
108 epithelial cells, and both cytokines cooperate for the induction of IFN-stimulated genes and the
109 control of rotavirus infection in mice [27]. IL-22 dramatically alters epithelial cell expression of a
110 variety of genes, ultimately stimulating proliferation and migration of intestinal epithelial cells
111 toward villus tips, driving increased extrusion of the highly differentiated enterocytes in which
112 rotavirus replicates [26, 30]. However, the antiviral activity of IL-22 is less well-understood compared
113 with its well-described function as a key mediator of anti-bacterial responses [31, 32]. IL-22
114 expression is controlled by the commensal microbiota [33] and, in turn, IL-22 can modulate the
115 composition of the microbiota [34].
116 To better understand the role of commensal bacteria in host defense against rotavirus, we
117 performed infections of microbiota-depleted mice and we evaluated the antiviral activity of IL-22
118 under gnotobiotic conditions. Using antibiotic-treated and germ-free mice we found that the
119 microbiota strongly repressed rotavirus replication in the intestinal tract. Our data indicate that
120 rotavirus susceptibility of microbiota-depleted mice results from poor expression of IL-22 in the
121 intestine. We further show that IL-22 confers rotavirus resistance in microbiota-depleted mice by a
122 mechanism that is independent of IFN-λ or STAT1 signaling, fucosyl-transferase 2, or mediators of
123 inducible cell death.

124 **MATERIAL & METHODS**

125 Mice: Conventional specific pathogen free (SPF) C57BL/6J mice of both sexes were purchased from
126 Janvier Labs. Germ-free (GF) C57BL/6 mice were obtained from the Clean Mouse Facility of the
127 University of Bern, Bern, Switzerland. GF mice were kept in autoclaved individually ventilated cages,
128 and sterile water and food was provided. GF mice were handled exclusively in a biosafety cabinet.
129 C57BL/6J mice lacking functional *Fut2*, *Ripk3* or *Casp1/11* genes [35-37] as well as mutant B6.A2G-
130 *Mx1-Ifnlr1*$^{-/-}$ [38], B6.A2G-*Mx1-Ifnlr1*$^{-/-}$*Il22*$^{-/-}$ [27], B6.A2G-*Mx1-Stat1*$^{-/-}$ [39] and corresponding
131 B6.A2G-*Mx1* wild-type mice [38] were bred and housed in the animal facilities of the University
132 Medical Center Freiburg. Other C57BL/6J mice originally purchased from Jackson Laboratories (stock
133 000664; Jackson Laboratories, Bar Harbor, ME) were bred and housed in animal facilities of
134 Washington University in Saint Louis under specific-pathogen-free (including murine norovirus-free)
135 conditions. Generation of *Ifnlr1*$^{-/-}$ mice was previously described [40]; briefly, these mice were
136 established by interbreeding *Ifnlr1*$^{tm1a(EUCOMM)Wtsi}$ mice and deleter-Cre mice, followed by backcrossing
137 by speed congenics onto a C57BL/6J background. *Ifnar1*$^{-/-}$ (B6.129.Ifnar1tm1) were crossed with
138 these *Ifnlr1*$^{-/-}$ mice to generate *Ifnar1*$^{-/-}$*Ifnlr1*$^{-/-}$ double knockout mice. Sentinel animals of our
139 housing facilities were routinely checked for unwanted pathogens by serological and molecular
140 technologies. These observations yielded no evidence for spontaneous infections of our mice with
141 rotavirus.
142
143 Ethics statement: All experiments with mice were carried out in accordance with the guidelines of
144 the Federation for Laboratory Animal Science Associations and the national animal welfare body.
145 Experiments were in compliance with the German animal protection law and were approved by the
146 animal welfare committee of the Regierungspräsidium Freiburg (permit G-16/98). All experiments at
147 Washington University were conducted according to regulations stipulated by the Washington
148 University Institutional Animal Care and Use Committee and to animal protocol 20190126, approved
149 by the Washington University Animal Studies Committee.
150
151 Antibiotic treatment of mice: To deplete the commensal microbiota, four- to six-week old mice
152 received drinking water *ad libitum* containing 1 mg/ml Cefoxitin (Santa Cruz Biotechnology, Inc), 1
153 mg/ml gentamycin sulfate (Sigma-Aldrich), 1 mg/ml metronidazole (Sigma-Aldrich) and 1 mg/ml
154 vancomycin (HEXAL®) for 4 weeks. For experiments at Washington University, six-week old mice
155 received drinking water with 1 mg/ml ampicillin, 1 mg/ml metronidazole, 1 mg/ml neomycin, 0.5
156 mg/ml vancomycin (Sigma, St. Louis, MO) in 20 mg/ml grape Kool-Aid (Kraft Foods, Northfield, IL), or
157 with Kool-Aid alone, *ad libitum* for 9-14 days. In most experiments we confirmed the absence of live
158 bacteria in fecal samples by plating the material on suitable agar plates.

159

160 Virus stocks and infections: To produce the rotavirus used in Freiburg, six day old suckling BALB/c
161 mice were infected orally with 680 infectious dose 50 (ID_{50}) (5 µl of a 1:250 dilution) from a stock
162 containing $3.4x10^7$ ID_{50}/ml of murine rotavirus strain EDIM [23]. Four days post-infection, mice were
163 sacrificed and colon samples with content were homogenized twice for 18 sec at 6 m/s using a
164 FastPrep®-12 homogeniser (MP Biomedicals). The homogenate was cleared by centrifugation at
165 5,000 rpm for 15 min. Supernatants were pooled and filtered using a 10 ml syringe and 4.5 µm sterile
166 filters. The filtrate was aliquoted and stored at -80 °C. Viral protein VP6 was quantified by
167 RIDASCREEN® ELISA in comparison to the parental stock. The infectious dose 50 (ID_{50}) was
168 determined by orally infecting groups of six C57BL/6 suckling mice with 5 µl samples of 10-fold serial
169 dilutions, ranging from 10^{-3} to 10^{-7}. Mice were sacrificed 24 h post-infection and intestines were
170 analyzed for the presence of RNA encoding for VP6 by RT-qPCR. Adult mice (8-10 week old) were
171 orally infected with $2.4x10^4$ ID_{50} in 100 µl by oral gavage using a gavage needle (19G).
172 To produce the rotavirus used in Saint Louis, four day old suckling CD-1 mice (Charles River) were
173 infected orally with 400 diarrhea dose 50 (5 µl of a 1:10 dilution in PBS + Evan's blue) from a stock of
174 murine rotavirus strain EC-RV received from Estes Lab at Baylor College of Medicine. Three days post-
175 infection, mice were sacrificed and the entire small intestine and colon with contents were harvested
176 and pooled for weighing. A 20% homogenate was prepared in homogenization media of DMEM
177 (Gibco 11995-040) supplemented with 1% penicillin/streptomycin (Gibco 15140-122) by three rounds
178 of homogenization on ice (homogenize for 1 min, then allow to settle for 30 sec). Homogenate was
179 pooled on ice, aliquoted, and then stored at -80˚C until use. The shedding dose 50 (SD_{50}) of the stock
180 was determined by orally infecting groups of five adult mice (6-8 weeks old), of both BALB/c and
181 C57BL/6 genotypes, with 100 µl of 10-fold serial dilutions, ranging from 10^{-5} to 10^{-9} for BALB/c and
182 10^0 to 10^{-5} for C57BL/6, and analyzing fecal pellets for RV genome copies by RT-qPCR. Adult mice (8-
183 10 weeks old) were orally infected with 10^4 SD_{50} in 100 µl by oral gavage, preceded by 100 µl of
184 sterile 1.33% sodium bicarbonate solution.

185

186 Viral protein quantification in feces by RIDASCREEN® Rotavirus ELISA: Fecal samples were
187 homogenized according to weight with RIDASCREEN® sample dilution buffer 1 and homogenized
188 twice for 11 sec with 6 m/s using a FastPrep®-12 homogenizer. Homogenates were cleared by
189 centrifugation [5,000 rpm, 5 min, 4 °C] and 100 µl of fecal homogenate supernatants were used for
190 RIDASCREEN® Rotavirus ELISA. ELISA was performed according to the manufacturer's instructions.
191 Optical density was measured at 450 nm wave length by using a Tecan Infinite 200 plate reader.

192

193 RNA isolation and RT-qPCR: RNA was isolated using the Direct-zol™ RNA MiniPrep Kit (Zymo
194 Research). Two-three cm pieces of ilea were homogenized in 1 ml TriFast™ twice for 18 sec with 6
195 m/s using a FastPrep®-12 homogenizer (MP Biomedicals). Homogenates were centrifuged at 12,000 g
196 for 5 min and supernatant were diluted 1:10 in TriFast™. RNA was then isolated following the
197 manufacturer's instructions. LunaScript™ RT SuperMix Kit (New England Biolabs) was used to
198 generate cDNA from 850 ng of total RNA following the manufacturer's instructions. Resulting cDNA
199 served as template for the amplification of transcripts from *Ubc* (QT00245189, QuantiTect Primer
200 Assay, Qiagen), *Hprt* (mm00446968_m1, Applied Biosystems), *Il22* (forward: 5'-
201 CATGCAGGAGGTGGTACCTT -3'; reverse: 5'- CAGACGCAAGCATTT CTCAG -3'), *Reg3b* (forward: 5'-
202 GCTGGAAGTTGGACACCTCAA -3'; reverse: 5'- GACATAGGGCAACTTCACCTCACA -3'), *Reg3g* (forward:
203 5'- TTCCTGTCCTCCATGATCAAAA -3'; reverse: 5'- CATCCACCTCTGTTGGGTTCA -3'), *Saa1* (forward: 5'-
204 AAATCAGTGATGGAAGAGAGGC -3'; reverse: 5'- CAGCACAACCTACTGAGCTA -3'), *Fut2* (forward: 5'-
205 ACCTCCAGCAACGAATAGTGA -3'; reverse: 5'- GCCGATGGAATTGATCGTGAA -3'), *Rps29 (*forward 5'-
206 GCAAATACGGGCTGAACATG-3'; reverse 5'-GTCCAACTTAATGAAGCCTATGTC-3') by real-time PCR using
207 TaqMan Gene Expression Assays (Applied Biosystems), Universal PCR Master Mix (Applied
208 Biosystems) and the QuantStudio 5 Real-Time PCR system (Applied Biosystems by Thermo Fisher
209 Scientific). Increase in transcript levels were determined by the $2^{-\Delta Ct}$ method relative to expression of
210 the indicated housekeeping genes.
211
212 Intestinal epithelial cell enrichment: Small intestines were collected and cut into 3-6 cm long pieces,
213 mechanically cleaned, rinsed with PBS using a gavage needle (19G) and inverted on customized
214 inoculating loops. Samples were incubated in 30 mM EDTA for 10 min at 37 °C. Intestinal epithelia
215 cells were detached from organ pieces by centripetal force using a customary electric drill
216 [approximately 1,000 rpm]. Samples were centrifuged with 2-3 short impulses in 50 ml PBS with 5 %
217 FCS. After 1 h of sedimentation, the lower half of the suspension was isolated, cells were pelleted
218 [1,300 rpm, 10 min, 4 °C], lyzed in TriFast™ and total RNA isolated using the Direct-zol™ RNA
219 MiniPrep Kit (Zymo Research).
220
221 Transcriptome analysis: RNA for transcriptome analysis was isolated using the RiboPure™ Kit
222 (Invitrogen) according to the manufacturer's instructions. In brief, 2-3 cm pieces of ilea were
223 homogenized in 1 ml TriFast™ twice for 18 sec with 6 m/s using a FastPrep®-12 homogenizer (MP
224 Biomedicals). Homogenates were centrifuged with 12,000 g for 10 min at 4 °C, 600 µl of supernatant
225 mixed with 500 µl TriFast™, centrifuged again with 12,000 g for 10 min at 4 °C and 1 ml of
226 supernatant was mixed with 100 µl of 1-bromo-3-chloropropane and incubated for 5 min at room
227 temperature. Phase separation was achieved by centrifugation with 12,000 g for 10 min at 4 °C. 400

228 µl of the aqueous phase was then mixed with 200 µl of ethanol and passed through the filter
229 cartridge by centrifugation (12,000 g for 1 min), the filter cartridge was then washed twice with 500
230 µl wash solution and RNA was finally eluted with 100 µl elution buffer.
231
232 MicroArray: Total RNA harvested from intestinal epithelia cells was hybridized using Illumina Mouse
233 WG-6_V2_0_R0_11278593_A arrays. The raw intensity values for each entity were preprocessed by
234 RMA normalization against the median intensity in mock-treated samples. Using GeneSpring 11.5, all
235 transcripts were then filtered based on flags (present or marginal, in at least 2 out of 10 samples).
236 Moderate t-test (IL22-treated versus mock-treated) was performed to identify genes differentially
237 expressed relative to controls (≥5-fold change; $p<0.01$, Benjamini-Hochberg multiple test correction).
238 Raw microarray data can be accessed at GEO under accession number GSE166400.
239
240 Statistical analysis: For statistical analyses the GraphPad Prism 8 software (GaphPad Software, USA)
241 was used. Testing for statistical significance was performed on log-transformed values by using
242 ordinary one-way ANOVA with Tukey's multiple comparisons or the unpaired Student's t-test.
243 Asterisks indicate p-values: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.001$.
244
245
246 **RESULTS**
247 **Germ-free and antibiotic-treated mice are highly susceptible to oral infection with rotavirus**
248 To investigate the effect of the microbiota on rotavirus susceptibility, we monitored the levels of
249 rotavirus antigen in the feces of three experimentally infected groups of adult C57BL/6 mice, namely
250 conventionally-housed specific pathogen-free (SPF) animals, SPF animals treated with an antibiotic
251 cocktail (Abx), and germ-free (GF) animals. During the 6-day observation period following oral
252 infection with rotavirus, viral antigen was nearly undetectable in fecal samples of SPF mice by ELISA
253 (Fig. 1). In contrast, fecal samples from the majority of infected Abx and GF mice contained high
254 levels of rotavirus antigen (Fig. 1), indicating that the microbiota has an inhibitory effect on rotavirus
255 replication.
256 Since an earlier study indicated that the microbiota promotes rather than inhibits rotavirus
257 replication [19], we sought to confirm this finding in an unrelated colony of C57BL/6 wild-type mice
258 at a second independent institution, using an independently prepared rotavirus stock. By measuring
259 viral RNA in fecal samples on day 5 post-infection by RT-qPCR analysis we again found that wild-type
260 (WT) mice with undisturbed microbiota shed only low amounts of virus whereas virus shedding of
261 antibiotic-treated WT mice was strongly enhanced (Fig. 2). Prior work indicated that the type I and
262 the type III IFN systems inhibit the replication of rotavirus in the mouse intestine [24, 25]. Therefore,

263 we assessed the possibility that microbiota-mediated rotavirus resistance might depend on IFN
264 signaling. In agreement with earlier studies [24, 25], we observed that animals lacking functional
265 receptors for type I IFN (*Ifnar1*$^{-/-}$), for type III IFN (*Ifnlr1*$^{-/-}$) or for both IFN types (DKO) were more
266 susceptible to rotavirus infection compared with WT mice (Fig. 2). Importantly, however, treating
267 these mutant mice with antibiotics also enhanced rotavirus levels on average at least 50-fold (Fig. 2),
268 demonstrating that enhanced intestinal replication of rotavirus in mice with reduced microbiota is
269 not only due to impaired activation of type I IFN and/or type III IFN pathways by commensal bacteria.
270 Rotavirus shedding by untreated mutant mice was surprisingly heterogeneous (Fig. 2), raising the
271 possibility that the microbiota composition of individual animals could matter. Since our animal
272 facilities are not free of segmented filamentous bacteria (SFB) which are known to confer rotavirus
273 resistance [20], we speculate that the degree of SFB colonization in individual animals could influence
274 intrinsic rotavirus susceptibility.
275
276 **IL-22 confers rotavirus resistance in antibiotic-treated mice via a mechanism that is not dependent**
277 **on IFN-λ or STAT1 signaling**
278 To identify IFN-independent antiviral factors which are present in the intestine of SPF mice but
279 absent or strongly diminished in GF mice, we performed RT-qPCR analysis of cytokines previously
280 linked to rotavirus resistance [24-26]. We found that expression of *Il22* was on average about 200
281 fold lower in ileum samples from uninfected GF (Fig. 3A) or Abx mice (Fig. 3B) than in samples from
282 uninfected SPF mice. Furthermore, compared with SPF mice, expression of the IL-22-induced *Reg3b*
283 and *Reg3g* genes was on average 10-20 fold reduced in the ileum of GF (Fig. 3A) and Abx mice (Fig.
284 3B), demonstrating that the high rotavirus susceptibility of animals with diminished microbiota
285 correlates with diminished baseline expression of IL-22.
286 We next tested whether IL-22 can confer rotavirus resistance by treating Abx mice with recombinant
287 IL-22 one day before and on days 0, 1, 2 and 4 after oral infection with rotavirus, then monitoring
288 fecal virus shedding by ELISA for the next 12 days. All saline-treated control mice acutely shed high
289 amounts of viral antigen, whereas all IL-22-treated animals exhibited low levels of fecal viral antigen
290 during the first 5 days post-infection (Fig. 3C). However, four of the five IL-22-treated mice shed virus
291 with delayed kinetics, indicating that the protective effect of exogenous IL-22 was short-lived.
292 Therefore, in a second experiment, the IL-22 treatment period was extended and daily injections of
293 IL-22 were continued until day 8 post-infection. Under these more stringent conditions, IL-22-
294 mediated suppression of virus shedding was very effective (Fig. 3D).
295 As synergistic activity between IL-22 and IFN-λ to control rotavirus has previously been reported [27],
296 we assessed whether the rotavirus-inhibitory effect of IL-22 was direct or mediated by IFNs. We
297 repeated the IL-22 treatment study using mice in which either IFN-λ (*Ifnlr1*$^{-/-}$) or all IFN subtypes

298 (*Stat1*$^{-/-}$) are no longer functional, and found that daily injections of IL-22 were still highly effective at
299 suppressing rotavirus shedding in antibiotic-treated *Ifnlr1*$^{-/-}$ (Fig. 3E) and *Stat1*$^{-/-}$ (Fig. 3F) mice. Thus,
300 IL-22 confers virus resistance in microbiota-depleted mice by a mechanism that is not dependent on
301 IFN-λ or STAT1 signaling.
302
303 **Microbes modulate the IL-22 response of the intestinal epithelium**
304 To identify IL-22-induced effector molecules which confer rotavirus resistance in microbiota-depleted
305 mice, we compared gene expression profiles in the ileum of antibiotic-treated *Ifnlr1*$^{-/-}$ mice which
306 received either saline or IL-22. We identified 22 genes that showed at least 5-fold increased
307 expression and 3 genes with at least 5-fold decreased expression after IL-22 treatment compared
308 with mock-treated mice (Fig. 4A). Enhanced expression of several genes, including *Saa1*, *Fut2* and
309 *Reg3g,* in tissue of IL-22-treated animals was confirmed by RT-qPCR (Fig. 4B).
310 The *Fut2* gene was of particular interest because it encodes the enzyme fucosyl-transferase 2 (FUT2)
311 that participates in the biosynthesis of histo-blood group antigens which can be recognized by
312 rotavirus [41]. To investigate whether IL-22 inhibits rotavirus growth in the intestine of antibiotic-
313 treated mice by regulating FUT2 expression, we first determined whether *Fut2*$^{-/-}$ mice with normal
314 gut flora were more susceptible to oral infection with rotavirus than standard wild-type mice, using
315 highly susceptible *Ifnlr1*$^{-/-}$*Il22*$^{-/-}$ mice as a positive control (Fig. 4C). *Fut2*$^{-/-}$ mice exhibited no defects in
316 rotavirus control. Next, we tested whether IL-22 might no longer be able to inhibit rotavirus
317 replication in antibiotic-treated *Fut2*$^{-/-}$ mice, but found IL-22 treatment to be highly effective in these
318 animals (Fig. 4D). We thus concluded that IL-22-mediated upregulation of *Fut2* in microbiota-
319 depleted mice does not play a decisive role in rotavirus resistance.
320 Since SFB increase the turnover of intestinal epithelial cells that results in rotavirus resistance [20],
321 microbiota-mediated acceleration of infected epithelial cell death might be responsible for the
322 intrinsically high rotavirus resistance of mice with intact microbiota. Therefore, we first evaluated the
323 possibility that the antiviral activity of IL-22 in antibiotic-treated mice results from accelerated death
324 of intestinal epithelial cells by necroptosis [42]. However, we found that IL-22 was effective in
325 inhibiting rotavirus growth in antibiotic-treated *Ripk3*$^{-/-}$ mice (Fig. 5A), in which cell death by
326 necroptosis cannot occur [43]. This finding excluded the possibility that IL-22 induces premature
327 death of rotavirus-infected cells by necroptosis in antibiotic-treated mice. An alternative form of
328 induced cell death is pyroptosis, in which caspase-1 plays a key role [44]. To evaluate the possibility
329 that IL-22 acts by inducing pyroptosis in infected cells, possibly via regulation of IL-18 expression [26,
330 45], we tested IL-22-induced antiviral effects in antibiotic-treated *Casp1/11*$^{-/-}$ mice. We found that IL-
331 22 remained active in these mice (Fig. 5B), precluding the possibility that IL-22 inhibits rotavirus by
332 triggering pyroptotic cell death in antibiotic-treated mice.

333 **DISCUSSION**

334 Our work revealed that the microbiota protects against rotavirus infection and inhibits virus
335 replication in the small intestine. These results are distinct from conclusions of an earlier study [19],
336 which indicated that antibiotic treatment renders mice resistant to rotavirus infection. Of interest,
337 our observation of a protective effect of the microbiota against rotavirus recapitulates observations
338 in neonatal gnotobiotic piglets, wherein the microbiota has been shown to protect against diarrhea
339 and viral shedding [46]. Our use of mice from two separate animal facilities located on different
340 continents, as well as two independently-generated rotavirus stocks of EDIM and EC, makes it
341 unlikely that housing conditions or unrecognized mutations in the virus genome are responsible for
342 this discrepancy. We speculate that delayed passage of content through the intestines of antibiotic-
343 treated mice [5, 12, 47] may contribute to differences in virus excretion between microbiota-replete
344 and -depleted mice, which ultimately resulted in differential interpretations regarding the influence
345 of the microbiota on virus resistance. Of note, our results are highly consistent with recent work that
346 attributed the presence of particular SFB with rotavirus resistance of mice [20].
347 Our study revealed that the rotavirus susceptibility phenotype of antibiotic-treated mice cannot be
348 explained by the involvement of known mediators of rotavirus resistance such as IFN-λ or type I IFN.
349 We confirmed earlier findings [24, 25] that mice with defective IFN receptors are intrinsically more
350 susceptible to rotavirus infection than wild-type mice. Importantly, however, when such mutant mice
351 were treated with antibiotics, their susceptibility to infection with rotavirus still increased, indicating
352 that poorly defined additional factors also contribute to rotavirus resistance. Our analysis suggests
353 that IL-22 plays an important role in this phenomenon. First, we observed that poor expression of IL-
354 22 and IL-22-regulated genes in the ileum correlates with rotavirus susceptibility of antibiotic-treated
355 mice. This observation is consistent with previous work demonstrating decreased intestinal IL-22
356 expression in the intestine in germ-free or antibiotics-treated mice that can be rescued with short-
357 chain fatty acid administration [48], which drives IL-22 production by CD4+ T cells and innate
358 lymphoid cells [49, 50].
359 Second, we found that application of IL-22 readily restored rotavirus resistance of antibiotic-treated
360 mice. Interestingly, unlike in mice with undisturbed microbiota in which the antiviral activity of IL-22
361 was strongly dependent on IFN-λ [27], the anti-rotavirus effect of IL-22 in antibiotic-treated mice was
362 not dependent on IFN-λ or transcription factor STAT1. Thus, IL-22 can negatively affect rotavirus
363 replication by at least two mechanisms, of which only one is dependent on IFN. The IFN-independent
364 mechanism plays a dominant role in antibiotic-treated mice, whereas the IFN-λ-dependent
365 mechanism dominates in mice with undisturbed microbiome.
366 We currently do not understand how IL-22 inhibits the replication of rotavirus in antibiotic-treated
367 mice. Our transcriptome analysis showed that *Fut2* is strongly induced by IL-22 under such

368 conditions. This finding is in good agreement with earlier work indicating that antibiotic-mediated
369 down-regulation of *Fut2* alters the glycosylation pattern of intestinal epithelial cells [51, 52]. Such
370 changes in protein glycosylation resulted in enhanced susceptibility of mice to infection with several
371 pathogenic bacteria, including *Citrobacter rodentium* [53], Helicobacter pylori [36, 52] and
372 Salmonella typhimurium [51]. However, our experiments with *Fut2*-deficient mice indicate that
373 antiviral defense is not reduced in these animals and that fucosyltransferase-2 is not the elusive IL-
374 22-induced mediator of rotavirus resistance.
375 IL-22 is known to act through the STAT3 transcription factor in intestinal epithelial cells to drive
376 expression of numerous antimicrobial genes, though it remains unclear which are particularly critical
377 for antiviral effects against rotavirus [26, 54]. Since the intestinal epithelium consists of short-lived
378 cells, we envisaged the possibility that IL-22 might inhibit rotavirus replication by inducing premature
379 death of infected cells. Yet, we found that IL-22 effectively blocked rotavirus replication in antibiotic-
380 treated mice lacking key factors for necroptosis or pyroptosis, rendering such a mechanism unlikely.
381 However, since these programmed cell death pathways are highly interconnected, we cannot
382 exclude the possibility of compensatory effects [55]. Available data suggest that SFB prevents and
383 cures rotavirus infection by enhancing the proliferation of infected epithelial cells in the terminal
384 ileum, promoting their shedding from the tissue [20]. We speculate that IL-22 may provide similar
385 growth-promoting stimuli to epithelial cells in antibiotic-treated mice and that enhanced cell
386 proliferation might explain the antiviral effect of IL-22 that we observed. It has also been suggested
387 that IL-22 may induce extrusion of intestinal epithelial cells [30], which remains a possibility in the
388 context of microbiota-mediated regulation of rotavirus infection. Recently, reversal of viral
389 appropriation of autophagic flux has been suggested as an antiviral mechanism for IL-22 in control of
390 respiratory syncytial virus, a possibility that remains open for its regulation of rotavirus [56]. Our
391 study reveals a clear role for the microbiota in maintenance of a homeostatic antiviral state, driven
392 by IL-22, against rotavirus infection, and future studies delineating the specific pathways required for
393 this activity may help reveal additional therapeutic approaches against this pathogen.
394

395 **ACKNOWLEDGEMENTS**

396 We thank the Clean Mouse Facility (CMF), University of Bern, Bern, Switzerland, for providing GF
397 mice, Ana Magalhães, University of Porto, Portugal for providing *Fut2*-deficient mice, and Otto Haller
398 for helpful comments on the manuscript.
399 This work was supported by a grant from the Deutsche Forschungsgemeinschaft to P.S., as well as
400 grants from the NIH (R01 AI141716 and R01 OD024917), the Children's Discovery Institute of
401 Washington University and St. Louis Children's Hospital Interdisciplinary Research Initiative (MI-II-
402 2019-790), and The Mathers Foundation to M.T.B.

403 **REFERENCES**

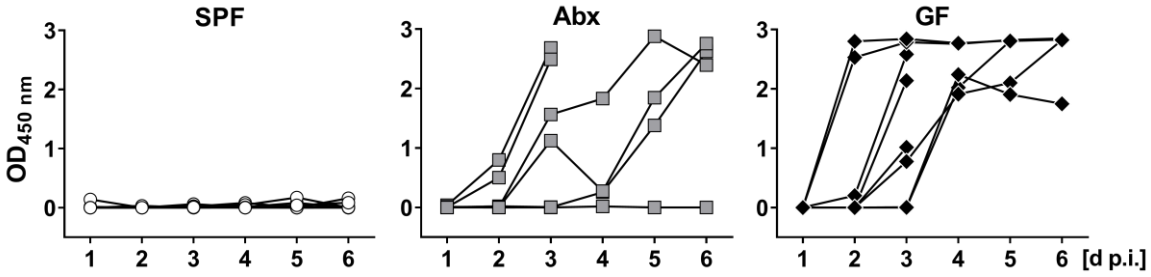
404 1. Shi Z, Gewirtz AT. Together Forever: Bacterial-Viral Interactions in Infection and Immunity.
405 Viruses. 2018;10(3). doi: 10.3390/v10030122. PubMed PMID: 29534424; PubMed Central
406 PMCID: PMCPMC5869515.
407 2. Karst SM. The influence of commensal bacteria on infection with enteric viruses. Nat Rev
408 Microbiol. 2016;14(4):197-204. doi: 10.1038/nrmicro.2015.25. PubMed PMID: 26853118;
409 PubMed Central PMCID: PMCPMC5198578.
410 3. Robinson CM, Pfeiffer JK. Viruses and the Microbiota. Annu Rev Virol. 2014;1:55-69. doi:
411 10.1146/annurev-virology-031413-085550. PubMed PMID: 25821837; PubMed Central PMCID:
412 PMCPMC4373533.
413 4. Abt MC, Osborne LC, Monticelli LA, Doering TA, Alenghat T, Sonnenberg GF, et al. Commensal
414 bacteria calibrate the activation threshold of innate antiviral immunity. Immunity.
415 2012;37(1):158-70. doi: 10.1016/j.immuni.2012.04.011. PubMed PMID: 22705104; PubMed
416 Central PMCID: PMCPMC3679670.
417 5. Kuss SK, Best GT, Etheredge CA, Pruijssers AJ, Frierson JM, Hooper LV, et al. Intestinal microbiota
418 promote enteric virus replication and systemic pathogenesis. Science. 2011;334(6053):249-52.
419 doi: 10.1126/science.1211057. PubMed PMID: 21998395; PubMed Central PMCID:
420 PMCPMC3222156.
421 6. Robinson CM, Jesudhasan PR, Pfeiffer JK. Bacterial lipopolysaccharide binding enhances virion
422 stability and promotes environmental fitness of an enteric virus. Cell Host Microbe.
423 2014;15(1):36-46. doi: 10.1016/j.chom.2013.12.004. PubMed PMID: 24439896; PubMed Central
424 PMCID: PMCPMC3920179.
425 7. Erickson AK, Jesudhasan PR, Mayer MJ, Narbad A, Winter SE, Pfeiffer JK. Bacteria Facilitate
426 Enteric Virus Co-infection of Mammalian Cells and Promote Genetic Recombination. Cell Host
427 Microbe. 2018;23(1):77-88 e5. doi: 10.1016/j.chom.2017.11.007. PubMed PMID: 29290575;
428 PubMed Central PMCID: PMCPMC5764776.
429 8. Kane M, Case LK, Kopaskie K, Kozlova A, MacDearmid C, Chervonsky AV, et al. Successful
430 transmission of a retrovirus depends on the commensal microbiota. Science.
431 2011;334(6053):245-9. doi: 10.1126/science.1210718. PubMed PMID: 21998394; PubMed
432 Central PMCID: PMCPMC3519937.
433 9. Wilks J, Lien E, Jacobson AN, Fischbach MA, Qureshi N, Chervonsky AV, et al. Mammalian
434 Lipopolysaccharide Receptors Incorporated into the Retroviral Envelope Augment Virus
435 Transmission. Cell Host Microbe. 2015;18(4):456-62. doi: 10.1016/j.chom.2015.09.005. PubMed
436 PMID: 26468748; PubMed Central PMCID: PMCPMC4795803.
437 10. Jones MK, Watanabe M, Zhu S, Graves CL, Keyes LR, Grau KR, et al. Enteric bacteria promote
438 human and mouse norovirus infection of B cells. Science. 2014;346(6210):755-9. doi:
439 10.1126/science.1257147. PubMed PMID: 25378626; PubMed Central PMCID:
440 PMCPMC4401463.
441 11. Madrigal JL, Bhar S, Hackett S, Engelken H, Joseph R, Keyhani NO, et al. Attach Me If You Can:
442 Murine Norovirus Binds to Commensal Bacteria and Fungi. Viruses. 2020;12(7). doi:
443 10.3390/v12070759. PubMed PMID: 32674489; PubMed Central PMCID: PMCPMC7412252.
444 12. Baldridge MT, Nice TJ, McCune BT, Yokoyama CC, Kambal A, Wheadon M, et al. Commensal
445 microbes and interferon-lambda determine persistence of enteric murine norovirus infection.
446 Science. 2015;347(6219):266-9. doi: 10.1126/science.1258025. PubMed PMID: 25431490;
447 PubMed Central PMCID: PMCPMC4409937.
448 13. Grau KR, Zhu S, Peterson ST, Helm EW, Philip D, Phillips M, et al. The intestinal regionalization of
449 acute norovirus infection is regulated by the microbiota via bile acid-mediated priming of type III
450 interferon. Nat Microbiol. 2020;5(1):84-92. doi: 10.1038/s41564-019-0602-7. PubMed PMID:
451 31768030; PubMed Central PMCID: PMCPMC6925324.
452 14. Ganal SC, Sanos SL, Kallfass C, Oberle K, Johner C, Kirschning C, et al. Priming of natural killer
453 cells by nonmucosal mononuclear phagocytes requires instructive signals from commensal

454 microbiota. Immunity. 2012;37(1):171-86. doi: 10.1016/j.immuni.2012.05.020. PubMed PMID:
455 22749822.
456 15. Bradley KC, Finsterbusch K, Schnepf D, Crotta S, Llorian M, Davidson S, et al. Microbiota-Driven
457 Tonic Interferon Signals in Lung Stromal Cells Protect from Influenza Virus Infection. Cell Rep.
458 2019;28(1):245-56 e4. doi: 10.1016/j.celrep.2019.05.105. PubMed PMID: 31269444.
459 16. Kim AH, Hogarty MP, Harris VC, Baldridge MT. The Complex Interactions Between Rotavirus and
460 the Gut Microbiota. Front Cell Infect Microbiol. 2020;10:586751. doi:
461 10.3389/fcimb.2020.586751. PubMed PMID: 33489932; PubMed Central PMCID:
462 PMCPMC7819889.
463 17. Harris VC, Haak BW, Handley SA, Jiang B, Velasquez DE, Hykes BL, Jr., et al. Effect of Antibiotic-
464 Mediated Microbiome Modulation on Rotavirus Vaccine Immunogenicity: A Human,
465 Randomized-Control Proof-of-Concept Trial. Cell Host Microbe. 2018;24(2):197-207 e4. doi:
466 10.1016/j.chom.2018.07.005. PubMed PMID: 30092197.
467 18. Vlasova AN, Shao L, Kandasamy S, Fischer DD, Rauf A, Langel SN, et al. Escherichia coli Nissle
468 1917 protects gnotobiotic pigs against human rotavirus by modulating pDC and NK-cell
469 responses. Eur J Immunol. 2016;46(10):2426-37. doi: 10.1002/eji.201646498. PubMed PMID:
470 27457183; PubMed Central PMCID: PMCPMC5201163.
471 19. Uchiyama R, Chassaing B, Zhang B, Gewirtz AT. Antibiotic treatment suppresses rotavirus
472 infection and enhances specific humoral immunity. J Infect Dis. 2014;210(2):171-82. doi:
473 10.1093/infdis/jiu037. PubMed PMID: 24436449; PubMed Central PMCID: PMCPMC4399425.
474 20. Shi Z, Zou J, Zhang Z, Zhao X, Noriega J, Zhang B, et al. Segmented Filamentous Bacteria Prevent
475 and Cure Rotavirus Infection. Cell. 2019;179(3):644-58 e13. doi: 10.1016/j.cell.2019.09.028.
476 PubMed PMID: 31607511.
477 21. Munoz JA, Chenoll E, Casinos B, Bataller E, Ramon D, Genoves S, et al. Novel probiotic
478 Bifidobacterium longum subsp. infantis CECT 7210 strain active against rotavirus infections. Appl
479 Environ Microbiol. 2011;77(24):8775-83. doi: 10.1128/AEM.05548-11. PubMed PMID:
480 22003027; PubMed Central PMCID: PMCPMC3233071.
481 22. Duffy LC, Zielezny MA, Riepenhoff-Talty M, Dryja D, Sayahtaheri-Altaie S, Griffiths E, et al.
482 Effectiveness of Bifidobacterium bifidum in mediating the clinical course of murine rotavirus
483 diarrhea. Pediatr Res. 1994;35(6):690-5. doi: 10.1203/00006450-199406000-00014. PubMed
484 PMID: 7936820.
485 23. Pott J, Mahlakoiv T, Mordstein M, Duerr CU, Michiels T, Stockinger S, et al. IFN-lambda
486 determines the intestinal epithelial antiviral host defense. Proc Natl Acad Sci U S A.
487 2011;108(19):7944-9. doi: 10.1073/pnas.1100552108. PubMed PMID: 21518880; PubMed
488 Central PMCID: PMCPMC3093475.
489 24. Mahlakoiv T, Hernandez P, Gronke K, Diefenbach A, Staeheli P. Leukocyte-derived IFN-
490 alpha/beta and epithelial IFN-lambda constitute a compartmentalized mucosal defense system
491 that restricts enteric virus infections. PLoS Pathog. 2015;11(4):e1004782. doi:
492 10.1371/journal.ppat.1004782. PubMed PMID: 25849543; PubMed Central PMCID:
493 PMCPMC4388470.
494 25. Lin JD, Feng N, Sen A, Balan M, Tseng HC, McElrath C, et al. Distinct Roles of Type I and Type III
495 Interferons in Intestinal Immunity to Homologous and Heterologous Rotavirus Infections. PLoS
496 Pathog. 2016;12(4):e1005600. doi: 10.1371/journal.ppat.1005600. PubMed PMID: 27128797;
497 PubMed Central PMCID: PMCPMC4851417.
498 26. Zhang B, Chassaing B, Shi Z, Uchiyama R, Zhang Z, Denning TL, et al. Viral infection. Prevention
499 and cure of rotavirus infection via TLR5/NLRC4-mediated production of IL-22 and IL-18. Science.
500 2014;346(6211):861-5. doi: 10.1126/science.1256999. PubMed PMID: 25395539; PubMed
501 Central PMCID: PMCPMC4788408.
502 27. Hernandez PP, Mahlakoiv T, Yang I, Schwierzeck V, Nguyen N, Guendel F, et al. Interferon-
503 lambda and interleukin 22 act synergistically for the induction of interferon-stimulated genes
504 and control of rotavirus infection. Nat Immunol. 2015;16(7):698-707. doi: 10.1038/ni.3180.
505 PubMed PMID: 26006013; PubMed Central PMCID: PMCPMC4589158.

506 28. Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, et al. IFN-lambdas
507 mediate antiviral protection through a distinct class II cytokine receptor complex. Nat Immunol.
508 2003;4(1):69-77. doi: 10.1038/ni875. PubMed PMID: 12483210.
509 29. Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, et al. IL-28, IL-29
510 and their class II cytokine receptor IL-28R. Nat Immunol. 2003;4(1):63-8. doi: 10.1038/ni873.
511 PubMed PMID: 12469119.
512 30. Zhang Z, Zou J, Shi Z, Zhang B, Etienne-Mesmin L, Wang Y, et al. IL-22-induced cell extrusion and
513 IL-18-induced cell death prevent and cure rotavirus infection. Sci Immunol. 2020;5(52). doi:
514 10.1126/sciimmunol.abd2876. PubMed PMID: 33008915.
515 31. Zheng Y, Li T. Interleukin-22, a potent target for treatment of non-autoimmune diseases. Hum
516 Vaccin Immunother. 2018;14(12):2811-9. doi: 10.1080/21645515.2018.1509649. PubMed PMID:
517 30335564; PubMed Central PMCID: PMCPMC6343613.
518 32. Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, et al. Interleukin-22 mediates early
519 host defense against attaching and effacing bacterial pathogens. Nat Med. 2008;14(3):282-9.
520 doi: 10.1038/nm1720. PubMed PMID: 18264109.
521 33. Sanos SL, Bui VL, Mortha A, Oberle K, Heners C, Johner C, et al. RORgammat and commensal
522 microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells.
523 Nat Immunol. 2009;10(1):83-91. doi: 10.1038/ni.1684. PubMed PMID: 19029903; PubMed
524 Central PMCID: PMCPMC4217274.
525 34. Keir M, Yi Y, Lu T, Ghilardi N. The role of IL-22 in intestinal health and disease. J Exp Med.
526 2020;217(3):e20192195. doi: 10.1084/jem.20192195. PubMed PMID: 32997932.
527 35. Li P, Allen H, Banerjee S, Franklin S, Herzog L, Johnston C, et al. Mice deficient in IL-1 beta-
528 converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic
529 shock. Cell. 1995;80(3):401-11. doi: 10.1016/0092-8674(95)90490-5. PubMed PMID: 7859282.
530 36. Magalhaes A, Gomes J, Ismail MN, Haslam SM, Mendes N, Osorio H, et al. Fut2-null mice display
531 an altered glycosylation profile and impaired BabA-mediated Helicobacter pylori adhesion to
532 gastric mucosa. Glycobiology. 2009;19(12):1525-36. doi: 10.1093/glycob/cwp131. PubMed
533 PMID: 19706747; PubMed Central PMCID: PMCPMC2782244.
534 37. Newton K, Sun X, Dixit VM. Kinase RIP3 is dispensable for normal NF-kappa Bs, signaling by the
535 B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2 and 4. Mol
536 Cell Biol. 2004;24(4):1464-9. doi: 10.1128/mcb.24.4.1464-1469.2004. PubMed PMID: 14749364;
537 PubMed Central PMCID: PMCPMC344190.
538 38. Mordstein M, Kochs G, Dumoutier L, Renauld JC, Paludan SR, Klucher K, et al. Interferon-lambda
539 contributes to innate immunity of mice against influenza A virus but not against hepatotropic
540 viruses. PLoS Pathog. 2008;4(9):e1000151. doi: 10.1371/journal.ppat.1000151. PubMed PMID:
541 18787692; PubMed Central PMCID: PMCPMC2522277.
542 39. Mahlakoiv T, Ritz D, Mordstein M, DeDiego ML, Enjuanes L, Muller MA, et al. Combined action of
543 type I and type III interferon restricts initial replication of severe acute respiratory syndrome
544 coronavirus in the lung but fails to inhibit systemic virus spread. J Gen Virol. 2012;93(Pt
545 12):2601-5. doi: 10.1099/vir.0.046284-0. PubMed PMID: 22956738.
546 40. Baldridge MT, Lee S, Brown JJ, McAllister N, Urbanek K, Dermody TS, et al. Expression of Ifnlr1
547 on Intestinal Epithelial Cells Is Critical to the Antiviral Effects of Interferon Lambda against
548 Norovirus and Reovirus. J Virol. 2017;91(7). doi: 10.1128/JVI.02079-16. PubMed PMID:
549 28077655; PubMed Central PMCID: PMCPMC5355594.
550 41. Gozalbo-Rovira R, Ciges-Tomas JR, Vila-Vicent S, Buesa J, Santiso-Bellon C, Monedero V, et al.
551 Unraveling the role of the secretor antigen in human rotavirus attachment to histo-blood group
552 antigens. PLoS Pathog. 2019;15(6):e1007865. doi: 10.1371/journal.ppat.1007865. PubMed
553 PMID: 31226167; PubMed Central PMCID: PMCPMC6609034.
554 42. Aden K, Tran F, Ito G, Sheibani-Tezerji R, Lipinski S, Kuiper JW, et al. ATG16L1 orchestrates
555 interleukin-22 signaling in the intestinal epithelium via cGAS-STING. J Exp Med.
556 2018;215(11):2868-86. doi: 10.1084/jem.20171029. PubMed PMID: 30254094; PubMed Central
557 PMCID: PMCPMC6219748.

558 43. Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, et al. Phosphorylation-driven
559 assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced
560 inflammation. Cell. 2009;137(6):1112-23. doi: 10.1016/j.cell.2009.05.037. PubMed PMID:
561 19524513; PubMed Central PMCID: PMCPMC2727676.
562 44. Man SM, Karki R, Briard B, Burton A, Gingras S, Pelletier S, et al. Differential roles of caspase-1
563 and caspase-11 in infection and inflammation. Sci Rep. 2017;7:45126. doi: 10.1038/srep45126.
564 PubMed PMID: 28345580; PubMed Central PMCID: PMCPMC5366862.
565 45. Munoz M, Eidenschenk C, Ota N, Wong K, Lohmann U, Kuhl AA, et al. Interleukin-22 induces
566 interleukin-18 expression from epithelial cells during intestinal infection. Immunity.
567 2015;42(2):321-31. doi: 10.1016/j.immuni.2015.01.011. PubMed PMID: 25680273.
568 46. Kumar A, Vlasova AN, Deblais L, Huang HC, Wijeratne A, Kandasamy S, et al. Impact of nutrition
569 and rotavirus infection on the infant gut microbiota in a humanized pig model. BMC
570 Gastroenterol. 2018;18(1):93. doi: 10.1186/s12876-018-0810-2. PubMed PMID: 29929472;
571 PubMed Central PMCID: PMCPMC6013989.
572 47. Wichmann A, Allahyar A, Greiner TU, Plovier H, Lunden GO, Larsson T, et al. Microbial
573 modulation of energy availability in the colon regulates intestinal transit. Cell Host Microbe.
574 2013;14(5):582-90. doi: 10.1016/j.chom.2013.09.012. PubMed PMID: 24237703.
575 48. Bajic D, Niemann A, Hillmer AK, Mejias-Luque R, Bluemel S, Docampo M, et al. Gut Microbiota-
576 Derived Propionate Regulates the Expression of Reg3 Mucosal Lectins and Ameliorates
577 Experimental Colitis in Mice. J Crohns Colitis. 2020;14(10):1462-72. doi: 10.1093/ecco-
578 jcc/jjaa065. PubMed PMID: 32227170.
579 49. Fachi JL, Secca C, Rodrigues PB, Mato FCP, Di Luccia B, Felipe JS, et al. Acetate coordinates
580 neutrophil and ILC3 responses against C. difficile through FFAR2. J Exp Med. 2020;217(3). doi:
581 10.1084/jem.20190489. PubMed PMID: 31876919; PubMed Central PMCID: PMCPMC7062529.
582 50. Yang W, Yu T, Huang X, Bilotta AJ, Xu L, Lu Y, et al. Intestinal microbiota-derived short-chain fatty
583 acids regulation of immune cell IL-22 production and gut immunity. Nat Commun.
584 2020;11(1):4457. doi: 10.1038/s41467-020-18262-6. PubMed PMID: 32901017; PubMed Central
585 PMCID: PMCPMC7478978.
586 51. Goto Y, Obata T, Kunisawa J, Sato S, Ivanov, II, Lamichhane A, et al. Innate lymphoid cells
587 regulate intestinal epithelial cell glycosylation. Science. 2014;345(6202):1254009. doi:
588 10.1126/science.1254009. PubMed PMID: 25214634; PubMed Central PMCID:
589 PMCPMC4774895.
590 52. Magalhaes A, Rossez Y, Robbe-Masselot C, Maes E, Gomes J, Shevtsova A, et al. Muc5ac gastric
591 mucin glycosylation is shaped by FUT2 activity and functionally impacts Helicobacter pylori
592 binding. Sci Rep. 2016;6:25575. doi: 10.1038/srep25575. PubMed PMID: 27161092; PubMed
593 Central PMCID: PMCPMC4861914.
594 53. Pham TA, Clare S, Goulding D, Arasteh JM, Stares MD, Browne HP, et al. Epithelial IL-22RA1-
595 mediated fucosylation promotes intestinal colonization resistance to an opportunistic pathogen.
596 Cell Host Microbe. 2014;16(4):504-16. doi: 10.1016/j.chom.2014.08.017. PubMed PMID:
597 25263220; PubMed Central PMCID: PMCPMC4190086.
598 54. Xue M, Zhao J, Ying L, Fu F, Li L, Ma Y, et al. IL-22 suppresses the infection of porcine enteric
599 coronaviruses and rotavirus by activating STAT3 signal pathway. Antiviral Res. 2017;142:68-75.
600 doi: 10.1016/j.antiviral.2017.03.006. PubMed PMID: 28322925; PubMed Central PMCID:
601 PMCPMC7113769.
602 55. Doerflinger M, Deng Y, Whitney P, Salvamoser R, Engel S, Kueh AJ, et al. Flexible Usage and
603 Interconnectivity of Diverse Cell Death Pathways Protect against Intracellular Infection.
604 Immunity. 2020;53(3):533-47 e7. doi: 10.1016/j.immuni.2020.07.004. PubMed PMID: 32735843.
605 56. Das S, St Croix C, Good M, Chen J, Zhao J, Hu S, et al. Interleukin-22 Inhibits Respiratory Syncytial
606 Virus Production by Blocking Virus-Mediated Subversion of Cellular Autophagy. iScience.
607 2020;23(7):101256. doi: 10.1016/j.isci.2020.101256. PubMed PMID: 32580124; PubMed Central
608 PMCID: PMCPMC7317237.

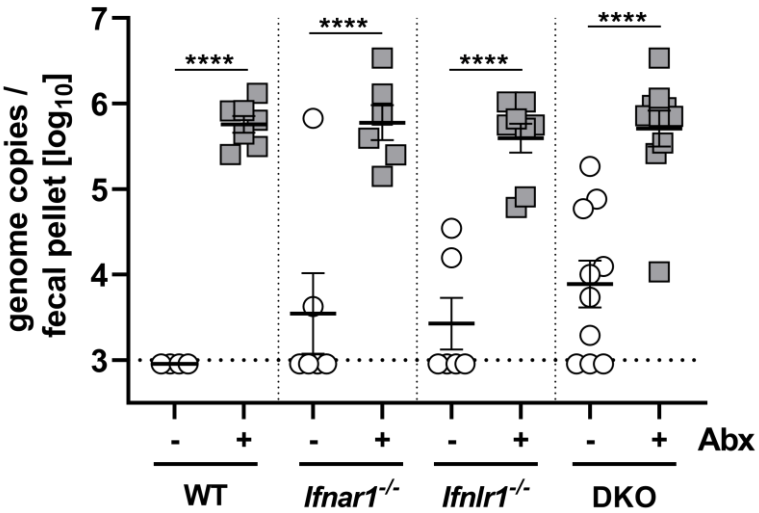
609



610

611 Figure 1: Germ-free and antibiotic-treated mice are highly susceptible to oral infection with rotavirus.
612 Conventional specific pathogen free (SPF, n = 10), antibiotic-treated (Abx, n = 9) and germ-free (GF, n
613 = 8) C57BL/6 mice were infected orally with 2.4×10^4 ID_{50} of murine rotavirus strain EDIM. Fecal
614 pellets of individual mice were collected daily and levels of viral antigen were determined by ELISA.
615 Four animals of the Abx and the GF groups were sacrificed on day 3 post-infection for analysis of
616 tissue in the context of a different project.

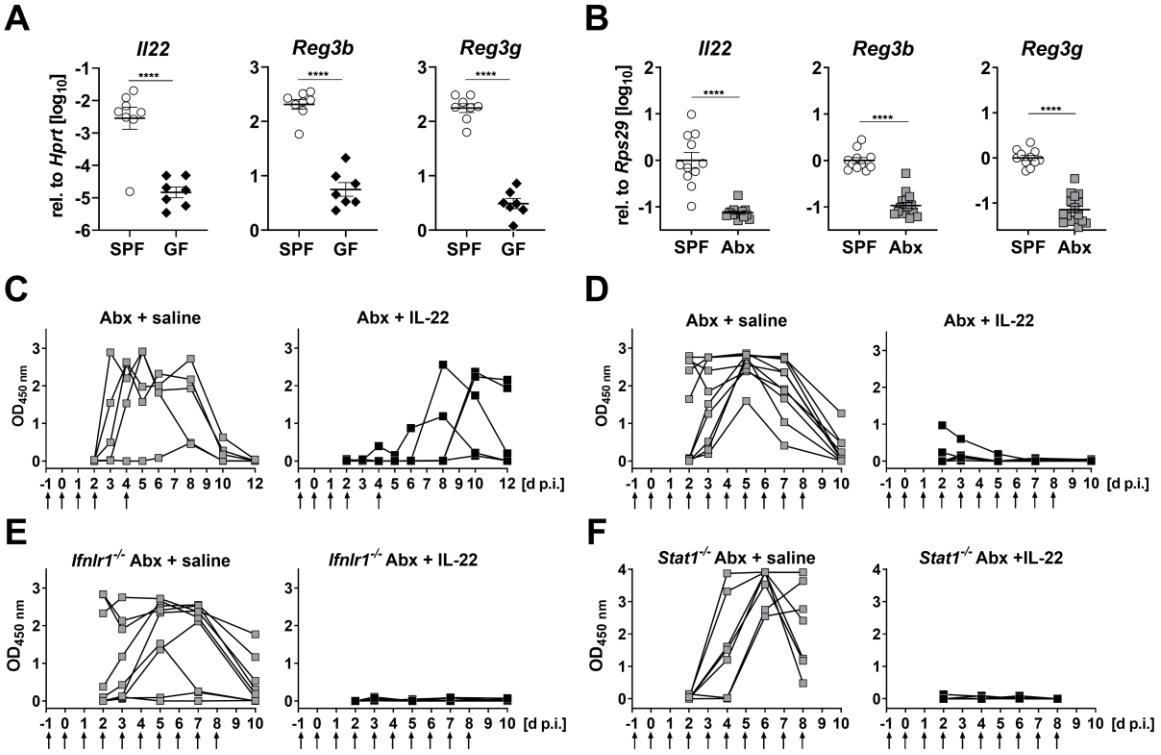
617



618

619 Figure 2: Rotavirus susceptibility of wild-type and IFN receptor-deficient C57BL/6 mice is greatly
620 enhanced if the microbiome is depleted with antibiotics. WT (n = 7), *Ifnar1*$^{-/-}$ (n = 6), *Ifnlr1*$^{-/-}$ (n = 8)
621 and *Ifnar1*$^{-/-}$ *Ifnlr1*$^{-/-}$ (DKO, n = 10) mice were left untreated or treated with antibiotics (Abx) before
622 oral infection with 10^4 SD_{50} of the EC strain of murine rotavirus. On day 5 post-infection, fecal pellets
623 were collected and viral RNA levels were determined by RT-qPCR. Symbols represent individual mice,
624 and bars represent means ± SEM. Line represents limit of detection of the RT-qPCR assay. Statistical
625 analysis: Ordinary one-way ANOVA with Tukey's multiple comparisons; ****$p<0.0001$.

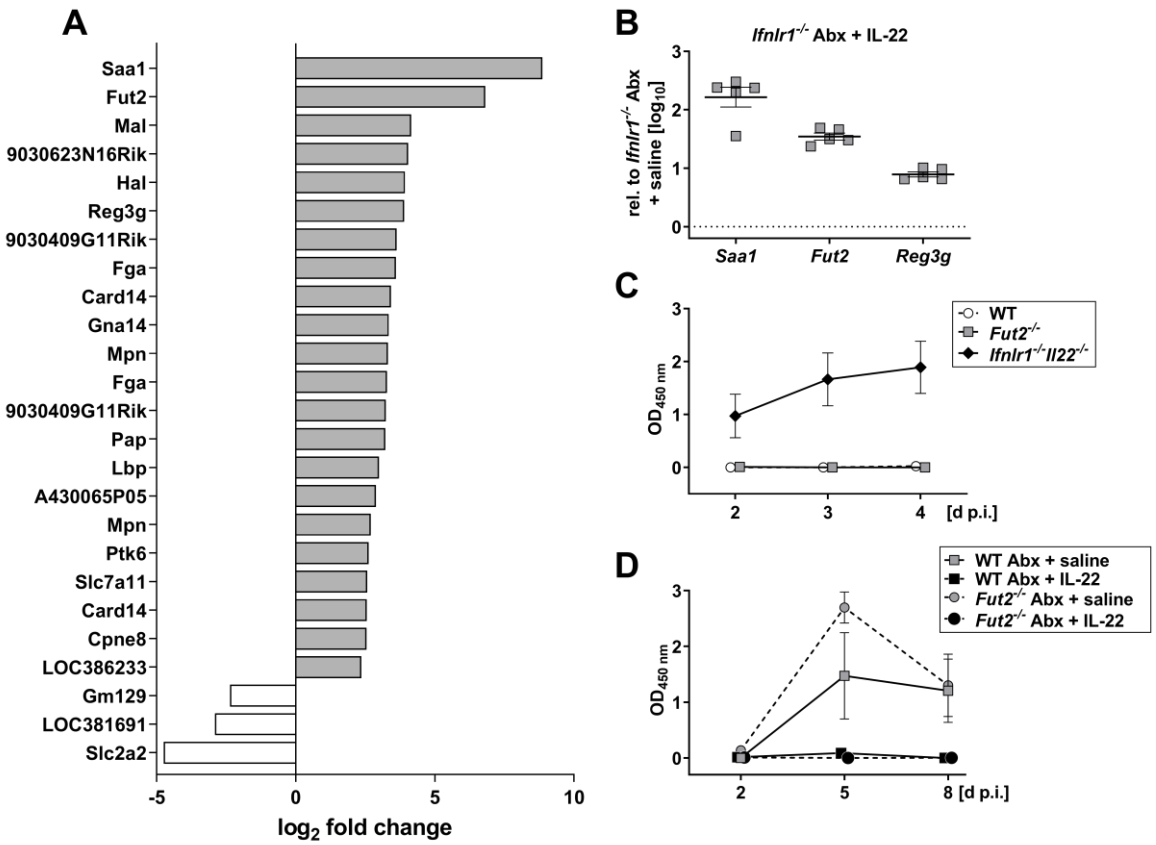
626



627

628 Figure 3: IL-22 confers short-lived rotavirus resistance in antibiotic-treated C57BL76 mice via a
629 mechanism that is not dependent on IFN signaling. (A, B) RT-qPCR analysis of tonic expression of *Il22*
630 and IL-22-regulated genes *Reg3b* and *Reg3g* in enriched intestinal epithelial cell fractions of (A) germ-
631 free (GF) or in ileum samples of (B) antibiotic-treated (Abx) mice compared to animals with
632 undisturbed microbiota (SPF). (C, D) Antibiotic-treated WT mice were subjected to brief (n = 5) or
633 extended (n = 10) IL-22 treatment regimens (1 µg per injection, time points indicated by arrows)
634 before infection with 2.4×10^4 ID_{50} of murine rotavirus strain EDIM by oral gavage. Control animals (C,
635 n = 5; D, n = 9) were treated with saline. Fecal pellets were collected at the indicated time points and
636 viral antigen in the samples was quantified by ELISA. (E, F) Same experimental setup as in panel D,
637 except that (E) *Ifnlr1*$^{-/-}$ (saline n = 9; IL-22 n = 10) or (F) *Stat1*$^{-/-}$ (saline n = 7; IL-22 n = 9) mice were
638 used. Symbols represent individual mice, and bars in (A) and (B) represent means ± SEM. Statistical
639 analysis: Unpaired t-test; ****p<0.0001.

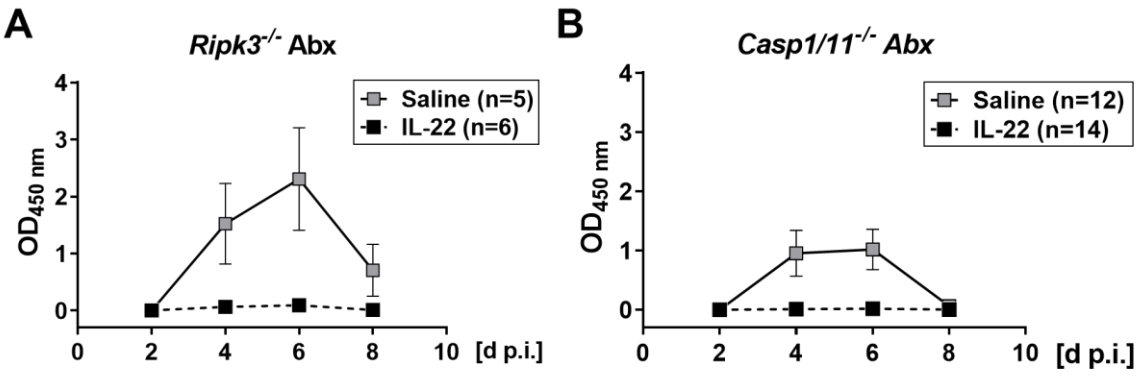
640



641

642 Figure 4: IL-22 regulates a distinct set of genes in antibiotic-treated *Ifnlr1*$^{-/-}$ mice, but the *Fut2* gene
643 plays no decisive role in rotavirus resistance. (A) Four h prior to preparation of ileum samples for
644 transcriptome analysis, antibiotic-treated *Ifnlr1*$^{-/-}$ mice (n = 5 per group) were treated with either
645 buffer or 1 µg of IL-22. Genes up- or downregulated at least 5-fold on average in IL-22-treated mice
646 are listed. For all listed genes, variation between samples from the different mice was small (p value
647 <0.01 for every data point) and was part of the original filter used to acquire the data. (B) Verification
648 of IL-22-mediated induction of *Saa1*, *Fut2* and *Reg3g* by RT-qPCR. Symbols represent individual mice,
649 and bars represent means ± SEM. (C) *Fut2*$^{-/-}$ mice with undisturbed microbiota (n = 6) were orally
650 infected with 2.4×10^4 ID_{50} of murine rotavirus strain EDIM and viral antigen levels in fecal samples
651 were analyzed by ELISA. WT (n = 6) and *Ifnlr1*$^{-/-}$ *Il22*$^{-/-}$ (n = 6) mice served as negative and positive
652 controls, respectively. (D) *Fut2*$^{-/-}$ mice with depleted microbiota (n = 6 per group) were treated daily
653 with saline or IL-22 (extended treatment as described in figure 3) prior to infection with 2.4×10^4 ID_{50}
654 of murine rotavirus strain EDIM. Antibiotic-treated WT mice (n = 4-5 per group) served as controls.
655 Symbols in (C) and (D) represent means ± SEM.

656



657

658 Figure 5: No role for RIPK3-induced necroptosis or caspase 1-mediated death in IL-22-mediated
659 clearance of rotavirus from antibiotic-treated mice. Antibiotic-treated *Ripk3*$^{-/-}$ (A) and *Casp1/11*$^{-/-}$ (B)
660 mice were treated with saline or IL-22 and infected with rotavirus strain EDIM as in the experiment
661 described in figure 3D. Viral antigen levels in fecal samples were analyzed by ELISA. Numbers of
662 animals in each group are indicated. Symbols represent means ± SEM.