Supplemental Data

Supplementary methods

Patients and samples

Clinical samples were obtained from CLL patients and normal volunteers at the First Affiliated Hospital of Nanjing Medical University, Jiangsu Province Hospital (Nanjing, China) with informed consent, in accordance with the Declaration of Helsinki and approved by the National Research Ethics Committee. Blood samples were collected in heparinized tubes, centrifuged at 1,500 g for 10 min, and the supernatant collected and stored at -80 ℃ until use. PBMCs were isolated by density gradient centrifugation with Lymphoprep™ (Stemcell Technologies, Vancouver, Canada) to generate produce single cell suspensions.

Single-cell stemness estimation and metabolic pathway enrichment analysis

The stemness score of each single cell was estimated using CytoTRACE [1] tool with default parameters. After the B cells had been classified into IBR, IBS, and shared clusters groups, we performed single-cell metabolic pathway analysis using single sample gene set enrichment analysis [2] and calculated the metabolic pathway scores of each group to identify pathways specifically activated in IBR clusters.

Gene set enrichment analysis

We identified DEGs between IBR and IBS/shared clusters using “FindMarkers (only.pos = FALSE, min.pct = 0.25, logfc.threshold = 0)” in Seurat. A gene rank list was generated based on average fold change scores and used for gene set enrichment analysis based on signatures from the MsigDB hallmark gene set using ClusterProfiler [3] (v3.14.3). Significant pathways were selected using a false discovery rate (FDR) < 0.05.

Pseudo-time trajectory analysis

Monocle2 [4] (v2.14.0) was used to perform pseudo-time trajectory analysis.

Trajectories were visualized as 2D tSNE plots using “plot_cell_trajectory” and B cells from IBR and IBS/Shared clusters were mapped to the trajectory. Dynamic changes of gene expression were visualized by heatmap using “plot_pseudotime_heatmap”. LGALS1, LAG3 and PTMS were visualized by “plot_genes_in_pseudotime”.

Cellular interaction analysis

We performed cell-cell communication analysis among IBR B, IBS B, monocyte, NK, and T cells using CellphoneDB [5] (v2.1.1) with default parameters. Ligand-receptor pairs with $p < 0.05$ were considered significant.

Bulk RNA-seq data analysis

We identified DEGs from PBMC bulk RNA-seq data from 6 IBR and 40 IBS patients with CLL using the Wilcoxon rank-sum test. Delta mean was defined as the difference between the mean gene expression in the IBR and IBS groups.

Gene ontology (GO) pathway enrichment analysis

We selected genes that co-expressed with LGALS1 or LAG3 with Rho ≥ 0 and $p < 0.05$. Only the top 50 genes were visualized in heatmap. GO enrichment analyses were performed using “enrichGO (ont = 'BP', pAdjustMethod = 'BH', pvalueCutoff = 0.05, qvalueCutoff = 0.2)” for all co-expressed genes in the ClusterProfiler R package [3].

Survival analysis

Microarray and survival data for CLL patients were downloaded from the Gene Expression Omnibus (GEO) database, numbered as GSE22762, and performed in GPL570 platform. Survival outcomes were plotted using the Kapan-Meier method in survminer (v0.4.6). Differences were assessed using the log-rank test in the survival R package (v2.44.1.1).

Cell culture and compounds

The MEC-1 CLL cell line purchased from Cobioer (Nanjing, China) and primary cells

were cultured in RPMI-1640 medium (Gibco, Grand Island, USA) supplemented with 10 % fetal bovine serum (Gibco) and 100 μg/mL penicillin/streptomycin (Gibco) at 37 °C in a humidified atmosphere (5 % CO_2). Ibrutinib was purchased from Selleck Chemicals (Houston, TX, USA). OTX008 was purchased from MedChemExpress (Monmouth Junction, NJ, USA).

RNA isolation and qRT-PCR

Total RNA was isolated using TRIzol reagent (Life Technologies, Carlsbad, USA) according to the manufacturer's instructions and reverse transcribed using HiScript reverse transcriptase (Vazyme, Nanjing, China). qPCR was performed using Hieff qPCR SYBR Green master mix (Yeasen, Shanghai, China) with GAPDH mRNA as a control. LGALS1 and LAG3 mRNAs expression were detected with LGALS1 primer: 5′-AGCTTCAATCATGGCCTGTGGTC-3′ (forward) and 5′-TCCCAGGTTCAGCACAAAGCTC-3′ (reverse), LAG3 primer: 5′-TCACAGTGACTCCCAAATCCT-3′ (forward) and 5′-GCTCCACACAAAGCGTTCTT-3′ (reverse).

Cell counting kit-8 (CCK-8) assay

Cell proliferative ability was detected using Cell Counting Kit-8 (APExBIO, Houston, TX, USA). Briefly, cells were seeded in 96-well plates, incubated with CCK8 solution (10 μL/well) for 2 h, and the absorbance was measured at 450 nm.

Western blot

Cells were lysed in radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China) supplemented with a protease and phosphatase inhibitor cocktail (Bimake, Houston, TX, USA). Proteins present in the lysates were stored in sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) loading buffer (Beyotime, Shanghai, China) before being resolved on 4–20 % gradient SDS-PAGE gels (Genscript, Nanjing, China) and transferred to polyvinylidene difluoride (Immobilon-P) membranes. The membranes,

blocked with skimmed milk, were immunoblotted using antibodies against Gal-1 (16858-1-AP, Proteintech, Chicago, USA), LAG3 (16616-1-AP, Proteintech), and β-actin (AF0003, Beyotime). Corresponding secondary antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). After 1 h's incubation with the indicated antibodies, proteins were detected using a chemiluminescent substrate (Millipore, Darmstadt, Germany). Protein levels were normalized using β-actin.

Enzyme-linked immunosorbent assay (ELISA)

ELISA kits for Gal-1 and LAG3 (R&D Systems, Minneapolis, MN, USA) were used according to the manufacturer's instructions. Briefly, a 96-well microplate was coated with the capture antibody, incubated overnight at 4°C, washed three times with wash buffer, and blocked using diluent reagent (1:1-1:1000) for 1 h. After three further washes, the samples or standards were incubated for 2 h at room temperature, washed three times, incubated with the detection antibody for 2 h at room temperature, and washed three more times. The samples were then incubated with streptavidin-HRP in the dark at room temperature for 20 min the reaction stopped using the stop solution. Absorbance at 450 and 570 nm was determined using a Multiskan MS microplate reader (Thermo Fisher Scientific, USA).

Apoptosis assay

The collected cells were stained using propidium iodine/Annexin V-FITC (FcMACS, Nanjing, China) and detected using flow cytometry to determine the extent of apoptosis. Assays were performed in triplicate for each cell line.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Between-group differences were estimated using student's *t*-tests, non-parametric (Mann-Whitney) tests, or one-way ANOVA with Bonferroni's correction. All analyses were performed using GraphPad prism v8.0 (La Jolla, CA, USA) or SPSS Statistics v25 (Armonk, NY, USA) software. Two-tailed *p* values of * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, and **** ≤ 0.0001

were considered significant. All experiments were performed independently at least three times.

References

1. Gulati GS, Sikandar SS, Wesche DJ, Manjunath A, Bharadwaj A, Berger MJ, Ilagan F, Kuo AH, Hsieh RW, Cai S, et al: **Single-cell transcriptional diversity is a hallmark of developmental potential.** *Science* 2020, **367:**405-411.
2. Xiao ZT, Dai ZW, Locasale JW: **Metabolic landscape of the tumor microenvironment at single cell resolution.** *Nature Communications* 2019, **10**.
3. Yu G, Wang LG, Han Y, He QY: **clusterProfiler: an R package for comparing biological themes among gene clusters.** *Omics* 2012, **16:**284-287.
4. Trapnell C, Cacchiarelli D, Grimsby J, Pokharel P, Li S, Morse M, Lennon NJ, Livak KJ, Mikkelsen TS, Rinn JL: **The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells.** *Nat Biotechnol* 2014, **32:**381-386.
5. Efremova M, Vento-Tormo M, Teichmann SA, Vento-Tormo R: **CellPhoneDB: inferring cell-cell communication from combined expression of multi-subunit ligand-receptor complexes.** *Nat Protoc* 2020, **15:**1484-1506.

Supplementary Figure Legend

Supplementary Figure 1. Clinical information of CLL patients.

Supplementary Figure 2. Marker-gene expression and distribution among cells. Blue to gray: high to low expression.

Supplementary Figure 3. Visualization of genes related to LAG3 expression. Red represents genes highly expressed in bulk-RNA data. Blue represents genes expressed at low levels in bulk-RNA data.

Supplementary Figure 4. Functional enrichment analysis of LAG3-related genes. Dot size represents the number of enriched genes in the pathway. Color gradient represents adjusted *p*-value.

Supplementary Figure 5. Relative Gal-1 and LAG3 expression in CLL patients. A Quantitative detection of Gal-1 and LAG3 protein expression in IBS and IBR CLL patients. **B** ELISA detection of plasma Gal-1 and LAG3 levels in healthy donors (HD, $n = 10$), newly diagnosed (ND, $n = 10$), and relapse and refractory CLL patients (R/R, $n = 10$). *$p \leq 0.05$, ****$p \leq 0.0001$.

sampling time

S1

1964-07 — 2016-07 CLL

2018-08 Ibrutinib — CR

S2

1950-05 — 2018-06 CLL

No treatment

S3

1958-06 — 2018-06 CLL

No treatment

S4

1960-03 — 2018-08 CLL

2018-11 Ibrutinib — CR

R1

1953-09 — 2016-06 CLL

2017-11 Ibrutinib — PR — 2019-03 PD；ABT199

R2

1972-11 — 2013-08 CLL

2013-09 hyper-CVAD — PR — 2014-09 PD

2015-10 Ibrutinib — PD — 2014-12 Clinical trail (rituximab)

PD — 2018-09 ABT199

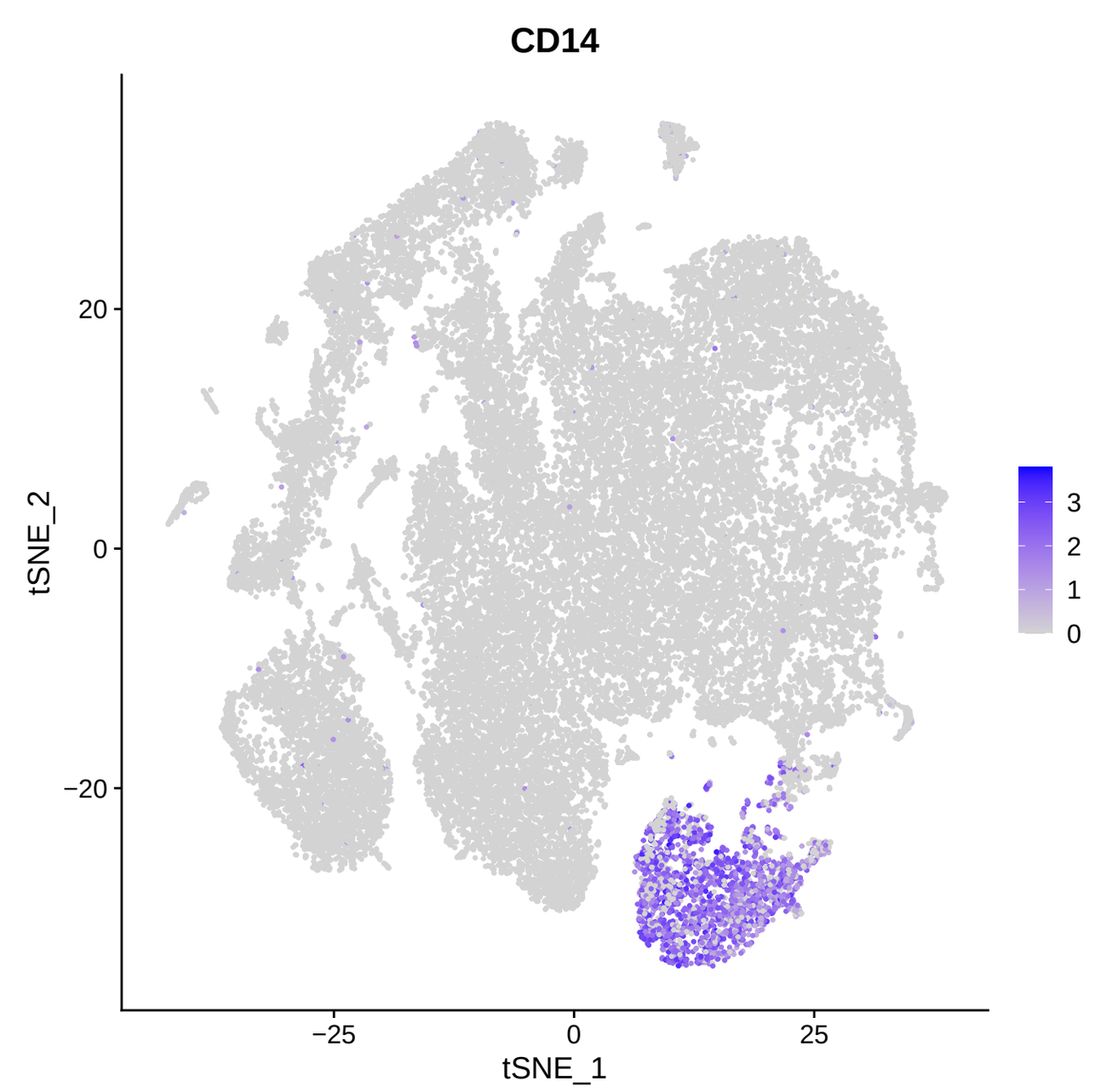
2019-04 Ibrutinib+ABT199 — 2019-07 Richter transformation. Death

R3

1977-12 — 2018-07 CLL

2018-12 Ibrutinib — PR — 2019-08 PD, Ibrutinib+Prednisone

2019-09 FFR+R+Modified HyperCVAD1+ABT199 — 2020-09 Death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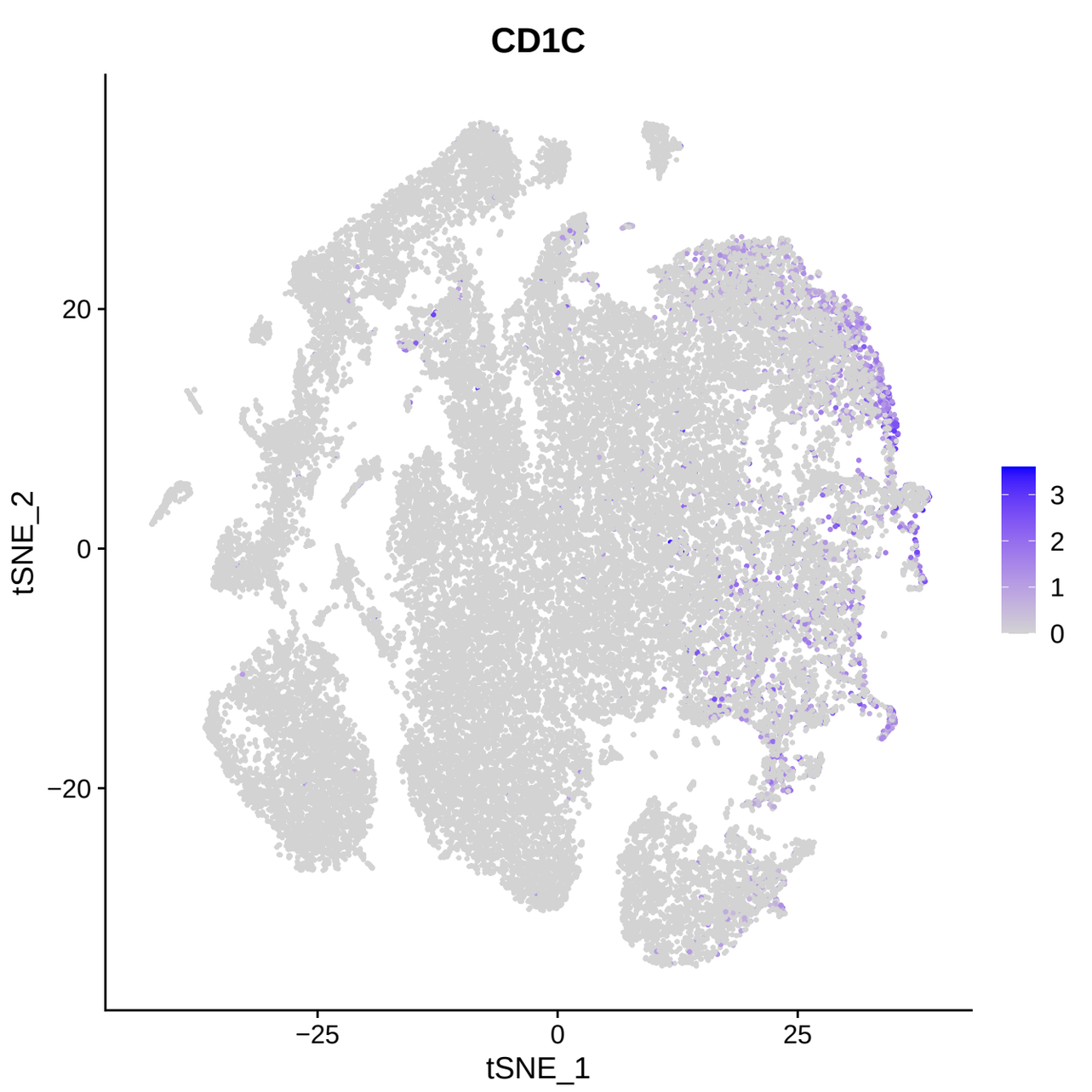
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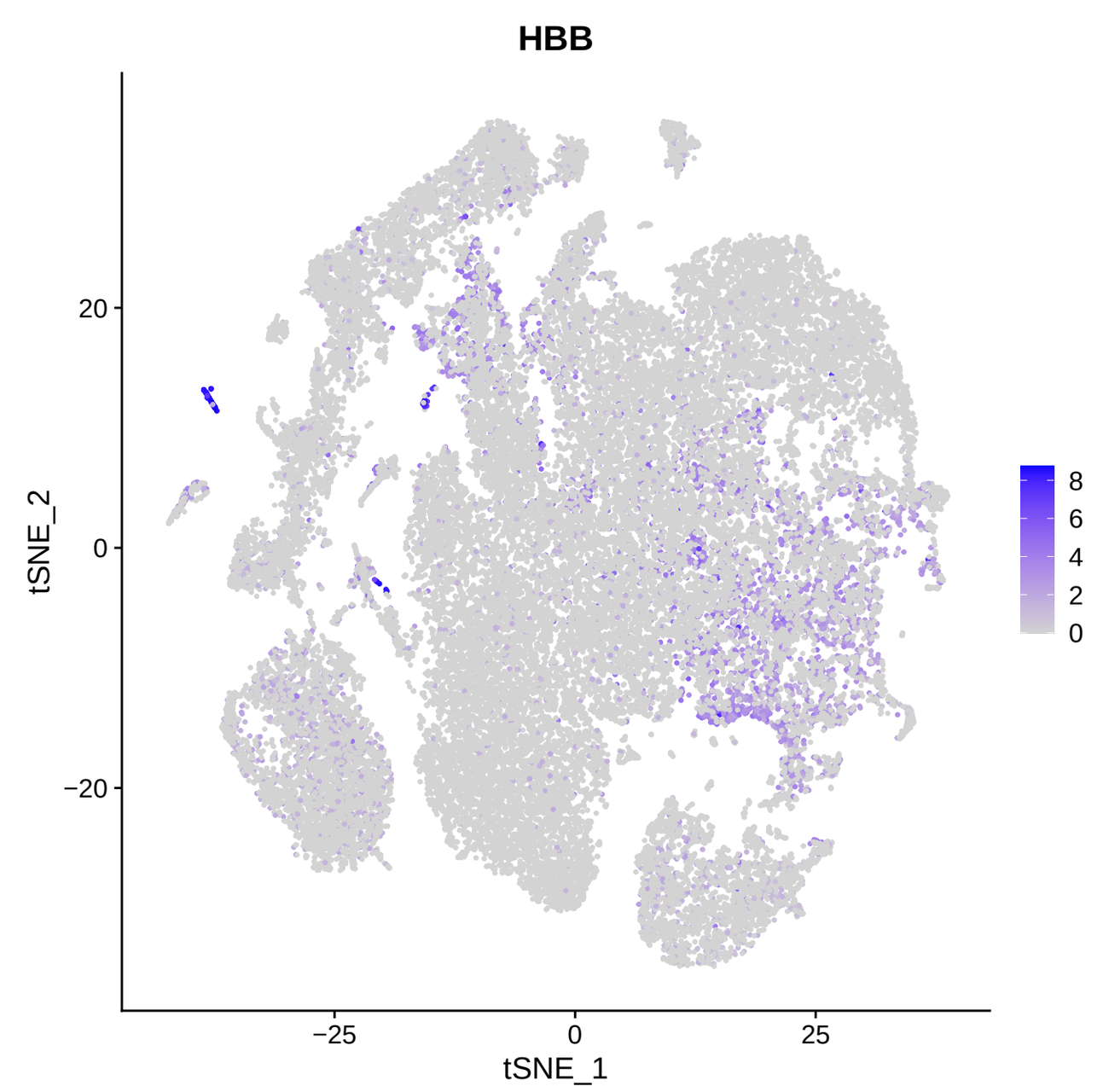
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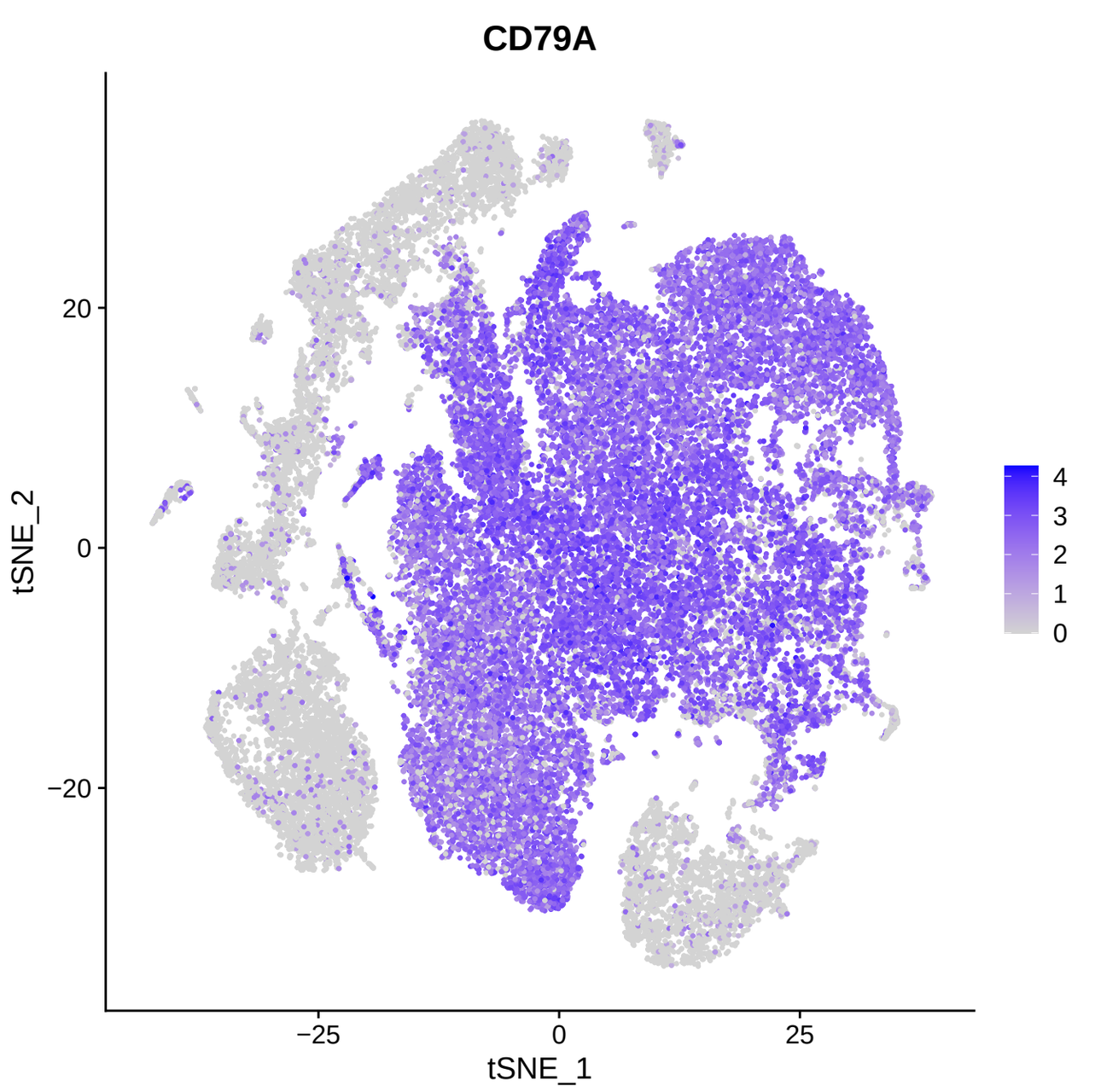
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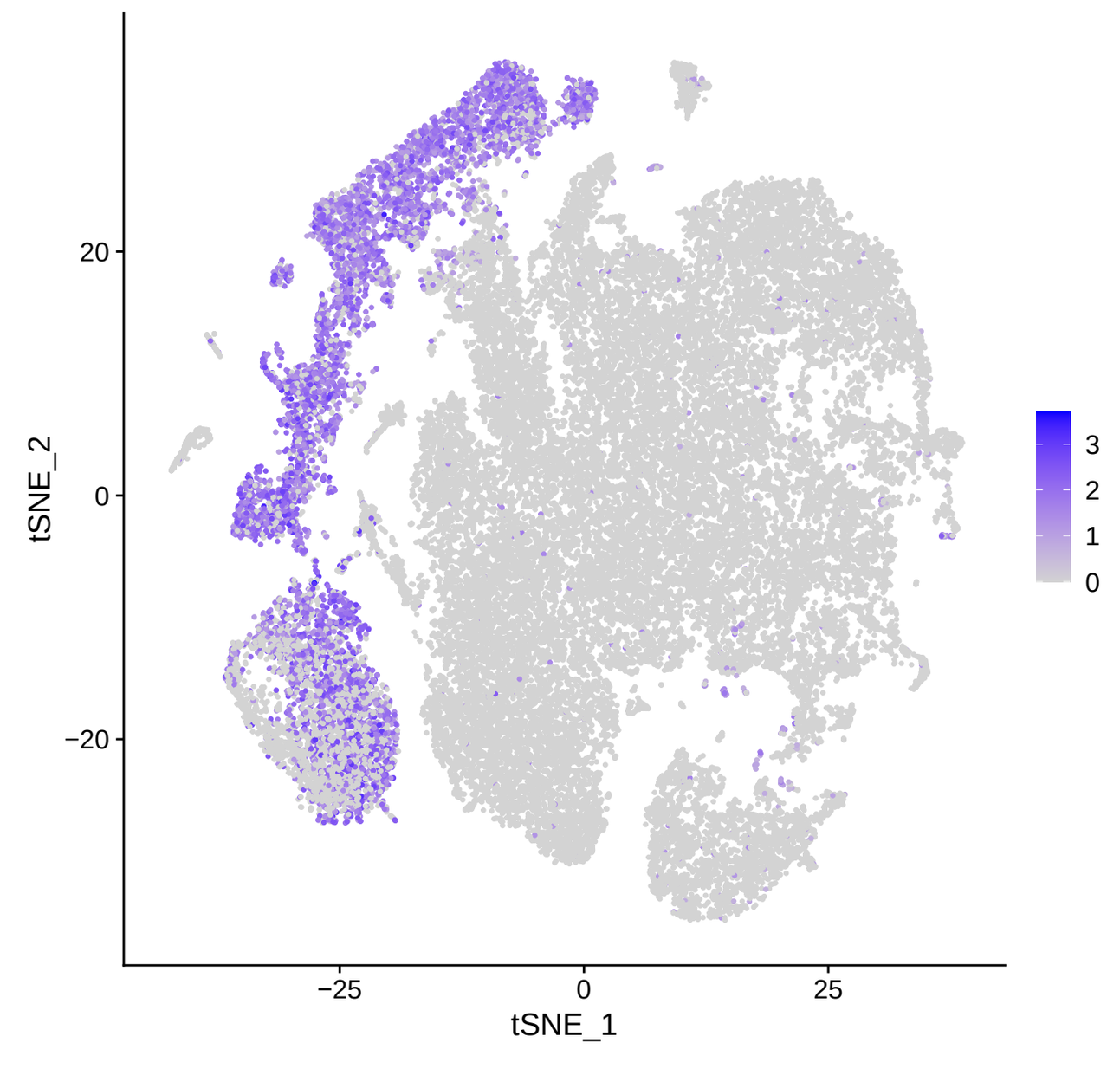
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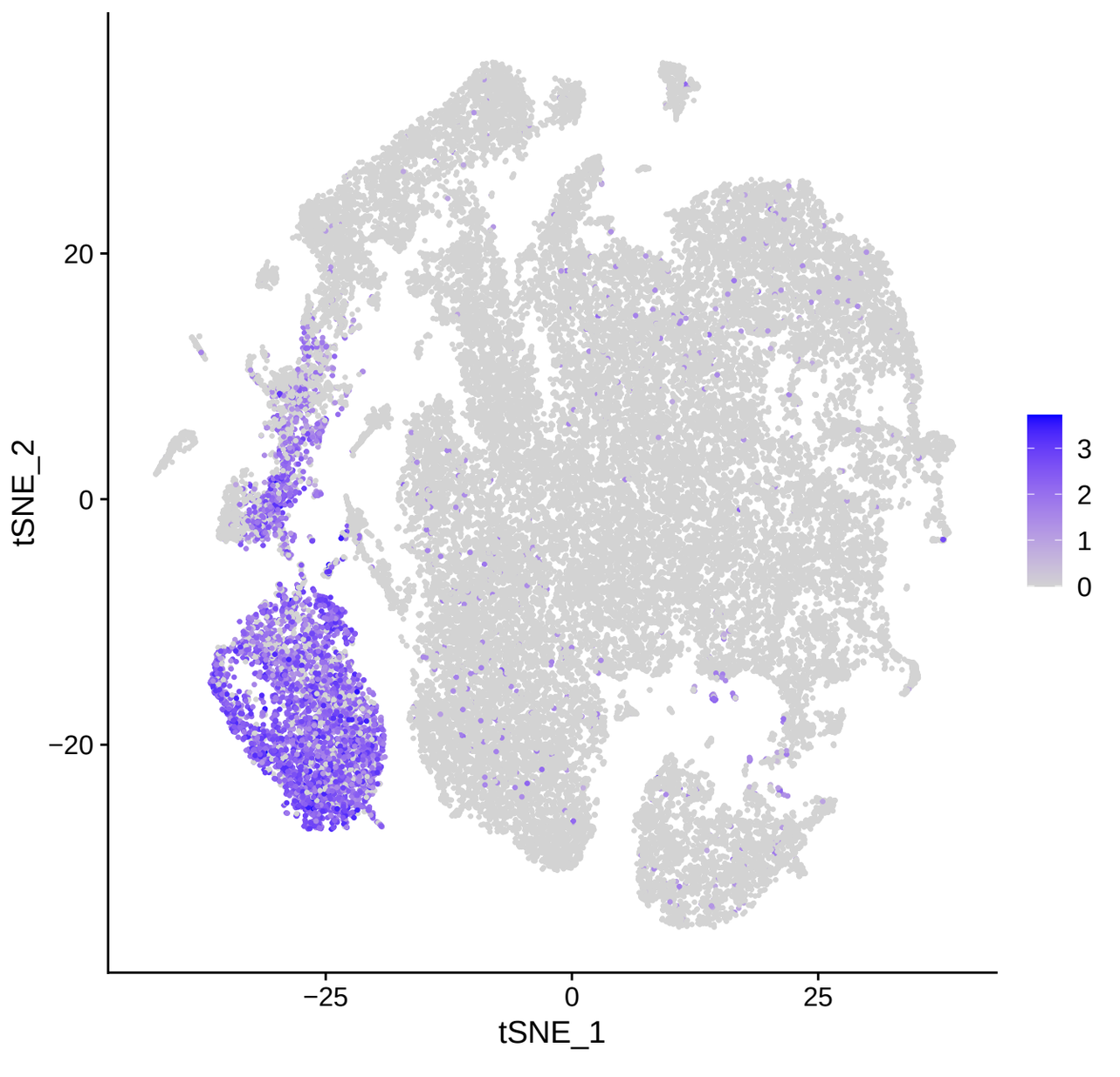
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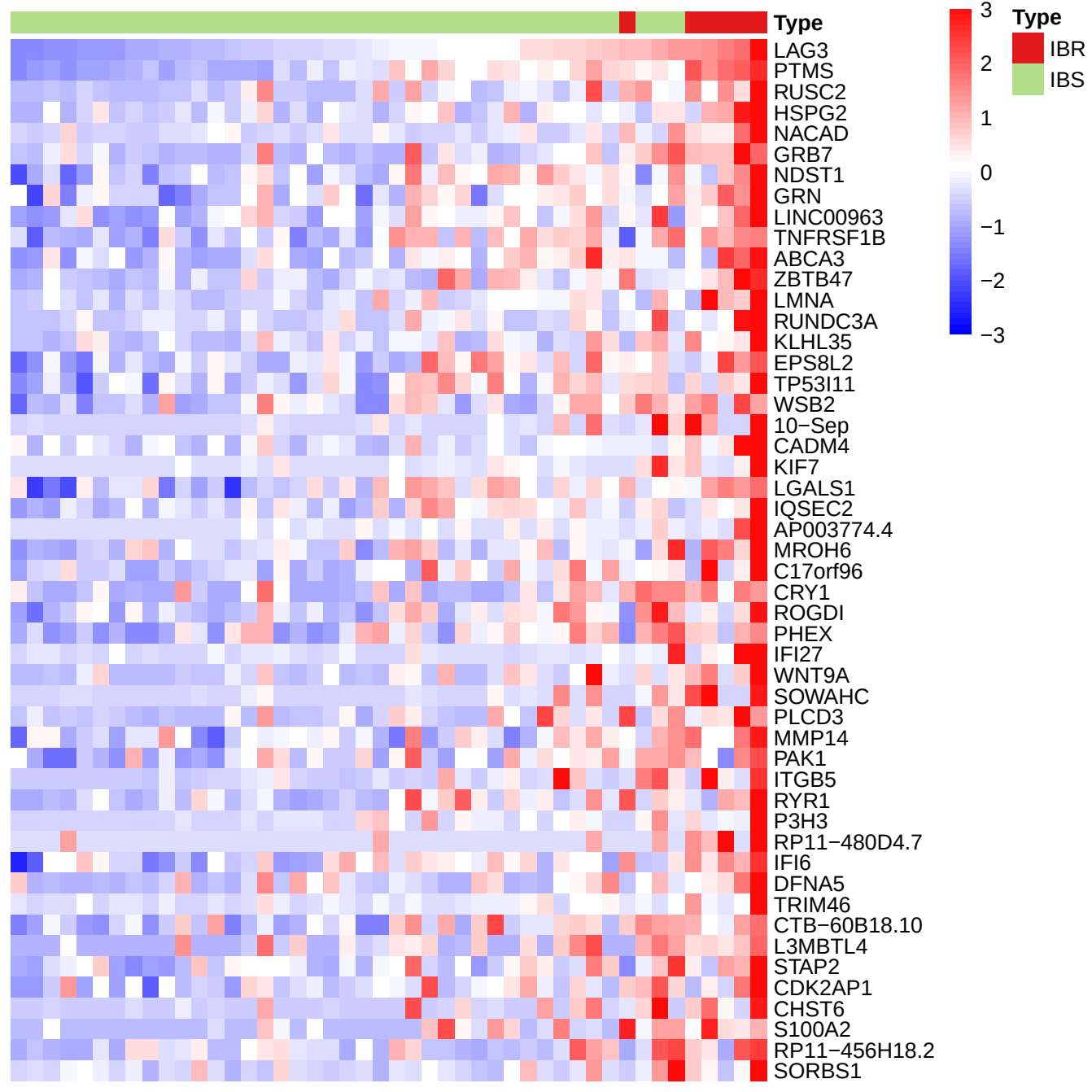
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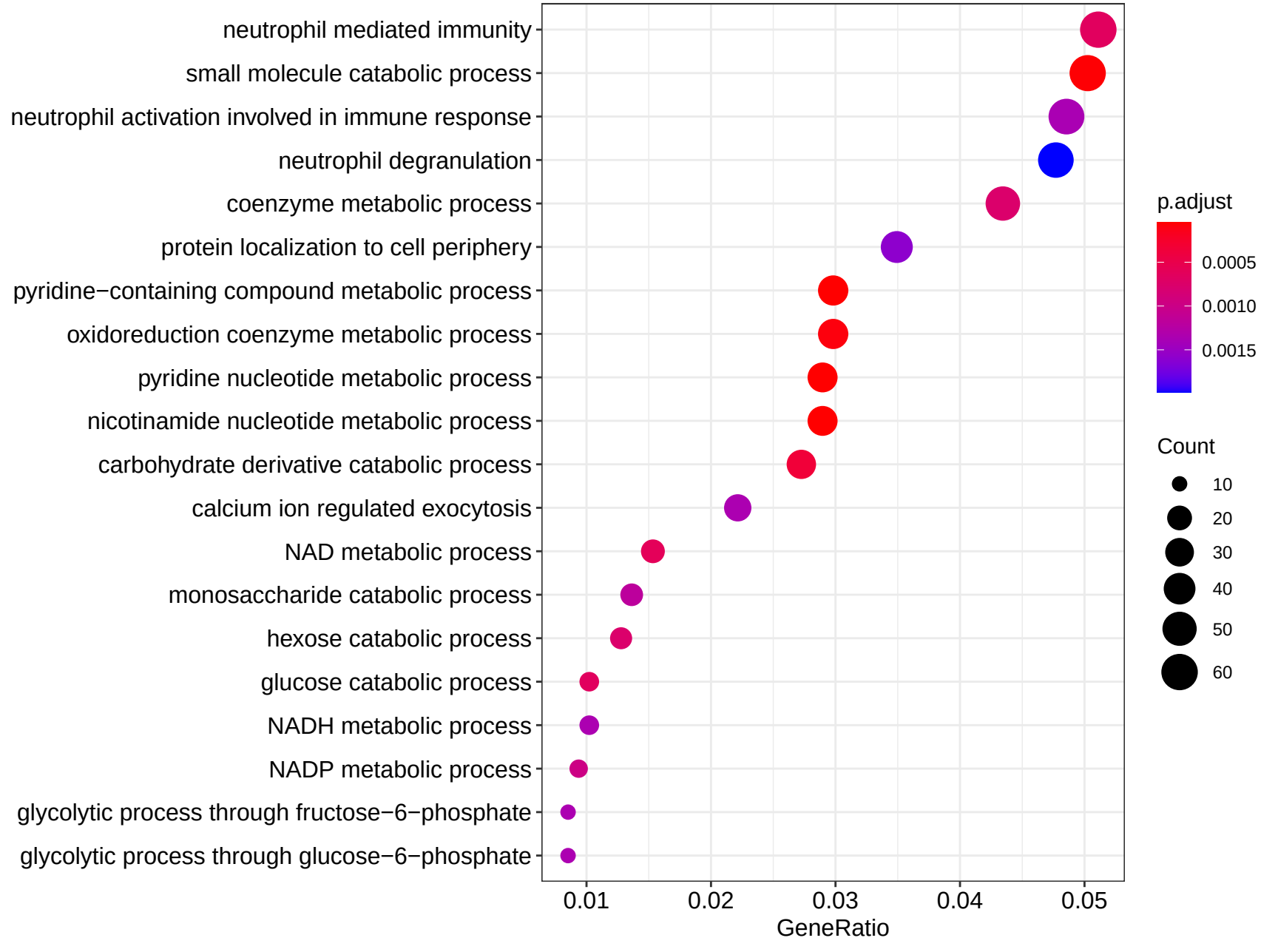
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neutrophil activation involved in immune response
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coenzyme metabolic process
protein localization to cell periphery
pyridine−containing compound metabolic process
oxidoreduction coenzyme metabolic process
pyridine nucleotide metabolic process
nicotinamide nucleotide metabolic process
carbohydrate derivative catabolic process
calcium ion regulated exocytosis
NAD metabolic process
monosaccharide catabolic process
hexose catabolic process
glucose catabolic process
NADH metabolic process
NADP metabolic process
glycolytic process through fructose−6−phosphate
glycolytic process through glucose−6−phosphate
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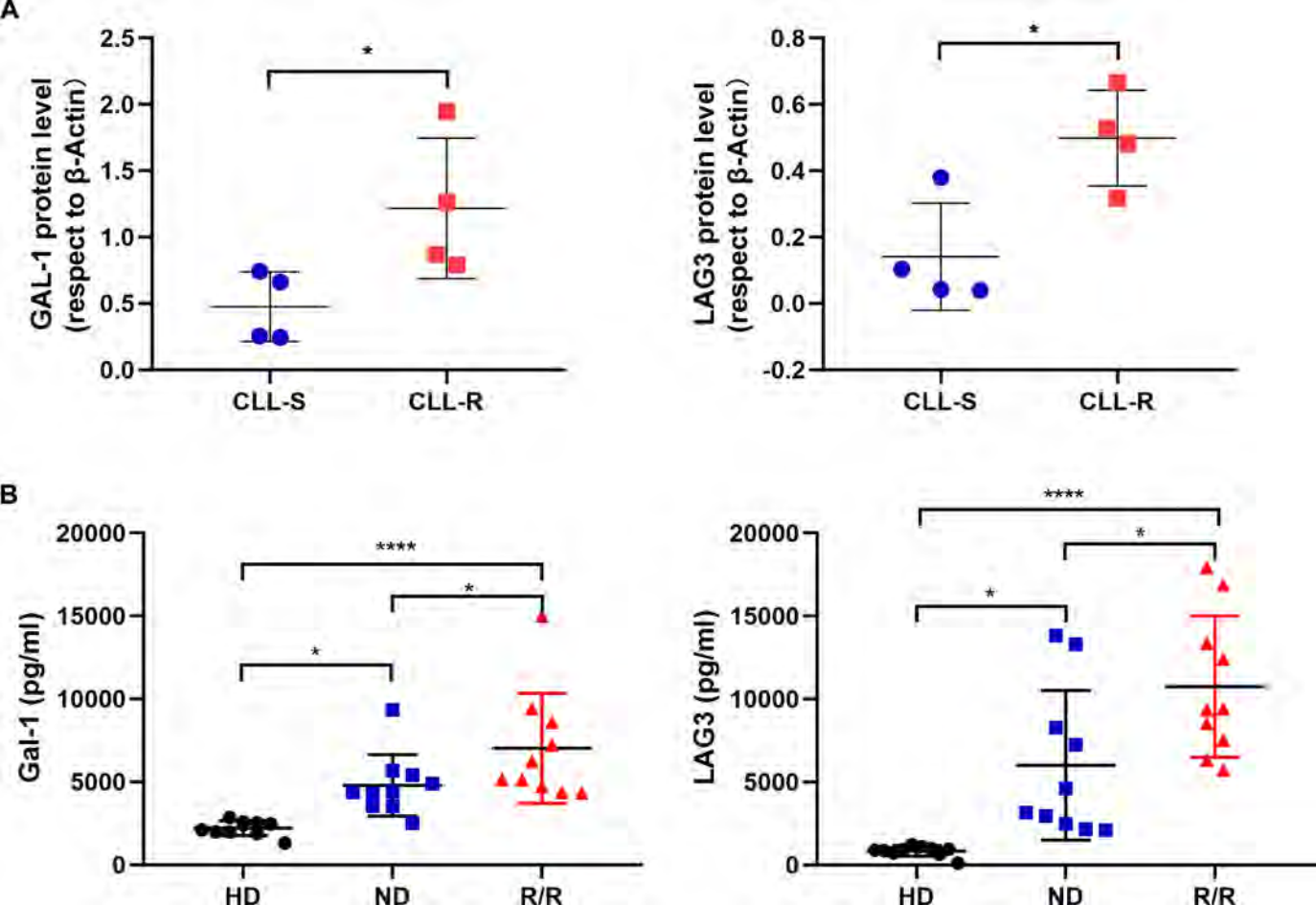
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2.0
1.5
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0.5
0.0
*
CLL-S
CLL-R
LAG3 protein level (respect to β-Actin)
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0.6
0.4
0.2
0.0
-0.2
*
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CLL-R
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15000
10000
5000
0
****
*
*
HD
ND
R/R
LAG3 (pg/ml)
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15000
10000
5000
0
****
*
*
HD
ND
R/R