An enteric virus can replace the beneficial function of commensal bacteria

Elisabeth Kernbauer1,2, Yi Ding3,4 & Ken Cadwell1,2

Intestinal microbial communities have profound effects on host physiology. Whereas the symbiotic contribution of commensal bacteria is well established, the role of eukaryotic viruses that are present in the gastrointestinal tract under homeostatic conditions is undefined. Here we demonstrate that a common enteric RNA virus can replace the beneficial function of commensal bacteria in the intestine. Murine norovirus (MNV) infection of germ-free or antibiotic-treated mice restored intestinal morphology and lymphocyte function without inducing overt inflammation and disease. The presence of MNV also suppressed an expansion of group 2 innate lymphoid cells observed in the absence of bacteria, and induced transcriptional changes in the intestine associated with immune development and type I interferon (IFN) signalling. Consistent with this observation, the IFN-α receptor was essential for the ability of MNV to compensate for bacterial depletion. Importantly, MNV infection offset the deleterious effect of treatment with antibiotics in models of intestinal injury and pathogenic bacterial infection. These data indicate that eukaryotic viruses have the capacity to support intestinal homeostasis and shape mucosal immunity, similarly to commensal bacteria.

Despite significant limitations in the ability to detect and annotate enteric viruses present in the gastrointestinal tract, recent deep-sequencing efforts reveal the existence of a complex enteric virome. Members of this viral component of the intestinal microbiota could include pathogens such as noroviruses that continue to persist after disease is resolved, Anelloviridae and Circoviridae family members that are ubiquitously detected in healthy individuals, and uncharacterized viruses that display little sequence identity with known viruses. Also, examination of the enteric virome of rhesus monkeys infected with simian immunodeficiency virus (SIV) suggest that many viruses are present at low levels and are kept in check by the immune system. Evidence that such viruses in the intestine can contribute to physiology beyond acute diarrhoeal disease is provided by studies examining MNV, a positive-strand RNA virus of the Caliciviridae family that is endemic in mouse facilities. MNV displays tropism for myeloid cells and can establish persistent infection without causing obvious disease in immuno-competent mice. We recently demonstrated that persistent infection by the MNV strain CR6 (MNV.CR6) induces intestinal pathologies in mice deficient in the inflammatory bowel disease gene Atg16l1 (ref. 15). The observation that MNV induces inflammatory pathologies in a genetically susceptible host resembles similar observations made with commensal bacteria. However, the bacterial component of the microbiota also provides considerable benefit to the host by generating metabolites, promoting the development of the mucosal immune system, and preventing colonization by pathogenic microorganisms. It is unknown whether eukaryotic viruses in the intestine interact with the host in an analogous symbiotic manner.

Much of our knowledge on the role of commensal bacteria in mucosal immunity comes from the characterization of germ-free (GF) mice, which show aberrant intestinal morphology and deficiencies in the lymphocyte compartment due to the absence of bacteria. Thus, we used GF mice as a reductionist model to determine whether MNV infection can provide developmental cues that have been mainly attributed to bacteria. GF mice were mono-associated with MNV.CR6 (GF+MNV) in a gnotobiotic isolator by infecting breeding pairs with 3 × 10^3 plaque-forming units (p.f.u.) and allowing the virus to be naturally propagated to offspring in subsequent generations, analogous to newborns that inherit commensal bacteria from parents (Extended Data Fig. 1a). Despite persistent presence of the virus, there were no signs of overt inflammation in the intestine (Extended Data Fig. 1b–d).

While GF mice had thin villi containing few CD4+ T cells and narrow crypts, the general appearance of the small intestine in GF+MNV mice resembled that of conventional mice (Fig. 1a–f). The presence of virus in GF mice also partially restored the number of granules and lysozyme expression in Paneth cells (Extended Data Fig. 2a–d). These changes in appearance due to MNV.CR6 were associated with a significant increase in the overall cellularity of the lamina propria and mesenteric lymph nodes (MLNs) (Fig. 1g, h). Consistent with the ability of certain bacterial species to promote lymphocyte differentiation, GF mice had reductions in the numbers of CD4+ T cells in the lamina propria and MLNs, CD8+ T cells in MLNs, IFN-γ expression by these cells, and mucosal and serum antibody production (Extended Data Fig. 2e–p). By contrast, GF+MNV mice displayed increases in these factors, in some cases to an extent similar to or in excess of conventional mice (Extended Data Fig. 2e–p). These effects of the virus were not dependent on neonatal infection since a reversal of abnormalities was observed when adult GF mice were infected with MNV.CR6 for 10 days (Extended Data Fig. 3). Also, the effects of MNV.CR6 cannot be explained by uncontrolled viral replication because GF mice harbour less virus compared with conventional mice upon infection (Extended Data Fig. 1b), as seen with other intestinal viruses. Importantly, these changes in response to MNV.CR6 are specific to conditions in which bacteria are absent. MNV.CR6 had little or no effect on the appearance of the intestine and lymphocytes in conventional mice (Extended Data Fig. 4). Therefore, there is considerable overlap between the responses to MNV and to commensal bacteria.

The host response evoked by MNV could depend on anatomical location or on the strain being examined. Abnormalities in the morphology of the colon of GF mice were not as apparent as in the small intestine, but we detected a significant increase in natural killer (NK) T cells. While MNV mono-assocation had little effect on tissue morphology or on the number of NK T cells, the presence of the virus increased the frequencies of both CD4- and CD8- T cells in the colon (Extended Data Fig. 5). To determine whether the effect of MNV is specific to the CR6 strain, we infected GF mice with MNV.CW3, a strain that is considered more relevant to human noroviruses because it displays increased virulence compared with MNV.CR6 and establishes an acute infection. Unlike conventional mice, which clear the virus between days 5–7 after infection, we found that a low amount of MNV.CW3 could be detected in the stool of GF mice on day 10 after infection (Extended Data Fig. 1b), and that the virus strain was able to induce similar effects on intestinal morphology and the lymphocyte compartment as MNV.CR6 (Extended Data

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1Kimmel Center for Biology and Medicine at the Skirball Institute, New York University School of Medicine, New York, New York 10016, USA. 2Department of Microbiology, New York University School of Medicine, New York, New York 10016, USA. 3New York Presbyterian Hospital, New York, New York 10065, USA. 4Department of Pathology, New York University School of Medicine, New York, New York 10016, USA.

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Figure 1 | MNV reverses intestinal abnormalities in GF mice.

**a–d.** Representative small intestinal sections from GF, GF+MNV or conventional (Conv) mice stained with haematoxylin and eosin (H&E) (**a, b**) or anti-CD3 antibody (**c, d**). Scale bars, 100 μm (**a, d**); 10 μm (**c, d**).

**e, f.** Quantification of villus width (**e**) and CD3+ cells (**f**) per villus. Fifty villi were quantified from 6–8 mice per group. **g, h.** Total number of cells in the small intestine lamina propria (SILP) and MLNs. **i.** Flow cytometry analysis of small intestine lamina propria cells for Gata3, Ror-γt, IL-22 and IL-13 expression in live, Lin− cells (CD11b+, CD19− and CD3−). j–m, Per cent Gata3+ (**j**) and Ror-γt+ (**k**) cells, and ratio of Ror-γt+ to Gata3+ (**l**) and IL-22+ to IL-13+ (**m**) in Lin− cells from i. **n.** Summary of comparisons between GF, GF+MNV and Conv mice. GF+MNV mice have similar villus width, T-cell numbers in small intestine lamina propria cells and MLNs, and antibody levels compared with conventional mice, and are designated with double plus signs, indicating restoration to the maximal value. GF+MNV mice show partial increases in the number of Paneth cell granules, lysozyme expression and IFN-γ expression. For ILC2 cells, GF mice display the maximal value and GF+MNV mice are similar to conventional mice. NS, not significant. **P < 0.05,** **PP < 0.01,** **PPP < 0.001,** **PPPP < 0.0001.** Analysis of variance (ANOVA) with Holm–Sidak correction. Graphs show means ± standard error of the mean (s.e.m.) from at least two independent experiments.

Fig. 6b–h). Next, we isolated virus directly from a naturally infected mouse housed in the vivarium (MNV.SKI, described in Methods). Alignment of the capsid sequence showed that MNV.SKI is a previously uncharacterized strain (Extended Data Fig. 6a). MNV.SKI was also able to reverse abnormalities in GF mice (Extended Data Fig. 6b–h). Although all three MNV strains induce qualitatively similar alterations in intestinal morphology and lymphocytes, the effects are quantitatively distinct, especially when comparing T-cell subsets. Nevertheless, the general ability of MNV to evoke a response in GF mice appears to be strain independent.

Innate lymphoid cells (ILCs) are emerging as important participants in mucosal immunity, but the role of the microbiota in maintaining this compartment is less understood. Unlike the skin and lungs, the intestine has been shown to contain reduced numbers of Gata3+ ILC type 2 (ILC2) cells relative to Ror-γt+ ILC3 cells3. We found that GF mice had an increase in the proportion of ILC2 cells in the small intestine compared with conventional mice (Fig. 1i–m). This expansion of ILC2 cells due to the absence of bacteria was reversed in GF+MNV mice (Fig. 1i–m). Consistent with this finding, MNV.CR6 infection increased the proportion of ILCs expressing interleukin (IL)-22 relative to IL-13, effector cytokines expressed by ILC3 cells and ILC2 cells, respectively (Fig. 1i, m). The proportion of the T-bet+ ILC1 subset was similar across conditions (Extended Data Fig. 2q). Thus, we find that commensal bacteria prevent expansion of intestinal ILC2 cells, and MNV.CR6 infection mimics this function of commensal bacteria. When taken together, these results indicate that the presence of a single virus can reverse many of the abnormalities that arise in the complete absence of the commensal bacterial community (Fig. 1n).

GF mice lack bacteria from birth and represent an extreme condition. We found that adult mice treated with a cocktail of antibiotics for 2 weeks display several of the abnormalities described earlier in GF mice, including aberrant intestinal morphology and a reduction in the number of total CD4+ T cells as well as IFN-γ-expressing CD4+ and CD8+ T cells.
(Fig. 2a–j and Extended Data Fig. 7a–d). Thus, we examined the ability of MNV infection to reverse these abnormalities induced by antibiotics treatment as another assay to demonstrate functional redundancies between this virus and commensal bacteria (Fig. 2a and Extended Data Fig. 1b). By day 10 after infection, MNV.CR6 infection had led to increased villus width, Paneth cell granules, intestinal T cells, and IFN-γ expression by CD4+ T cells (Fig. 2c–m). For comparison, mice were treated with antibiotics for 2 weeks and then inoculated with 1 × 10^9 colony-forming units (c.f.u.) of the representative commensals Bacteroides thetaiotaomicron, Lactobacillus johnsonii, or left alone after cessation of antibiotic treatment (Extended Data Fig. 7e–h). B. thetaiotaomicron and L. johnsonii restored the morphology of the intestine to a similar degree to MNV.CR6, but had varying effects on the T-cell compartment. B. thetaiotaomicron increased T-cell numbers when measured by CD3 staining of small intestinal sections, whereas L. johnsonii induced an IFN-γ response in CD4+ and CD8+ T cells without affecting their overall numbers (Extended Data Table 1). Pathway analysis of the transcripts induced by commensal bacteria from conventional mice (Fig. 3a, Extended Data Table 1 and Supplementary Table 1). Pathway analysis of the transcripts induced by commensal bacteria indicated alterations in the metabolic state, immunity and blood vessel morphogenesis. MNV.CR6 induced a more limited gene expression pattern associated with lymphoid cell development and immune responses, many of which are related to the antiviral type I IFN (IFN-I) response (Extended Data Table 1a). Consistent with the shared ability of MNV and bacteria to reverse abnormalities in GF mice, the overlapping gene set contained genes associated with the development and function of haematopoietic lineage cells (Fig. 3a and Extended Data Table 1c). The IFN-I signature induced by MNV.CR6 prompted us to examine whether the effect of viral infection was dependent on IFN-I signalling. The intestine of untreated IFN-γ receptor knockout mice (Ifnar1−/−) displayed a normal appearance, while antibiotics treatment of these mice led to similar abnormalities in intestinal morphology and T-cell numbers as described earlier (Fig. 3b–h). However, MNV.CR6

**Figure 3** MNV-induced changes are dependent on a type I IFN response. 

a, RNA-seq analysis of small intestine tissue from GF mice inoculated with MNV.CR6, conventionalized with bacteria (Conv), or left untreated. Venn diagram represents number of transcripts displaying >1.4 fold enrichment upon conventionalization (blue), inoculation with MNV.CR6 (yellow), or in both conditions (green), compared with untreated mice. Bar graphs represent Gene Ontology (GO) terms displaying association with the above gene sets. n = 3–4 mice. AG, antigen; dev., development; inflamm., inflammatory; med., mediated; pos., positive; reg., regulation. b–d, Quantification of villus width (b), CD3+ cells per villus (c) and granules per Paneth cells (d) of Ifnar1−/− mice that received antibiotics (ABX), antibiotics plus MNV.CR6 (ABX+MNV), or that were left untreated. Fifty villi or 30 crypts per mouse were quantified. n = 6–10 mice per group. e–h, Total number of CD4+ (e) and CD8+ (f) T cells, and IFN-γ (g) and CD4+ (h) T cells in the small intestine lamina propria of Ifnar1−/− mice that received antibiotics, antibiotics plus MNV.CR6, or that were left untreated. I. MNV.CR6 p.f.u. per cm tissue or per g stool from antibiotic-treated wild-type (WT) and Ifnar1−/− mice 10 days after infection. Dashed line denotes limit of detection. n = 3 mice per group. NS, not significant. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ANOVA with Holm–Sidak correction. Graphs show means ± s.e.m.
infection did not have a detectable effect on antibiotic-treated Ifnar1−/− mice despite productive replication (Fig. 3i and Extended Data Fig. 1b), indicating that virus-mediated reversal of these abnormalities is dependent on IFN-I signalling. Inducing IFN-I gene expression through polyinosinic-polycytidylic acid (poly(I:C)) injection was sufficient to increase villus width in GF mice but did not have a noticeable effect on intestinal T cells (Extended Data Fig. 8). Thus, IFN-I signalling probably functions in conjunction with other pathways to confer the full effect of MNV infection. Indeed, transcriptional analyses indicate a large overlap between the genes induced by MNV.CR6 and by bacterial colonization of GF mice that are not associated with IFN-I (Extended Data Table 1c).

The depletion of commensal bacteria is considered a major health hazard of indiscriminate use of antibiotics. Because MNV can reverse abnormalities observed upon bacterial depletion, we tested the possibility that viral infection can provide protection against intestinal damage. We found that antibiotic treatment increased sensitivity to chemical injury of the intestine by dextran sodium sulphate (DSS) (Fig. 4a). Remarkably, all three MNV strains examined were able to improve survival in antibiotic-treated mice receiving DSS (Fig. 4a). MNV.CR6, in particular, conferred robust protection and prevented shortening of the colon (Fig. 4a, b). MNV.CR6 was unable to improve survival in antibiotic-treated Ifnar1−/− mice, indicating that IFN-I signalling was necessary (Fig. 4c).

Next, we used inflammation induced by Citrobacter rodentium to test the protective effect of MNV, because antibiotic pre-treatment was shown to exacerbate the disease induced by this model enteric pathogen without altering bacterial burden22. In mice pre-treated with antibiotics, MNV.CR6 did not alter the amount of C. rodentium, which was the dominant bacterium in the gut (Fig. 4f and Extended Data Fig. 9a, b). However, MNV.CR6 ameliorated weight loss, diarrhoea and histopathology (Fig. 4g–k). This effect of the virus on bacterial gene expression and the protection from inflammation are probably due to the ability of MNV.CR6 to enhance the immune response to C. rodentium, which includes increased levels of antibodies as well as antimicrobial and regulatory cytokines (Extended Data Fig. 9c, d). Thus, MNV enhances protection against both infectious and non-infectious secondary challenges to the gastrointestinal tract in antibiotic-treated mice.

Figure 4 | MNV protects antibiotic-treated mice from intestinal injury and C. rodentium superinfection. a. Survival after DSS treatment of conventional mice (Conv), mice receiving antibiotics (ABX) only, and mice receiving antibiotics and infected with indicated MNV strains. n = 12 mice per group. Statistical significance represents comparisons between antibiotic-treated mice infected with indicated MNV strains versus the antibiotics-only group. b. Colon length of conventional, antibiotics-only and antibiotics plus MNV.CR6 mice on day 6 of DSS treatment. n = 3–5 mice per group. c. Survival of conventional wild-type (WT) and Ifnar1−/− mice, and antibiotic-treated wild-type and Ifnar1−/− mice with and without MNV.CR6 infection, treated with DSS. n = 7 mice per group. d. Schematic of MNV.CR6 and C. rodentium co-infection in antibiotic-treated mice. Antibiotic treatment was stopped before C. rodentium infection. e, f. Quantification of weight loss (e) and c.f.u. in stool (f) in mice receiving treatment as illustrated in d on day 9 and 11 after infection with C. rodentium, n = 5 mice per group. g. Diarrhoea from the mice described earlier on day 9 after infection. h–k. Representative H&E-stained caecal sections (h) from the mice described earlier (arrows span hyperplasia and stars denote ulceration and oedema), quantification of hyperplasia (i), and histopathology score at day 9 and 11 (see Methods) (j, k). Scale bar, 150 μm (n = 5 mice per group). *P < 0.05, **P < 0.01, ***P < 0.001. a, e, g, i. Log-rank Mantel–Cox test (a), ANOVA with Holm–Sidak correction (b, e, i), unpaired two-tailed t-test (g). Bar graphs show means ± s.e.m. and bars in e and f represent mean from at least two independent experiments.

Received 5 June; accepted 13 October 2014.
Published online 19 November 2014.

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**Supplementary Information** is available in the online version of the paper.

**Acknowledgements** We would like to thank S. Koralov and P. Loke for advice on the manuscript, E. Venturini for assistance with deep sequencing, S. Brown and Z. Tang for data analysis, L. Ciriboga for CD3 staining, the flow cytometry and histopathology cores (Cancer Center Support Grant, P30CA16087) for assistance with sample preparation and analyses, M. Alva and D. Littman for assistance with breeding and maintaining GF mice, and H. Moura Silva for sample collection for MNV isolation. This research was supported by National Institutes of Health grant R01 DK093668 (K.C.) and a New York University Whitehead Fellowship (K.C.), Vilcek Fellowship (E.K.) and Erwin Schrödinger Fellowship from the Austrian Science Foundation (E.K.).

**Author Contributions** E.K. performed all the experiments, Y.D. analysed and scored histological sections, K.C. and E.K. designed the study and wrote the manuscript.

**Author Information** RNA-seq data have been deposited in the Gene Expression Omnibus under accession number GSE60163. The MNV.SKI capsid sequence has been deposited in the NCBI Reference Sequence database under accession number KM463105. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to K.C. (ken.cadwell@med.nyu.edu).
METHODS

Mice. GF mice on a C57BL/6j background were bred and housed in flexible film isolators in the New York University (NYU) School of Medicine Gnotobiatics Animal Facility. Absence of fecal bacteria and fungi was confirmed by culture absence in brain infiltration, salubration and nutrient broth (Sigma) and qPCR for bacterial 16S (UniF340 5’-ACTCTACGAGGAGGCAGGT-3’; UniR341 5’-A TTACCGCGGCTGCTGGC-3’) and eukaryotic 18S ribosomal RNA genes (B2F 5’-ACTTCTGATGAGCAGTGAT-3’; BRF 5’-TGGATGTCTTGGATCCTCA-3’) through sampling of stool from individual cages in each isolator on a monthly basis. Mono-association with MNV as described in Extended Data Fig. 1 was performed within a dedicated isolator by inoculating three male and three female GF mice with 3×10^3 p.f.u. of MNV CR6 from an endotoxin-free stock prepared as described later. MNV mono-associated GF mice (GF+MNV) were bred to each F0 generation, and successful transmission to offspring was confirmed by performing a plaque assay on stool collected from 8–10-week-old adult progeny (4–5 weeks after weaning) (Extended Data Fig. 1). Progeny (F1) was used for experimental analyses or further breeding to generate additional mice for analyses (F2). Absence of bacteria and fungi was confirmed as described earlier. Control GF mice were bred and maintained in a separate isolator free of MNV. Unless stated otherwise, GF and GF+MNV mice remained in isolators until the time of analyses. Conventional C57BL/6j wildtype and Ifnar−/− (B6.129S2-Ifnar1tm1(Ma)) mice were purchased from Jackson Laboratory in the USA and Holecobacter-negative specific-pathogen-free (SPF) animal facility. The absence of segmented filamentous bacteria (SFB) was determined by PCR (SFB736F 5’-GAGCTGAGGATGAGACAT-3’; SFB844R 5’-GAGCGCGATTGTTATC-3’). Age (6–9 weeks) and gender-matched mice were used in all experiments and assigned randomly to experimental groups. Sample size for animal experiments was chosen based on prior 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 (IACUC).

Antibiotics and DSS treatment. Mice were gavaged with 100 mg streptomycin (Sigma) and the drinking water was immediately replaced with filter-sterilized water for 7 days. Enteropathogenic E. coli (EPEC) was cultured in peptone yeast glucose broth for 36 h in an anaerobic incubator and centrifugation at 12 000 g for 5 min at 4 °C to get the bacterial inoculum. Bacterial inocula were determined by serial dilution plating on MacConkey plates. For induction of colitis, mice were transferred to pre-assembled autoclaved cages with autoclaved food and water. L. johnsonii and colonic tissue on bacteroides bile esculin (BBE agar, Anaerobe Systems) plates. For indicated experiments in which adult GF mice were infected with MNV, C. rodentium 91–91 (∼10^9 c.f.u.) was inoculated per mouse. Mean values were calculate for each mouse and used as individual data points. Sections were imaged on a Zeiss Axiophot microscope. All analyses of slides were performed blind and quantified using ImageJ software. The histopathology score for individual mice is the sum of three parameters, including villus hyperplasia and crypt architectural distortion, degree of inflammation and oedema thickness in the lamina propria and submucosa. The scoring criteria are as follows: villus hyperplasia and crypt architectural distortion: 0 = no; 1 = mild; 2 = moderate; 3 = severe; inflammation: 0 = no; 1 = focal; 2 = multifocal; oedema thickness: 0 = 0 μm; 1 = 1–100 μm; 2 = 101–200 μm; 3 = 201–300 μm, giving a maximal score of 9. The mean of the score for individual mice was calculated. Blind histopathology scoring was performed by a pathologist (Y.D.).

Flow cytometry. Small intestinal or colonic tissue was flushed with PBS, fat and Peyer’s patches were removed and the tissue was incubated in HBSS with 5 mM EDTA and 1 mM dithiothreitol (DTT) at 37 °C for 20 min, followed by HBSS and 5 mM EDTA for 10 min. The tissue was digested using collagenase (Sigma) for 30 min, followed by a Percoll (Fisher) gradient centrifugation using 40% and 80% Percoll. Single-cell suspensions of MLNs were prepared by passing MLNs through 100 μm cell strainers (BD) and subsequent resuspension in PBS. Total cell numbers per ml were determined by counting recovered cells in a haemocytometer. For intracellular cytokine staining, cells were stimulated using the eBioscience cell stimulation cocktail for 4 h at 37 °C. Cells were fixed and permeabilized using the Biolegend fixation and permeabilization buffers. Samples were stained with a fixable live dead stain (Invitrogen) and live gating was performed before any other gating for all samples. The following antibodies (clones) were used for staining: CD4 (GK1.5), CD8 (53.6.7), CD3 (145-2C11), CD11b (M1/70), TCR-β (H57-597), T-bet (4B10), IFN-γ (XMG1.2), IL-17 (TC11-18H101), CD19 (6D5), Fc block (TruStainXfc), all from Biolegend; GATA3 (L50-823) from BD bioscence and IL-12 (IHPWSR), IL-13 (eBio13A), B220 (RA3-6B2) from eBioscience. The National Institutes of Health tetramer facility provided the mCD1d tetramer. FlowJo v.10 was used to analyse flow cytometry data.

RNA-seq. RNA was extracted from 2 cm of snap-frozen small intestinal tissue with TRIzol reagent (Fisher BioScience) using the manufacturer’s protocol. An RNA library was prepared using the Illumina TruSeq RNA sample preparation kit and sequenced with the Illumina Hiseq2000 using the TruSeq RNA v2 protocol. Illumina CASAVA v.1.8.2 was used to generate FASTQ files containing 29.5–53.5 million qualified reads per sample. Alignment and gene expression count were computed using default settings, which aligns reads to the union of all RefSeq annotated exons for each gene. Fifty-nine per cent to 78% of reads were aligned to mouse genome version mm9. All genes expressed below one base pair per million (b.p.m) in the majority of replicates in one of the biological replications were filtered out of the data set. The false discovery rate was used to find differentially expressed genes with a false discovery rate (FDR) below 5% (ref. 33). The Database of Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resource was used to assign differentially regulated genes to GO functional categories and determine enrichment scores and significance by a modified Fisher’s exact test. Gene set enrichment analysis (GSEA; Broad institute) was performed in Gene Pattern using the GSEA_Perranked module and a Gene set file created by the Bader laboratory for mouse (http://download.baderlab.org/EM_Genestset/current_release/Mouse/Entrezgene) with RNA-seq gene expression results ranked by fold change of MNV treated versus uninfected and of conventional versus antibiotic-treated mice.

Enzyme-linked immunosorbent assay. Two centimetres of small intestinal tissue was homogenized in 1 ml PBS containing protease inhibitor cocktail or serum and was collected. Samples were diluted 1:100 and levels of IgA and IgG2c (ng/ml^{-1}) were determined according to the manufacturer’s protocol (Southern Biotech).

Isolation of MNV from vivarium. Stool from Rag1−/− mice housed in a MNV-positive room within the Skirball vivarium was tested for the presence of MNV with previously described primers and nested PCR condition. Stool from the same mice was homogenized in 1 ml of DMEM, and the supernatant was cleared by centrifugation and filtered through a 0.2 μm filter. Fifty microlitres were added to a well of a 96-well microplate with 100 μl per well. The plates were incubated at 37 °C for 72 h. After 5 days, the plates were read on a 96-well microplate reader (SPECTRAmax 340M, Molecular Devices, Sunnyvale, California) at 570 nm.

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Strand cDNA synthesis kit (New England Biolabs) and random hexamers. The capsid region was amplified and sequenced as previously described. Capsid sequences from various MNV strains were aligned using Clustal Omega (EMBL-EBI).

**qRT–PCR.** Total RNA from colonic tissue was isolated using TRIzol and standard protocols, followed by cDNA synthesis using ProtoScript M-MuLV First Strand cDNA synthesis kit (New England Biolabs) and OligodTs. qPCR was performed on a Roche480II Lightcycler using the following primers:

- **Gbp2**, Fwd 5′- TGCT AAACCTCGGAACAGG-3′, Rev 5′- GAGCTTGCCAGAAGGTGG-3′; Il10, Fwd 5′- GGCGTACATTCTTCGTCAT-3′, Rev 5′- GCCTCCCTATATGGCCTCATT-3′; Il10, Fwd 5′- ATGAACGCGTACACAGC-3′, Rev 5′- CCATCTTTGCCAGTTCCC-3′; Gbadh, Fwd 5′- TGCCCCCATGTTTGTGATG-3′, Rev 5′- TGCATGGAGCCCTTCTCC-3′. Relative expression of the respective genes to Gapdh expression was calculated using the ΔΔCt method and values were expressed as fold change from antibiotic-treated mice. Total RNA from stool was isolated with TRIzol followed by cDNA synthesis with random hexamer primers for quantification of C. rodentium virulence factor expression with the following primers:

- ler, Fwd 5′- AATATACCTGATGTTGGCCTT-3′, Rev 5′- TTCTTCCATTCAATAATGGCTTCTT-3′; tir, Fwd 5′- TACACATTCGTTGTTACAGCAG-3′, Rev 5′- GACATCCACCTTTCAGCATA-3′; and recA, Fwd 5′- GCAGTCTGGCATTACCCACCC-3′, Rev 5′- TCGGTGAAAATCTACGGACCGGA-3′.

**Statistical analysis.** Analyses except for RNA-seq data used Graphpad Prism v.6. An unpaired two-tailed t-test was used to evaluate differences between two groups where data was distributed normally with equal variance between conditions. An analysis of variance (ANOVA) with Holm–Sidak multiple comparisons test was used to evaluate experiments involving multiple groups. The log-rank Mantel–Cox test was used for comparison of mortality curves.

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Extended Data Figure 1 | Mono-association of GF mice with MNV does not lead to uncontrolled viral replication or disease. **a,** Schematic of MNV mono-association procedure (see Methods for additional details). GF breeder pairs (F0) within a gnotobiotic isolator were infected with $3 \times 10^6$ p.f.u. of MNV.CR6, which was allowed to transmit naturally to offspring (F1). Weaned offspring were maintained in isolators until adulthood, and then either used for analysis or as breeders to generate additional experimental animals (F2). **b,** Successful transmission to offspring and the persistent presence of the virus in mono-associated GF mice (GF-MNV) was confirmed by performing a plaque assay using stool harvested from 8-week-old offspring (~1 month after weaning). The amount of virus in stool from GF mice, antibiotic (ABX)-treated wild-type (WT) and Ifnar1$^{-/-}$ mice, and conventional (Conv) mice infected with $3 \times 10^6$ p.f.u. of the indicated strains of MNV for 10 days are also shown, n = 5 mice per group. **c–f,** Mice receiving the indicated treatments did not display marked histopathology in the small intestine (c, e) and colon (d, f) based on blind quantification of H&E-stained sections using a previously described scoring system$^{37}$. Mice receiving a pathology score of 1 displayed mild blunting of villi (additional details in Methods). No histopathology was detected in spleens, and no other signs of disease were noted. Note that a previous publication in which mice were reported to display pathologies after MNV infection used a different strain of MNV, an early time point (24 h after infection), and mice on a different background (129/Sv)$^{38}$. The lack of pathology in C57BL/6 mice persistently infected with MNV.CR6 is consistent with our previous publication$^{15}$. n = 5–7 mice per group. All graphs show means ± s.e.m.
Extended Data Figure 2 | MNV improves several deficiencies related to intestinal immunity in GF mice. a, b, Representative images of crypts from small intestinal tissue sections stained with H&E (a) and an anti-lysozyme antibody (b) harvested from GF, GF + MNV (mono-association) and conventional (Conv) mice. Scale bar, 1 μm. c, d, Quantification of the above images shows an increase in granules per Paneth cell (c) and lysozyme-positive cells per crypt (d) in GF + MNV mice, indicating that the presence of MNV partially reverses Paneth cell abnormalities due to the absence of bacteria. n = 5 mice per group. e–h, MNV mono-association of GF mice increases the number of CD4⁺ (e, g) and CD8⁺ (f, h) T cells (TCR-β⁺) in small intestinal (SI) lamina propria cells and MLNs. i–n, Flow cytometry analysis indicates that MNV mono-association of GF mice also increases the number of IFN-γ-expressing CD4⁺ and CD8⁺ T cells in small intestine lamina propria (i, k) and MLNs (j, l). IL-17 expression by CD4⁺ T cells is also influenced by the presence of MNVs (m, n). n = 10. o, p, GF + MNV mice display increased IgA levels in small intestine tissue (o) and IgG2c levels in serum (p). n = 5 mice per group. q, Percentage of T-bet⁺ cells in the small intestine lamina propria after gating on live and Lin⁻ cells remain unchanged by MNV infection of GF mice. n = 10 mice per group. NS, not significant. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ANOVA with Holm–Sidak correction. All graphs show means ± s.e.m. from at least two independent experiments.
Extended Data Figure 3 | MNV infection of adult GF mice has similar effects to mono-association of GF mice from birth. **a–f,** Six- to eight-week old adult GF mice were infected with MNV.CR6 for 10 days and examined for reversal of intestinal abnormalities. a, b, Quantification of villus width (a) and granules per Paneth cell (b) in H&E-stained small intestinal sections. n = 5 mice per group. c, Quantification of the number of CD3⁺ T cells in the small intestine lamina propria (LP) by flow cytometric analysis. n = 6 mice per group. d, e, Quantification of IFN-γ⁺-producing CD3⁺ CD4⁺ (d) and CD3⁺ CD8⁺ (e) T cells by flow cytometry. f, Quantification of small intestinal IgA by enzyme-linked immunosorbent assay (ELISA). n = 6 mice per group. **P < 0.05. Unpaired two-tailed t-test. All graphs show means ± s.e.m. from at least two independent experiments.
Extended Data Figure 4 | The effect of MNV is specific to mice depleted of bacteria. a–h, Conventional mice were infected with \(3 \times 10^6\) p.f.u. of MNV.CR6 to determine the effect of MNV in the presence of commensal bacteria. a,b, Representative images of H&E-stained small intestinal sections of conventional (Conv and Conv mice infected with MNV (Conv + MNV) mice showing no aberrant changes after MNV infection. Scale bar, 100 \(\mu\)m (a); 1 \(\mu\)m (b). c,d, Villus width (c) and Paneth cell granules (d) were quantified from at least 50 villi and 30 crypts of 2–5 mice per group. e–h, Cell numbers of CD4\(^{+}\)TCR\(\beta^{+}\) (e), CD8\(^{+}\)TCR\(\beta^{+}\) (f), IFN-\(\gamma^{+}\) producing CD4\(^{+}\) (g) and IFN-\(\gamma^{+}\) producing CD8\(^{+}\) T cells in small intestine lamina propria (h). \(n = 6\) mice per group. NS, not significant. *\(P < 0.05\). Unpaired two-tailed t-test. All graphs show means ± s.e.m. from at least two independent experiments.
Extended Data Figure 5 | MNV mono-association of GF mice increases colonic lymphocyte populations. a, Representative images of H&E-stained colonic small intestinal sections of GF, GF + MNV (mono-association with MNV.CR6) and conventional (Conv) mice. Scale bar, 100 μm. b, In these mice the crypt height was measured, showing a significant difference between GF and conventional mice. c, Percentages of NK T cells (CD1d+, TCR-β+) in colonic lamina propria of GF, GF + MNV and conventional mice. d–g, Percentages of CD4+ TCR-β+ (d) and CD8+ TCR-β+ cells (f), and percentages of IFN-γ-producing CD4+ (e) and CD8+ T cells (g) in the colonic lamina propria of GF, GF + MNV and conventional mice. n = 5 mice per group. NS, not significant. *P < 0.05, **P < 0.01. ANOVA with Holm–Sidak correction. All graphs show means ± s.e.m. from at least two independent experiments.
Extended Data Figure 6 | The effect of MNV on the small intestine of GF mice is not strain specific.  

**a**, Phylogenetic tree of the capsid sequences of the indicated MNV strains.  

**b–d**, Quantification of the villus width (b), granules (c) and CD3$^+$ cells (d) in small intestinal sections prepared from conventional (Conv) mice, GF mice, and GF mice infected with the indicated strains of MNV for 10 days.  

**e–h**, Percentages of CD4$^+$ TCR-β$^+$ (e) and CD8$^+$ TCR-β$^+$ cells (g), and percentages of IFN-γ-producing CD4$^+$ (f) and CD8$^+$ T cells (h) in the small intestine lamina propria of the indicated mice. n = 6 mice per group. NS, not significant. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ANOVA with Holm–Sidak correction. All graphs show means ± s.e.m. from at least two independent experiments.
Extended Data Figure 7 | Antibiotics treatment induces intestinal abnormalities that can be reversed by B. thetaiotaomicron, L. johnsonii or MNV.CR6. a–g, Percentages of CD4⁺ TCRβ⁺ (a) and CD8⁺ TCRβ⁺ cells (b), and percentages of IFN-γ-producing CD4⁺ (c) and CD8⁺ (d) T cells in small intestine lamina propria in conventional (Conv) mice with and without antibiotic (ABX) treatment. n = 5–10 mice per group. e, Schematic of antibiotic treatment for introducing bacteria. After 14 days, antibiotic-containing water was replaced by regular water. Mice were then inoculated with B. thetaiotaomicron (B. theta), L. johnsonii, or left untreated for 10 days before analyses. f–h, Bacterial loads of colon (f), small intestine (g) and stool (h) of antibiotic-treated mice inoculated with B. thetaiotaomicron or L. johnsonii for 10 days. i, J. Small intestinal sections stained with H&E (i) or anti-CD3 antibody (j) indicating that inoculation with MNV.CR6, B. thetaiotaomicron or L. johnsonii have similar effects on intestinal morphology. k–p. Mice that received antibiotics during the whole course of the experiment with or without MNV (ABX and ABX + MNV) were compared with mice treated as in e using the previously described measurements: quantification of villus width (k), CD3⁺ cells per villi (l), and percentages of CD4⁺ (m), CD8⁺ (o), IFN-γ CD4⁺ (n) and IFN-γ CD8⁺ (p) T cells in the small intestine lamina propria. n = 8 mice per group. NS, not significant. *P < 0.05, **P < 0.01, ***P < 0.001. a–d, k–p. Unpaired two-tailed t-test (a–d), ANOVA with Holm–Sidak correction (k–p). All graphs show means ± s.e.m. from at least two independent experiments.
Extended Data Figure 8 | Type I IFN induction by poly(I:C) changes small intestinal architecture without affecting the T-cell compartment. a, qRT–PCR quantification of the IFN-I inducible gene Mx2 in small intestinal tissue of untreated GF mice, GF mice injected for 10 days with poly(I:C), MNV.CR6 mono-associated GF mice (GF + MNV), and conventional (Conv) mice indicate that the poly(I:C) injection procedure induces an IFN-I response. Values represent fold induction of Mx2 compared with untreated GF mice after normalizing to Gapdh. b–d, Quantification of the villus width (b), granules (c) and CD3⁺ cells in the small intestine of the same mice as in a of H&E-stained small intestinal sections. e–h, Percentages of CD4⁺ TCR-β⁺ (e), CD8⁺ TCR-β⁺ cells (g) and IFN-γ-producing CD4⁺ (f) and CD8⁺ (h) T cells from the small intestine lamina propria. n = 6 mice per group. NS, not significant. ***P < 0.001, ****P < 0.0001. a–h, ANOVA with Holm–Sidak correction (a–d), unpaired two-tailed t-test (e–h). All graphs show means ± s.e.m. from at least two independent experiments.
Extended Data Figure 9 | MNV infection alters the host response to a superinfection with *C. rodentium*. a, Taxon-specific 16S qRT–PCR for Lactobacilli, Enterobacteriaceae, Fusobacteria, Bacteroides and *C. rodentium* normalized to total 16S gene expression in stool of mice infected for 9 days with *C. rodentium* after antibiotic (ABX) pre-treatment with or without MNV.CR6 infection, showing similar colonization of mice throughout the groups. b, c, Fold induction of *C. rodentium* virulence factors *tir* (b) and *ler* (c) compared with the antibiotic group after normalization to *recA* in stool of indicated mice on day 9 after *C. rodentium* infection. d–f, MNV.CR6 infection of antibiotic-treated mice before *C. rodentium* infection increases IgA levels in colonic tissue (d) and stool (e), and IgG2c levels in serum (f) at day 9 after *C. rodentium* infection. g–i, At day 9 after *C. rodentium* infection, antibiotics plus MNV mice display elevated expression of IFN-γ (g), Gbp2 (h) and IL-10 (i) in colonic tissue compared with antibiotics-only mice. *n* = 5 mice per group. ND, not detectable. *P* < 0.05, **P* < 0.01, ***P* < 0.001. Unpaired two-tailed t-test. All graphs show means ± s.e.m. from at least two independent experiments.
Extended Data Table 1 | Genes displaying increased expression after MNV infection or conventionalization of GF mice

| Genes | pValue |
|-------|--------|
| >1.4 fold upregulated in GF+MNV compared to GF | |
| immune response | 7.15E-10 |
| CSF2, EPAS1, IKZF1, SLC37A4, PTPN22, ZBTB16, SOX6, IL15, PLSCR1, DOCK2, HIF1A, CXCL13, MFSD7B, LCK, VEGFA, BCL6, JAK2, HEPH, LETS | |
| positive regulation of immune system processes | 1.16E-05 |
| MBL2, TIGAL, ICOSL, IL27RA, IFI2F, BK1, ICOS, CD43, PTPN22, IL15, TNFRSF4, B2M, CD91, CD11A, CD37, CD19, KLHL6, LXAX1, LCK, BCL8, CD79B, SASH3 | |
| negative regulation of immune system processes | 2.12E-06 |
| MBL2, IFI2F, IL27RA, BK1, CRP, CCL3, RSAD2, PPI10, TR7, CD43, TNFRSF4, LEAP2, CXCL10, B2M, SEP2, IFIH1, ISG15, IRF7, OAS1B, RSAD2, OAS1A, MX1, MX2, LTR7 | |
| defense response | 6.90E-05 |
| MBL2, IFH1, IL27RA, BK1, CRP, CCL3, RSAD2, PPI10, TR7, CD43, TNFRSF4, LEAP2, CXCL10, B2M, SEP2, IFIH1, ISG15, IRF7, OAS1B, RSAD2, OAS1A, MX1, MX2, LTR7 | |
| response to virus | 7.39E-04 |
| PLSCR1, IFH1, ISG15, IFIH1, ISG15, IRF7, OAS1B, RSAD2, OAS1A, MX1, MX2, LTR7 | |
| antigen receptor mediated signaling pathway | 9.40E-04 |
| KLHL6, CD19, LXAX1, CD247, LCK, PTPN22, CD79B | |
| immune system development | 1.46E-03 |
| CSF2, ICOSL, EPAS1, IKZF1, SLC37A4, PTPN22, ZBTB16, SOX6, IL15, PLSCR1, DOCK2, HIF1A, CXCL13, MFSD7B, LCK, VEGFA, BCL6, JAK2, HEPH | |
| hemopoietic or lymphoid organ development | 1.96E-03 |
| CSF2, EPAS1, ICF1, SLC37A4, PTPN22, ZBTB16, IL15, SOX6, PLSCR1, DOCK2, HIF1A, CXCL13, MFSD7B, LCK, VEGFA, BCL6, JAK2, LPHN, RHX | |
| >1.4 fold upregulated in GF+conv compared to GF | |
| carboxylic acid transport | 2.18E-05 |
| SLC27A1, SLC38A1, SLC7A9, PRR9D, SLC38A3, XKL, SLC7A9, CADN48, SLC7A4, SLC7A5, SLC7A14, SLC8A5, SLC28A5, SLC5A, SLC25A2, SLC7A2, SLC4A1 | |
| amino acid transport | 3.38E-05 |
| SLC27A1, SLC7A9, SLC38A4, SLC7A9, XKL, CADN48, SLC7A19, SLC7A14, SLC8A5, SLC28A5, SLC4A1, SLC26A1 | |
| glucose transport | 5.33E-05 |
| PRKAA2, SLC22A3, SLC5A8, SLC22A2, SLC22A5, SLC22A3, SLC7A9, EDN1, SLC37A4, KLF15 | |
| response to hypoxia | 7.16E-05 |
| ATPH1, IFIH1, IL27RA, RXR, EDN1, BNP3, TRF, CID2, SLC24A8, VEGFA, PRKX2, HIF3A, NOS2, LCT | |
| enzyme linked receptor protein signaling pathway | 7.15E-04 |
| FGF1, WDF5X2D, E2D, NOG, ERBB3, SLC2A2, IKBKIP1, PRKX1, PRKX2, HOX1, SLC24A2, HOX1, SLC24A1, SLC24A1 | |
| vitamin A metabolic process | 1.61E-04 |
| RD9H, RBP4, PRAR, LRAT, DHRS8, RDH16, BCO2 | |
| acute inflammatory response | 2.53E-03 |
| REG3B, ORM1, C3, CFB, EPB2, VIN1, CFI, REG3G, CFD, TRF, CD163, C80 | |
| blood vessel morphogenesis | 2.62E-03 |
| FGF1, EPAS1, EGFL7, SMAD7, IL18, EDN1, CD40, CID2, APOB, HEY1, HAND2, NTRK2, NOTCH4, VEGFA, PLCO1, RHOB, SOX18, NOS3, NOS2, SOX17, FGF | |
| >1.4 fold upregulated common genes | |
| glucose transport | 2.14E-04 |
| PRKAA2, SLC22A3, SLC26A1, SLC37A4, KLF15 | |
| erythrocyte differentiation | 0.0017248 |
| EPAS1, VEGFA, BCEL, HEPH, SOX6 | |
| steroid metabolic process | 0.0017271 |
| RDH9, HSD3B1, APOA1, OSBPL3, HMGCS2, OSBPL1A, SLC37A4, SLC7A1 | |
| hemopoiesis | 0.0057204 |
| EPAS1, SLC37A4, VEGFA, PTPN22, BCEL, HEPH, LIP, SOX6, ZBTB16 | |
| homeostasis of number of cells | 0.0074214 |
| EPAS1, SLC37A4, VEGFA, BCEL, HEPH, SOX6 | |
| immune system development | 0.0109743 |
| EPAS1, SLC37A4, VEGFA, PTPN22, BCEL, HEPH, LIP, SOX6, ZBTB16 | |
| oxidation reduction | 3.14E-04 |
| ACOX2, BCMO1, HSD3B1, PTGR1, CYP3A13, CYP24A4, CYP24A1, CYP4V3, MOSC1, KMD, BBOX1, RHDD, AKRIB, CYP27A1, HEPH, RHDD, CYP27A1, MOSC1, KMD, BBOX1, RHDD, AKRIB, CYP27A1, HEPH, RHDD, CYP27A1, MOSC1, KMD | |
| myeloid cell differentiation | 0.0184445 |
| EPAS1, VEGFA, BCEL, HEPH, SOX6 | |

Genes upregulated more than 1.4 fold in the RNA-seq experiment were subjected to DAVID pathway analysis (Fig. 3), which assigned genes to GO categories. a–c, Individual genes are shown with their GO category for GF+MNV (a) and for conventionalized GF (GF+conv) (b), and genes that overlap the GF+MNV and GF+conv conditions are shown (c, d). The same data set was analysed using gene set enrichment analysis. NES, normalized enrichment score.