IFN-I and IL-22 mediate protective effects of intestinal viral infection

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Products derived from bacterial members of the gut microbiota evoke immune signalling pathways of the host that promote immunity and barrier function in the intestine. How immune reactions to enteric viruses support intestinal homeostasis is unknown. We recently demonstrated that infection by murine norovirus (MNV) reverses intestinal abnormalities following depletion of bacteria, indicating that an intestinal animal virus can provide cues to the host that are typically attributed to the microbiota. Here, we elucidate mechanisms by which MNV evokes protective responses from the host. We identify an important role for the viral protein NS1/2 in establishing local replication and a type I interferon (IFN-I) response in the colon. We further show that IFN-I acts on intestinal epithelial cells to increase the proportion of CCR2-dependent macrophages and interleukin (IL)-22-producing innate lymphoid cells, which in turn promote pSTAT3 signalling in intestinal epithelial cells and protection from intestinal injury. In addition, we demonstrate that MNV provides a striking IL-22-dependent protection against early-life lethal infection by Citrobacter rodentium. These findings demonstrate novel ways in which a viral member of the microbiota fortifies the intestinal barrier during chemical injury and infectious challenges.
strain, the CR6 strain of MNV confers greater protection from DSS to ABX-treated mice\(^6\). Although MNV CR6 and CW3 share ~95% amino acid sequence identity, they display significant differences in their virulence. The VP1 major capsid protein of MNV CW3 accounts for the ability of this strain to cause lethality when introduced into immunocompromised Stat1\(^{−/−}\) mice\(^{13–15}\). The less-virulent CR6 strain evades recognition by lymphocytes to establish persistent infection\(^6\), which is attributed to sequence variation in NS1/2\(^1\). We used recombinant complementary DNA clones of MNV, in which the VP1 and NS1/2 regions were swapped between CR6 and CW3 (Fig. 1a), to determine which of these factors contribute to protection from DSS.

We first used GF mice to verify our previous observations made in ABX-treated mice. Unlike mice infected with CW3, GF mice infected with CR6 displayed similar survival levels to conventional mice following the administration of DSS, indicating that the CR6 strain has the ability to compensate for the complete absence of bacteria (Fig. 1b and Supplementary Fig. 1a). Although no chimaeric virus was able to induce the same level of protection as CR6, we observed significant differences. Sequence variation in VP1 did not contribute to protection as the CR6 mutant harbouring VP1 from CW3 (CR6.VP1\(^{CW3}\)), but not the converse (CW3.VP1\(^{CR6}\)), improved the survival of GF mice (Fig. 1c). In contrast, the CR3 mutant harbouring NS1/2 from CR6 (CW3.NS1/2\(^{CR6}\)) gained the ability to promote survival, whereas the CR6 mutant harbouring NS1/2 from CW3 (CR6.NS1/2\(^{CW3}\)) lost this ability (Fig. 1d). We previously demonstrated that protection against DSS correlates with the ability of MNV to restore the width of small intestinal villi in the absence of villi in bacterially depleted mice\(^1\). We found that the strains that enhanced survival during DSS treatment (MNV CR6, CR6.VP1\(^{CW3}\) and CW3.NS1/2\(^{CR6}\)) increased the villus width of GF mice (Fig. 1e,f). Therefore, NS1/2 contributes to the differential response to MNV CR6 and CW3 infection.

We detected all virus strains in the stool at day 10 post infection, which is indicative of productive infection, albeit at lower levels than conventional mice, consistent with the role of bacteria in promoting MNV infection\(^6\). However, the parental CR6 strain replicated to higher titres (Fig. 1g). We did not detect any correlation between extra-intestinal spread and survival following DSS treatment (Supplementary Fig. 1b). A more efficient intestinal infection could explain why GF mice infected with CR6 display the highest degree of survival, even when compared with CW3.NS1/2\(^{CW3}\) infection. Importantly, CW3.NS1/2\(^{CW3}\) (which protects against DSS) and the original CW3 strain (which does not provide protection) display similar degrees of shedding, indicating that additional factors are important for determining the effect of MNV infection on intestinal injury.

### Protection from DSS by MNV is dependent on IL-22

We and others have shown that MNV infection increases the numbers of IL-22-producing group 3 innate lymphoid cells (ILC3s) and IFN-γ-T cells in GF or ABX-treated mice\(^6\). MNV CR6 retained the ability to improve the survival of ABX- and DSS-treated IFN-γ receptor (Ifngr\(^{−/−}\)) mice but was unable to protect similarly treated Il22\(^{−/−}\) mice (Fig. 3a–c). Il22\(^{−/−}\) mice that received DSS without ABX displayed 100% survival, indicating that IL-22 is dispensable at this particular dose and duration of DSS treatment when intestinal bacteria are intact, and that this cytokine is especially necessary to overcome the heightened sensitivity to injury when bacteria are depleted. The loss of protection in Il22\(^{−/−}\) mice could not be explained by lower levels of intestinal virus (Fig. 3e). Increased villus width was observed following CR6 infection in ABX-treated WT, and to a lesser extent Ifngr\(^{−/−}\) mice, but not IL-22\(^{−/−}\) mice (Fig. 3d). These observations support the idea that the widening of villi reflects an increase in leukocytes and IEC proliferation downstream of an immune response to local viral replication.

We next examined whether MNV induces phosphorylation of STAT3 (pSTAT3) in the colon, as activation of this transcription factor downstream of IL-22 signalling mediates wound repair following DSS treatment\(^6\). We detected an IL-22-dependent increase in pSTAT3\(^{+}\) cells in the colon following infection with MNV CR6, whereas the positive control pSTAT1 (induced by IFN-I, IFN-λ, and IFN-γ) was detectable in all conditions (Fig. 3f–h). Interestingly, we found that MNV was also unable to induce pSTAT3 staining in the colon of Ifnr\(^{−/−}\) mice (Fig. 3h). MNV CR6 infection still induced significant ISG expression in Il22\(^{−/−}\) mice (Supplementary Fig. 3a), indicating that IFN signalling is not dependent on IL-22. MNV CR6 infection increased the proportion and absolute numbers of IL-22-producing ILCs in the colonic lamina propria in ABX- and DSS-treated WT but not Ifnr\(^{−/−}\) mice (Fig. 3i–j and Supplementary Fig. 3b,c and e). Viral infection increased the IFN-γ-CD4\(^+\) T cell populations (but not IL-22\(^+\) or IL-13\(^+\) CD4\(^+\) T cells) in an IFNAR1-dependent manner (Fig. 3i,j and Supplementary Fig. 3d); however, our earlier results (Fig. 3b) show that IFN-γ was not involved in protection from injury. MNV CR6 evoked similar responses in GF mice following DSS administration (Supplementary Fig. 3g,h). IL-22 secretion in colonic explants and pSTAT3 staining in colonic...
**Fig. 1 | Sequence variation in NS1/2 contributes to protection from intestinal injury.**

**a.** Illustration of the parental and chimaeric MNV strains. **b.** Survival rates of uninfected GF, MNV-infected GF mice (CR6 and CW3) and conventional (Conv) mice following the administration of 3% DSS in the drinking water for 5 d. **c,d.** Survival rates of GF mice infected with VP1 (c) or NS1/2 (d) chimaeric viruses followed by DSS administration. The mice were infected for 10 d before administration of 3% DSS for 5 d. The survival experiments with the parental strains and chimaeric viruses were conducted concurrently and therefore the uninfected GF survival curves shown in b,c,d are identical. **e.** Representative haematoxylin and eosin staining images of small intestinal villi (e) and the quantification of villus width (f) at 10 d post infection (d.p.i.). Scale bars, 50 μm. CR6 and CR6.VP1.CWS, n = 8 mice; all other groups, n = 7 mice. Yellow arrows in e indicate villus width. **g.** Infectious MNV in the stool as determined by plaque assays at 10 d.p.i. CW3, n = 18; CW3.VP1.CWS, n = 15; CW3.NS1/2.CWS, n = 17; CR6, n = 18; CR6.VP1.CWS, n = 17; and CR6.NS1/2.CWS, n = 18 mice. The survival curves were analysed using the log-rank Mantel–Cox test. Villus width and log10-transformed stool titres were analysed using an analysis of variance (ANOVA) with Dunnett’s multiple comparisons test to compare to GF mice and CR6-infected GF mice, respectively. All bars represent the mean and the error bars represent the s.e.m. All P values are shown; ns, not significant.
Fig. 2 | NS1/2 sequence variation contributes to intestinal persistence, IFN-I signalling and epithelial cell proliferation following intestinal injury. a, OasL2 (left), Mx2 (middle) and Isg15 (right) messenger RNA expression relative to uninfected GF mice versus the log_{10} viral genomes in the colon. Data represent four independent experiments. CW3, n = 8; CR6, n = 11; CW3.NS1/2^{CR6}, n = 11 and CR6.NS1/2^{CR6}, n = 10 mice. b, Viral genome levels in sorted colonic IECs (left) and LPLs (right). LOD, limit of detection. Data represent two independent experiments each with two mice per group. c, OasL2 mRNA expression relative to uninfected GF mice versus the log_{10} viral genomes in IECs (left) and LPLs (right). Data represent two independent experiments each with two mice per group. d, Representative images of the colon stained for Ki67 (left) and the corresponding fold change in Ki67+ IECs per crypt was analysed using an ANOVA with Dunnett’s multiple comparisons test. All bars represent the mean and the error bars represent the s.e.m.

DSS injury in ABX-treated Ccr2−/− mice (Fig. 4b, c). We observed an increase in several CCR2-dependent MHCI+ myeloid populations in the colon following infection with MNV CR6 (Supplementary Fig. 4a and Fig. 4d). Among these infiltrating cells, MNV-induced increases in CD11b+/CD11c+CD103− cells were observed in GF and ABX-treated WT mice but not ABX-treated Ifnar1−/− mice (Fig. 4e and Supplementary Fig. 4b). Other MHCI+ populations were not dependent on IFN-I signalling and recruitment of all sub-sets following infection remained intact in Il22−/− mice (Fig. 4e). Therefore, MNV-mediated protection from injury is associated with increases in several CCR2-dependent MHCI+ myeloid populations in the colon following infection with MNV CR6 (Supplementary Fig. 4a and Fig. 4d). Among these infiltrating cells, MNV-induced increases in CD11b+/CD11c+CD103− cells were observed in GF and ABX-treated WT mice but not ABX-treated Ifnar1−/− mice (Fig. 4e and Supplementary Fig. 4b). Other MHCI+ populations were not dependent on IFN-I signalling and recruitment of all sub-sets following infection remained intact in Il22−/− mice (Fig. 4e). Therefore, MNV-mediated protection from injury is associated
Fig. 3 | Protection of ABX-treated mice from intestinal injury is dependent on IL-22. a–c, Survival rates of WT (a), Ifngr<sup>−/−</sup> (b) and IL22<sup>−/−</sup> (c) mice following DSS administration. The mice were treated with ABX for at least 10 d before CR6 infection and given DSS at 10 d.p.i. for 6 d. d,e, Small intestinal villus width (d) and virus levels in the stool, colon and small intestine (SI) (e) at 10 d.p.i. with CR6. In the ABX, ABX + CR6 and Conv groups analysed for villus width: WT, n = 5, 5 and 4; Ifngr<sup>−/−</sup>, n = 9, 11 and 6; and IL22<sup>−/−</sup>, n = 10, 11 and 8 mice, respectively. In the groups analysed for virus titre in the stool, colon and small intestine: WT, n = 36, 4 and 17; Ifngr<sup>−/−</sup>, n = 12, 6 and 6; and IL22<sup>−/−</sup>, n = 25, 11 and 11 mice, respectively. f, Representative images of pSTAT1 and pSTAT3 expression in the colon of ABX-treated WT mice. Scale bars, 100 μm. g,h, Expression scores of pSTAT1 (g) and pSTAT3 (h) in the colon at day 6 post DSS administration. WT ABX, n = 9 and 9; WT ABX + CR6, n = 10 and 9; Ifnar1<sup>−/−</sup> ABX, n = 4 and 8; Ifnar1<sup>−/−</sup> ABX + CR6, n = 5 and 9; IL22<sup>−/−</sup> ABX, n = 6 and 5; and IL22<sup>−/−</sup> ABX + CR6, n = 6 and 6 mice for g and h, respectively. i,j, Proportion (i) and absolute numbers (j) of IL-22-expressing ILCs (CD19<sup>−</sup>CD11b<sup>−</sup>CD90.2<sup>−</sup>CD3<sup>−</sup>TCR<sup>+</sup>αβ<sup>−</sup>; left) and CD4<sup>+</sup> T cells (CD19<sup>−</sup>CD11b<sup>−</sup>CD90.2<sup>−</sup>CD3<sup>−</sup>TCR<sup>+</sup>αβ<sup>+</sup>; right) in the colon of ABX-treated WT and Ifnar1<sup>−/−</sup> mice at day 6 post DSS administration. WT ABX, n = 15 and 6; WT ABX + CR6, n = 15 and 6; Ifnar1<sup>−/−</sup> ABX, n = 12 and 9; and Ifnar1<sup>−/−</sup> ABX + CR6, n = 14 and 10 mice for i and j, respectively. The survival curves were analysed using the log-rank Mantel–Cox test. An ANOVA with Dunnett’s multiple comparisons test was used to analyse the villus width and log<sub>10</sub>-transformed MNV titres compared with ABX and WT mice, respectively. A two-tailed Student’s t-test was used to analyse the STAT expression levels. An ANOVA with Tukey’s multiple comparisons test was used to analyse the flow cytometry data. All bars represent the mean and the error bars represent the s.e.m. All P values are shown.
with IFN-dependent alterations to myeloid populations upstream of IL-22 expression by ILC3s.

**IFN-1 signalling in IECs is required for protection.** Although deletion of *Ifnar1* in IECs alters the composition of the bacterial microbiota, other studies have shown that IECs are unresponsive to IFN-1 and implicate a dominant role for IFN-λ signalling in inhibiting enteric RNA viruses. In contrast to *Ifnar1* control mice, MNV infection of ABX-treated *Ifnar1* mice; *Villin-Cre* mice in which *Ifnar1* is deleted in IECs (*Ifnar1IEC*) did not enhance survival following DSS injury or restore villi width (Fig. 5a–c). We did not observe an IEC-specific role for IFNAR1 in restricting MNV burden (Fig. 5d). The ability of MNV CR6 to induce pSTAT3 staining was eliminated in *Ifnar1IEC* but not, *Ifnar1* mice (Fig. 5e).
Recombinant IFN-β induced Mx2 expression in human and mouse intestinal organoids (Fig. 6a–c), confirming that IECs can directly respond to IFN-I. According to our model, STAT3 is activated by IL-22 directly and IFN-I indirectly during MNV infection. We found that IL-22, but not IFN-β, induced pSTAT3 and REG3β in organoids (Fig. 6d,e and Supplementary Fig. 6). These findings show that IECs respond to IFN-I and that STAT3 phosphorylation is an independent event. MNV-induced increases in IL-22+ and IFN-γ+ ILCs and MHCII+CD11b+CD11c+CD103+ cells were absent in Iftar<sup>1<sub>−<sub>−</sub></sup> mice (Fig. 6f–g and Supplementary Fig. 4c,d).
Fig. 6 | IECs react to IFN-I stimulation and promote an IL-22 response. a–c. Representative images of murine small intestine (a), murine colon (b) and human colon (c) organoids that were either unstimulated or stimulated with 500 U ml⁻¹ IFN-β for 48 h (left) and the expression levels of Mx2 mRNA relative to unstimulated organoids (right). Scale bars, 50 μm. n = 7 (a), 3 (b) and 3 (c) independent experiments. The log_{10}-transformed Mx2 expression data were analysed using a two-tailed paired Student’s t-test. d. Phosphorylation of STAT3 at 2 h in small-intestine organoids. Images are representative of four independent experiments. e. Representative images of Reg3β expression (left) and representative mean fluorescence intensity (MFI) of individual Lin− cells (% right) in ABX-treated Ifnα/Ifnβ−/− mice at day 6 post DSS administration. f. Ifnar1−/− ABX, n = 16; Ifnar1−/− ABX + CR6, n = 19; Ifnar1−/− ABX + CR6, n = 15 and Ifnar1+/− ABX + CR6, n = 15 mice. g. Ifnar1−/− ABX, n = 18; Ifnar1−/− ABX + CR6, n = 20; Ifnar1+/− ABX, n = 14 and Ifnar1+/− ABX + CR6, n = 14 mice. An ANOVA with Tukey’s multiple comparisons test was used to analyse the cell populations. h. Gene expression analysis of IECs from MNV CR6-infected Ifnar1−/− (n = 3) and Ifnar1+/− (n = 3) mice compared with uninfected mice (Ifnar1−/−, n = 3 and Ifnar1+/−, n = 3) at day 6 post DSS administration. The circles in the Venn diagram represent the number of transcripts that are enriched following MNV infection in the mice with the indicated genotypes. Pathway analysis was performed on the non-overlapping gene set and P values were determined by ingenuity pathway analysis. All bars represent the mean and the error bars represent the s.e.m. All P values are shown.
We then performed RNA sequencing analysis on sorted IECs from Ifnar1Δ/Δ and Ifnar1Δ/ΔC mice following DSS administration, with or without viral infection. MNV CR6 infection led to an increased expression of genes representing cell-cycle regulation and DNA damage response pathways in a manner dependent on epithelial IFNAR1 (Fig. 6h). This IFNAR-dependent gene expression pattern is consistent with MNV-induced IEC proliferation (Fig. 2d) and is remarkably similar to a recently described IL-22-induced transcriptional response that prevents genotoxic damage to epithelial stem cells in the colon. Together, these data indicate that IFNAR signalling in the epithelium mediates the beneficial effect of MNV infection and decouples the role of IFN-I in antiviral versus injury responses.

**MNV protects against enteric bacterial infection during development.** Over time, the gastrointestinal tract becomes exposed to diverse animal viruses after birth. It is unclear how the presence of these viruses affects disease caused by enteric bacterial pathogens, which are also a common occurrence during childhood. To examine the consequences of coinfection, three-week-old WT mice were challenged with the Gram-negative bacterial pathogen *Citrobacter rodentium*, with or without concurrent MNV CR6 infection. In the absence of the virus, WT mice failed to grow and succumbed to *C. rodentium* infection, whereas almost all of the MNV CR6-infected mice survived and gained weight (Fig. 7a). MNV CR6 did not provide any benefit to three-week-old Il22−/− mice (Fig. 7b). Next, we took advantage of the observation that lymphocyte-deficient Rag1−/− mice have a compensatory increase in IL-22-producing ILC3s during a similar developmental period as the above coinfection model. We hypothesized that this increase in ILC3s would serve a similar purpose to MNV infection and thus Rag1−/− mice will no longer be dependent on MNV for survival during *C. rodentium* infection. Rag1−/− mice were indeed protected from bacterial infection irrespective of MNV infection, which was associated with an overall increase in ILC3s (Supplementary Fig. 5a,b). Therefore, enhancing IL-22+ ILC3s through viral infection or genetically is associated with protection against secondary bacterial infection in juvenile animals.

IL-22 is more important for reducing intestinal inflammation than for reducing the burden of intestinal *C. rodentium* in adult mice. Consistent with this observation, MNV CR6 infection of WT mice led to a modest or no reduction in bacterial burdens (Fig. 7c–e). Furthermore, similar numbers of *C. rodentium* were recovered from the stools of MNV-infected WT and Il22−/− mice. We instead observed that MNV CR6 infection reduced intestinal pathology in an IL-22-dependent manner (Supplementary Fig. 5c). Protection from *C. rodentium* infection was independent of the MNV strain, as CW3-infected WT mice showed increased survival and weight gain (Supplementary Fig. 5d). Protection from *C. rodentium* infection was also maintained in Ifnar1Δ/Δ mice (Supplementary Fig. 5e). Thus, sequence variation in NS1/2 and IFNAR1 signalling does not contribute to protection from bacteria-induced injury.

It is possible that MNV protects young mice by accelerating the development of the microbiota because the compositional shift in intestinal bacterial communities that occurs during neonatal development is key to *C. rodentium* resistance. However, we did not detect significant changes in the gut microbiota due to MNV infection or absence of IL-22 (Supplementary Fig. 5f). Instead, MNV-mediated protection from *C. rodentium* was associated with a general increase in the proportion of CD4+ and CD8+ T cells in the colon, which was not observed in MNV-infected Il22−/− mice (Fig. 7f). In addition, MNV CR6 increased the proportion of IFN-γ-expressing CD8+ T cells and ILCs in an IL-22-dependent manner and, similar to the DSS model, the presence of the virus was associated with IL-22+ ILCs (Fig. 7g,h).

**Discussion**

The bacterial members of the gut microbiota acquired during development promote the maturation of the immune system and intestinal barrier. This mutualistic host–microbiota relationship can be disrupted in adulthood by ABX treatment, diet or other factors that alter bacterial community structures. Tremendous effort has thus been placed in understanding the mechanistic basis of this relationship and clinical trials are attempting to remodel the microbiota in diseased individuals by transplanting isolated microorganisms or faeces from healthy donors. Despite accumulating evidence of the presence of a dynamic enteric virome that begins to take shape after birth, it is unknown how intestinal viruses impact the resilience of the intestinal barrier to damage caused by environmental agents. Using two distinct models, we show that MNV infection improves the outcome of intestinal injury through IL-22.

In the chemical injury model, we demonstrate that compensation for the absence of beneficial bacteria by MNV is associated with the NS1/2 region of the viral genome. NS1/2 from mouse and human noroviruses binds the host molecule VAMP-associated protein A (VAPA), which potentially mediates membrane remodelling during viral replication. NS1/2 is structurally disordered and subject to cleavage, suggesting that it can adopt additional conformations with alternate functions that are currently unknown. NS1/2 from the protective MNV CR6 strain confers the ability to replicate and persist in colonic IECs, leading to a local IFN-I response that is probably downstream of MAVS-dependent IFN-β production in neighbouring immune cells, although we cannot rule out an epithelial contribution of IFN-β. Our extensive analyses of Ifnar1Δ/Δ mice indicate that IECs are the IFN-responding cell type.

We further found that MNV induces IL-22-producing ILC3s in an IFNAR1- and CCR2-dependent manner. Unlike IFN-λ, which acts on neutrophils to protect against DSS, we identified a role for IFN-I acting on IECs to mediate the recruitment or activation of CCR2-dependent cells during MNV infection. Recently, we showed that enhanced IFN-I signalling due to autophagy inhibition in IECs alters the function of CCR2-dependent monocytes. Thus, it is also plausible that augmenting IFN-I signalling through MNV CR6 infection also induces functional changes to these populations. A role for macrophages in the activation of ILC3 has been previously demonstrated following *C. rodentium* infection. We therefore hypothesize that alterations in the proportions and potentially the function of macrophage populations, through IFNAR1-mediated responses in IECs, affects the ability of these cells to promote IL-22 production by ILC3s.

MNV-mediated protection of young mice from *C. rodentium* is also IL-22-dependent. The mechanism is distinct from another study that reported that MNV confers resistance to vancomycin-resistant *Enterococcus* in ABX-treated adult mice involving a virus-dependent reduction in bacterial colonization. Vancomycin-resistant *Enterococcus* is a Gram-positive bacterium that is sensitive to antimicrobial REG molecules produced in response to IL-22, whereas other functions, such as epithelial repair, may be more important for protecting against Gram-negative bacteria such as *C. rodentium*, especially in young mice. It was recently shown that MNV recruits CCR2-dependent monocytes to the mesenteric lymph node (MLN) in an IFNAR1-independent manner to support systemic persistence. Although we identified a subset of myeloid cells that were IFNAR1 dependent, this was not universal across all subsets. Therefore, unlike the DSS model, accumulation of IFNAR1-independent macrophages may be sufficient to provide protection following *C. rodentium* infection, which would explain why IFNAR1 is dispensable and MNV CW3 is able to provide protection. Due to its increased virulence compared with other strains, infection of mice with MNV CW3 has been used as a surrogate for pandemic human norovirus GII.4 strains. Given that up to 30% of asymptomatic infants and children are positive for norovirus RNA
in the stool\(^{61}\), concurrent infection with bacterial pathogens is likely to be commonplace. Our results raise the possibility that underlying norovirus infections may have a protective role when the virus is not evolving diarrheal disease.

The inter-strain diversity between human noroviruses is far greater than the sequence differences between MNV strains, yet little is known about how different noroviruses impact the mucosal immune system of the human gut. This lack of knowledge applies to other enteric viruses as well. Elegant work combining observations in mice and humans implicates reoviruses and noroviruses in the onset of celiac disease in a virus strain-dependent manner\(^{65,66}\). Cytomegalovirus can reactivate in the gastrointestinal tract and contribute to inflammatory bowel disease flares but was shown to enhance epithelial turnover in mice, which presumably improves resistance to injury\(^ {62}\). These observations resemble some aspects of our findings with MNV showing that an intestinal virus can be either beneficial or adverse depending on the strain and properties of the host. Finally, we wish to note that few studies investigating the effect of the bacterial microbiota exclude the possibility that results are driven by the presence of MNV or other enteric viruses, such as astroviruses, that are common in mouse facilities. We suggest that the host immune response to enteric viral infections requires attention beyond its antiviral function and needs to be considered in microbiota studies.

**Methods**

**Mice.** GF mice on a C57BL/6J background were bred and maintained in flexible-film isolators in the New York University (NYU) School of Medicine Gnotobiotics Animal Facility. Absence of fecal bacteria was confirmed monthly, as before\(^ {67}\). For experiments, GF mice were housed in Techniplast Bioexclusion cages with access to sterile food and water. Conventional C57BL/6J WT, Ifnar1\(^{-/-}\), Ifng\(^{-/-}\), Ccr2\(^{-/-}\), Mass \(^{-/-}\), Rag1\(^{-/-}\), Ifnar1\(^{-/-}\) and Villin-cre mice were purchased from Jackson Laboratories. Il22\(^{-/-}\) mice were provided by S. Koralov (NYUMC). Ifnar1\(^{-/-}\) mice were bred to Villin-cre mice to produce cre\(^{-/-}\) and cre\(^{-/-}\) littersmates. Mass\(^{-/-}\) mice on a B6129S/EJ background were bred to C57BL/6J mice to produce Mavs\(^{-/-}\) mice that were crossed to each other to produce Mavs\(^{-/-}\) and Mavs\(^{-/-}\) littersmates. All other knockout strains were on the C57BL/6J background and C57BL/6J mice were used as WT controls. All mice were bred onsite in an MNV and Helicobacter-negative specific-pathogen free animal facility. Randomly assigned age- (8–10 weeks) and gender-matched mice were used for the DSS experiments, in which MNV-infected mice were compared with uninfected littersmates to control for potential microbiota differences between mice originating from different breeding pairs. Age-matched 12–14-day-old litters, which were generated from two pregnant

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**Fig. 7 | MNV protects young mice from enteric bacterial infection.** a, b. Survival (left) and weight (right) of B6 (a) and Il22\(^{-/-}\) (b) mice infected with C. rodentium at the age of 21 d. The survival curves were analysed using the log-rank Mantel–Cox test. For the weight curves, the area under the curve was determined for each mouse and the differences between the uninfected and CR6-infected groups were determined by a two-tailed Student’s t-test. All bars represent the mean and the error bars represent the s.e.m. All P values are shown.
dams that were co-housed and the subsequent litters separated approximately 2–4 d before infection with MNV, were used for the C. rodentium experiments. The dams were litermates from the previous generation. All young mice were weighed daily throughout the experiment. The sample size for animal experiments was chosen based on previous data generated in the laboratory. All animal studies were performed according to protocols approved by the NYU School of Medicine Institutional Animal Care and Use Committee.

**ABX and DSS treatment.** Mice were given filter-sterilized water containing ampicillin (1 g l⁻¹; American Bioanalytical), vancomycin (0.5 g l⁻¹; Sigma), neomycin (1 g l⁻¹; Sigma), metronidazole (1 g l⁻¹; Sigma) and 1% sucrose (Sigma) for the ABX treatments. ABX-containing water was replaced at least once a week. The mice were treated with this ABX cocktail for at least 10 d before infection. The mice were given 3% DSS (TdB Consultancy), with or without the ABX cocktail, in their drinking water for 6 d, followed by regular or ABX-containing drinking water for the remainder of the experiment. The GF mice were given 3% DSS in filter-sterilized water for 5 d. The mice were monitored daily for survival.

**MNV and bacterial inoculation.** Plasmids for chimaeric MNV strains were previously described. Two viruses were on a CR6 backbone containing the indicated gene from CRW3: CR6-VPI^mOv and CR6.NSI1^mOv. Two viruses were on a CR3W backbone containing the indicated gene from CRW6: CR3W-VPI^mOv and CR3W.NSI1^mOv. Chimaeric viruses, CRW3 and CR6 were prepared and titrated as described previously. The mice were infected orally by pipette with 3 x 10⁷ plaque-forming units (p.f.u.) resuspended in PBS (Corning). C. rodentium ∆BSO100 was grown overnight in Luria–Bertani broth with shaking at 37 °C and diluted 1:100, followed by an additional 4 h of growth until the bacteria were at an optical density of 2. The mice were gavaged with 2 x 10⁶ colony-forming units (c.f.u.). Bacterial inocula were determined by serial dilution plating on MacConkey plates.

**Detection of infectious MNV and C. rodentium in organs.** Stools, ileum (2 cm), ascending colon (2 cm), lungs, spleens and MLNs were harvested from MNV-infected mice at 10 d.p.i. The organs were weighed and homogenized in PBS and serial dilutions were plated onto BMDCs or Corning. The titres of MNV were determined by plaque assays as previously described. The titres are shown as p.f.u. g⁻¹ or p.f.u. cm⁻¹. Stools, lungs and spleens were harvested from C. rodentium-infected mice at 6 d.p.i. The organs were weighed and homogenized in PBS and serial dilutions were plated onto MacConkey plates. The titres are shown as c.f.u. g⁻¹.

**Organoid culture.** Murine small intestinal and colonic organoids were cultured as described previously. At day 5 (small intestine) or day 3 (colon), organoids were stimulated with either 500 U ml⁻¹ IFN-β (PBL Assay Science), 5 ng ml⁻¹ β-2M (PBL Assay Science), 5 ng ml⁻¹ IFN-γ (XMG1.2) from Biolegend, and IL-22 (1H8PWSR) from eBioscience cell stimulation cocktail for 4 h at 37 °C. The cells were fixed and permeabilized using the Biolegend fixation and permeabilization buffer. The cells were stained with anti-mouse phospho-Stat1 (Tyr701; Cell Signaling Technology), rabbit anti-mouse phospho-Stat3 (Tyr705; Cell Signaling Technology) or rabbit anti-mouse Ki67 (MIB1) and used as individual data points. Sections were imaged on the Evos FL Auto cell imaging system (Thermo Fisher Scientific). All analyses were performed using ImageJ software.

**Flowerometry.** Lamina propria cells from colonic tissue were harvested as before. For intracellular cytokine staining, cells were stimulated using the eBioscience cell stimulation cocktail for 4 h at 37 °C. The cells were fixed and permeabilized using the Biolegend fixation and permeabilization buffer. The following antibodies (clones) were used for staining: CD45 (30-F11) from Biolegend, CD11b (M1/70), CD90.2 (53–2.1), CD3 (145-2C11), TCR-β (PBL Assay Science), 5 ng ml⁻¹ IFN-γ (XMG1.2), 5 ng ml⁻¹ IL-13 (eBio13A) from eBioscience. All samples were blocked with Fc Block (TruStain FcX) and stained with a fixable live/dead stain (Invitrogen). Flow cytometry was used to analyse the flowerometry data. IECs were harvested by incubation at 37 °C with 2 mM dithiothreitol (Sigma), followed by two incubations with 5 mM EDTA and then digested with Dispase (Sigma) and Dnase (Sigma). The IECs were sorted as PI-CD45-EP-Cam⁺ using the following antibody clones: CD45 (30-F11) and EP-Cam (G8.8) from Biolegend on a FACSAria II (BD Biosciences). The purity of the IECs was >95%.

**Colon explant culture and IL-22 detection.** The colon tissue (1 cm) was opened longitudinally, washed with PBS and cultured for 4 h in 500 μl complete RPMI containing 2% l-glutamine (Corning), penicillin/streptomycin solution (Gibco), 5% (v/v) FBS (Corning), 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Corning) and 1 mM sodium pyruvate (Corning) and β-mercaptoethanol (Gibco). The IL-22 in supernatants was measured using the mouse IL-22 ELISA MAX Deluxe kit (Biolegend) according to the manufacturer’s instructions.

**RNA isolation and qPCR.** RNA from BMDCs and colonic tissue (1 cm) homogenized using a TissueRuptor (Qiagen) was isolated using the Qiagen RNasey mini kit with DNP treatment (Qiagen). RNA from sorted IECs and LPLs was isolated using the RNasey micro kit with DNP treatment (Qiagen). RNA from colon tissues was isolated using TRIzol reagent (Fisher Scientific) as per the manufacturer’s protocol and the DNA was digested using RNase-free DNase (Thermo Fisher Scientific). Synthesis of cDNA was conducted with the ProtoScript M-MuLV first strand cDNA synthesis kit (New England Biolabs). Quantitative PCR was performed using SybrGreen (Roche) on a Roche480II Lightcycler using the following primers: mHpr (accession no. J003423), Fwd 5'-GTCATGCGCATCGCTGACCTA-3' and Rev 5'-CCGCTGACCGTTGTTGTTGCG-3' (mHpr6s) (accession no. NR_003728), Fwd 5'-TAGAGGGCAACAGGGCCATTTCC-3' and Rev 5'-CCGTCGACGCGCTGACCC-3'; mHf1 (accession no. X14455), Fwd 5'-TCAGAGATGAGTTGTTGTTGCG-3' and Rev 5'-GGCTTTCAAGCTGAGTTAGTACA-3'; mHf2 (accession no. NM_011854), Fwd 5'-GGGATGGCTGGGAGAATGCT-3' and Rev 5'-TGCGCTGCTGCTGAGGACCTG-3'; mIg15 (accession no. NM_017583), Fwd 5'-GGTGTTGCGTACTAATCTCCT-3' and Rev 5'-TGGAAGGCTAGACCGCTGCT-3'; mIF-h (accession no. NM_0011019), Fwd 5'-GCTTACGCGACCGGCCTC-3' and Rev 5'-CCGACGATGCTGTTGCTA-3'; and mIg2 (accession no. NM_002463), Fwd 5'-AACTCTTGCGACGACCGTAAAGG-3' and Rev 5'-ACCATGCTGCTAGGTTAAGC-3'. Relative expression of the respective genes to either Hprt (murine organs), 18S (murine colon or sorted cells) or β-actin (human organoids) expression was calculated using the 2^ΔΔCt method.
and the values were expressed as fold change from uninfected mice or untreated organoids. Absolute MNV genome copies were determined using a standard curve and the following primers: Fwd 5′-CAGATCACATGCTTCCCCAC-3′ and Rev 5′-AGACACAAAAAGACTCATCAC-3′.

**Immunoblotting.** Organoids were isolated and processed for immunoblotting as previously described. Briefly, protein was run through a 4–12% gradient gel (BioRad), transferred onto a PVDF membrane and blocked using Odyssey blocking buffer (LI-COR). The membranes were stained with mouse anti-STAT3 and rabbit anti-pSTAT3 (Ty705) and detected using IRDye 680RD goat anti-rabbit IgG and IRDye 800CW goat anti-mouse IgG (LI-COR) on an Odyssey CLx near-infrared-fluorescence imaging system.

**RNA deep sequencing.** The cDNA libraries were prepared with the Ovation Trio Low Input RNA-Seq System V2 (NuGEN) and sequenced on Illumina HiSeq platform using a paired-end protocol at the NYU Genomics Core. On average, each sample yielded 25 × 10^6 paired reads, which passed FASTQC quality control. The sequencing reads were aligned with STAR v2.6.1d against the GRCh38.p6 genome assembly, resulting in an average of 20 × 10^6 aligned pairs per sample and normalized read counts were calculated using Cufflinks v.2.2.1 against the same reference genome. Differential gene expression was determined with Cuffdiff and gene ontology analysis performed using Qiagen’s Ingenuity Pathway Analysis.

**16S library preparation and sequencing analysis.** DNA was isolated from stool samples using the Nucleospin Soil kit (Macherey-Nagel). Bacterial DNA was isolated from stool gene sequencing was performed on the Illumina MiSeq system. The sequencing reads were processed using the DADA2 pipeline in the QIMEM software package. Beta diversity was calculated using unweighted UniFrac distance. Principle coordinate analysis was performed on the UniFrac distance matrix and visualized with EMPeror.

**Statistical analysis.** All data were analysed using GraphPad Prism v.7. All graphs show the mean and s.e.m. The number of samples per group is indicated in the figure legends. The log-rank Mantel–Cox test was used for the comparison of survival curves. An unpaired two-tailed Student’s t-test was used to evaluate the differences between two groups. Welch’s correction was used when variances were significantly different between groups. An ANOVA with Dunnett’s or Tukey’s multiple comparisons test was used to evaluate experiments involving multiple groups. To analyse bacterial and viral titres, data were log_{10}-transformed. Correlations were analysed using Pearson’s r. The weight curves were analysed using area under the curve followed by a two-tailed t-test. All P values are shown in the figures.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The data that support the findings of this study are available from the corresponding author on request. FASTQ files corresponding to the RNA–seq and 16S rRNA sequencing have been deposited in a public database (RNA–seq accession no. GSE129384 and 16S accession no. PRJNA532632).

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Author contributions

J.A.N. and K.C. formulated the original hypothesis and designed the study. J.A.N. performed the experiments and analyses, and received assistance from E.K.-H. (DSS experiments), S.S. (in vitro MNV responses) and Y.M.-I. (organoids), S.L.S. and M.V. processed and analyzed the samples for 16S rRNA sequencing. A.G.N. performed the histopathology analysis. P.L., D.H. and A.H. provided the human colon biopsies. S.D. performed the sorting of IECs. T.L.N. provided the cDNA clones of MNV and advice. J.A.N. and K.C. wrote the manuscript. All authors commented on the manuscript, data and conclusions.

Competing interests

K.C. has consulted for PureTech Health and AbbVie Inc. and is an inventor on US patent application 62/608,404.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41564-019-0470-1.

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- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated
- Clearly defined error bars
  State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

FACSDiva was used to collect Flow cytometry data. RNASeq data was obtained using the Ovation Trio Low Input RNA–seq System V2 (NuGEN) and Illumina’s HiSeq platform. Bacterial 16S rRNA gene was amplified at the V4 region using primer pairs and paired-end amplicon sequencing was performed on the Illumina MiSeq system.

Data analysis

All analysis except for 16S sequencing and RNASeq was performed using Graphpad Prism v8. ImageJ 1.49v was used for histology analysis. FloJo v 10 was used to analyze all flow cytometry experiments. Bacterial 16S rRNA sequencing reads were processed using the DADA2 pipeline in the QIIME2 software package. Beta diversity was calculated using unweighted UniFrac distance. Principle Coordinate Analysis (PCoA) was performed on the UniFrac distance matrix and visualized with EMPeror. RNASeq reads were aligned with STAR v2.6.1d against the GRCm38.p6 genome assembly, resulting in an average of 20 million aligned pairs per sample, and normalized read counts were calculated using Cufflinks v2.2.1 against the same reference genome. Differential gene expression was determined with Cuffdiff and gene ontology analysis performed using Qiagen’s Ingenuity Pathway Analysis (IPA, Qiagen).

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

The number of animals used in the experiments in this study is estimated based on a power analysis with the following assumptions: standard deviation will be ~20% of the mean, p-value will be under 0.05 when the null hypothesis is false, the effect size (Cohen’s d) is between 1.0-2.0. The minimal number of mice required under these conditions ranges between 6-28 for in vivo experiments. Additionally, we have carefully chosen the sample size listed below based on empirical evidence of what is necessary for interpretation of the data and statistical significance.

Data exclusions

No data was excluded from the analysis.

Replication

In all experiments, no attempts at replication failed. To confirm the reproducibility of experiments, each independent experiment was analyzed to confirm results before combining multiple replicate experiments.

Randomization

Groups were established based on genotype and infection status. All other aspects were randomized.

Blinding

Blinding was used for analysis for all histological analysis by assigning numbers to the samples in place of genotype or infection status. Blinding was not performed for flow cytometry analysis. Blinding was not possible for flow cytometry experiments because all samples were collected and processed on the same day by the same person and strain and infection status were indicated on the mouse cage.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a
- Involved in the study
- Unique biological materials
- Antibodies
- Eukaryotic cell lines
- Palaeontology
- Animals and other organisms
- Human research participants

Methods

n/a
- Involved in the study
- ChIP-seq
- Flow cytometry
- MRI-based neuroimaging

Antibodies

Antibodies used

For flow cytometry: CD19 (6D5, Biolegend, 115530), CD11b (M1/70, Biolegend, 101226), CD90.2 (53-2.1, Biolegend, 140322), CD3 (145-2C11, Biolegend, 100310), TCR-β (H57-597, Biolegend, 109222), CD4 (RM4-5, Biolegend, 100559), CD8 (53-6.7, Invitrogen, 56-0081-82), IL-22 (1H8PSR, ebioscience, 12-7221-82), IFN-γ (XMG1.2, Biolegend, 505814), IL-13 (eBio13Ae, eBiocience, 53-7133-82), CD45 (30-F11, Biolegend, 103132), MHCII (M5/114.15.2, Biolegend, 107631), CD11c (N418, Biolegend, 117334), CD103 (2E7, Biolegend, 121414) and Gr-1(RB6-85, Biolegend, 108406).

For Histology: Unconjugated rabbit anti-mouse phospho-Stat1 (Y701, clone DB6, Cell Signaling Technology #9167), rabbit anti-mouse phospho-Stat3 (Y705, Cell Signaling Technology #9131) or rabbit anti-mouse Ki67 (M3062, Spring Biosciences), sheep anti-mouse Reg3β antibody (R&D systems, AF5110) and anti-sheep Ig NLS57 (R&D systems, NL010).
For western blot: mouse anti–STAT3 (#9139, Cell Signaling Technology) and rabbit anti–pSTAT3 (#9131, Tyr705, Cell Signaling Technology) and detected using IRDye 680RD goat anti–rabbit IgG and IRDye 800CW goat anti–mouse IgG (LI-COR).

Validation
All antibodies used in this study were validated by the supplier using mouse cells or have been used in prior publications (Kernbauer, E., Ding, Y. & Cadwell, K. An enteric virus can replace the beneficial function of commensal bacteria. Nature 516, 94-98 (2014) and Martin, P. K. et al. Autophagy proteins suppress protective type I interferon signalling in response to the murine gut microbiota. Nat Microbiol 3, 1131-1141 (2018).

Eukaryotic cell lines
Policy information about cell lines
Cell line source(s)
RAW 264.7 (ATCC® TIB-71™) and 293T (ATCC® CRL-3216™)
Authentication
No cell lines were authenticated
Mycoplasma contamination
All lines tested negative for mycoplasma contamination
Commonly misidentified lines
None of the cell lines used in this study are listed on the ICLAC database (See ICLAC register)

Animals and other organisms
Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research
Laboratory animals
Laboratory mice were used in this study. The following strains were used: C57BL/6J WT, Ifnar1−/−, Ifngr−/−, Ccr2−/−, Mavs−/−, Rag1−/−, Ifnar1fl, Villin-cre and IL22−/− mice. Experiments were conducted on both males and females. For adult experiments, mice between 8-10 weeks of age were used. For young mouse experiments, 12-14 day old mice were used.
Wild animals
Wild animals were not used in this study
Field-collected samples
Field-collected samples were not used in this study

Flow Cytometry
Plots
Confirm that:
☑️ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☑️ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
☑️ All plots are contour plots with outliers or pseudocolor plots.
☑️ A numerical value for number of cells or percentage (with statistics) is provided.
Methodology
Sample preparation
Colon was processed for the isolation of intestinal epithelial cells and lamina propria leukocytes
Instrument
BD LSR II and BD FACSAria II
Software
BD FACSDiva for data collection and FlowJo v.10 for analysis
Cell population abundance
Purity of samples was determined at the time of sorting by running a sorted sample on the FACSAria. All samples tested were more than 95% pure.
Gating strategy
Cells were gated on FCS-A and SSC-A. Doublets were removed by gating FSC-A v FSC-H. Live cells were gated as Zombie-Dye negative. Innate lymphoid cells were gated as CD11b-CD19-CD90.2+CD3-TCRb- and CD4+ T cells were gated as CD11b-CD19-CD90.2+CD3+TCRb+CD4+CD8-. Gates for IL-13, IL-22 and IFNg were set using an antibody isotype control sample. For MHCII+ populations, cells were gated as CD45+CD19-CD90.2+CD3+TCRb-Gr-1lowMHCII+ followed by gating of populations based on expression of CD11b, CD11c and CD103. For sorting for epithelial cells, cells were gated as PI-CD45-EpCam+

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