The major targets of acute norovirus infection are immune cells in the gut-associated lymphoid tissue

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Noroviruses are the leading cause of food-borne gastroenteritis outbreaks and childhood diarrhoea globally, estimated to be responsible for 200,000 deaths in children each year1–4. Thus, reducing norovirus-associated disease is a critical priority. Development of vaccines and therapeutics has been hindered by the limited understanding of basic norovirus pathogenesis and cell tropism. While macrophages, dendritic cells, B cells and stem-cell-derived enteroids can all support infection of certain noroviruses in vitro5–9, efforts to define in vivo norovirus cell tropism have generated conflicting results. Some studies detected infected intestinal immune cells8–12, other studies detected epithelial cells13, and still others detected immune and epithelial cells13–15. Major limitations of these studies are that they were performed on tissue sections from immunocompromised or germ-free hosts, chronically infected hosts where the timing of infection was unknown, or following non-biologically relevant inoculation routes. Here, we report that the dominant cellular targets of a murine norovirus inoculated orally into immunocompetent mice are macrophages, dendritic cells, B cells and T cells in the gut-associated lymphoid tissue. Importantly, we also demonstrate that a norovirus can infect T cells, a previously unrecognized target, in vitro. These findings represent the most extensive analyses to date of in vivo norovirus cell tropism in orally inoculated, immunocompetent hosts at the peak of acute infection and thus they significantly advance our basic understanding of norovirus pathogenesis.

Previous efforts to identify norovirus-infected cells along the gastrointestinal (GI) tract relied on the detection of viral structural or nonstructural proteins, and the results of these studies have generally reported a low number of infected cells8–10,11–16. This is incongruous with the extremely high amount of virus shed in the stool of norovirus-infected hosts17–20. Thus, we hypothesized that rare pockets of robust norovirus infection exist along the GI tract that had yet to be detected either due to insufficient sensitivity of the detection methods, insufficient sampling of the target tissue and/or confounding issues with high background signal inherent to intestinal tissue. RNAscope in situ hybridization (ISH) was an attractive approach to address these possibilities due to its exquisite sensitivity and specificity. The mouse model of norovirus infection is well established, has been widely used to gain insight into general norovirus pathogenesis, and was ideally suited to our purposes as wild-type mice are susceptible to oral murine norovirus infection17. We first validated probes specific to plus- and minus-strand murine norovirus 1 (MNV-1) RNA (the latter being a replication intermediate only produced during intracellular replication and therefore indicative of productive infection of target cells), using a murine macrophage (Mφ) cell line (RAW264.7) known to be permissive to MNV-1. As expected, nearly all infected cells were positive for both plus- and minus-strand genomes, while minimal signal was observed in mock-inoculated cells (Supplementary Fig. 1a). To next test whether virus-specific probes could detect infected cells in vivo, we inoculated wild-type B6 mice with 1×107 TCID50 (tissue culture infectious dose 50) units of MNV-1 by the peroral route and collected intestinal tissue at 24 h post-infection (h.p.i.), representing the peak of acute infection21. Three consecutive pieces comprising the entire small intestine (SI-1, SI-2 and SI-3) and the colon (CO) were processed as described in the Methods (Supplementary Fig. 1b). Confirming the RNA integrity of our samples, intestinal sections hybridized with a positive control probe specific to the housekeeping peptidyl-prolyl cis-trans isomerase B (PPIB) transcript displayed a strong and uniform signal throughout the intestine, whereas sections hybridized with a nonspecific negative control probe targeting the bacterial dapB transcript were devoid of signal (Supplementary Fig. 1c). Confirming the specificity of virus probes, intestinal sections from naive mice hybridized with either the plus- or minus-strand viral probes were also devoid of signal (Supplementary Fig. 1d). Tissue sections from naive or mock-inoculated mice were probed in parallel with virus-infected tissue sections in every experiment of this study to ensure the absence of background signal.

We next hybridized sections from swiss rolls of MNV-1-infected mice at 24 h.p.i. with the plus-strand virus probe. Consistent with virus titre data (Supplementary Fig. 2a), viral foci were observed in SI-2 and SI-3 of all infected mice; however, there were minimal to no viral foci detected in SI-1 or CO rolls (see Supplementary Fig. 2b for all rolls and layers from a representative experiment, with viral foci boxed). Importantly, virus-positive signal was not...
uniformly detected in the five layers of a given roll, even in SI-2 and SI-3 (Supplementary Fig. 2b). The localization of minus-strand viral RNA mirrored that of plus-strand RNA, although at expectedly reduced levels (Supplementary Fig. 2c). The paucity of signal in SI-1 and CO rolls was surprising given the modest but detectable virus titres in these intestinal segments (Supplementary Fig. 2a). One possible explanation is that virus titres reflect both tissue-associated and mucosa-bound virus and are thus not necessarily an accurate reflection of infection, whereas ISH detects only tissue-associated virus. However, we observed no reduction in virus titres upon removal of the mucosa (Supplementary Fig. 3), and we previously reported that nearly all virus titred from intestinal segments at 24 h.p.i. reflects newly synthesized virus rather than residual input virus. An alternative possibility is that the sporadic nature of infection minimizes the likelihood of detecting viral foci in regions with modest levels of infection and that more extensive sampling of SI-1 and CO is required to detect existing viral foci by RNAscope ISH. We speculate that this nonuniform nature of infection contributed to the difficulty that we and others have had in previous attempts to detect norovirus-infected cells in intestinal sections. In spite of its patchy nature, a clear pattern of infection emerged. A majority of virus-positive signal was detected in the gut-associated lymphoid tissue (GALT), comprising Peyer’s patches and isolated lymphoid follicles (ILFs). Overall, these data indicate that the GALT within the distal two-thirds of the small intestine (the jejunum and ileum) is the primary target of MNV-1, while the proximal third of the small intestine (the duodenum) and the colon support modest levels of infection.

To begin determining the types of cells targeted by MNV-1, intestinal sections hybridized with plus- and minus-strand viral probes were analysed at higher magnification. Although plus-strand genomes were detected throughout the GALT, minus-strand antigenomes were preferentially detected in the subepithelial dome with minimal signal in the B-cell-rich germinal centres (Fig. 1a). Productive infection, as indicated by the presence of minus-strand antigenomes, was typically confined to subepithelial cells (Fig. 1b, left inset), although, in very rare instances, minus-strand antigenomes were also observed in the follicle-associated epithelium (FAE) (Fig. 1b, right inset). To gain insight into the relative contribution of FAE versus subepithelial cell infection, cells positive for minus-strand viral RNA were enumerated in each layer of each intestinal segment from six infected mice. No minus-strand viral RNA was detected in CO and very few dots were observed in SI-1 (Supplementary Fig. 2c). On the other hand, there were an average of 335 subepithelial cells, 64 cells in the FAE and 33 undefinable cells positive for viral minus-strand RNA in layers of SI-2 and SI-3 (Fig. 1b). We infrequently detected intestinal villi with substantial numbers of lamina propria cells positive for plus-strand genomes and even less frequently for minus-strand genomes (Fig. 1c). These data suggest the predominant targets of acute norovirus infection are immune cells within the GALT.

To discern the specific types of cells productively infected with the GALT, we next performed multi-probe hybridizations with plus- and minus-strand viral probes. A panel of cell-specific probes (CD11c+CD4− for dendritic cells (DCs) and CD11c+CD4− for Mψs5; CD19+ for B cells; CD3+ for T cells; and EpCAM+ for epithelial cells) using RNAscope fluorescent ISH (FISH). The expected distribution of each cell type was observed throughout the intestinal tissue, and infected cells were predominately observed in the GALT (Fig. 2a and Supplementary Fig. 4), consistent with chromogenic assays, although the FISH assay was notably less sensitive than the chromogenic assay (Supplementary Fig. 5). Viral minus-strand RNA was detected in CD11c+CD4− DCs (Fig. 2b), CD11c+CD4− Mψs (Fig. 2c), CD19+ B cells (Fig. 2d) and CD3+ T cells (Fig. 2e). Although we occasionally detected viral minus-strand RNA in the FAE in chromogenic assays (Fig. 1b), we did not observe EpCAM+ positive infected cells by RNAscope FISH. It is possible that this is explained by the difference in chromogenic and fluorescent assay sensitivities (Supplementary Fig. 5). Alternatively, these cells could be intraepithelial lymphocytes (IELs), a possibility that will be investigated in future studies. The absence of substantial epithelial cell infection along the GI tract is consistent with previous reports by ourselves and others that epithelial cells do not support MNV infection in vitro5,22,23 (Fig. 4a). Because Peyer’s patches were observed to be the major target of MNV-1 infection, we next measured virus titres in Rag1−/− mice, which lack Peyer’s patches. Indeed, virus titres were significantly reduced in all intestinal regions of Rag1−/− mice compared to wild-type B6 mice (Fig. 3).
To this end, we next demonstrated to support norovirus infection in vitro5,6, productive norovirus infection in vitro (Fig. 2). NS-2, SI-2, and SI-3 are outlined in white. Mock-inoculated mice (a) were tested in parallel (b–e). In b–e, individual cells are outlined in white. Mock-inoculated mice (n = 2) were tested in parallel and no virus signal was observed (for example, Supplementary Fig. 1d). The entire experiment was repeated twice. Scale bars, 50 μm (a) and 5 μm (b–e).

Although DCs, MqRs, and B cells have been previously demonstrated to support norovirus infection in vitro24, productive norovirus infection of T cells has not been reported. To this end, we next infected the murine T-cell line EL4 with MNV-1 at a multiplicity of infection (MOI) of 5 and analyzed the virus growth kinetics. The murine Mq cell line RAW264.7 and murine B cell lines M12 and WEHI served as positive controls, while the murine intestinal epithelial cell line CMT-93 served as negative control7. Peak titres in RAW264.7 cells were observed at 24 h.p.i., at which point virus decay was observed (Fig. 4a), coinciding with nearly complete loss in cell viability (Fig. 4b). Peak titres in M12 and WEHI cells were observed at 72 and 24 h.p.i., respectively (Fig. 4a). These cell lines displayed variable levels of cytopathicity, with M12 cultures displaying a modest loss in viability over 96 h of infection and the WEHI cells displaying a more substantial initial loss in viability but recovering by 72 h.p.i. (Fig. 4b). The CMT-93 cells failed to support MNV-1 infection (Fig. 4a). These results are all consistent with our previously published findings5. T-cell line EL4 was permissive to MNV-1, albeit reaching reduced peak titres compared to all other permissive cell lines, and the kinetics of infection were similar to those in M12 cells (Fig. 4a). Remarkably, there was no reduction in viability of infected EL4 cultures compared to mock-inoculated cultures (Fig. 4b), demonstrating that T cell infection is nonlytic, in spite of substantial release of progeny virus into the supernatant. Because the MNV receptor has recently been identified as the CD300lf molecule26,27, we tested whether permissivity in cell lines directly correlated with receptor levels (Fig. 4a–c and Supplementary Fig. 7). Indeed, RAW264.7 and WEHI cell lines displayed the highest frequency (86% and 92%, respectively) and abundance of CD300lf expression, with M12 and EL4 lines expressing these molecules at reduced frequencies (26% and 19%, respectively) and abundance. Nonpermissive CMT-93 cells did not express appreciable levels of CD300lf, in contrast to varying degrees, while epithelial cells expressed minimal CD300lf. To further substantiate the correlation between receptor expression and permissivity, we identified another CD300lf-negative cell line, Neuro2A, which failed to support MNV infection (Fig. 4a–c and Supplementary Fig. 7). Finally, we measured CD300lf expression frequencies on Peyer’s patch cell types. Consistent with in vitro observations, permissive immune cells expressed the receptor to varying degrees, while epithelial cells expressed minimal CD300lf (Fig. 4d and Supplementary Fig. 8c). Overall, these data demonstrate that T cells are a novel target of MNV-1 infection in vivo.
vitro and in vivo, and that viral infection of T cells is nonlytic in vitro. Interestingly, viral capsid protein was detected in intestinal T cells of biopsy tissue from immunocompromised patients chronically infected with a human norovirus, suggesting that T cells are a common but previously unrecognized target of noroviruses.

In summary, our data prove that the cell tropism of a norovirus is predominantly distal ileum GALT immune cells, including Mφ, DC, B and T cells, as well as rare cells within the FAE. Importantly, these data were generated in immunocompetent hosts following oral inoculation at the peak of acute infection. Our results are consistent with those of Karandikar and colleagues, in which biopsies from chronically infected immunocompromised people were analysed for the presence of viral protein. In these subjects, a majority of viral antigen was detected in the intestinal lamina propria, which contains a variety of immune cells, while epithelial cells were a minor target of infection (supplementary table 2 in ref. 14). This consistency between human and murine norovirus cell tropism lends significant weight to the power of the mouse model of infection for general pathogenesis studies. Infection of the GALT was not absolute in our model because there were numerous Peyer’s patches and ILFs in the SI-2 and SI-3 of infected mice that were devoid of signal. The sporadic nature of infection provides an explanation for the difficulty in detecting infected cells in previous studies. Moreover, these data suggest that a yet to be defined host factor either promotes infection at infrequent regions of the gut, or that an inhibitory factor may prevent infection of a majority of the tissue. For example, M cells overlying the FAE display regional and species-specific variability in glycan expression patterns.

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**Fig. 4 | MNV-1 infects T cells nonlytically in vitro, and infection correlates with CD300lf expression frequency.** a, b, Duplicate wells of the indicated cell lines were infected with mock inoculum or MNV-1 at an MOI of 5. Virus titres were determined by TCID<sub>50</sub> assay on culture supernatants (a) and cell viability was assessed by propidium iodide staining (b) at the indicated time points. Error bars denote s.e.m. c, The frequency and abundance of cells expressing the MNV-1 receptor CD300lf were determined on naive cells by flow cytometric staining using a matched isotype antibody as a negative control. MFI, mean fluorescence intensity. d, Peyer’s patch cells from B6 mice (n = 6) were stained with two panels of antibodies (see Methods). The frequency and abundance of DC/Mφ (CD45<sup>+</sup>CD11c<sup>+</sup>), B cells (CD45<sup>+</sup>CD19<sup>+</sup>B220<sup>+</sup>), T cells (CD45<sup>+</sup>CD3<sup>+</sup>) and intestinal epithelial cells (CD45<sup>−</sup>EpCAM<sup>+</sup>) expressing CD300lf were determined. For all panels, experiments were performed three times. N/A, not applicable.
that noroviruses are well established to bind host glycans\(^\text{29}\) and to exploit M cells for virus entry\(^\text{24,25}\), this could represent a critical

### Methods

#### Cells and viruses.

The RAW264.7 (ATCC), 293T (provided by F. Zhu, Florida University), M12 (provided by S. Tibbetts, University of Florida), WEHI (provided by S. Tibbetts, University of Florida), EL4 (provided by D. Avram, University of Florida), CMT-93 (ATCC) and Neuro2A (provided by D. Bloom, University of Florida) cell lines tested negative for mycoplasma. Cell lines were not authenticated by our laboratory. RAW264.7, 293T, EL4 and CMT-93 lines were grown in Dulbecco’s modified Eagle media (DMEM, Corning) supplemented with 10% fetal bovine serum (FBS, Omega), 100 U ml\(^{-1}\) penicillin, 100 \(\mu\)g ml\(^{-1}\) streptomycin and 2 mM l-glutamine. EL4 cells were also supplemented with 0.05 mM 2-mercaptoethanol. ME2 and WEHI lines were cultured in Roswell Park Memorial Institute 1640 media (RPMI, Corning) supplemented with 10% FBS, 100 U ml\(^{-1}\) penicillin, 100 \(\mu\)g ml\(^{-1}\) streptomycin, 2 mM l-glutamine and 0.05 mM 2-mercaptoethanol. Neuro2A cells were grown in Eagle’s minimum essential media (MEM, Corning) supplemented with 10% FBS, 100 U ml\(^{-1}\) penicillin, 100 \(\mu\)g ml\(^{-1}\) streptomycin, 1.5 \(g\) l\(^{-1}\) sodium bicarbonate and 2 mM l-glutamine. Stocks of recombinant MNV-1 (GenBank accession no. KC782764) were generated as previously described\(^\text{30}\). In brief, 293T cells were transfected with 5\(\mu\)g of infectious clone plasmid DNA per 10\(^{5}\) cells using Lipofectamine 2000 (Life Technologies) and used in freeze-thawing and post-transfection. 293T lysates were used to infect RAW264.7 cells at an MOI of 0.05. RAW264.7 lysates were prepared when ~90% of cells displayed cytopathic effect (CPE) and were purified through a 25% sucrose cushion. Viral stocks were titred using a standard TCID\(_{50}\) assay\(^\text{31}\).

#### Mouse infections.

Specific pathogen-free (SPF) mice used in this study were bred and housed in animal facilities at the University of Florida. All animal experiments were performed in strict accordance with federal and university guidelines. The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Florida (study no. 201408668). Experiments were not performed in a blinded fashion and nor were randomization used. Eight- to ten-week-old, sex-matched C57BL/6J (Jackson no. 002216, referred to as B6) or B6–Rag1\(^{-/-}\) (Jackson no. 000664, referred to as B6Rag1) mice were inoculated perorally (p.o.) with the indicated dose of MNV-1. Haematoyxlin staining of the tissue revealed the differentiation of subepithelial cells and cells within the FAE, because the single layer of epithelial cells and interspersed IELs lining the entire gut wall has a defined location and morphology. In cases where the infected cell type was questionable, we counted these as undefined. In three instances, a Peyer’s patch had too many subepithelial cells positive for M cells, and the viral signal to count reliably, these were given a value of 0.5, which is a conservative estimate. The values for each layer of a given roll (for example, L1–L5 for SI-3) were averaged, and the SI-2 and SI-3 values for individual mice were then averaged.

### RNAseq FISH.

An RNAscope Multiplex Fluorescent Detection Kit v2 (Amplification Kit, murine panel 1, designed to perform dual or triple hybridizations with the MNV-1 minus-strand probe and cell-specific probes (CD11c and CD4 for DCs [CD11c+CD4+] and Mps [CD11c+CD4+]; CD19 for B cells; CD3 for T cells; and EpCAM for epithelial cells) on FFPE tissue sections. Serial sections from individual layers were hybridized with the viral minus-strand probe and one or two of the five cell-specific probes. After the addition of horseradish peroxidase (HRP) to the probes, a tryamide signal amplification (TSA)-conjugated cyanine 5 (Cy5) fluorophore (Perkin Elmer) was used to visualize viral minus-strand RNA, and a TSA-conjugated Cy3 (CD11c, CD19, CD3 and EpCam) or fluorescein (FITC; CD4) fluorophore was used to visualize the cell-specific transcript. Tissues were counterstained with DAPI and mounted using ProLong Gold Antifade Mountant (ThermoFisher Scientific). Images for FISH were acquired using a Carl Zeiss LSM710 confocal microscope using the ZEN Digital Imaging software (Zeiss). Z-stacks were taken at x63 magnification using 0.5 or 1 \(\mu\)m intervals. Single colour controls, as well as positive and negative control probes (PPIB and DapB, respectively), were stained in parallel.

#### Virus growth curves and viability assays.

RAW264.7, M12, WEHI, EL4, CMT-93 and Neuro2A cells were infected with mock inoculum or MNV-1 at an MOI of 5 and incubated for 1 h at room temperature. Cells were then washed once with PBS to remove unbound virus and incubated at 37°C. Supernatants were collected from duplicate wells per condition at 0, 24, 48, 72 and 96 h.p.i., and virus titres were determined by standard TCID\(_{50}\) assay, as previously described\(^\text{20}\). From the same infections, cells at each time point were incubated with propidium iodide (BD Pharlmgen) at a final concentration of 2.5 \(\mu\)g ml\(^{-1}\) for 5 min at room temperature. Flow cytometric analysis was performed on a FACS Calibur instrument with BD CellQuest Pro software (BD Biosciences), and FCSExpress software was used for analysis. Data are reported as the percentage of cells in infected wells that did not incorporate dye compared to the percentage of cells in mock-inoculated wells at the same time point that did not incorporate dye (relative viability). Unstained cells were used to set the gate (Supplementary Fig. 6).

#### Flow cytometric assay using cell lines.

Cells were resuspended in FACS buffer containing 1% BSA and 0.5\(\mu\)g ml\(^{-1}\) anti-mouse CD16/CD32 (eBioscience) to block nonspecific binding. All cell lines were double stained with a polyclonal rabbit anti-MNV-1 antibody (R&D Systems, AF27788) or appropriate isotype control antibody (R&D Systems, AB-108-C) at 0.1 \(\mu\)g per 10\(^{5}\) cells and then with donkey anti-goat IgG secondary antibody conjugated to AlexaFluor 488 (Abcam, ab150129) at 0.05 \(\mu\)g per 10\(^{6}\) cells. Cells were washed extensively and resuspended in FACS buffer for data acquisition on a FACSCalibur instrument with BD CellQuest Pro software. Data were analysed using BD Flowjo (De Novo Software) as previously described\(^\text{34}\). Viral staining was calculated as the frequency of CD300lf+ positive cells and the mean fluorescence intensity (MFI) as a measure of receptor abundance. Gates were set using isotype control antibody staining (Supplementary Fig. 7).

#### Flow cytometric assay using in vivo samples.

Splenic and Peyer’s patches were collected from three male or three female B6 mice per experiment into 10% DMEM containing 1 \(mg\) ml\(^{-1}\) collagenase D (Sigma-Aldrich, 11088858001) and incubated on a shaker at 37°C for 30 min before mechanical disruption through
a 70 μm cell strainer to generate single-cell suspensions. Cells were washed in PBS and incubated with Live/Dead Fixable Aqua dye (ThermoFisher, L34965). Fc receptors were then blocked as described above and cells were stained with a primary antibody against CD300lf (BD Biosciences, #561702) and CD45 (BD Biosciences, #555213) followed by a secondary antibody conjugated to Alexa Fluor 647 (Molecular Probes, A-21203). All antibodies were diluted in 2% FBS in PBS and used at 1:2000 dilution.

**Life Sciences Reporting Summary**
Further information on experimental design and reagents is available in the Life Sciences Reporting Summary.

**Data availability.**
All relevant data are available from the authors or are included in the manuscript or Supplementary Information. The RNAscope probes used in this study were designed by Advanced Cell Diagnostics.

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**Author contributions**

K.R.G., A.N.R., S.Z. and S.M.K. designed the study and analysed results. K.R.G. performed and analysed RNAscope-based FISH assays and quantified chromogenic assays. S.Z. and A.H. performed mouse infections, harvests and plaque assays, and S.Z. performed RNAscope-based chromogenic assays. A.N.R. performed and analysed in vitro infections and viability assays on cell lines as well as CD300lf expression on cell lines and Peyer’s patch cells. N.C. and M.M. assisted with fluorescence microscopy. N.C. and B.B.D. performed flow cytometric analyses of in vivo samples guided by the expertise of S.M.W. and M.M. D.T.P.P. performed TCI0 assays and analysed data. C.R. and B.G. assisted with analysing chromogenic assays using a slide scanner. K.R.G., A.N.R. and S.M.K. prepared the manuscript. M.M. and S.Z. edited the manuscript.

**Competing interests**

The authors declare no competing financial interests.

**Additional information**

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Experimental design

1. Sample size
   Describe how sample size was determined.
   
   For quantitative analyses, we used power analysis to obtain a power of 80% at alpha = 0.05. For qualitative analyses (e.g., RNAscope assays), we used 4 mock and 6 infected mice reasoning that the extensive sampling of each mouse (5 layers from each of 4 intestinal segments, multiple serial sections of each layer) would provide a definitive representation of cell tropism of MNV-1.

2. Data exclusions
   Describe any data exclusions.
   
   No data were excluded from this study.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   
   We clearly state the number of experimental replicates in each figure legend. For quantitative analyses, we average data for all replicates. For visual analyses, we selected the most representative images available. In all cases, findings were reliably reproduced.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   
   Mice were age- and sex-matched for all animal experiments.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   
   Investigators were not blinded to group allocation during data collection or analysis.

   Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a
   
   □ ☒ Confirmed

   □ ☒ The exact sample size \( n \) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)

   □ ☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

   □ ☒ A statement indicating how many times each experiment was replicated

   □ ☒ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)

   □ ☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons

   □ ☒ The test results (e.g. \( P \) values) given as exact values whenever possible and with confidence intervals noted

   □ ☒ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)

   □ ☒ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Describe the software used to analyze the data in this study.

Several software programs were used to analyze RNAscope images depending on what microscope was used to acquire images. The Axiovision 4.7 software was used when analyzing chromogenically stained slides individually; Aperio ImageScope software was used when images were collected on a slide scanner; and the ZEN Digital Imaging software from Zeiss was used for FISH assays. FCSExpress 4 and BD CellQuest Pro software were used to analyze flow cytometric data. GraphPad Prism was used to generate graphs and for performing statistical analysis in Figure 5.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

RNAscope probes are proprietary and available only by purchase from Advanced Cell Diagnostics.

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

anti-mouse CD300f antibody (R&D Systems, AF2788), anti-mouse CD19 PE (clone 103, ebioscience, 12-0193), anti-mouse B220 PE-Cy7 (clone RA3-6B2, BioLegend, 103221), anti-mouse CD4 PerCP (clone RM4-5, BioLegend, 100537), anti-mouse CD11c FITC (clone N418, BioLegend, 117302), anti-mouse CD45 Pacific Blue (clone 30-F11, BioLegend, 103125), anti-mouse CD3 PE (clone 145-2C11, ebioscience, 12-0031-82), anti-mouse EpCAM PerCP-Cy5.5 (clone G8.8, BioLegend, 118219), anti-mouse NK1.1 Alexa Fluor 488 (clone PK136, BioLegend, 108717), and anti-mouse CD45 Pacific Blue (clone 30-F11, BioLegend, 103125). All antibodies were obtained from the indicated vendors, and recommended isotype antibodies used to set negative gates.

Eukaryotic cell lines

State the source of each eukaryotic cell line used.

RAW264.7 (ATCC), 293T (provided by Fanxiu Zhu, Florida State University), M12 (provided by Scott Tibbetts, University of Florida), WEHI (provided by Scott Tibbetts, University of Florida), EL4 (provided by Dorina Avram, University of Florida), CMT-93 (ATCC), and Neuro2A (provided by David Bloom, University of Florida)

Describe the method of cell line authentication used.

Cell lines were not authenticated.

Report whether the cell lines were tested for mycoplasma contamination.

All cell lines tested negative for mycoplasma contamination.

If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

N/A

Animals and human research participants

Provide details on animals and/or animal-derived materials used in the study.

C57BL/6j (Jackson no. 000664; referred to as B6) and B6RAG1-/- (Jackson no. 002216; referred to as Rag1-/-) strains were used in these studies.
12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

N/A