HHS Public Access

Author manuscript

Science. Author manuscript; available in PMC 2018 October 13.

Published in final edited form as:

Science. 2018 April 13; 360(6385): 204–208. doi:10.1126/science.aar3799.

Tropism for tuft cells determines immune promotion of norovirus pathogenesis

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Abstract

Complex interactions between host immunity and the microbiome regulate norovirus infection. However, the mechanism of host immune promotion of enteric virus infection remains obscure. The cellular tropism of noroviruses is also unknown. Recently, we identified CD300lf as a murine norovirus (MNoV) receptor. Here we show that tuft cells, a rare type of intestinal epithelial cell, express CD300lf and are the target cell for MNoV in the mouse intestine. We found that type 2 cytokines, which induce tuft cell proliferation, promote MNoV infection *in vivo*. These cytokines can replace the effect of commensal microbiota in promoting virus infection. This is the first report of viral infection of tuft cells and provides insight into how the immune system and microbes can coordinately promote enteric viral infection.

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Author contributions: CBW, SL, LYH, MM, DB, CY, XZ, YTW, DM, DK, KC, and MTB performed experiments. CBW, SL, RCO, CH, KC, PA, MRH, SAH, MC, MTB, and HWV designed the project. CBW, SL, LYH, RCO, CD, BLH, TF, JRB, HWV, and SAH analyzed data. CBW wrote the paper. All authors read and edited the manuscript.

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Main Text

Human noroviruses (HNoVs) are the leading cause of acute viral gastroenteritis worldwide causing up to 700 million infections and 200,000 deaths annually (1). Despite this disease burden, it is unknown what cell type(s) mediate transmission and in some individuals chronic infection (2). Murine norovirus (MNoV) represents a model for HNoV pathogenesis and immunity. More broadly, MNoV serves as a tractable system to uncover novel virus-host interactions such as the capacity of MNoV infection to trigger human-relevant pathology in genetically susceptible animals and the role of intestinal bacteria in promoting enteric viral infection (3–8). Identifying the cell tropism of MNoV could provide mechanistic insight into such phenomena and thereby shed light on enteric immunity and the genotype-phenotype relationship.

Norovirus tropism is not fully understood in either immunocompetent mice or humans. Recently, we showed that a small population of epithelial cells are the reservoir for chronic MNoV infection, and this epithelial cell tropism was determined in part by the MNoV non-structural protein NS1 (9). However, the reason for selective intestinal epithelial cell infection, how infected cells differ from adjacent cells in the intestinal epithelium, and why we seldom observed adjacent infected epithelial cells is unknown. We recently identified CD300lf as a protein receptor for MNoV (10, 11). CD300lf is both necessary and sufficient for infection *in vitro*, and *Cd300lf*$^{-/-}$ animals are resistant to fecal-oral transmission of persistent MNoV infection (10). Here, we used this finding to identify the target cell of MNoV *in vivo.*

Because MNoV readily replicates in explanted macrophages and dendritic cells which express CD300lf (12), we first sought to determine if bone marrow-derived myeloid cells were responsible for infection by performing bone marrow transplants between *Cd300lf*$^{-/-}$ and wild type (WT) littermates followed by oral infection with MNoV strain CR6 (MNoV^{CR6}). MNoV^{CR6} infection is characterized by robust fecal-oral transmission, persistent enteric infection resistant to adaptive immune clearance, and prolonged fecal shedding (13). Such persistent MNoV strains replicate predominantly in the distal small intestine and colon and can be detected in mesenteric lymph nodes (MLN) but infection is scant in the spleen (14).

WT mice receiving WT bone marrow remained susceptible to MNoV^{CR6} while *Cd300lf*$^{-/-}$ mice receiving *Cd300lf*$^{-/-}$ bone marrow were resistant to MNoV infection as measured by fecal shedding of MNoV (Fig 1A) and tissue levels of viral genomes 21 days after infection (Fig 1B–E). Surprisingly, WT mice that received *Cd300lf*$^{-/-}$ bone marrow were susceptible to MNoV^{CR6} while *Cd300lf*$^{-/-}$ animals receiving WT bone marrow were resistant to infection. Viral titers in the ileum and colon correlated with those in feces (Fig 1A–C). WT mice that received either WT or *Cd300lf*$^{-/-}$ bone marrow transplants remained susceptible to MNoV^{CR6}. Splenic infection was minimal in all groups examined, consistent with prior studies in non-irradiated WT animals (Fig 1D)(13). In the mesenteric lymph nodes (MLN), MNoV^{CR6} genomes were undetectable in *Cd300lf*$^{-/-}$ mice receiving either WT or *Cd300lf*$^{-/-}$ bone marrow, while viral genomes were detected at similar levels in WT mice receiving either WT or *Cd300lf*$^{-/-}$ bone marrow transplants (Fig 1E). Thus, recipient *Cd300lf*

genotype was the primary determinant of $MNoV^{CR6}$ intestinal replication and shedding, indicating that radiation-resistant cells were responsible for $MNoV^{CR6}$ enteric infection. In contrast to $MNoV^{CR6}$, both radiation-sensitive and resistant cells contributed to infection with MNoV strain CW3 ($MNoV^{CW3}$), which causes acute systemic infection (14–16). The inability of $MNoV^{CW3}$ to infect epithelial cells, to be shed in the feces, and to establish chronic infection maps to the viral NS1 protein, which is required to counteract IFN-λ signaling (5, 9, 13). We focused further efforts on identifying the tropism responsible for enteric infection and shedding of $MNoV^{CR6}$.

Consistent with our bone marrow transplant data, we recently identified that rare isolated intestinal epithelial cells are infected by $MNoV^{CR6}$ during chronic infection, though the identity of the cell was not defined (9). Together with the bone marrow transplantation experiments above, these findings indicate that a radiation-resistant epithelial cell must express the MNoV receptor (9). However, CD300lf is an immunoregulatory protein thought to be expressed on hematopoietic cells, particularly myeloid cells (17, 18). Notably, expression of CD300lf on epithelial cells has not been described previously. We therefore performed immunofluorescence microscopy on uninfected WT mice and observed a rare population of CD300lf expressing cells throughout the ileum and colon (Fig 2A–B). Given the amphora-like morphology and scarcity of CD300lf expressing epithelial cells, we hypothesized that they were tuft cells, a rare chemosensory epithelial cell type in the hollow organs of mammals including mice and humans (19). These cells, also known as brush, caveolated, multivesicular, or fibrillovesicular cells, contain a long apical “tuft” of microvilli, which protrudes into the intestinal lumen, and were recently discovered to be the primary source of IL-25, a cytokine that initiates a type 2 immune response upon intestinal helminth or parasite infection (20–22). Indeed, all observed $CD300lf^{+}$ epithelial cells expressed the tuft cell markers doublecortin-like kinase 1 (DCLK1) and cytokeratin 18 (CK18; Fig 2A–B) (23). We also confirmed tuft cell-specific expression of *Cd300lf* transcripts in previously reported single cell RNAseq data from mouse intestinal enteroids (24, 25). Next, we assessed CD300lf expression on intestinal epithelial cells ($EpCAM^{+}CD45^{-}$) in a tuft cell-specific fluorescent reporter mouse (Gfi1b-GFP) (26). There was near perfect concordance between Gfi1b-GFP and CD300lf expression in both the ileum and colon, confirming that tuft cells are unique among epithelial cells in their expression of CD300lf (Fig 2C).

Given these findings, we assessed whether $MNoV^{CR6}$ infects tuft cells. Immunofluorescence microscopy on intestines of WT mice infected with $MNoV^{CR6}$ revealed rare cells expressing the MNoV non-structural protein NS6/7 (Fig 3A). These cells were in direct contact with the intestinal lumen and observed in the surface epithelium of the colon and both the villi and crypts of the ileum. All MNoV NS6/7 positive cells co-expressed the tuft cell marker DCLK1. No viral antigen positive cells were observed in the lamina propria or immune cells. Similar histologic findings and viral tropism were identified in WT germ-free mice (Fig S1) indicating that intestinal bacteria are not required for either CD300lf expression by tuft cells or $MNoV^{CR6}$ infection of tuft cells. To confirm and quantify $MNoV^{CR6}$ infection of tuft cells, we performed flow cytometry of colonic epithelial cells from infected Gfi1b-GFP mice. $MNoV^{CR6}$ infection did not significantly reduce tuft cell frequency (Fig 3B). Infected cells were defined as those expressing two independent viral non-structural proteins

(NS1/2 and NS6/7) (9). We observed 128±33 (mean±SEM) infected tuft cells per million live epithelial cells ($EpCAM^{+}CD45^{-}$). We did not observe infection of non-tuft epithelial cells (Fig 3C–D). Overall, 1.4%± 0.37% of Gfi1b-GFP+ tuft cells were MNoV infected. Together our immunofluorescence and flow cytometric analyses indicate that tuft cells are the physiologic target cell of MNoV in WT animals. This likely explains why we did not observe clusters of infected cells in the intestine since tuft cells are isolated from one another, being surrounded by other intestinal epithelial cells (9).

Given the role of tuft cells in type 2 immunity, we hypothesized that there might be an intimate relationship between type 2 immunity and enteric norovirus infection. The type 2 cytokines IL-4 and IL-25 induce tuft cell hyperplasia (20–22). Therefore we assessed whether these cytokines augmented MNoV transmission. WT mice were treated with IL-4, IL-25, or a PBS control prior to peroral challenge with a low dose of $MNoV^{CR6}$ (4.25×10^{4} plaque forming units (PFU) per mouse) insufficient to establish robust infection in the majority of control mice. Both IL-4 and IL-25 treated animals were significantly more likely to be productively infected than PBS treated animals as measured by viral genomes in the feces seven days-post challenge (Fig 4A). This shows that type 2 immune responses can enhance enteric viral transmission. We therefore asked whether type 2 cytokines affect $MNoV^{CR6}$ fecal shedding during persistent infection. WT mice were challenged perorally with a high-dose of $MNoV^{CR6}$ (10^{6} PFU/mouse), which is sufficient to infect all animals. After at least 21 days of infection, IL-4 or a PBS control was injected intraperitoneally and fecal shedding of virus was monitored. IL-4 significantly increased $MNoV^{CR6}$ fecal shedding as observed one day after the second and final IL-4 injection (Fig 4B). As observed in WT animals, IL-4 increased MNoV shedding in *Rag1*$^{-/-}$ and *Ifnlr1*$^{-/-}$ mice persistently infected with MNoV (Fig 4B), indicating that cytokine promotion of infection was not caused by effects on T cell or B cells and is independent of IFN-λ-induced innate immune signaling, a potent regulator of intestinal norovirus infection (5). In addition, we demonstrate that IFN-λ treatment did not alter tuft cell abundance in the intestine (Fig S3).

The murine parasite *Trichinella spiralis* induces type 2 inflammation and augments $MNoV^{CR6}$ infection (7). The mechanism of action was hypothesized to be increased viral replication in alternatively activated macrophages exposed to type 2 cytokines such as IL-4 and IL-13 (7). However, here we show that tuft cells and not macrophages are the target cell for $MNoV^{CR6}$. Thus, we tested whether the enhanced $MNoV^{CR6}$ infection resulting from IL-4 treatment was mediated by effects of this cytokine on epithelial cells. To test this hypothesis, we generated epithelial cell-specific IL-4 receptor alpha (Il4rα) conditional knockout mice ($Il4r\alpha^{f/f}$ x VillinCre) (27). Mice were infected with MNoV for at least 21 days after which IL-4 was administered. IL-4 enhanced $MNoV^{CR6}$ shedding in $Il4r\alpha^{f/f}$ x VillinCre- but not $Il4r\alpha^{f/f}$ x VillinCre+ animals, demonstrating that IL-4 signals through its receptor on epithelial cells (Fig 4C) (22). These data suggest that IL-4 promotes norovirus infection via effects on tuft cells, the only epithelial cell infected with the virus.

Prior work showed that the bacterial microbiome is required for efficient establishment of enteric $MNoV^{CR6}$ infection (6). Specifically, broad-spectrum antibiotics that deplete intestinal bacteria prevent $MNoV^{CR6}$ transmission and persistent infection (6). The mechanism for this effect is incompletely understood. We therefore asked whether antibiotic

treatment affected expression of tuft cell specific transcripts. RNAseq was performed on control and antibiotic-treated mice, and the expression of a curated tuft cell gene list was used to assess for differences in tuft cell-specific genes (24). Antibiotic treatment resulted in a decrease in tuft cell specific genes in the colon (Normalized Enrichment Score 2.23; $P<0.001$; False Discovery Rate <0.001; Fig 4D); changes in tuft cell genes did not reach statistical significance in the ileum. Consistent with the RNAseq gene set enrichment analysis, antibiotics decreased $DCLK1^+$ cells in the colon but not the ileum (Fig 4E–F). IL-4 and IL-25 induced tuft cell hyperplasia in the ileum of antibiotic-treated mice, whereas colonic tuft cells were not increased in number by IL-4 or IL-25 (Fig 4E–F). This indicates that both type 2 cytokines and intestinal bacteria regulate tuft cells, albeit in a tissue-specific manner (22). The observation that intestinal bacteria contribute to tuft cell regulation *in vivo* raised the question of whether the antiviral role of antibiotics could be overcome with administration of type 2 cytokines that act on epithelial cells to control MNoV infection. WT mice were pre-treated with antibiotics for two weeks prior to challenge with a high dose of 10^6 PFU of $MNoV^{CR6}$. Consistent with prior findings, antibiotics significantly reduced $MNoV^{CR6}$ infection (Fig 4G) (6). Importantly, IL-4 or IL-25 administration, prior to $MNoV^{CR6}$ challenge, rescues viral infection in antibiotic-treated mice. Indeed, both IL-4 and IL-25 significantly increased both the proportion of mice infected with virus and the magnitude of fecal shedding (Fig 4G). The differential regulation of tuft cells by type 2 cytokines and antibiotics in the ileum and colon, respectively, suggests that a threshold number of tuft cells may matter more than the anatomic location of tuft cells within the intestine.

Here, we identify intestinal tuft cells as the physiologic target cell of MNoV. This discovery has important implications for our understanding of transkingdom interactions, and the pathogenesis of persistent intestinal infection. Norovirus infection triggers inflammatory bowel disease-like phenotypes in genetically susceptible hosts (3, 4). It is interesting to consider, now that we have defined the tropism of norovirus to be tuft cells, whether tuft cells regulate inflammatory bowel disease-like phenotypes. Second, tuft cell tropism links the proviral effects of helminths and commensal bacteria, which both increase tuft cells in the ileum and colon, respectively (20–22). Importantly, noroviruses can persist in the intestine for months in both mice and humans (2, 28–30). This persistent infection is resistant to both antibody and $CD8^+$ T cell mediated clearance, yet the mechanism of immune evasion is unknown (31). Our identification of MNoV tropism for tuft cells suggests that tuft cells represent an immune privileged site for enteric viral infection in mice. Possibly, other viruses may infect tuft cells, enabling them to take advantage of type 2 immune responses to promote infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to acknowledge Jun Guo, Xin Zhang, Anthony Orvedahl, Annie Mayer, Tim Schaiff, Maxim Artyomov, George Broze, and Nina Lasky for helpful discussions and technical support. We would also like to thank Wandy Beatty at the Molecular Microbiology Imaging Facility and Erica Lantelme and Dorjan Brinja at the

Flow Cytometry and Fluorescence Activated Cell Sorting Core at Washington University School of Medicine. This work was supported by NIH grants K08 AI128043 (CBW), K22 AI127846 (MTB), K99 DK116666 (RCO), T32 AI007163 (CCY), R01 AI127552 (HWV), U19 AI109725 (HWV), DK093668 (JAN, KC), DK103788 (JAN, KC), HL123340 (JAN, KC), R01 AI121244 (JAN, KC). In addition, SL was supported by the Basic Sciences Research Program through the National Research Foundation of Korea funded by the Ministry of Education (NRF-2016R1A6A3A03012352). MTB was supported DDRCC grant P30 DK052574. JAN was supported by a Vilcek Fellowship and Sir Keith Murdoch Fellowship. CBW, RCO, and HWV are inventors on a patent application submitted by Washington University entitled “Receptor for norovirus and uses thereof” (U.S. Provisional Application 62/301,965). The data from this study are tabulated in the main paper and supplementary materials. All reagents are available from HWV under a material transfer agreement with Washington University. All data and code to understand and assess the conclusions of this research are available in the main text, supplementary materials and via the following repositories: European nucleotide Archive accesion PRJEB23132

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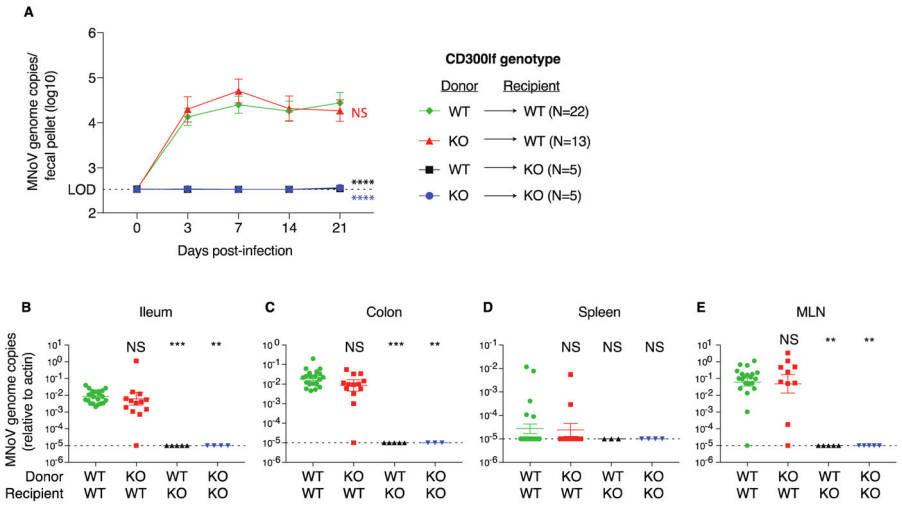

Fig. 1. Fecal-oral MNoV transmission requires radiation-resistant cells

Reciprocal bone marrow transplants were performed among *Cd300lf*$^{+/+}$ (WT) and *Cd300lf*$^{-/-}$ (KO) littermates. Mice were then challenged perorally with MNoV^{CR6}, which establishes persistent enteric infection in WT animals. (A) WT mice remained susceptible to MNoV as measured by viral genomes in feces at indicated time points. In contrast KO mice did not shed MNoV^{CR6} whether they received WT or KO bone marrow. (B–E) 21 days post-challenge MNoV viral genomes were determined in the ileum (B), colon (C), spleen (D), and mesenteric lymph nodes (MLN) (E). WT recipients had significantly more viral genomes than KO recipients. There was no significant difference between WT recipients of either WT or KO bone marrow. Fecal samples were analyzed by repeated-measures ANOVA. Tissue samples were analyzed by one-way ANOVA. Significant differences for both fecal and tissue samples were compared to WT→WT control as indicated. Shown are means ± SEM. NS, not significant; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. L.O.D., limit of detection. Data is pooled from three independent experiments. The number of mice per group is indicated in (B).

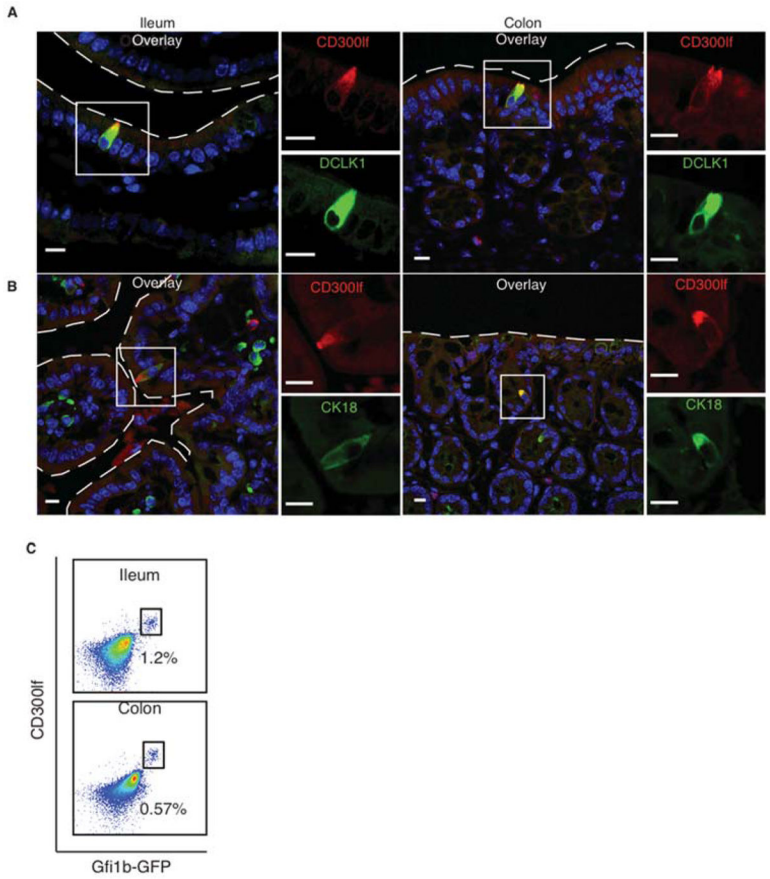

Fig. 2. CD300lf is expressed on tuft cells but not other intestinal epithelial cells

(A–B) The MNoV receptor CD300lf is detectable on rare intestinal epithelial cells with morphology consistent with tuft cells. CD300lf colocalizes with tuft cell markers (A) DCLK1 and (B) CK18 in mouse ileum and colon. CD300lf is apically polarized towards the intestinal lumen. (C) CD300lf is expressed on Gfi1b-GFP^{+} tuft cells, but not other intestinal epithelial cells as measured by flow cytometry. Events shown are Singlets^{+}Live $^{+}$CD45^{-}EpCAM^{+}. Images and FACS plots are representative of one of at least three independent experiments. Dashed lines represent the epithelial barrier. White boxes in the overlaid image reflect the magnified inset images. Scale bars, 10 microns.

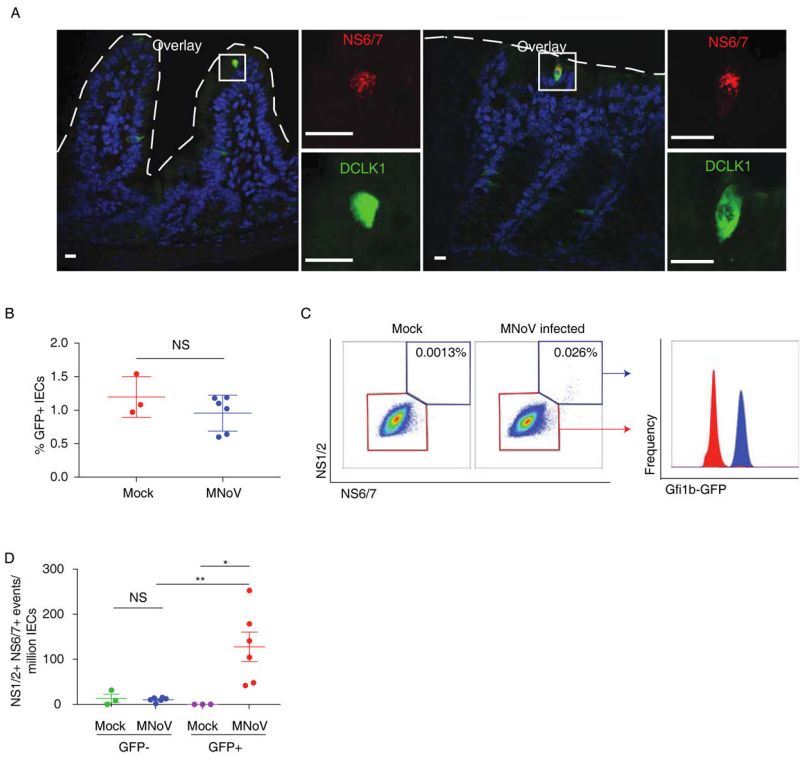

Fig 3. MNoV^{CR6} specifically infects CD300lf-expressing intestinal tuft cells

(A) MNoV non-structural protein NS6/7 colocalizes with DCLK1 in the ileum and colon of WT mice infected with MNoV^{CR6} at seven days post-infection. NS6/7 expression is punctate and cytoplasmic consistent with the viral replication complex. (B) Flow cytometry of intestinal epithelial cells (Singlet^{+}Live^{+}CD45^{-}EpCAM^{+}) from Gfi1b-GFP+ tuft cell reporter mice reveal similar frequencies of tuft cells between infected and uninfected mice. (C) A rare population of cells that co- express the MNoV non-structural proteins NS1/2 and NS6/7 was observed. These MNoV-positive cells are Gfi1b-GFP^{+} demonstrating they are tuft cells. (D) NS1/2+NS6/7+ events were significantly enriched among GFP+ cells. NS1/2+NS6/7+ events were at background levels among non-tuft cells. Data is pooled from three independent experiments with one to two mice per group. Shown are means ± SEM. NS, not significant; *P<0.05; **P<0.01. Dashed lines represent the epithelial barrier. White boxes in the overlaid image reflect the magnified inset images. Scale bars, 10 microns.

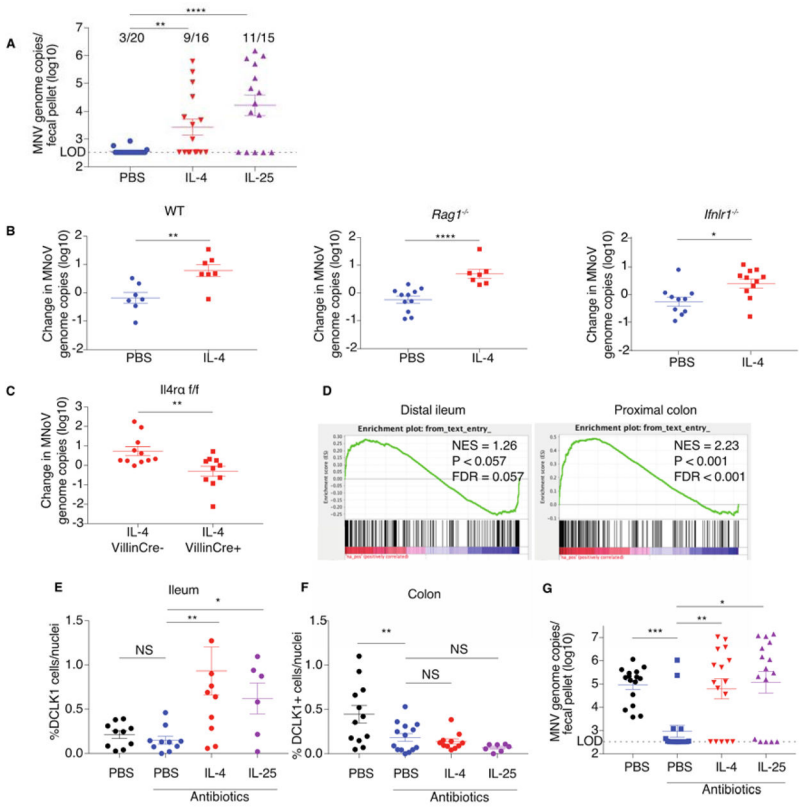

Fig. 4. Tuft cell tropism determines transkingdom interactions of MNoV

(A) WT mice were injected intraperitoneally with PBS, IL-4, or IL-25 prior to peroral challenge with a low dose (4.25×10^4 PFU) of $MNoV^{CR6}$. Both IL-4 and IL-25 increase MNoV transmission as measured by detection of MNoV genomes in feces seven days post-infection. The number above each column reflects the infected animals relative to total animals per group (Chi-square <0.0015). (B) WT, *Rag1*$^{-/-}$, and *Ifnlr1*$^{-/-}$ mice chronically infected with high dose (10^6 PFU) $MNoV^{CR6}$ for 21 days were administered PBS or IL-4. MNoV fecal shedding significantly increased after IL-4 injection (24 days post infection) compared to PBS in WT, *Rag1*$^{-/-}$, and *Ifnlr1*$^{-/-}$ mice. (C) IL-4 enhancement of MNoV fecal shedding during chronic infection requires Il4rα expression on VillinCre-expressing epithelial cells. (D) Broad spectrum antibiotics (vancomycin, neomycin, ampicillin, metronidazole), which prevent $MNoV^{CR6}$ infection, significantly reduce tuft cell specific gene transcripts as measured by RNAseq in the colon but not the ileum. (E–F) $DCLK1^+$ tuft cells were quantified by immunofluorescent microscopy. Antibiotics reduce $DCLK1^+$ tuft cells in the colon but not the ileum. IL-4 and IL-25 increase $DCLK1^+$ tuft cells in the ileum but not the colon. (G) Antibiotic pre-treatment prevents $MNoV^{CR6}$ infection. This antiviral state can be reversed with IL-4 or IL-25 administration prior to $MNoV^{CR6}$ challenge. Shown are means ± SEM. NS, not significant; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. L.O.D., limit of detection. Data in mouse experiments is pooled from at least three independent experiments with 2–6 mice per group except for the *Ifnlr1*$^{-/-}$ study in which data is pooled from two independent experiments. Each dot in (E–F) represents the tuft cell

frequency in one mouse. At least ten independent low power images were averaged per mouse. Data was analyzed by Mann-Whitney test unless otherwise indicated.