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Single-cell RNA-seq provides insight into the underdeveloped immune system of germ-free mice

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ABSTRACT

Germ-free mice exhibit profound immunological immaturity. Despite recent studies emphasizing the role of specific bacterium-derived metabolites in immune cell development and differentiation, the mechanisms linking microbiota absence to systemic immune deficits remain incompletely defined. Here, droplet-based single-cell RNA sequencing of bone marrow and peripheral blood from both germ-free and specific pathogen-free mice was performed, identifying 25 transcriptionally distinct cell types. Neutrophil apoptosis was elevated in germ-free mice, potentially due to the absence of niacin dehydrogenase, a metabolite primarily produced by *Pseudomonas*. In addition, germ-free mice exhibited increased excretion of 5'-methylthioadenosine, enhanced ERK activation driven by reactive oxygen species, and disruption of bone marrow stromal antigen 2 signaling. Monocytes and CD8⁺ T cells from germ-free mice showed diminished responses to interferon- β and interferon- γ , consistent with heightened viral susceptibility. These findings establish a microbiota-dependent regulatory pathway linking immunodeficiency to microbial absence in germ-free mice, confirmed through complementary validation techniques.

Keywords: Germ-free mice; Microbiota; Single-cell RNA sequencing; Underdeveloped immune system

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INTRODUCTION

Eukaryotic hosts and their microbiota do not exist in isolation, but rather form an integrated biological organism, the holobiont, that orchestrates essential physiological functions, including metabolism, immune maturation, and neural development (Sommer & Bäckhed, 2013). Through diverse compositional configurations, microbial communities generate, modify, and degrade a wide array of metabolites that extend and complement host metabolic capacity. For example, many complex proteins and carbohydrates resistant to host enzymatic digestion can be degraded by the microbiota (Chen et al., 2018; Nicholson et al., 2012). In addition to nutrient processing, microbial metabolites engage in bidirectional signaling with host cells through a series of innate immune receptors (Bassler & Losick, 2006). Notably, emerging evidence suggests that microbiota-derived metabolites are essential coordinators of host physiology and pathophysiology, influencing not only a wide range of metabolic and inflammatory pathways but also behavioral phenotypes (Hsiao et al., 2013).

The extensive co-evolutionary history between mammals and their microbiota led Louis Pasteur to postulate that life in the absence of microbes would be unsustainable (Pasteur, 1885). This notion was later challenged by the successful derivation of healthy germ-free (GF) rats by Reyniers and colleagues in 1946 (Reyniers et al., 1946). Since then, GF animal models have been instrumental in delineating the indispensable roles of microbes in mammalian development and health. GF mice are completely devoid of detectable microbial life and must be housed under a tightly monitored

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and controlled gnotobiotic environment to prevent contamination. These animals exhibit pronounced immunological underdevelopment (Smith et al., 2007), a condition that can be substantially reversed by reintroducing species-specific microbiota in adulthood (Chung et al., 2012). Early studies already documented heightened viral susceptibility in GF mice, including increased vulnerability to influenza A virus (Dolowy & Muldoon, 1964), coxsackie B virus (Schaffer et al., 1963), and Friend virus (Mirand & Grace, 1963). Although the underlying mechanisms remain incompletely understood, several studies have suggested that microbiota-derived metabolites, such as short-chain fatty acids (Balmer et al., 2014; Erny et al., 2015; Khosravi et al., 2014; Morrison & Preston, 2016), tryptophan metabolites (Lee et al., 2011; Li et al., 2011; Shi et al., 2007) and retinoic acid (Bakdash et al., 2015; Kjer-Nielsen et al., 2012; Kunisawa et al., 2012; Qiu et al., 2012; Spencer et al., 2014), are essential modulators of immune cell development and functional differentiation (Levy et al., 2016).

While recent studies have employed single-cell RNA sequencing (scRNA-seq) to compare immune phenotypes between GF and specific pathogen-free (SPF) mice, these investigations have primarily focused on specific immune subsets within peripheral tissues, such as CD4⁺ T cells in the colon (Kiner et al., 2021) and microglia in the brain (Matcovitch-Natan et al., 2016). However, comprehensive immune profiling of peripheral blood (PB) and bone marrow (BM) in GF versus SPF mice remains unexplored. To address this gap, the present study systematically mapped the immune landscape of adult GF and SPF mice and elucidated the mechanisms underlying immune impairment in the absence of microbiota. A two-stage analytical framework was employed. In the discovery stage, hematological parameters were assessed, followed by high-resolution scRNA-seq of approximately 35 000 immune cells from PB and BM to resolve transcriptional differences between conditions. In the validation stage, independent datasets from non-targeted metabolomics, single-cell transcriptomics, and spatial transcriptomics across multiple tissues were used to confirm and extend our findings. Our comprehensive analysis reveals specific molecular pathways disrupted by microbiota absence, including altered neutrophil apoptosis through niacin metabolism dysregulation and impaired antiviral responses mediated by BST2 signaling disruption, providing mechanistic insights into the profound immunodeficiency observed in germ-free mice.

MATERIALS AND METHODS

Ethics statement

All animal experiments were approved by the Institutional Review Board of BGI (BGI-IRB A21028) and Institutional Animal Care and Use Committee of Huazhong Agricultural University (HZAUMO-2020-0050) based on the principles of animal welfare and ethics.

Study design

This study investigated the mechanistic basis of immune underdevelopment in GF mice by comprehensively profiling immune cells in PB and BM using scRNA-seq. Experiments were conducted under rigorously controlled conditions to ensure microbial sterility in GF mice. Over 35 000 high-quality single cell transcriptomes were analyzed to compare immune

cell composition and gene expression between GF and SPF mice, with the aim of delineating microbiota-dependent immune regulation. Findings were validated using multiple complementary approaches.

Mice

Male Kunming (KM) mice (10 weeks), both GF and SPF, were provided by the Huazhong Agricultural University Experimental Animal Center (China). The GF mice displayed the classical features of microbial absence, including an enlarged cecum and a profoundly underdeveloped immune system. The KM mouse strain, originally derived from Swiss albino mice, is widely used in biomedical research in China due to its high disease resistance, large litters, and rapid growth (Li et al., 2020; Yu et al., 2014). The choice of KM mice in this study was based on their suitability for examining microbial influence on immune function rather than any strain-specific genetic traits. Notably, KM mice demonstrate immune responses consistent with those of other strains, enhancing the generalizability of our findings. For example, KM and C57BL/6 mice show comparable neurological symptoms following Zika virus infection (Yu et al., 2017) and respond similarly to high-fat diets in terms of weight gain, liver fat accumulation, and glucose dysregulation (Li et al., 2020), supporting their use in immunological studies.

GF mice were maintained in sterile isolators with strict exclusion of microbial exposure. SPF mice were housed in a dedicated pathogen-free isolator in a specific pathogen-free environment to prevent cross-contamination. All mice were kept on a 12 h light:dark cycle under controlled conditions (temperature: 25±2°C, humidity: 45%–60%) with *ad libitum* access to water and food. Both GF and SPF mice were fed gamma-irradiated diets (Co60-γ, 50 kGy) to ensure complete elimination of microorganisms. Peripheral blood and bone marrow were collected following euthanasia by cervical dislocation.

Sample preparation and cell isolation for scRNA-seq

Mice were euthanized by cervical dislocation, and blood was collected via retroorbital puncture. Following disinfection with 75% alcohol, an incision was made through the skin and muscle between the hip joints using sterile ophthalmic scissors. Lower limbs were detached at the hip, and surrounding muscle tissues were carefully removed with scissors and cleaned with gauze. Femurs and tibias were excised by cutting at the ankle and approximately 1 cm distal to the tibiofibular junction to expose the marrow cavity. Cartilage at both ends was trimmed to expose the red marrow, which was flushed from the bone cavity using Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, USA). Fresh blood and bone marrow aspirates were transported and stored at 4°C for 24 h.

For cell isolation, samples were filtered through 40-μm sterile strainers and centrifuged at 300 *g* and 4°C for 5 min. Supernatants were discarded, and cell pellets were resuspended in 1 mL of phosphate-buffered saline (PBS, Invitrogen, USA). To remove red blood cells (RBCs), 3 mL of RBC lysis buffer (eBioscience, USA) was added, and the cells were incubated at 25°C for 5 min. The reaction was terminated by diluting with 8 mL of PBS, followed by centrifugation at 300 × *g* and 4°C for 5 min. Bone marrow cells were washed twice with PBS and centrifuged at 300 *g* and 4°C for 5 min, after which the supernatant was discarded. Single cells were resuspended in 0.04% bovine serum

albumin (BSA) in PBS and loaded on a 10× Chromium chip. Cell viability was assessed using trypan blue staining (Invitrogen, USA) and visualized under an inverted microscope (Zeiss, Germany).

Library preparation

ScRNA-seq was performed using the 10x Genomics Chromium Next GEM Single Cell 3' Reagent Kit v.3.1, followed by library construction. Approximately 16 000 cells were loaded per sample for gel bead-in-emulsion (GEM) generation and barcoding. Final libraries, ranging from 200 to 9 000 bp, were size-selected. The average fragment size, used as the insert size for library quantification, was determined by analyzing 1 µL of a 1:10 diluted sample using an Agilent Bioanalyzer High Sensitivity chip (Agilent Technologies, USA). Libraries were sequenced using the MGISEQ-2000 system (BGI, China) with paired-end reads.

Statistical analysis

All *P*-values were calculated using two-sided Student's *t*-tests under the assumption of equal variances to compare means between two independent groups. Statistical analyses were performed using R software (v.4.0.5).

Non-targeted metabolomics

Non-targeted metabolomic profiling was conducted on cecal content, feces, and serum collected from GF (*n*=11) and SPF (*n*=10) mice. Fecal samples were harvested within one hour of food intake, while serum and cecal contents were harvested after overnight fasting. Metabolite profiling was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a high-resolution Q Exactive HF mass spectrometer (Thermo Fisher Scientific, USA), acquiring data in both positive and negative ion modes to enhance metabolite coverage. Raw LC-MS/MS data were processed using Compound Discoverer v.3.1 (Thermo Fisher Scientific, USA) to perform peak extraction, peak alignment, and compound identification. Data preprocessing, statistical analysis, metabolite classification annotations, and functional annotations were performed using the metaX R package v.2.0.0 (Wen et al., 2017) and an established metabolomic bioinformatic pipeline. Principal component analysis (PCA) was applied to reduce the dimensionality of multivariate data and to visualize group separation, trends, and outliers among the observed variables. Partial least squares discriminant analysis (PLS-DA) was then used to identify metabolites based on variable importance in projection (VIP) scores from the first two principal components (PCs), integrated with variance assessment, fold change analysis, and Student's *t*-tests.

Preprocessing of scRNA-seq data

Single-cell RNA sequencing reads were aligned to the mm10 mouse reference genome using Cell Ranger v.6.0.2 (10x Genomics, USA). Unique molecular identifier (UMI)-based gene expression profiles were generated for each cell that passed quality control thresholds. Only confidently mapped, non-polymerase chain reaction (PCR) duplicates with valid barcodes and UMIs were retained for further analysis. From PB, 10 504 cells were derived from GF mice and 11 323 cells were obtained from SPF mice. From BM, 10 693 cells were derived from GF mice and 9 247 cells were obtained from SPF mice. Cells with UMI/gene numbers beyond three standard deviations from the mean, or with mitochondrial gene expression exceeding 10%, were filtered out to remove low-

quality, likely multiplet, or dying captures. After filtering, 18 344 high-quality PB cells (median: 1 426 genes per cell) and 16 537 high-quality BM cells (median: 1 391 genes per cell) were retained for downstream analyses. UMI counts were normalized using the NormalizeData function in the Seurat (v.4.0.3) package, applying a natural logarithmic transformation (base e).

Data integration and dimensionality reduction

To integrate datasets across conditions, canonical correlation analysis (CCA) was applied using the FindIntegrationAnchors and IntegrateData functions in Seurat (v.4.0.3) (Butler et al., 2018). PCA was then performed on variable genes using the RunPCA function in Seurat, followed by dimensionality reduction via uniform manifold approximation and projection (UMAP) using the umap-learn (v.0.5.1) package in Python (v.3.8.11). UMAP visualization was generated using the top 30 PCs with a resolution parameter of 1.0 (Supplementary Figure S1A). Cell clusters were annotated manually based on canonical markers curated from the CellMarker database (Zhang et al., 2019) and published articles.

Quantification of differences between major clusters

To quantify differences in cell-type distributions between GF and SPF mice, Bhattacharyya distances were calculated for each cell identity. Analysis was restricted to clusters containing more than 400 cells in both groups (Cillo et al., 2020). PCA embeddings derived from highly variable genes were computed using the top 30 PCs. For each pairwise comparison, 50 cells were randomly sampled from each cluster 100 times. To generate a background distribution for statistical comparison, another 50 cells were randomly sampled twice without replacement from a pooled set of the two clusters, simulating two artificial groups with no true difference. Significance was assessed by comparing the GF versus SPF distances to the null distribution using two-sided Student's *t*-tests.

Differential expression analysis

Cluster-specific marker genes were identified using the FindAllMarkers function in Seurat, applying the Wilcoxon rank-sum test. For direct comparisons between GF and SPF mice, differentially expressed genes (DEGs) were identified using the MAST (v.1.18.0) method via the FindMarkers function. DEGs with |log fold change|>0.26 and *P*≤0.05 were considered significant.

Gene functional annotation

Functional enrichment analysis of DEGs was performed using the clusterProfiler package (v.4.0.5) (Yu et al., 2012). Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were analyzed using biological process annotations from the org.Mm.eg.db database. Gene ratio was calculated as the proportion of DEGs annotated to a specific GO term compared with the proportion of genes in the whole genome annotated to that term. *P*-values were calculated with the enrichGO function in the clusterProfiler package and adjusted via the Benjamini-Hochberg method for multiple testing.

Pseudotime inference

To infer cellular differentiation trajectories, Monocle3 (v.1.3.1) (Cao et al., 2019) was used to process all immune cell subsets. Seurat objects were first converted to Monocle format, followed by dimensionality reduction and clustering

procedures implemented in Monocle3. The learn_graph function was used with use_partition=FALSE to construct trajectory graphs. Pseudotime scores were assigned to individual cells to quantify their progression along inferred differentiation paths. All visualizations were generated using built-in plotting functions in Monocle3.

Cell-cell interaction analysis

Cell-cell communication was assessed using CellChat (v.1.1.3) (Jin et al., 2021) to compare intercellular interactions between GF and SPF mice based on scRNA-seq data. GF and SPF datasets were merged following the standard CellChat workflow, and a unified CellChat object was constructed using the CellChatDB.mouse database. To assess the influence of microbiota on cell-cell interactions, key signaling pathways, such as BST2, ICAM, SELPLG, TGFb, and THBS, were identified in each group and visualized as chord plots.

RESULTS

Underdeveloped immune system confirmed by routine blood tests in GF mice

PB was collected from GF (n=11) and SPF mice (n=10) for routine hematological analysis (Table 1; Figure 1A). GF mice exhibited a significantly lower white blood cell (WBC) count compared to SPF mice (mean: 1.80×10⁹/L and 3.40×10⁹/L; P=0.040). Specifically, monocytes and neutrophil counts were significantly reduced under germ-free conditions (P=0.027 and P=0.014, respectively), whereas the reduction in lymphocyte

count did not reach statistical significance (P=0.097). Recent research has reported reduced platelet counts in GF mice relative to SPF controls, including both wild-type and IL-1 receptor 1 knockout strains (Kovtonyuk et al., 2022). In the present study (Table 1), platelet count was not significantly different between groups (P=0.233); however, both mean platelet volume (MPV) and platelet distribution width (PDW) were significantly decreased in GF mice.

Major transcriptional changes in GF and SPF mice revealed by single-cell profiling

To investigate how the microbiota shapes immune cell composition and transcriptional regulation, a comprehensive single-cell transcriptomic atlas of the hematopoietic system was generated from adult GF and SPF mice using scRNA-seq of PB and BM (Figure 1A, D). A total of 21 827 PB cells and 19 940 BM cells were captured from one GF and one SPF mouse. Following stringent quality control and data integration, 18 344 high-quality PB cells (median: 1 426 genes per cell) and 16 537 high-quality BM cells (median: 1 391 genes per cell) were retained for downstream analyses.

Unsupervised clustering of the combined dataset (34 881 cells) resolved 18 major cell identities (Supplementary Figure S1B), which were further refined into 25 subpopulations (Figure 1B). Cell types were annotated based on known marker genes for hematopoietic lineages (Figure 1C; Supplementary Figure S2). Eosinophils were not detected in the scRNA-seq data, consistent with their absence in routine blood analysis (Table 1).

To assess transcriptional divergences between GF and SPF

Table 1 Routine blood tests in germ-free (GF) and specific pathogen-free (SPF) Kunming mice

Test (unit)	GF (n=11)	SPF (n=10)	P-value
WBC (10 ⁹ /L)	1.80±1.08	3.40±1.98	0.040
LYM (10 ⁹ /L)	1.44±0.96	2.45±1.56	0.097
MON (10 ⁹ /L)	0.06±0.03	0.17±0.14	0.027
NEU (10 ⁹ /L)	0.30±0.14	0.78±0.48	0.014
EOS (10 ⁹ /L)	0.00±0.00	0.00±0.00	N/A
BAS (10 ⁹ /L)	0.00±0.00	0.00±0.00	N/A
LYM% (%)	77.77±6.57	69.66±12.05	0.080
MON% (%)	3.44±2.17	5.72±2.57	0.042
NEU% (%)	18.81±6.46	24.62±10.61	0.156
EOS% (%)	0.00±0.00	0.00±0.00	N/A
BAS% (%)	0.00±0.00	0.00±0.00	N/A
RBC (10 ¹² /L)	10.98±0.84	10.68±1.05	0.477
HGB (g/L)	14.44±1.31	13.76±1.69	0.324
HCT (%)	51.62±6.29	50.62±5.70	0.705
MCV (fL)	47.00±3.58	47.50±2.80	0.724
MCH (pg)	13.15±0.34	12.89±0.82	0.361
MCHC (g/L)	28.12±1.65	27.23±1.94	0.277
RDWc (%)	20.13±0.99	19.43±0.93	0.113
RDWs (fL)	35.37±3.45	34.53±1.68	0.482
PLT (10 ⁹ /L)	464.55±144.97	540.00±135.75	0.233
MPV (fL)	6.78±0.28	7.77±1.13	0.023
PCT (%)	0.32±0.10	0.43±0.15	0.073
PDWc (%)	31.89±2.48	35.03±3.30	0.026
PDWs (fL)	10.47±1.98	13.66±3.80	0.033

WBC, white blood cell count; LYM, lymphocyte count; MON, monocyte count; NEU, neutrophil count; EOS, eosinophil count; BAS, basophil count; LYM%, lymphocyte ratio; MON%, monocyte ratio; NEU%, neutrophil ratio; EOS%, eosinophil ratio; BAS%, basophil ratio; RBC, red blood cell count; HGB, hemoglobin; HCT, hematocrit; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; RDWc, red cell distribution width (CV); RDWs, red cell distribution width (SD); PLT, platelet count; MPV, mean platelet volume; PCT, plateletcrit; PDWc, platelet distribution width (CV); PDWs, platelet distribution width (SD); N/A, not applicable. P-values were calculated using Student's t-test.

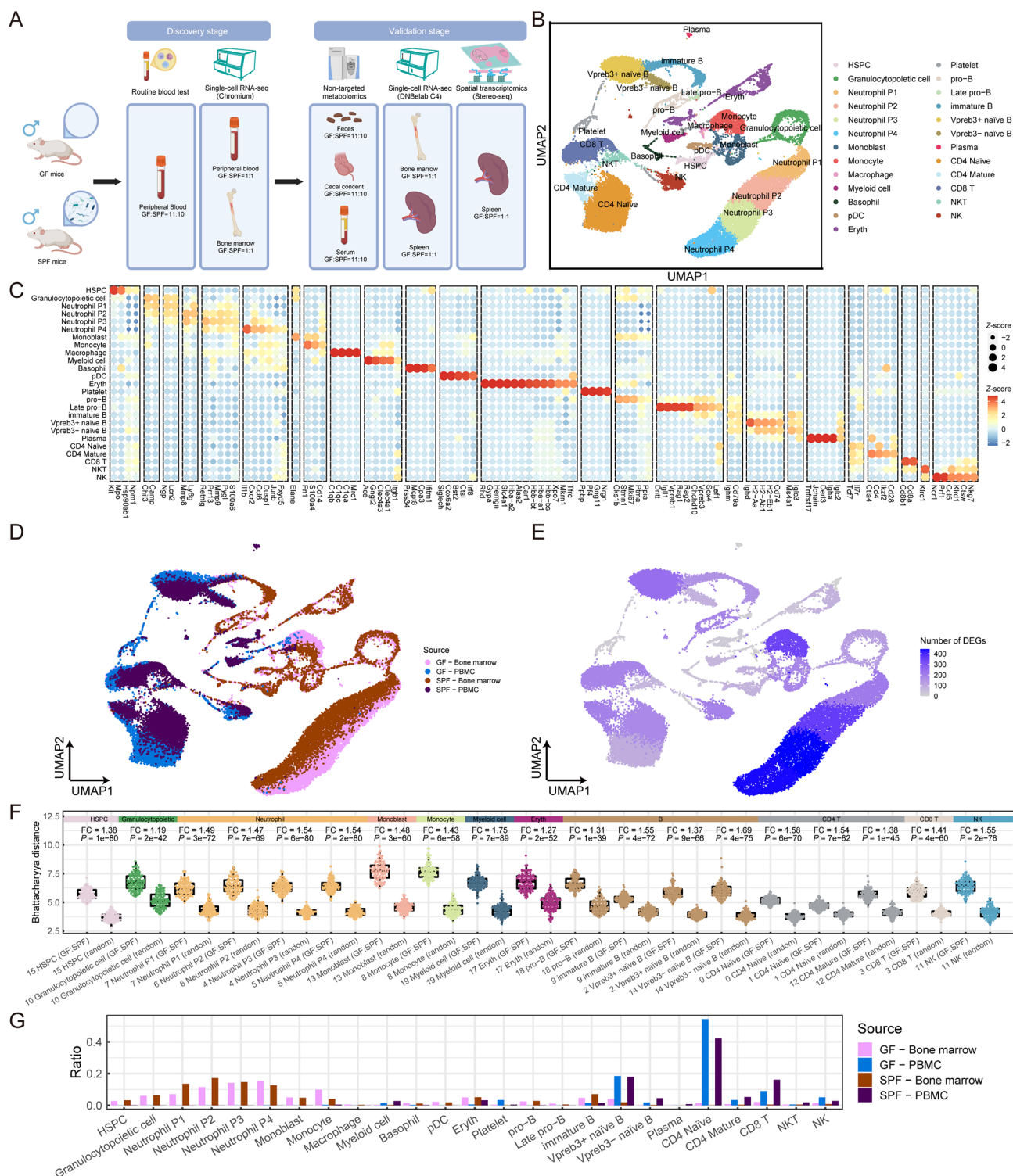


Figure 1 Transcriptional and cellular landscape differences between germ-free (GF) and specific pathogen-free (SPF) mice in peripheral blood (PB) and bone marrow (BM)

A: Schematic overview of entire study. Illustration was created with BioRender. **B:** Uniform manifold approximation and projection (UMAP) visualization of 27 cell subpopulations. HSPC, hematopoietic stem and progenitor cells; pDCs, plasmacytoid dendritic cells; Eryth, erythrocytes; pro-B, progenitor B cells; NK, natural killer cells; NKT, natural killer T cells. **C:** Dot plot showing expression of key marker genes across all cell subpopulations. Dot size and color denote Z-score values. **D:** UMAP showing the tissue origin of each cell. PBMCs, peripheral blood mononuclear cells. **E:** UMAP highlighting number of differentially expressed genes (DEGs) between GF and SPF mice within each cluster. **F:** Quantification of transcriptional divergence in major immune cell types between GF and SPF mice. Each dot represents a subsample of 50 cells in PCA space. Horizontal axis represents cluster identity. *P*, *P*-value by Student's *t*-test. **G:** Bar plot showing relative proportions of each immune subpopulation in GF and SPF samples.

mice, two complementary strategies were applied. DEGs were first identified for each immune cell identity, and their distribution was visualized using UMAP (Figure 1E). Widespread transcriptional changes were observed, with the most substantial differences detected in neutrophils, monocytes, and B cells. The number of DEGs increased along differentiation trajectories, particularly within neutrophil lineages (Supplementary Figure S1D). To further quantify transcriptional dissimilarity, the Bhattacharyya distance was calculated across major immune cell identities (Cillo et al., 2020). Clusters with fewer than 400 cells were excluded due to limited statistical robustness (Materials and Methods). Significant expression differences were identified in neutrophils, monoblasts, monocytes, myeloid cells, B cells, CD4⁺ T cells, and natural killer (NK) cells (Figure 1F). All comparisons yielded consistent significance across 100 resampling replicates, with fold changes ranging from 1.82-fold (myeloid cells) to 1.19-fold (granulocytopoietic cells). Although less prominent, changes in hematopoietic stem and progenitor cells were also observed, indicating that this population may be directly or indirectly regulated by the microbiota and its metabolites.

Together, these data define key transcriptional and compositional differences between GF and SPF mice, with pronounced alterations in neutrophils and monocytes (Figure 1E, F, G). These findings are consistent with reductions in these cell types observed in routine blood tests and support a role for the microbiota in regulating immune cell gene expression, potentially through modulation of proliferation or apoptosis.

HCAR2 activation as a potential mechanism regulating neutrophil abundance

Bone marrow continuously generates neutrophils through cytokine signaling (such as G-CSF), with subsequent release into blood circulation (Furze & Rankin, 2008). Previous studies have shown that the microbiota influences neutrophil production and function throughout life (Kramer et al., 2014; Manz & Boettcher, 2014). However, the precise molecular mechanisms, pathways, and genes involved remain unclear. To better understand the role of microbiota in bone marrow neutrophil development, neutrophils were extracted from the scRNA-seq dataset for downstream analysis. Clustering resolved four distinct neutrophil populations (P1–P4, Figure 2A), corresponding to progressive developmental stages based on established marker genes (Grieshaber-Bouyer et al., 2021). These populations were arranged according to their developmental progression (Supplementary Figure S1D), with neutrophil P1 representing the earliest stage derived from granulocytopoietic cells and neutrophil P4 representing a late-stage population approaching transcriptional profiles characteristic of mature neutrophils.

Differential expression analysis between GF and SPF mice identified stage-specific up-regulated and down-regulated genes. GO enrichment analysis revealed activation of interleukin-1 beta (IL-1 β)-related pathways, consistent with more potent antibacterial effects in GF mice. Specifically, *Arg2*, *Egr1*, and *Tlr2*, key genes involved in IL-1 β signaling, were up-regulated in GF mice (Figure 2B–D). In contrast, down-regulated genes were enriched in actin-related pathways, including actin cytoskeleton organization and actin filament-based processes (Supplementary Figure S3), especially in the early stages, suggesting impaired mobility

and division in early-stage neutrophils under germ-free conditions.

Among genes differentially expressed in mature neutrophils (P4), *Hcar2* was significantly up-regulated in GF mice compared to SPF mice (Figure 2B, D). HCAR2 (also known as GPR109A) is a high-affinity receptor for both nicotinic acid (NA; or niacin) and (D)-beta-hydroxybutyrate and mediates NA-induced apoptosis in mature neutrophils (Taggart et al., 2005). NA binding to HCAR2 reduces intracellular cAMP levels, suppressing protein kinase A activity and triggering downstream phosphorylation of target proteins, leading to neutrophil apoptosis (Kostylina et al., 2008). Thus, we hypothesized that *Hcar2* expression is regulated by NA or related microbial metabolites. To validate the up-regulation of *Hcar2*, scRNA-seq was performed on BM cells from GF ($n=1$) and SPF ($n=1$) mice using the DNBelab C4 platform (BGI, China). Results confirmed a significant reduction in neutrophils and a robust up-regulation of *Hcar2* in GF mice ($P<0.0001$, Supplementary Figure S4C). Furthermore, a recent study using scRNA-seq and spatial transcriptomics in the spleen of GF ($n=1$) and SPF ($n=1$) mice similarly reported reduced neutrophil abundance in GF mice (Figure 2E, F) (Zhang et al., 2023). Spatial transcriptomic analysis of the spleen revealed distinct expression patterns of three neutrophil marker genes—*Il1b*, *Ccl6*, and *Ly6g*—highlighting a marked reduction in neutrophil abundance in GF mice compared to SPF controls (Figure 2F). Notably, scRNA-seq of splenic tissue also confirmed a significant up-regulation of *Hcar2* in neutrophils from GF mice (Figure 2E).

To further investigate alterations in NA metabolism, non-targeted metabolomics was conducted on cecal contents, feces, and serum from GF ($n=11$) and SPF mice ($n=10$) (Figures 1A, 2G; Supplementary Data). Fecal samples from GF mice exhibited markedly reduced levels of 6-hydroxynicotinic acid, a key NA degradation product generated by niacin dehydrogenase—an enzyme primarily produced by *Pseudomonas putida* KT2440 (Jimenez et al., 2008; Yang et al., 2009). NA levels were also diminished in cecal content. In contrast, levels of nicotinamide (NAM), a downstream product resulting from the conversion of NA when it is in excess, and trigonelline, a product of NA metabolism typically excreted in mammalian urine, were both elevated in GF samples.

These findings indicate that the absence of microbiota in GF mice suppresses the nicotinate degradation pathway, likely due to the loss of microbial enzymes such as niacin dehydrogenase. As a result, excess NA is redirected toward alternative metabolic products, including trigonelline, NAM, and nicotinuric acid (NUA). The accumulation of NA and its derivatives may contribute to the up-regulation of *Hcar2*, a receptor previously shown to promote apoptosis in mature neutrophils, thus representing a potential mechanism linking microbial absence to neutrophil depletion.

Enhanced bacterial recognition and impaired antiviral responses in GF mouse monocytes

Monocytes and macrophages are critical mediators of immune regulation, contributing to immunosuppression, tissue repair, resolution of inflammation, bacterial clearance, atherosclerosis, and fibrosis. Monoblasts were distinguished from monocytes based on elevated expression of *Irf8* and *Elane* (Figure 1C), and both monoblasts and monocytes from PB and BM were extracted for further analysis. Macrophages

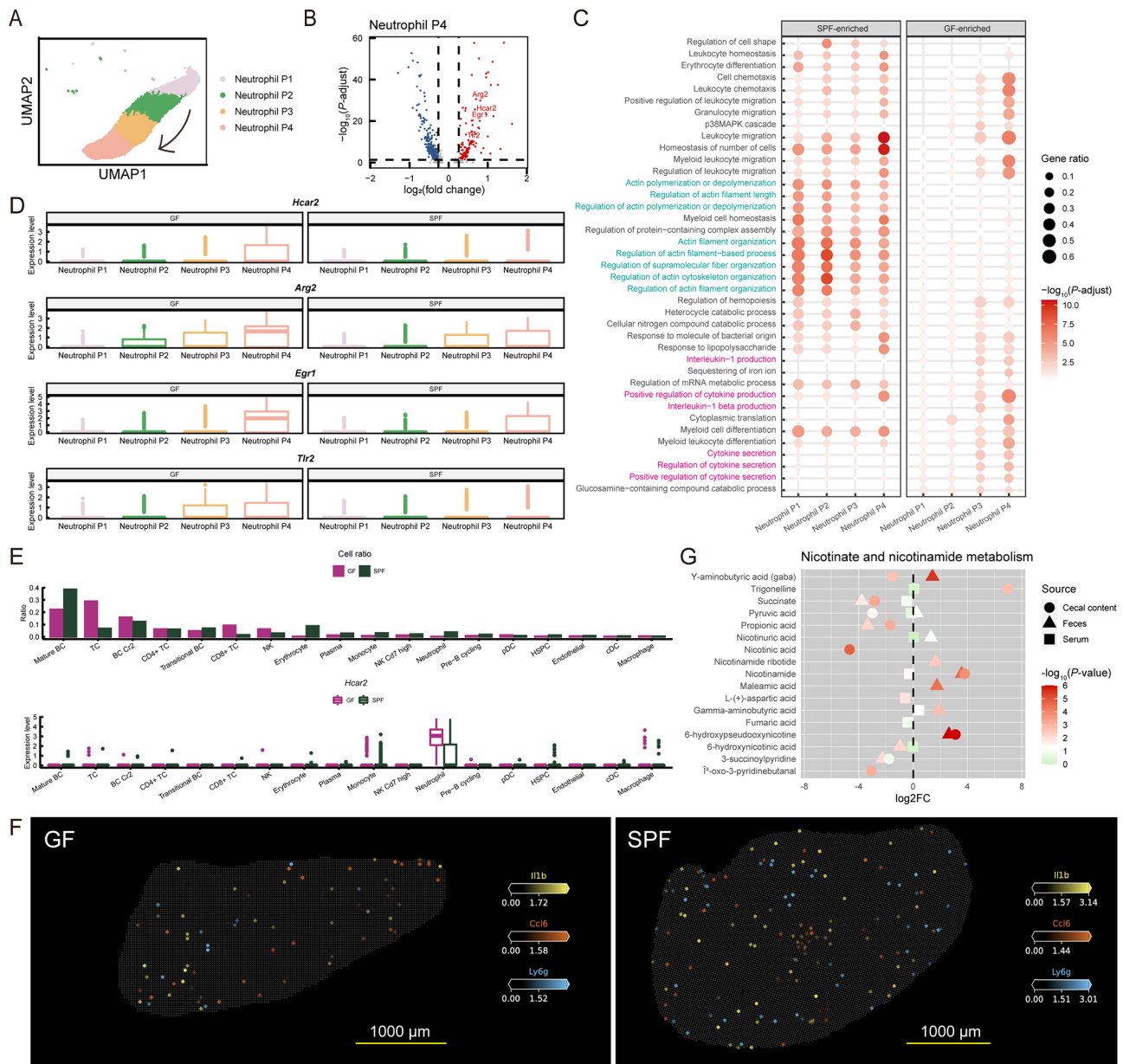


Figure 2 HCAR2 signaling as a potential regulator of neutrophil abundance in GF mice

A: UMAP plot showing partitioning of neutrophils into four developmental periods (P1–P4). **B:** Volcano plot showing up- and down-regulated DEGs in neutrophils of GF mice versus SPF mice based on fold change and P -value thresholds. **C:** GO enrichment analysis comparing DEGs between GF and SPF mice across neutrophil stages. Blue entries are actin-related functions enriched in SPF mice, pink entries are interleukin-1-related functions enriched in GF mice. **D:** Box plots showing *Hcar2*, *Arg2*, *Egr1*, and *Tlr2* expression in neutrophils across four developmental stages in GF and SPF mice. **E:** Bar plot showing cell-type proportions and *Hcar2* up-regulation in spleen immune populations from GF and SPF mice. BCs, B cells; TCs, T cells; Pre-B, precursor B cells; cDCs, conventional dendritic cells. **F:** Spatial transcriptomic maps of three neutrophil marker genes in spleen sections, visualizing neutrophil distribution in GF versus SPF mice. **G:** Differential abundance of metabolites in the nicotinate and nicotinamide metabolism pathways in GF versus SPF mice across sample types, based on non-targeted metabolomics.

were excluded due to insufficient cell numbers for statistical comparison (Figure 3A).

Differential expression analysis revealed elevated expression of versican (*Vcan*) in both monoblasts and monocytes of GF mice (Figure 3B). *Vcan* encodes a large chondroitin sulfate/dermatan sulfate proteoglycan in the aggrecan/lexican family (Islam & Watanabe, 2020), which has been implicated in inflammatory responses and tumor progression (Islam & Watanabe, 2020; Wight et al., 2020). *Vcan* is also known to impair cell migration, slow proliferation, inhibit cytodifferentiation, and enhance monocyte adhesion to the extracellular matrix (ECM) (Kang et al., 2017), suggesting

that its up-regulation may contribute to altered monocyte function and impaired development in GF mice. Moreover, *Cd14* was highly expressed in monocytes but not in monoblasts in GF mice (Figure 3B). CD14 functions as a coreceptor for bacterial lipopolysaccharide (LPS) and other pathogen-associated molecular patterns, including lipoteichoic acid. The soluble form, sCD14, extends LPS responsiveness to cells lacking *Cd14* expression and serves as a marker of monocyte activation (Tapping & Tobias, 2000). The observed up-regulation of *Cd14* suggests increased microbial recognition in GF mice, which may reflect a predisposition toward inflammatory activation. Elevated *Cd14* expression

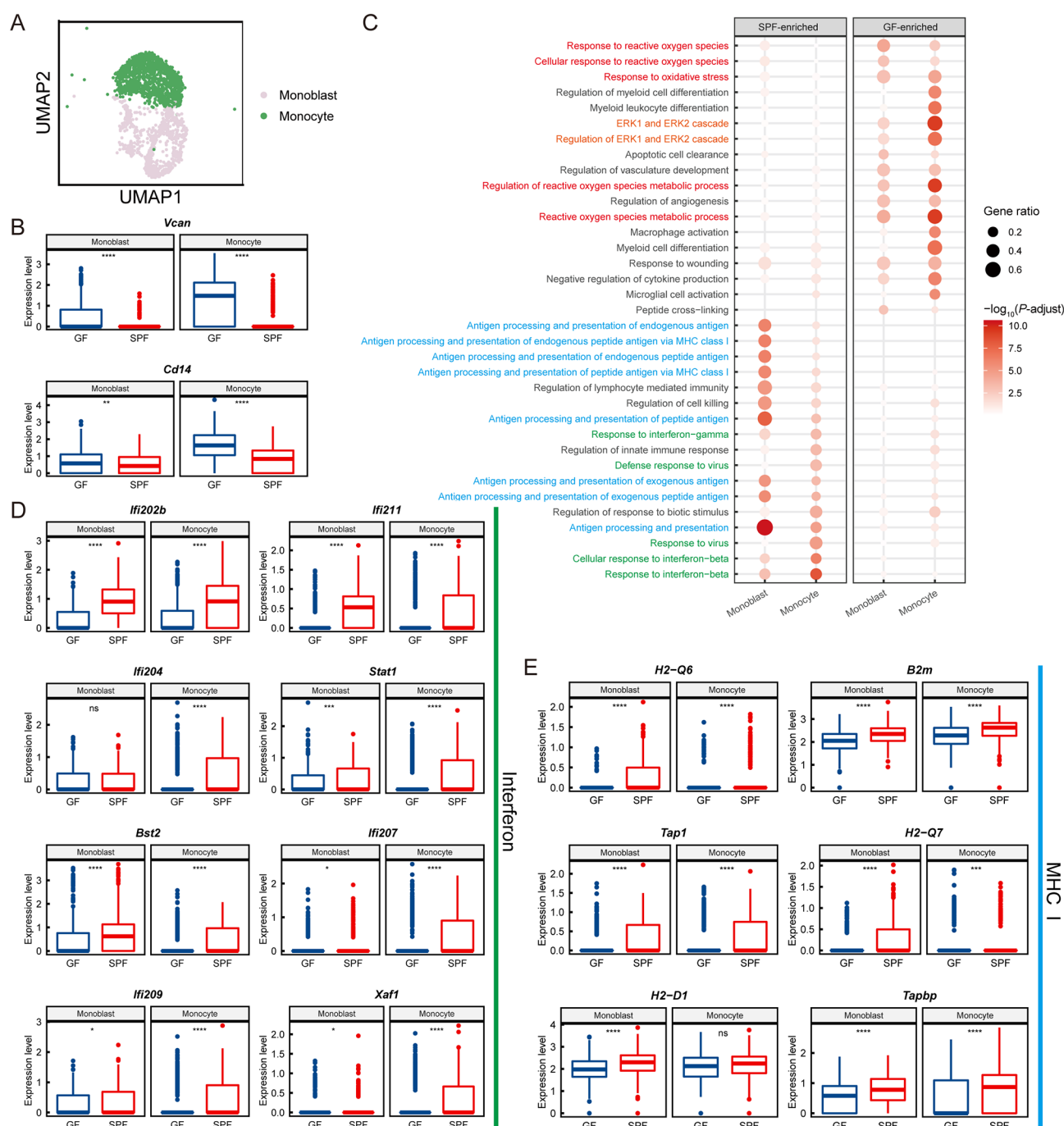


Figure 3 Increased bacterial recognition and impaired viral defense in monocytes of GF mice

A: UMAP plot separating monocytes into two subtypes: monoblasts and monocytes. B: Box plots showing up-regulated expression of *Vcan* and *Cd14* in GF monoblasts and monocytes. ns: Not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$. C: GO enrichment analysis of DEGs between GF and SPF mice in monoblasts and monocytes. D: Box plots showing down-regulated expression of genes involved in interferon pathways in monoblasts and monocytes of GF mice. E: Box plots showing down-regulated expression of genes involved in antigen processing and presentation in GF monoblasts and monocytes. MHC I: Major histocompatibility complex class I.

was also detected in neutrophil populations (P1–P4). Supporting these findings, differential expression analysis of an independent cecal transcriptome dataset (Chai et al., 2024) revealed significant up-regulation of *Cd14* in enterocytes from GF mice compared to SPF mice (fold change=1.28, $P=6.65\text{e-}17$).

GO enrichment of up-regulated genes in GF monocytes revealed significant association with reactive oxygen species (ROS) pathways, ERK1 and ERK2 signaling cascades,

myeloid differentiation, and apoptotic cell clearance (Figure 3C). While ROS are well-known inducers of inflammation (Mittal et al., 2014) and ERK signaling promotes cell proliferation and survival (Bahar et al., 2023), their combined activation under oxidative stress typically induces cell cycle arrest and apoptosis (Kim & Wong, 2009; Mebratu & Tesfaigzi, 2009), potentially explaining the reduced monocyte count observed in GF mice. In contrast, down-regulated genes were enriched in pathways related to antigen processing and

presentation as well as interferon signaling (Figure 3D, E), indicating a diminished capacity for antiviral defense. This includes impaired responses to interferon- β (IFN- β) and interferon- γ (IFN- γ), both of which are essential for antiviral immunity and endogenous peptide antigen presentation. These transcriptomic findings are consistent with previous reports of increased susceptibility of GF mice to viral infections, including influenza A virus (Dolowy & Muldoon, 1964), coxsackie B virus (Schaffer et al., 1963), and Friend virus (Mirand & Grace, 1963).

Elevated 5'-methylthioadenosine (MTA) excretion is associated with immunodeficiency

MTA is a sulfur-containing nucleoside present in all mammalian tissues and is synthesized primarily via the polyamine biosynthetic pathway from S-adenosylmethionine (Sauter et al., 2013). MTA functions as a potent regulator of multiple enzymatic processes and has been implicated in the regulation of gene expression, cell proliferation, differentiation, and apoptosis (Avila et al., 2004). Elevated MTA excretion has previously been reported in children with severe combined immunodeficiency syndrome (SCID) (Mills & Mills, 1985). Consistent with these observations, significantly elevated MTA levels were detected in GF mice with underdeveloped immune systems. Specifically, MTA concentrations were increased in serum (fold change=1.5304, $P=0.0121$), feces (fold change=2.3591, $P=0.0211$), and cecal contents (fold change=4.4906, $P=0.0023$), suggesting a potential link between elevated MTA and immunodeficiency under microbiota-depleted conditions.

Disruption of BST2-mediated signaling may inhibit immune activation in GF mice

Given the transcriptomic alterations observed in genes encoding receptor-ligand pairs in response to microbial absence, cell-cell communication was inferred using CellChat (Jin et al., 2021) to identify global signaling changes across all immune cell identities in PB and BM, excluding plasma cells, which were absent in GF mice. The total number and overall strength of inferred intercellular interactions were markedly reduced in GF mice compared to SPF controls (Figure 4A). Notably, signaling directed toward natural killer T (NKT) cells was diminished or abolished across all sending populations, indicating a potentially impaired capacity of NKT cells in GF mice to receive extracellular cues. Similarly, CD8⁺ T cells exhibited substantially reduced interaction strength with other lymphocyte subsets, including NK cells, suggesting a broader disruption of lymphoid network integrity. In contrast, macrophages showed increased bidirectional communication, implying a compensatory shift in signaling dynamics under microbial depletion (Figure 4B).

To further quantify differences in intercellular communication, CellChat was used to calculate pathway-level signaling distance among the 24 cell groups in the GF and SPF datasets (Figure 4C). The BST2 signaling pathway emerged as the most significantly altered. The ligand gene *Bst2*, expressed by BM stromal cells, plays a critical role in promoting pre-B cell growth in mice (Ishikawa et al., 1995), with *Pira2* identified as its receptor. In GF mice, BST2 signaling was markedly inhibited in basophils, platelets, Vpreb3-naïve B cells, and all T cell subpopulations due to the down-regulation of *Bst2* expression in these populations (Figure 4D, E). Given its essential role in antiviral immunity, reduced BST2 may underlie the impaired T cell responses and

increased susceptibility to viral infections observed in GF mice (Urata et al., 2018). Reduced *Bst2* expression suggests a diminished immune response, indicating that microbial stimuli are essential for effective immune defense. These findings highlight the critical role of microbial exposure in shaping robust antiviral responses and immune defenses.

Despite widespread *Bst2* down-regulation in GF mice, plasmacytoid dendritic cells (pDCs) retained high *Bst2* expression levels in both GF and SPF mice (Figure 4E). Previous studies have shown that *Bst2* is constitutively expressed in mature B cells, plasma cells, and pDCs, while its expression in other immune cell types is largely stimulus-dependent, particularly through interferon signaling (El-Sherbiny et al., 2020; Le Tortorec et al., 2011). Importantly, BST2 engagement with ILT7 (human homolog of *Pira2*) in pDCs negatively regulates Toll-like receptor 7 and 9 (TLR7/TLR9) activation, thereby modulating IFN-I secretion (Cao et al., 2009). Collectively, these results suggest that loss of microbiota suppresses BST2-dependent signaling in key immune lineages, contributing to compromised antiviral capacity in GF mice.

DISCUSSION

Although GF rodent colonies have been in use for nearly eight decades, providing considerable understanding of microbial function (Kennedy et al., 2018), many aspects of host-microbiota interactions, particularly those involving the immune system, remain unresolved due to their complexity and context-dependent nature. This study represents the first integrated application of scRNA-seq and non-targeted metabolomics to characterize how the absence of microbiota alters immune cell development and transcriptional programming in GF mice. Our data not only reinforce the long-established view that GF mice exhibit systemic immune system underdevelopment but also provide preliminary insights into possible molecular and cellular mechanisms contributing to this phenotype. Routine hematological profiling revealed substantial reductions in circulating immune cells in GF mice, with neutrophils and monocytes reduced to approximately 40% of levels observed in SPF mice. However, lymphocyte counts showed no significant change. Notably, scRNA-seq failed to detect plasma cells in either PB or BM of GF mice, raising the possibility that gut microbial signals are required for their differentiation or maintenance. Together, these results indicate widespread deficits across immune lineages, with cell type-specific variation in sensitivity to microbiota absence.

Among myeloid cells, neutrophils and monocytes were especially depleted in GF mice, consistent with previous reports from antibiotic-treated models (Khosravi et al., 2014). Neutrophils are essential for the innate immune response against bacteria and fungi, with their development proceeding through post-mitotic maturation over 5–6 days in the BM before release into circulation (Macallan et al., 1998), after which they undergo spontaneous apoptosis and removal by other phagocytes, a default process altered by interactions with microbes and their products (Kobayashi et al., 2017). Our findings suggest that the accumulation of niacin and its derivatives, potentially resulting from the absence of niacin microbial dehydrogenase, may promote neutrophil apoptosis, although this hypothesis requires validation in a larger cohort. *Hcar2*, which encodes the high-affinity niacin receptor HCAR2, was also significantly up-regulated in GF mice and has been

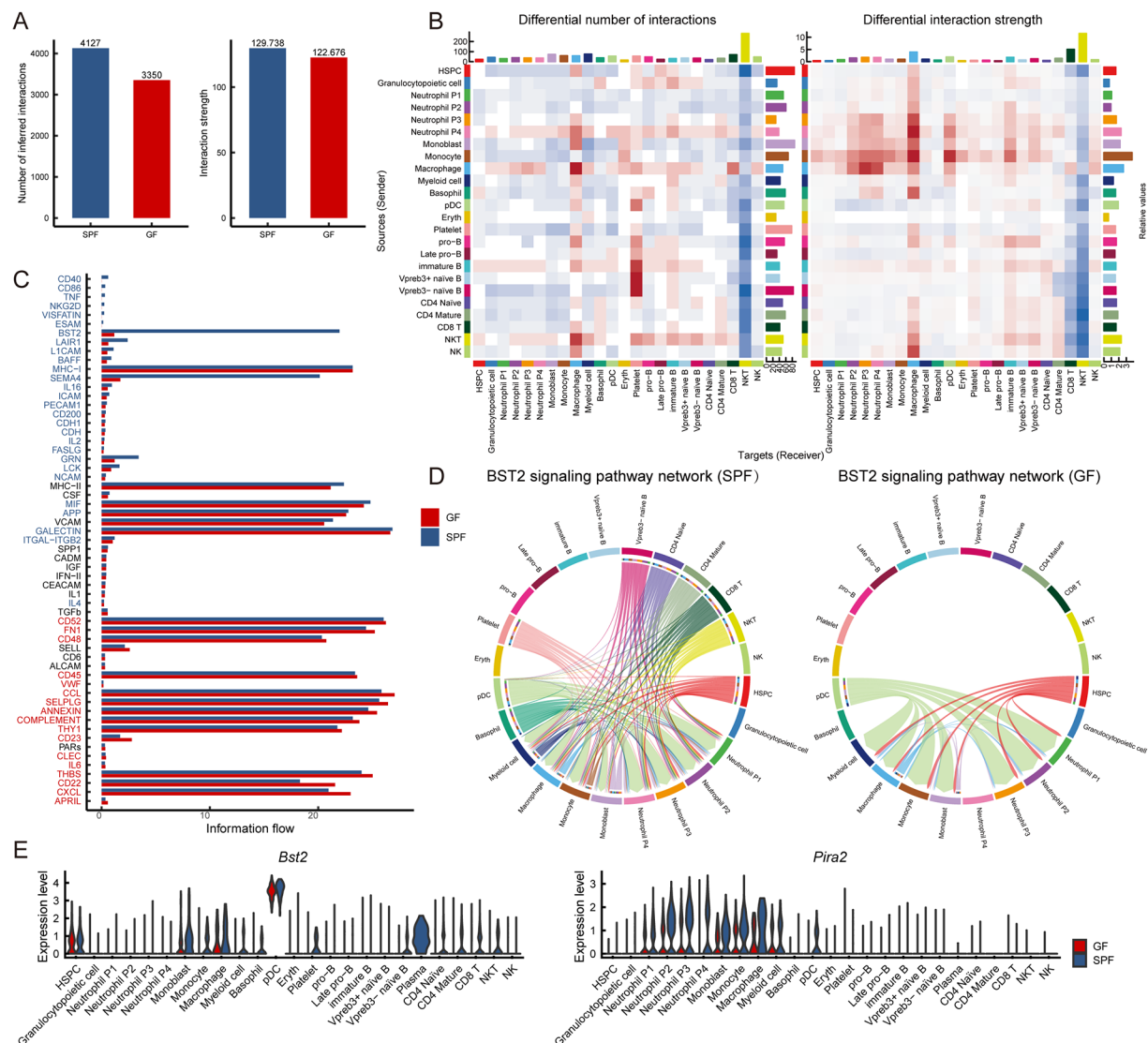


Figure 4 Major differences in the BST2 signaling pathway between GF and SPF mice

A: Bar plots showing differences in the number and strength of inferred intercellular interactions between GF and SPF mice. B: Heatmap showing differential interaction counts and strengths between immune cell types. Top and right plots represent the sums of the columns and rows (incoming and outgoing signals), respectively. Red (blue) represents increased (decreased) signaling in GF mice compared with SPF mice. C: Bar plot showing differences in information flow for each signaling pathway between GF and SPF mice. Information flow is defined as the sum of communication probabilities among all pairs of cell groups in the inferred network (i.e., total weight in the network). Significant signaling pathways were ranked based on differences in overall information flow within the inferred networks between GF and SPF mice. Top signaling pathways in red are enriched in SPF mice, and in blue are enriched in GF mice. D: Chord plots showing detailed BST2 pathway interactions. Edge colors are consistent with sources as senders, and edge weights are proportional to interaction strength. Thicker edge lines indicate stronger signals. Inner bar colors represent targets receiving signals from the corresponding outer bar. Size of the inner bar is proportional to signal strength received by the target. E: Violin plots illustrating *Bst2* (ligand) and *Pira2* (receptor) expression within the BST2 signaling pathway in GF mice versus SPF mice.

reported to mediate niacin-induced neutrophil apoptosis (Taggart et al., 2005). It is important to note that the P4 neutrophil population identified in this study reflects a near-mature stage restricted to the bone marrow, rather than fully mature circulating neutrophils.

In antibiotic-treated mice, monocytes exhibit reduced migratory capacity and decreased abundance in peripheral tissues (Emal et al., 2017; Zhang et al., 2015), despite their numbers in BM and PB remaining largely unchanged (Josefsdottir et al., 2017; Zhang et al., 2015). However, our study revealed significant monocyte deficiencies in GF mice, with routine blood tests showing markedly reduced counts (Figure 1G). While scRNA-seq indicated increased monocyte

representation, absolute cell numbers from single-cell data are sensitive to various factors, such as cell viability, library preparation, sequencing depth, and data processing. Consequently, we did not use absolute cell numbers in scRNA-seq to compare different samples, especially when better and more direct methods (e.g., routine blood tests) were available. Based on our observations, two plausible mechanisms may underlie the decreased monocyte counts in GF mice: impaired development due to ROS-induced ERK activation and restricted migration associated with up-regulation of *Vcan* expression.

Analysis of intercellular signaling further implicated impaired antiviral immunity in GF mice. CellChat-based inference

revealed broad reductions in signaling activity across multiple immune cell populations, with marked suppression of BST2-mediated communication. *Bst2*, which encodes tetherin, is highly expressed in pDCs and supports IFN-I production critical for antiviral responses (Ishikawa et al., 1995; Cao et al., 2009). In GF mice, *Bst2* was down-regulated in basophils, T cells, platelets, and naïve B cells, suggesting widespread disruption of BST2-dependent signaling pathways. Although *Bst2* expression remained high in pDCs, reduced responsiveness in other immune compartments may contribute to the heightened viral susceptibility previously reported in GF mice (Dolowy and Muldoon, 1964; Schaffer et al., 1963; Mirand and Grace, 1963). Future work will include functional validation of BST2 pathway disruption, with a particular focus on the role of pDCs in orchestrating IFN-I-driven antiviral immunity.

How microbiota-derived metabolites affect the development of immune cells such as neutrophils through systemic circulation was also explored. While fecal and cecal samples from GF mice displayed pronounced alterations in metabolic composition, serum metabolite differences were modest. This discrepancy likely reflects dilution of localized metabolic changes in whole-body serum pools, suggesting that microbiota–host interactions may exert regionally restricted effects—particularly within the gastrointestinal tract or liver—that are not readily detected in systemic circulation.

In summary, GF mice exhibit broad immunodeficiency across multiple immune lineages. This study provides a foundational framework for understanding immune underdevelopment in the absence of microbiota. The resulting reference dataset serves as a valuable resource for studying microbiota–host immune system crosstalk and provides a reference for future efforts to design interventions targeting immune dysfunction driven by microbial dysbiosis.

LIMITATIONS

This study has several limitations to mention. First, the sample size was relatively small, with only GF and one SPF mouse used in both the discovery and validation stages. However, both PB and BM samples were collected from the same individuals in the discovery cohort, ensuring that there was no sample bias between tissue types. Second, the scRNA-seq data generated using the droplet-based 10× Genomics Chromium platform exhibited incomplete coverage, especially with respect to neutrophils in the PB subset. This limitation is inherent to the platform, which often underrepresents mature neutrophils and other granulocytes due to their low RNA content, high RNAase activity, and other inhibitory compounds, leading to the detection of fewer transcripts and sequencing reads.

CONCLUSIONS

This study provides preliminary insights into the potential mechanisms underlying immune system underdevelopment in GF mice based on integrated scRNA-seq and non-targeted metabolomic analyses, warranting further investigation with larger sample sizes. Results showed a marked decline in immune cell abundance, particularly neutrophils and monocytes, in GF mice. Analyses suggested that the reduction in neutrophils may be associated with changes in niacin metabolism, potentially related to the absence of microbial niacin dehydrogenase. In addition, *Hcar2* was up-

regulated in GF mice, potentially promoting neutrophil apoptosis. Gene expression analysis of monocytes showed enhanced bacterial recognition but diminished viral defense. Analysis of cell–cell interactions demonstrated reduced BST2 signaling activity in GF mice, which may inhibit immune response activation. Collectively, these findings highlight microbiota-dependent regulation of immune cell development and function and establish a foundation for future studies aimed at understanding and therapeutically targeting immune deficiencies associated with microbial dysbiosis.

DATA AVAILABILITY

The scRNA-seq raw data, expression matrix, and metadata generated using 10× Chromium, as well as the raw data from non-targeted metabolomics, have been deposited in the CNGB Nucleotide Sequence Archive (CNSA) under accession code CNP0002818 (<https://db.cngb.org/cnsa/>). The raw and processed scRNA-seq data generated in this study are also available from the NCBI database (GSE295200), China National Center for Bioinformation database of Genome Sequence Archive (GSA) (CRA024883), and Science Data Bank database (<https://doi.org/10.57760/sciencedb.j00139.00203>). The spleen scRNA-seq and spatial transcriptomic data used in this study are available via CNSA under accession number CNP0003930, while the cecum scRNA-seq data can be found under CNP0004192. The non-targeted metabolomic results are provided in the Supplementary Materials. All datasets were analyzed using standard programs and packages, as detailed above. Additional information, code, and resources supporting the findings of this study are available from the corresponding author upon reasonable request.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Y.F.S., W.C., H.W., L.J.H., and X.D.F. contributed to the conception and design of the study. J.S., Y.F.S., R.Z.Z., T.L.C., X.H., and S.S.P. participated in experimental design. W.C.'s team was responsible for sampling. Y.F.S., Y.Z., Q.J.L., C.W., W.N.H. and B.W. performed statistical analyses. Y.F.S. wrote the draft of the manuscript. J.S., Q.J.L., L.J.H., Y.R.Z., R.X.C., J.P.M., and X.D.F. revised the manuscript. All authors read and approved the final version of the manuscript.

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REFERENCES

- Avila MA, García-Trevijano ER, Lu SC, et al. 2004. Methylthioadenosine. *The International Journal of Biochemistry & Cell Biology*, **36**(11): 2125–2130.
- Bahar E, Kim HJ, Kim DR. 2023. Targeting the RAS/RAF/MAPK pathway for cancer therapy: from mechanism to clinical studies. *Signal Transduction and Targeted Therapy*, **8**(1): 455.
- Bakdash G, Vogelpoel LT, Van Capel TM, et al. 2015. Retinoic acid primes human dendritic cells to induce gut-homing, IL-10-producing regulatory T cells. *Mucosal Immunology*, **8**(2): 265–278.
- Balmer ML, Schürch CM, Saito Y, et al. 2014. Microbiota-derived compounds drive steady-state granulopoiesis via MyD88/TICAM signaling.

The Journal of Immunology, **193**(10): 5273–5283.

Bassler BL, Losick R. 2006. Bacterially speaking. *Cell*, **125**(2): 237–246.

Butler A, Hoffman P, Smibert P, et al. 2018. Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nature Biotechnology*, **36**(5): 411–420.

Cao JY, Spielmann M, Qiu XJ, et al. 2019. The single-cell transcriptional landscape of mammalian organogenesis. *Nature*, **566**(7745): 496–502.

Cao W, Bover L, Cho M, et al. 2009. Regulation of TLR7/9 responses in plasmacytoid dendritic cells by BST2 and ILT7 receptor interaction. *Journal of Experimental Medicine*, **206**(7): 1603–1614.

Chai TL, Shen J, Sheng YF, et al. 2024. Effects of flora deficiency on the structure and function of the large intestine. *iScience*, **27**(2): 108941.

Chen YE, Fischbach MA, Belkaid Y. 2018. Skin microbiota-host interactions. *Nature*, **553**(7689): 427–436.

Chung H, Pamp SJ, Hill JA, et al. 2012. Gut immune maturation depends on colonization with a host-specific microbiota. *Cell*, **149**(7): 1578–1593.

Cillo AR, Kürten CHL, Tabib T, et al. 2020. Immune landscape of viral- and carcinogen-driven head and neck cancer. *Immunity*, **52**(1): 183–199. e9.

Dolowy WC, Muldoon RL. 1964. Studies of germfree animals. I. Response of mice to infection with influenza a virus. *Proceedings of the Society for Experimental Biology and Medicine*, **116**: 365–371.

El-Sherbiny YM, Yusof YM, Psarras A, et al. 2020. B cell tetherin: a flow cytometric cell-specific assay for response to type I interferon predicts clinical features and flares in systemic lupus erythematosus. *Arthritis & Rheumatology*, **72**(5): 769–779.

Emal D, Rampanelli E, Stroo I, et al. 2017. Depletion of gut microbiota protects against renal ischemia-reperfusion injury. *Journal of the American Society of Nephrology*, **28**(5): 1450–1461.

Erny D, Hrabě De Angelis AL, Jaitin D, et al. 2015. Host microbiota constantly control maturation and function of microglia in the CNS. *Nature Neuroscience*, **18**(7): 965–977.

Furze RC, Rankin SM. 2008. Neutrophil mobilization and clearance in the bone marrow. *Immunology*, **125**(3): 281–288.

Grieshaber-Bouyer R, Radtke FA, Cunin P, et al. 2021. The neutrotome transcriptional signature defines a single continuum of neutrophils across biological compartments. *Nature Communications*, **12**(1): 2856.

Harper A, Vijayakumar V, Ouwehand AC, et al. 2021. Viral infections, the microbiome, and probiotics. *Frontiers in Cellular and Infection Microbiology*, **10**: 596166.

Hsiao EY, McBride SW, Hsien S, et al. 2013. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell*, **155**(7): 1451–1463.

Ishikawa J, Kaisho T, Tomizawa H, et al. 1995. Molecular cloning and chromosomal mapping of a bone marrow stromal cell surface gene, BST2, that may be involved in pre-B-cell growth. *Genomics*, **26**(3): 527–534.

Islam S, Watanabe H. 2020. Versican: a dynamic regulator of the extracellular matrix. *Journal of Histochemistry & Cytochemistry*, **68**(11): 763–775.

Jiménez JI, Canales Á, Jiménez-Barbero J, et al. 2008. Deciphering the genetic determinants for aerobic nicotinic acid degradation: the *nic* cluster from *Pseudomonas putida* KT2440. *Proceedings of the National Academy of Sciences of the United States of America*, **105**(32): 11329–11334.

Jin SQ, Guerrero-Juarez CF, Zhang LH, et al. 2021. Inference and analysis of cell-cell communication using CellChat. *Nature Communications*, **12**(1): 1088.

Josefsdottir KS, Baldrige MT, Kadmon CS, et al. 2017. Antibiotics impair murine hematopoiesis by depleting the intestinal microbiota. *Blood*, **129**(6): 729–739.

Kang I, Harten IA, Chang MY, et al. 2017. Versican deficiency significantly reduces lung inflammatory response induced by polyinosine-polycytidylic acid stimulation. *Journal of Biological Chemistry*, **292**(1): 51–63.

Kennedy EA, King KY, Baldrige MT. 2018. Mouse microbiota models: comparing germ-free mice and antibiotics treatment as tools for modifying gut bacteria. *Frontiers in Physiology*, **9**: 1534.

Khosravi A, Yáñez A, Price JG, et al. 2014. Gut microbiota promote hematopoiesis to control bacterial infection. *Cell Host & Microbe*, **15**(3): 374–381.

Kim J, Wong PK. 2009. Oxidative stress is linked to ERK1/2-p16 signaling-mediated growth defect in ATM-deficient astrocytes. *Journal of Biological Chemistry*, **284**(21): 14396–14404.

Kiner E, Willie E, Vijaykumar B, et al. 2021. Gut CD4⁺ T cell phenotypes are a continuum molded by microbes, not by T_H archetypes. *Nature Immunology*, **22**(2): 216–228.

Kjer-Nielsen L, Patel O, Corbett AJ, et al. 2012. MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature*, **491**(7426): 717–723.

Kobayashi SD, Malachowa N, Deleo FR. 2017. Influence of microbes on neutrophil life and death. *Frontiers in Cellular and Infection Microbiology*, **7**: 159.

Kostyliina G, Simon D, Fey MF, et al. 2008. Neutrophil apoptosis mediated by nicotinic acid receptors (GPR109A). *Cell Death & Differentiation*, **15**(1): 134–142.

Kovtonyuk LV, Caiado F, Garcia-Martin S, et al. 2022. IL-1 mediates microbiome-induced inflammaging of hematopoietic stem cells in mice. *Blood*, **139**(1): 44–58.

Kramer PA, Ravi S, Chacko B, et al. 2014. A review of the mitochondrial and glycolytic metabolism in human platelets and leukocytes: implications for their use as bioenergetic biomarkers. *Redox Biology*, **2**: 206–210.

Kunisawa J, Hashimoto E, Ishikawa I, et al. 2012. A pivotal role of vitamin B9 in the maintenance of regulatory T cells *in vitro* and *in vivo*. *PLoS One*, **7**(2): e32094.

Le Tortorec A, Willey S, Neil SJD. 2011. Antiviral inhibition of enveloped virus release by tetherin/BST-2: action and counteraction. *Viruses*, **3**(5): 520–540.

Lee JS, Cella M, McDonald KG, et al. 2012. AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch. *Nature immunology*, **13**(2): 144–151.

Levy M, Thaiss CA, Elinav E. 2016. Metabolites: messengers between the microbiota and the immune system. *Genes & Development*, **30**(14): 1589–1597.

Li JL, Wu HS, Liu YT, et al. 2020. High fat diet induced obesity model using four strains of mice: Kunming, C57BL/6, BALB/c and ICR. *Experimental Animals*, **69**(3): 326–335.

Li Y, Innocentin S, Withers DR, et al. 2011. Exogenous stimuli maintain intraepithelial lymphocytes via aryl hydrocarbon receptor activation. *Cell*, **147**(3): 629–640.

Macallan DC, Fullerton CA, Neese RA, et al. 1998. Measurement of cell proliferation by labeling of DNA with stable isotope-labeled glucose: studies *in vitro*, in animals, and in humans. *Proceedings of the National Academy of Sciences of the United States of America*, **95**(2): 708–713.

Manz MG, Boettcher S. 2014. Emergency granulopoiesis. *Nature Reviews Immunology*, **14**(5): 302–314.

Matcovitch-Natan O, Winter DR, Giladi A, et al. 2016. Microglia development follows a stepwise program to regulate brain homeostasis. *Science*, **353**(6301): aad8670.

Mebratu Y, Tesfagzi Y. 2009. How ERK1/2 activation controls cell proliferation and cell death: is subcellular localization the answer?. *Cell Cycle*, **8**(8): 1168–1175.

Mills GC, Mills JS. 1985. Urinary excretion of methylthioadenosine in immunodeficient children. *Clinica Chimica Acta*, **147**(1): 15–23.

Mirand EA, Grace Jr JT. 1963. Responses of germ-free mice to friend virus. *Nature*, **200**(4901): 92–93.

Mittal M, Siddiqui MR, Tran K, et al. 2014. Reactive oxygen species in

- inflammation and tissue injury. *Antioxidants & Redox Signaling*, **20**(7): 1126–1167.
- Morrison DJ, Preston T. 2016. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes*, **7**(3): 189–200.
- Nicholson JK, Holmes E, Kinross J, et al. 2012. Host-gut microbiota metabolic interactions. *Science*, **336**(6086): 1262–1267.
- Pasteur L. 1885. Observations relatives à la note précédente de M. Duclaux. *Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences*, **100**: 68.
- Qiu J, Heller JJ, Guo XH, et al. 2012. The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. *Immunity*, **36**(1): 92–104.
- Reyniers JA, Trexler PC, Ervin RF. 1946. Rearing germ-free albino rats. *Lobund Reports*, (1): 1–84.
- Sauter M, Moffatt B, Saechao MC, et al. 2013. Methionine salvage and S-adenosylmethionine: essential links between sulfur, ethylene and polyamine biosynthesis. *Biochemical Journal*, **451**(2): 145–154.
- Schaffer J, Beamer PR, Trexler PC, et al. 1963. Response of germ-free animals to experimental virus monocontamination. I. Observation on Cocksackie B virus. *Proceedings of the Society for Experimental Biology and Medicine*, **112**: 561–564.
- Shi LZ, Faith NG, Nakayama Y, et al. 2007. The aryl hydrocarbon receptor is required for optimal resistance to *Listeria monocytogenes* infection in mice. *The Journal of Immunology*, **179**(10): 6952–6962.
- Smith K, McCoy KD, Macpherson AJ. 2007. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Seminars in Immunology*, **19**(2): 59–69.
- Sommer F, Bäckhed F. 2013. The gut microbiota — masters of host development and physiology. *Nature Reviews Microbiology*, **11**(4): 227–238.
- Spencer SP, Wilhelm C, Yang Q, et al. 2014. Adaptation of innate lymphoid cells to a micronutrient deficiency promotes type 2 barrier immunity. *Science*, **343**(6169): 432–437.
- Taggart AKP, Kero J, Gan XD, et al. 2005. (D)- β -Hydroxybutyrate inhibits adipocyte lipolysis via the nicotinic acid receptor PUMA-G. *Journal of Biological Chemistry*, **280**(29): 26649–26652.
- Tapping RI, Tobias PS. 2000. Soluble CD14-mediated cellular responses to lipopolysaccharide. *Chemical Immunology*, **74**: 108–121.
- Urata S, Kenyon E, Nayak D, et al. 2018. BST-2 controls T cell proliferation and exhaustion by shaping the early distribution of a persistent viral infection. *PLoS Pathogens*, **14**(7): e1007172.
- Wen B, Mei ZL, Zeng CW, et al. 2017. metaX: a flexible and comprehensive software for processing metabolomics data. *BMC Bioinformatics*, **18**(1): 183.
- Wight TN, Kang I, Evanko SP, et al. 2020. Versican-a critical extracellular matrix regulator of immunity and inflammation. *Frontiers in Immunology*, **11**: 512.
- Yang Y, Yuan S, Chen T, et al. 2009. Cloning, heterologous expression, and functional characterization of the nicotinate dehydrogenase gene from *Pseudomonas putida* KT2440. *Biodegradation*, **20**(4): 541–549.
- Yu GC, Wang LG, Han YY, et al. 2012. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS: A Journal of Integrative Biology*, **16**(5): 284–287.
- Yu JH, Liu XL, Ke CW, et al. 2017. Effective suckling C57BL/6, kunming, and BALB/c mouse models with remarkable neurological manifestation for zika virus infection. *Viruses*, **9**(7): 165.
- Yu SM, Yan XR, Liu HH, et al. 2014. Improved establishment of embryonic stem (ES) cell lines from the Chinese Kunming mice by hybridization with 129 mice. *International Journal of Molecular Sciences*, **15**(3): 3389–3402.
- Zhang DC, Chen G, Manwani D, et al. 2015. Neutrophil ageing is regulated by the microbiome. *Nature*, **525**(7570): 528–532.
- Zhang XX, Lan YJ, Xu JY, et al. 2019. CellMarker: a manually curated resource of cell markers in human and mouse. *Nucleic Acids Research*, **47**(D1): D721–D728.
- Zhang Y, Shen J, Cheng W, et al. 2023. Microbiota-mediated shaping of mouse spleen structure and immune function characterized by scRNA-seq and Stereo-seq. *Journal of Genetics and Genomics*, **50**(9): 688–701.
- Zheng DP, Liwinski T, Elinav E. 2020. Interaction between microbiota and immunity in health and disease. *Cell Research*, **30**(6): 492–506.