

A Non-Canonical Effector Transcriptional Programme Characterises IFN- γ - Mediated Hyperinflammation in MIS-C

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Abstract

Background

Multisystem inflammatory syndrome in children (MIS-C) is a severe hyperinflammatory disorder that develops weeks after SARS-CoV-2 infection. IFN- γ is consistently implicated as a central mediator, yet the transcriptional programme and upstream signalling axis that sustain IFN- γ production in MIS-C remain poorly defined, and it is unclear whether they engage the canonical IL-12/STAT4/T-bet pathway or proceed through alternative effector routes.

Methods

We studied paired flare and remission samples from paediatric patients with MIS-C using an integrated multi-omic approach: single-cell multiome profiling (simultaneous single-cell RNA sequencing and chromatin accessibility profiling by ATAC-seq), bulk RNA sequencing in a discovery and an independent validation cohort, plasma cytokine profiling, and whole-exome sequencing. Within-patient flare–remission comparisons and cross-cohort replication were used to identify reproducible transcriptional and chromatin-level features.

Results

We characterise a non-canonical IFN- γ effector programme in MIS-C. CD8⁺ effector-memory T cells and natural killer cells showed IFNG upregulation alongside coordinated induction of BATF, STAT1, and PRDM1, confirmed in per-patient analyses across CD8⁺ effector-memory T, natural killer, and $\gamma\delta$ T cells, with broader lymphoid engagement extending to naive populations. STAT4 was downregulated across both bulk cohorts and in natural killer cells, and plasma IL-12p70 was not elevated, indicating limited engagement of the classical IL-12/STAT4 axis, whereas IL6ST (gp130) was upregulated, consistent with a gp130-associated programme converging on the BATF/STAT1 axis. The IFN- γ –regulatory long non-coding RNA IFNG-AS1 was downregulated despite sustained IFNG transcription, indicating chromatin-level decoupling. Checkpoint receptors (CTLA4, HAVCR2) were upregulated without induction of canonical exhaustion factors (NR4A2, NR4A3), indicating a persistent effector state distinct from exhaustion. CD8⁺ effector-memory and $\gamma\delta$ T cells converged on this output through distinct regulatory architectures. No monogenic predisposition was identified.

Conclusions

These observations associate MIS-C with a gp130-linked, BATF/STAT1/PRDM1-anchored non-canonical IFN- γ effector programme, consistent with acquired post-viral transcriptional reprogramming rather than monogenic predisposition. The findings nominate gp130-associated signalling, JAK1/2 inhibition, and

IFN- γ -directed therapy as candidate avenues for preclinical investigation in MIS-C and broader post-viral hyperinflammation.

BACKGROUND

Multisystem Inflammatory Syndrome in Children (MIS-C) is a rare, severe post-infectious inflammatory disorder that emerges weeks after SARS-CoV-2 infection, characterised by systemic hyperinflammation and multi-organ involvement^{1–3}. Despite its declining incidence, MIS-C has become an important clinical model for understanding how post-viral immune priming drives sustained hyperinflammation, a phenomenon central to conditions including long COVID^{4–6}. Both MIS-C and long COVID involve persistent immune activation, endothelial dysfunction, and delayed resolution of inflammation, underscoring the broader scientific relevance of continued MIS-C investigation for fundamental immunology⁷.

Early studies established that MIS-C flares are dominated by elevated IFN- γ , IL-6, IL-18, and TNF- α , distinguishing MIS-C from Kawasaki disease, which is driven primarily by IL-1 β and Th17 pathways^{8,9}. Comparative profiling further revealed that MIS-C is marked by transient but pronounced immune activation with heightened IFN- γ and IL-10, in contrast to the sustained hyperinflammation of severe adult COVID-19¹⁰. Notably, while paediatric COVID-19 is dominated by type I interferon signatures, MIS-C is distinguished by prominent IFN- γ (type II IFN)-dependent responses^{11–13}. Sacco et al. reported elevated IL-18 and IFN- γ alongside expanded activated CD8⁺ and CD4⁺ T cells in MIS-C patients¹⁴.

Despite this consensus on IFN- γ centrality, the transcriptional programmes and upstream signalling axes that sustain IFN- γ production in MIS-C remain poorly defined. By default, IFN- γ production is attributed to the IL-12/STAT4/T-bet axis in which IL-12 signalling activates STAT4. STAT4 can directly promote *IFNG* transcription and also induces T-bet (TBX21), which further enhances *IFNG* expression^{15,16}. However, alternative routes to IFN- γ exist, since IL-27 signalling through IL-27R α and gp130 activates STAT1 and STAT3 and can support type 1 effector gene expression, while BATF cooperating with IRF4 shapes effector T cell transcriptional programs that can promote *IFNG* expression in parallel to the canonical IL-12 STAT4 axis^{17,18}. Whether MIS-C utilises such non-canonical effector programmes has not been investigated.

A related unresolved question concerns the chromatin-level regulation of sustained IFN- γ production. IFN- γ signalling promotes histone acetylation, enhances chromatin accessibility, and can reshape three-dimensional genome architecture, establishing epigenetic states that persist beyond the initial stimulus^{19,20}. *IFNG-AS1*, a long non-coding RNA enhancer of the *IFNG* locus, normally fine-tunes IFN- γ output in a T-bet-dependent manner²¹. Whether this canonical enhancer mechanism operates normally during MIS-C, or whether chromatin-level bypass occurs, has not been explored, in part because no study has simultaneously profiled transcription and chromatin accessibility at single-cell resolution in MIS-C.

Immune checkpoints (CTLA-4, PD-1) and regulatory cytokines such as TGF- β normally constrain effector responses after infection^{22,23}. Recent work has implicated TGF- β -mediated suppression in MIS-C²⁴, but how checkpoint and TGF- β regulation interact with sustained IFN- γ signalling in this context remains unclear. Whether such dysregulation reflects monogenic predisposition or post-infectious transcriptional reprogramming is uncertain²⁵.

The limited understanding of the upstream axis sustaining IFN- γ production, the chromatin-level regulation of the *IFNG* locus, and the interplay of regulatory feedback with effector signalling motivated an integrated, multi-modal investigation of the MIS-C flare. We therefore set out to determine whether the IFN- γ -dominated hyperinflammation of MIS-C engages the canonical IL-12/STAT4/T-bet axis or proceeds through alternative effector routes, and how this programme is regulated at the transcriptional and chromatin-accessibility levels.

To investigate this, we combined single-cell multiome profiling (simultaneous RNA-seq and ATAC-seq), bulk RNA-seq, plasma cytokine profiling, and whole-exome sequencing on longitudinal paired flare-remission samples from paediatric MIS-C patients (Fig. 1). While prior studies have applied single-cell transcriptomics and CITE-seq to MIS-C¹⁴, to our knowledge this is the first study combining simultaneous single-cell RNA-seq and ATAC-seq on paired longitudinal samples, enabling integrated analysis of transcriptional and chromatin-level immune dynamics²⁶. Our findings characterise a non-canonical IFN- γ effector programme in MIS-C, identify candidate immunomodulatory nodes for further investigation, and position $\gamma\delta$ T cells as previously underappreciated contributors to MIS-C pathophysiology.

METHODS

This prospective, observational study aimed to characterise the immune transcriptional programme sustaining IFN- γ -mediated hyperinflammation in multisystem inflammatory syndrome in children (MIS-C), and to determine whether it engages the canonical IL-12/STAT4/T-bet axis or proceeds through alternative effector routes. Paediatric patients diagnosed with MIS-C according to WHO criteria were recruited at the University Children's Hospital, University Medical Centre Ljubljana, Slovenia. Peripheral blood was sampled at two timepoints: during acute flare (before immunomodulatory treatment) and in clinical remission (6–12 months post-illness) and analysed by an integrated multi-omic approach comprising single-cell multiome profiling, bulk RNA sequencing in a discovery and an independent validation cohort, plasma cytokine profiling, and whole-exome sequencing.

Study design and ethical approval

Informed consent

was obtained from the legal guardians of all participants, and the study was approved by the National Ethics Committee for Research in Medicine, Slovenia (approval number 0120–536/2020/3). The primary

cohort included 10 patients (median age 8.4 years; 40% male), while the validation cohort comprised 9 patients (median age 11 years; 55% male). All participants presented with fever and gastrointestinal symptoms, with frequent mucocutaneous and neurologic involvement. Samples were collected at two timepoints: during acute flare (prior to immunomodulatory treatment) and in clinical remission (6–12 months post-illness). At MIS-C onset, all participants were seropositive for SARS-CoV-2. Acute virologic testing at presentation was largely negative: RT-PCR was negative in 8/10 (2 not tested) in the main cohort and in 6/9 in the validation cohort. Evidence of recent exposure was documented in most cases: confirmed SARS-CoV-2 infection within ~ 3–6 weeks (or parental infection) in 6/10 (including 2 parental) in the main cohort and 6/9 (including 3 parental) in the validation cohort; 4/10 and 3/9, respectively, had no confirmed PCR-positive test.

Sample Collection and Biobanking

Peripheral blood samples were obtained in EDTA and heparin tubes. PBMCs were isolated using Ficoll-Paque density gradient centrifugation, resuspended in FBS with 10% DMSO, and cryopreserved for later use. Total RNA was preserved using PAXgene Blood RNA tubes and stored at – 80°C. Genomic DNA was extracted using the Qiagen FlexGene DNA kit and stored at 4°C.

Plasma Cytokine Profiling

Plasma cytokines were quantified by multiplex bead-based immunoassay using the Human COVID-19 Cytokine Storm Panel 1 and Panel 2 (LEGENDplex, BioLegend), following the manufacturer's instructions. Samples were incubated with antibody-coated beads, washed, and incubated with detection reagent as specified. Data acquisition was performed on a BD FACSLyric flow cytometer (BD Biosciences), and cytokine concentrations were calculated using LEGENDplex Data Analysis Software Suite (BioLegend). Cytokine concentrations below the assay detection limit were reported as provided by the analysis software. For analytes with high rates of values below detection in remission samples (notably IFN- γ : valid results in 92.3% of flare vs. 37.5% of remission samples), formal paired statistical testing was precluded; these analytes are reported with observed magnitudes and consistency across patients with valid results in both conditions. For all other cytokines, paired Wilcoxon signed-rank tests with Benjamini-Hochberg correction were applied.

Single-cell Multiome Library Preparation and Sequencing

Cryopreserved PBMCs were used to prepare nuclei suspensions for the Chromium Single Cell Multiome ATAC + Gene Expression assay (10x Genomics). Nuclei isolation followed the optimized protocol "Isolation of Nuclei for Single Cell RNA Sequencing" provided by 10x Genomics.

Cells were rapidly thawed in a 37°C water bath for 1–2 minutes, ensuring a small ice crystal remained. After transfer to a 50 mL conical tube, cells were rinsed with pre-warmed RPMI + 10% FBS and gradually

diluted to prevent osmotic shock. Cells were centrifuged at $300 \times g$ for 5 minutes at room temperature, resuspended in granulocyte buffer (PBS + 0.5% BSA + 2 mM EDTA), and kept on ice. Granulocytes were depleted using CD66abce-Biotin and Anti-Biotin MicroBeads (Miltenyi Biotec) followed by magnetic separation using LD Columns (Miltenyi Biotec). Unlabeled cells were treated with DNase I (5-minute incubation on ice) to remove extracellular DNA. Cells were washed in PBS + 0.04% BSA and counted using a DeNovix counter with AO/PI staining. For nuclei extraction, 800,000 cells were pelleted ($300 \times g$, 5 min, 4°C), resuspended in chilled 0.1X lysis buffer, and incubated on ice for 10 minutes. Lysed cells were washed three times in chilled wash buffer and centrifuged at $500 \times g$ for 5 minutes. The final nuclei suspension was filtered through a $40 \mu\text{m}$ Flowmi strainer and resuspended in 20 μL of Diluted Nuclei Buffer. Nuclei quality and concentration were verified using AO/PI staining on a DeNovix counter. Library construction followed the manufacturer's Multiome ATAC + Gene Expression workflow. Paired-end sequencing was performed on an Illumina NovaSeq 6000 Sequencing System (Illumina), targeting approximately 20,000 read pairs per cell for 10,000 cells per sample.

Single-Cell Multiome Data Analysis

Sequencing data were processed using the 10x Genomics Cell Ranger ARC Analysis Pipeline (v2.0) to generate counts. Quality control was performed using Seurat (v5.1.0)^{26,55}, SeuratObject (v5.0.2), Signac (v1.13.0)⁵⁶, and scDbfFinder (v1.8.0)⁵⁷. Cell type annotation used reference-based mapping with the Azimuth human PBMC reference atlas, constructed from multimodal CITE-seq data and integrated using weighted nearest neighbour (WNN) analysis, as previously described⁵⁸. This reference incorporates joint transcriptomic and surface protein information to improve immune cell state resolution. Filters included total fragments in peaks (1,000–100,000), TSS enrichment (> 1), nucleosome signal (< 4), UMI counts (> 500), gene features (> 250), and mitochondrial RNA content ($< 20\%$). Doublets and low-quality cells were removed (Additional File 1: Figure S1). Each patient dataset was preprocessed separately (Additional file 2: Table S3). ATAC data were normalised using TF-IDF and reduced via SVD; RNA data were normalised via SCTransform and reduced via PCA. Harmony (v1.2.0) was used to correct batch effects in both assays⁵⁹. Datasets were merged in Seurat using canonical correlation analysis (CCA), clustered at resolution 0.6, and visualised with UMAP (Additional File 1: Figure S2). Cell types were annotated using Seurat's FindTransferAnchors against the Azimuth reference atlas (Additional File 1: Figure S3). Differential expression testing used presto (v1.0.0) for accelerated Wilcoxon rank-sum computation.

Cell type composition shifts were tested using paired Wilcoxon signed-rank tests with Benjamini-Hochberg correction for multiple comparisons across all cell types (Additional file 2: Table S4). Compared to compositional tools such as scCODA, this approach enabled clear matched-pair interpretation within each patient. To assess robustness of the cohort-level findings to extreme per-sample cell yields, we performed a leave-out sensitivity analysis in which the paired analysis was repeated on the remaining eight paired patients after excluding P6 and P8 (Additional file 2: Table S4a). The two excluded patients sit at opposite extremes of the per-sample cell-yield distribution after quality control: P6 contributed only 702 cells in the flare phase (~ 3 -fold below the next-lowest flare sample)

while P8 contributed 11,692 cells in the flare phase (~ 2-fold above the next-highest). Removing both patients therefore probes the robustness of cohort-level findings to the lowest- and highest-yield outliers simultaneously, without selectively retaining outliers in one direction. All visualisations were generated using ggplot2 (v3.5.1) with patchwork (v1.2.0) for multi-panel layout.

Differential expression analysis.

Three complementary analyses were performed to assess robustness at both the cell and patient levels. *First*, an integrated analysis across all patients was performed after Harmony batch correction, in which composite identities (cell type × condition) were generated and DE analysis was computed using Seurat FindMarkers (Wilcoxon rank-sum test) on the merged dataset. We note that cell-level Wilcoxon tests in the integrated analysis treat cells as independent observations and may inflate statistical significance; integrated p-values should therefore be interpreted alongside the patient-level analyses described below. *Second*, within-patient directional consistency was assessed by computing differential expression independently within each patient using Seurat FindMarkers (Wilcoxon rank-sum test on cells from flare vs remission within that patient). For each gene, the proportion of patients showing the same direction of change was calculated. This within-patient cell-level test provides a measure of directional consistency across individuals rather than a formal patient-level effect estimate. *Third*, to provide a formal patient-level effect estimate, we performed a one-sample Wilcoxon signed-rank test on per-patient log₂FC values for key genes, testing whether the distribution of within-patient effects was significantly shifted from zero. This approach treats each patient as a single observation (n = 7–10 depending on gene detection), avoiding the pseudoreplication inherent in cell-level testing. P-values were adjusted using Benjamini-Hochberg correction (Additional File 1: Figure S4).

Key transcription factor and pathway findings reported in the main text were confirmed across all three analytical approaches where applicable, with integrated p-values, within-patient directional consistency, and patient-level signed-rank statistics reported together. Independent bulk RNA-seq validation in both the discovery and external validation cohorts provides additional confirmation. GSEA was conducted using fgsea (v1.31.0) for each cell type on the integrated DE results.

Regulatory Network and DORC Analysis

Chromatin-level regulatory architecture associated with the IFN-γ effector programme was examined by inferring gene regulatory networks (GRNs) using FigR (v1.0.1; Kartha et al., 2022) independently in two cytotoxic effector populations, CD8⁺ effector-memory T cells (CD8 TEM) and γδ T cells. Within each cell type, FigR was applied separately to single cells from MIS-C flare samples and from the matched remission samples of the same patients, and a post-hoc comparison of the two runs was used to identify regulatory edges associated specifically with the flare condition. FigR identifies correlations between transcription-factor motif accessibility and chromatin-level target-gene expression at domains of regulatory chromatin (DORCs); these are statistical associations rather than demonstrated causal regulatory relationships, and are interpreted as candidate regulatory wiring throughout.

RNA and ATAC matrices were extracted from harmonised Seurat objects, split by cell type and condition. Chromatin accessibility topics (1–50) were defined using *cisTopic* (v0.3.0)⁶⁰ and the optimal model was selected by likelihood. A k-nearest-neighbour graph ($k = 40$) was built from topic distributions using the *FNN* package (CRAN) and used for smoothing. Peak–gene correlations were computed with *FigR::runGenePeakcorr* (hg38, 100 permutations); peaks reaching peak–gene p -value z -score ≤ 0.05 were aggregated into DORC scores using *FigR::getDORCScores* and smoothed over the kNN graph; smoothed RNA values were obtained analogously. Motif accessibility–DORC expression correlations and their permutation p -values were then computed with *FigR::runFigRGRN*. Significant TF–DORC interactions were defined as those reaching $Corr.P < 0.05$ within each run, and were classified as candidate activators ($Corr \geq 0$) or candidate repressors ($Corr < 0$).

Per-cell-type FigR runs were filtered to three pre-specified DORC gene panels capturing the mechanistic axes of the manuscript (an IFN- γ effector panel, a TGF- β pathway panel and a checkpoint/exhaustion panel; full per-cell-type gene lists in Additional file 2: Table S8). The panels were curated with reference to the differential-expression findings reported in this manuscript and are therefore not statistically independent of the transcriptomic signal; the chromatin-level results based on these panels should be read as complementary mechanistic evidence rather than as an independent statistical confirmation. The IFN- γ and Checkpoint panels share a common core gene set across cell types and add a small number of cell-type-specific genes reflecting the divergent effector biology of CD8 TEM and $\gamma\delta$ T cells; the TGF- β panel is identical across cell types because the canonical TGF- β signalling cascade does not differ between them at the gene-membership level.

To identify regulatory edges associated specifically with the MIS-C flare condition, the four resulting tables for each cell type (flare activators, flare repressors, remission activators, remission repressors) were intersected on exact (motif, DORC) pairs. An edge was retained as flare-exclusive if and only if it reached $Corr.P < 0.05$ in the flare run and the corresponding (motif, DORC) pair did not reach $Corr.P < 0.05$ in the matched remission run; activators and repressors were intersected separately to avoid sign-flipping artefacts. The concatenated flare-exclusive activator and repressor sets are reported in Additional file 2: Table S8 (1,714 edges across CD8 TEM and $\gamma\delta$ T; 942 in CD8 TEM and 772 in $\gamma\delta$ T).

Top transcription-factor hubs were defined within each cell type by ranking transcription-factor motifs by the number of distinct significant target DORCs they regulate across the three pathway panels combined; these rankings underlie the heatmap row order in Fig. 6 panels A and B and the bar-plot selection in panels C and D. For Fig. 6, an additional $|correlation| \geq 0.3$ filter and a top-30 motifs-by-edge-count cap were applied to the rows of Additional file 2: Table S8 to produce a tractable and visually legible figure; readers wishing to inspect the full evidence underlying any single transcription factor or DORC may consult Additional file 2: Table S8 directly.

Bulk RNA Sequencing

RNA sequencing was performed for both the primary and validation cohorts. Total RNA was extracted using the PAXgene Blood RNA Kit (PreAnalytiX, Cat# 762165). Libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep Kit (New England Biolabs, Cat# E7760S) with prior ribosomal RNA depletion using the NEBNext rRNA Depletion Kit (New England Biolabs, Cat# E6310). Quality control was assessed on the Agilent 2100 Bioanalyzer with the Bioanalyzer RNA 6000 Pico Assay. Paired-end sequencing (2 × 150 bp) was performed on a NovaSeq 6000 Sequencing System (Illumina).

Reads were aligned to the GRCh38 reference genome using the STAR aligner⁶¹. Differential gene expression was computed using DESeq2 (v1.38.3)⁶² and edgeR (v3.36.0)⁶³. Annotation infrastructure used AnnotationDbi, GenomicRanges, and rtracklayer (Bioconductor). Enrichment analysis was conducted using the fgsea (v1.31.0) R package with MSigDB Hallmark gene sets^{64,65}. The complete code is available on GitHub: <https://github.com/CISLD/urnades-universal-rna-differential-expression-scripts>.

To assess reproducibility of the differential-expression signal, per-gene log₂ fold-changes (flare vs remission) were compared between the main (n = 10 paired samples) and external validation (n = 9 paired samples) cohorts, restricted to genes detected in both. Genome-wide concordance was quantified as the Pearson correlation of log₂ fold-changes across all commonly detected genes and, separately, across genes reaching significance (adjusted p < 0.05) in both cohorts. Each gene was assigned a concordance class from its per-cohort significance and direction: not significant in either cohort; significant in the main cohort only; significant in the validation cohort only; significant in both cohorts with concordant direction (separated into concordantly up- and down-regulated); or significant in both with opposite direction (discordant). Directional concordance among genes significant in both cohorts was expressed as the percentage sharing the same sign of log₂ fold-change (Additional File 1: Figure S5). For targeted visualisation, manuscript-relevant genes were curated into panels spanning the transcription factors, IFN-γ-axis components, interferon-stimulated genes, checkpoint ligands, alarmins, and effector molecules referenced in the Results, together with all single-cell-derived and FigR DORC-identified regulators. A wide panel of 113 genes (111 expressed in the bulk data) and a focused 39-gene panel matching the single-cell pathway organisation (Additional file 2: Table S6) were defined; their per-gene log₂ fold-changes, adjusted p-values, and cross-cohort concordance status are reported in Additional file 2: Table S7 (wide and focused panels on separate sheets). A 15-gene subset spanning four functional categories (non-canonical / gp130-associated transcription factors; the STAT1 IFN-γ core module and downstream ISGs; the canonical IL-12/STAT4/T-bet axis; and IFN-γ effector output) was displayed as a forest plot showing both cohorts' per-gene estimates. All bulk differential-expression statistics in these analyses derive from the DESeq2 Wald test with BH correction; concordance analysis and figure generation were performed in R using ggplot2.

Whole-Exome Sequencing and Variant Analysis

Genomic DNA was processed using the IDT xGen Exome Research Panel v1.0 (Integrated DNA Technologies, Cat# 1056113). Libraries were prepared using tagmentation and hybrid capture. Paired-end sequencing (2 × 150 bp) was performed on a NovaSeq 6000 Sequencing System (Illumina). Reads were aligned using BWA-MEM to the hg19 reference genome⁶⁶. Variant calling was performed with GATK HaplotypeCaller⁶⁷. Variant consequences were annotated with the Ensembl Variant Effect Predictor⁶⁸, and population allele frequencies were obtained from gnomAD v2.1.1⁶⁹. WES analysis focused on gene-level rare variant identification (exonic and splice-site variants within a curated gene panel) rather than coordinate-level integration with single-cell data; since variant interpretation was performed at the gene symbol level, which is consistent across genome builds, gene-level conclusions are not affected by the use of hg19 for WES versus GRCh38 for transcriptomic analyses. Variants were filtered to retain rare (MAF < 0.01) exonic, nonsense, and splice-site variants in a curated 1,091-gene immune panel (Additional file 2: Table S9) spanning: (i) genes from the Human Phenotype Ontology associated with autoinflammatory diseases, primary immunodeficiencies, and monogenic inflammatory bowel disease (n = 578); (ii) candidate genes identified in a multicentre study of rare variants in MIS-C, MIS-A, Kawasaki disease, and severe COVID-19⁷⁰ (n = 84 not already covered above); (iii) HALLMARK_INTERFERON_GAMMA_RESPONSE genes (n = 139); (iv) HALLMARK_TGF_BETA_SIGNALING genes (n = 49); and (v) transcription factors identified in our FigR regulatory network analysis (n = 230). Variants were further prioritised if present in at least five patients.

Statistical Analysis

All statistical analyses were performed in R (v4.3.0). Plasma cytokine comparisons used paired Wilcoxon signed-rank tests with Benjamini-Hochberg correction for multiple testing. Single-cell differential expression used the Wilcoxon rank-sum test via Seurat FindMarkers, performed both per-patient (paired) and on the integrated dataset; per-patient directional consistency was assessed by computing the proportion of patients showing the same direction of change for each gene, and a one-sample Wilcoxon signed-rank test on per-patient log₂FC values provided formal patient-level effect estimates. GSEA was performed using fgsea (v1.31.0) with Benjamini-Hochberg correction. Cell type composition changes were tested with paired Wilcoxon signed-rank tests with Benjamini-Hochberg correction. Bulk RNA-seq DE used DESeq2 (Wald test) with Benjamini-Hochberg correction. Data manipulation used dplyr (v1.1.4), tibble (v3.2.1), and data.table (v1.15.4); structured data matrices used SummarizedExperiment (Bioconductor). For all analyses, statistical significance was defined as adjusted p < 0.05 unless otherwise stated. Large language models (Claude, Anthropic; ChatGPT, OpenAI) were used to assist with code drafting and language editing; all outputs were reviewed and approved by the authors.

RESULTS

Among 88 children with MIS-C enrolled in the broader clinical cohort²⁷, 19 patients with paired biospecimens collected during acute flare (before treatment) and in remission (6–12 months later) were

included in the study. The main cohort comprised 10 patients (40% male; median age 8.4 years) who underwent multiomic single-cell RNA and ATAC sequencing, along with bulk RNA-seq. The validation cohort comprised 9 patients (55% male; median age 11 years) for independent bulk RNA-seq replication (Fig. 1). Plasma cytokine profiling was performed on 11 patients with available samples, including 9 from the main cohort and 2 additional patients from the broader clinical cohort who were not included in the sequencing analyses.

At MIS-C onset, all participants had positive SARS-CoV-2 serology and negative or absent SARS-CoV-2 PCR at presentation, consistent with the post-infectious timing characteristic of MIS-C. Documented SARS-CoV-2 infection 3–6 weeks prior to MIS-C onset was confirmed in 6/10 (main) and 6/9 (validation) cases, with parental infection documented in 2 and 3 additional cases respectively. Inferred prior infection from parental exposure should be interpreted as supportive but not definitive evidence. All patients presented with fever and gastrointestinal symptoms, with frequent occurrence of rash, conjunctivitis, lymphadenopathy, and neurological symptoms. One patient per cohort required ICU care. Inflammatory markers (CRP, ferritin), cardiac injury markers (troponin, BNP), and coagulation indicators (D-dimer) were elevated during flare in both cohorts. All patients received intravenous immunoglobulin (IVIG) and corticosteroids; anticoagulation therapy was administered in 70% (main) and 55% (validation) of cases, and one patient in the validation cohort received biologic therapy. Detailed clinical characteristics are provided in Additional file 2: Table S1.

Plasma Cytokine Profiles Reveal IFN- γ Elevation Without Significant IL-12p70 Induction

To define the systemic inflammatory milieu of MIS-C flares, we quantified 26 plasma cytokines in paired flare–remission samples from 11 patients (Fig. 2; Additional file 2: Table S2). Twelve cytokines were significantly elevated during flares, dominated by IFN- γ signalling with concurrent myeloid activation and limited regulatory counter-response.

IFN- γ was the most prominently elevated cytokine (~ 30 -fold; 2184 vs. 71 pg/mL), with 62.5% of remission values below detection. CXCL10 (IP-10), a canonical IFN- γ -induced chemokine recruiting CXCR3⁺ effectors²⁸, was likewise markedly elevated (4063 vs. 103 pg/mL; adj.p = 0.019). IL-18, which potentiates cytotoxicity with IFN- γ ^{29,30} and the IL-1 receptor antagonist IL-1RA³¹ were both significantly increased (995 vs. 260 and 3917 vs. 451 pg/mL; both adj.p = 0.019). T cell activation, acute-phase, and myeloid trafficking markers were also elevated, including CD25/IL-2R α , IL-6, VEGF, CCL2, G-CSF, CCL4, CXCL8, and CCL5/RANTES (all adj.p < 0.05; Additional file 2: Table S2), VEGF aligning with the endothelial dysfunction characteristic of MIS-C⁷. IL-10, a counter-regulatory cytokine induced by IFN- γ ³², was significantly increased (~ 12 -fold; adj.p = 0.019) but did not restrain IFN- γ -associated activation.

Critically, cytokines expected to drive IFN- γ production through classical pathways were not elevated: IL-12p70, the upstream activator of the IL-12/STAT4/T-bet axis, did not differ between conditions (adj.p = 0.52), and IL-2, IL-15, and IFN- α 2 were similarly unchanged, indicating that neither systemic γ c-cytokine

signalling nor type I interferon drives MIS-C effector activation, though local contributions cannot be excluded. TNF- α was also not elevated, consistent with an IFN- γ -dominated rather than TNF- α /NF- κ B-centred profile. This dissociation between IFN- γ elevation and absent IL-12p70 induction motivated integrated single-cell transcriptomic and chromatin accessibility profiling to identify the non-canonical programmes sustaining IFN- γ production in MIS-C.

Figure 2. Plasma cytokine profiling reveals IFN- γ -associated inflammation with limited IL-12/STAT4 engagement in MIS-C flares.

Paired plasma cytokine measurements from MIS-C patients ($n = 11$) during acute flare and remission. Boxplots show selected cytokines including IFN- γ , CXCL10, IL-18, IL-6, VEGF, IL-2R α , IL-10, and IL-12p70. IFN- γ , CXCL10, IL-18, IL-6, IL-2R α , and IL-10 were significantly elevated during flare, whereas IL-12p70 was not ($p = 0.46$), consistent with limited engagement of the classical IL-12/STAT4 axis. Paired Wilcoxon signed-rank tests with Benjamini-Hochberg correction; for IFN- γ (valid results: 92.3% flare vs. 37.5% remission), observed magnitudes are reported in place of formal paired testing.

Single-Cell Transcriptomics Reveals Expansion of Proliferative Effector Compartments and Decreased Peripheral Representation of Unconventional T Cells

In total, 89,154 individual nuclei isolated from PBMCs were analysed after quality filtering and doublet removal. Of these, 37,842 cells were obtained during acute flare (prior to treatment) and 51,312 during remission, with an average of 3,784 (SD = 3,128) and 5,131 (SD = 1,626) cells per patient, respectively (Fig. 3A and B, Additional file 2: Table S3).

Analysis of single-cell transcriptomic data revealed significantly altered immune cell proportions during MIS-C flares compared to remission (Fig. 3C; Additional file 2: Table S4). The most prominent compositional changes involved cytotoxic and proliferative compartments: CD8⁺ proliferating cells (adj. $p = 0.014$) and NK proliferating cells (adj. $p = 0.014$) were present at increased frequency in peripheral blood during flares, while CD8⁺ TEM cells (adj. $p = 0.041$), MAIT cells (adj. $p = 0.014$), and CD4⁺ CTLs (adj. $p = 0.042$) were reduced. B naïve cells were increased (adj. $p = 0.035$), and plasmablasts showed a trend toward increased frequency (adj. $p = 0.051$). Frequencies of cDC2 were significantly reduced (adj. $p = 0.015$), while pDCs and CD16⁺ monocytes showed modest reductions (adj. $p > 0.05$).

Simultaneous expansion of CD8⁺ and NK proliferating populations alongside reduction of CD8⁺ TEM and MAIT cells in peripheral blood is consistent with continuous effector generation paired with emigration to inflamed tissues³³, supported by elevated CXCL10 (~ 40-fold increase). The CD4⁺/CD8⁺ T cell ratio shift ($p = 0.031$; Fig. 3D) may reflect total CD8⁺ tissue redistribution rather than total CD4⁺ expansion.

Figure 3. Single-cell profiling reveals altered immune composition and CD4⁺/CD8⁺ ratio in MIS-C flares.

Single-cell transcriptomic landscape of paired MIS-C flare and remission PBMC samples. Joint UMAP of 89,154 harmony-integrated nuclei from 10 paediatric MIS-C patients (37,842 flare; 51,312 remission). (A) Cells coloured by cell-type annotation (30 PBMC populations resolved across adaptive and innate

compartments). (B) Same embedding coloured by disease state (red, flare; teal, remission). (C) Paired proportions of immune cell subsets identified by single-cell transcriptomics during flare (red) and remission (blue) across 10 MIS-C patients. Box plots show median and interquartile range; individual points represent per-patient proportions. Significance: * adj.p < 0.05, ** adj.p < 0.01, *** adj.p < 0.001 (paired Wilcoxon signed-rank test with Benjamini-Hochberg correction). (D) CD4⁺/CD8⁺ T cell ratio in peripheral blood during MIS-C flare compared with remission (paired Wilcoxon signed-rank test).

The proliferative expansion of CD8⁺ and NK populations occurs against a cytokine background that lacks the classical drivers of lymphocyte proliferation. Neither IL-2 nor IL-15, the classical γ -cytokine drivers of lymphocyte proliferation^{34,35}, were significantly elevated in plasma, whereas IFN- γ and its downstream mediators were substantially increased. This dissociation is consistent with IFN- γ –associated effector proliferation rather than conventional γ -cytokine–mediated expansion, in line with previous observations in MIS-C³⁶. Transcriptional profiling of these proliferating populations confirmed upregulation of E2F targets, G2M checkpoint genes, MYC targets, and metabolic reprogramming pathways (Fig. 4B), further supporting active cell-cycle engagement in the context of inflammatory rather than homeostatic signalling.

Figure 4. Hallmark pathway enrichment across immune compartments in MIS-C flares.

(A) Bulk RNA-seq GSEA of MSigDB Hallmark pathways in the main and validation cohorts. Bars show normalised enrichment scores (NES); filled bars indicate FDR < 0.05. IFN- γ , IFN- α and inflammatory response pathways are consistently enriched in flare versus remission. (B) Bubble plot of Hallmark enrichment across bulk RNA-seq and single-cell RNA-seq immune subsets. Bubble size shows $-\log_{10}(\text{FDR})$, and colour indicates enrichment direction. IFN response pathways are broadly upregulated, whereas TGF- β and TNF- α /NF- κ B signalling are reduced in cytotoxic effector compartments. (C) Differential expression of manuscript-curated genes grouped by mechanistic pathway. The 39 genes shown are a curated subset selected to represent the principal mechanistic claims of the non-canonical IFN- γ effector programme. Left: bulk RNA-seq main and validation cohorts. Right: harmony-integrated single-cell DE across six PBMC populations. Dot size shows $-\log_{10}(\text{adjusted } p)$, colour shows $\log_2$ fold-change, open circles indicate non-significance, and grey squares indicate undetected genes.

The significant reduction of cDC2 frequencies during flare, combined with the broader reduction of antigen-presenting cell subsets, might be consistent with prior reports of dendritic cell dysfunction in MIS-C^{9,14}. The reduction of key APC populations, occurring alongside IFN- γ –associated cytotoxic activation, may contribute to altered regulatory control of effector responses^{37,38}. CD4⁺ TCM cells showed a trend toward expansion ($p = 0.067$), and may reflect a memory reservoir being primed under sustained inflammatory conditions. The expansion of B naïve cells may reflect enhanced lymphopoiesis or mobilisation, while the trend toward increased plasmablasts is consistent with terminal B cell differentiation and polyclonal antibody production reported in prior MIS-C studies^{10,39}.

IFN- γ Pathway Activation Is Pervasive Across Immune Compartments and Is Associated with a Non-Canonical BATF/STAT1/PRDM1 Transcriptional Profile

Single-cell transcriptional profiling revealed pervasive upregulation of the IFN- γ response pathway across multiple immune compartments during MIS-C flares (Fig. 4C). Significant enrichment of the IFN- γ response pathway (GSEA, FDR < 0.05) was detected across 11 cell types spanning both adaptive and innate immunity, including CD8⁺ TEM cells, NK cells, CD8⁺ proliferating T cells, proliferating NK cells, CD4⁺ naïve and TCM cells, CD8⁺ TCM cells, B naïve and memory cells, CD14⁺ monocytes, and CD16⁺ monocytes (Fig. 4; Additional file 2: Table S5). This breadth is consistent with paracrine IFN- γ signalling activating JAK1/2–STAT1 across immune compartments²⁰. A conserved core module comprising *GBP1*, *IFITM1*, *STAT1*, *BATF*, *PARP9*, and *SOCS1* was recurrently and significantly elevated across cell types (Additional file 2: Table S6), defining a shared IFN- γ –responsive signature that characterises MIS-C flares. *STAT3* was concordantly but modestly upregulated in most cell types, consistent with engagement of gp130-family signalling alongside the IFN- γ response. Notably, the non-canonical transcription factor programme was not restricted to cytotoxic effector populations (Fig. 4C). CD4⁺ naïve cells exhibited the strongest *BATF* (+ 3.45), *PRDM1* (+ 2.54), and *IRF4* (+ 1.55) induction in the entire dataset, alongside robust *SOCS3* upregulation (+ 2.23) and the strongest *IFNG-AS1* downregulation across any cell type (– 2.17; all harmony adj.p < 1e-30; paired padj = 0.012–0.015). CD8⁺ naïve-like cells showed a parallel pattern (*BATF* + 3.18, *PRDM1* + 1.75, *IRF4* + 1.31, *IFNG-AS1* – 2.02; all harmony adj.p < 1e-7; paired padj ≤ 0.015). The non-canonical transcription factor axis was robustly validated by bulk RNA-seq in both cohorts (Fig. 4C, Additional file 2: Table S7). *BATF* (main cohort log₂FC = + 2.27, adj.p = 1.2×10⁻³³; validation log₂FC = + 1.69, adj.p = 6.7×10⁻⁵), *STAT1* (main + 1.64, adj.p = 6.5×10⁻¹⁵; validation + 0.94, adj.p = 3.8×10⁻³) and *STAT3* (main + 0.97, adj.p = 7.4×10⁻²⁴; validation + 0.73, adj.p = 5.2×10⁻³) were strongly upregulated, while *STAT4* was concordantly downregulated in both cohorts (main – 0.93, adj.p = 2.5×10⁻⁹; validation – 0.89, adj.p = 2.2×10⁻⁵), confirming the dissociation between non-canonical effector TF induction and the canonical IL-12/STAT4 axis at the bulk level. *PRDM1* reached significance only in the main cohort (+ 0.79, adj.p = 6.2×10⁻¹⁰), consistent with the expected attenuation of cell-type-specific changes when single-cell signals from cytotoxic effector populations are diluted in bulk PBMC profiling. Of 113 key TF and pathway genes nominated by single-cell analysis and detected in bulk RNA-seq, 67 (60%) were significantly differentially expressed in both cohorts with concordant direction (Additional file 2: Table S7, Additional File 1: Figure S4). Consistent with previous reports¹⁴, the alarmins S100A8 and S100A9 were broadly upregulated across nearly all immune cell types profiled (CD14⁺ and CD16⁺ monocytes, NK, $\gamma\delta$ T, CD8⁺ TEM, CD4⁺ TCM, CD4⁺ and CD8⁺ naïve cells; harmony adj.p < 1e-8) in the integrated analysis, although patient-level paired Wilcoxon testing did not reach significance (paired padj > 0.07), reflecting between-patient heterogeneity in alarmin induction, co-occurring with IFN- γ pathway activation.

Having established the breadth of IFN- γ pathway activation, we investigated which transcriptional programmes are associated with IFN- γ production in the representative effector populations: CD8⁺ TEM, NK, and $\gamma\delta$ T cells. Classically, IFN- γ induction is attributed to the IL-12/STAT4/T-bet axis⁴⁰. However, our

data reveal a different transcription factor profile. *BATF* was among the most strongly upregulated transcription factors across all three cytotoxic effector populations: CD8⁺ TEM (+ 1.33), NK (+ 1.26), and $\gamma\delta$ T cells (+ 2.40; all harmony adj.p < 1e-10; paired padj $\leq$ 0.079). *STAT1* and *PRDM1* followed the same pattern across CD8⁺ TEM, NK, and $\gamma\delta$ T cells (*STAT1*: +0.79, + 1.07, + 1.14; *PRDM1*: +1.78, + 1.31, + 1.05; all harmony adj.p < 1e-3; paired padj $\leq$ 0.065 in CD8⁺ TEM and NK; in $\gamma\delta$ T cells *STAT1* and *PRDM1* trended toward significance but did not reach the patient-level threshold (paired padj = 0.052 and 0.065). *IRF4* showed cell-type-specific induction, reaching significance only in CD8⁺ TEM (+ 0.93, harmony adj.p = 0.013; paired padj = 0.020) but not in NK or $\gamma\delta$ T cells (paired padj = 0.27 and 0.20), indicating that *IRF4* contributes to the non-canonical programme in CD8⁺ TEM but is not uniformly engaged across all cytotoxic effector populations. *STAT3*, the gp130-coupled transcription factor, was concordantly upregulated in CD8⁺ TEM (+ 0.30, harmony adj.p = 2.6e-3; paired padj = 0.014) and $\gamma\delta$ T cells (+ 0.58, harmony adj.p = 7.7e-13; paired padj = 0.052), and showed a patient-level trend in NK cells (paired padj = 0.056), supporting receptor-proximal gp130 engagement alongside the *BATF/STAT1/PRDM1* axis. In contrast, *STAT4*, the canonical IL-12 signal transducer, was significantly downregulated in the integrated analysis across all three effector populations (CD8⁺ TEM - 0.44, NK - 0.47, $\gamma\delta$ T - 0.69; all harmony adj.p < 1e-15) but only NK cells reached patient-level significance (paired padj = 0.022; CD8⁺ TEM and $\gamma\delta$ T paired padj > 0.7). This downregulation occurred despite IL-12 receptor upregulation in NK (*IL12RB1* + 0.62, harmony adj.p = 1.6e-6; paired padj = 0.022). Plasma IL-12p70 was not significantly elevated during flares (p = 0.46), consistent with the classical IL-12/STAT4 axis not being the primary route of IFN- γ production in MIS-C. Notably, while IL-12 receptor components showed selective upregulation (*IL12RB1* in NK as above; *IL12RB2* + 1.00 in CD4⁺ naive (harmony adj.p = 3.4e-6; paired padj = 0.026) and + 0.85 in CD4⁺ TCM (harmony adj.p = 9.4e-8; paired padj = 0.042)), the IL-12 ligand subunits *IL12A* (p35) and *IL12B* (p40) were transcriptionally undetectable in PBMCs (*IL12A*: n_sig = 0 across all profiled cell types; *IL12B*: not detected in any cell type at the DE threshold), further indicating that the canonical IL-12 ligand–receptor–STAT4 axis is not engaged in MIS-C (Fig. 4C, Additional file 2: Table S6).

The transcription factor and receptor profile is consistent with a gp130-associated signalling programme. *IL6ST* (encoding gp130, the shared signal-transducing subunit of IL-27, IL-6, and other gp130-family cytokines⁴¹) was significantly upregulated in CD8⁺ TEM (+ 0.83), $\gamma\delta$ T (+ 1.10), and CD14⁺ monocytes (+ 0.64) (all harmony adj.p < 1e-10). Patient-level paired Wilcoxon testing reached significance in NK (paired padj = 0.032), CD4⁺ naive (paired padj = 0.020), and CD8⁺ naive cells (paired padj = 0.016); $\gamma\delta$ T trended toward significance (paired padj = 0.052) and CD8⁺ TEM was borderline (paired padj = 0.18). Gp130-family cytokines, including IL-27, activate *STAT1* and *STAT3* and can induce *BATF/IRF4*. *IL27* mRNA was detectable in only one patient's CD14⁺ monocytes reflecting the low baseline expression of *IL27*, precluding robust patient-level testing; *EBI3*, the IL-27 subunit partner, was sparsely detected with no significant patient-level signal. γ c-cytokine receptor components were significantly upregulated specifically in cytotoxic effector compartments: *IL2RG* (γ c) in CD8⁺ TEM (+ 0.55, harmony adj.p = 9.4e-5; paired padj = 0.040) and NK (+ 0.77, harmony adj.p = 2.2e-27; paired padj = 0.028), with $\gamma\delta$ T cells showing a similar harmony-level signal (+ 0.65, harmony adj.p = 5.0e-4) but borderline patient-level support (paired padj = 0.065); and *IL15RA* in NK cells (+ 1.10, harmony adj.p =

5.7e-11; paired padj = 0.022), suggesting receptor-level priming for transient or local $\gamma\delta$ -cytokine pulses despite no systemic IL-2 or IL-15 elevation in plasma.

Among all cell types profiled, $\gamma\delta$ T cells exhibited the highest *BATF* upregulation (+ 2.40; harmony adj.p < 1e-10; paired padj = 0.079, 7/7 detectable patients) and engaged the broader non-canonical effector programme: *STAT1* (+ 1.14), *PRDM1* (+ 1.05), *STAT3* (+ 0.58), *IL6ST* (+ 1.10), *GZMA* (+ 1.30), and *GZMB* (+ 1.46) (all harmony adj.p < 1e-3). At the patient level, *STAT1*, *STAT3*, *IL6ST*, and *GZMA* showed concordant trends (paired padj = 0.052), while *PRDM1* was borderline (paired padj = 0.065) and *GZMB* did not reach trend-level threshold (paired padj = 0.104). *IFNG* mRNA showed substantial fold-change in the integrated analysis (+ 0.98) but did not reach harmony-level significance (harmony adj.p = 1.00) and was variable at the patient level (paired padj = 0.42), consistent with $\gamma\delta$ T cells engaging the non-canonical programme through gp130-associated chromatin-level mechanisms rather than uniform per-patient *IFNG* transcription. *STAT4* downregulation was significant in the integrated analysis (– 0.69, harmony adj.p < 1e-15) but variable across patients (paired padj = 0.92). $\gamma\delta$ T cell proportions were significantly reduced in peripheral blood during flares, consistent with redistribution to tissues paralleling the pattern observed for MAIT cells³³.

Sustained *IFNG* expression in cytotoxic effectors co-occurred with significant downregulation of *IFNG-AS1*, the *IFNG*-regulatory lncRNA that normally fine-tunes IFN- γ output (Fig. 5A-B). *IFNG-AS1* was significantly downregulated in CD8⁺ TEM (– 1.49), $\gamma\delta$ T (– 1.61), NK (– 1.46), CD4⁺ naive (– 2.17), CD8⁺ naive cells (– 2.02), B naive, B memory, and CD8⁺ TCM cells (all harmony adj.p < 1e-5; paired padj = 0.009–0.035 in lymphoid compartments). This downregulation was independently confirmed in bulk RNA-seq from both the main cohort (log₂FC = – 2.14, adj.p = 1.05E-31) and the validation cohort (– 1.4, adj.p = 1.76E-5) (Fig. 5C). Whether this decoupling has a chromatin-level basis is examined in the integrated multiome regulatory network analysis below.

Figure 5. Expression- and chromatin-level decoupling of *IFNG* and *IFNG-AS1* in MIS-C flares.

(A) Single-cell log₂ fold-changes for ****IFNG**** and ****IFNG-AS1**** across immune populations. ****IFNG**** is upregulated in CD8⁺ TEM, CD8⁺ proliferating and NK cells, whereas ****IFNG-AS1**** is downregulated across multiple lymphoid subsets. Red, upregulated in flare; blue, downregulated. *** adj.p < 0.001; ns, not significant. (B) Per-patient mean ****IFNG**** and ****IFNG-AS1**** expression in flare versus remission across cell types. Half-violins show distributions; lines connect paired patient samples. (C) Paired bulk RNA-seq expression of ****IFNG**** and ****IFNG-AS1**** in main and validation cohorts. ****IFNG**** increases during flare, whereas ****IFNG-AS1**** does not show a concordant increase. (D) TBX21 motif-level chromatin correlations with ****IFNG**** and ****IFNG-AS1**** DORCs in CD8⁺ TEM and $\gamma\delta$ T cells during flare. In CD8⁺ TEM, TBX21 correlates positively with ****IFNG**** accessibility and negatively with ****IFNG-AS1**** accessibility; no significant correlations are detected in $\gamma\delta$ T cells. r, correlation coefficient; * p < 0.05.

CD16⁺ monocytes displayed a paradoxically distinct transcriptional profile: despite robust upregulation of IFN- γ and type I interferon response pathways, they exhibited significant downregulation of canonical inflammatory programmes, including TNF- α signalling via NF- κ B and the broader HALLMARK

inflammatory response gene set, a pattern not observed in CD14⁺ monocytes. This divergence suggests monocyte subsets adopt distinct functional states during MIS-C, potentially reflecting chronic interferon exposure or tolerisation. CD14⁺ monocytes showed strong *STAT1* upregulation (+ 3.10, harmony adj.p = 2.3e-171, paired padj = 0.252) alongside induction of PD-L1 (*CD274* + 2.12, harmony adj.p = 5.45e-3, paired padj = 0.564) and the TIM-3 ligand galectin-9 (*LGALS9* + 1.64, harmony adj.p = 1.25e-5, paired padj = 0.94 in CD16⁺ monocytes), consistent with IFN- γ -activated monocytes simultaneously amplifying and attempting to regulate effector responses through checkpoint ligand expression.

Hallmark gene set enrichment analysis revealed cell-type-specific downregulation of TGF- β signalling in cytotoxic effectors during MIS-C flare. Hallmark TGF- β signalling was significantly downregulated by GSEA in all four cytotoxic effector populations profiled: CD8⁺ TEM (NES = - 2.05, FDR = 2.5 $\times$ 10⁻³), CD4⁺ CTL (NES = - 1.90, FDR = 0.031), $\gamma\delta$ T cells (NES = - 1.85, FDR = 0.014), and NK cells (NES = - 1.73, FDR = 0.030; Fig. 4B; Additional file 2: Table S5), but did not reach significance in CD4⁺ or CD8⁺ naive populations (FDR > 0.10). This cell-type-specific pattern is consistent with selective uncoupling of TGF- β -mediated regulatory restraint within the cytotoxic compartments engaged in the active effector response, while preserving TGF- β signalling tone in naive lymphoid populations. The pathway-level reduction was concomitant with patient-variable single-gene heterogeneity at the TGF- β ligand and SMAD level (Additional File 1: Figure S4), and contrasted with the TGF- β receptor and inhibitory SMAD7 chromatin correlations identified by FigR (described below), indicating that the TGF- β programme is altered at the pathway level rather than uniformly silenced across all components.

Integrated Multiome Regulatory Network Analysis Reveals Divergent Chromatin Architectures Converging on the Same Effector Output

To resolve the broader regulatory network, we integrated single-cell RNA-seq and ATAC-seq data from CD8⁺ TEM and $\gamma\delta$ T cells using FigR⁴², identifying flare-specific gene-peak correlations across three programmes: IFN- γ signalling, TGF- β regulation, and checkpoint receptors (Fig. 6; Additional file 2: Table S8, S8a). In CD8⁺ TEM cells (942 flare-specific TF-DORC edges: 351 IFN- γ , 284 TGF- β , 307 checkpoint), the analysis revealed two opposing transcription factor modules: an effector/activator module anchored by *TBX21*, *NFIL3*, *ZEB2*, *STAT1*, and *NFATC2*, showing positive correlations with IFN- γ and checkpoint gene chromatin accessibility, and a progenitor/repressor module (*LEF1*, *BACH2*, *ARID5B*, *TCF7*, *ZEB1*) showing negative correlations, consistent with reduced progenitor/stem-like CD8⁺ T cell identity during sustained inflammatory stimulation.

To examine the chromatin-level architecture of the non-canonical IFN- γ effector programme, we first tested whether the IFNG/IFNG-AS1 decoupling observed transcriptionally has a chromatin-level basis. In CD8⁺ TEM cells, *TBX21* motif activity showed a significant positive correlation with *IFNG* chromatin accessibility ($r = 0.52$, $p < 0.05$) and a significant negative correlation with *IFNG-AS1* accessibility ($r = -0.39$, $p < 0.05$) (Fig. 5D), consistent with *TBX21*-anchored accessibility at the *IFNG* locus co-occurring

with reduced accessibility at *IFNG-AS1*. No significant *TBX21* correlations were detected in $\gamma\delta$ T cells, indicating a distinct chromatin-level regulatory architecture for the $\gamma\delta$ T IFN- γ programme.

TBX21 (T-bet), the canonical master transcription factor of the CD8⁺ effector chromatin programme¹⁶, emerged as the most broadly connected activating TF in CD8⁺ TEM during MIS-C flare (31 significant target pairs across all three programmes, tied with *FOSB*, Fig. 6 left panels). The presence of a *TBX21*-anchored chromatin programme alongside limited engagement of the upstream *IL-12/STAT4* axis is consistent with downstream effector chromatin architecture being maintained through transcription factor inputs other than *STAT4*, specifically the *BATF/STAT1/PRDM1* programme associated with gp130-family signalling. *TBX21* showed strong positive correlations with effector-programme targets including *IFNG* ($r = 0.518$, $p = 0.032$), *GZMB* ($r = 0.661$, $p = 0.009$), *STAT1* ($r = 0.561$, $p = 0.026$), *BHLHE40* ($r = 0.700$, $p = 0.016$), and *ZEB2* ($r = 0.682$, $p = 0.030$). *BHLHE40*, a critical modulator of T cell effector function⁴³, showed high network centrality with strong *TBX21* correlation among effector targets. Notably, *TBX21* motif activity was also positively correlated with chromatin accessibility at the *STAT4* locus, despite *STAT4* mRNA being downregulated and plasma IL-12p70 being unaltered (Fig. 2). This dissociation of open chromatin at *STAT4* but suppressed transcription is consistent with *TBX21* establishing and maintaining a broad effector chromatin landscape that keeps the *STAT4* locus permissive for transcription, while transcription itself is gated by upstream cytokine signalling that is not engaged in MIS-C (canonical IL-12/STAT4 induction). Multiome profiling of paired chromatin and transcript states is well-suited to reveal such uncoupling between chromatin accessibility and transcriptional output. In contrast, *IFNG-AS1* was negatively correlated with *TBX21* ($r = -0.386$, $p = 0.025$, Fig. 5D), consistent with a model in which *TBX21*-associated chromatin remodelling at the *IFNG* locus reduces the requirement for *IFNG-AS1*-mediated regulation under sustained inflammatory stimulation. *TBX21* simultaneously showed positive correlations with checkpoint targets *CTLA4* ($r = 0.393$, $p = 0.027$), *TNFRSF9/4-1BB* ($r = 0.487$, $p = 0.045$), and *CD38* ($r = 0.583$, $p = 0.010$), consistent with co-expression of activation markers and checkpoint receptors that characterises recently activated rather than terminally exhausted CD8⁺ T cells⁴⁴. Critically, the canonical exhaustion TFs *NR4A2* and *NR4A3* were not consistently induced at the patient level in CD8⁺ TEM (paired $p_{adj} = 0.29$ and 0.62 ; significant only in NK for *NR4A2*), and the progenitor/repressor module showed negative correlations with effector gene accessibility, distinguishing the MIS-C effector state from canonical T cell exhaustion^{45,46}.

Within the TGF- β regulatory network in CD8⁺ TEM, *TBX21* positively correlated with *TGFBR1* ($r = 0.638$, $p = 0.024$) and *TGFBR3* ($r = 0.580$, $p = 0.005$) and with the inhibitory *SMAD7* ($r = 0.538$, $p = 0.018$). The chromatin-level co-association of *TBX21* with both TGF- β receptors and the inhibitory *SMAD7* suggests a regulatory architecture in which effector cells maintain TGF- β receptor accessibility alongside chromatin-level priming of inhibitory feedback components, contributing to the overall pathway-level reduction observed by GSEA despite mixed gene-level directions. *TBX21* also negatively correlated with tolerance-associated regulators *FOXP3* ($r = -0.472$, $p = 0.022$), *ID3* ($r = -0.483$, $p = 0.033$), and *ZEB1* ($r = -0.558$, $p = 0.021$), consistent with chromatin-level disengagement from regulatory programmes.

Parallel FigR analysis in $\gamma\delta$ T cells identified 772 flare-specific TF–DORC edges (353 IFN- γ , 166 TGF- β , 253 checkpoint Additional file 2: Table S8a), a scale of chromatin remodelling comparable to CD8⁺ TEM. However, the transcription factor architecture differed substantially: *FOSB* remained the most connected TF (26 edges), but *TBX21* was less connected (17 edges, ranked 9th) and targeted a largely non-overlapping gene set, with $\gamma\delta$ T *TBX21* correlating with innate-like effector genes (*CX3CR1*, *ID2*). *NFIL3* and *STAT1*, prominent hubs in CD8⁺ TEM, had no detectable IFN- γ programme edges in $\gamma\delta$ T cells; in their place, $\gamma\delta$ T networks were characterised by AP-1 family members (*FOSB*, *FOS*), innate-like TFs (*MAF*, *CEBPD*), the lineage factor *BCL11B* (18 edges), and *SMAD3* (16 edges). $\gamma\delta$ T *TBX21* also showed positive correlations with *TGFB1* ($r = 0.755$, $p = 0.018$), *SMAD7* ($r = 0.716$, $p = 0.031$), *NFATC2* ($r = 0.763$, $p = 0.029$), *CX3CR1* ($r = 0.740$, $p = 0.026$), and *PDCD1* ($r = 0.614$, $p = 0.016$), and negative correlations with *IL6ST* ($r = -0.771$, $p = 0.017$), *ID3* ($r = -0.741$, $p = 0.030$) (Fig. 6 right panel), a regulatory architecture distinct from CD8⁺ TEM in both target identity and correlation polarity, consistent with $\gamma\delta$ T cells integrating TGF- β and gp130-family inputs through a fundamentally different chromatin-level wiring.

CD8⁺ TEM and $\gamma\delta$ T cells therefore converge on a similar effector transcriptional output through partially distinct chromatin architectures, *TBX21*-dominated in CD8⁺ TEM and AP-1/innate-like (*FOSB*, *MAF*, *CEBPD*) in $\gamma\delta$ T cells, with *FOSB* as a shared anchor.

Whole-Exome Sequencing Reveals No Monogenic Drivers of MIS-C Susceptibility

To investigate whether the non-canonical IFN- γ effector programme reflects inherited genetic predisposition, we performed whole-exome sequencing on the main cohort. Variants were filtered against a curated panel of 1,091 unique immunological genes (Additional file 2: Table S9) spanning: (i) autoinflammatory and primary immunodeficiency disorders from the Human Phenotype Ontology; (ii) candidate genes from a multicentre study of rare variants in MIS-C, MIS-A, Kawasaki disease, and severe COVID-19 (Reis et al. 2023) (iii) HALLMARK_INTERFERON_GAMMA_RESPONSE genes; (iv) HALLMARK_TGF_BETA_SIGNALING genes; and (v) transcription factors identified in our FigR regulatory network analysis. After standard rare variant filtering ($MAF < 0.01$; exonic variants present in ≥ 5 patients), no recurrent pathogenic rare variants with clear MIS-C association were identified. This is consistent with prior work showing that rare variants in interferon-regulatory pathways contribute to susceptibility in only a subset of patients²⁵ and supports a model in which the non-canonical IFN- γ effector programme in MIS-C reflects acquired chromatin-level changes rather than inherited defects in interferon or TGF- β signalling pathways.

DISCUSSION

Our integrated single-cell multiome, bulk transcriptomic, and plasma cytokine analyses define a non-canonical IFN- γ effector programme in MIS-C, characterised by a *BATF/STAT1/PRDM1* axis (with *IRF4* additionally engaged in CD8⁺ TEM and naive lymphoid populations) and limited engagement of the classical IL-12/STAT4/T-bet pathway. This effector programme co-occurs with chromatin-level

decoupling of *IFNG* transcription from *IFNG-AS1*-mediated regulation and with upregulation of checkpoint receptors (*CTLA4*, *HAVCR2*) in CD8⁺ TEM cells. However, this checkpoint induction occurs without concordant patient-level upregulation of the NR4A exhaustion module (*NR4A2*, *NR4A3*), indicating a state of altered regulation rather than classical terminal exhaustion. A proposed mechanistic model is presented in Fig. 7. All findings are observational and require functional validation.

The unexpected finding is the dissociation between robust IFN- γ production and the classical IL-12/STAT4 axis: across CD8⁺ TEM, NK, and $\gamma\delta$ T cells, *STAT4* was downregulated, IL-12 receptor regulation was cell-type-specific rather than uniformly upregulated and *IL12A/IL12B* ligands were transcriptionally undetectable. The emerging profile *BATF*, *STAT1*, *PRDM1*, and *STAT3* with *IL6ST* (gp130) upregulation is consistent with a gp130-associated signalling programme. IL-27 is one candidate gp130 family cytokine because it activates STAT1/STAT3 and can promote IFN- γ . It may support IFN- γ production in contexts where STAT4 activity is not dominant. *BATF* (often with *IRF4*) can promote effector chromatin remodeling and differentiation, functioning in concert with other effector TFs that may include T-bet depending on context^{13,17,18,47}. *PRDM1* induction in cytotoxic effectors is consistent with terminal effector differentiation, where *PRDM1* cooperates with T-bet to drive *IFNG* and cytotoxic gene expression^{48,49}. The strong *PRDM1* induction in CD4⁺ naive, CD4⁺ TCM, and CD8⁺ naive lymphocytes is more difficult to interpret, as *PRDM1* in CD4⁺ T cells additionally promotes Tr1-like IL-10 production⁵⁰; whether this reflects effector commitment, regulatory differentiation, or both will require longitudinal phenotyping. IL-27 protein was not measured, gp130 is shared across multiple cytokines, and phospho-STAT data were not collected; we therefore cannot definitively distinguish among gp130 ligands or exclude residual IL-12/STAT4 contribution. To our knowledge, this *BATF*-anchored programme has not been described in a post-viral hyperinflammatory syndrome, and the framework warrants investigation in long COVID and viral-associated haemophagocytic syndromes.

A second distinctive observation is the paradoxical downregulation of *IFNG-AS1*, an *IFNG*-regulatory lncRNA that normally fine-tunes IFN- γ output²¹, despite sustained *IFNG* transcription. Our chromatin accessibility analysis provides a candidate mechanism: *TBX21* motif activity in CD8⁺ TEM cells correlates positively with *IFNG* accessibility but negatively with *IFNG-AS1* accessibility. This pattern is consistent with *TBX21*-anchored chromatin remodelling at the *IFNG* locus that reduces the requirement for *IFNG-AS1*-mediated regulation. No equivalent *TBX21* correlation was detected in $\gamma\delta$ T cells, indicating a distinct chromatin architecture for the $\gamma\delta$ T IFN- γ programme. To our knowledge, this represents the first observation of *IFNG/IFNG-AS1* expression decoupling in a human inflammatory disease, enabled by the simultaneous single-cell RNA and ATAC-seq approach. This interpretation rests on correlative chromatin data; reporter assays or CRISPR-mediated perturbation at the *IFNG-AS1* locus would be required to confirm a causal regulatory relationship.

Cross-cell-type FigR analysis revealed that CD8⁺ TEM and $\gamma\delta$ T cells achieve a similar effector output through partially distinct chromatin architectures: *TBX21*-dominated in CD8⁺ TEM, AP-1/innate-like (*FOSB*, *MAF*, *CEBPD*) in $\gamma\delta$ T cells, with comparable scales of flare-specific chromatin remodelling. This convergence-through-divergence may reflect the distinct developmental origins of adaptive and innate-

like effector populations and suggests that the non-canonical programme can be engaged through multiple regulatory routes. $\gamma\delta$ T cells, previously underappreciated in MIS-C, exhibited the highest *BATF* induction in our dataset and a non-canonical effector profile comparable to or exceeding that of CD8⁺ TEM cells. The non-canonical aspect of the MIS-C programme therefore lies in the upstream cytokine signalling route, while downstream effector chromatin anchoring is retained (*TBX21* in CD8⁺ TEM) or replaced by lineage-appropriate alternatives (*AP-1* in $\gamma\delta$ T). The non-canonical TF programme was also strongly engaged in CD4⁺ and CD8⁺ naive lymphocytes, consistent with broad lymphoid priming. Whether these primed naive cells contribute to the persistent effector pool through accelerated differentiation will require longitudinal sampling and TCR clonality analysis.

Checkpoint receptors (*CTLA4*, *HAVCR2*, *LAG3*) were robustly upregulated in CD8⁺ TEM cells without consistent induction of canonical exhaustion transcription factors *NR4A2* and *NR4A3*. The chromatin-level data indicate coordinated *TBX21*- and *NFIL3*-driven correlations linking effector activation to checkpoint receptor accessibility without engaging the *NR4A*-dependent exhaustion programme, consistent with a recently activated rather than terminally exhausted effector state^{18,51}. This distinction is clinically relevant: terminally exhausted T cells may benefit from checkpoint blockade, whereas the metabolically active, proliferative effectors observed in MIS-C might respond unfavourably. Together with concurrent monocyte induction of checkpoint ligands (PD-L1, galectin-9), this configuration is consistent with a failed regulatory feedback loop that may contribute to flare persistence.

The cell-type-specific TGF- β pathway reduction in cytotoxic effectors, combined with patient-variable single-gene heterogeneity, raises the possibility of distinct MIS-C immunotypes within a shared non-canonical IFN- γ framework. Our findings are consistent with features of an emerging spectrum of acquired interferonopathies, in which environmental or infectious triggers induce sustained interferon dysregulation in the absence of monogenic predisposition⁵². The robust IFN- γ signature, the chromatin-level patterns from our multiome analysis, and the absence of monogenic drivers in our WES analysis collectively support a model of post-infectious transcriptional reprogramming. Formal classification within the interferonopathy spectrum would require demonstration of sustained IFN- γ signalling beyond the acute flare and systematic comparison with established interferonopathy signatures. These findings suggest several therapeutic candidates warranting preclinical investigation, including anti-IFN- γ therapy (e.g., emapalumab⁵³), gp130-pathway modulation pending identification of the specific upstream ligand, and JAK1/2 inhibition, which targets both classical IFN- γ signalling and STAT1/3 downstream of gp130⁵⁴. More broadly, MIS-C provides a clinical model for how post-viral immune priming establishes non-canonical transcriptional programmes that sustain hyperinflammation, mechanisms potentially relevant to long COVID and related post-infectious conditions⁵.

Strengths and limitations.

This is, to our knowledge, the first study combining simultaneous single-cell RNA-seq and ATAC-seq on longitudinal paired MIS-C samples, with cross-validation across four data layers and replication in an independent bulk RNA-seq cohort. Within-patient comparisons minimise inter-individual confounding.

Limitations include: (i) the correlative nature of all observations, requiring functional validation through phospho-STAT1/STAT3/STAT4 flow cytometry, IL-27/IL-6/IL-11 protein measurement with paired IL-12p70 and p35/p40 subunit confirmation, gp130-ligand neutralisation, SMAD2/3 phosphorylation assays, *IFNG-AS1* perturbation, longitudinal CD4⁺ naive phenotyping to distinguish Tr1-like (IL-10⁺) from effector commitment, and ex vivo polyfunctionality testing; (ii) modest cohort size (10 main, 9 validation), potentially underpowered for rare subsets including $\gamma\delta$ T cells; (iii) FigR cross-cell-type comparisons sensitive to differences in cell number and chromatin landscape; (iv) restriction to peripheral blood, which may not reflect tissue-specific dynamics; (v) inability to fully exclude transient type I or III interferon contributions, since many ISGs respond to multiple IFN classes; and (vi) single-site Slovenian origin limiting ethnic diversity. Replication in multi-centre cohorts and direct functional validation are essential next steps.

CONCLUSIONS

Through integrated single-cell multiome, bulk RNA-seq, plasma cytokine, and exome profiling, this study characterises a non-canonical IFN- γ effector programme in MIS-C anchored by a *BATF/STAT1/PRDM1* transcriptional axis, with limited engagement of the classical IL-12/STAT4 pathway. IFNG/IFNG-AS1 chromatin decoupling, divergent regulatory architectures converging on a shared effector output across CD8⁺ TEM and $\gamma\delta$ T cells, and checkpoint receptor upregulation without canonical exhaustion together define a transcriptional state distinct from Kawasaki disease and severe COVID-19. The absence of monogenic drivers and the chromatin-level patterns observed are consistent with acquired post-viral transcriptional reprogramming. These findings nominate gp130-associated signalling, JAK1/2 inhibition, and IFN- γ -directed therapy as candidates for preclinical investigation, and position MIS-C as a model system for understanding sustained hyperinflammation in post-viral syndromes including long COVID.

Abbreviations

MIS-C
multisystem inflammatory syndrome in children
IFN- γ
interferon gamma
scRNA-seq
single-cell RNA sequencing
ATAC-seq
assay for transposase-accessible chromatin with sequencing
WES
whole-exome sequencing
TEM
effector-memory T cell
NK
natural killer

PBMC
peripheral blood mononuclear cell
lncRNA
long non-coding RNA
TF
transcription factor
DORC
domain of regulatory chromatin
ISG
interferon-stimulated gene
GEO
Gene Expression Omnibus.

Declarations

Ethics approval and consent to participate

The study was approved by the National Ethics Committee for Research in Medicine, Slovenia (approval number 0120-536/2020/3). Written informed consent was obtained from the legal guardians of all participants.

Consent for publication

Not applicable.

Availability of data and materials

Processed single-cell multiome and bulk RNA-seq data have been deposited in GEO under accession number GSE301727. All analyses were performed using publicly available tools and packages as detailed in the Methods section. The code is available at: <https://github.com/CISLD/urnades-universal-rna-differential-expression-scripts> for bulk RNA-seq and at: https://github.com/LabenaBioinf/10X_multiome-analysis for multiomic single-cell GEX-ATAC analysis.

Competing interests

The authors declare no competing interests.

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Authors' contributions

B.J.B. (Barbara Jenko Bizjan): Conceptualization; Methodology; Supervision; Project administration; Writing – original draft. J.K. (Jernej Kovač): Conceptualization; Methodology; Supervision; Writing – review & editing. T.T. (Tine Tesovnik): Investigation (single-cell and cytokine wet lab); Methodology (wet lab assays); Data curation. R.Š. (Robert Šket): Investigation (single-cell wet lab); Methodology (single-cell library preparation). M.D. (Maruša Debeljak): Conceptualization; Writing – review & editing. M.Z.A. (Mojca Zajc Avramovič): Resources; Patient recruitment and inclusion; Investigation. N.E. (Nina Emeršič): Resources; Patient recruitment and inclusion; Investigation. B.V. (Blaž Vrhovšek): Formal analysis; Bioinformatics (bulk RNA-seq); Data curation. I.S. (Iva Sabolić): Formal analysis; Bioinformatics (single-cell analysis); Visualization. A.S. (Anže Smole): Conceptualization; Writing – review & editing. T.A. (Tadej Avčin): Conceptualization; Clinical oversight; Writing – review & editing. All authors read and approved the final manuscript.

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Declaration of generative AI and AI-assisted technologies

During preparation of this manuscript the authors used Claude (Anthropic) and ChatGPT (OpenAI) to assist with code writing, drafting and language polishing, and used Elicit and Consensus to assist with literature searching and evidence synthesis. All AI-assisted outputs were reviewed, edited, and approved by the authors, who take full responsibility for the final content.

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Figures

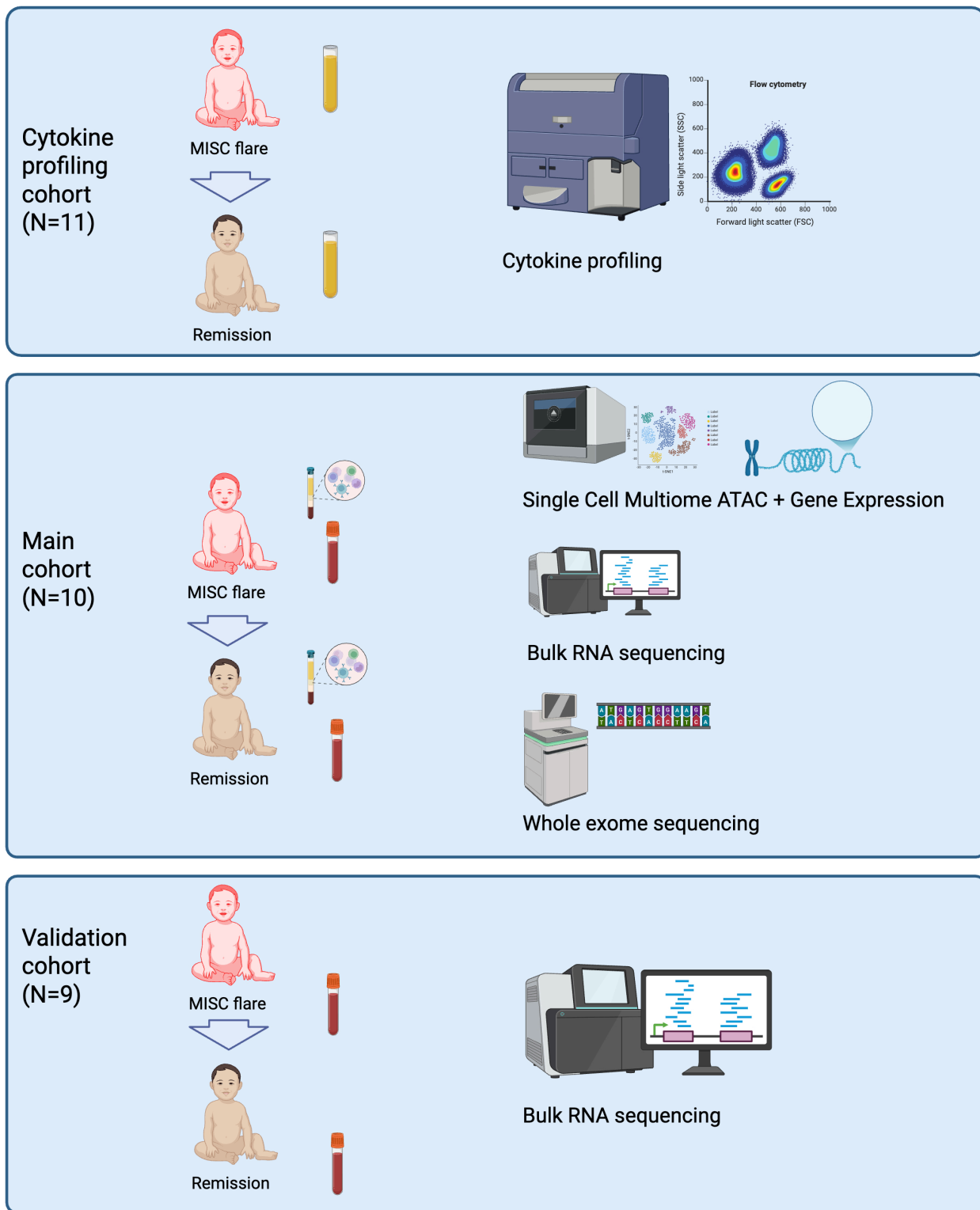


Figure 1

Study design and cohort overview.

Paired flare and remission sampling across three MIS-C cohorts. The cytokine profiling cohort (n=11) provided plasma for multiplex cytokine measurement. The main cohort (n=10) provided peripheral blood mononuclear cells for single-cell multiome profiling (simultaneous ATAC and gene expression), bulk RNA

sequencing, and whole-exome sequencing. The validation cohort (n=9) provided an independent bulk RNA-sequencing dataset. All participants were sampled during acute flare, prior to immunomodulatory treatment, and again in clinical remission six to twelve months after illness onset. Created with BioRender.

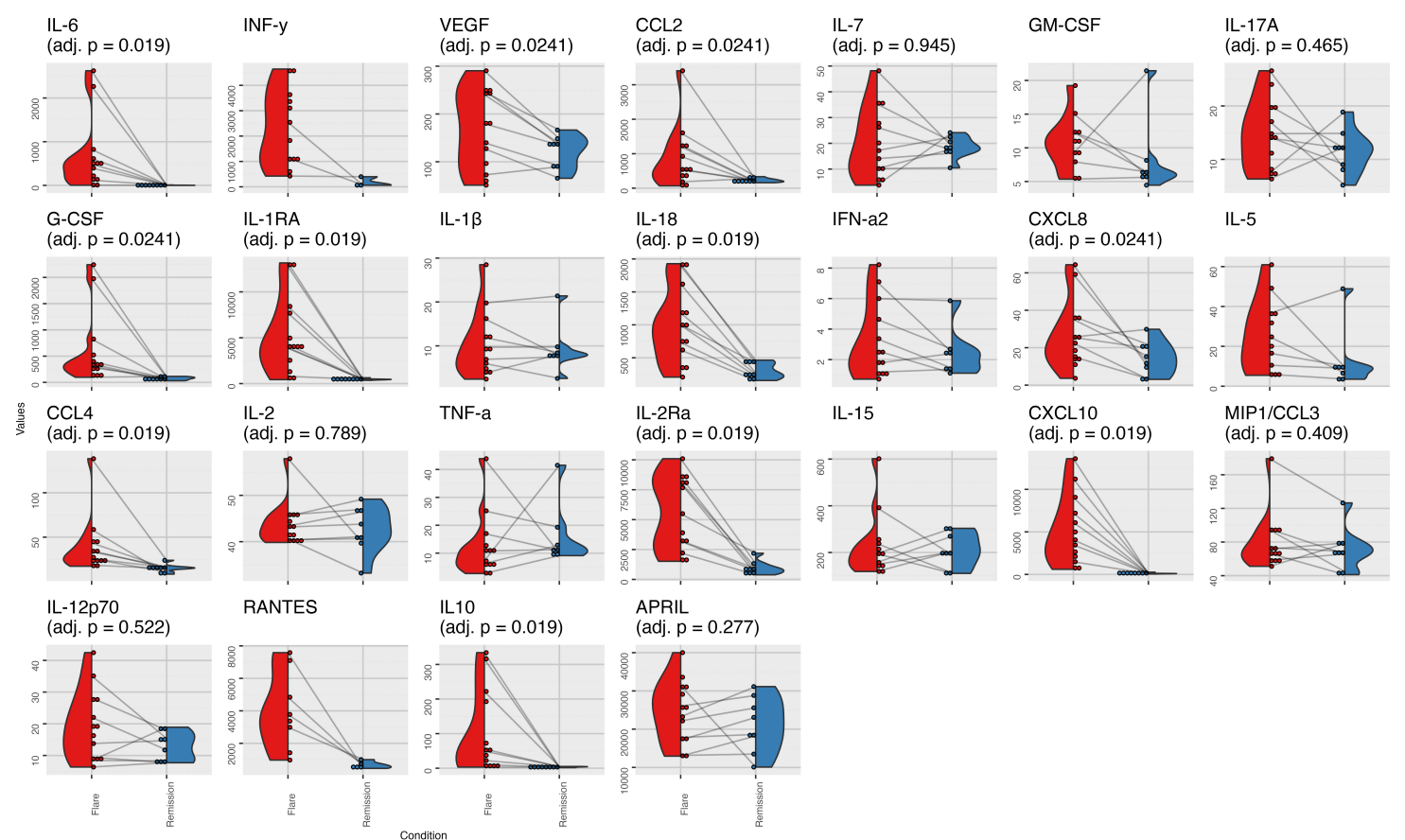


Figure 2

Plasma cytokine profiling reveals IFN-γ-associated inflammation with limited IL-12/STAT4 engagement in MIS-C flares.

Paired plasma cytokine measurements from MIS-C patients (n=11) during acute flare and remission. Boxplots show selected cytokines including IFN-γ, CXCL10, IL-18, IL-6, VEGF, IL-2Ra, IL-10, and IL-12p70. IFN-γ, CXCL10, IL-18, IL-6, IL-2Ra, and IL-10 were significantly elevated during flare, whereas IL-12p70 was not (p = 0.46), consistent with limited engagement of the classical IL-12/STAT4 axis. Paired Wilcoxon signed-rank tests with Benjamini-Hochberg correction; for IFN-γ (valid results: 92.3% flare vs. 37.5% remission), observed magnitudes are reported in place of formal paired testing.

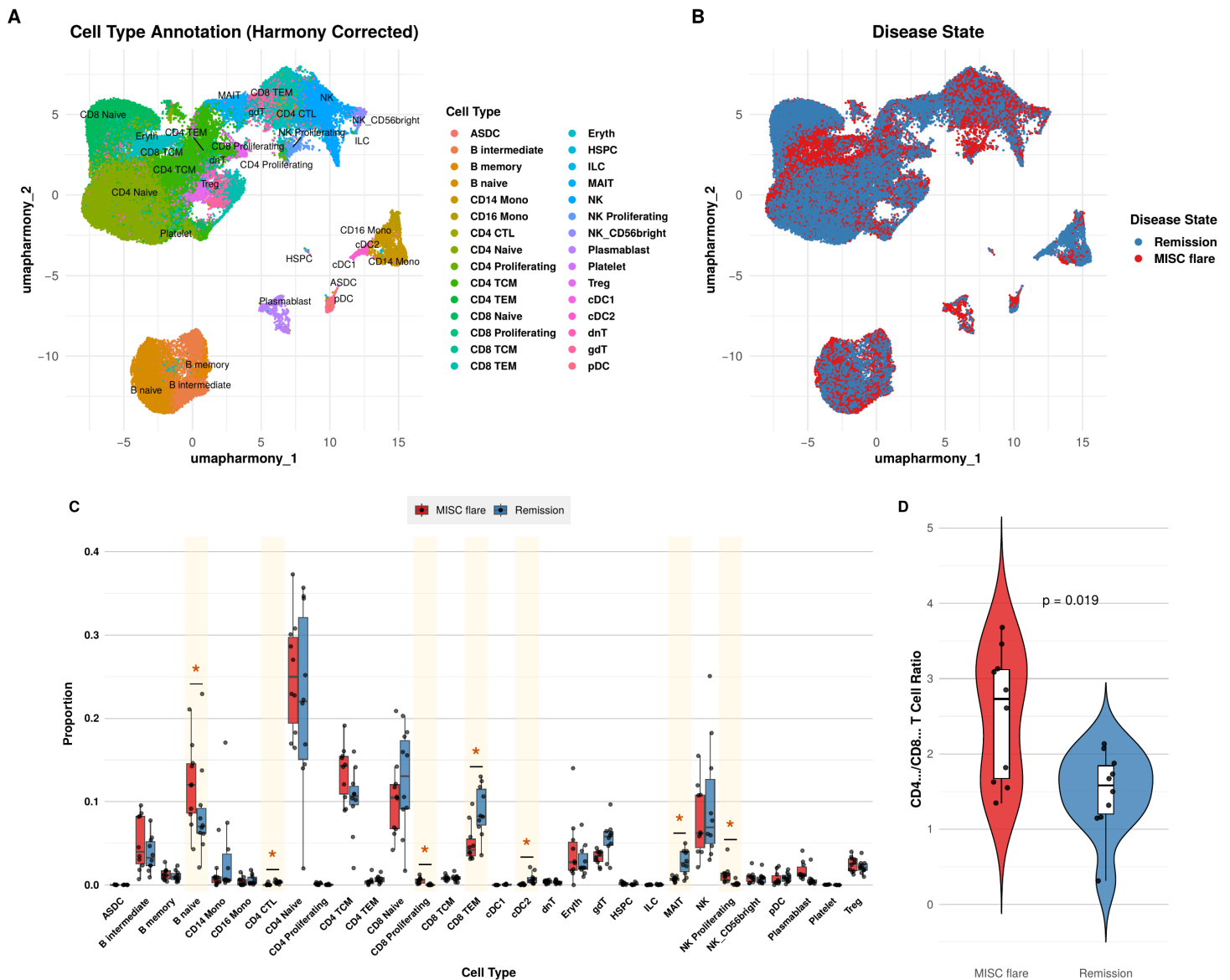


Figure 3

Single-cell profiling reveals altered immune composition and CD4⁺/CD8⁺ ratio in MIS-C flares.

Single-cell transcriptomic landscape of paired MIS-C flare and remission PBMC samples. Joint UMAP of 89,154 harmony-integrated nuclei from 10 paediatric MIS-C patients (37,842 flare; 51,312 remission). (A) Cells coloured by cell-type annotation (30 PBMC populations resolved across adaptive and innate compartments). (B) Same embedding coloured by disease state (red, flare; teal, remission). (C) Paired proportions of immune cell subsets identified by single-cell transcriptomics during flare (red) and remission (blue) across 10 MIS-C patients. Box plots show median and interquartile range; individual points represent per-patient proportions. Significance: * adj.p < 0.05, ** adj.p < 0.01, *** adj.p < 0.001 (paired Wilcoxon signed-rank test with Benjamini-Hochberg correction). (D) CD4⁺/CD8⁺ T cell ratio in peripheral blood during MIS-C flare compared with remission (paired Wilcoxon signed-rank test).

Inflammation-Related Hallmark Pathways

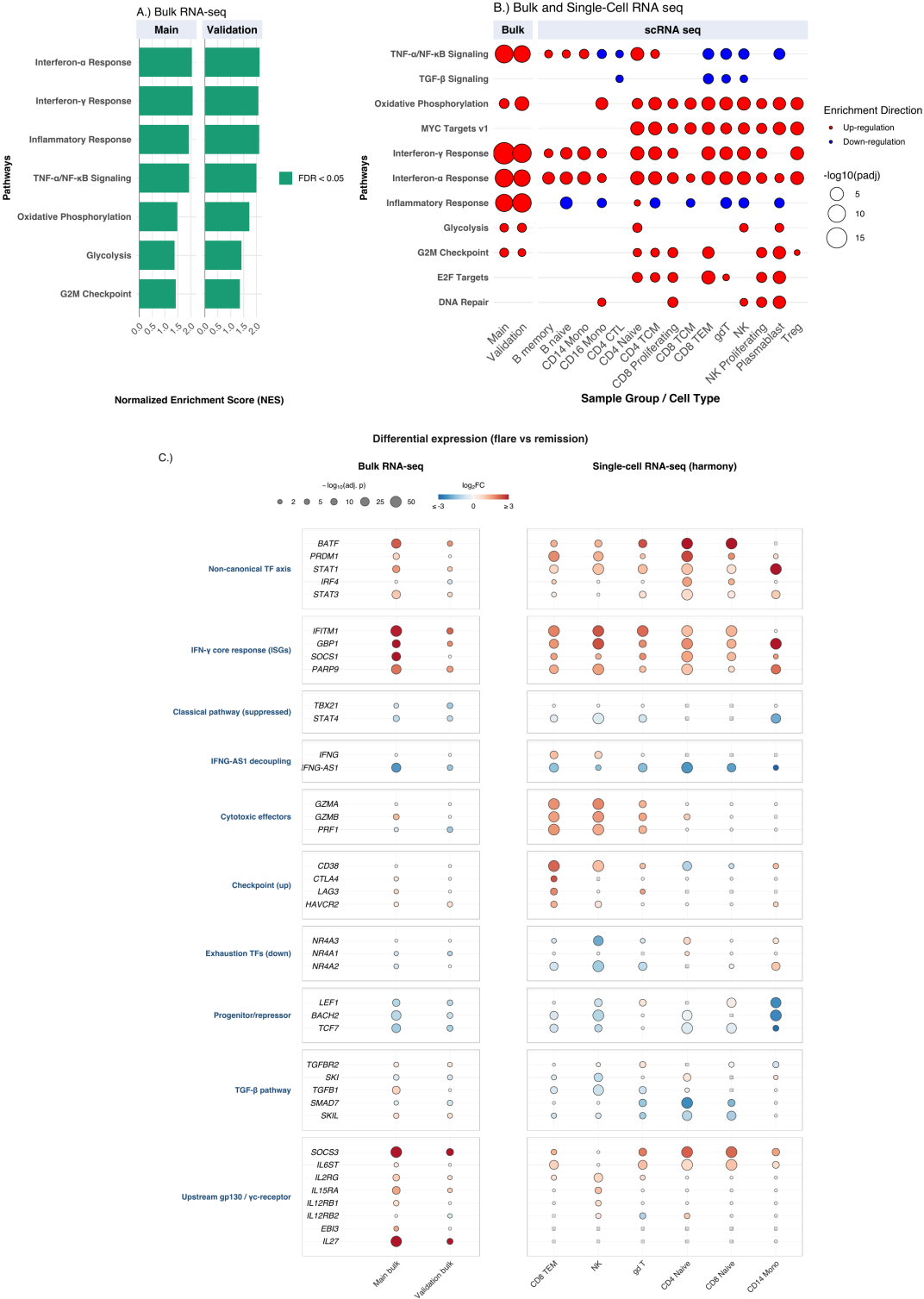


Figure 4

Hallmark pathway enrichment across immune compartments in MIS-C flares.

(A) Bulk RNA-seq GSEA of MSigDB Hallmark pathways in the main and validation cohorts. Bars show normalised enrichment scores (NES); filled bars indicate FDR < 0.05. IFN- γ , IFN- α and inflammatory response pathways are consistently enriched in flare versus remission. (B) Bubble plot of Hallmark

enrichment across bulk RNA-seq and single-cell RNA-seq immune subsets. Bubble size shows $-\log_{10}(\text{FDR})$, and colour indicates enrichment direction. IFN response pathways are broadly upregulated, whereas TGF- β and TNF- α /NF- κ B signalling are reduced in cytotoxic effector compartments. (C) Differential expression of manuscript-curated genes grouped by mechanistic pathway. The 39 genes shown are a curated subset selected to represent the principal mechanistic claims of the non-canonical IFN- γ effector programme. Left: bulk RNA-seq main and validation cohorts. Right: harmony-integrated single-cell DE across six PBMC populations. Dot size shows $-\log_{10}(\text{adjusted } p)$, colour shows $\log_2$ fold-change, open circles indicate non-significance, and grey squares indicate undetected genes.

IFNG and IFNG-AS1 expression and chromatin regulation in MIS-C

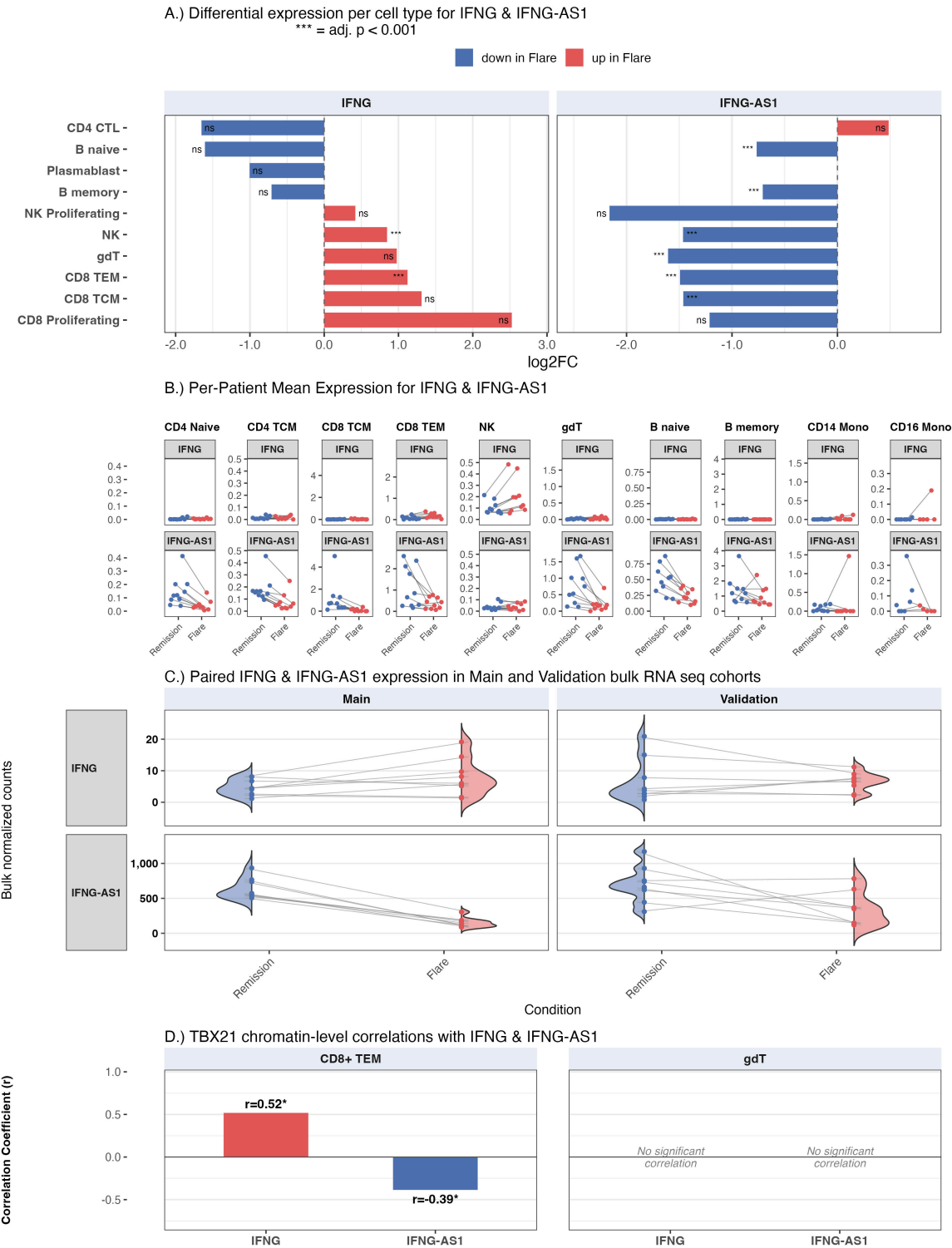


Figure 5

Expression- and chromatin-level decoupling of IFNG and IFNG-AS1 in MIS-C flares.

(A) Single-cell log₂ fold-changes for **IFNG** and **IFNG-AS1** across immune populations. **IFNG** is upregulated in CD8⁺ TEM, CD8⁺ proliferating and NK cells, whereas **IFNG-AS1** is downregulated across multiple lymphoid subsets. Red, upregulated in flare; blue, downregulated. *** adj.p < 0.001; ns,

not significant. (B) Per-patient mean **IFNG** and **IFNG-AS1** expression in flare versus remission across cell types. Half-violins show distributions; lines connect paired patient samples. (C) Paired bulk RNA-seq expression of **IFNG** and **IFNG-AS1** in main and validation cohorts. **IFNG** increases during flare, whereas **IFNG-AS1** does not show a concordant increase. (D) **TBX21** motif-level chromatin correlations with **IFNG** and **IFNG-AS1** DORCs in CD8⁺ TEM and $\gamma\delta$ T cells during flare. In CD8⁺ TEM, **TBX21** correlates positively with **IFNG** accessibility and negatively with **IFNG-AS1** accessibility; no significant correlations are detected in $\gamma\delta$ T cells. *r*, correlation coefficient; * *p* < 0.05.

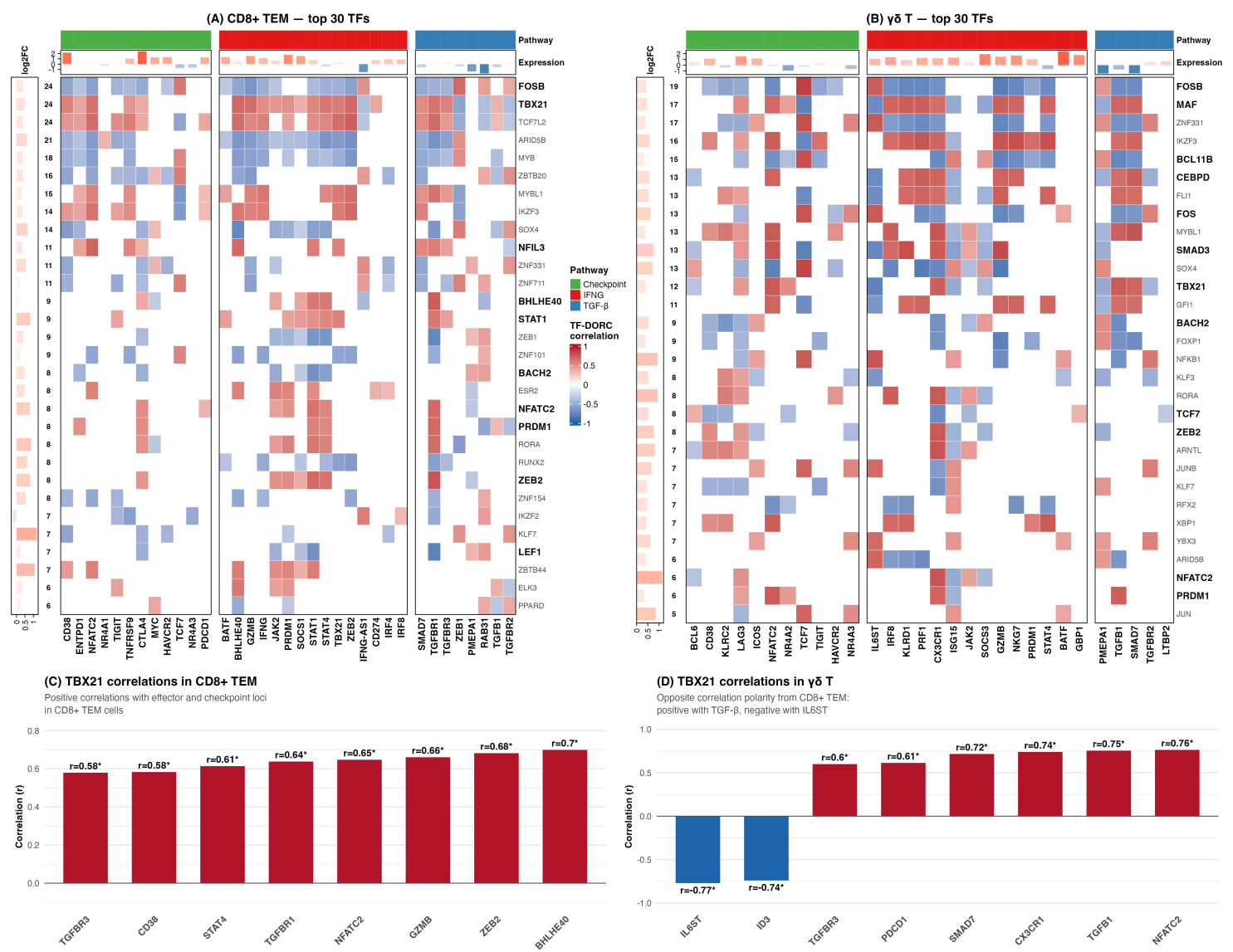


Figure 6

Divergent chromatin architectures converge on a shared effector output in MIS-C. (A, B) In CD8⁺ TEM and $\gamma\delta$ T cells during flare, FigR-derived flare-specific TF–DORC correlation heatmaps for the top 30 transcription factors (ranked by edge count) in CD8⁺ TEM (A) and $\gamma\delta$ T (B) cells. Rows: transcription factor motifs (key TFs in bold); columns: DORC (domains of regulatory chromatin) target genes grouped by pathway (Checkpoint, IFN- γ signalling, TGF- β regulation; coloured bars at top). Cell color indicates

Figure 7

Per-cell-type mechanistic model of the non-canonical IFN- γ effector programme in MIS-C. Monocyte-derived gp130-family cytokines are proposed to engage three cytotoxic effector populations (CD8⁺ TEM, NK, and $\gamma\delta$ T cells) through partially distinct chromatin architectures that converge on sustained IFN- γ output. CD14⁺ monocytes are a candidate cytokine source, secreting IL-6 (elevated in plasma) and expressing IL27 mRNA only (IL-27 protein not measured), and are themselves IFN- γ -responsive (STAT1 +3.10***, PD-L1/CD274 +2.12), consistent with a failed regulatory feedback loop. In CD8⁺ TEM and NK cells, gp130/IL6ST signalling (+0.83 and +0.16, respectively) is associated with STAT1 phosphorylation and a TBX21-anchored programme (in CD8⁺ TEM, TBX21 is the top activating hub, 31 TF–DORC edges; TBX21–IFNG chromatin correlation $r = +0.52$), with coordinated induction of BATF, STAT1, PRDM1, and IRF4. In $\gamma\delta$ T cells, the highest IL6ST signal (+1.10) accompanies dual STAT1/STAT3 phosphorylation and a distinct AP-1/innate-like architecture, with the strongest BATF induction of any profiled population (+2.40) and significant JUNB induction (+0.58), while TBX21 is bypassed. Across all populations the canonical IL-12/STAT4 axis is non-dominant: IL-12 receptor components are present but STAT4 mRNA is reduced and plasma IL-12p70 is not elevated ($p = 0.46$). The three architectures converge on sustained IFNG transcription and broad paracrine IFN- γ release; effector cells upregulate checkpoint receptors and cytotoxic effectors without induction of the exhaustion transcription factors NR4A2 or NR4A3, defining a persistent effector state distinct from terminal exhaustion. Green arrows denote engaged gp130/STAT signalling; grey dashed arrows and faded boxes denote canonical components with limited or non-dominant engagement. Values are $\log_2$ fold-changes (flare vs remission) from Harmony-integrated single-cell differential expression; *** $p_{adj} < 0.001$. The model is correlative and presented as a working hypothesis; direct validation will require phospho-STAT quantification, direct measurement of IL-27 and IL-6, and gp130-ligand neutralisation.

Supplementary Files

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