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Nasopharyngeal Cancer Stem Cells Drive Immune Paralysis via the MIF, CD96, and CD99 Axis Despite Intact Antigen Presentation

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Abstract

Nasopharyngeal carcinoma (NPC) is characterized by significant intratumoral heterogeneity and a propensity for recurrence driven by therapy-resistant Cancer Stem Cells (CSCs). While the genomic landscape of adult NPC is increasingly understood, the cellular architecture of paediatric NPC and its distinct stem cell hierarchies remain largely unexplored. To overcome the limitations of short-read sequencing in resolving complex transcriptomes, we performed high-resolution, long-read single-cell RNA sequencing (scRNA-seq) using Oxford Nanopore Technologies on fresh biopsy specimens from a paediatric and an adult NPC patient. Transcriptional profiling and intercellular communication networks were reconstructed to dissect the tumour microenvironment (TME). Our analysis revealed fundamental differences in stemness programs dependent on patient age. Adult NPC stem cells exhibited a classic basal-like phenotype (*KRT15*hi/*TP63+*/*KRT5+*), relying on basement membrane interactions. In contrast, paediatric CSCs formed a distinct quiescent reservoir (*SOX2+*/*ALDH1A1+*/*MKI67*neg) that was transcriptionally separated from a transient amplifying progenitor population. Despite these structural differences, both stem cell subtypes orchestrated a conserved, multi-layered immune evasion strategy. Network analysis identified NPC stem cells as the dominant hubs for (1) CD99 signalling, which facilitates structural cohesion and suppresses innate immunity via the PILRα inhibitory receptor; (2) MIF secretion, promoting tolerogenic myeloid polarization; and (3) the NECTIN1-CD96 checkpoint axis, which drives T-cell exhaustion despite the presence of intact MHC Class I and II antigen presentation machinery. Disruption of the CD96, CD99, and MIF signalling axes represents a promising universal therapeutic approach to restore anti-tumor immunity in high-risk NPC.

Keywords: Nasopharyngeal carcinoma, Single-cell RNA sequencing, Cancer stem cells, CD96, CD99, MIF

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Introduction

Nasopharyngeal carcinoma (NPC) is a malignant epithelial tumour arising from the nasopharyngeal mucosal lining, characterized by a unique geographical distribution and a strong etiological association with the Epstein-Barr virus (EBV) (Gondhowiardjo *et al*., 2021). While advances in intensity-modulated radiation therapy (IMRT) and concurrent chemotherapy have improved survival rates, locoregional recurrence and distant metastasis remain significant clinical challenges, leading to treatment failure in approximately 20–30% of patients (Zhang *et al*., 2023; Wong *et al*., 2025). These failures are increasingly attributed to the inherent heterogeneity of the tumour microenvironment (TME) and the persistence of a subpopulation of cells known as Cancer Stem Cells (CSCs).

CSCs possess the capacity for self-renewal, differentiation, and inherent resistance to conventional cytotoxic therapies (Lee *et al*., 2025). In the context of NPC, these cells act as the "seeds" of recurrence, surviving initial treatment to repopulate the tumour. However, the molecular identity of NPC stem cells remains a subject of debate. Furthermore, the majority of genomic and transcriptomic studies have focused exclusively on adult NPC (Chow *et al*., 2022; Handoko *et al*., 2024; Handoko *et al*., 2025a; Handoko *et al*., 2025b; Morales *et al*., 2025). Paediatric NPC, while rare, presents with distinct clinical behaviours and outcomes. It remains largely unknown whether the stem cell hierarchies and immune landscapes of paediatric tumours mirror those of their adult counterparts or if they are driven by distinct developmental

programs. Understanding these differences is critical for tailoring age-specific therapeutic strategies.

Dissecting the complexity of the NPC ecosystem requires high-resolution genomic tools. Single-cell RNA sequencing (scRNA-seq) has revolutionized our understanding of tumour heterogeneity; however, most studies rely on short-read sequencing technologies that may miss complex transcriptomic features. Third-generation sequencing platforms, such as Oxford Nanopore Technologies (ONT), offer the advantage of long-read sequencing, enabling the capture of full-length transcripts and providing a more comprehensive view of the gene expression landscape (Sereika *et al*., 2022; Novak & Dvorak, 2025).

In this study, we leveraged Nanopore-based single-cell transcriptomics to construct a comparative cellular atlas of paediatric and adult NPC. We aimed to delineate the transcriptomic signatures of NPC stem cells, identifying distinct "quiescent" versus "basal-like" stem cell phenotypes dependent on patient age. Furthermore, by reconstructing intercellular communication networks, we sought to map the immunosuppressive crosstalk between stem cells and the immune niche. Our findings reveal a conserved axis of immune evasion, mediated by the CD96 immune checkpoint, CD99 homophilic interactions, and the Macrophage Migration Inhibitory Factor (MIF) pathway, that facilitates tumour survival despite robust antigen presentation, offering new targets for therapeutic intervention.

Materials and Methods

Patient Recruitment and Sample Collection

Fresh biopsy specimens were obtained from two patients diagnosed with Nasopharyngeal Carcinoma (NPC) at Cipto Mangunkusmo Hospital and Dharmais Hospital. The cohort consisted of one male paediatric patient, 10 years old (Patient 1) and one male adult patient, 49 years old (Patient 2). Histopathological confirmation of NPC was performed for both cases prior to processing. Informed consent was obtained from the adult patient and the guardians of the paediatric patient in accordance with the institutional review board (IRB) guidelines with ethical approval number KET-149/UN2.F1/ETIK/PPM.00.02/2023.

Tissue Dissociation and Single-Cell Suspension

Fresh tumour tissues were immediately processed into single-cell suspensions following an optimized enzymatic dissociation protocol. Briefly, tissues were minced and incubated in a dissociation buffer containing 0.2% Collagenase Type IV (Gibco) in Phosphate Buffered Saline (PBS) at 37°C for 4 hours in a 5% CO2 incubator. The enzymatic reaction was ceased with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) Fetal Bovine Serum (FBS). The digested tissue was filtered with 70µm and 40µm cell strainers to remove debris and undigested remnant tissue. Red blood cell lysis was performed to eliminate erythrocyte contamination. The resulting cell suspension was washed with PBS containing no bovine serum albumin (BSA), no calcium, and no magnesium. Cell viability and concentration were assessed microscopically using Trypan Blue exclusion staining. Only samples with >80% viability were processed for library preparation.

Single-Cell Partitioning and cDNA Synthesis

Single-cell partitioning and barcoding were performed using the Chromium X Series platform (10x Genomics, Pleasanton, CA) with the Chromium Single Cell 3' Reagent Kit v4, adhering strictly to the manufacturer's instructions (User Guide CG000731). Approximately 10,000 cells were loaded per channel to target a recovery of ~6,000 cells.

Crucially, the protocol was followed through Step 1 (GEM Generation and Barcoding) and Step 2 (Post GEM-RT Cleanup & cDNA Amplification) to generate full-length, barcoded cDNA. The fragmentation and dual-index library construction steps typical for short-read sequencing were omitted to preserve full-length transcript information for long-read sequencing.

Nanopore Library Preparation and Sequencing

Full-length cDNA amplicons generated from the 10x Genomics workflow were used as input for Oxford Nanopore Technologies (ONT) library preparation. The sequencing library was constructed using the Ligation Sequencing Kit (SQK-LSK114) (Oxford Nanopore Technologies, Oxford, UK) according to the specific protocol for "Single-cell transcriptomics with cDNA prepared using 10x Genomics" (Document SST_9198_v114).

Sequencing was performed on a PromethION device using PromethION Flow Cells (FLO-PRO114M). Each patient sample was sequenced on a dedicated flow cell for a runtime of 96 hours to maximize read depth and unique molecular identifier (UMI) recovery.

Data Acquisition and Pre-processing

Raw sequencing data were acquired in POD5 format. Basecalling was performed using the super-accuracy model (dna_r10.4.1_e8.2_400bps_sup@v5.2.0). Downstream data processing, including demultiplexing, barcode assignment, and UMI counting, was conducted using the EPI2ME Single Cell workflow (Oxford Nanopore Technologies). Reads were aligned to the human reference genome (hg38) to generate feature-barcode matrices containing gene expression counts.

Dimensionality Reduction and Clustering

The gene expression matrices were imported into R (v5.0) and processed using the Seurat package (Hao *et al*., 2024). Low-quality cells (characterized by low feature counts or high mitochondrial gene content) were filtered out. Data were normalized using the LogNormalize method, and highly variable features were identified for downstream analysis. Principal Component Analysis (PCA) was performed for dimensionality reduction, followed by unsupervised clustering using the Louvain algorithm. Visualization of cell clusters was achieved using t-Distributed Stochastic Neighbor Embedding (t-SNE).

Cell Type Annotation and Marker Identification

Initial cell type annotation was performed in an automated fashion using SingleR (Aran *et al*., 2019), utilizing the Human Primary Cell Atlas as a reference (Almeida & Moreira, 2025; Parums, 2025). These annotations were subsequently refined manually by assessing the expression of canonical lineage markers (e.g., *EPCAM* for epithelial cells, *CD3D* for T cells, *MS4A1* for B cells) and top differentially expressed genes identified via the FindAllMarkers function in Seurat.

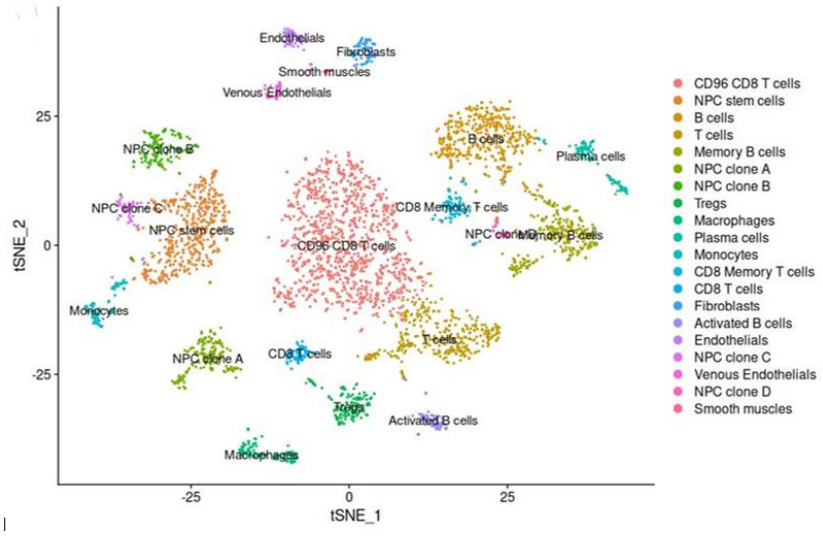
Intercellular Communication Analysis

To decipher the cell-cell communication networks within the tumour microenvironment, we utilized the CellChat R package (Abdullah *et al*., 2025; Jin *et al*., 2025). The ligand-receptor interaction database was used to infer intercellular signalling pathways. We specifically analysed the signalling roles of NPC stem cell clusters in the context of the MHC-I, MHC-II, MIF, CD99, and NECTIN-CD96 pathways. Network centrality scores were calculated to identify dominant "Sender," "Receiver," and "Mediator" cell populations within the signalling networks.

Results and Discussion

Transcriptomic Atlas and Cellular Heterogeneity of Paediatric and Adult NPC

To dissect the cellular heterogeneity of Nasopharyngeal Carcinoma (NPC), we performed unsupervised clustering on the gene expression matrices derived from the Nanopore-sequenced single-cell libraries. Visualized via t-Distributed Stochastic Neighbour Embedding (t-SNE), this analysis revealed distinct cellular ecosystems for the paediatric (Patient 1) and adult (Patient 2) cases, identified as 20 and 17 distinct clusters, respectively (**Figures 1a and 2a**).



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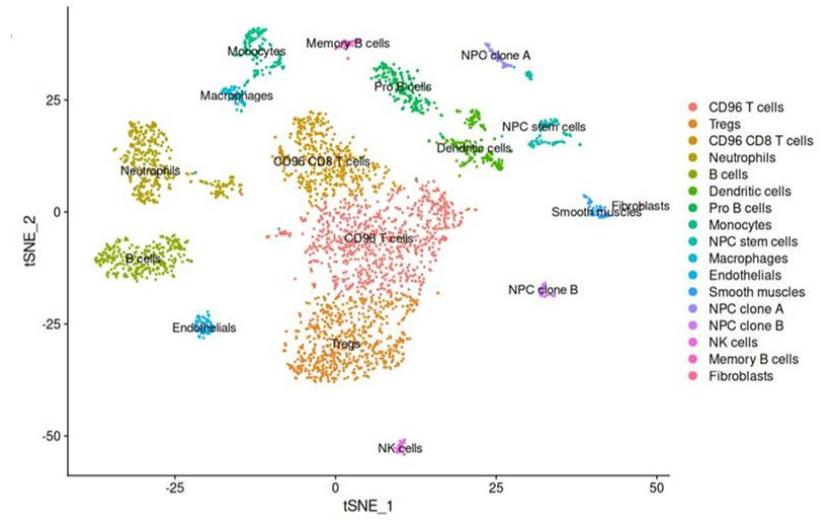

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Figure 1. tsne graph from unsupervised clustering of nasopharyngeal cancer biopsy samples: a) Patient 1 paediatric; b) Patient 2 adult.

Cell type annotation was conducted using a two-step approach. First, automated annotation was performed using SingleR against the Human Primary Cell Atlas to establish broad lineage identities. Second, these identities were refined manually by examining the top differentially expressed genes (DEGs) for each cluster. This robust annotation strategy allowed us to resolve the tumour microenvironment (TME) into four major compartments: malignant epithelial cells, T/NK cells, B/Plasma cells, and myeloid/stromal populations. The final clustering annotation table is shown in **Table 1**.

Table 1. Annotated clusters

Patient 1		Patient 2	
Clusters	**Cell Types**	**Clusters**	**Cell Types**
0	CD8 CD96 T cells	**0**	CD96 T cells
1	NPC stem cells	**1**	T regulatory cells
2	B cells	**2**	CD96 CD8 T cells
3	T cells	**3**	Neutrophils
4	Memory B cells	**4**	B cells
5	NPC clone A	**5**	Dendritic cells
6	NPC clone B	**6**	Pro B cells
7	T regulatory	**7**	Monocytes
8	Macrophages	**8**	NPC stem cells
9	Plasma cells	**9**	Macrophages
10	Monocytes	**10**	Endothelials
11	CD8 Memory T cells	**11**	Smooth muscles
12	CD8 T cells	**12**	NPC clone A
13	Fibroblasts	**13**	NPC clone B
14	Activated B cell	**14**	Natural killer cells
15	Endothelials	**15**	Memory B cells
16	NPC clone C	**16**	Fibroblasts
17	Venous Endothelial Cells		
18	NPC clone D		
19	Smooth muscles		

Identification of Distinct NPC Stem Cell Clones

Within the epithelial compartment, we identified multiple malignant clones exhibiting varying degrees of differentiation. Crucially, both patients harboured a distinct subpopulation of cells exhibiting a molecular signature consistent with NPC Cancer Stem Cells (CSCs), which we validated based on established stemness markers.

In the paediatric case (Patient 1), Cluster 1 was identified as the putative NPC Stem Cell. This cluster showed high expression of the pluripotency transcription factors SOX2 and TP63, alongside the functional stem cell marker ALDH1A1, which is associated with chemotherapy resistance. Notably, Cluster 1 lacked expression of proliferation markers (e.g., *MKI67*, *TOP2A*), suggesting these cells reside in a quiescent, dormant state typical of therapy-resistant stem cell reservoirs. This is in contrast to Cluster 16, which expressed similar stemness factors but was highly enriched for cell-cycle genes, representing a transient amplifying progenitor population driving rapid tumour growth.

In the adult case (Patient 2), Cluster 8 was identified as the CSC population. This cluster displayed a classic "Basal Stem" phenotype, characterized by the exceptionally high expression of KRT15 (Log_2FC > 6.0), a specific marker of epithelial stem cell quiescence, as well as KRT5, KRT14, SOX2, and ALDH1A1. The specific enrichment of these basal markers suggests that in the adult patient, the stem cell niche is strictly maintained via interactions with the basement membrane, distinct from the more heterogeneous stem-like populations observed in the paediatric sample.

Immune Landscape and Dysfunctional Regulatory Phenotypes

The TME of both patients was heavily infiltrated by immune cells, yet the composition revealed signs of significant immunosuppression. We identified multiple T-cell clusters, including cytotoxic CD8+ T cells (Cluster 0 in P1; Cluster 2 in P2) expressing *GZMB* and *NKG7*. However, their potential anti-tumor activity appears compromised by the presence of highly suppressive regulatory T cells (Tregs).

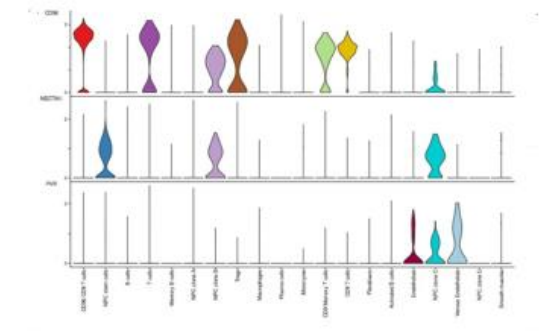
In the adult patient, Cluster 1 represented a prominent Treg population marked by the canonical expression of FOXP3 and IL2RA (CD25). Crucially, these cells co-expressed high levels of CTLA4, TIGIT, BATF, CCR8, and LAYN. The expression of *LAYN* and *CCR8* specifically demarcates a subset of highly activated, tumour-infiltrating Tregs known to suppress inflammation and inhibit cytotoxic T-cell function potently. Similarly, in the paediatric patient, Cluster 7 was identified as Tregs expressing *FOXP3* and *CTLA4*. The presence of these specialized immunosuppressive cells suggests that the NPC stem cells exist within a "cold" immune niche, actively shielded from inflammatory surveillance.

The CD96-NECTIN1 Immune Checkpoint Axis Facilitates Immune Evasion of NPC Stem Cells

To delineate the mechanisms of T-cell dysfunction within the NPC microenvironment, we investigated the expression of co-inhibitory receptors on tumour-infiltrating lymphocytes (TILs). In both the paediatric and adult patients, we identified a specific subpopulation of cytotoxic T cells exhibiting an "exhausted" phenotype, characterized by the aberrant upregulation of CD96.

In the paediatric patient (Patient 1), differential expression analysis identified Cluster 0 (CD8+ T cells) as the primary reservoir of CD96+ cells (**Figure 2a**). In the adult patient (Patient 2), high *CD96* expression was localized to Cluster - 2 (CD8+ T and Tregs) (**Figure 2b**). To understand the drivers of this exhaustion, we performed ligand-receptor interaction analysis using CellChat.

We identified NECTIN1 (CD111) as the dominant ligand driving CD96 signalling in both patients. Strikingly, the source of this inhibitory ligand was directly mapped to the malignant epithelial compartments.



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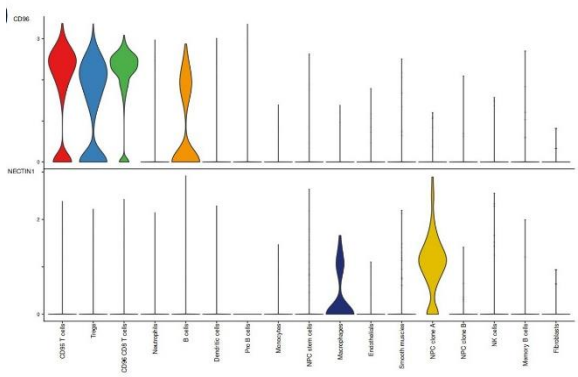

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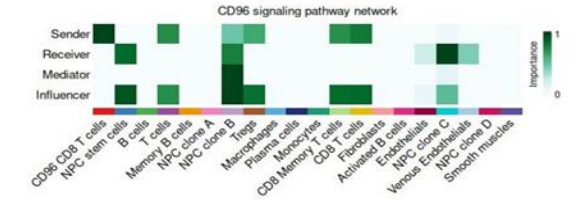

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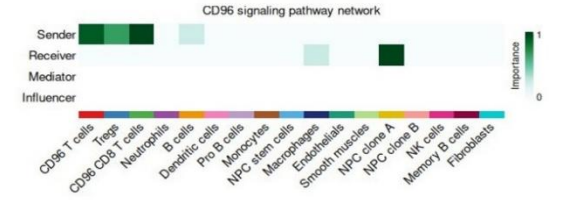

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Figure 2. Violin plot showing CD96 pathway differential expression across all cluster cell types: a) Patient 1 paediatric; b) Patient 2 adult. CD96 network centrality analysis: c) Patient 1 paediatric; d) Patient 2 adult.

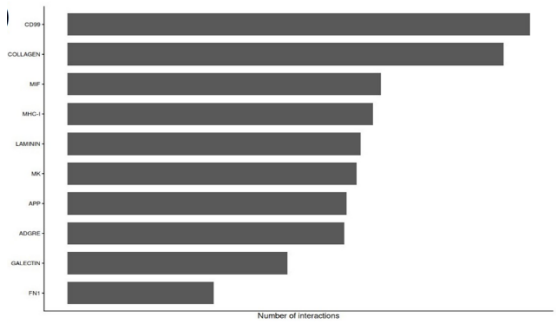
In the adult patient, the NPC clone A (Cluster 12) exhibited the highest expression of *NECTIN1* (**Figure 2b**). Network centrality analysis confirmed a strong unidirectional flow of signaling from the CD8+ T cells (Sender) to the NPC cells clusters (Receiver) (**Figures 2c and 2d**). This suggests that the NPC cells actively shield themselves from immune surveillance by engaging the CD96 checkpoint on T cells.

Similarly, in the paediatric patient, *NECTIN1* expression was enriched in the Proliferating NPC clone A cells (Cluster 5), NPC clone B Cluster (Cluster 6) and NPC clone C cluster (Cluster 16) (**Figure 2a**). The "Signalling Role" heatmap revealed that these tumour clusters act as the dominant outgoing sources of NECTIN1, targeting the CD96+ T cells in Cluster 0 (**Figures 2c and 2d**).

Collectively, these data define a conserved immunosuppressive axis in NPC where malignant cells, specifically the stem and proliferating compartments, hijack the NECTIN1-CD96 pathway to block CD226-mediated co-stimulation physically, thereby inducing functional exhaustion in cytotoxic T cells.

The CD99 Axis: A Dominant Signal Promoting Tumour Cohesion and Myeloid Suppression

Analysis of the global communication network revealed that the CD99 signalling pathway was the single most significant contributor to intercellular crosstalk in both the paediatric and adult patients, surpassing even MHC-I and MIF signalling (**Figures 3a and 3b**). CD99 is a cell surface glycoprotein with a context-dependent "dual role" in malignancy, capable of acting as either a tumour suppressor or an oncogenic driver depending on the cellular context and ligand engagement. In our NPC cohort, the pathway activity suggests a strong pro-tumorigenic function mediated through two distinct mechanisms: structural cohesion and immune evasion.



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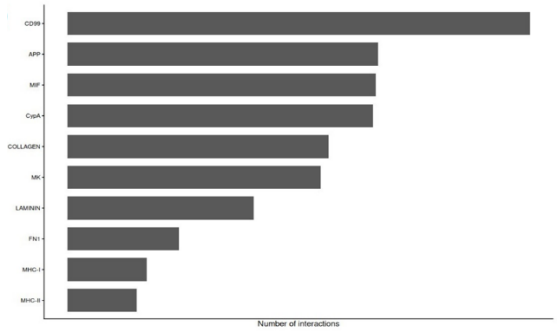

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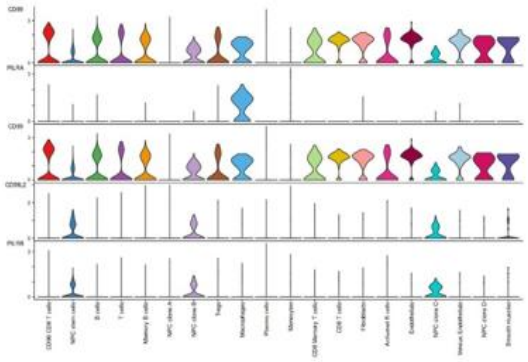
Figure 3. Top 10 contributors from network pathway analysis: a) Patient 1 paediatric b) Patient 2 adult.

Homophilic CD99 Signalling Maintains Tumour Architecture

In both patients, the majority of CD99 signalling was mediated via homophilic (CD99-CD99) interactions. Violin plot analysis showed high expression of *CD99* ubiquitously across malignant epithelial clusters and T-cells, but significantly lower expression in B-cells and fibroblasts (**Figures 4a and 4b**). Network analysis indicated that the NPC Stem Cell clusters (Cluster 1 in Patient 1; Cluster 8 in Patient 2) together with CD8 T cells, macrophages and stromal cells were major hubs of this autocrine and paracrine signalling loop. This homophilic binding is critical for facilitating cell-cell adhesion and transendothelial migration, suggesting that high CD99 activity in these samples may support the physical integrity of the tumour nests and their potential for metastatic invasion.



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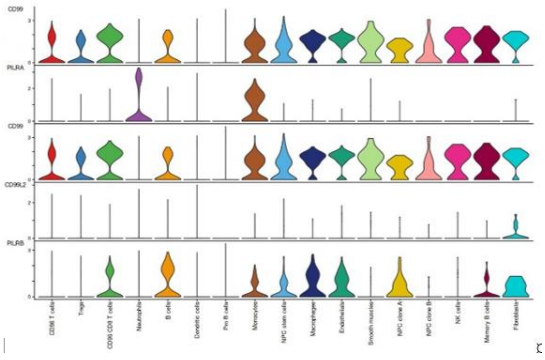

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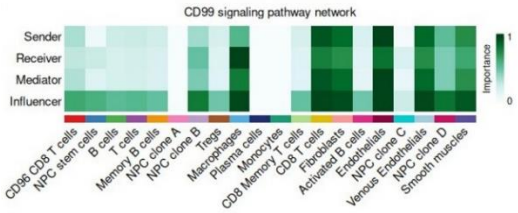

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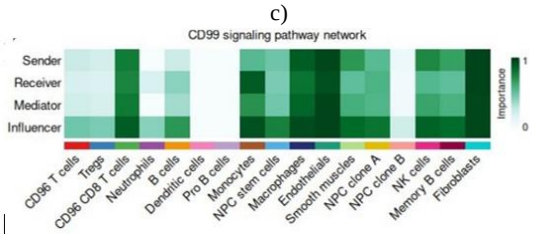

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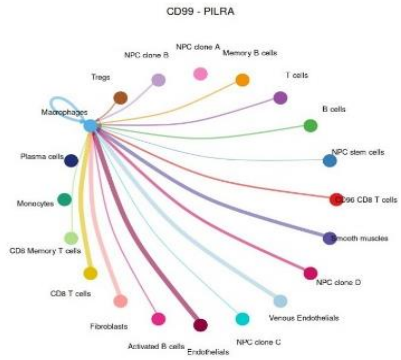

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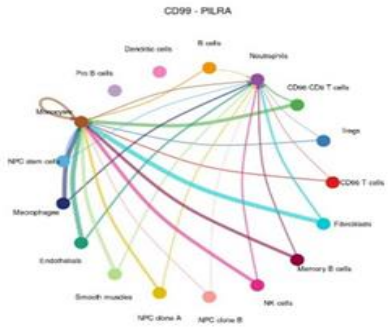

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Figure 4. Violin plot showing differential expression of the CD99 pathway: a) Patient 1 paediatric; b) Patient 2 adult. CD99 network centrality analysis: c) Patient 1 paediatric; d) Patient 2 adult. Ligand receptor interaction analysis from all cell types/clusters from e) Patient 1 paediatric; f) Patient 2 adult.

Heterophilic CD99-PILRα Interaction Drives Innate Immune Evasion

Beyond structural support, we identified a critical immune checkpoint mechanism in the adult patient (Patient 2) involving the heterophilic interaction between CD99 and the Paired Immunoglobulin-like Type 2 Receptor Alpha (PILRα).

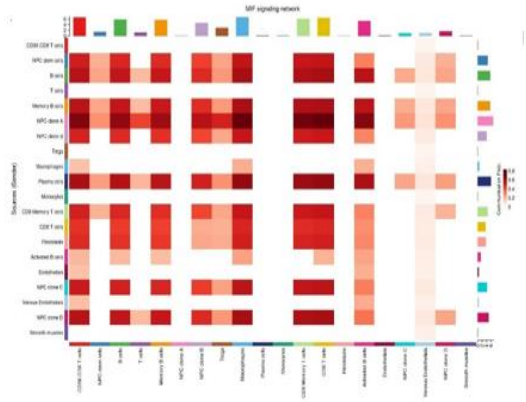
While *CD99* was expressed on the tumour cells, its ligand *PILRA* was restricted exclusively to the myeloid compartment (Neutrophils / Macrophages / Monocytes) (**Figures 4a and 4b**). Ligand-Receptor analysis confirmed a strong signalling trajectory from the Tumour (Sender) to Myeloid cells (Receiver) (**Figures 4c-4f**). *PILRα* is an inhibitory receptor containing ITIM motifs that, upon binding to CD99, suppresses myeloid cell activation. This finding indicates that in adult NPC, CD99 functions as a "Don't Eat Me" signal, actively engaging PILRα on tumour-associated macrophages to inhibit phagocytosis and promote an immunosuppressive microenvironment. In contrast, this specific inhibitory axis was present albeit less prominent in the paediatric patient, pointing to potential age-dependent differences in innate immune evasion strategies.

NPC Stem Cells Orchestrate an Immunosuppressive Microenvironment via the MIF-CD74 Axis

Beyond direct cell-cell contact mechanisms like CD96 and CD99, we investigated soluble signalling mediators within the tumour microenvironment (TME). Our analysis identified the Macrophage Migration Inhibitory Factor (MIF) signalling pathway as a highly active, conserved communication axis in both paediatric and adult NPC, specifically originating from the stem cell compartments.

MIF is Constitutively Expressed by NPC Stem Cells

In the adult patient (Patient 2), the NPC Stem Cell population (Cluster 8) exhibited the highest expression of the *MIF* ligand among all epithelial clusters (**Figure 5b**). Network centrality analysis revealed that all NPC cells clusters act as the dominant "Sender" (outgoing signal source) for the entire MIF network, transmitting strong signals to the myeloid and lymphoid compartments (**Figures 5c and 5d**).



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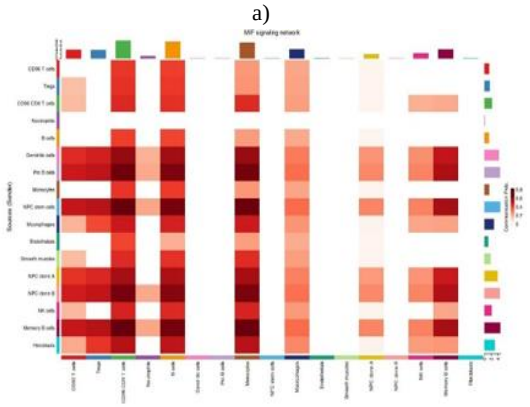

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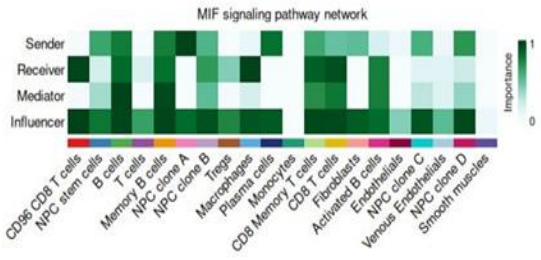

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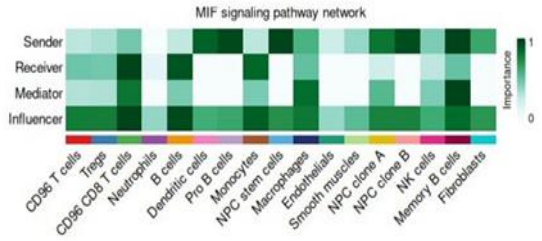

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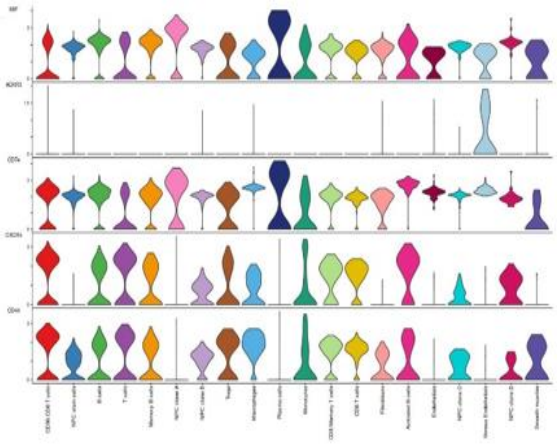

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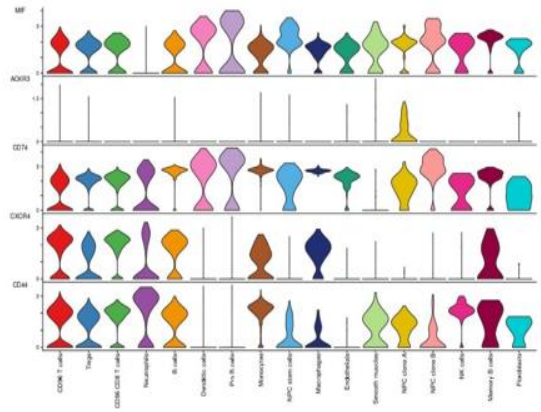


f)

Figure 5. Heatmap showing MIF signalling network strength across clusters/cell types. a) Patient 1 paediatric; b) Patient 2 adult. MIF network centrality analysis: c) Patient 1 paediatric; d) Patient 2 adult. Violin plot of differentially expressed genes from MIF pathways across all clusters/cell types: e) Patient 1 paediatric; f) Patient 2 adult.

Similarly, in the paediatric patient (Patient 1), strong *MIF* expression was observed in the Quiescent Stem Cell (Cluster 1) and the Proliferating Tumour (Cluster 5) populations (**Figure 5a**). This suggests that MIF secretion is a fundamental property of the malignant state in NPC, regardless of patient age, and is maintained as stem cells enter the cell cycle.

Targeting of Myeloid and T Cells to Promote Tolerance

Ligand-receptor analysis and "Heatmaps" **(Figures 5a and 5b)** mapped the downstream targets of this stem-cell-derived MIF. In both patients, the primary "Receivers" were Macrophages/Monocytes and T Cells, which expressed high levels of the MIF receptors CD74 and CXCR4 (**Figures 5c-5f**).

The binding of MIF to CD74/CXCR4 on macrophages is a well-documented driver of M2 polarization, shifting them towards an anti-inflammatory, tissue-repair phenotype that supports tumour growth rather than attacking it. Furthermore, the reception of MIF signals by T cells (as seen in the heatmap for Patient 2, Cluster 2) is known to suppress T-cell activation.

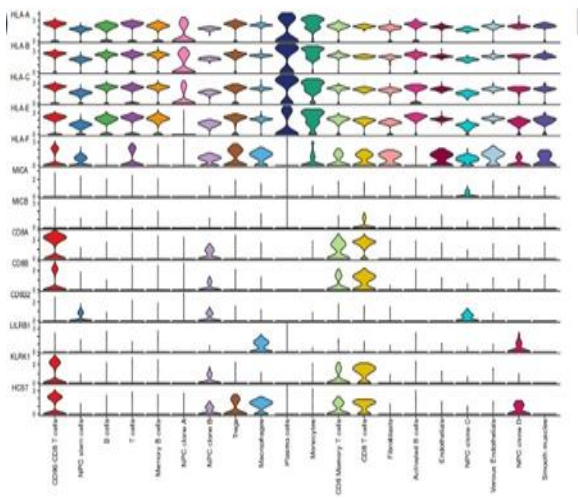
By acting as the central hub of MIF signalling, NPC stem cells appear to sculpt a "tolerogenic" niche actively. This data supports a model where the stem cells do not merely reside in the tumour but actively release cytokine barriers to turn off local immune surveillance.

Sustained MHC-I Antigen Presentation and the Upregulation of Non-Classical HLA-E

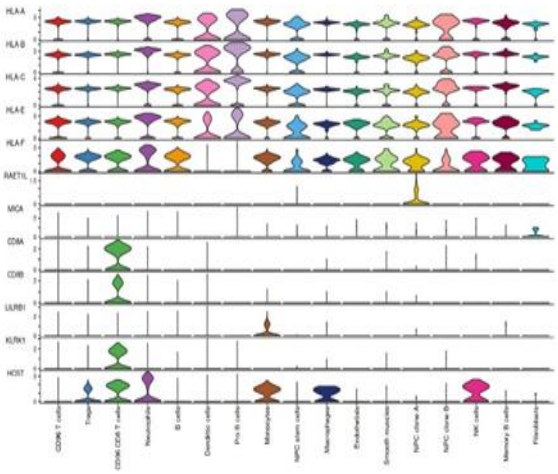
To determine if NPC stem cells utilize "invisibility" (downregulation of MHC-I) as an immune evasion strategy, we profiled the expression of the Major Histocompatibility Complex Class I (MHC-I) machinery. Contrary to the "missing self" hypothesis often seen in solid tumours, our analysis revealed robust and sustained expression of MHC-I genes across the malignant compartment in both patients.

Preservation of Antigen Presentation Machinery

In both patients, the NPC Stem Cell population and the Malignant NPC cell cluster showed high expression of classical MHC-I genes (*HLA-A, HLA-B, HLA-C*) as well as the non-classical *HLA-E* (**Figures 6a and 6b**). Network centrality analysis confirmed that the Tumour clusters, among other immune cells, act as the dominant "Sender" hubs for the MHC-I signalling network (**Figures 6c and 6d**), actively engaging CD8+ T cells within the microenvironment.



a)



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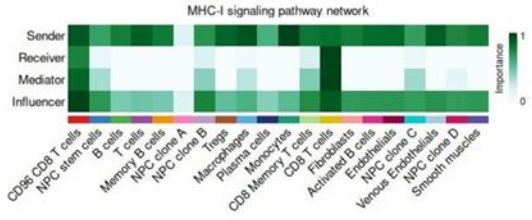
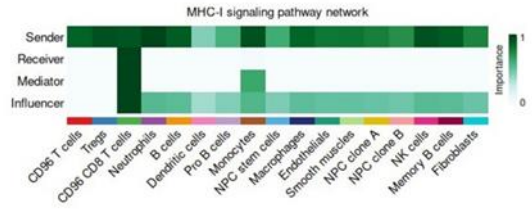
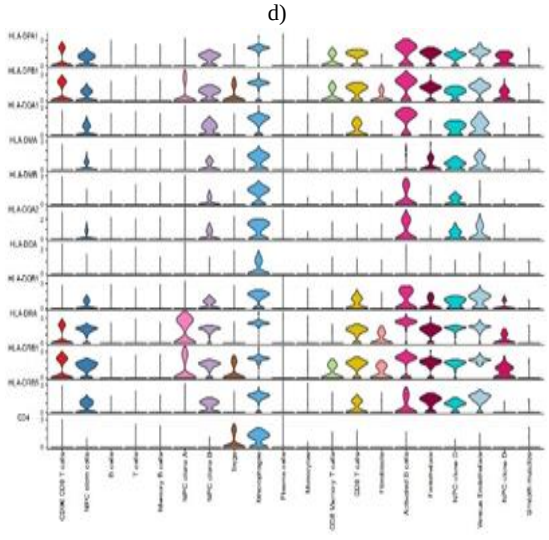


c)



d)



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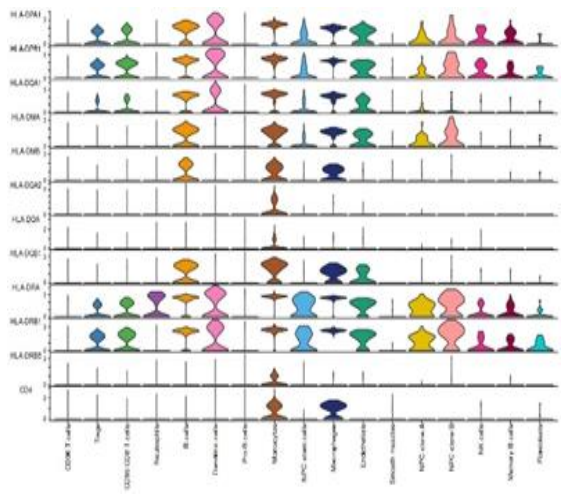
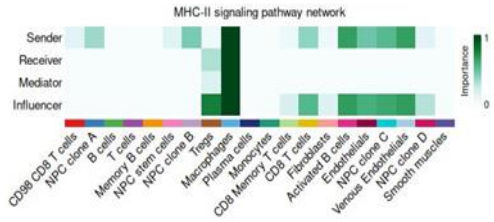
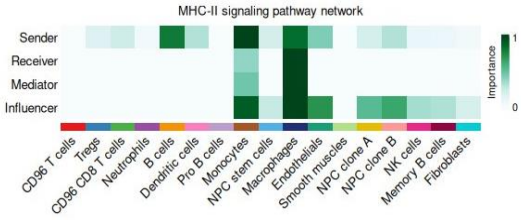


f)



g)



h)

Figure 6. a-d) Violin plot of differentially expressed genes from MHC-I pathways across all clusters / /cell types. a) Patient 1 paediatric b) Patient 2 adult. MHC-I network centrality analysis: c) Patient 1 paediatric; d) Patient 2 adult. e, f) Violin plot of differentially expressed genes from MHC-II pathways across all clusters / cell types e) Patient 1 paediatric f) Patient 2 adult. MHC-II network centrality analysis g) Patient 1 paediatric h) Patient 2 adult.

Similarly, in the paediatric patient (Patient 1), the Quiescent Stem Cell (Cluster 1) retained high expression of the MHC-I complex (**Figure 6a**). This indicates that NPC stem cells do not evade the immune system by hiding their antigens; rather, they remain visible to cytotoxic lymphocytes.

The Inhibitory Role of HLA-E

While the preservation of classical HLA-A/B/C suggests potential for tumour recognition, the concurrent high expression of HLA-E (visible in violin plots for both patients) points to a specific evasion mechanism **(Figures 6a and 6b)**. HLA-E is a non-classical MHC molecule that, instead of presenting tumour antigens for killing, binds to the NKG2A (KLRC1) receptor on NK and CD8+ T cells to deliver a potent inhibitory signal.

Ligand-Receptor analysis confirmed significant interactions between the MHC-I on tumour cells and the receptors on cytotoxic lymphocytes. The coexistence of robust MHC-I signalling with the previously identified exhaustion markers (CD96+) and immunosuppressive cytokines (MIF) suggests a complex scenario where NPC stem cells are "visible" to the immune system but actively paralyze the effector response through inhibitory ligand engagement (HLA-E) and checkpoint activation.

Intact Professional Antigen Presentation Suggests Immune Recognition Overwhelmed by Tolerance

To determine if the functional exhaustion of T cells was due to a failure of initial antigen recognition ("immune ignorance"), we analysed the MHC Class II signalling network. MHC-II is critical for priming CD4+ T cells and sustaining the anti-tumor immune response. Unlike the tumour-driven MHC-I and MIF signals, our analysis revealed that MHC-II signalling is preserved and driven by professional Antigen Presenting Cells (APCs) rather than the tumour itself.

Professional APCs Drive Robust CD4+ T Cell Engagement

In the adult patient (Patient 2), high expression of MHC-II genes (*HLA-DRA, HLA-DRB1, HLA-DPA1*) was localized to B-cells (Cluster 4) and Dendritic Cells (DCs, Cluster 5), and malignant NPC cell clusters **(Figure 6f)**. Network centrality analysis identified these professional APCs as the dominant "Senders" **(Figure 6h)**, directing strong signals among themselves and possibly also toward CD4+ Helper T cells (contained within Cluster 0/2) via the *HLA-DR – CD4* axis.

A similar landscape was observed in the paediatric patient (Patient 1), where Activated B-cells (Cluster 14) and Myeloid cells such as Macrophages (Cluster 8) formed the primary signalling hubs, actively communicating with each other and other T-cell populations **(Figures 6e and 6g)**.

The "Failed Response" Hypothesis

The presence of robust MHC-II signalling indicates that the tumour microenvironment is not immunologically "silent." The immune system has successfully detected the presence of antigens, and APCs are actively attempting to prime T cells. However, the coexistence of this active recognition signal with the potent immunosuppressive mediators released by the Stem Cells (MIF, CD99, NECTIN1-CD96) suggests a dominance of peripheral tolerance.

Essentially, the "Go" signal provided by MHC-II is being overridden by the "Stop" signals (Checkpoints and MIF) orchestrated by the NPC stem cells. This highlights that the therapeutic bottleneck in these patients is not the lack of tumour recognition, but the active suppression of the effector response.

The cellular heterogeneity and immunosuppressive landscape of Nasopharyngeal Carcinoma (NPC) have long been recognized as barriers to effective treatment, particularly in recurrent and

metastatic disease (Gong *et al*., 2021; Salem *et al*., 2025). By leveraging the long-read capabilities of Nanopore sequencing, our study provides a high-resolution transcriptomic atlas of the NPC microenvironment, revealing fundamental differences in the stem cell architecture between paediatric and adult patients while identifying a conserved, multi-layered strategy of immune evasion.

A pivotal finding of this study is the identification of distinct Cancer Stem Cell (CSC) phenotypes driven by patient age and disease context. In the adult patient, the stem cell compartment (Cluster 8) exhibited a classic "basal-like" signature (*KRT15+, TP63+, ITGA6+*), suggesting that these cells rely on signals from the basement membrane and cell-matrix interactions to maintain their undifferentiated state (Feng & Wang, 2024; Sadu Murari *et al*., 2025; Wang *et al*., 2025). This aligns with the established model of epithelial carcinomas where stemness is tightly coupled to the basal niche (Garg, 2017; Loh & Ma, 2024; Bello & Musa, 2026).

In contrast, the paediatric tumour displayed a more complex, hierarchical organization. We identified a specific "Quiescent Stem Cell" reservoir (Cluster 1) characterized by high *SOX2* and *ALDH1A1* expression but a lack of proliferation markers (Mirzaei *et al*., 2022; Lindell *et al*., 2023; Nguyen *et al*., 2025). These cells appear to feed into a separate "Transient Amplifying" population (Cluster 16) that drives rapid tumour growth. This distinction is clinically significant; quiescent stem cells are notoriously resistant to chemotherapy and radiation, which target dividing cells (Paul *et al*., 2022; Lindell *et al*., 2023; Sa-Couto *et al*., 2025). The presence of this dormant reservoir may explain the aggressive nature and recurrence patterns often observed in paediatric malignancies, suggesting that therapeutic strategies must go beyond anti-proliferative agents to target the quiescent stem cell pool directly (Truskowski *et al*., 2023; Mulyani *et al*., 2026).

A prevailing theory in cancer immunology is "immune ignorance," where tumours evade detection by downregulating MHC Class I molecules (Gondhowiardjo *et al*., 2020; Handoko *et al*., 2024; Skoda *et al*., 2026). However, our data challenge this paradigm in NPC. We observed robust expression of MHC-I machinery in the stem cell compartments of both patients, and the presence of professional antigen-presenting cells (B cells and DCs) driving active MHC-II signalling. This indicates that the immune system successfully *recognizes* the tumour, yet fails to eliminate it.

Our network analysis elucidates the mechanism behind this failure: a coordinated "paralysis" of the effector response orchestrated directly by the NPC stem cells. We identified a tripartite evasion strategy:

- Physical Exclusion and Inhibition: The dominance of homophilic CD99 signalling facilitates tumour cohesion, while the heterophilic interaction with PILRα on macrophages delivers a potent "Don't Eat Me" signal, effectively suppressing innate immunity (Manara *et al*., 2025; Thach, 2026).
- T-Cell Exhaustion via CD96: By upregulating *NECTIN1*, NPC stem cells physically block the costimulatory receptor CD226 on cytotoxic T cells, instead engaging the inhibitory receptor CD96 (Chan *et al*., 2014; Holmes *et al*., 2019). This checkpoint axis appears critical for maintaining the exhausted phenotype observed in the tumour-infiltrating CD8+ T cells (Mittal *et al*., 2019; Wang *et al*., 2022; Basaad *et al*., 2026).
- Cytokine-Mediated Suppression: The secretion of MIF by stem cells serves as a soluble barrier, binding to CD74/CXCR4 on macrophages to promote an anti-inflammatory M2 phenotype and further suppress T-cell activation (Simpson *et al*., 2012; Balogh *et al*., 2018; Mora Barthelmess *et al*., 2023; Ryan *et al*., 2024; Liu *et al*., 2025).

The identification of these conserved checkpoints highlights potential therapeutic vulnerabilities. While PD-1/PD-L1 blockade has shown mixed results in NPC (Hsu *et al*., 2017; Chan *et al*., 2023; Chong *et al*., 2025), our data suggest that targeting "upstream" evasion mechanisms may be more effective. Specifically, disrupting the CD96-NECTIN1 axis or blocking MIF signalling could release the "brakes" on the pre-existing, antigen-aware immune cells. Furthermore, the distinct stem cell phenotypes suggest that paediatric patients might benefit from therapies directed against ALDH+ quiescent cells, whereas adult patients might respond better to agents disrupting basal cell-matrix interactions (e.g., anti-integrin therapies) (Bergonzini *et al*., 2022; Paulus & Sewald, 2024; Chitren *et al*., 2025; Kariya & Nishita, 2025).

While the use of Nanopore sequencing provided superior transcript resolution, our study is limited by the small sample size. Validation of these findings in a larger cohort and spatial transcriptomics to map the physical colocalization of these immune interactions will be essential. Additionally, functional assays are required to confirm that disrupting the CD99 or MIF axes restores T-cell cytotoxicity in vitro.

Conclusion

In summary, this study unravels the transcriptomic complexity of NPC, demonstrating that while the fundamental machinery of immune evasion, mediated by CD99, MIF, and CD96, is conserved, the stem cell hierarchy differs significantly between paediatric and adult disease. These findings provide a molecular rationale for age-stratified therapeutic approaches and highlight novel checkpoint targets for next-generation immunotherapy in NPC.

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Conflict of interest: None

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