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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Supplementary Materials and Methods

Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Harvard University and the Broad Institute and were in compliance with all relevant ethical regulations. The KOMP and Neo deleted *C9orf72* loss of function strains were generated as described previously[7]. Mice were housed with nestlet bedding, red hut for enrichment, provided water ad libitum and fed ad libitum either Prolab Isopro RMH 3000 (Harvard BRI) or PicoLab Rodent Diet 20 (Broad Institute) and kept on a 12 hour light-dark cycle. Embryo rederivation was performed by collecting embryos from super-ovulated *C9orf72* +/- females, washing embryos, then surgical transfer using aseptic technique into the reproductive tract of pseudopregnant recipient females. For experiments involving antibiotics, animals were cohoused for at least a week prior to

initiation of dosing. Animals were administered either vehicle (water) or a freshly prepared cocktail of four antibiotics including Ampicillin sodium salt (200 mg/kg/day), Neomycin trisulfate salt hydrate (200 mg/kg/day), Metronidazole (200 mg/kg/day), and Vancomycin hydrochloride from *Streptomyces orientalis* (100 mg/kg/day) (all from Sigma) administered by twice daily gavage. N, sex and ages of the animals used in each study are described in figure legends or text. Power calculations (G*Power 3.1.9.2) using the mean and standard error of endophenotype data was used to estimate necessary cohort sizes for antibiotics and fecal transplant studies. Before administration of antibiotics, animals were assessed for systemic inflammatory measures and mice were allocated into groups so that no significant differences were present prior to treatment initiation.

Motor behavior

Naïve animals were trained on the rotarod at constant speed of 4 RPM for 300 seconds at least one day before competitive assessment. For performance trials, the rotarod accelerated from 4 to 40 RPM over 300 seconds using Ugo Basile mouse RotaRod NG (Harvard FAS BRI) or Panlab Rota Rod (Broad Institute). Each trial day consisted of three tests per mouse, with each test separated by at least 20 minutes. Operator was blinded to animal genotype during trials.

Fecal transplantation

Using sterilized forceps, donor fecal pellets were collected directly from the anus or donor upper and lower intestinal contents were isolated from euthanized animals and immediately frozen on dry ice. Recipient mice received antibiotics twice daily by gavage for two weeks, then two day secession of antibiotics, then fecal transplantation once per day for two days. Feces pellets and intestinal contents from donor mice were weighed, pooled,

diluted to 200 mg/mL in degassed PBS and administered by oral gavage to recipient mice at 2mg feces/g body weight. All cage changes were performed in HEPA filtered hoods with freshly autoclaved cages, bedding and enrichment.

Blood and cytokine measures

Peripheral blood was collected via mandible puncture into EDTA-coated tubes. Blood counts were assessed using a Hemavet (Abaxis). Samples were then centrifuged to pellet cells and plasma harvested from supernatant. Plasma was diluted 1:2 for luminex-based multiplexed fluorescence assay to assess 36 cytokines and chemokines. Plasma was diluted 1:200 to assess mouse anti-dsDNA total IgG autoantibodies (Alpha Diagnostic International).

Tissue preparation

Animals were anesthetized with isofluorane followed by transcardial perfusion with HBSS supplemented with 10 U/mL heparin. Spleens were dissociated by repeated trituration with glass pipetteman in HBSS, subjected to 10-minute RBC lysis (eBioscience), washed in autoMACS (Miltenyi), filtered (40 μm) and counted using a Countess (Invitrogen) for antibody staining. For flow cytometry of the CNS, spinal cords were digested by papain and DNase diluted in EBSS (Worthington) for 10 minutes at 37°C, triturated with glass pipettman to generate large tissue chunks then allowed to digest for 20 minutes at 37°C.DMEM supplemented with glutamax was added, samples triturated to single cells, ovomucoid (Worthington) and DNase diluted in EBSS added to inhibit protease activity, cells filtered, washed in autoMACS buffer, and pelleted at 500xg for 15 minutes at 4°C. Cell pellets brought up in isotonic Percol Plus (Sigma) diluted to 30% in autoMACS and spun for 15 minutes at room temperature with no brake. Floating myelin was gently removed using

plastic transfer pipette. Cell pellets were resuspended, filtered, washed in autoMACS and re-pelleted at 4°C. Cells were fixed in 4% paraformaldehyde (PFA; Electron Microscopy Sciences) either before or after antibody staining depending on need. Samples collected on BD LSRII. Data analyzed using Flowjo and/or Cytobank. For immunofluorescence experimenets, following HBSS perfusion animals were perfused with 4% PFA and CNS tissue post-fixed in 4% PFA overnight at 4°C. The next day samples were washed with PBS overnight at 4°C. Tissue was submerged in 30% sucrose for two days. After cryoprotection, lumbar regions were mounted in OCT and cryostat sectioned at 30µm.

Immunofluorescence

Spinal cord sections were washed three times in PBS to remove residual OCT. Sections were incubated in a blocking solution (10% donkey serum, 0.1M glycine, 0.1% Tween20 or 0.3% Triton X100, PBS, Image-iT FX Signal Enhancer (Thermo) for 1 hour at room temperature. Following blocking, sections were incubated with primary antibodies for two days on a rocker at 4°C. Primary antibodies include: Rat-CD11b-FITC 1:200 (M1/70, BioLegend), rabbit-Cathepsin B 1:400 (D1C7Y CST), rat-CD45-488 1:200 (30-F11 BioLegend), guinea pig-Iba1 1:500 (234004 Synaptic Systems), rat-Lamp1 1:200 (1D4B SCB), rat-Ccr9-FITC (9B1 Biolegend), rat-Dectin1/Clec7a (mabg-mdect Invivogen), mouse-Lpl (ab21356 Abcam). Sections were then washed with 0.1% Tween20 in PBS (for stains with CD11b, CD45, Ccr9, Cathepsin B) or 0.3% TritonX100 in PBS (for stains with Iba1, Lamp1, Clec7a/Dectin1, Lpl) at least five times. Secondary antibodies include: Donkey-anti-rat-AlexaFluor-488, -mouse IgG-555, -rabbit-555, -rabbit-647, -rat-647, -guinea pig-647, all 1:500 dilution (Invitrogen), for 2 hours at room temperature. Sections were washed again, mounted on microscope slides in Fluoromount for curing overnight. Spinal cords were

imaged on a ZEISS LSM700 with either a 10x and 40x objective or Axio scan Z.1 at 20x objective. Images were stitched and processed on ZEISS ZEN 2.6 image processing software and Bitplane Imaris 9.2. All comparative stains between control and mutant animals were acquired using identical laser and microscope settings and images processed with viewer blinded to genotype.

Flow cytometry

Dissociated single cells were stained in autoMACS on ice using the following antibodies (BioLegend): CD45-BV421 or APC-Cy7 1:200 (30-F11), rabbit-Cathepsin B 1:100 (D1C7Y CST) and goat-anti-rabbit-AlexaFluor-488 1:500 (Invitrogen), Ccr9-FITC 1:200 (9B1), F4/80-PE-Cy5 1:400 (BM8), CD11b-AlexaFluor-700 1:400 (M1/70 Invitrogen), Lamp1-APC-Cy7 1:400 (1D4B), TruStain FcX 1:250 (93), CD39-PE 1:400 (Duha59), Ly6G-PE-Cy7 1:600 (1A8), Ly6C-AlexaFluor-647 (HK1.4). To retrieve the cathepsin B epitope, fixed cells were slowly permeabilized in 90% methanol prior to staining for cathepsin B.

16S sequencing, PRIA

DNA was isolated by Powersoil (QIAGEN, Germantown, MD) per the manufacturer's protocol and recovery yield and DNA quality was determined by fluorometric analysis. DNA concentration was standardized and amplified using 16s rRNA primers spanning the V3 and V4 regions (Illumina). Resulting amplified PCR products were analyzed on a Bioanalyzer (Agilent Technologies, Santa Clara, CA) then purified and amplified with primers containing unique sample nucleotide barcodes (Illumina). PCR products were analyzed with the Bioanalyzer for product quality control and also by SYBR green PCR to determine the quantity. All samples were pooled and standardized to a final concentration of 4.0 nM representation for each sample. The 16S PCR product pool was denatured with

sodium hydroxide then adjusted to 4.0 pM and combined with 5% PhiX control DNA prior to loading onto a sequencing flow cell (Illumina) with 300 bp paired ends and a unique molecular tag for each sample. Following the sequencing run, the sequence data was separated based on the nucleotide bar code and then compared to the Greengenes database[38]. Relative abundance, alpha diversity, beta diversity and principal coordinate analysis was performed using QIIME analysis software[39]. PCR assays for rodent infectious agents (PRIA) were performed as described[40].

PCR

Fecal DNA was isolated from fecal pellets using QIAmp Fast DNA Stool Mini Kit (Qiagen). *Helicobacter spp.* 16S rRNA was amplified using primers 5'-CTATGACGGGTATCCGCC-3' and 5'-ATTCCACCTACCTCTCCCA-3'. Total Eubacteria 16S rRNA amplified using primers 5'-TCCTACGGGAGGCAGCAG-3' and 5'-GGACTACCAGGGTATCTAATCCTGTT -3'. Tritrichomonas muris 28S rRNA was amplified using primers 5'-GCTTTTGCAAGCTAGGTCCC-3' and 5'-TTTCTGATGGGGCGTACCAC -3'. RNA was isolated from tissue by dissociating cortex in Trizol LS (Thermo) using pellet pestle and reverse transcriptase with iScript (Biorad). qRT-PCR was performed using SYBR (Biorad). Ly6C was amplified using primers 5'-TACTGTGTGCAGAAAGAGCTCAG-3' and 5'- TTCCTTCTTTGAGAGTCCTCAATC-3'. Gapdh was amplified using primers 5'-TGCGACTTCAACAGCAACTC-3' and 5'-GCCTCTCTTGCTCAGTGTCC-3'.

Bone marrow derived macrophages

Two femus and tibias were stripped of musculature, flushed and cultured in IMDM supplemented with 10% FCS, NEAA, Glutamax, pen/strep and 20 ng/mL murine M-csf (PeproTech). Media was changed on day 3 and cells plated for experiments after 6 days.

Cells plated at 4E4 per 96 well and allowed to attach overnight, followed by stimulation with microbial moieties (Invivogen) including Pam_3csk_4 (10-1000 ng/mL; tlrl-pms), Zymosan (1 ug/mL; tlrl-zyn), HMW Poly(I:C) (10 ug/mL; tlrl-pic), LPS (10 ng/mL; tlrl-peklps), R848 (20 ng/mL; tlrl-r848), CpG ODN (25 ug/mL; tlrl-1826), or PGN (20 ug/mL; tlrl-pgnb3). For fecal stimulations, previously frozen feces were thawed, diluted to 200 mg/mL in PBS, passed through 40 um filter, quick spun, and supernatant collected and kept on ice. Bacterial DNA was isolated from each sample using QIAmp Fast DNA Stool Mini Kit (Qiagen) and total Eubacteria 16S rDNA abundance determined by qPCR. The more concentrated sample was diluted in PBS to normalize the relative Eubacteria abundance, which was confirmed again by bacterial DNA isolation and qPCR. Dilution curves were prepared for each normalized fecal sample and added to macrophage cultures. Pen/strep added to cultures after 2 hours, then media harvested after 18 hours for testing by Tnf alpha ELISA at 1:2 and 1:10 dilution (BioLegend).

Statistics

Statistical calculations were performed using Graphpad prism 8.0. Tests between two groups used two-tailed Student *t* test. A Bonferroni corrected T test was used to assess differentially abundant bacterial species between pro-inflammatory and pro-survival environments. Tests between multiple groups used one-way analysis of variance (ANOVA) with either Tukey or Sidak multiple comparisons. Tests between multiple groups over time used two-way ANOVA with Dunnett multiple comparisons. Survival curves were evaluated by generalized Wilcoxon.

Additional References

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Figure Legends

Fig. 1 | Environment governs survival, inflammation and autoimmunity in *C9orf72* LOF mice.

a, Aseptic embryo transfer of *C9orf72* neo deleted allele from Harvard BRI to Broad Institute. Males and females were aged for survival or tissue harvest. Survival of mice at **b**, Harvard BRI (*C9orf72* +/+ n=55; +/- n=114; -/- n=62) or **c**, Broad Institute (*C9orf72* +/+ n=22; +/- n=36; -/- n=23) (Gehan-Breslow-Wilcoxon). ns not significant. Age-matched (48-week-old) mice reared at Harvard BRI (*C9orf72* +/+ n=12; +/- n=13; -/- n=10) or Broad Institute (*C9orf72* +/+ n=12; +/- n=18; -/- n=11) were assessed for **d**, spleen weight, **e,** blood neutrophil count, **f**, blood platelet count measured at 0ºC and **g**, plasma anti-double stranded (ds)DNA antibody activity. **d-g**, One way ANOVA with Sidak multiple comparisons. Each dot represents one animal.

Fig. 2 | Lifelong suppression of gut microflora prevents inflammation and autoimmunity in *C9orf72* LOF mice

a, Male and female *C9orf72*^{Harvard} +/+ and -/- neo deleted mice of weaning age were co-housed by treatment group, then administered vehicle (+/+ n=7; -/- n=11) or antibiotics