# SUPPLEMENTAYR INFORMATION

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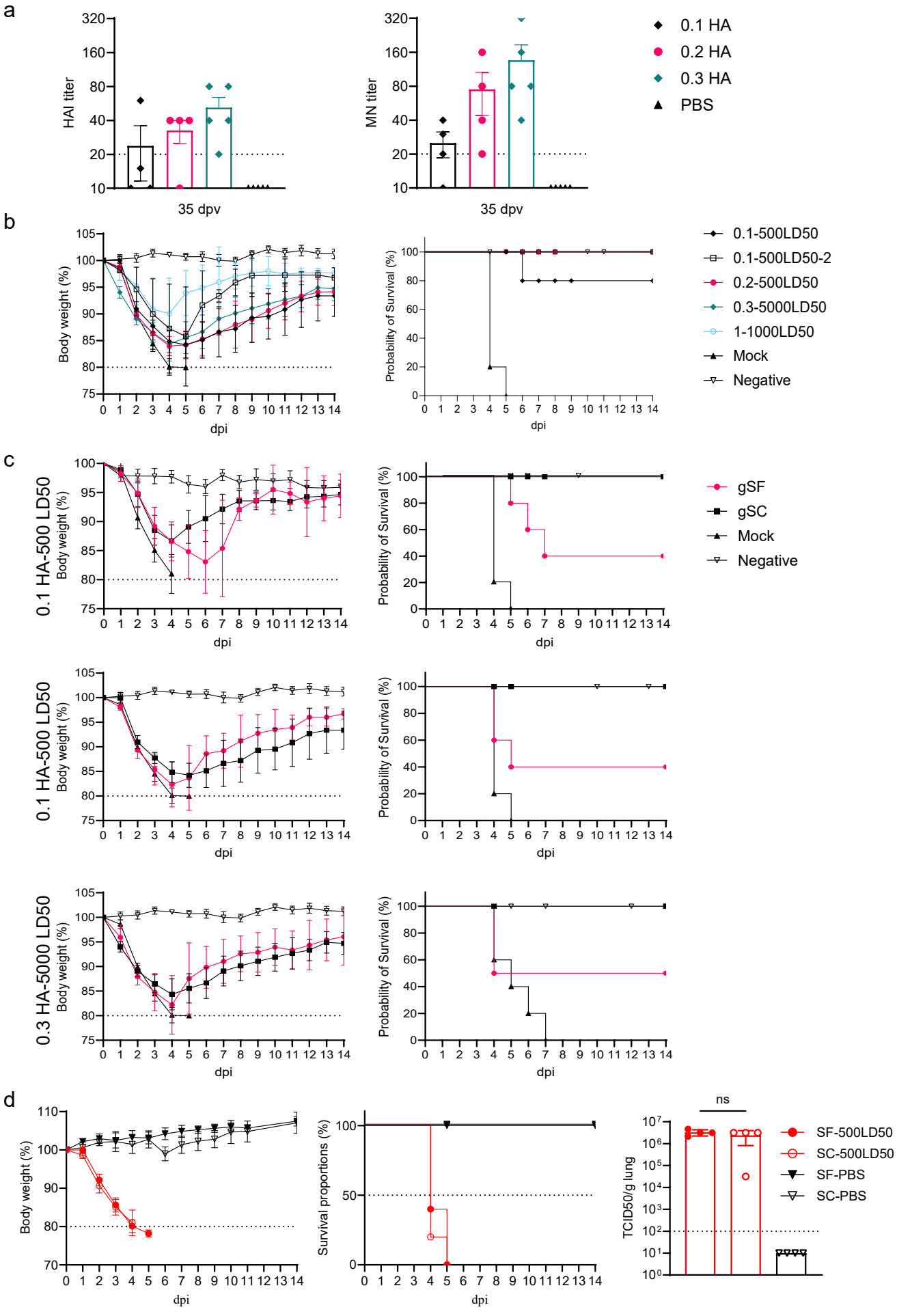
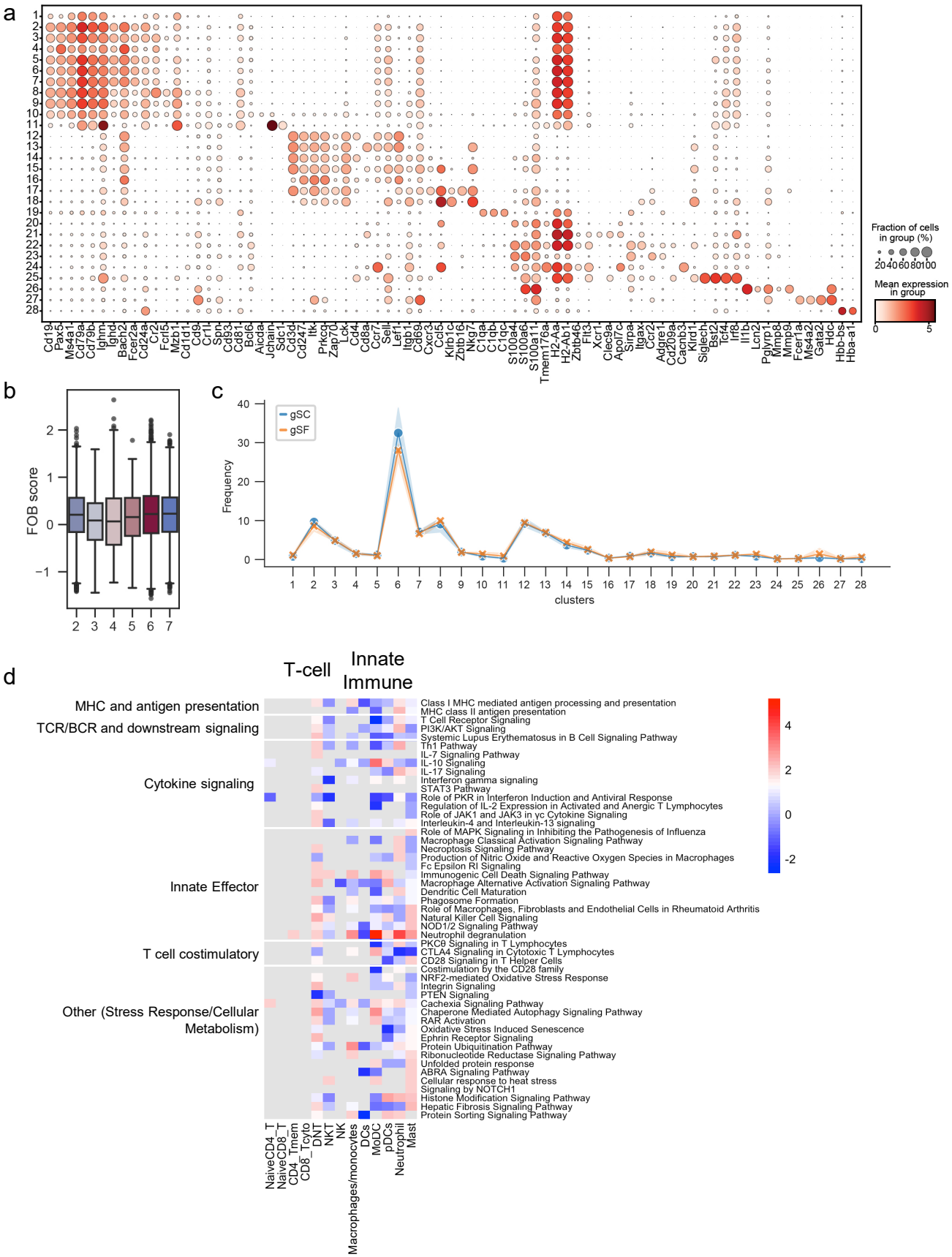
Supplementary Figure S1



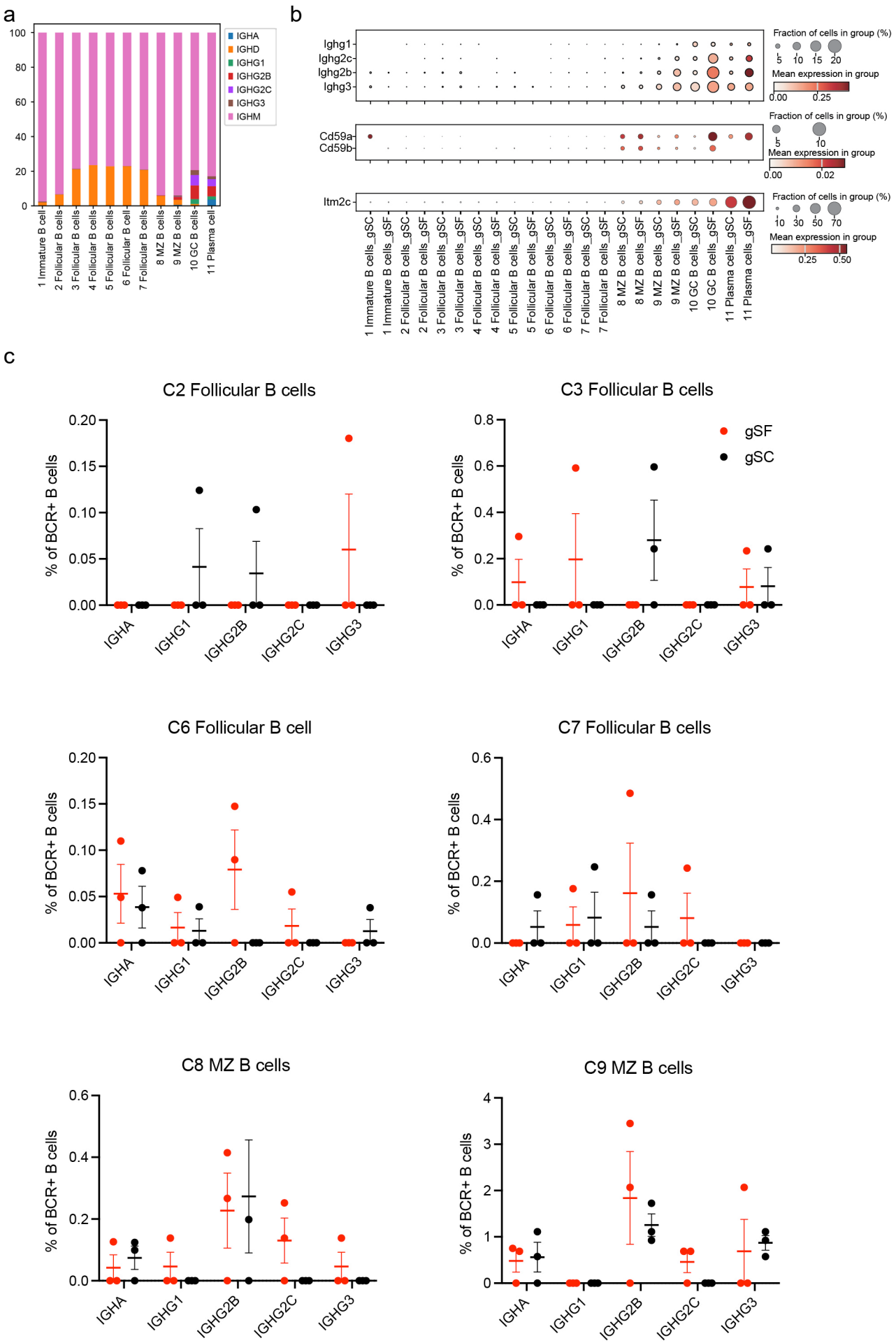
Supplementary Fig. S1. Vaccine dose optimization and infection-only control experiments under chronic sleep fragmentation (CSF). a) Hemagglutination inhibition (HAI) and microneutralization (MN) titers in mice immunized intramuscularly with 0.1, 0.2, or 0.3 μg of inactivated A/California/07/2009 (H1N1) (CA/07) hemagglutinin (HA) antigen, or PBS as control. Each datapoint represents an individual animal, and bars indicate mean ± SD. b) Body weight loss and survival rates for mice vaccinated with varying doses (0.1, 0.2, 0.3, and 1.0 μg) of inactivated CA/07 HA antigen or PBS, followed by lethal challenge with 500–5000 $LD_{50}$ of mouse-adapted CA/04 (CA/04-MA) virus. In the body weight loss figure, points indicate mean ± SD. c) Body weight loss and survival rates from three independent experiments using different vaccine–challenge dose combinations: two independent experiments with 0.1 μg HA – 500 $LD_{50}$ and one experiment with 0.3 μg HA – 5,000 $LD_{50}$. Experiment 1: 0.1 μg HA – 500 $LD_{50}$; gSF (n = 9), gSC (n = 10), Mock (n = 7), Negative (n = 9); Experiment 2: 0.1 μg HA – 500 $LD_{50}$; gSF (n = 5), gSC (n = 5), Mock (n = 5), Negative (n = 5); Experiment 3: 0.3 μg HA – 5000 $LD_{50}$; gSF (n = 5), gSC (n = 5), Mock (n = 5), Negative (n = 4). CSF was induced in gSF groups beginning 14 days prior to vaccination and continued through challenge, while gSC groups maintained normal sleep. Mock groups received PBS in place of vaccine, and negative controls were neither vaccinated nor challenged. In body weight loss figures, points indicate mean ± SD. d) Infection-only control experiment to assess baseline susceptibility to influenza under CSF. Two groups were subjected to 8 weeks of CSF, while two control groups were maintained under standard conditions prior to infection, following the same timeline used for the vaccination experiments. Mice were infected with 500 $LD_{50}$ CA/04-MA or administered PBS. Lung tissues were collected at 3 days post-infection (dpi) for viral load quantification to evaluate whether CSF alone alters infection outcomes independent of vaccination. In the body weight loss figure, points indicate mean ± SD. In the lung viral titer figure, each datapoint represents an individual animal, and bars indicate mean ± SD; Group comparisons (n=4) were performed using two-sided Mann–Whitney U tests. n.s., not statistically significant ($P>0.05$). In all experiments, mice were housed under standard conditions in ABSL-2 containment. Source Data are provided in the Source Data file.

Supplementary Figure S2



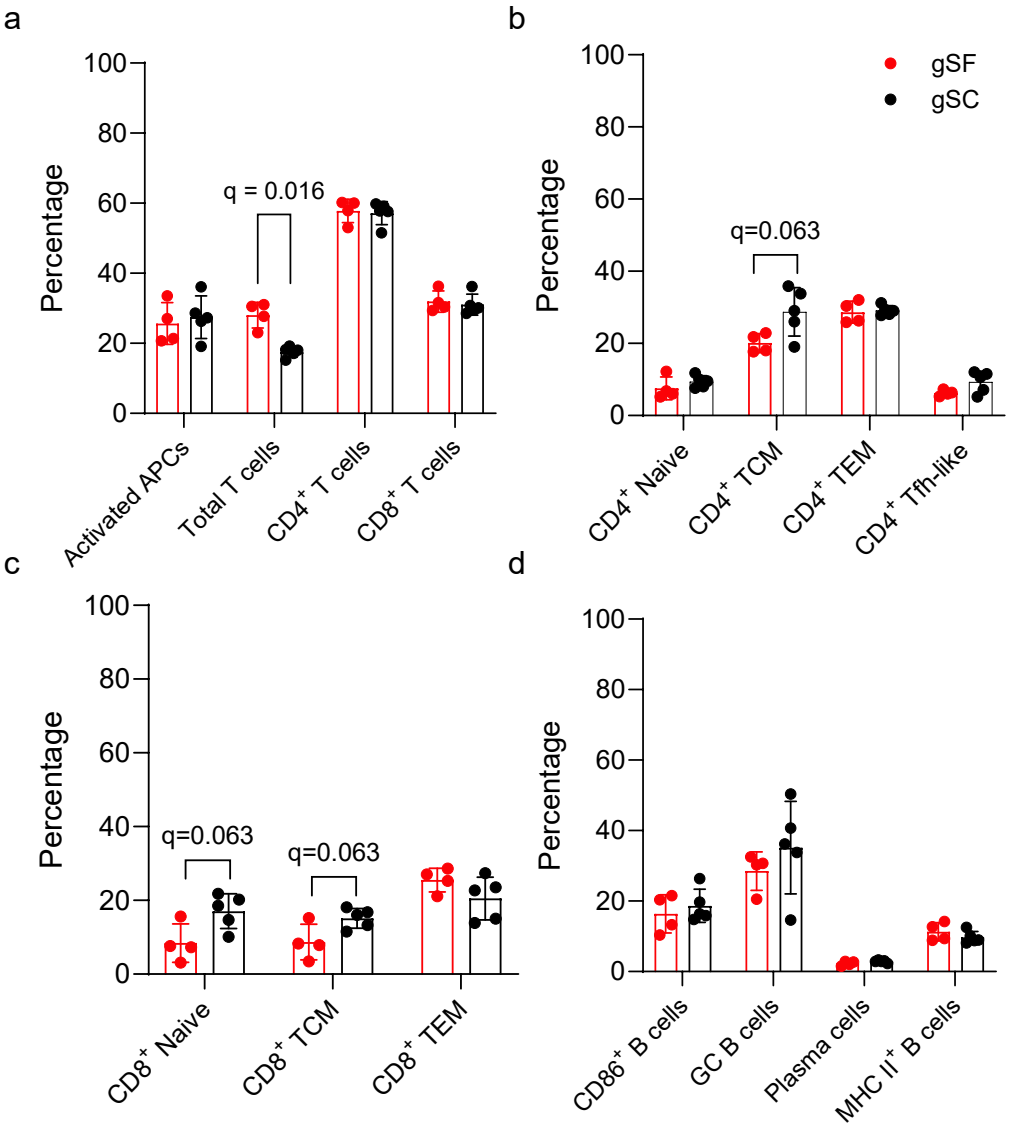
Supplementary Fig. S2. Single-cell analyses of innate immune cells and T-cells following CSF exposure. a) Canonical marker gene expression across splenic immune cell clusters. Dot plot showing the expression of canonical lineage and functional marker genes across 28 transcriptionally defined cell clusters identified by single-cell RNA sequencing of splenocytes from normal-sleep control (gSC) and CSF-exposed (gSF) mice. Each row corresponds to a distinct cell cluster, and each column represents a selected marker gene. Dot size reflects the percentage of cells in each cluster expressing the gene, while color intensity indicates the mean expression level of the gene within that cluster (see scale bar). b) Box-and-whisker plots show the distribution of standardized follicular gene set (*Fcer2a*, *Cd24a*, *Ighd*) expression score (FOB) for mice in clusters 2–7. Boxes represent the interquartile range (IQR), horizontal lines indicate medians, whiskers extend to 1.5× IQR, and individual points denote outliers. Statistical comparisons were performed using two-sided Kruskal–Wallis test. No statistically significant differences were observed, and comparisons with $P > 0.05$ are not labelled. c) Distribution of immune cell cluster frequencies in CSF and normal-sleep control mice. Line plot showing the relative frequencies of 28 transcriptionally defined immune cell clusters in splenocytes from CSF-exposed (gSF) and control (gSC) mice. Frequencies are expressed as the percentage of total cells assigned to each cluster. Data points and lines indicate group means, and shaded areas denote standard deviation. Overall, cluster distributions were broadly conserved between groups, indicating comparable cellular composition despite transcriptional reprogramming in select subsets. d) Ingenuity Pathway Analysis (IPA) of T-cell and innate immune subsets. Analyses were performed using the QIAGEN IPA platform (two-sided Fisher's exact test). Core Analysis function was used to identify canonical pathways significantly associated with the DEGs, applying a p-value threshold of 0.05. Pathways were considered significant if they met a $-\log(\text{p-value}) > 1.3$ (equivalent to $p < 0.05$) and a Z-score $> 2$ (activation) or $< -2$ (inhibition). Only pathways with a Z-score $\geq 2$ or $\leq -2$ in at least one cell type were included. Positive Z-scores (red) indicate pathway upregulation in CSF-treated mice. Pathways were grouped into six categories: 1) MHC and antigen presentation, 2) TCR/BCR and downstream signaling, 3) Cytokine signaling, 4) Innate effector functions, 5) T-cell costimulatory signals, 6) Other such as stress response and cellular metabolism. Source Data are provided in the Source Data file.

Supplementary Figure S3



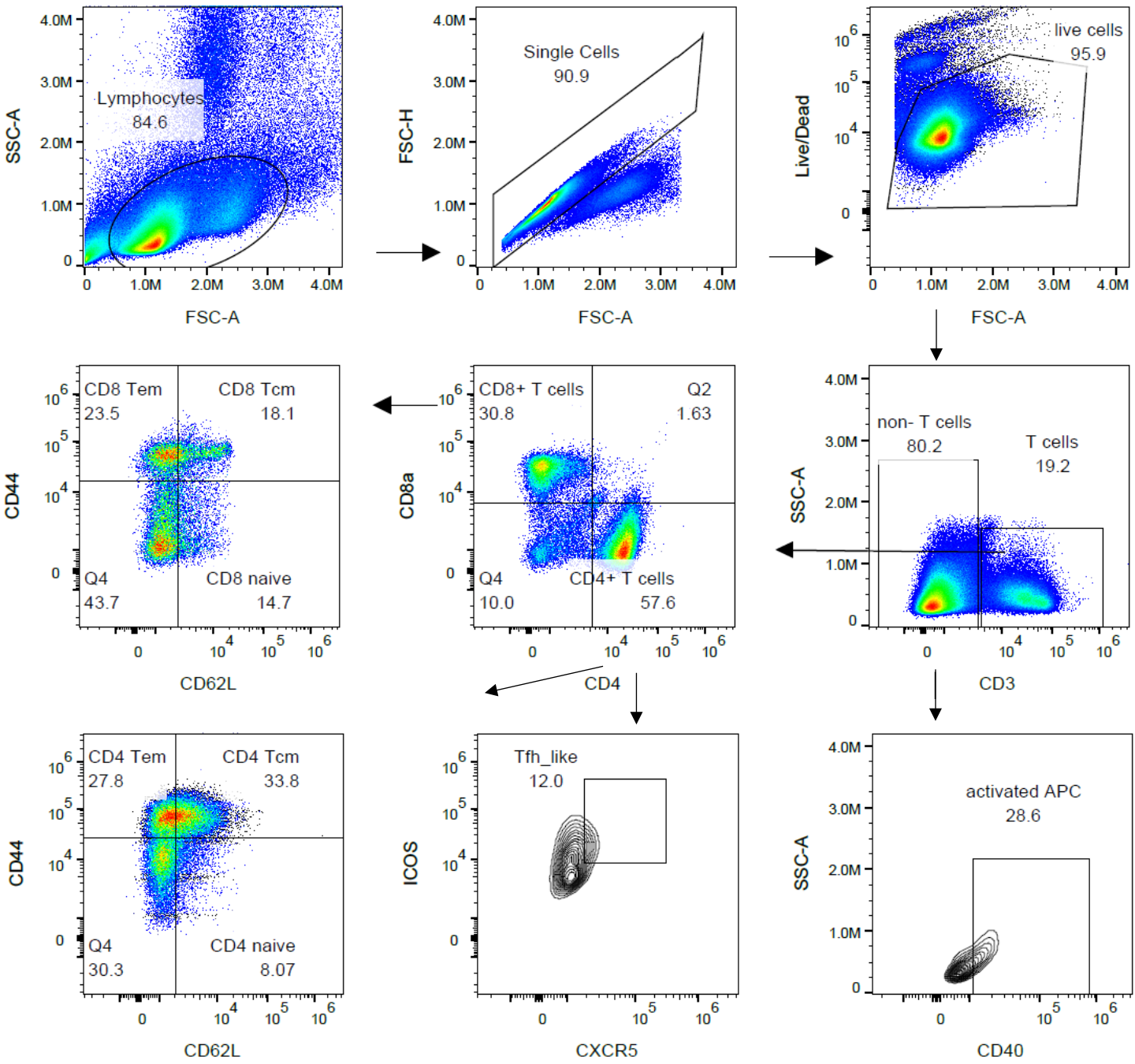
Supplementary Fig. S3. Distribution of immunoglobulin isotypes across B-cell subsets in CSF and normal-sleep control mice. a) Stacked bar plot showing the relative abundance of immunoglobulin isotypes (*IGHM*, *IGHD*, *IGHG1*, *IGHG2B*, *IGHG2C*, *IGHG3*, and *IGHA*) among $BCR^+$ cells across transcriptionally defined B-cell subsets. Immature B-cell cluster (C1), Follicular B-cell clusters (C2–C7), marginal zone (MZ) B-cells (C8–C9), germinal center (GC) B-cells (C10), and plasma cells (C11) are shown. Most early B-cell subsets (e.g., immature and follicular B-cells) are dominated by *IGHM*, whereas GC and plasma cells show greater isotype diversity. b) Dot plot showing the mean expression of selected genes in B cell clusters across treatment groups. c) Dot plots show the percentage of $BCR^+$ cells expressing each isotype (y-axis) in individual follicular B-cell clusters and marginal zone B-cells, comparing CSF-exposed (gSF) and normal-sleep control (gSC) mice. Each dot represents one mouse; bars indicate mean ± SEM. Statistical comparisons were made using unpaired two-tailed t-tests; $p < 0.05$ was considered significant. Exact P values are indicated in the figures where applicable. Comparisons with $P > 0.05$ are not labelled. Source Data are provided in the Source Data file.

Supplementary Figure S4



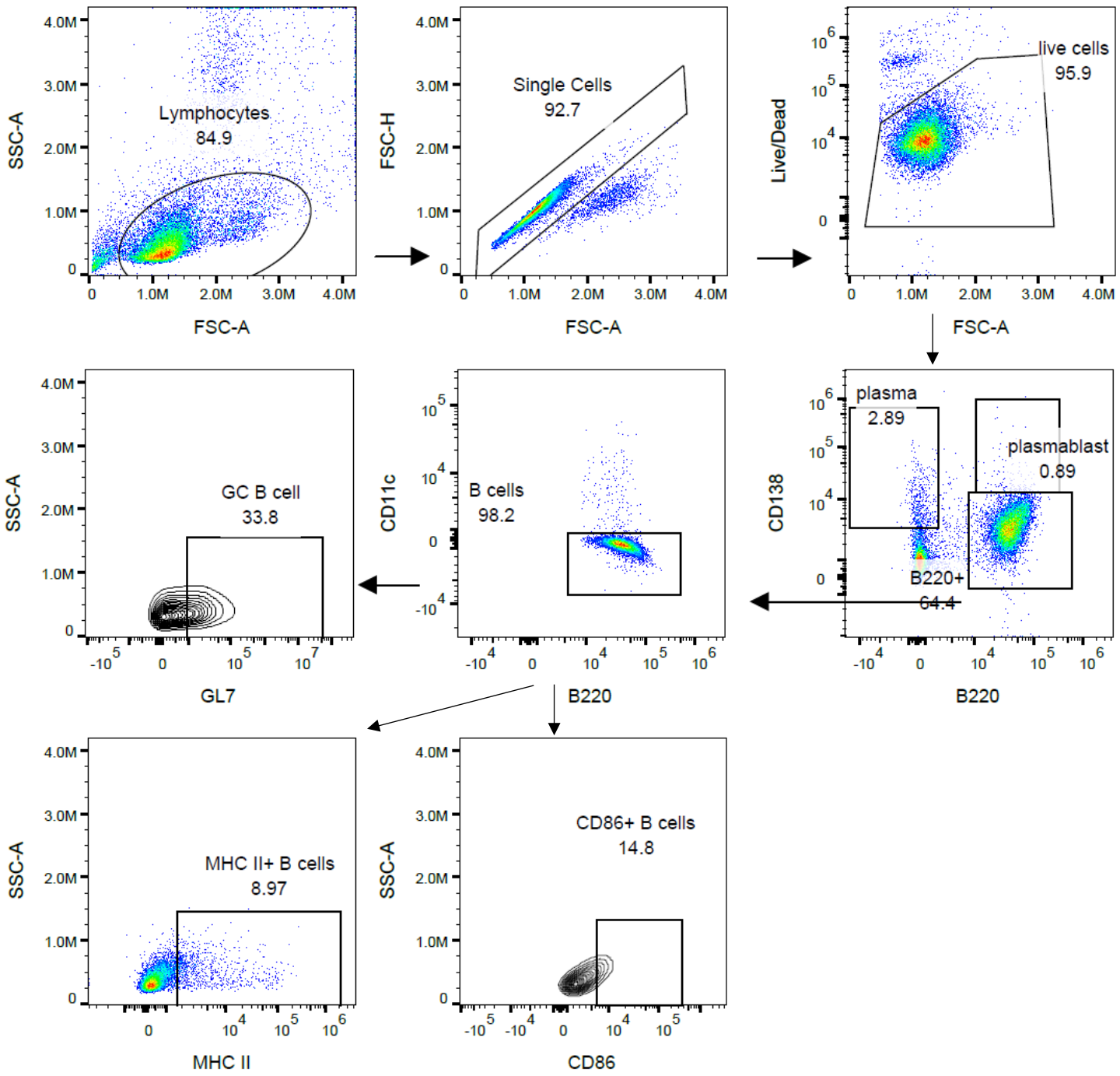

Supplementary Fig. S4. Flow cytometric comparison of lymphocyte and antigen-presenting cell subsets in the spleen at 35 days post vaccination between gSC and gSF groups. Frequencies of immune cell populations in the spleen were quantified by flow cytometry and expressed as percentage of parent population. Panels show comparisons between gSF and gSC groups for: a) activated antigen-presenting cells (APCs, non-T cells), total T cells, $CD4^+$ T cells, and $CD8^+$ T cells; b) $CD4^+$ T-cell subsets including naïve, central memory (TCM), and effector memory (TEM) cells; c) $CD8^+$ T-cell subsets including naïve, TCM, TEM, and Tfh-like cells. Each dot represents an individual sample, with bars indicating group means ± standard deviation (SD); and d) B-cell subsets including $CD86^+$ B cells, germinal center (GC) B cells, plasmablast/plasma cells (antibody-secreting cells) and MHC $II^+$ B cells; and Statistical comparisons between gSF and gSC were performed using two-sided Mann–Whitney U tests. For each panel, p-values were adjusted for multiple comparisons using false discovery rate (FDR) correction with the two-stage step-up method of Benjamini, Krieger, and Yekutieli, controlling the FDR at 5% (Q = 0.05). A corrected p-value (q value) < 0.05 was considered statistically significant. Exact P values are indicated in the figures where applicable. Each data point represents an individual animal, and bars indicate mean ± SD. Source Data are provided in the Source Data file.

Supplementary Figure S5




Supplementary Fig. S5. Flow cytometry gating strategy for identification of T-cell subsets. Representative gating strategy used to identify T-cell populations from splenocytes. Cells were first gated on lymphocytes (FSC-A vs SSC-A), followed by singlets (FSC-A vs FSC-H) and live cells. T cells were defined as $CD3^+$ cells and further subdivided into $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells were further classified into naïve, central memory (TCM), and effector memory (TEM) subsets based on CD62L and CD44 expression, as well as T follicular helper (Tfh)-like cells based on CXCR5 and ICOS expression. $CD8^+$ T cells were similarly subdivided into naïve, TCM, and TEM subsets. Non-T cells were analyzed for activated antigen-presenting cells (APCs). Percentages indicate the frequency of each population within the parent gate. Data were analyzed using FlowJo software (v10.10.1). Representative plots are shown.

Supplementary Figure S6




Supplementary Fig. S6. Flow cytometry gating strategy for identification of B-cell subsets. Representative gating strategy used to identify B-cell populations from splenocytes. Cells were first gated on lymphocytes (FSC-A vs SSC-A), followed by singlets (FSC-A vs FSC-H) and live cells. Cells were then subdivided based on B220 and CD138 expression into $B220^{+}$ cells and $CD138^{+}$ plasma cells/plasmablasts. Within the $B220^{+}$ population, CD11c was used to exclude myeloid cells, and total B cells were defined accordingly. Germinal center (GC) B cells were identified based on GL7 expression. Expression of MHC class II and the costimulatory molecule CD86 was further assessed within the B cell population. Percentages indicate the frequency of each population within the parent gate. Data were analyzed using FlowJo software (v10.10.1). Representative plots are shown.