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Supplementary Materials for

Scratching promotes allergic inflammation and host defense via neurogenic mast cell activation

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The PDF file includes:

Figs. S1 to S9

Other Supplementary Material for this manuscript includes the following:

MDAR Reproducibility Checklist

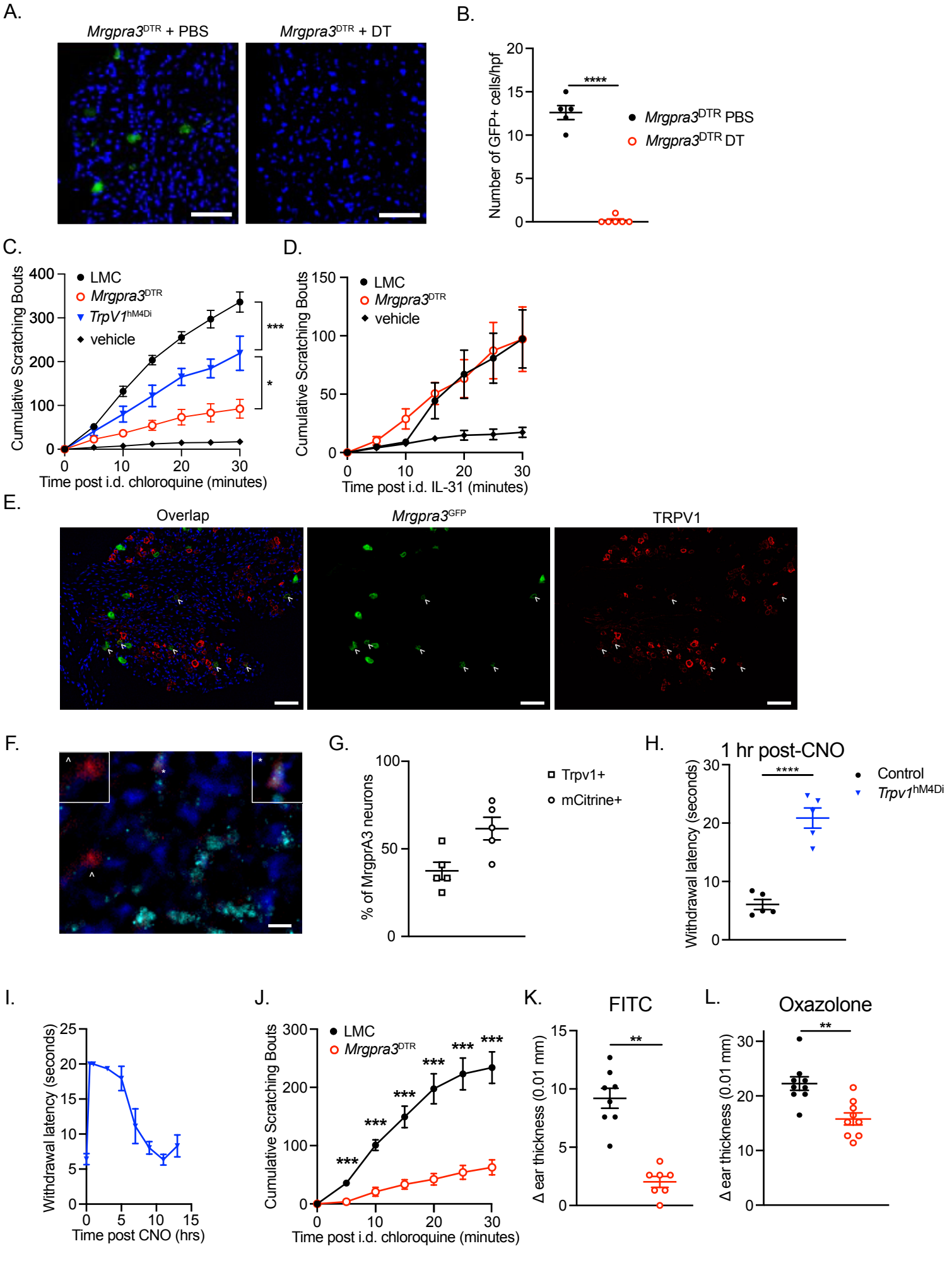

A.
Mrgpra3DTR + PBS
Mrgpra3DTR + DT
B.
Number of GFP+ cells/hpf
****
Mrgpra3DTR PBS
Mrgpra3DTR DT
C.
Cumulative Scratching Bouts
LMC
Mrgpra3DTR
TrpV1hM4Di
vehicle
***
*
Time post i.d. chloroquine (minutes)
D.
Cumulative Scratching Bouts
LMC
Mrgpra3DTR
vehicle
Time post i.d. IL-31 (minutes)
E.
Overlap
Mrgpra3GFP
TRPV1
F.
G.
% of MrgprA3 neurons
Trpv1+
mCitrine+
H.
1 hr post-CNO
Withdrawal latency (seconds)
****
Control
Trpv1hM4Di
I.
Withdrawal latency (seconds)
Time post CNO (hrs)
J.
Cumulative Scratching Bouts
LMC
Mrgpra3DTR
Time post i.d. chloroquine (minutes)
K.
FITC
Δ ear thickness (0.01 mm)
**
L.
Oxazolone
Δ ear thickness (0.01 mm)
**

Fig. S1. Validation of MrgprA3-DTR loss-of-function mouse model
(A) Immunofluorescent visualization of dorsal root ganglia cell bodies (DAPI, blue) and MrgprA3 (GFP, green) after 6 total 300 ng DT treatments in litter mate control (LMC, black circle) and *Mrgpra3*^{DTR} (red circle) mice quantified in (B) (*Mrgpra3*^{DTR} PBS n = 5 and *Mrgpra3*^{DTR} DT n = 6) (C) Total scratching behavior at the indicated time points following injection of either PBS vehicle (black diamond) or 0.2 mg chloroquine (CQ) or (D) 0.8 µg IL-31 i.d. in the nape in LMC, *TrpV1*^{hM4Di} (blue triangle), and *Mrgpra3*^{DTR} mice (PBS control n = 4, LMC n = 8-16, *Mrgpra3*^{DTR} n = 10-11, TrpV1^{hM4Di} n = 7). All mice were treated with 6 i.p. injections of 300 ng DT separated by 3 days. (E) Representative images from immunofluorescent visualization of dorsal root ganglia cell bodies (DAPI, blue, MrgprA3 (GFP, green) and TrpV1 (RFP, red) in MrgprA3^{Cre} Rosa26.YFP mice. MrgprA3^{YFP} cells that co-express TrpV1 are indicated with white arrow heads. (F) Representative images of RNAscope visualization of DRG cell bodies (DAPI, blue) identifying mRNA for mCitrine (cyan) and *Mrgpra3* (red) isolated from unmanipulated *TrpV1*^{hM4Di} mice which have mCitrine expressed in tandem with hM4Di. Asterix identifies Mrgpra3-expressing cells that co-express mCitrine and have undergone cre-mediated recombination. Arrowhead identifies cells not expressing mCitrine that have not undergone cre-mediated recombination. (G) A quantification of the percent of MrgprA3-expressing cells from (E) that co-express TrpV1 and *Mrgpra3*-expressing cells from (F) that co-express mCitrine is shown. Each symbol represents average values from 3 high powered fields from a single animal (n = 5). (H) Thermal withdrawal latency measured in CNO-injected LMC or *TrpV1*^{hM4Di} mice 1 hour following CNO injection and (I) at the indicated time points (LMC and TrpV1^{hM4Di} n = 5). (J) Total scratching behavior over 5 minute intervals at the indicated time points following injection of CQ in LMC and *Mrgpra3*^{DTR} mice after 2 i.p. injections of 600 ng DT (LMC n = 7, *Mrgpra3*^{DTR} n = 8). (K) Ear thickness 1 day after FITC and (L) oxazolone challenge in hapten-sensitized LMC and *Mrgpra3*^{DTR} that were given 2 i.p. injections of 600 ng DT after sensitization and before challenge (FITC LMC n = 8, *Mrgpra3*^{DTR} n = 7; oxazolone LMC and *Mrgpra3*^{DTR} n = 9). Scale bar in (A and E) 200 µm and in (F) 50 µm. Results in C-D and I-J are displayed as mean +/- SEM and are representative of 3 independent experiments. Each symbol in B, H and K-L represent data from an individual animal. Significance was calculated using a one-way ANOVA with multiple comparisons (C and D), Mann-Whitney (H, J), or unpaired Student's t-test (B, K-L). *p<0.05, **p<.01, ***p<.001, ****p<.0001.

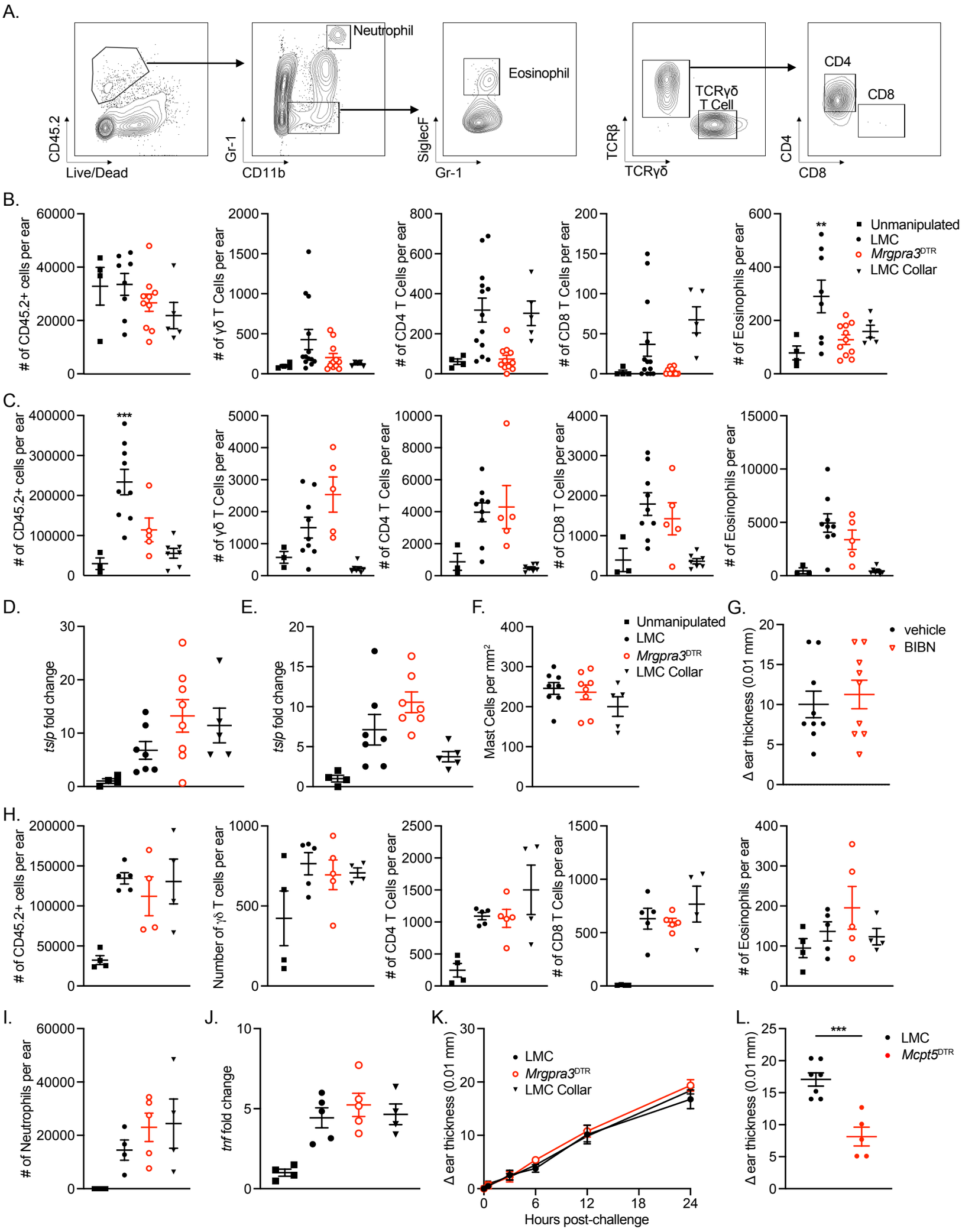

A.
CD45.2
Live/Dead
Neutrophil
Gr-1
CD11b
Eosinophil
SiglecF
Gr-1
TCRγδ T Cell
TCRβ
TCRγδ
CD4
CD8
CD4
CD8
B.
# of CD45.2+ cells per ear
# of γδ T Cells per ear
# of CD4 T Cells per ear
# of CD8 T Cells per ear
# of Eosinophils per ear
Unmanipulated
LMC
Mrgpra3DTR
LMC Collar
C.
# of CD45.2+ cells per ear
# of γδ T Cells per ear
# of CD4 T Cells per ear
# of CD8 T Cells per ear
# of Eosinophils per ear
D.
tslp fold change
E.
tslp fold change
F.
Mast Cells per mm2
Unmanipulated
LMC
Mrgpra3DTR
LMC Collar
G.
Δ ear thickness (0.01 mm)
vehicle
BIBN
H.
# of CD45.2+ cells per ear
Number of γδ T cells per ear
# of CD4 T Cells per ear
# of CD8 T Cells per ear
# of Eosinophils per ear
I.
# of Neutrophils per ear
J.
tnf fold change
K.
Δ ear thickness (0.01 mm)
LMC
Mrgpra3DTR
LMC Collar
Hours post-challenge
L.
Δ ear thickness (0.01 mm)
LMC
Mcpt5DTR

Fig. S2. Cellular infiltrate of FITC and Oxazolone-challenged ears.
(A) Representative gating strategy for immune cells from single cell suspensions of whole skin. Indicated cell populations in ears from unmanipulated (black square) or hapten-challenged LMC (black circle), *Mrgpra3*^{DTR} (red circle), and LMC collared (black triangle) mice 1 day following (B) FITC or (C) oxazolone challenge. *Tslp* mRNA expression in whole ear tissue from LMC, *Mrgpra3*^{DTR}, and LMC collared mice 1 day following (D) FITC or (E) oxazolone challenge (Unmanipulated n = 4 LMC n = 8-13, *Mrgpra3*^{DTR} n = 5-11, collar n = 5). (F) Total number of mast cells quantified in ear skin 10 hours post-FITC challenge (LMC and *Mrgpra3*^{DTR} n = 8, collar n = 5). (G) 80 ng BIBN4096 (red triangle) or vehicle (black circle) was given i.p. to FITC sensitized mice 1 hour prior to FITC challenge (vehicle and BIBN n = 10). Ear thickness was measured 24 hours post-challenge. (H) Indicated cell populations and (I) neutrophils in ears from unmanipulated or LMC, *Mrgpra3*^{DTR}, and LMC collared mice 1 day following DNFB challenge of sensitized mice (Unmanipulated n = 4, LMC n = 5, *Mrgpra3*^{DTR} n = 4, collar n = 4). (J) *Tnf* mRNA expression in whole ear tissue from unmanipulated or LMC, *Mrgpra3*^{DTR}, and LMC collared mice 1 day following DNFB challenge. (K) Ear thickness at the indicated timepoints after DNFB challenge. (L) 120 ng DT was injected i.d. at the ear pinna in FITC-sensitized *Mcpt5*^{DTR} (red closed circle) mice 6 and 2 days before FITC challenge (LMC n = 7, *Mcpt5*^{DTR} n = 5). Ear thickness was measured 24 hours post-challenge. Results in K are displayed as mean +/- SEM and are representative of 3 independent experiments. Each symbol in A-J and L represent data from an individual animal. Significance was calculated using a one-way ANOVA with multiple comparisons, *p<0.05, **p<.01, ***p<.001, ****p<.0001.

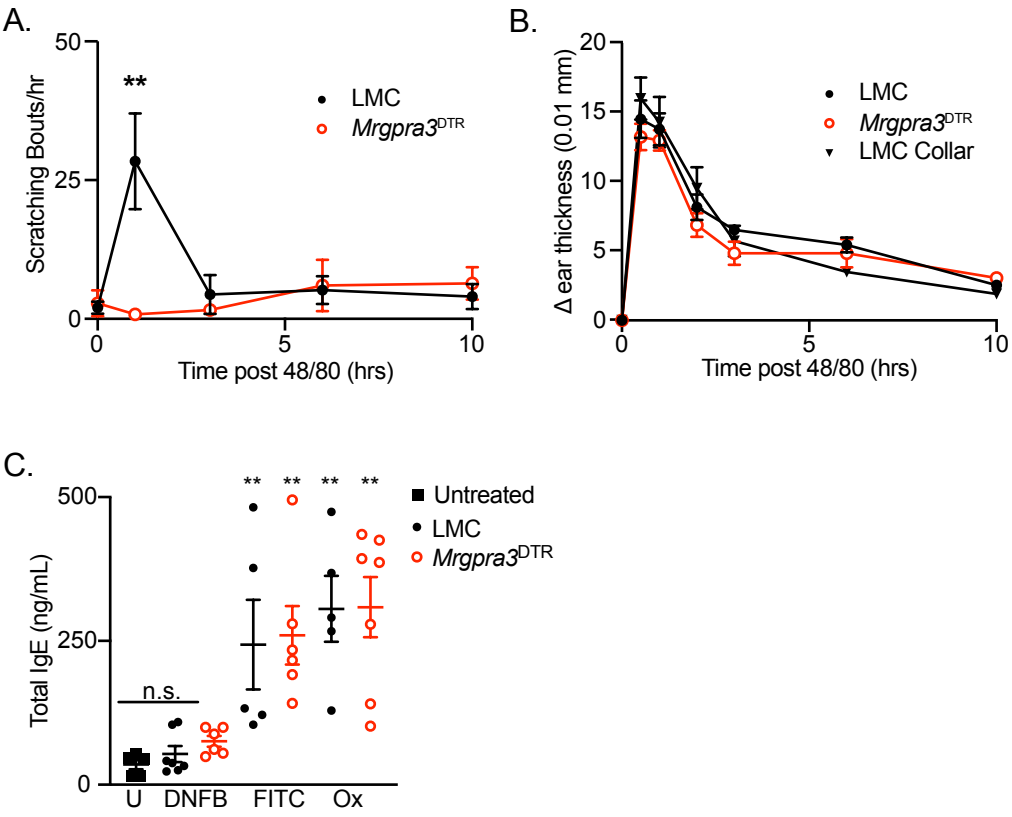

A.
Scratching Bouts/hr
50
25
0
**
LMC
Mrgpra3DTR
0
5
10
Time post 48/80 (hrs)
B.
Δ ear thickness (0.01 mm)
20
15
10
5
0
LMC
Mrgpra3DTR
LMC Collar
0
5
10
Time post 48/80 (hrs)
C.
Total IgE (ng/mL)
500
250
0
n.s.
**
**
**
**
Untreated
LMC
Mrgpra3DTR
U
DNFB
FITC
Ox

Fig. S3. Compound 48/80 phenocopies SP *in vivo* and Serum IgE is elevated in FITC and Ox, but not DNFB CHS

(A) The number of scratching bouts per hour in DT-treated littermate control (LMC, black circle), *Mrgpra3*^{DTR} (red circle) after intradermal injection of 200ng compound 48/80 into the ear is shown (LMC and *Mrgpra3*^{DTR} n = 5). (B) Ear thickness in DT-treated litter mate control (LMC), *Mrgpra3*^{DTR}, and collared LMC mice at the indicated time following intradermal injection of compound 48/80 into the ear is shown (LMC and *Mrgpra3*^{DTR} n = 5, Collar n = 4). (C) Total IgE levels taken from serum of untreated (black square) mice and DT-treated, hapten-sensitized LMC and *Mrgpra3*^{DTR} mice 10 hours after DNFB, FITC, and oxazolone challenge (Unmanipulated n = 5, LMC n = 5-7, *Mrgpra3*^{DTR} n = 6-7). Results in A-B are displayed as mean +/- SEM and are representative of 3 independent experiments. Each symbol in C represent data from an individual animal. Significance was calculated using a Mann-Whitney test (A) or by one-way ANOVA with multiple comparisons (B-C), *$p<0.05$, **$p<.01$.

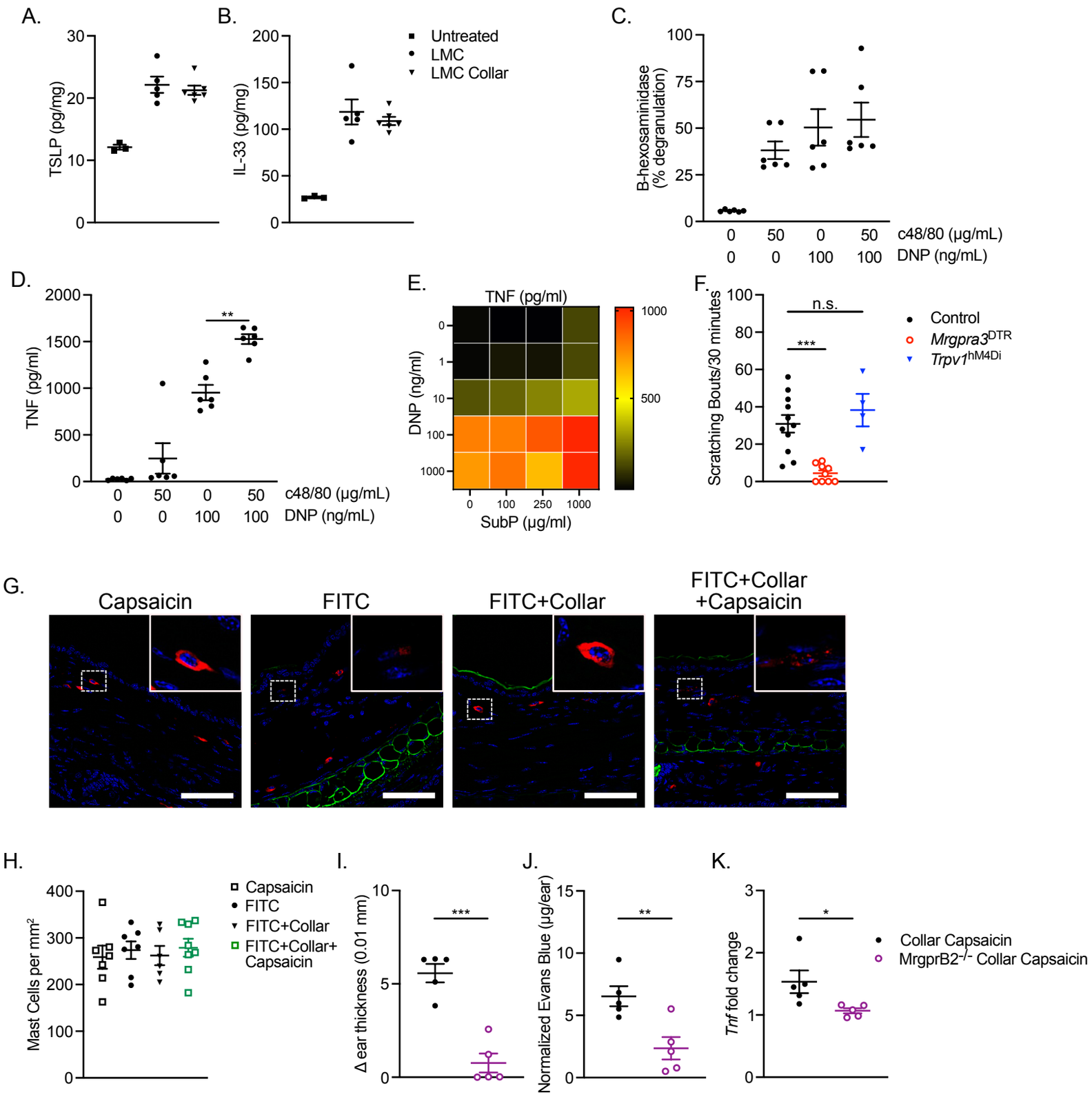
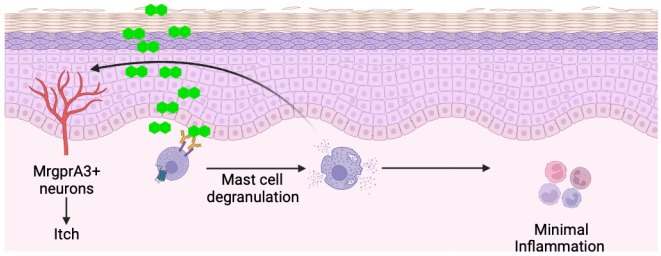

A.
TSLP (pg/mg)
B.
IL-33 (pg/mg)
Untreated
LMC
LMC Collar
C.
B-hexosaminidase (% degranulation)
c48/80 (µg/mL)
DNP (ng/mL)
D.
TNF (pg/ml)
**
c48/80 (µg/mL)
DNP (ng/mL)
E.
TNF (pg/ml)
DNP (ng/ml)
SubP (µg/ml)
F.
Scratching Bouts/30 minutes
n.s.
***
Control
Mrgpra3DTR
Trpv1hM4Di
G.
Capsaicin
FITC
FITC+Collar
FITC+Collar +Capsaicin
H.
Mast Cells per mm2
Capsaicin
FITC
FITC+Collar
FITC+Collar+ Capsaicin
I.
Δ ear thickness (0.01 mm)
***
J.
Normalized Evans Blue (µg/ear)
**
K.
Tnf fold change
*
Collar Capsaicin
MrgprB2-/- Collar Capsaicin

Fig. S4. IgE cross linking and MrgprB2 agonism synergize for TNF release
(A) TSLP and (B) IL-33 protein levels in whole ear skin from untreated (black square) mice and DNP-specific IgE sensitized LMC (black circle) and collared LMC (black triangle) mice 10 hours after DNP challenge (Untreated n = 3, LMC and collar n = 5). (C) β-hexosaminidase and (D) TNF protein levels from the culture supernatants of DNP-specific IgE sensitized cultured PMCs from 6 mice across 3 experiments 6 hours following treatment with the indicated doses of DNP and compound 48/80. (E) TNF protein levels from the supernatants of DNP-specific IgE sensitized cultured PMCs 6 hours following treatment with the indicated dose of DNP and Substance P. (F) Scratching behavior over 30 minutes after FITC challenge in sensitized LMC, *Mrgpra3*^{DTR}, and *TrpV1*^{hM4Di} mice (LMC n = 11, *Mrgpra3*^{DTR} n = 9, TrpV1^{hM4Di} n = 4). (G) Immunofluorescent visualization of ear skin 10 hours after FITC challenge in capsaicin-treated (black square), FITC-challenged (black circle), collared FITC-challenged (black triangle), and collared capsaicin-treated and FITC-challenged (green square) mice with the total number of mast cells quantified in (H) (capsaicin and FITC n = 7, collar n = 6, FITC capsaicin collar n = 8). (I) Ear thickness, (J) quantification of Evans Blue extravasation, and (K) *Tnf* mRNA measured at 10 hours following DNP and capsaicin treatment in collared WT (black circle) and MrgprB2$^{-/-}$ (purple circle) DNP-specific IgE sensitized mice (WT and MrgprB2$^{-/-}$ n = 5). Scale bar in (H) = 200 μm. Each symbol in A-D, F and H-K represent data from an individual animal. Significance was calculated using a one-way ANOVA with multiple comparisons (C, D, F, H), or unpaired Student's t-test (I-K) *$p<0.05$, **$p<.01$.

A.


MrgprA3+
neurons
Itch
Mast cell
degranulation
Minimal
Inflammation

B.

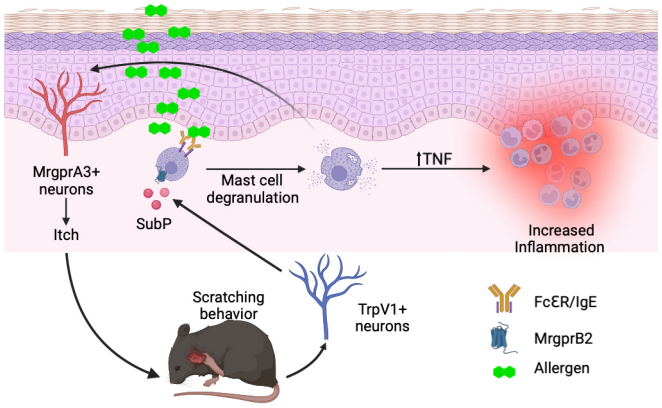
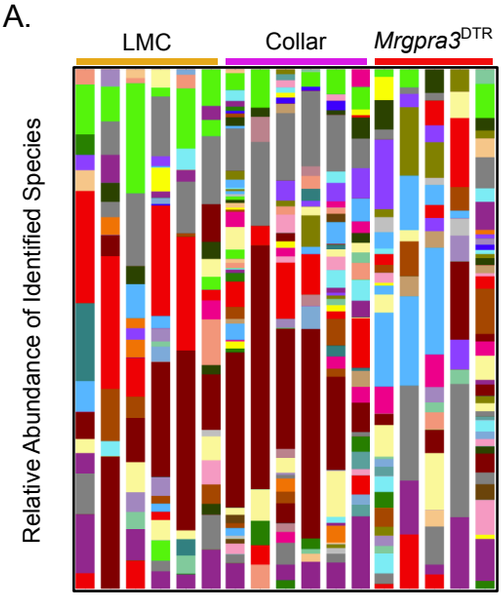
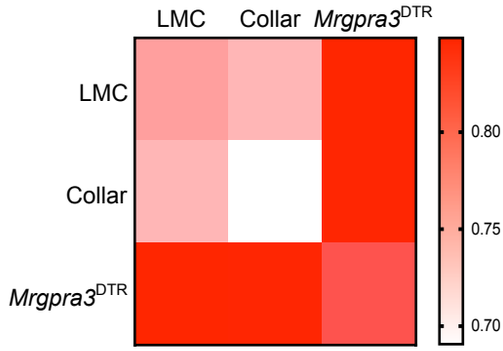

MrgprA3+
neurons
Itch
SubP
Mast cell
degranulation
↑TNF
Increased
Inflammation
Scratching
behavior
TrpV1+
neurons
FcεR/IgE
MrgprB2
Allergen

Fig. S5. Model
(A) Following allergen challenge in an immunized host, mast cells are activated via FcεRI cross linking, resulting in degranulation and release of pruritogens that activate MrgprA3-expressing itch-sensing neurons. Mediators are also released, leading to edema (not pictured) and a small degree of inflammation. (B) Scratching in response to activation of MrgprA3-sensing neurons activates TRPV1-expressing nociceptors and the release of Substance P. Activation of mast cells via FcεRI cross linking combined with SP agonism of MrgprB2 results in increased production of TNF and exaggerated neutrophil recruitment and inflammation. Created with Biorender.com.

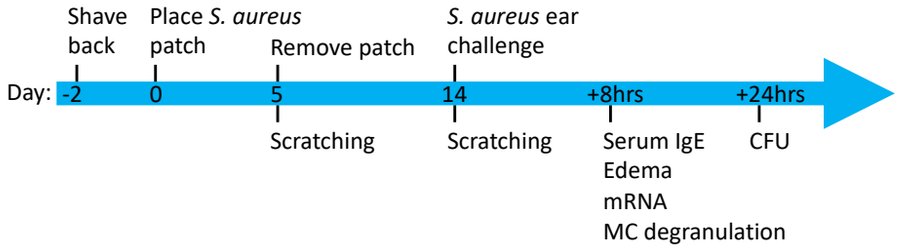
A.


LMC
Collar
Mrgpra3DTR
Relative Abundance of Identified Species

B.


LMC
Collar
Mrgpra3DTR
LMC
Collar
Mrgpra3DTR
0.80
0.75
0.70

C.


Shave back
Place S. aureus patch
Remove patch
S. aureus ear challenge
Day:
-2
0
5
14
+8hrs
+24hrs
Scratching
Scratching
Serum IgE
Edema
mRNA
MC degranulation
CFU

D.

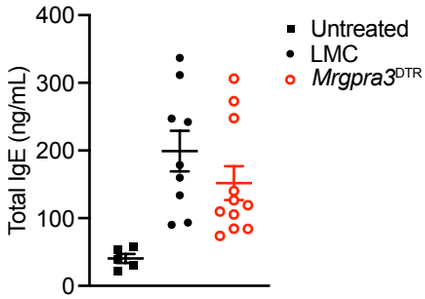
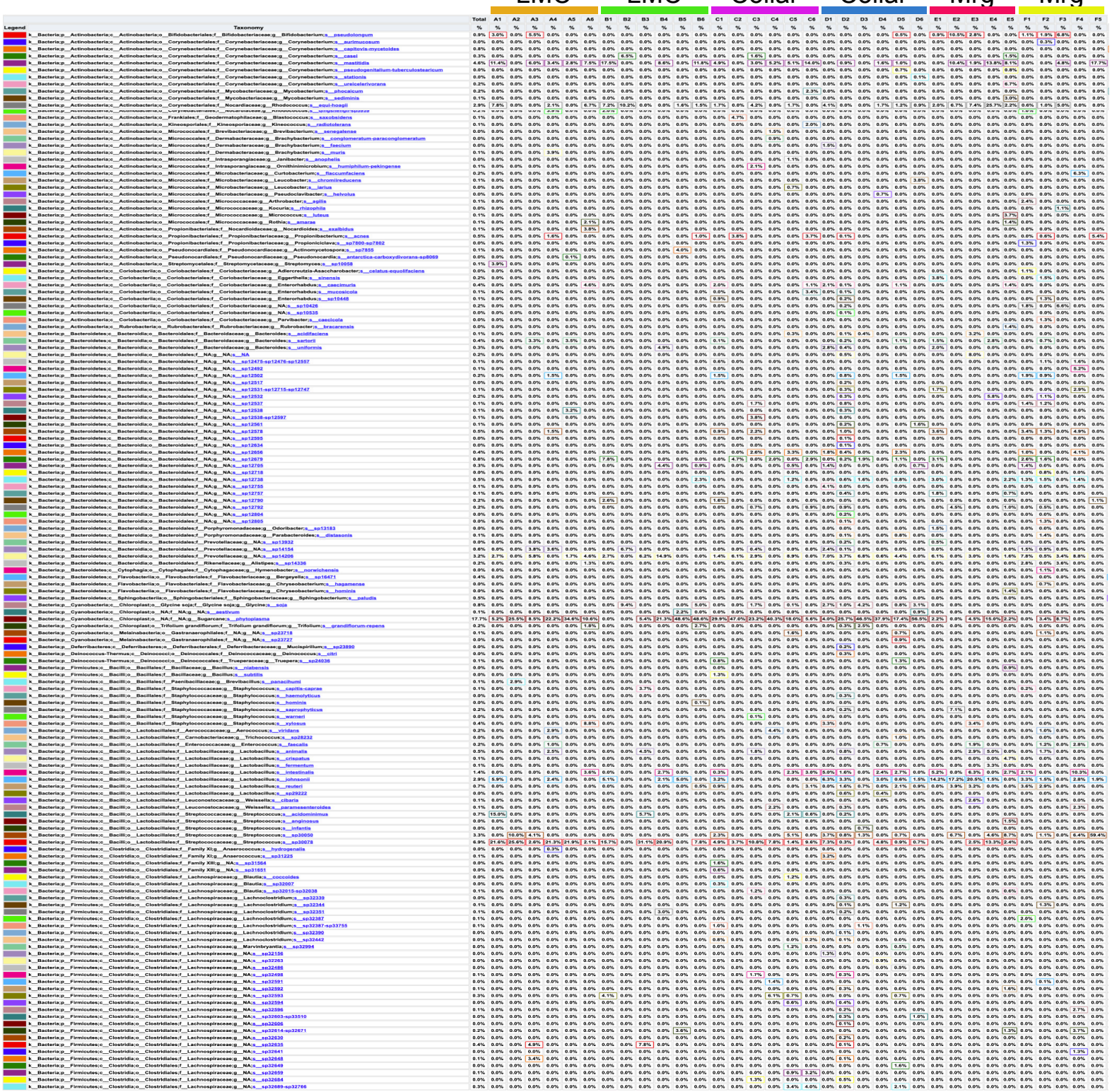

Total IgE (ng/mL)
400
300
200
100
0
Untreated
LMC
Mrgpra3DTR

Fig. S6. Experimental scheme for *S. aureus* infections and serum IgE
(A) The relative abundance of 16S rRNA reads associated with specific bacterial species from unmanipulated ears from the indicated group is shown. The species associated with each color and its relative abundance are shown in Supplemental Figure 7. (B) Bray Curtis beta diversity comparing taxa isolated from unmanipulated ears from the indicated group is shown. (C) Experimental scheme for Figure 5. (D) Total IgE levels taken from serum of untreated (black square), LMC (black circle) and *Mrgpra3*^{DTR} (red circle) mice 14 days after epicutaneous *S. aureus* immunization (Untreated n = 5, LMC n = 9, *Mrgpra3*^{DTR} n = 11). Each symbol in D represent data from an individual animal..


LMC
FITC LMC
Collar
FITC Collar
Mrg
FITC Mrg

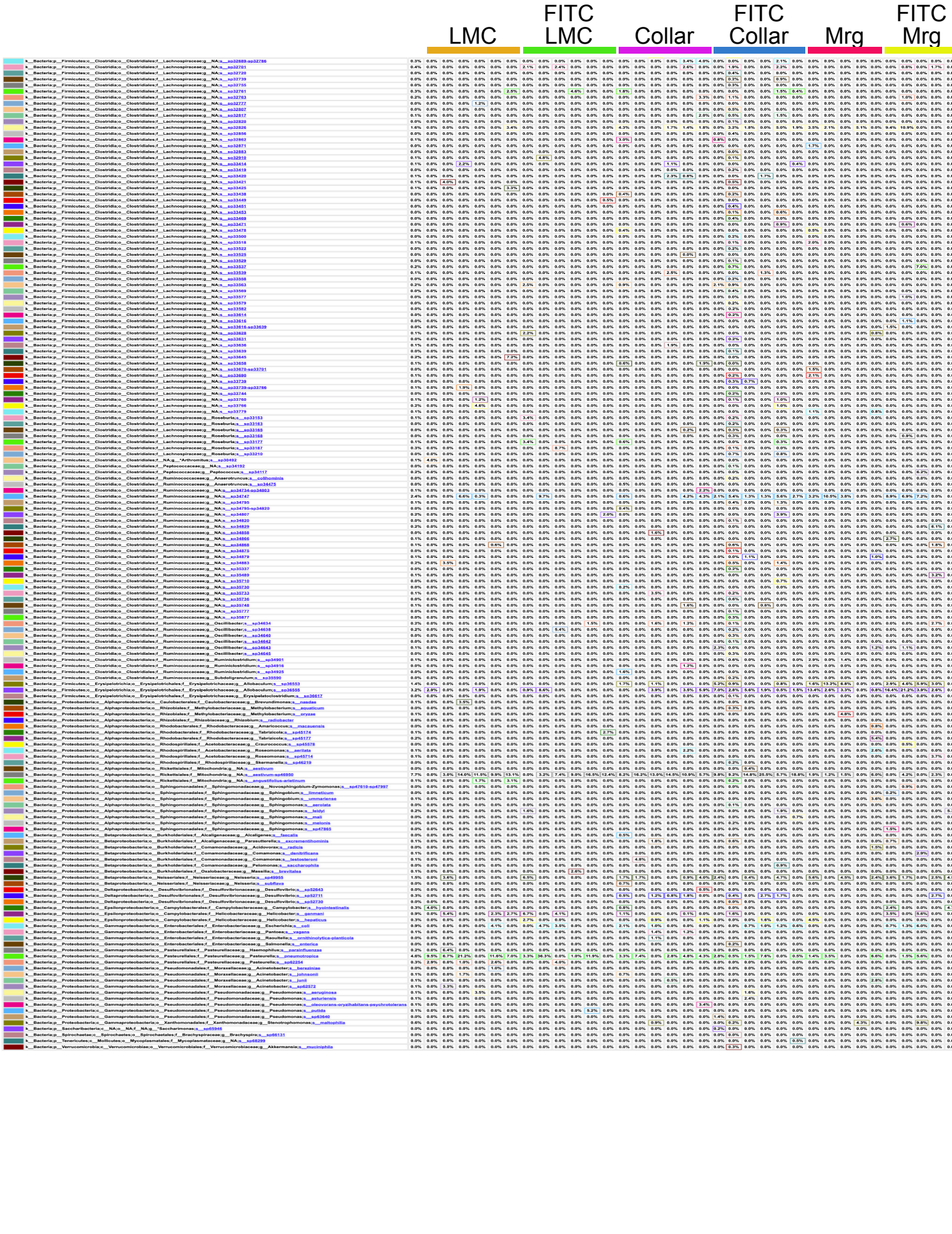

LMC
FITC LMC
Collar
FITC Collar
Mrg
FITC Mrg

Fig. S7. Bacterial species and percent abundance of 16S rRNA reads from Figure S6A

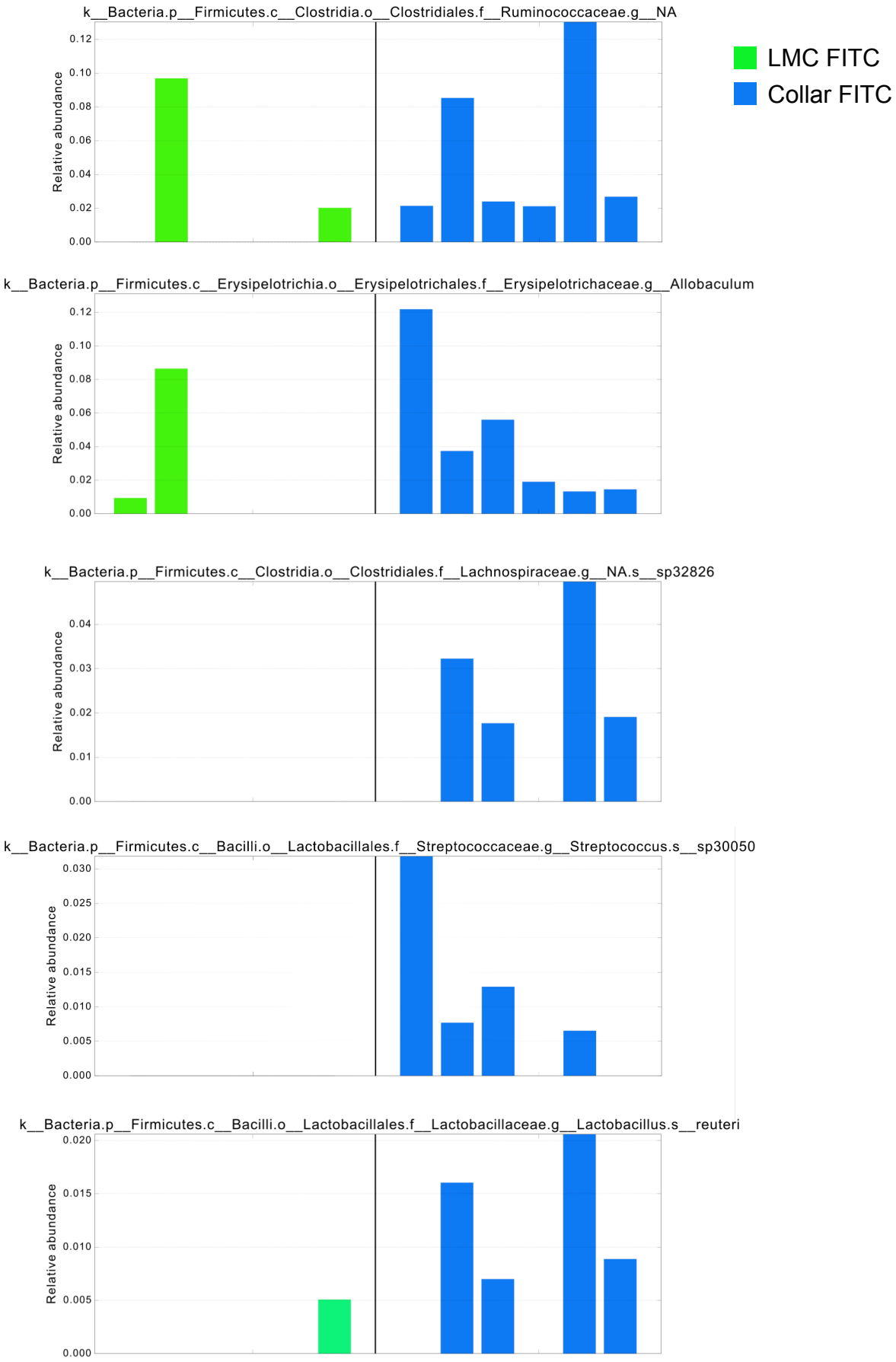

k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__NA
Relative abundance
0.12
0.10
0.08
0.06
0.04
0.02
0.00
LMC FITC
Collar FITC
k__Bacteria.p__Firmicutes.c__Erysipelotrichia.o__Erysipelotrichales.f__Erysipelotrichaceae.g__Allobaculum
Relative abundance
0.12
0.10
0.08
0.06
0.04
0.02
0.00
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp32826
Relative abundance
0.04
0.03
0.02
0.01
0.00
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Streptococcaceae.g__Streptococcus.s__sp30050
Relative abundance
0.030
0.025
0.020
0.015
0.010
0.005
0.000
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Lactobacillaceae.g__Lactobacillus.s__reuteri
Relative abundance
0.020
0.015
0.010
0.005
0.000

Fig. S8. Individual 16SrRNA reads associated with decreased taxa from scratching identified by LEfSe analysis

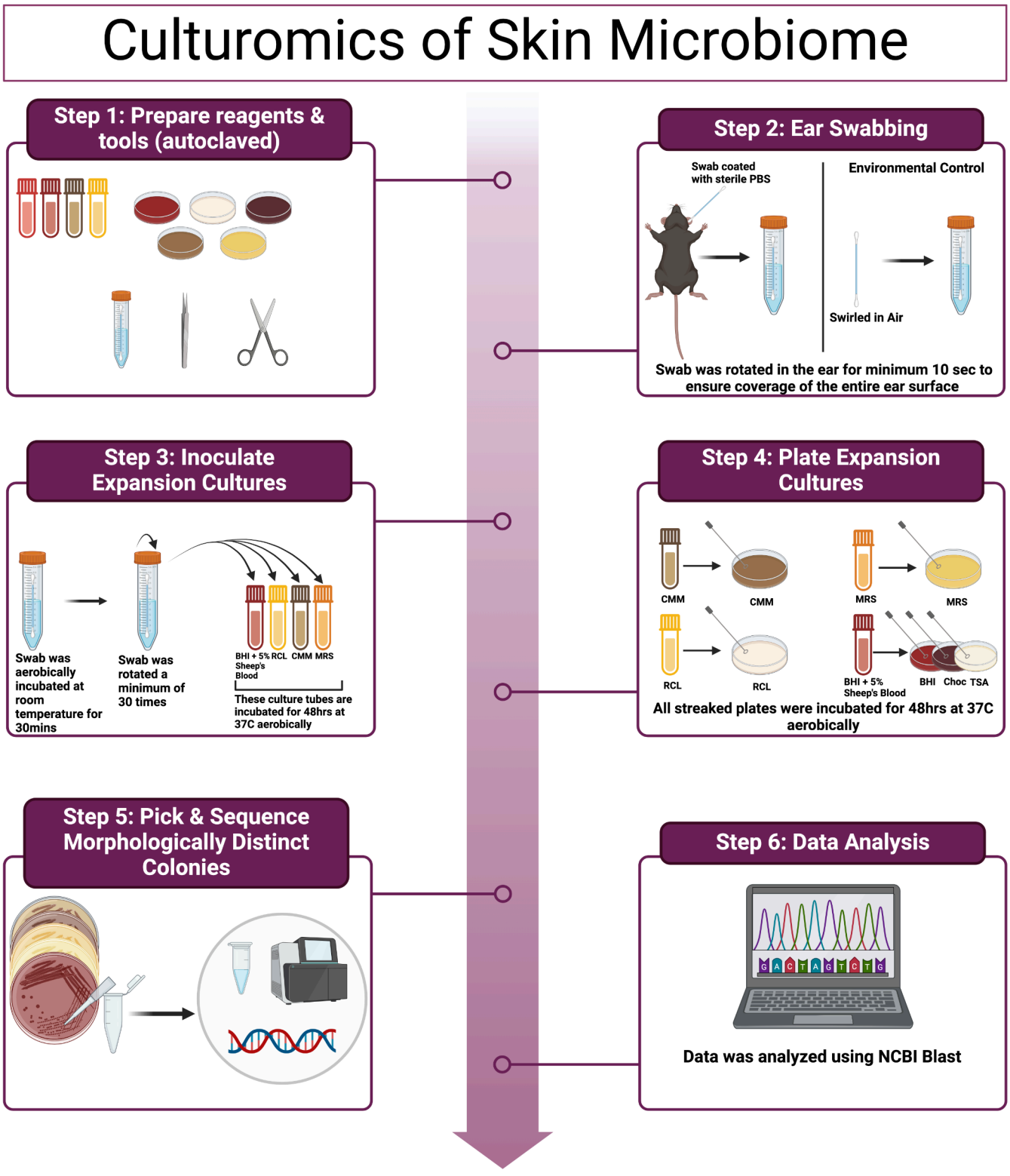

Culturomics of Skin Microbiome
Step 1: Prepare reagents & tools (autoclaved)
Step 2: Ear Swabbing
Swab coated with sterile PBS
Environmental Control
Swirled in Air
Swab was rotated in the ear for minimum 10 sec to ensure coverage of the entire ear surface
Step 3: Inoculate Expansion Cultures
Swab was aerobically incubated at room temperature for 30mins
Swab was rotated a minimum of 30 times
BHI + 5% Sheep's Blood
RCL
CMM
MRS
These culture tubes are incubated for 48hrs at 37C aerobically
Step 4: Plate Expansion Cultures
CMM
CMM
MRS
MRS
RCL
RCL
BHI + 5% Sheep's Blood
BHI
Choc
TSA
All streaked plates were incubated for 48hrs at 37C aerobically
Step 5: Pick & Sequence Morphologically Distinct Colonies
Step 6: Data Analysis
Data was analyzed using NCBI Blast

Figure S9. Schema of Culturomics approach
Created with Biorender.com