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# New insights into the tumour immune microenvironment of nasopharyngeal carcinoma

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ABSTRACT

Nasopharyngeal carcinoma (NPC) is unique among head and neck cancers for its strong causative association with Epstein Barr-Virus and high levels of immune infiltration that play a role in pathogenesis. As such, immunotherapy for the treatment of NPC is a promising area of research in the pursuit of improving patient outcomes. Understanding the tumour immune microenvironment (TIME) of NPC is the key to developing targeted immunotherapies and stratifying patients to determine optimal treatment regimens. Recent research has uncovered distinct characteristics of the TIME in NPC as well as important differences between the different disease subtypes; however, reviewing the state of the field reveals a further need for the application of novel techniques like multiplexed hyperspectral imaging and mass cytometry. These techniques can be used to identify spatial, compositional, and functional aspects of the TIME in NPC such as immune cell sociology, novel immune populations, and differences in immune-related signalling pathways in NPC in order to identify clinically relevant characteristics for targeted immunotherapy development and biomarker discovery.

## 1. Introduction

Nasopharyngeal carcinoma (NPC), a cancer of the head and neck, arises from squamous epithelial cells of the nasopharynx. Its unique etiology is thought to be driven by a combination of genetic susceptibility, environmental factors, and association with the Epstein-Barr Virus (EBV) in most cases (Wong et al., 2021). NPC is characterized by very high stromal and immune infiltration, which is likely due to proximity to lymphoid structures in the nasopharynx and the close association with EBV infection (Gong et al., 2021). A key feature of NPC is the immune contribution to pathogenesis, where the immunosuppressive microenvironment provides a favourable environment for tumour cells (Gong et al., 2021). Chemoradiation is currently the standard of care for NPC, but recurrence occurs in approximately 20% of patients at which point the 5-year overall survival drops from 70% to 41% (Howlett et al., 2021). Thus, significant focus has been placed on the use of novel strategies, in particular immunotherapy, in NPC to improve patient outcomes (Gong et al., 2021). It has been shown that subtypes of NPC delineated by immune features of the tumour microenvironment can predict survival and response to immunotherapy (Chen et al., 2021). Recently, high throughput technologies suitable for studying the tumour microenvironment of NPC at a single cell resolution have facilitated further insights into the immune contribution to pathogenesis, paving the way to optimize treatment by identifying patients likely to respond to specific immunotherapies.

## 2. The tumour immune microenvironment (TIME)

The tumour microenvironment (TME) is the immediate region surrounding a solid tumour and includes stromal cells such as fibroblasts, endothelial cells, and immune cells in addition to non-cellular components such as the extracellular matrix. Once co-opted by the tumour, the TME plays a significant role in supporting tumour growth and progression.

In contrast, the tumour immune microenvironment (TIME) consists of the immune component of the TME minus the other stromal and extracellular components (Fig. 1). The three main cell lineages in the TIME are the T lymphocytes, B lymphocytes, and myeloid cells, which include myeloid-derived suppressor cells (MDSCs), macrophages, and dendritic cells (Gong et al., 2021). Within the TIME, two functional

*Abbreviations:* NPC, Nasopharyngeal carcinoma; EBV, Epstein-barr virus.

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subcategories of cells can be distinguished: immunostimulating cells, which facilitate the anti-cancer immune response, and immunosuppressive cells, which inhibit the anti-cancer immune response to promote tumour progression. Immunostimulating cells include tumour-infiltrating lymphocytes (TILs), lymphoid cells (ILCs) including natural killer (NK) cells, dendritic cells, and eosinophils. Immunosuppressive cells include MDSCs, mast cells, regulatory T cells (Tregs), and macrophages (specifically M2-polarized tumour-associated macrophages) (Salemme et al., 2021). The balance of immunostimulating and immunosuppressive cells in the TIME is often specific to the type of tumour and can influence tumour progression, for example by suppressing the immune response to facilitate the unhindered growth of cancer cells.

## 3. Features of the TIME in NPC

Recently, with the use of novel high throughput methods, the TIME in NPC has been shown to exhibit increased immune infiltration within a tolerogenic setting, due to suppression of the anti-cancer immune response (Fig. 2). Recruitment of immune cells, both immunostimulating and immunosuppressive, is mediated in part by increased expression of chemokines by tumour cells, macrophages, and dendritic cells (Chen et al., 2020). Immune escape can be mediated by direct communication between the tumour and immune cells, for instance via inhibitory ligands/receptors and/or mutations of antigen presentation machinery, and/or indirect communication through chemokines/cytokines (Jiang and Ying, 2022).

One immune cell type that exhibits unique characteristics in the TIME of NPC is macrophages. These macrophages may be recruited directly by secreted chemokines or can differentiate from monocytes that are recruited, as evidenced by similar patterns of transcription factors between these macrophages and monocytes (Chen et al., 2020). Macrophages in the TIME have altered properties in comparison with macrophages in the tissue or circulation, and in NPC have been shown to exhibit a unique co-expression of M1 and M2-associated genes, possibly indicating an intermediate phenotype between the traditionally tumour-suppressing M1 and tumour-promoting M2 subtypes (Jin et al., 2020; Gong et al., 2021).

A distinctive dendritic cell subtype marked by lysosome-associated membrane glycoprotein 3 (LAMP3) has also been described in NPC. These LAMP3+ dendritic cells interact with Tregs via cytotoxic T-lymphocyte-associated protein 4 (CTLA4), leading to downregulation of antigen processing and presentation in the LAMP3+ dendritic cells and proliferation of the Tregs, in addition to expressing programmed death ligand 1 (PD-L1), which interacts with PD-1 on cytotoxic T cells to cause apoptosis (Liu et al., 2021).

The TIME of NPC is also characterized by higher enrichment of TILs than most other solid tumors, although these lymphocytes express both effector markers, including interleukin-2 and interferon gamma (IFN-γ), and exhaustion markers, including CTLA4 and PD-1, likely indicating that their effector functions are being suppressed (Liu et al., 2021). Evidence for an increased number of Tregs points to suppression of anti-cancer effector functions of TILs as a result (Jin et al., 2020), mediated in part by inhibitory molecules such as programmed cell death

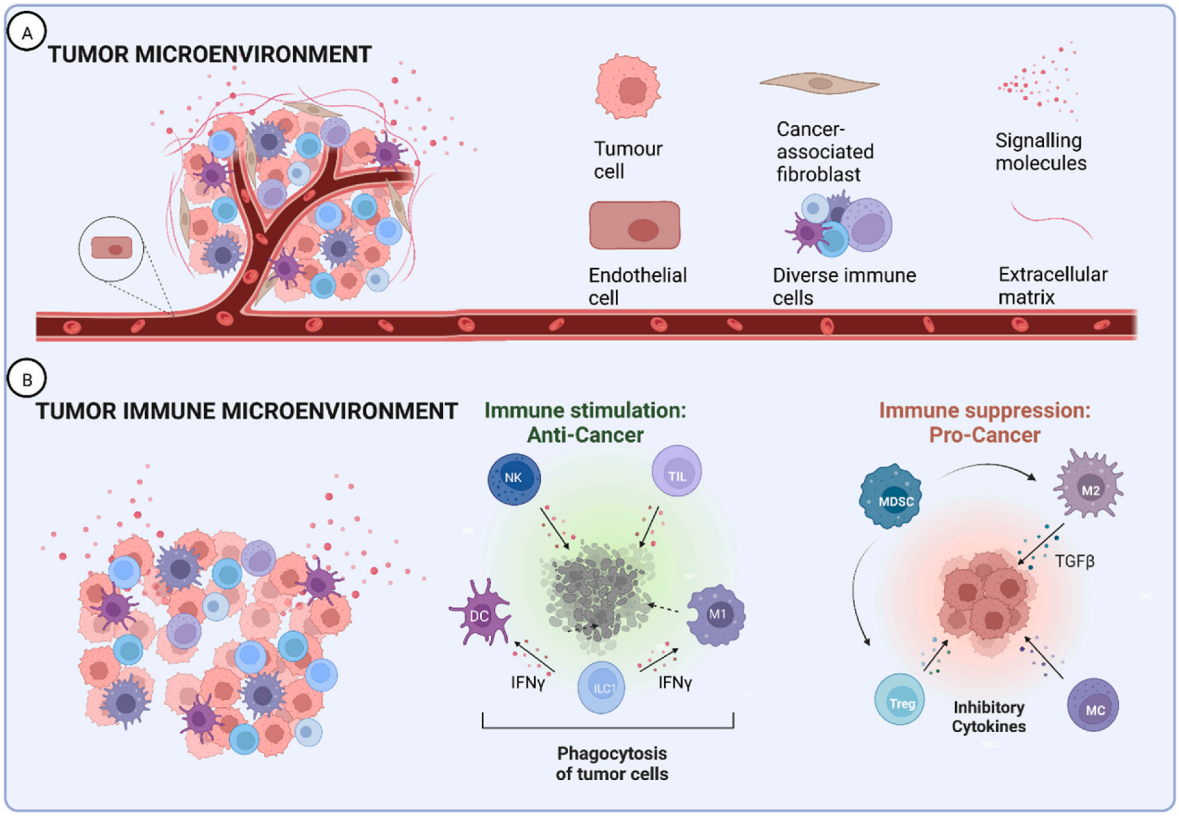


**Fig. 1.** The tumour microenvironment versus the tumour immune microenvironment.
The tumour immune microenvironment is an important component of the larger tumour microenvironment that can prevent or promote tumour progression. A: The tumour microenvironment (TME) is composed of tumour cells; stromal cells such as cancer-associated fibroblasts (CAFs), endothelial cells, and a diverse population of immune cells; and non-cellular components including signalling molecules and extracellular matrix. B: The tumour immune microenvironment (TIME) is the immune subpopulation of the TME, which can be subdivided into two functional groups. The immunostimulatory cells promote immune surveillance and thus killing of cancer cells and include natural killer (NK) cells, tumour-infiltrating lymphocytes (TILs), M1-polarized macrophages, dendritic cells (DCs), and type 1 innate lymphoid cells (ILC1). In contrast, the immunosuppressive cells facilitate a permissive environment for cancer progression. These include myeloid-derived suppressor cells (MDSCs), M2-polarized macrophages, regulatory T cells (Tregs), and mast cells (MCs).

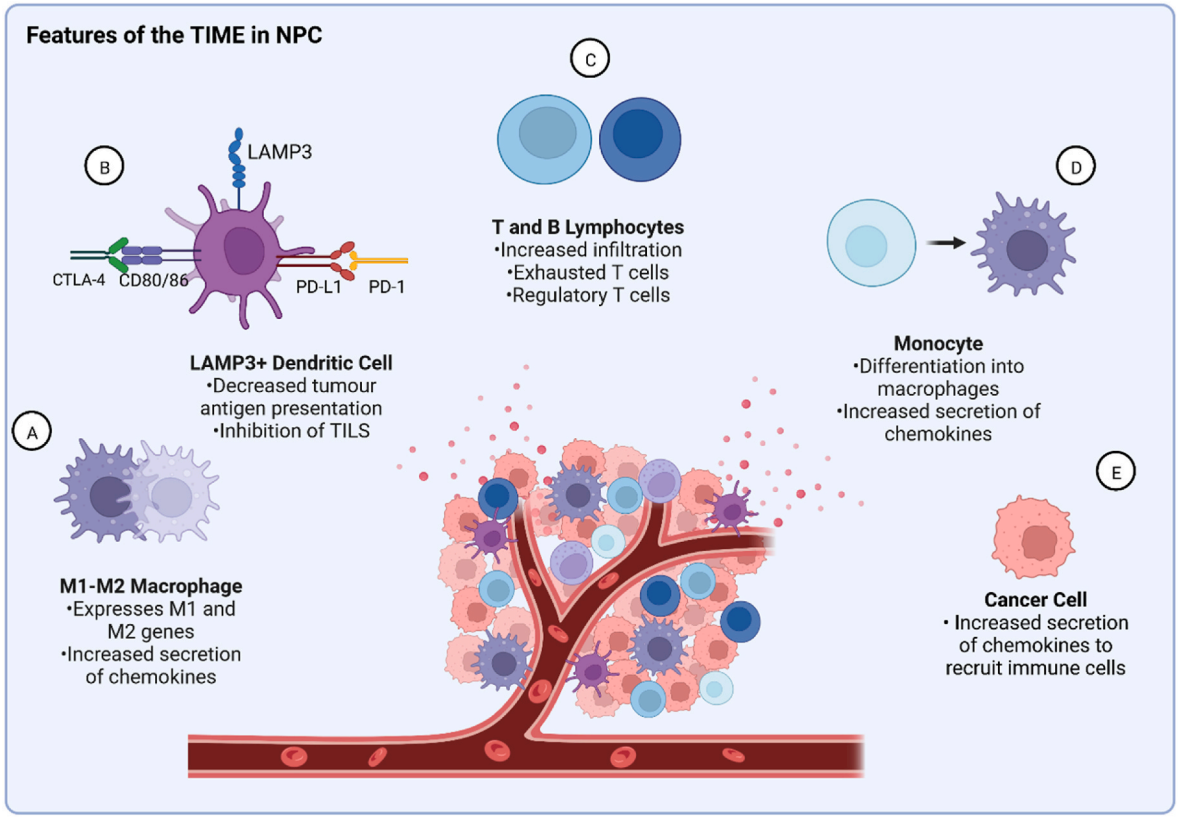


**Fig. 2.** Features of the tumour immune microenvironment in NPC.
The TIME in NPC is characterized by increased recruitment of immune cells and formation of a tolerogenic environment. A: Macrophages co-express anti-inflammatory M1 and pro-inflammatory M2 gene signatures, forming an intermediate phenotype known to secrete elevated amounts of chemokines to recruit immune cells to the TIME. B: LAMP3+ dendritic cells are a unique population in the TIME of NPC that express PD-L1 and the ligand for CTLA4, leading to downregulation of antigen processing in the dendritic cell and inhibition of the effector function of tumour-infiltrating lymphocyte. C: Increased recruitment of B and T cells is mediated in part by increased chemokines secreted by other components of the TIME. Enrichment of regulatory T cells contributes to the high number of exhausted T cells observed.D: Monocytes differentiate into macrophages, which then secrete chemokines to recruit immune cells. E: Increased secretion of chemokines from cancer cells facilitates recruitment of immune cells.

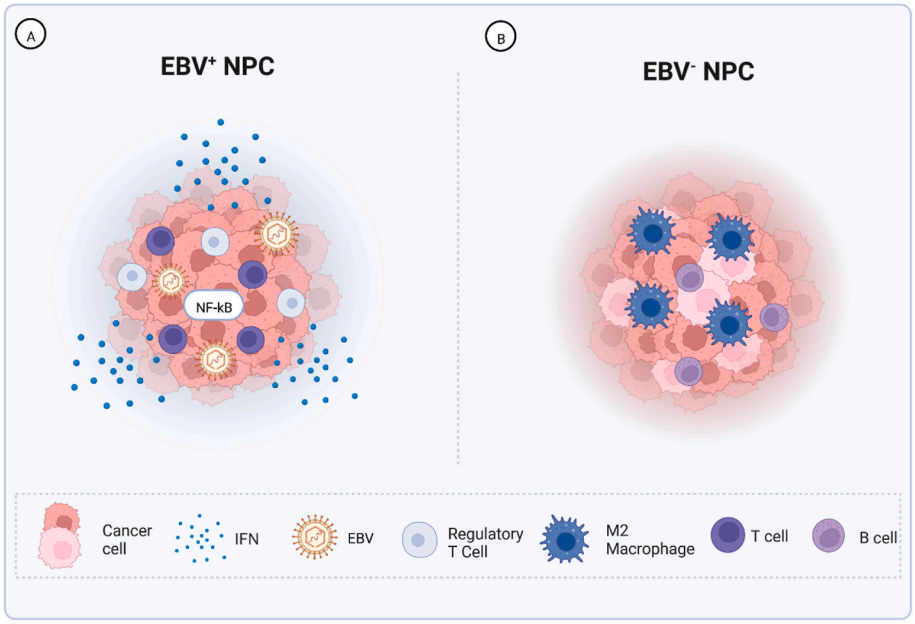


**Fig. 3.** Differences in the TIME between EBV-positive (EBV+) and EBV-negative (EBV-) NPC.
Differences between the TIME of EBV+ and EBV- NPC. A: EBV+ NPC shows a relative enrichment of effector and regulatory T cells and is dominated by constitutive activation of NF-κB and classically anti-cancer interferon-mediated signaling. B: EBV- NPC is relatively enriched in B cells and M2-polarized macrophages, and cancer cells display increased heterogeneity.

protein 1 (PD-1) and Hepatitis A Virus Cellular Receptor 2 (HAVCR2) on cells in the TIME (Liu et al., 2021).

Finally, the TIME of recurrent NPC has recently been profiled and demonstrates similar features to primary NPC, albeit to a greater extent. Namely, increased enrichment of immunosuppressive cells, particularly Tregs, LAMP3+ dendritic cells and M2-polarized macrophages, and an even stronger effector-exhaustion signal for TILs have been observed (Peng et al., 2022). In combination, these features in both primary and recurrent NPC indicate a strongly immunosuppressive microenvironment.

## 4. Differences in the TIME between EBV+ and EBV- NPC

Within the framework of general characteristics of the TIME in NPC, differences in certain features have been observed between EBV-positive (EBV+) and EBV-negative (EBV-) tumors (Fig. 3). These differences have yet to be synthesized into a subtype-specific definition of the TIME but can still provide insights into the immune-related pathogenesis of NPC in the presence or absence of EBV.

In EBV + NPC, recent whole genome sequencing has revealed constitutive nuclear factor kappa beta (NF-κB) activation, driven by viral expression of latent membrane protein 1 (LMP1) or somatic mutations. Downstream signalling leads to increased secretion of immunomodulatory molecules such as interleukin-6 (IL-6), interleukin-8 (IL-8), and leukemia inhibitory factor (LIF) which facilitate the recruitment of immune cells to establish a chronic inflammatory milieu (Bruce et al., 2021; Lo et al., 2021). In addition, the EBV + TIME is characterized by high levels of the pro-inflammatory cytokine IFN-γ that is produced in response to the viral infection. Classically, IFN-γ is considered an anti-tumour cytokine, in part by increasing the efficacy of NK cells and TILs, but persistent upregulation as seen in EBV + NPC can promote an immunosuppressive TIME through multiple mechanisms including expression of inhibitory molecules like PDL-1/CTLA-4 (Mojic et al., 2017; Jin et al., 2020). Overall, the EBV + TIME is an immunosuppressed environment characterized by a large population of Tregs, exhausted TILs, and MDSCs (Ooft et al., 2018).

In contrast, mechanisms underlying the composition of the EBV- TIME have not been well researched. However, it has been shown that the EBV- TIME shows a comparative enrichment of B cells over T cells (Zhao et al., 2020), with fewer Tregs and myeloid-derived cells present, and M2 macrophages more commonly observed (Ooft et al., 2018). EBV- tumors also exhibit increased intratumoral heterogeneity, as seen by an increased number of cell populations when classified using gene expression status from scRNA-seq data and by increased diversity in the tumour cells themselves (Zhao et al., 2020). Subtype-specific research is required to uncover characteristics of immune pathogenesis in NPC that are independent of EBV infection.

## 5. New methods in characterizing the TIME

Investigating the composition, localization, and function of components of the TIME between and within tumors is an emerging field driven by novel high-throughput technologies (Fig. 4). These characteristics are highly relevant in highly immune-infiltrated tumors such as NPC, as

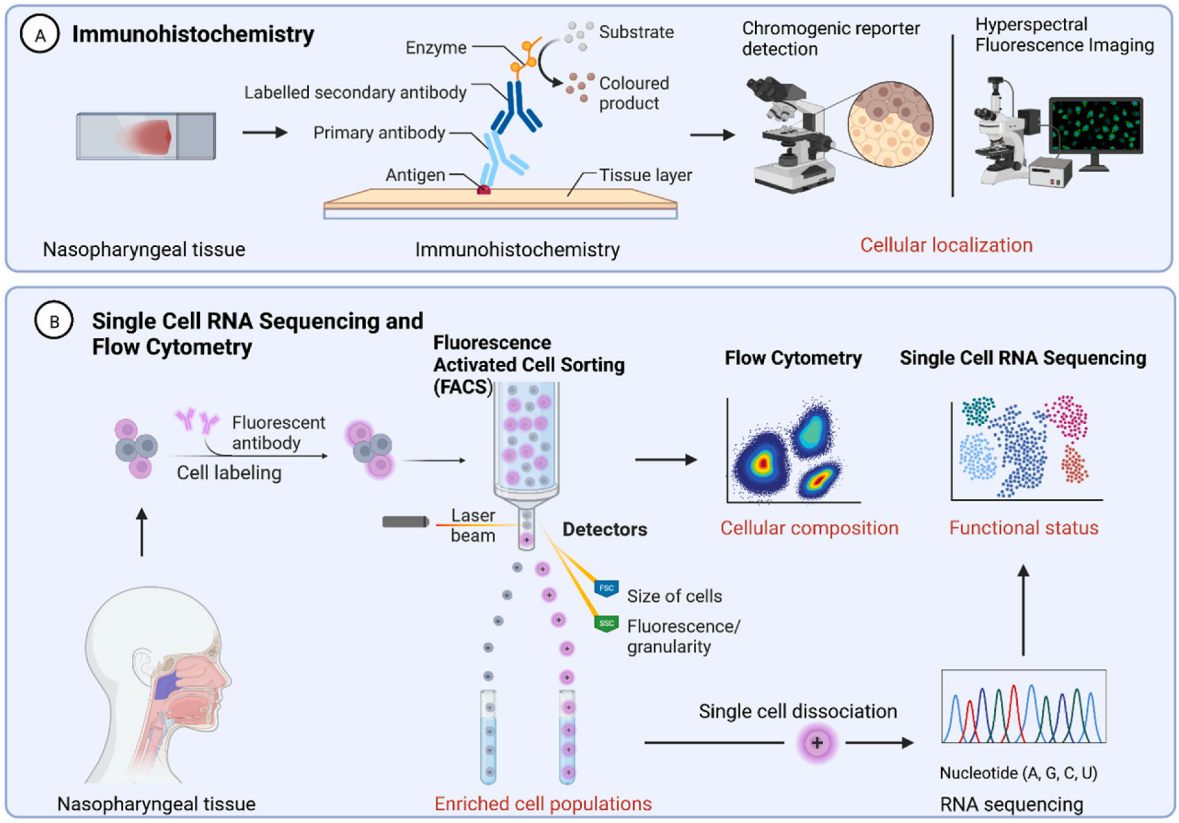


**Fig. 4.** Techniques used to characterize the TIME.
Multiple techniques can be used to characterize different aspects of the TIME. A: Immunohistochemistry is a classical technique to investigate the localization of cells on tissue sections using tagged antibodies against antigens, or markers, for specific cell types. Antibodies that have bound to their antigen can then be detected by colour change or fluorescence microscopy. B: Flow cytometry requires cells to be labeled with fluorescent-tagged antibodies against specific markers, at which point the suspension can be passed through a flow cytometer or specialized Fluorescence-Activated Cell Sorter (FACS) which separates cells by the presence of fluorescence when a laser is shone upon the single cell passing through. This facilitates analysis and separation of different cellular populations present in a sample. Single cell RNA sequencing occurs downstream of FACs, using a single cell dissociation which is then subjected to RNA sequencing to give insight as to the functional status of cells by their transcriptome.

they can be used to stratify subtypes of tumors, elucidate immune-driven mechanisms of pathogenesis, and potentially guide immunotherapy (Binnewies et al., 2018).

Cellular localization is conventionally examined using immunohistochemistry (IHC) on tissue sections, which is commonly employed in clinical pathology settings for differential diagnosis when histopathology is inconclusive. In NPC, IHC for cytokeratin and *in situ* hybridization against EBV-encoded RNA (EBER) can support a diagnosis of squamous cell carcinoma of nasopharyngeal origin, respectively (Chan, 2017). Multiplexed IHC, which has enabled sequential staining with marker panels on the same tissue section to provide spatial context (Koh et al., 2020), has been combined with hyperspectral imaging, which analyzes a wide spectrum of light, to facilitate single cell characterization, allowing cell neighbor analyses to determine tumour-immune cell interactions and single-cell network analyses for delineating clonal expansion (Enfield et al., 2019). These cell sociology features have proven indicative of disease behavior in other solid tumors (Enfield et al., 2019), and may provide opportunities for developing new markers for NPC classification.

Identifying and quantifying the frequency of different immune cell populations present within the TIME is traditionally investigated using flow cytometry, which analyzes single cells in suspension based on the presence or absence of a panel of fluorescent tags against specific features (McKinnon, 2018). Recently, mass cytometry has been developed to overcome the limitations on the number of parameters that can be measured with flow cytometry, which arises due to overlap between the colour spectrums of fluorescent tags, by tagging antibodies with unique metal isotopes that can be separated based on atomic weight. This allows the analysis of up to 50 parameters per cell to effectively profile immune populations in high throughput from a single sample (Gonder et al., 2020).

Functional status of individual cells can be examined using single-cell RNA sequencing (scRNA-seq) (Zhao et al., 2020). Beyond cellular composition of the TIME, single cell RNA sequencing (scRNAseq) provides insight into the functional status of single cells based on their RNA transcriptome. Coupled with dimensionality reduction techniques, which are required to analyze the thousands of RNA reads generated, scRNAseq is a powerful tool that can identify functional subpopulations of cells within the TIME (Chen et al., 2020; Gong et al., 2021). In combination, information gained from these techniques allows classification of the TIME at a high resolution, providing a foundation for further investigation into clinical applications such as potential biomarkers or treatment stratification based on immunological subtypes.

## 6. Conclusions

Nasopharyngeal carcinoma is a solid tumour characterized by high levels of immune infiltration and the formation of a tolerogenic immune microenvironment that facilitates tumour progression. Significant attention has been placed on studying the TIME in NPC in recent years, particularly at the single-cell resolution using newer technologies like scRNA-seq. As a result, unique characteristics of the TIME in NPC are currently being translated into the clinical sphere to improve existing immunotherapies and to guide treatment. For instance, the prevalence of the inhibitory molecules PD-1 and HAVCR2 in the NPC TIME has motivated a Phase II clinical trial that is currently underway targeting both molecules to overcome resistance to PD-1 blockade alone (Desai et al., 2020). Another example is the immune signature generated by Liu et al. using multiplex IHC and consisting of the immune markers PD-L1+, CD163+, CXCR5, and CD117 that was shown to predict the risk of distant metastasis in NPC patients, potentially providing a tool to identify cohorts for differential treatment strategies (Liu et al., 2020). A recent publication also showed that spatial heterogeneity of TILs in NPC, determined using digital pathology and deep learning algorithms, had prognostic value, in that so-called “immune hotspots” significantly correlated with improved patient outcomes (Wang et al., 2021). Although our current understanding of the distinct immune features of NPC is stimulating potential clinical applications, there is a need to for further research using novel high throughput techniques such as mass cytometry and hyperspectral imaging-coupled multiplex IHC to uncover novel immune cell populations, cell sociology, and signalling pathways in the NPC, with a particular focus on distinguishing characteristics that differ between the EBV+ and EBV- microenvironment. Elucidating these characteristics of the TIME in NPC will provide the foundation for biomarker discovery, the development of targeted immunotherapies, and personalized treatment strategies to improve patient outcomes.

## CRediT authorship contribution statement

**Aisling Forder:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Greg L. Stewart:** Writing – review & editing, Visualization. **Nikita Telkar:** Writing – review & editing, Visualization. **Wan L. Lam:** Conceptualization, Supervision, Writing – review & editing. **Cathie Garnis:** Supervision, Writing – review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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