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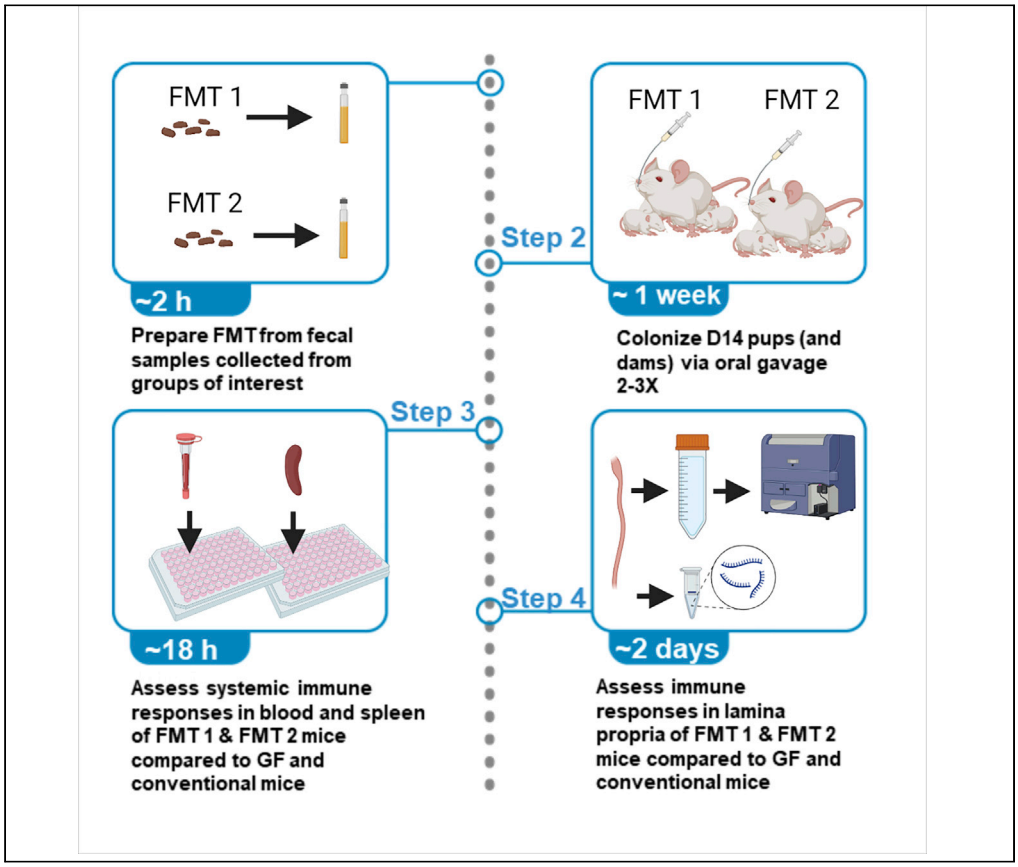
Protocol to colonize gnotobiotic mice in early life and assess the impact on early life immune programming



Understanding how changes in gut microbiota in early life impact immune programming can be difficult to study due to variations in the assembly of the microbiota. In this protocol, we describe how to colonize gnotobiotic/germ-free mice in early life with different microbiota community types (e.g., PAMI and PAMII). We detail several assays to determine whether differential colonization alters immune programming in early life. We also describe how to propagate mouse fecal microbiota transplant material if the donor fecal sample is limited.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol to colonize infant germ-free mice by fecal microbiome transplant

Steps to propagate FMT material if initial fecal sample is limited

Assessment of how differential colonization impacts immune imprinting

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Protocol to colonize gnotobiotic mice in early life and assess the impact on early life immune programming

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SUMMARY

Understanding how changes in gut microbiota in early life impact immune programming can be difficult to study due to variations in the assembly of the microbiota. In this protocol, we describe how to colonize gnotobiotic/germ-free mice in early life with different microbiota community types (e.g., PAMI and PAMII). We detail several assays to determine whether differential colonization alters immune programming in early life. We also describe how to propagate mouse fecal microbiota transplant material if the donor fecal sample is limited.

For complete details on the use and execution of this protocol, please refer to Lynn et al. (2021).[1]

BEFORE YOU BEGIN

Disruption to the normal gut microbiota homeostasis in early life has been associated with a range of metabolic and immune-mediated diseases including metabolic disease, obesity and allergy.[2] We have recently reported that differences in the composition of the gut microbiota following antibiotic exposure in early life impacted host health and longevity in later life.[1] In our study, we found that mice colonized with a specific microbiota community type following antibiotic exposure (which we termed post-antibiotics microbiota II (PAM II)) had a significantly shorter lifespan and metabolic dysfunction in later life compared to mice colonized with a different community type, (post-antibiotics microbiota I (PAM I)) or mice that were not treated with antibiotics. Recently, it has been reported that at weaning, the intestinal microbiota induces a strong immune response peaking in 21-day-old mice, which has been termed a "weaning reaction".[3] Furthermore, if disrupted, this weaning reaction can lead to pathological immune imprinting that drives pathology in later life. To investigate whether colonization with the PAM I or PAM II microbiota alters this weaning reaction, we colonized 14-day-old gnotobiotic/germ-free (GF) mice with either the PAM I or PAM II microbiota and assessed markers of the weaning reaction and other immune responses at day 21 of life compared to GF and SPF mice.

In this protocol, we describe how to colonize GF mice in early life with different microbiota community types (such as PAM I and PAM II) and describe several assays to assess whether differential colonization alters immune programing in early life. We also describe how to propagate mouse fecal microbiota transplant (FMT) material if the donor fecal sample is limited. All animal procedures need to be approved by the appropriate Institutional Animal Care and Use Committee. All experiments in this protocol were approved by the South Australia Health and Medical Research Institute Animal Ethics Committee.

Preparation of reagents for fecal microbiome transplant protocols

Timing: 2 h preparation time, 18 h incubations

Here, instructions for sterilization of solutions and materials for fecal microbiome transplant protocol are detailed.

1. Prepare reagents and materials needed for the fecal microbiome transplant (FMT); anaerobic glycerol, 1× PBS and sterile Hungate tubes in advance.
   a. Prepare 30% glycerol solution.
      i. Add 150 mL glycerol to 350 mL 1× PBS and autoclave.
   b. Aliquot 50 mL 1× PBS and 30% glycerol into separate 50 mL falcon tubes.
   c. Place solutions in an anaerobic chamber for 18 h to deplete oxygen.
   d. Prepare Hungate tubes for sterilization.
      i. Individually wrap rubber seals in aluminum foil.
      ii. Place tubes, wrapped rubber seals and screw cap lids into an autoclave bag.
      iii. Autoclave.

Note: We recommend 3–4 Hungate tubes per preparation.

Note: Our dry autoclave cycle conditions are 122°C for 20 min and 122°C for 40 min for fluid cycle conditions.

Note: Our anaerobic chamber uses a gas mix of CO_2 (10%), H_2 (10%), N_2(80%).

Preparation of materials for germ-free mouse work

Timing: 2–4 h preparation time

Here, instructions for sterilization of materials for germ-free mouse work are detailed.

2. Prepare the Clidox-S® (Pharmacol Research Laboratories) disinfectant.

Clidox-S® disinfectant

Reagent	Final concentration	Amount
Base	1×	600 mL
H_2O	3×	1,800 mL
Activator	1×	600 mL
Total		3 L

⚠ CRITICAL: Clidox-S® is a chlorine dioxide sterilant. Use in a well ventilate space. Wear appropriate PPE (eye protection, respirator, gloves, gown and enclosed shoes). Adhere to institutional safety regulations.

⚠ CRITICAL: Clidox-S® requires a 10 min contact time to disinfect.

Note: Clidox-S® needs to be prepared 15 min in advance of use. Clidox-S® is effective for 3–4 h.

Note: Clidox-S® should be deactivated using Deactivator reagent, 13 mL per liter of Clidox-S®, prior to disposal.

Note: Clidox-S® is highly corrosive and requires effective cleaning to prevent oxidization damage to surfaces. Use paper towel to absorb any residual Clidox-S®, spray down all surfaces with 80% ethanol to wipe clean.

3. Prepare gas sterilant for items not suitable for autoclaving.
   a. Prepare F10SC, a broad-spectrum disinfectant.

F10SC disinfectant		
Reagent	Final concentration	Amount
F10SC	1:125	4 mL
H_2O	3×	500 mL
Total		500 mL

⚠ CRITICAL: F10SC requires a 10 min contact time to disinfect.

Note: F10SC solution is effective at disinfecting materials for 3–4 months according to the manufacturer.

4. Bag and autoclave the following items in preparation for GF mouse work:
   a. Prepare sterile gowns for research personnel conducting the experiment.
      i. Fold suitably sized gown in large autoclave bag with strings near opening.
      ii. Seal autoclave bag with double fold.
      iii. Place autoclave tape on seal and autoclave.

   Note: Use a single autoclave bag per gown.

   b. Prepare bedding for a single cage.
      i. Place enough bedding material and nesting materials for 1 cage into small brown paper bag.
      ii. Seal bag with double fold.
      iii. Place autoclave tape on seal.
      iv. Place inside another autoclaved bag, double fold, place autoclave tape on seal and autoclave.
   c. Prepare food.
      i. Place a 2 week supply of autoclavable food in a small brown paper bag (e.g., 2 cups chow/cage).
      ii. Seal bag with double fold.
      iii. Place autoclave tape on seal.
      iv. Place inside another autoclave bag, double fold, place autoclave tape on seal and autoclave.
   d. Prepare water.
      i. Fill Duran® membrane screw cap glass bottle or equivalent with drinking water.
      ii. Place autoclave tape on seal and autoclave.
   e. Prepare cages.
      i. Wrap cage base and cage lid separately in an autoclave bag or autoclavable cloth.
      ii. Place autoclave tape on seal and autoclave.
   f. Prepare Teri® wipe (or equivalent).
      i. Place 2 sheets of Teri® wipe or equivalent paper towel per cage into small brown paper bag.
      ii. Seal bag with double fold.
      iii. Place autoclave tape on seal and autoclave.

g. Prepare microcentrifuge tubes.
   i. Label microcentrifuge tubes with experimental number, time point, group and mouse identification number on lid and side of tube.
   ii. Place prelabeled microcentrifuge tubes with the cap open into an autoclave bag.
   iii. Seal with autoclave tape and autoclave.

Note: Our dry autoclave cycle conditions are 122°C for 20 min and 122°C for 40 min for fluid cycle conditions.

5. Bag and gas sterilize the following items in preparation for germ-free work.
   a. Prepare gavage tubing.
      i. Place single tube for each gavage into a small autoclave bag.
      ii. Seal with gas sterilization indicator tape.
      iii. Sterilize with F10SC gas using an atomizer in a biosafety cabinet (BSC).
   b. Gas sterilize blood collection tubes.
      i. Label blood collection tubes with experimental number, time point, group and mouse identification number on lid and side of tube.
      ii. Place prelabeled tubes with the cap open into autoclave bag.
      iii. Seal with autoclave tape and gas sterilize.
6. Directly immerse the following items into Clidox-S™.
   a. Immerse plastic environmental enrichment items such as "teepees" for directly into Clidox-S™.
   b. Place items in a BSC for 10 min of contact time.
   c. Immerse plastic holding containers (for individually holding germ-free mice during procedures) directly into Clidox-S™.
   d. Place items in a BSC for 10 min of contact time.

Note: The BSC must be properly prepared for germ-free work prior to commencing. A 20 min UV decontamination cycle must be performed. Fogging with F10SC sterilant using an atomizer for a 10 min contact time is also highly recommended. All BSC surfaces, and airflow grills need to be wiped down with a Clidox-S® soaked Chux® cloth or equivalent with a 10 min contact time before commencing work.

Note: Have two research personnel available when setting up sterile conditions for germ-free work within the BSC. Person one will work outside of the BSC to pass sterile items to person two who will be performing the germ-free work. Person one will be dressed in appropriate personal protective equipment (PPE) including gown, mask/respirator, hair net, booties, and sterile gloves. Person one will assist person two to put on PPE in a sterile manner to perform the germ-free work. This individual will need to wear a mask/respirator, hair net, booties, a sterile gown and sterile gloves.

Note: To put on sterile gown. Open autoclaved package containing sterile gown. Pull gown out using ties only and place arms into position in gown. Pull gown over shoulders using ties only and tie at base of neck.

7. To put on sterile gloves:
   a. Touching only the packaging, open sterile gloves packet onto bench top (Figure 1).
   b. Open inner package which contains left and right glove.
   c. Place right hand into sterile glove, touching only the inside or "skin side" of the glove with left hand.
   d. Place the left hand into the left sterile glove; right gloved hand may assist by touching only the sterile side of the left glove.
   e. Insert left gloved fingers into the fold on the "sterile" side of the right glove.

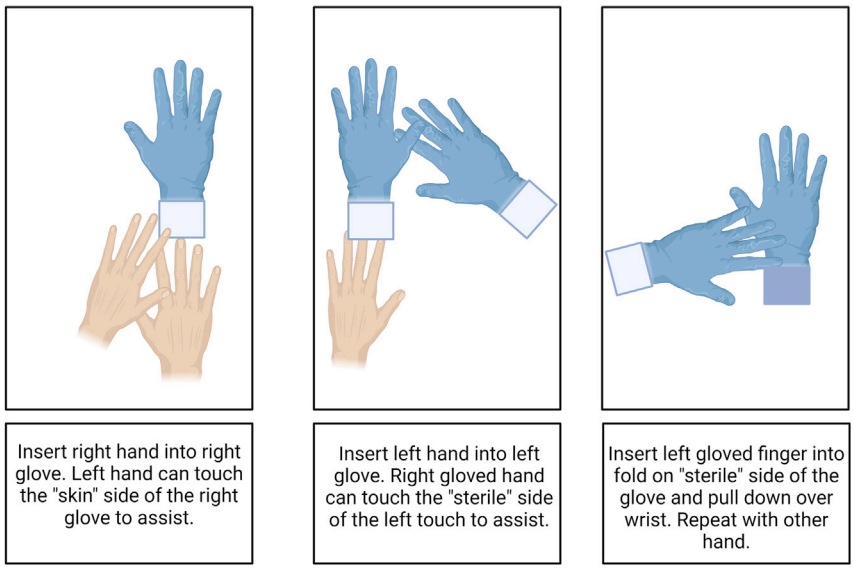

Figure 1. Schematic representation of donning sterile gloves
Created in Biorender.com.

f. Pull the right glove sleeve down over the top of the sterile gown using the sterile glove.
g. Repeat with the other hand.

Note: One researcher can pass materials using sterile gloves into the cabinet and the second person can receive items sterilely. For doubly autoclave bagged items: the research personnel outside the BSC can dip the seal of the outer bag in Clidox®, and open seal at entrance to the BSC and the inner bag should be carefully dropped into the BSC or pulled sterilely into BSC by the second researcher performing the germ-free work. We recommend items which can be opened and dropped directly into the cabinet such as Teri® wipes, cage bases, cage lids, microcentrifuge tubes to be singularly bagged. We recommend that food, bedding, and cage enrichment items are doubly bagged.

Note: For singularly autoclave bagged items: Large items such as cage base and lid can be opened directly into the BSC. Similarly, small items like Teri® wipes can be dropped sterilely into BSC.

Preparation of culturing media

Timing: 1 h preparation time, 18 h incubations

Samples collected from GF mice are cultured on brain heart infusion agar to confirm germ-free status. This agar can be purchased from most suppliers including Oxoid, Sigma-Aldrich, and Thermo Fisher Scientific.

8. Prepare Brain Heart Infusion (BHI) agar plates.
   a. Prepare and autoclave the BHI agar according to manufacturer's instructions, 47 g/L.
   b. Pour approximately 20 mL of BHI agar in bacterial petri dishes and cool.
   c. Store at 4°C until use.

Note: Ideally, the agar plates should be prepared the day before culturing, so that the agar media can cool/set on the plates before use (100 mL = 5 agar plates).

Alternatives: Blood agar or equivalent may be utilized.

Preparation of solutions for immunological assays

Timing: 1 h preparation time

Prepare the following solutions for the immunological assays listed in (Part 3: Assessment of immune response alterations in colonized germ-free mice).

BD Pharm Lyse™ Buffer		
Reagent	Final concentration	Amount
BD Pharm Lyse™	1×	5 mL
H_2O	N/A	45 mL
Total		**50 mL**

Wash Buffer		
Reagent	Final concentration	Amount
RPMI	N/A	500 mL
Fetal bovine serum	~1%	5 mL
Total		**505 mL**

Complete Media		
Reagent	Final concentration	Amount
RPMI	N/A	500 mL
Fetal bovine serum	10%	50 mL
Penicillin-streptomycin	1×	5 mL
MEM non-essential amino acids	1×	5 mL
HEPES	200 μM	5 mL
GlutaMAX™	1×	5 mL
β-Mercaptoethanol	55 nM	500 μL
Total		**570.5 mL**

⚠ CRITICAL: β-mercaptoethanol is a hazardous reagent and must be contained appropriately. Once diluted the working stock can be handled in ordinary cell culture conditions.

9. Prepare the following solutions for the lamina propria extraction.

Note: Each mouse requires 30 mL Media A, 10 mL Media B, 5 mL Media C.

Preparation of Supplemented RPMI		
Reagent	Final concentration	Amount
RPMI	N/A	500 mL
Penicillin-streptomycin	1×	5 mL
GlutaMAX™	1×	5 mL
Total		**510 mL**

Preparation of Media A		
Reagent	Final concentration	Amount
Supplemented RPMI	N/A	30 mL
Fetal bovine serum	3%	900 μL
500 mM EDTA	2 mM	120 μL
Total		**~31 mL**

Preparation of Media B		
Reagent	Final concentration	Amount
Supplemented RPMI	N/A	10 mL
Fetal bovine serum	3%	300 µL
500 mM EDTA	2 mM	40 µL
1, 4- dithiothreitol (DTT)	0.154 mg/mL	1.54 mg
Total		**~10 mL**

Preparation of Media C		
Reagent	Final concentration	Amount
Supplemented RPMI	N/A	30 mL
Fetal bovine serum	3%	900 µL
500 mM EDTA	2 mM	120 µL
Collagenase IV	1 mg/mL	30 mg
DNase I	200 µg/mL	6 mg
Total		**~31 mL**

44% Percoll gradient solutions		
Reagent	Final concentration	Amount
1× PBS	N/A	4.48 mL
Isotonic Percoll	44%	3.52 mL
Total		**8 mL**

⚠ CRITICAL: Isotonic Percoll is required. To make isotonic Percoll, calculate the amount of Percoll needed for the gradient as 9/10 of the solution and add the final tenth using 10× PBS. For example, if 10 mL of Percoll is needed, make isotonic Percoll by adding 1 mL 10× PBS to 9 mL of 100% Percoll. This '100%' isotonic Percoll is used in the amounts stated in tables.

Note: Each mouse requires 8 mL 44% Percoll and 6 mL 66% Percoll.

66% Percoll gradient solutions		
Reagent	Final concentration	Amount
Hank's Balanced Salt solution (HBSS)	N/A	2.04 mL
Isotonic Percoll	66%	3.96 mL
Total		**6 mL**

Note: Make the 44% Percoll solution in 1× PBS and the 66% Percoll solution in HBSS. This allows for a color contrast to assist with interface identification following the spin step.

FACS buffer		
Reagent	Final concentration	Amount
1× PBS	N/A	500 mL
10% BSA solution	0.1%	5 mL
500 mM EDTA solution	10 mM	2 mL
Total		**507 mL**

Perm/Wash buffer			
Reagent	Initial concentration	Final concentration	Amount
BD Perm/Wash buffer	10×	1×	1 mL
H_2O	N/A	N/A	9 mL
Total			10 mL

75% Ethanol Wash			
Reagent	Initial concentration	Final concentration	Amount
Ethanol	100%	75%	7.5 mL
Nuclease-free H_2O	N/A	25%	2.5 mL
Total			10 mL

Suitability of mice for FMT

Timing: >10 days

Our early life gnotobiotic mouse model involves colonization of pups before weaning. The suitability of mice for the model should be considered before commencing these studies.

Note: Our mice are housed under strict gnotobiotic protocols using an ISO-P caging system (Techniplast) with access *ad libitum* to autoclaved commercial food pellets (2018SX Teklad global 18% protein, Envigo) and autoclaved water. Standardized housing conditions were maintained with 12 h day/night cycle as well as regulated temperature and humidity.

10. All animal procedures need to be approved by the Institutional Animal Care and Use Committee. All experiments in this protocol were approved by the South Australia Health and Medical Research Institute Animal Ethics Committee (SAM#20-08).
11. Consider appropriate mice for propagation of FMT material if starting fecal sample is limited. Either male or female germ-free weaners (aged 3–4 weeks) are recommended for use.
12. Consider appropriate female mice for the time-mated aspect of study. Virgin or experienced germ-free dams may be utilized.
13. Consider choice of male mice as studs. Practiced germ-free studs, housed individually for 1–2 weeks prior, should be utilized for timed-mating.

Note: Timed-mated germ-free dams can be requested from your gnotobiotic provider.

Note: If it is not possible to breed germ-free dams in your facility, pregnant dams may be shipped to your location. Germ-free mice are shipped in germ-free shipping containers (available from specialist suppliers such as Taconic) and remain germ-free.

Note: It is best to ship the dams as early into pregnancy as possible to minimize the chance of pregnancy loss, as dams find the shipping and handling process very stressful. By day 14 of pregnancy, it should be possible to confirm which dams are pregnant. Alternatively, the dams and pups can be shipped after birth. We suggest shipping ~7 days after birth of the pups, to minimize any loss of pups.

Note: Germ-free dams and pups should be housed in cages especially designed for gnotobiotic animals (e.g., Techniplast ISO-P cages or alternative) or inside suitable gnotobiotic isolators (e.g., Class Biologically clean (CBC)). If this is not possible, it may be possible to house a small number of mice in conventional IVCs for a short period of time, providing cages are held

in Biosafety cabinets and especially designed protocols to limit contamination, vibration and noise are utilized. See Lange et al. (2019) for more information.[4]

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD8 (clone 53-6.7), 200×	BD Biosciences	Cat#557654; RRID:AB_396769
CD4 (clone RM4-5), 400×	BioLegend	Cat#100527; RRID:AB_312728
CD3 (145-2C11), 200×	BD Biosciences	Cat#562600; RRID:AB_11153670
CD44 (Clone IM7), 200×	BD Biosciences	Cat#559250; RRID:AB_398661
TNFα (Clone MP6-XT22), 40×	Miltenyi Biotec	Cat#130-102-386; RRID:AB_2661141
IFNγ clone (XMG1.2), 400×	BD Biosciences	Cat#554411; RRID:AB_395375
Chemicals, peptides, and recombinant proteins		
Clidox-S activator	PRL Phamacal	Cat#95120F
Clidox-S base	PRL Phamacal	Cat#96125F
Deactivator reagent	PRL Phamacal	Cat#97120F
Brain heart infusion agar	Oxoid via Thermo Fisher Scientific (TFS)	Cat#CM1136B
Dulbecco's 1× PBS	Merck	Cat#D8537-500 mL
Dulbecco's 10× PBS	Merck	Cat#D1408-500 mL
BD Pharm Lyse	BD Biosciences	Cat#555899
RPMI1640	Merck	Cat#R8758
Fetal bovine serum (French origin)	Assay Matrix, Australia	Cat#ASFBS-FR
MEM non-essential amino acids solution (100×)	Thermo Fisher Scientific	Cat#11140050
GlutaMAX™ supplement	Thermo Fisher Scientific	Cat#35050079
HEPES	Thermo Fisher Scientific	Cat#15630080
Penicillin/streptomycin	Gibco via TFS	Cat#15140122
β-Mercaptoethanol	Merck	Cat#M3148-250 mL
UltraPure™ 0.5 M EDTA, pH 8.0	Thermo Fisher Scientific	Cat#15575020
DTT	Merck	Cat#10197777001
Collagenase IV	Gibco via TFS	Cat#17104019
DNase I (for lamina propria extraction)	Roche Diagnostic	Cat#11284932
Percoll	BioStrategy	Cat#GEHE17-0891-01
Hanks' balanced salt solution (HBSS)	Gibco via TFS	Cat#24020117
Glycerol	Merck	Cat#G5516-500 mL
LPS	Merck	Cat#L2880-10MG
Phorbol 12-myristate 13-acetate	Sigma	Cat#P8139-1MG
Ionomycin calcium salt from *Streptomyces conglobatus*	Sigma	Cat#I0634-1MG
"Premium" bovine serum albumin (BSA)	AusGeneX	Cat#PBSA
GolgiPlug protein transport inhibitor	BD Biosciences	Cat#555029
Fixable Viability Stain 700	BD Biosciences	Cat#564997
Mouse BD Fc	BD Biosciences	Cat#423102
TRIzol™ reagent	Thermo Fisher Scientific	Cat#15596026
Chloroform	Merck	Cat#C2432-500 mL
Isopropanol	Merck	Cat#I9516-500 mL
Ethanol	Merck	Cat#1117271000
Glycogen	Thermo Fisher Scientific	Cat#R0561
DNA-free™ kit	Life Technologies	Cat#AM1906
ProtoScript® II First Strand cDNA Synthesis Kit	New England Biolabs	Cat#E6560S
Oligo(dT)$_{12-18}$ Primer	Invitrogen via TFS	Cat#18418012
Deoxynucleotide (dNTP) solution mix	New England Biolabs	Cat#N0447L
SYBR™ Green PCR Master Mix	Applied Biosystems via TFS	Cat#4309155
Critical commercial assays		
TNF alpha mouse uncoated ELISA kit	Invitrogen via TFS	Cat#88-7324-77
Fixation and permeabilization solution	BD	Cat#554722
RNA ScreenTape	Agilent	Cat#5067-5576

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
C57BL/6J germ-free mice (male/female) aged 3–24 weeks, pregnant dams aged 8–24 weeks	Translational Research Institute, QLD, AU	N/A
Oligonucleotides		
Ifng primers	Qiagen	Cat#PPM03121A
Tnfa primers	Qiagen	Cat#PPM03113G
Hsp90 Fwd 5«TTGGTTACTTCCCCGTGCTG -3	Lynn et al.[1]	N/A
Hsp90 Rev 5« GCCTTTTGCCGTAGGGTTTC -3	Lynn et al.[1]	N/A
Gapdh Fwd 5«CTCCCACTCTTCCACCTTCG -3	Lynn et al.[1]	N/A
Gapdh Rev 5«GCCTCTCTTGCTCAGTGTCC -3	Lynn et al.[1]	N/A
Hrpt Fwd 5«CTGGTGAAAAGGACCTCTCGAAG -3	Lynn et al.[1]	N/A
Hrpt Rev 5«CCAGTTTCACTAATGACACAAACG-3	Lynn et al.[1]	N/A
Software and algorithms		
GraphPad Prism 7.01	GraphPad Software Inc	version 7.01 https://www.graphpad.com
FlowJo software	Tree Star	version 10.6.1 https://www.flowjo.com
Other		
Stomacher 80 Biomaster	Seward	Cat#0080/000/AJ
Stomacher 80 Biomaster Strainer Bags	Thermo Fisher Scientific	Cat#SEWBA6040/STR
ISO-P caging system	Techniplast	Cat#1S030P
2018SX Teklad global 18% protein	Envigo	Cat#2018SX
Hungate tubes	Bellco	Cat#2047-16125
Plastic feeding tubes (22 ga × 25 mm)	Walker Scientific via Able Scientific	Cat#FTP-22-25
Microvette 500 EDTA KE3	Sarstedt	Cat#20.1341.100
Atomizer	Spraying Systems Co.	Cat#FDS-MPT2
Autoclave	Thermo Fisher Scientific	Cat#Tuttnauer 44, Cat# 4472
Anaerobic chamber	Coy	Cat#100115

MATERIALS AND EQUIPMENT

Alternatives: In this protocol, Clidox-S® (Pharmacol Research Laboratories) is used to sterilize materials and equipment for germ-free work. A suitable alternative is DEC-SPORE 200 Plus (Veltek). In this protocol we recommend F10SC, a veterinary grade disinfectant for use. Alternatively, Viraclean® (Whiteley), a hospital grade disinfectant, could be used.

Alternatives: This protocol utilizes the Stomacher 80 Biomaster (Seward) and compatible Stomacher 80 Biomaster Strainer Bags. Alternative paddle blenders include models by Seward and models such as the BagMixer® (Interscience).

Alternatives: This protocol utilizes the ISO-P™ caging system (Techniplast). Alternatives include small gnotobiotic isolators of any type including Mini-Q™ (Park Bioservices) and semi-rigid and flexible film isolators (Charles River).

STEP-BY-STEP METHOD DETAILS

Part 1: Prepare FMT

Timing: 2 h

Here, steps needed to prepare a microbiome transplant material (FMT) are detailed.

Table 1. Table of glycerol and PBS needed for FMT per fecal weight

Reagent						
Fecal sample	<1 g	1 g	2 g	3 g	4 g	5 g
1× PBS	1.3 mL	2.6 mL	5.2 mL	7.8 mL	10.4 mL	13 mL
30% glycerol	1.3 mL	2.6 mL	5.2 mL	7.8 mL	10.4 mL	13 mL
Total	**2.6 mL**	**5.2 mL**	**10.4 mL**	**15.6 mL**	**20.8 mL**	**26 mL**

1. Collect fecal samples from mice with microbiota of interest, at timepoint of interest.
   a. Place each mouse individually onto a sterilized cage base where, the mouse will spontaneously defecate.
   b. Pick up the fecal sample using a sterile toothpick/tip and place into a pre-labeled 2 mL sterile microcentrifuge tube.
   c. Place collected sample directly onto dry ice.
   d. Replace mouse into cage.
   e. Sterilize the cage base and gloved hands with an appropriate reagent (i.e., 70% ethanol/F10SC) to sterilize.

   Note: A light scruff will encourage any mouse not spontaneously defecating to do so.

   Note: Two or more fecal samples should be collected for each individual mouse at each timepoint of interest.

   ⚠ CRITICAL: Fecal samples must be placed on dry ice immediately to preserve the integrity of the sample.

   ⏸ **Pause point:** Fecal samples may be stored at −80°C until required.

2. Prepare FMT material.
   a. Identify fecal samples of interest to use in the FMT preparation.
   b. Weigh a sterile microcentrifuge tube.
   c. Place the fecal samples inside the pre-weighed tube using a disposable sterile toothpick or tip.
   d. Re-weigh the tube, noting the weight of the fecal sample.

   Note: 100 μg of fecal material is enough to gavage a single mouse twice. Use an appropriate amount of fecal material for the FMT preparation based on number of animals and number of repeat gavages.

   e. Transfer fecal pellets, autoclaved Hungate tubes and rubber seals, disposal transfer pipettes, stomacher bags, screw cap microcentrifuge tubes to the anaerobic chamber via port.
   f. Sterilize gloved hands inside the chamber.
   g. Place stomacher bag onto provided stomacher stand (purchased with stomacher).
      i. Hold bag open using forceps (or similar) and drop fecal material in between the stomacher bag lining, to the bottom of the stomacher bag.
      ii. Add appropriate volume of 1× PBS and 30% glycerol based on weight of fecal sample as listed in Table 1 to the bottom of the stomacher bag using disposal Pasteur pipettes.
      iii. Turn stomacher to the highest setting for 120 s.
      iv. Remove stomacher bag, and manually ensure that all material has been homogenized or repeat process until material is fully homogenized.
   h. Use a disposal Pasteur pipette to remove homogenized material from the outside of the mesh lining of the stomacher bag and dispense into Hungate tubes.
   i. Seal the Hungate tube without touching the rubber seal.

i. Unwrap the rubber seal (only touching aluminum foil).
ii. Place on top of Hungate tube and use screw lid to secure tightly into position.

Note: A small portion of the FMT material can be placed in a screw top microcentrifuge tube for DNA extraction and subsequent 16S rRNA gene sequencing.

⚠ CRITICAL: Everything needs to be sterile for the preparation of an uncontaminated FMT. Place clean gloves over the gloved hands on the inside of the anaerobic chamber before use. Use single-use disposal items only. Aliquot all reagents inside a biosafety cabinet to ensure sterility and place solutions for removal of oxygen into sterile containers with filter lids when equilibrating.

Pause point: FMT material can be stored at −80°C until required. Studies have shown that samples stored in this way are stable to at least 6 months.[5]

Part 2: Propagation of colonization material

Timing: 3–5 days (for step 3)

Timing: 1 d (for step 4)

Here, enough material for a large germ-free colonization study is prepared. A microbiome transplant material (FMT) prepared from limited fecal samples (Part 1, Prepare FMT #1), is used to colonize a small number of germ-free mice to generate a larger amount of FMT material.

3. Order germ-free mice from your gnotobiotic facility.
   a. Order 2 or more germ-free mice from your gnotobiotic facility for each microbiota that is required.
   b. House germ-free mice for different microbiota in separate ISO-P cages.

⚠ CRITICAL: Germ-free mice should be handled using specially designed germ-free protocols.

Note: It is possible to house mice in small isolators for this propagation, however, a single isolator is required for each microbiota of interest. Using ISO-P cage can be a more efficient way to conduct these types of studies.

Note: It is possible to colonize additional mice in the initial colonization if more gavage material is needed for subsequent studies.

   c. Prepare biosafety cabinet for procedure.
   d. Prepare Clidox-S® (before you begin #2).
      i. Immerse all items needed for unpacking and gavaging mice.
      ii. Immerse notching device (if required), mouse transport cages, ISO-P cages for (re-housing mice), autoclaved materials (before you begin #4–6); microcentrifuge tubes (for fecal sample collection), enrichment, bedding, nesting materials, single layer autoclavable food, holding box, Teri-wipe, gavage material, needle (19–25 gauge), 1 mL syringe, disposable gavage tubing in Clidox-S®.
      iii. Place into BSC using sterilized gloves.

Note: Moisture sensitive items are double bagged, where the outer is opened and the inner bag containing the moisture sensitive item is dropped sterilely into the BSC.

e. Wait 10 min before commencing work.

Note: Clidox-S® requires a 10 min contact time to sterilize items.

f. Dress for commencing germ-free work (before you begin #2–6).

Optional: Sterile gloved hands maybe be dipped into Clidox-S® (10 min contact time) as an additional precaution before commencing unpacking and gavage.

g. Unpack mice into ISO-P cages.
   i. Set up ISO-P cages with appropriate amounts of bedding and enrichment.
   ii. Open transport container and remove germ-free mice and place into holding container with a piece of sterile Teri® wipe for enrichment.

h. Prepare gavage material.
   i. Extract colonization material from Hungate tube using needle and 1 mL syringe.
   ii. Remove needle from syringe and place gavage tube in place.

i. Perform gavage.
   i. Place mouse on wire rack of cage and scruff.
   ii. Insert gavage needle into mouse's mouth and perform oral gavage procedure as per animal ethics approved SOP.
   iii. Repeat for entire group of mice.
   iv. Once complete, check each mouse for recovery and place inside the ISO-P cage.

j. Finish packing ISO-P cage.
   i. Fill water bottles with autoclaved mouse drinking water and place in position on wire rack.
   ii. Dispense food pellets into position on wire rack.
   iii. Close lid.
   iv. Place ISO-P cage on mouse ISO-P mouse rack.

Optional: Mice may be ear notched at this time, if appropriate. Fecal samples or swabs may be collected from transport boxes (to ensure the mice are germ-free upon unpacking).

Note: A second or third colonization may be performed 2–7 days apart if inoculation material is diverse in nature as additional time may be needed to establish the less abundant bacterial species.

4. Assessment of germ-free status of mice.
   Confirm germ-free status of germ-free mice prior to inoculation with FMT.
   a. Collect fecal pellet from germ-free mouse (Part 1, prepare FMT**#1**) using sterile toothpick or Teri® wipe into sterile pre-labeled microcentrifuge tube.

Pause point: Fecal pellet can be stored at −80°C until required.

   b. Prepare and culture fecal slurry to confirm the germ-free status of mice.
      i. Add 500 µL of sterile anaerobic 1× PBS to fecal pellet using a disposal Pasteur Pipette.
      ii. Vortex for 1–2 min.
      iii. Prepare 1 in 2 serial dilutions in 1× PBS in a 96 well plate.
      iv. Plate 1 drop (20 µL) from each dilution onto an agar plate.
      v. Include 1× PBS reagent control when plating out.
      vi. Incubate for 18 h in anaerobic conditions at 37°C.

Note: Samples are considered germ-free if no colonies are formed on plates incubated for 48 h or more.

Alternatives: Extract collected fecal material collected using the DNA extraction protocol outlined in another STAR Protocol from our laboratory entitled "Protocol to assess the impact of early life antibiotic exposure on murine longevity". Perform 16S rRNA gene qRT- PCR to confirm germ-free status as directed in this protocol.

Part 2b: Glycerol preparation II

Timing: 2 h

Colonized mice are humanely culled at designated timepoint (5–7 days post inoculation) and cecal contents are prepared with glycerol to make fecal microbiome transplant material.

5. Prepare fecal microbiome transplant (FMT) from colonized germ-free mice.
   a. Humanely cull colonized mice as per animal ethics approved SOP.
   b. Collect whole intestine including ceca in 15 mL tube containing 10 mL 30% anaerobic glycerol.
   c. Transfer tube containing intestines, pre-weighed 50 mL tube, sterile petri dishes, scalpel, autoclaved Hungate tubes and lids, disposal transfer pipettes, stomacher bags, and screw cap microcentrifuge tubes to the anaerobic chamber.
   d. Sterilize gloved hands inside the anaerobic chamber.
   e. Prepare ceca for collection of cecal contents.
      i. Empty tube containing mouse intestines into a petri dish.
      ii. Using scalpel cut off rest of intestine and place ceca into fresh petri dish.
      iii. Dispose of glycerol solution and rest of intestines.
      iv. Using scalpel, cut down length of ceca and scrape cecal contents away from ceca membrane and transfer into pre-weighed 50 mL tube.
      v. Repeat with remaining ceca.
      vi. Re-weigh the tube now containing all ceca and note weight of the ceca contents.
      vii. Place ceca contents into stomacher bag.
      viii. Add glycerol and 1× PBS based on ceca weight and continue with protocol as outlined Prepare FMT #1.
      ix. Collect 0.5 mL of homogenized material in screw cap microcentrifuge tube for 16S rRNA gene sequencing.

Note: Aliquot colonization material in appropriate volumes for initial and subsequent gavages. i.e., 50 μL of colonization material is needed to colonize mice less than 14 days old, whilst 100 μL is needed for mice aged ~21 days or 200 μL for mice aged over 4 weeks of older in line with recommended maximum volumes of 10 mL/kg body weight. Noting that recommended volumes may differ substantially depending on body weight in different mouse strains of the same age. Furthermore, depending on the complexity of the gavage material, up to 2–3 gavages/mouse may be required.

Pause point: Homogenized fecal/cecal material for FMT can be stored at −80°C until required.

Optional: Assessment of the composition of the material for FMT via 16S rRNA gene sequencing may be carried out prior to commencing experiments.

Extract cecal contents collected in screw cap tube during glycerol preparation using the DNA extraction protocol outlined in STAR Protocols entitled "Protocol to assess the impact of early life antibiotic exposure on murine longevity #10".

Perform 16S rRNA gene sequencing as directed in the same protocol, " #12".

Part 2: Colonize infant germ-free mice with prepared FMT material

Timing: ~6 weeks

Now that the material for colonization has been propagated and a sufficient amount of FMT material has been prepared, we can perform larger scale experiments using the FMT material.

6. Order pregnant dams from your germ-free facility.

Note: Request 2–4 GF dams per microbiota community type of interest depending on your experimental needs and to account for loss of pups.

   a. Prepare BSC to unpack pregnant dams as described (part 2: propagation of colonization material #3).
   b. Set up ISO-P cages with appropriate amounts of bedding and enrichment.
   c. Open transport container and remove germ-free mice and place into prepared ISO-P cage.
   d. Finish packing ISO-P cage.
      i. Fill water bottles with autoclaved drinking water.
      ii. Place in position on wire rack.
      iii. Dispense autoclaved food pellets into position on wire rack.
      iv. Close lid.
      v. Place ISO-P cage on ISO-P rack.
   e. Collect fecal samples from dams to confirm germ-free status of mice as described (Part 1, Prepare FMT #1).

Note: Pregnant germ-free dams, aged 8–24 weeks, may be housed singularly or two pregnant dams to a single cage. If co-housing, try to co-house dams that plugged on the same day. Co-housing pregnant dams may reduce stress and may help prevent loss of pups.

Note: If pregnant germ-free dams are first time parents or stressed in any way, there may be excessive loss of pups. On occasion we have experienced loss of pups from all but one or two litters following multiple pregnancies.

7. Monitor for birth of pups.
   a. Note date of birth and number of pups born.

Note: C57BL6/J pups are born at approximately day 19.5 of pregnancy. The gestation period for other strains may differ slightly.

8. Colonize germ-free mice.
   a. Prepare biosafety cabinet for procedure (before you begin #2–6).
      i. Immerse all items needed for gavaging mice in Clidox-S®; ISO-P cages, holding box, sterile Teri® wipe, gavage material, needle, 1 mL syringe, disposable gavage tubing and place into BSC using sterilized gloves.
   b. Prepare gavage material.
      i. Collect 50 µL of colonization material for each pup to be colonized using needle (19–24 gauge) and 1 mL syringe (if colonizing day 14 pups).
      ii. Remove needle and attach gavage tube to syringe.
   c. Perform oral gavage.
      i. Place mouse on wire rack of cage.
      ii. Scruff and insert gavage needle into mouse's mouth.
      iii. Perform oral gavage procedure as per AEC approved SOP.
      iv. Once complete, place mouse into designated ISO-P cage.

Note: Consider the appropriate age of mice for your experimental model. Pups can be successfully oral gavaged from approximately day 4–7 after birth. Use 50 μL of gavage material for mice aged 7–21 days, use 100 μL of gavage material for mice aged 21–28 days and up to 200 μL of gavage material for mice aged > 4 weeks as per AEC approved SOPs.

Note: To colonize mice from day 0 of life, perform oral gavage on dams. The inoculated microbiota will be effectively transferred to the pups. Use isoflurane to anesthetize dams whilst performing the oral gavage procedure. This will result in less stress for dams and should result in less loss of pups.

Optional: Collect fecal sample from mice prior to gavage to confirm colonization status.

Part 3: Assessment of immune responses alterations in early life colonized germ-free mice

Timing: 10 min per mouse

This part of the protocol describes how to assess the impact of colonization of GF mice with specific microbiota community types of interest on the weaning reaction, however, you may wish to assess other parameters/phenotypes following colonization. In our published study,[1] mice were humanely culled at day 21 of life following colonization on day 14 of life.

9. Humanely kill mice as per AEC approved SOP and collect organs as outlined below.
   a. Humanely kill mice as per approved SOP.
   b. Perform cardiac puncture to collect blood and place blood into microvette EDTA blood collection tubes.
   c. Sterilely collect spleen into 1.5 mL microcentrifuge tube containing 500 μL of 1× PBS and place on ice.
   d. Remove intestine.
      i. Isolate 20 mm section directly above ceca (ileum).
      ii. Clear any feces.
      iii. Place in 1.5 mL microcentrifuge tube.
      iv. Snap-freeze using dry ice or liquid nitrogen.
      v. Isolate small intestine as a single unit from stomach to ceca.
      vi. Place in petri dish containing Media A for lamina propria extraction.

Part 3a: Blood stimulations

Timing: 1–2 h, plus 18 h stimulation

To assess whether colonization with the transplanted microbiota induces differential immune responses in peripheral blood, blood is stimulated with Lipopolysaccharide (LPS) and the resultant cytokine responses (TNFα) are measured via ELISA.

10. Perform red cell lysis on blood and set up stimulations as outlined below.
    a. Aliquot 200 μL peripheral blood into 96 well tissue culture plate.
    b. Spin down at 350 × *g* for 5 min at 4°C.
    c. Discard supernatant.
    d. Incubate pellet with 100 μL 1× BD Pharm Lyse™ buffer for 60 s.
    e. Stop reaction with 200 μL 1× Wash buffer.
    f. Spin down at 350 × *g* for 5 min at 4°C and discard supernatant.
    g. Resuspend pellet in 300 μL 1× Wash buffer.
    h. Spin down at 350 × *g* for 5 min at 4°C and discard supernatant.
    i. Resuspend in 200 μL Complete media.

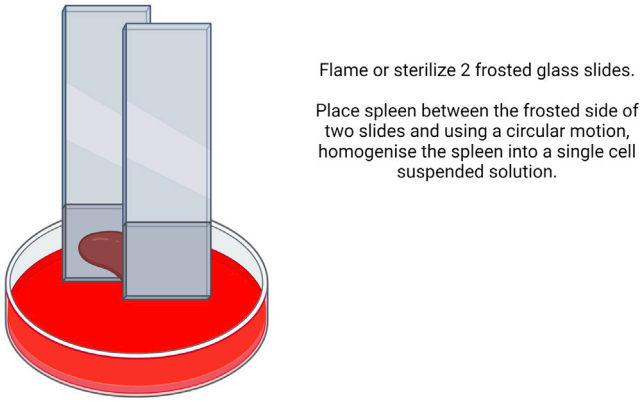

Figure 2. Schematic of manual homogenization
Created in Biorender.com.

j. Plate out 100 μL for each condition (stimulated/unstimulated).
k. Stimulate with 100 μL of 10 ng/mL LPS for 18 h in an incubator at 37°C, 5% CO_2.

Note: Store reconstituted 1× BD Pharm Lyse and 1× Wash buffer solution at 4°C for up to 4 weeks.

Pause point: Collect supernatant and store at −80°C.

Part 3b: Splenocyte stimulations

Timing: 2–4 h, plus 18 h stimulation

To assess whether colonization with the transplanted microbiome induces systemic differential immune responses, splenocytes are stimulated with Lipopolysaccharide (LPS) and the resultant cytokine responses (TNFα) are measured via ELISA.

11. Prepare single cell suspension from isolated spleen and stimulate with LPS as outlined below.
    a. Place single spleen into a well of a 6-well tissue culture plate with 3 mL of 1× Wash buffer.
    b. Perform manual homogenization with rough side of 2 sterile frosted glass slides (Figure 2).
    c. Filter homogenized suspension through 80 μM filter into sterile 10 mL tube to achieve single cell suspension.
    d. Spin down at 350 × *g* for 5 min at 4°C and discard supernatant.
    e. Perform red cell lysis with 1 mL of 1× BD Pharm Lyse™ buffer for 90 s.
    f. Stop with 4 mL of Wash buffer.
    g. Spin down at 350 × *g* for 5 min at 4°C and discard supernatant.
    h. Resuspend cells in 1 mL 1× Wash buffer.
    i. Bring volume to 5 mL with 1× Wash buffer.
    j. Spin down at 350 × *g* for 5 min at 4°C and remove supernatant.
    k. Repeat wash.
    l. Resuspend cells in 5 mL of 1× Wash buffer.
    m. Perform cell count using hemocytometer.
    n. Resuspend cells at 2 × 10^7 cells/mL in complete media.
    l. Stimulate cells with 100 μL 10 ng/mL LPS for 18 h in an incubator at 37°C, 5% CO_2.
    m. Include unstimulated control (100 μL cells at 2 × 10^7 cells/mL and add 100 μL of complete media).

Pause point: Collect supernatant and store at −80°C.

Part 3c: ELISA

Timing: 4 h, plus 18 h incubations

Supernatant was collected from whole blood and splenocytes following stimulation with LPS. TNFα levels were assessed by ELISA using the TNF alpha Mouse Uncoated ELISA kit as per manufacturer's instructions (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FMAN0017423_88-7324_MoTNFalpha-ELISA_PI.pdf).

Part 3d: Lamina propria extraction, stimulations and analysis

Timing: 2 d (for step 12)

Timing: ~4 h, 18 h fixation (for step 13)

To assess whether colonization with the transplanted microbiome induces differential immune responses in lamina propria, extracted cells are stimulated with PMA/ionomycin and the resultant intracellular expression of TNFα and IFNγ are measured via intracellular cytokine staining.

Note: Prepare buffers the day prior to the procedure (before you begin) and store at 4°C. Reconstituted medias can be stored at 4°C for up to 4 weeks. Percoll gradient solutions can be stored for 24 h at 4°C. Add the DTT, collagenase and DNase I on day of the procedure.

Note: Warm up Media A, Media B and Percoll solutions to 20°C–25°C. Warm Media C to 37°C (via water bath) and keep Supplemented Media at 4°C.

12. Perform Lamina propria extraction.
    a. Place small intestine on bench protector or equivalent.
    b. Remove all mesenteric fat, fat and Peyer's Patches from small intestine using scissors or scalpel.
    c. Open longitudinally and remove fecal material.
    d. Clean mucus and feces by gently scraping and flush with 1 mL of Media A.
    e. Place cleaned intestines into a new tube with 10 mL Media B.
    f. Cut intestines into ~1cm pieces and place in a 12 well plate with 3 mL of Media A on ice.

    Note: If working with multiple mice, prepare all intestines until step 12e then proceed.

    g. Incubate intestinal sections in orbital shaker at 150 × *g* for 10 min at 37°C.

    Note: Incubate tubes horizontally in orbital shaker for better results.

    h. Filter solution through 100 μM filter and spin down at 600 × *g* at 10 min at 4°C and remove supernatant to collect epithelial layer.
    i. Place filter containing tissue pieces onto a waste tube and wash with 5 mL of cold 1× PBS.

    Note: This step is used to wash away EDTA than can inhibit digestion of tissue.

    j. Transfer intestinal sections (located on top of 100 μM filter) into 1.5 mL microcentrifuge tube and add 1 mL Media C.
    k. Cut intestines into 1 mm sections.

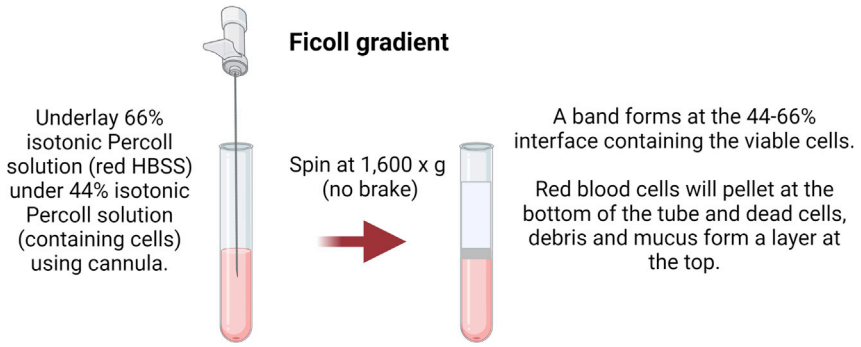

Figure 3. Schematic of sterile Percoll gradient preparation
Created in Biorender.com.

Note: Using scissors, roughly chop intestines until the desired intestinal length has been achieved.

l. Transfer intestines to a 15 mL tube.
m. Add 4 mL of Media C and incubate (horizontally) at 37°C in an orbital shaker at 150 × *g* for 20 min.
n. Filter suspension through a 70 μM filter into a 50 mL tube.

Note: The flow through will contain the lamina propria cells. Neutralize the flow through with 10 mL of media A.

Note: Repeat digest (k - m) until all tissue pieces have been dissolved. It usually requires 2–3 repeats to dissolve all tissue. Flow through from each subsequent digestion can be added to the 50 mL tube with the previous digestion and neutralized. The plunger of a syringe can help to manually push through remaining tissue.

o. Spin down at 600 × *g* at 10 min at 4°C and discard supernatant.
p. Add 1 mL of 44% gradient Percoll and resuspend cells.
q. Transfer to a new 15 mL tube and add 7 mL of 44% Percoll to a total of 8 mL of 44% Percoll gradient in a 15 mL tube.
r. Using blunted needle/cannula slowly underlay with 5 mL 66% isotonic Percoll solution (Figure 3).
s. Spin at 1,600 × *g* for 20 min at 4°C with no brake.

⚠ CRITICAL: Use of brake disrupts Percoll gradient interface.

Note: A band forms at the 44–66% interface containing the viable cells. Red blood cells will pellet at the bottom of the tube and dead cells, debris and mucous form a layer at the top.

t. Collect 2 mL of the opaque layer at the 44–66% interface and place into 50 mL tube.
u. Fill the 50 mL tube with 1× PBS (adding up to 48 mL of cold 1× PBS).
v. Spin at 600 × *g* for 10 min at 4°C and remove supernatant.
w. Resuspend cells in 100 μL Complete media.

13. Stimulate and stain lamina propria and measure intracellular IFNγ and TNFα using flow cytometry.

In this section isolated lamina propria are stimulated with PMA/ionomycin and stained to measure IFNγ and TNFα expressing $CD4^+$ and $CD8^+$ T cells using flow cytometry.

a. Stimulate 100 μL of isolated lamina propria cells with 100 μL of PMA/ionomycin stimulation solution for 4 h at 37°C.

PMA/ionomycin stimulation reagents			
Reagent	Initial concentration	Final concentration	Amount
Complete Media	N/A	N/A	10 mL
PMA	100 mg/mL	50 ng/mL	5 μL
Ionomycin	500 mg/mL	500 ng/mL	10 μL
Brefeldin A	1000×	1×	10 μL

b. Spin down cells in 96 well round bottom plate at 350 × *g* for 5 min at 4°C and remove supernatant.
c. Resuspend cell pellet in 200 μL of FACS buffer.
d. Spin down at 350 × *g* for 5 min at 4°C.
e. Prepare surface stain (Table 2).
f. Stain cell pellets in 30 μL staining solution/well.
g. Incubate in dark on ice for 30 min.

Note: Dilution factors for each antibody listed have been deduced from titration assays in our lab. Antibodies should be titrated for use in each lab as catalogues or equipment availability may vary from lab to lab.

h. Add 200 μL of FACS buffer and spin down at 350 × *g* for 5 min at 4°C and remove supernatant.
i. Resuspend cells in 80 μL BD Fixation/Permeabilization buffer.
j. Incubate cells in dark on ice for 30 min.
k. Spin down at 350 × *g* for 5 min at 4°C and remove supernatant.
l. Resuspend in 150 μL of 1× BD Perm/Wash buffer.
m. Spin down at 350 × *g* for 5 min at 4°C and remove supernatant.
n. Resuspend in 30 μL of intracellular stain.
o. Incubate cells in dark on ice for 30 min.

⚠ CRITICAL: Prepare intracellular stain in 1× Perm/Wash buffer.

p. Add 100 μL of 1× BD Perm/Wash buffer and spin down at 350 × *g* for 5 min at 4°C and remove supernatant.

Table 2. Table of stains for flow cytometry

Reagent	Fluorophore	Dilution factor	Amount
Surface staining			
FACS buffer	N/A	N/A	500 μL
Fc block	N/A	200×	2.5 μL
Fixable Viability Dye 700	700 nm	500×	1 μL
CD44	APC	200×	2.5 μL
CD8	BUV395	200×	2.5 μL
CD3e	PeCy7	200×	2.5 μL
CD4	PE/CY7	400×	1.25 μL
Intracellular staining			
Permeabilization buffer	N/A	N/A	500 μL
IFNy	FITC	400×	1.25 μL
TNFα	PE	40×	12.5 μL

q. Resuspend in 200 μL FACS buffer and spin down at 350 × *g* for 5 min at 4°C and remove supernatant.
r. Resuspend cells in 120 μL FACS buffer.
s. Run samples on flow cytometer.

Note: We used the BD FACSymphony™ to acquire our data.

Part 3e: Cytokine measurement in ileum

Timing: 2–3 days (for step 14)

Timing: 35 min (for step 15)

Timing: 35 min (for step 16)

Timing: 2 h (for step 17)

Timing: 2 h (for step 18)

To assess whether colonization with the transplanted microbiota induces differential immune responses in ileum, RNA was extracted from ileum and gene expression of *Tnfa* and *Ifng* were measured via qRT-PCR.

14. Prepare RNA from small intestine using TRIzol™ (Thermo Fisher Scientific) in accordance with manufacturer's instructions (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2Ftrizol_reagent.pdf).

Note: For best results autoclave mortar and pestle and place at −80°C for 18 h before use.

a. Place 20 mm section of ileum in mortar and grind into powder using pestle on dry ice.
b. Scrape powder into 1.5 mL microcentrifuge tube.
c. Resuspend powder in 500 μL of TRIzol™.
d. Incubate at 20°C–25°C for 5 min.

Note: Vortex RNA powder and TRIzol™ until suspension is prepared.

e. Add 200 μL of chloroform and shake vigorously for 15 s.
f. Spin at 12,000 × *g* for 15 min at 4°C to separate aqueous and organic layer.
g. Collect upper aqueous layer (containing RNA) into fresh 1.5 mL microcentrifuge tube.

Note: Hold microcentrifuge tube at 45° angle and collect upper layer. Be careful not to disturb interphase.

h. Add 250 μL of isopropanol, and 0.5 μL of glycogen and invert/vortex briefly (1–2 s) to mix.
i. Incubate at 20°C–25°C for 10 min.
j. Spin at 15,000 × *g* for 15 min at 4°C and remove supernatant.
k. Add 500 μL of freshly prepared 75% ethanol and invert.
l. Spin at 15,000 × *g* for 10 min at 4°C, remove supernatant and dry pellet.
m. Resuspend in 20 μL nuclease-free H_2O.

Note: To quickly dry RNA pellet, remove 480 μL of supernatant, spin again for 1 min at 15,000 × *g* and remove the residual solution with 20 μL tip.

Table 3. Preparation of reagents for DNase I treatment

Reagent	Final concentration	Amount
RNA	N/A	20 μL
10× DNase I buffer	1×	2 μL
DNase I		1 μL
Total	**N/A**	**23 μL**

Pause point: RNA may be stored at −80°C until next step.

15. DNase I treat extracted RNA.

Treat RNA samples with DNA-free™ kit (Ambion via Thermo Fisher Scientific) as per manufacturer's instructions (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2Fcms_055739.pdf).

a. Incubate 20 μL of RNA with 2 μL of DNase I buffer and 1 μL of DNase I (Table 3).
b. Incubate at 37°C for 30 min.
c. Add 4 μL of DNase inactivation reagent and incubate for 2 min, mixing regularly.
d. Spin down at 12,000 × *g* for 2 min and place treated RNA into fresh microcentrifuge tube.

Pause point: RNA may be stored at −80°C until next step.

16. Assess RNA quality and quantity using TapeStation (Agilent).

RNA samples are prepared as per manufacturer's instructions.

a. Assess 1 μL of RNA on the TapeStation to assess RNA quality (RNA integrity number, RIN) and quantity.

Note: If RIN value is less than 6, the RNA quality may be insufficient to proceed with qRT-PCR.

Pause point: RNA may be stored at −80°C until next step.

17. Make cDNA from the extracted RNA.

Make cDNA from the extracted RNA using the Protoscript® II first strand cDNA synthesis kit (New England Biolabs) as per manufacturer's instructions (https://www.nebiolabs.com.au/protocols/2016/04/26/first-strand-cdna-synthesis-standard-protocol-neb-m0368).

a. Mix 1 μg of RNA with buffer, Oligo dTs, dNTPs, as per manufacturer's instructions (Table 4).
b. Denature RNA on a heating block at 65°C for 5 min and incubate with Protoscript® II Reverse transcriptase, DTT buffer at 42°C for 60 min and 65°C for 20 min a per instructions (Table 5).

Pause point: cDNA may be stored at −20°C until next step.

18. Measure *Tnfa* and *Ifng* gene expression in lamina propria via qRT-PCR.

Table 4. Preparation of reagents for cDNA synthesis

Reagent	Final concentration	Amount
RNA	1 μg	Up to 12 μL
5× ProtoScript® II Reverse transcriptase buffer	1×	4 μL
Oligo $(dT)_{12-18}$ primer	0.5 μg	1 μL
dNTP	10 mM	1 μL
DTT	200 mM	2 μL
ProtoScript® II Reverse Transcriptase	2.5 units	1 μL
Nuclease-free H_2O	N/A	Bring up to 20 μL
Total	**N/A**	**20 μL**

Table 5. PCR cycling conditions

Steps	Temperature	Time
Initial Denaturation	65°C	5 min
Incubation	42°C	60 min
Extension	65°C	20 min

Measure *Tnfa* and *Ifng* gene expression in lamina propria via qRT-PCR. Calculate *Tnfa* and *Ifng* relative expression to the house-keeping genes e.g., *Gapdh*, *Hprt* and *Hsp90*.

Note: Calculate the amount of master mix components needed, multiplying volume by total number of reactions for each primer set (samples, controls and standards in duplicate and a few extra for pipetting errors, etc).

Note: In a template-free room, prepare the master mix solution for each primer set separately consisting of SYBR green PCR master mix, forward and reverse primer in a sterile 1.5 mL microfuge tube and keep on ice (Tables 5 and 6).

a. Combine master mix solution for each primer set separately (Tables 5 and 6).
b. Separately, prepare 1 in 10 dilutions of cDNA.
c. Add 3 μL diluted cDNA onto a PCR plate (96/384 depending on setup) first to the bottom of the well.
d. Add 7 μL primer specific master mix solution to each well.

⚠ CRITICAL: Use barrier tips to ensure no mixing of DNA.

Note: Load DNA first to the bottom of each well. Add master mix second to the side of the well. Then you can visualize that each well has been correctly loaded.

e. Include a no template DNA control (NTC), where DNA is substituted for 3 μL nuclease-free H_2O for each primer set.

Note: Combine 2 μL of each diluted sample in 1.5 mL microcentrifuge tube to create top standard for a standard curve. Create successive 1 in 4 serial dilutions. Repeat for 5 dilutions.

f. Briefly spin the PCR plate at 200 × *g* for 10 s to remove any air bubbles.
g. Load plate using following settings (Table 7).
h. Calculate the relative fold gene expression changes as described (Part 4: Calculate the relative fold gene expression of *Tnfa* and *Ifng*).

EXPECTED OUTCOMES

Fecal material prepared from limited fecal samples is faithfully propagated in a small germ-free experiment to generate enough FMT material for a larger scale germ-free colonization experiment.

Table 6. Preparation of reagents for qRT-PCR

Component	Volume
SYBR™ Green PCR Master Mix	5 μL
2 μM of forward primer	1 μL
2 μM of reverse primer	1 μL
cDNA template	3 μL
Total	10 μL

Table 7. PCR cycling conditions for qRT-PCR to assess *Tnfa* and *Ifng expression*

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	10 min	1
Denaturation	95°C	15 s	40 cycles
Annealing & Extension	60°C	60 s	
Hold	4°C	forever	

For instance, 16S rRNA gene sequencing reveals faithful propagation of two different low-diversity microbiota community types (Figure 4), which we referred to as post-antibiotic microbiota I (PAM I) and PAM II in our original study.[1] There appeared to be a very high level of congruence between the composition of the FMT material and that which was detected in the FMT recipient mice at day 21. At a detection threshold of 0.1% relative abundance there was a 100% match for the 16S rRNA gene sequences detected in both the PAMI and PAMII material and their corresponding GF+FMT mice.

Using the protocols described herein, blood and spleen derived immune cells from differentially colonized mice (GF, GF+PAM I, GF+PAM II and SPF) exhibited altered cytokine responses to LPS stimulation (Figure 5).

Using the protocols described herein, it is expected that detection of differential expression of key inflammatory immune markers, IFNγ and TNFα. SPF have significantly increased IFNy expression in lymphoid cells and *Ifng* and *Tfna* are significantly increased in ileum of SPF mice (Figure 6).

QUANTIFICATION AND STATISTICAL ANALYSIS

Part 4: Calculate the relative fold-change in gene expression of *Tnfa* and *Ifng*

For the *Ifng* and *Tnfa* quantitative RT-PCR, normalize Ct values to the mean Ct obtained from three house-keeping genes (*Gapdh*, *Hprt*, and *Hsp90*) for each sample.

1. Use the CT values obtained from the qRT-PCR to measure the relative gene expression fold change between control and treatment groups.
   a. Calculate the average technical replicates for each target gene [CT(Target gene)] and [CT(Housekeeping gene)] to calculate the average CT value.
   b. Calculate the average CT values from the 3 different housekeeping genes for the house-keeping gene CT value [CT(Housekeeping genes)].

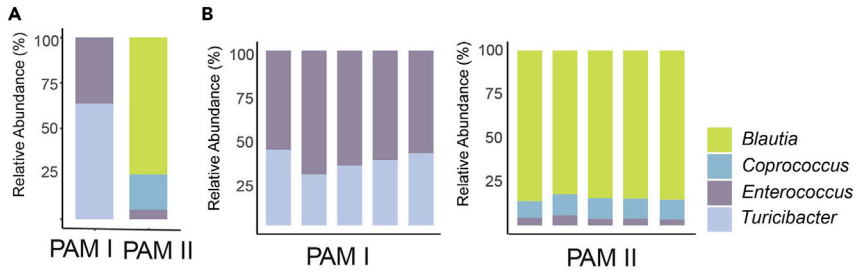

Figure 4. 16S rRNA gene sequencing reveals faithful propagation of two different low-diversity microbiota community types

(A) The composition of the post-antibiotic microbiota I (PAM I) and PAM II as generated in a small scale GF colonization experiment.

(B) The composition of the post-antibiotic microbiota I (PAM I) and PAM II as generated in a larger scale GF colonization experiment. The cecal contents of the GF+PAM I and GF+PAM II mice were profiled using 16S rRNA gene sequencing.

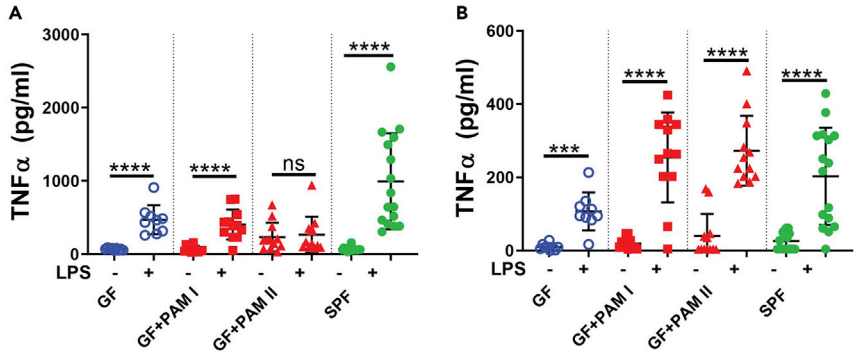

Figure 5. Differential TNFα responses to LPS stimulation in erythrocyte depleted blood and splenocytes

(A and B) (A) Blood and (B) splenocytes were collected from GF, GF+PAM I, GF+ PAM II and SPF mice at day 21 of age, erythrocyte depleted and stimulated with LPS (10 ng/mL) for 18 h. TNFα production in supernatant was assessed via ELISA. Data are represented as the mean ± SEM. Statistical significance in F-G was assessed using a Mann Whitney test. (ns = not significant, * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, **** $p< 0.0001$). Note: Reprinted from "The composition of the gut microbiota following early life antibiotic exposure affects host health and longevity in later life," by Lynn et al.[1] Reprinted with permission.

c. Calculate your ΔCT using the formula below for all your controls and treatment groups:

$$\Delta CT = CT(\text{Target gene}) - CT(\text{Housekeeping genes})$$

d. Calculate the average ΔCT from all samples in your control group to determine the average control ΔCT value, ΔCT(Control).

e. Calculate the difference of the target gene (Tnfa and Ifng) and your housekeeping genes (Gapdh, Hsp90, Hprt) as ΔCT(Control) or ΔCT(Treatment).

f. Calculate your ΔΔCT value using the formula:

$$\Delta\Delta CT = \Delta CT(\text{Control}) - \Delta CT(\text{Treatment})$$

g. Next, calculate the fold-change in gene expression using the formula below:

$$\text{Fold gene expression} = 2^{(-\Delta\Delta CT)}$$

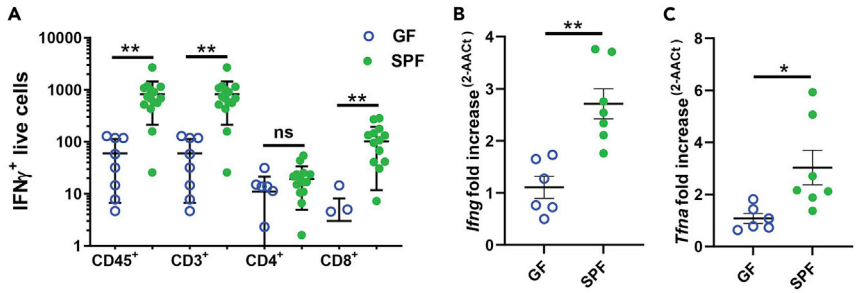

Figure 6. Differential IFNγ$^+$ expression in lymphoid cells and ileum in differentially colonized mice

(A) The number of IFNγ$^+$ lymphoid cells in small intestine lamina propria of GF and SPF mice. CD45$^+$ cells were gated on live cells, CD3$^+$ T cells were gated on CD45$^+$ cells, CD4$^+$ and CD8$^+$ T cells were gated on CD3$^+$CD45$^+$ cells. (B and C) (B) *Ifng* and (C) *Tnfa* gene expression in the ileum of GF and SPF mice was determined by qRT-PCR. Data are represented as the mean ± SEM. Statistical significance in D-E was assessed using a Kruskal-Wallis with Tukey's correction for multiple comparisons (ns = not significant, * $p< 0.05$, ** $p< 0.01$). Note: Reprinted from "The composition of the gut microbiota following early life antibiotic exposure affects host health and longevity in later life," by Lynn et al.[1] Reprinted with permission.

Note: For additional information regarding this method of calculating fold-change in gene expression, refer to Livak et al.[6]

LIMITATIONS

The quality of the initial colonization material is dependent on the quality of the fecal material that it is derived from. If the fecal material for the initial colonization has not been stored correctly at −80°C or preserved in glycerol (and stored at −80°C), it may not be possible to faithfully regenerate the microbiota with the same composition as the starting fecal sample.

There were only 3–4 bacterial taxa represented in the starting material for colonization of these mice. If using more complex starting material it may be necessary to include a third gavage to ensure that mice are colonized by low-abundance taxa. However repeated gavage may carry consequences for the health of the mice.[7]

Beyond establishing detection threshold to calculate the relative abundance of the faithful propagation of the microbiota, we would be hesitant to report any quantitative measure for success of the transplant for a number of reasons. First is the issue of accurately detecting rare taxa in sequencing experiments. For example, if we drop the detection threshold there are ~10 additional 16S ribosomal variants detected in the FMT material not detected in the mice which received an FMT. However, due to technical issues such as index hopping/bleed through during sequencing or extremely low levels of bacterial DNA in reagents it's possible these taxa are not from the FMT material at all and originated elsewhere. Second this type of sequencing approach cannot also not discern subgenus changes in taxonomy reliably, nor detect fungi/eukaryotes or viruses. Third, a similar composition does not necessitate a similar metabolic output which could lead to quite different consequences for the host.

Alternatively, propagation of mouse fecal material may be carried out using bacterial medias, however, not all medias will meet the nutritional requirements of the initial starting community. This will depend on the composition of the fecal material and the individual species nutrient and environmental requirements. If needed, one could explore the use of a fecal fermentation system or use more nutrient dense media (e.g., YCFA or Mega medium).[8]

Recent work has indicated that timing of colonization may be important for immune responses.[3] In this study germ-free mice were colonized at day 14 of life but depending on desired outcomes, timing of colonization may be changed.

This work aims to transplant and engraft a complete gut microbiome, however, we assume that the bacterial component of the microbiota is responsible for a phenotype of interest. Fungi, viruses or archaea in the gut may also contribute to immune phenotypes and this protocol is not optimized for their transplant and engraftment. Methodologies for the transfer of fecal viral communities are published elsewhere[9] and could be integrated into this protocol. In the case of fungal or archaeal transplant further consideration and method development is required.

Lamina propria extractions are a time and resource intensive process that will need the involvement of multiple persons to successfully incorporate the protocol into your workflow. Ensure thorough practice and optimizations are conducted to incorporate this protocol.

TROUBLESHOOTING

Problem 1

The colonization material does not faithfully reflect the starting material (part 2: propagation of colonization material).

Potential solution

Likely the FMT has been compromised and has become contaminated during the processes described herein. Check sterilization protocols for possible breaches. Most likely contamination of reagents occurred in the anaerobic chamber. Check if reagents (glycerol or PBS) are sterile. Contamination may also occur when handling germ-free mice, check sterilization protocols for possible breaches.

Problem 2

The 16S rRNA sequencing data shows some but not all of the desired taxa that colonize the germ-free mice (part 2: propagation of colonization material).

Potential solution

If the FMT material has a diverse composition, additional oral gavages may be required. Also ensure that the FMT material is not compromised during the gavaging process. During the gavaging procedure, the FMT material may encounter oxygen from the environment, which may comprise anaerobic bacteria viability. To limit oxygen exposure, aliquot sufficient FMT material for colonizing one cage at a time. Additionally, isoflurane may be utilized to speed-up the oral gavage process to reduce the time that anaerobic FMT material is exposed to oxygen.

Problem 3

Low yield/cell viability of lamina propria cells (Part 3: Assessment of immune response alterations in early life germ-free mice; part 3d: lamina propria extraction, stimulations and analysis).

Potential solution

The yield and viability of cells extracted from the lamina propria is highly dependent on the time between removal of the tissue from the mouse and the completion of the protocol. Ensure that a reasonable number of samples are being processed at any time; we recommend processing a maximum of 4–8 samples per researcher at any time. Keep tissues on ice whenever possible to maintain viability, i.e., when cleaning the intestines. Remove mucus and fecal material thoroughly from intestines prior to digestion steps as outlined herein. Warm Media C to 37°C prior to use for optimal digestion. Repeat the digest steps part 3d: lamina propria extraction, stimulations and analysis up to 3 times until all tissue pieces have been digested.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, David Lynn (David.lynn@sahmri.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published article includes all datasets/code generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS

Conceptualization, D.J.L.; Investigation, M.A.L., C.Y.T.; Writing – Original draft, M.A.L. and F.J.R; Writing – Review & editing, M.A.L., F.J.R., and D.J.L.; Funding acquisition, D.J.L.; Supervision, D.J.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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