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

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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 modifies the O-glycosylation pattern of rotavirus entry mediators. 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.

53 **INTRODUCTION**

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

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

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

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

88 shedding of a rotavirus live vaccine in adult volunteers [16], and gram-negative probiotics protected
89 against human rotavirus infection of gnotobiotic pigs [17]. However, antibiotic treatment has been
90 reported to suppress rotavirus infection of gut epithelial cells, with an overall reduction in the
91 incidence and duration of diarrhea in suckling mice [18]. Conversely, some mouse colonies have been
92 found to be highly resistant to rotavirus infection, a resistance that can be transferred to susceptible
93 mice via fecal microbial transplantation [19], indicating that a specific component of the microbiome
94 can mediate antiviral effects. Particular segmented filamentous bacteria (SFB) were shown to be
95 sufficient to protect mice against rotavirus infection and associated diarrhea [19]. Interestingly, SFB-
96 mediated protection was independent of known rotavirus-impeding factors such as IFN-λ [20-22],
97 interleukin-18 (IL-18) [23] and IL-22 [23, 24], and instead rotavirus resistance correlated with
98 accelerated epithelial cell turnover in this experimental system [19].
99 IFN-λ and IL-22 are both members of the IL-10 cytokine family. Their specific receptor chains (IFN-
100 λR1 and IL-22Rα, respectively) both associate with IL-10Rβ to form functional heterodimeric receptor
101 complexes, and the *Ifnlr1* and *Il22ra1* genes are close relatives located in adjacent positions in the
102 mouse and human genomes [25, 26]. The IFN-λ and the IL-22 receptors are highly expressed in
103 epithelial cells, and both cytokines cooperate for the induction of IFN-stimulated genes and the
104 control of rotavirus infection in mice [24]. However, the antiviral activity of IL-22 is poorly
105 understood compared with its well-described function as a key mediator of anti-bacterial responses
106 [27, 28]. IL-22 expression is controlled by the commensal microbiota [29] and, in turn, IL-22 can
107 modulate the composition of the microbiota [30].
108 To better understand the role of commensal bacteria in host defense against rotavirus, we
109 performed infections of microbiota-depleted mice and we evaluated the antiviral activity of IL-22
110 under gnotobiotic conditions. Using antibiotic-treated and germ-free mice we found that the
111 microbiota strongly repressed rotavirus replication in the intestinal tract. Our data indicate that
112 rotavirus susceptibility of microbiota-depleted mice results from poor expression of IL-22 in the
113 intestine. We further show that IL-22 confers rotavirus resistance in microbiota-depleted mice by a
114 mechanism that is independent of IFN-λ or STAT1 signaling, fucosyl-transferase 2, or mediators of
115 inducible cell death.
116
117

118 **MATERIAL & METHODS**

119 Mice: Conventional specific pathogen free (SPF) C57BL/6J mice of both sexes were purchased from
120 Janvier Labs. Germ-free (GF) C57BL/6 mice were obtained from the Clean Mouse Facility of the
121 University of Bern, Bern, Switzerland. GF mice were kept in autoclaved individually ventilated cages,
122 and sterile water and food was provided. GF mice were handled exclusively in a biosafety cabinet.
123 C57BL/6J mice lacking functional *Fut2*, *Ripk3* or *Casp1/11* genes [31-33] as well as mutant B6.A2G-
124 *Mx1-Ifnlr1*$^{-/-}$ [34], B6.A2G-*Mx1-Ifnlr1*$^{-/-}$*Il22*$^{-/-}$ [24], B6.A2G-*Mx1-Stat1*$^{-/-}$ [35] and corresponding
125 B6.A2G-*Mx1* wild-type mice [34] were bred and housed in the animal facilities of the University
126 Medical Center Freiburg. Other C57BL/6J mice originally purchased from Jackson Laboratories (stock
127 000664; Jackson Laboratories, Bar Harbor, ME) were bred and housed in animal facilities of
128 Washington University in Saint Louis under specific-pathogen-free (including murine norovirus-free)
129 conditions. Generation of *Ifnlr1*$^{-/-}$ mice was previously described [36]; briefly, these mice were
130 established by interbreeding *Ifnlr1*$^{tm1a(EUCOMM)Wtsi}$ mice and deleter-Cre mice, followed by backcrossing
131 by speed congenics onto a C57BL/6J background. *Ifnar1*$^{-/-}$ (B6.129.Ifnar1tm1) were crossed with
132 these *Ifnlr1*$^{-/-}$ mice to generate *Ifnar1*$^{-/-}$*Ifnlr1*$^{-/-}$ double knockout mice.
133
134 Ethics statement: All experiments with mice were carried out in accordance with the guidelines of
135 the Federation for Laboratory Animal Science Associations and the national animal welfare body.
136 Experiments were in compliance with the German animal protection law and were approved by the
137 animal welfare committee of the Regierungspräsidium Freiburg (permit G-16/98). All experiments at
138 Washington University were conducted according to regulations stipulated by the Washington
139 University Institutional Animal Care and Use Committee and to animal protocol 20190126, approved
140 by the Washington University Animal Studies Committee.
141
142 Antibiotic treatment of mice: Four to six week old mice were treated with a cocktail of antibiotics
143 consisting of Cefoxitin (Santa Cruz Biotechnology, Inc), Gentamycin sulfate (Sigma-Aldrich),
144 Metronidazole (Sigma-Aldrich) and Vancomycin (HEXAL®) for 4 weeks to deplete the commensal
145 microbiota. Drinking water containing 1 mg/ml of each antibiotic was provided *ad libitum*. For
146 experiments at Washington University, six-week old mice were treated with an antibiotic cocktail [1
147 mg/ml ampicillin, 1 mg/ml metronidazole, 1 mg/ml neomycin, 0.5 mg/ml vancomycin (Sigma, St.
148 Louis, MO)] in 20 mg/ml grape Kool-Aid (Kraft Foods, Northfield, IL), or with Kool-Aid alone, *ad*
149 *libitum* for 9-14 days.
150
151 Virus stocks and infections: To produce the rotavirus used in Freiburg, six day old suckling BALB/c
152 mice were infected orally with 680 infectious dose 50 (ID_{50}) (5 µl of a 1:250 dilution) from a stock

153 containing $3.4x10^7$ ID_{50}/ml of murine rotavirus strain EDIM [20]. Four days post-infection, mice were
154 sacrificed and colon samples with content were homogenized twice for 18 sec at 6 m/s using a
155 FastPrep®-12 homogeniser (MP Biomedicals). The homogenate was cleared by centrifugation at
156 5,000 rpm for 15 min. Supernatants were pooled and filtered using a 10 ml syringe and 4.5 µm sterile
157 filters. The filtrate was aliquoted and stored at -80 °C. Viral protein VP6 was quantified by
158 RIDASCREEN® ELISA in comparison to the parental stock. The infectious dose 50 (ID_{50}) was
159 determined by orally infecting groups of six C57BL/6 suckling mice with 5 µl samples of 10-fold serial
160 dilutions, ranging from 10^{-3} to 10^{-7}. Mice were sacrificed 24 h post-infection and intestines were
161 analyzed for the presence of RNA encoding for VP6 by RT-qPCR. Adult mice (8-10 week old) were
162 orally infected with $2.4x10^4$ ID_{50} in 100 µl by oral gavage using a gavage needle (19G).
163 To produce the rotavirus used in Saint Louis, four day old suckling CD-1 mice (Charles River) were
164 infected orally with 400 diarrhea dose 50 (5 µl of a 1:10 dilution in PBS + Evan's blue) from a stock of
165 murine rotavirus strain EC-RV received from Estes Lab at Baylor College of Medicine. Three days post-
166 infection, mice were sacrificed and the entire small intestine and colon with contents were harvested
167 and pooled for weighing. A 20% homogenate was prepared in homogenization media of DMEM
168 (Gibco 11995-040) supplemented with 1% penicillin/streptomycin (Gibco 15140-122) by three rounds
169 of homogenization on ice (homogenize for 1 min, then allow to settle for 30 sec). Homogenate was
170 pooled on ice, aliquoted, and then stored at -80˚C until use. The shedding dose 50 (SD_{50}) of the stock
171 was determined by orally infecting groups of five adult mice (6-8 weeks old), of both BALB/c and
172 C57BL/6 genotypes, with 100 µl of 10-fold serial dilutions, ranging from 10^{-5} to 10^{-9} for BALB/c and
173 10^0 to 10^{-5} for C57BL/6, and analyzing fecal pellets for RV genome copies by RT-qPCR. Adult mice (8-
174 10 weeks old) were orally infected with 10^4 SD_{50} in 100 µl by oral gavage, preceded by 100 µl of
175 sterile 1.33% sodium bicarbonate solution.
176
177 Viral protein quantification in feces by RIDASCREEN® Rotavirus ELISA: Fecal samples were
178 homogenized according to weight with RIDASCREEN® sample dilution buffer 1 and homogenized
179 twice for 11 sec with 6 m/s using a FastPrep®-12 homogenizer. Homogenates were cleared by
180 centrifugation [5,000 rpm, 5 min, 4 °C] and 100 µl of fecal homogenate supernatants were used for
181 RIDASCREEN® Rotavirus ELISA. ELISA was performed according to the manufacturer's instructions.
182 Optical density was measured at 450 nm wave length by using a Tecan Infinite 200 plate reader.
183
184 RNA isolation and RT-qPCR: RNA was isolated using the Direct-zol™ RNA MiniPrep Kit (Zymo
185 Research). Two-three cm pieces of ilea were homogenized in 1 ml TriFast™ twice for 18 sec with 6
186 m/s using a FastPrep®-12 homogenizer (MP Biomedicals). Homogenates were centrifuged at 12,000 g
187 for 5 min and supernatant were diluted 1:10 in TriFast™. RNA was then isolated following the

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

223 MicroArray: Total RNA harvested from intestinal epithelia cells was hybridized using Illumina Mouse
224 WG-6_V2_0_R0_11278593_A arrays. The raw intensity values for each entity were preprocessed by
225 RMA normalization against the median intensity in mock-treated samples. Using GeneSpring 11.5, all
226 transcripts were then filtered based on flags (present or marginal, in at least 2 out of 10 samples).
227 Moderate t-test (IL22-treated versus mock-treated) was performed to identify genes differentially
228 expressed relative to controls (≥5-fold change; $p<0.01$, Benjamini-Hochberg multiple test correction).
229 Raw microarray data can be accessed at GEO under accession number GSE166400.
230
231 Statistical analysis: For statistical analyses the GraphPad Prism 8 software (GaphPad Software, USA)
232 was used. Testing for statistical significance was performed on log-transformed values by using
233 ordinary one-way ANOVA with Tukey's multiple comparisons or the unpaired Student's t-test.
234 Asterisks indicate p-values: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.001$.
235

236 **RESULTS**

237 **Germ-free and antibiotic-treated mice are highly susceptible to oral infection with rotavirus**

238 To investigate the effect of the microbiota on rotavirus susceptibility, we monitored the levels of
239 rotavirus antigen in the feces of three experimentally infected groups of adult C57BL/6 mice, namely
240 conventionally-housed specific pathogen-free (SPF) animals, SPF animals treated with an antibiotic
241 cocktail (Abx), and germ-free (GF) animals. During the 6-day observation period following oral
242 infection with rotavirus, viral antigen was nearly undetectable in fecal samples of SPF mice by ELISA
243 (Fig. 1). In contrast, fecal samples from the majority of infected Abx and GF mice contained high
244 levels of rotavirus antigen (Fig. 1), indicating that the microbiota has an inhibitory effect on rotavirus
245 replication.

246 Since an earlier study indicated that the microbiota promotes rather than inhibits rotavirus
247 replication [18], we sought to confirm this finding in an unrelated colony of C57BL/6 wild-type mice
248 at a second independent institution, using an independently prepared rotavirus stock. Again, we
249 found that antibiotics treatment significantly increased rotavirus susceptibility, demonstrated by
250 strongly elevated levels of viral RNA in fecal samples of mice on day 5 post-infection as assessed by
251 RT-qPCR analysis (Fig. 2). Prior work indicated that the type I and the type III IFN systems inhibit the
252 replication of rotavirus in the mouse intestine [21, 22]. Therefore, we assessed the possibility that
253 microbiota-mediated rotavirus resistance might depend on IFN signaling. In agreement with earlier
254 studies [21, 22], we observed that animals lacking functional receptors for type I IFN (*Ifnar1*$^{-/-}$), for
255 type III IFN (*Ifnlr1*$^{-/-}$) or for both IFN types (DKO) were more susceptible to rotavirus infection
256 compared with wild-type (WT) mice (Fig. 2). Importantly, however, treating these mutant mice with
257 antibiotics also enhanced rotavirus levels on average at least 50-fold (Fig. 2), demonstrating that
258 enhanced intestinal replication of rotavirus in mice with reduced microbiota is not due to impaired
259 activation of type I IFN and/or type III IFN pathways by commensal bacteria.

260

261 **IL-22 confers rotavirus resistance in antibiotic-treated mice via a mechanism that is not dependent**
262 **on IFN-λ or STAT1 signaling**

263 To identify IFN-independent antiviral factors which are present in the intestine of SPF mice but
264 absent or strongly diminished in GF mice, we performed RT-qPCR analysis of cytokines previously
265 linked to rotavirus resistance [21-23]. We found that expression of *Il22* was on average about 200
266 fold lower in ileum samples from GF (Fig. 3A) or Abx mice (Fig. 3B) than in samples from SPF mice.
267 Furthermore, compared with SPF mice, expression of the IL-22-induced *Reg3b* and *Reg3g* genes was
268 on average 10-20 fold reduced in the ileum of GF (Fig. 3A) and Abx mice (Fig. 3B), demonstrating that
269 the high rotavirus susceptibility of animals with diminished microbiota correlates with diminished
270 baseline expression of IL-22.

271 We next tested whether IL-22 can confer rotavirus resistance by treating Abx mice with recombinant
272 IL-22 one day before and on days 0, 1, 2 and 4 after oral infection with rotavirus, then monitoring
273 fecal virus shedding by ELISA for the next 12 days. All saline-treated control mice acutely shed high
274 amounts of viral antigen, whereas all IL-22-treated animals exhibited low levels of fecal viral antigen
275 during the first 5 days post-infection (Fig. 3C). However, four of the five IL-22-treated mice shed virus
276 with delayed kinetics, indicating that the protective effect of exogenous IL-22 was short-lived.
277 Therefore, in a second experiment, the IL-22 treatment period was extended and daily injections of
278 IL-22 were continued until day 8 post-infection. Under these more stringent conditions, IL-22-
279 mediated suppression of virus shedding was very effective (Fig. 3D).
280 As synergistic activity between IL-22 and IFN-λ to control rotavirus has previously been reported [24],
281 we assessed whether the rotavirus-inhibitory effect of IL-22 was direct or mediated by IFNs. We
282 repeated the IL-22 treatment study using mice in which either IFN-λ (*Ifnlr1*$^{-/-}$) or all IFN subtypes
283 (*Stat1*$^{-/-}$) are no longer functional, and found that daily injections of IL-22 were still highly effective at
284 suppressing rotavirus shedding in antibiotic-treated *Ifnlr1*$^{-/-}$ (Fig. 3E) and *Stat1*$^{-/-}$ (Fig. 3F) mice. Thus,
285 IL-22 confers virus resistance in microbiota-depleted mice by a mechanism that is not dependent on
286 IFN-λ or STAT1 signaling.
287
288 **Microbes modulate the IL-22 response of the intestinal epithelium**
289 To identify IL-22-induced effector molecules which confer rotavirus resistance in microbiota-depleted
290 mice, we compared gene expression profiles in the ileum of antibiotic-treated *Ifnlr1*$^{-/-}$ mice which
291 received either saline or IL-22. We identified 22 genes that showed at least 5-fold increased
292 expression and 3 genes with at least 5-fold decreased expression after IL-22 treatment compared
293 with mock-treated mice (Fig. 4A). Enhanced expression of several genes, including *Saa1*, *Fut2* and
294 *Reg3g,* in tissue of IL-22-treated animals was confirmed by RT-qPCR (Fig. 4B).
295 The *Fut2* gene was of particular interest because it encodes the enzyme fucosyl-transferase 2 (FUT2)
296 that determines the O-glycosylation pattern of histo-blood group antigens which can serve as
297 rotavirus entry mediators [37]. To investigate whether IL-22 inhibits rotavirus growth in the intestine
298 of antibiotics-treated mice by regulating FUT2 expression, we first determined whether *Fut2*$^{-/-}$ mice
299 with normal gut flora were more susceptible to oral infection with rotavirus than standard wild-type
300 mice, using highly susceptible *Ifnlr1*$^{-/-}$*Il22*$^{-/-}$ mice as a positive control (Fig. 4C). *Fut2*$^{-/-}$ mice exhibited
301 no defects in rotavirus control. Next, we tested whether IL-22 might no longer be able to inhibit
302 rotavirus replication in antibiotic-treated *Fut2*$^{-/-}$ mice, but found IL-22 treatment to be highly
303 effective in these animals (Fig. 4D). We thus concluded that IL-22-mediated upregulation of *Fut2* in
304 microbiota-depleted mice does not play a decisive role in rotavirus resistance.

305 Since SFB increase the turnover of intestinal epithelial cells that results in rotavirus resistance [19],
306 microbiota-mediated acceleration of infected epithelial cell death might be responsible for the
307 intrinsically high rotavirus resistance of mice with intact microbiota. Therefore, we first evaluated the
308 possibility that the antiviral activity of IL-22 in antibiotic-treated mice results from accelerated death
309 of intestinal epithelial cells by necroptosis [38]. However, we found that IL-22 was effective in
310 inhibiting rotavirus growth in antibiotic-treated *Ripk3*$^{-/-}$ mice (Fig. 5A), in which cell death by
311 necroptosis cannot occur [39]. This finding excluded the possibility that IL-22 induces premature
312 death of rotavirus-infected cells by necroptosis in antibiotic-treated mice. An alternative form of
313 induced cell death is pyroptosis, in which caspase-1 plays a key role [40]. To evaluate the possibility
314 that IL-22 acts by inducing pyroptosis in infected cells, possibly via regulation of IL-18 expression [23,
315 41], we tested IL-22-induced antiviral effects in antibiotic-treated *Casp1/11*$^{-/-}$ mice. We found that IL-
316 22 remained active in these mice (Fig. 5B), precluding the possibility that IL-22 inhibits rotavirus by
317 triggering pyroptotic cell death in antibiotic-treated mice.
318

319 **DISCUSSION**

320 Our work revealed that the microbiota protects against rotavirus infection and inhibits virus
321 replication in the small intestine. These results are distinct from conclusions of an earlier study [18],
322 which indicated that antibiotic treatment renders mice resistant to rotavirus infection. Our use of
323 mice from two separate animal facilities located on different continents, as well as two
324 independently-generated rotavirus stocks makes it unlikely that housing conditions or unrecognized
325 mutations in the virus genome are responsible for this discrepancy. We speculate that differences in
326 timing of virus excretion between microbiota-replete and -depleted mice may have accounted for
327 differential interpretations of microbiota effects on virus resistance. Of note, our results are highly
328 consistent with recent work that attributed the presence of particular SFB with rotavirus resistance
329 of mice [19].
330 Our study revealed that the rotavirus susceptibility phenotype of antibiotic-treated mice cannot be
331 explained by the involvement of known mediators of rotavirus resistance such as IFN-λ or type I IFN.
332 We confirmed earlier findings [21, 22] that mice with defective IFN receptors are intrinsically more
333 susceptible to rotavirus infection than wild-type mice. Importantly, however, when such mutant mice
334 were treated with antibiotics, their susceptibility to infection with rotavirus still increased, indicating
335 that poorly defined additional factors also contribute to rotavirus resistance. Our analysis suggests
336 that IL-22 plays an important role in this phenomenon. First, we observed that poor expression of IL-
337 22 and IL-22-regulated genes in the ileum correlates with rotavirus susceptibility of antibiotic-treated
338 mice. Second, we found that application of IL-22 readily restored rotavirus resistance of antibiotic-
339 treated mice. Interestingly, unlike in mice with undisturbed microbiota in which the antiviral activity
340 of IL-22 was strongly dependent on IFN-λ [24], the anti-rotavirus effect of IL-22 in antibiotic-treated
341 mice was not dependent on IFN-λ or transcription factor STAT1. Thus, IL-22 can negatively affect
342 rotavirus replication by at least two mechanisms, of which only one is dependent on IFN. The IFN-
343 independent mechanism plays a dominant role in antibiotic-treated mice, whereas the IFN-λ-
344 dependent mechanism dominates in mice with undisturbed microbiome.
345 We currently do not understand how IL-22 inhibits the replication of rotavirus in antibiotic-treated
346 mice. Our transcriptome analysis showed that *Fut2* is strongly induced by IL-22 under such
347 conditions. This finding is in good agreement with earlier work indicating that antibiotic-mediated
348 down-regulation of *Fut2* alters the glycosylation pattern of intestinal epithelial cells [42, 43]. Such
349 changes in protein glycosylation resulted in enhanced susceptibility of mice to infection with several
350 pathogenic bacteria, including *Citrobacter rodentium* [44], Helicobacter pylori [32, 43] and
351 Salmonella typhimurium [42]. However, our experiments with *Fut2*-deficient mice indicate that
352 antiviral defense is not reduced in these animals and that fucosyltransferase-2 is not the elusive IL-
353 22-induced mediator of rotavirus resistance.

354 Since the intestinal epithelium consists of short-lived cells, we envisaged the possibility that IL-22
355 might inhibit rotavirus replication by inducing premature death of infected cells. Yet, we found that
356 IL-22 effectively blocked rotavirus replication in antibiotic-treated mice lacking key factors for
357 necroptosis or pyroptosis, rendering such a mechanism unlikely. However, since these programmed
358 cell death pathways are highly interconnected, we cannot exclude the possibility of compensatory
359 effects [45]. Available data suggest that SFB prevents and cures rotavirus infection by enhancing the
360 proliferation of infected epithelial cells in the terminal ileum, promoting their shedding from the
361 tissue [19]. We do not know whether IL-22 provides similar growth-promoting stimuli to epithelial
362 cells in antibiotic-treated mice and whether enhanced cell proliferation might explain the antiviral
363 effect of IL-22 that we observed. It has been suggested that IL-22 may induce extrusion of intestinal
364 epithelial cells [46], which remains a possibility in the context of microbiota-mediated regulation of
365 rotavirus infection.
366

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376 **Conflict of interest statement**

377 The authors have no conflict of interest

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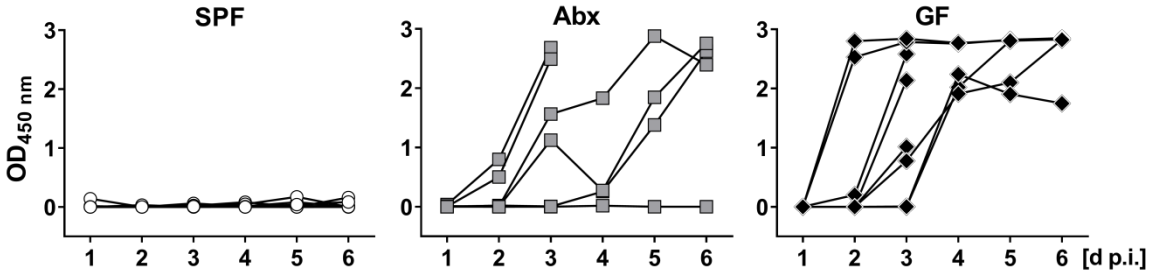

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550 Figure 1: Germ-free and antibiotic-treated mice are highly susceptible to oral infection with rotavirus.
551 Conventional specific pathogen free (SPF, n = 10), antibiotic-treated (Abx, n = 9) and germ-free (GF, n
552 = 8) C57BL/6 mice were infected orally with $2.4x10^4$ ID_{50} of murine rotavirus. Fecal pellets of
553 individual mice were collected daily and levels of viral antigen were determined by ELISA. Four
554 animals of the Abx and the GF groups were sacrificed on day 3 post-infection for analysis of tissue in
555 the context of a different project.

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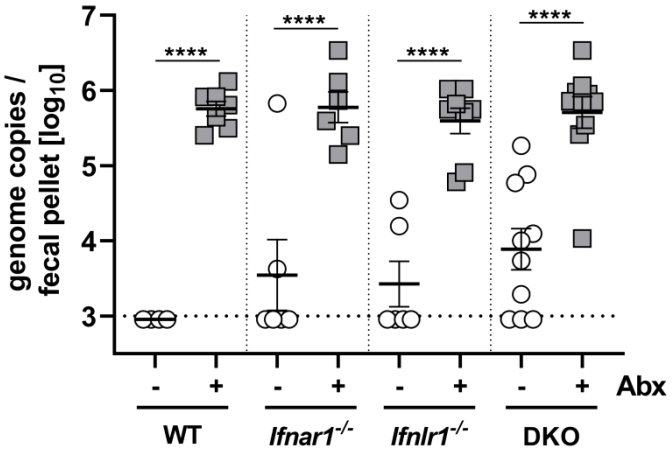

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560 Figure 2: Rotavirus susceptibility of wild-type and IFN receptor-deficient C57BL/6 mice is greatly
561 enhanced if the microbiome is depleted with antibiotics. WT (n = 7), *Ifnar1*$^{-/-}$ (n = 6), *Ifnlr1*$^{-/-}$ (n = 8)
562 and *Ifnar1*$^{-/-}$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

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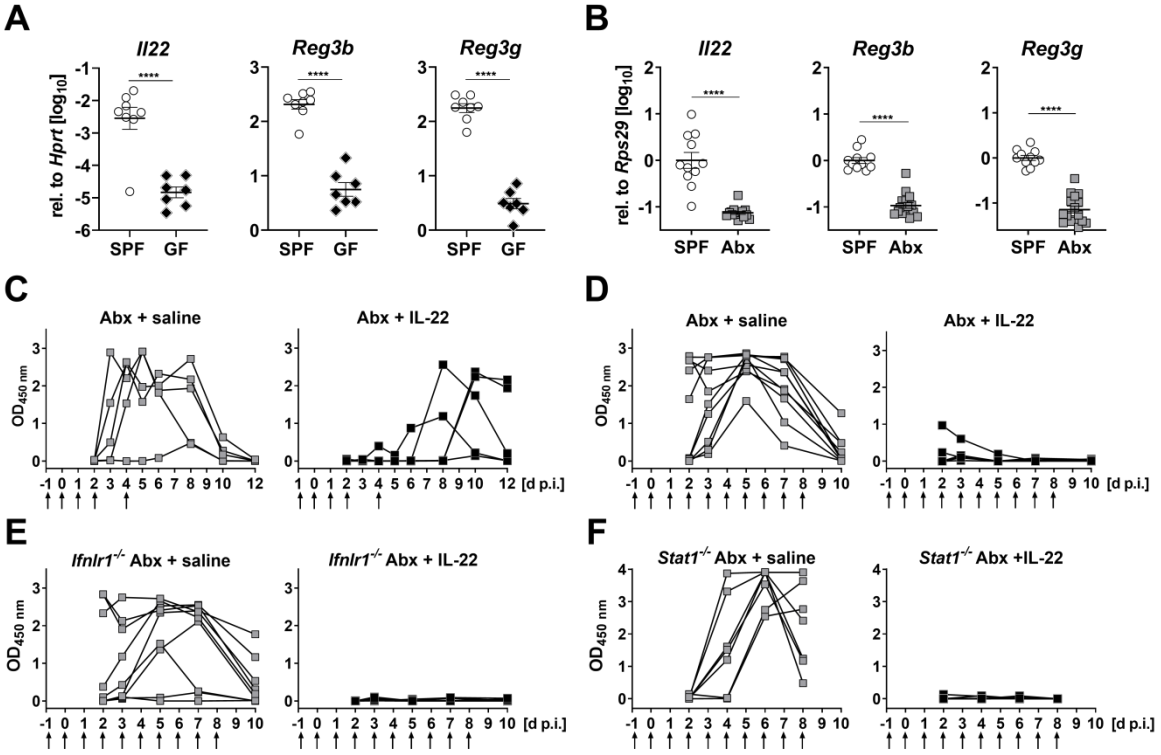

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571 Figure 3: IL-22 confers short-lived rotavirus resistance in antibiotic-treated C57BL76 mice via a
572 mechanism that is not dependent on IFN signaling. (A, B) RT-qPCR analysis of tonic expression of *Il22*
573 and IL-22-regulated genes *Reg3b*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

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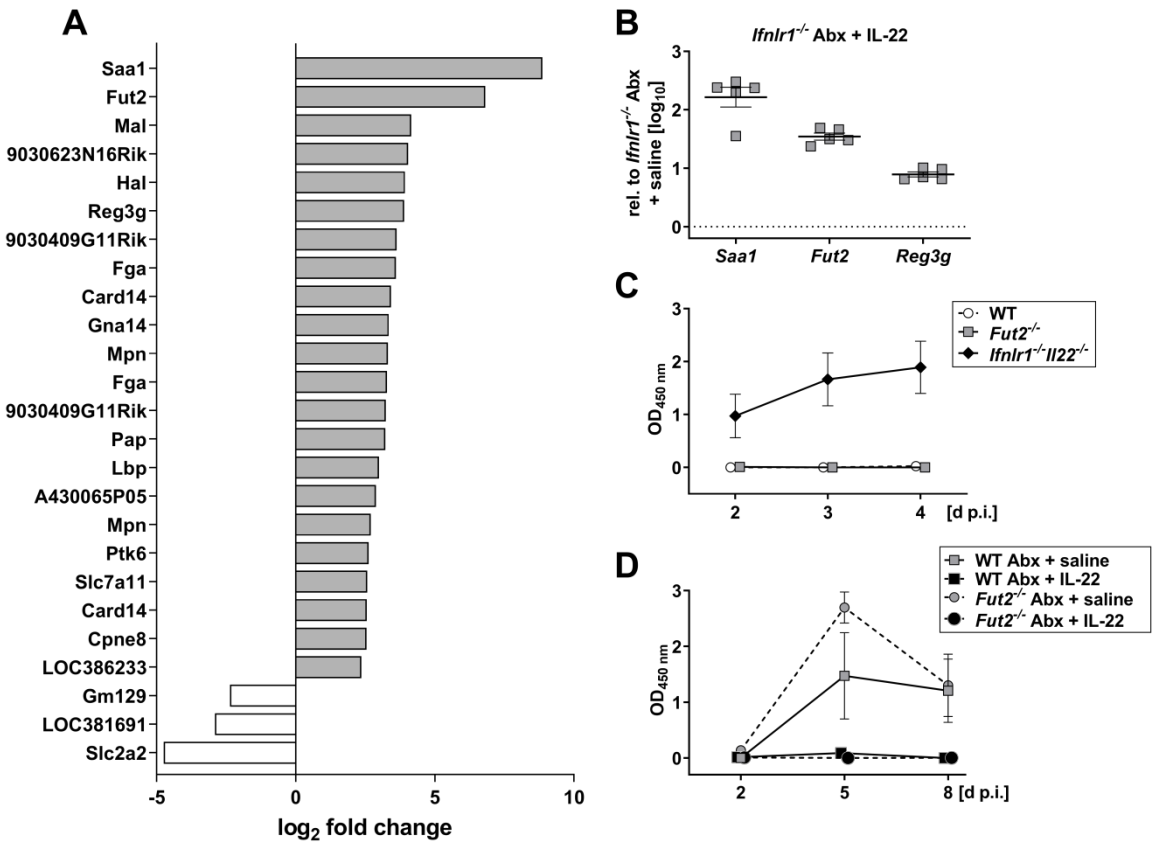
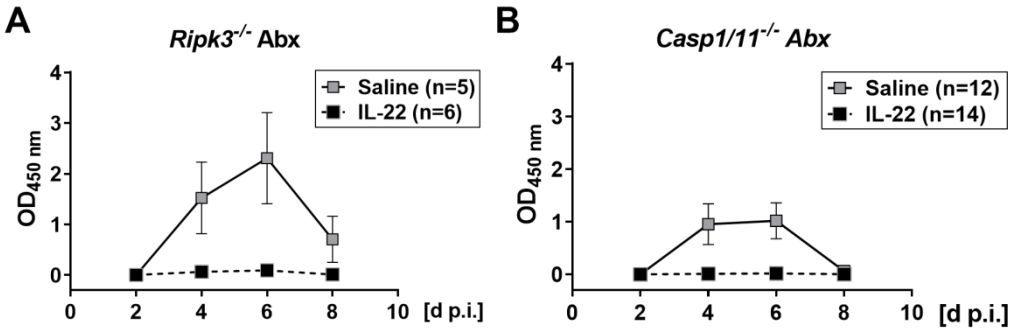


Figure 4: IL-22 regulates a distinct set of genes in antibiotic-treated *Ifnlr1*$^{-/-}$ mice, but the *Fut2* gene plays no decisive role in rotavirus resistance. (A) Four h prior to preparation of ileum samples for transcriptome analysis, antibiotic-treated *Ifnlr1*$^{-/-}$ mice (n = 5 per group) were treated with either buffer or 1 µg of IL-22. Genes up- or downregulated at least 5-fold on average in IL-22-treated mice are listed. For all listed genes, variation between samples from the different mice was small (p value <0.01 for every data point) and was part of the original filter used to acquire the data. (B) Verification of IL-22-mediated induction of *Saa1*, *Fut2* and *Reg3g* by RT-qPCR. Symbols represent individual mice, and bars represent means ± SEM. (C) *Fut2*$^{-/-}$ mice with undisturbed microbiota (n = 6) were orally infected with 2.4x10^4 ID_{50} of murine rotavirus and viral antigen levels in fecal samples were analyzed by ELISA. WT (n = 6) and *Ifnlr1*$^{-/-}$ *Il22*$^{-/-}$ (n = 6) mice served as negative and positive controls, respectively. (D) *Fut2*$^{-/-}$ mice with depleted microbiota (n = 6 per group) were treated daily with saline or IL-22 (extended treatment as described in figure 3) prior to infection with 2.4x10^4 ID_{50} of murine rotavirus. Antibiotic-treated WT mice (n = 4-5 per group) served as controls. Symbols in (C) and (D) represent means ± SEM.



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