PEGASuS
PEdiatric Glioma Architecture Single cell and Spatial mapping: from fundamental insights to novel immunotherapeutic approaches

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Introduction

Pediatric high-grade and non-resectable low grade gliomas (pHGG and nrpLGG) represent the main cause of cancer related morbidity and mortality in children. These heterogeneous disorders arise from non-neuronal glia (in particular astrocytes) in the brain or spine and remain difficult to treat.

For **pHGG**, clinical outcome is poor with 2-year survival ranging from 10-30% for hemispheric tumors (formerly described as pediatric glioblastoma) and dropping to less than 1% for Histone 3 mutated midline gliomas arising in the brain stem or thalamus. No effective therapy exists and radiotherapy remains the primary treatment. The difficulty to treat pHGG has been attributed to extensive heterogeneity of tumor cells as well as the interplay with the tumor microenvironment (TME). First, tumor cells demonstrate transcriptomic and (epi)genetic plasticity and can exist in different interchangeable cellular states[1-2] with specific phenotypic and functional properties, often leading the resistance to therapy. Second, pHGG arise in developing tissue where numerous interactions occur between tumor cells, stroma and resident and infiltrating immune cells, affecting tumor properties. Third, radiotherapy induces changes in both glioma (in particular a mesenchymal cellular state) and TME associated with radioresistance[3].

The main therapy for **progressive or symptomatic pLGG** is surgical resection. Completely resected tumors often don't need further treatment, and even partial resection can lead to tumor quiescence. If resection is not possible or if the tumor progresses after surgery, additional therapy is however needed. For instance, suprasellar pLGG tumors remain often unresectable and therefore require more extensive treatment. Radiation therapy has been used historically for pLGG treatment. However, it is associated with serious side effects such as cognitive decline, endocrine deficiencies, and secondary malignancies. Therefore, it is usually reserved for older patients or when other treatments have been exhausted. Chemotherapy was introduced in the 1980s as an alternative to radiation therapy for young children with pLGG, especially those with neurofibromatosis type-1. Chemotherapy regimens achieve 3-year progression-free survival rates ranging from 50% to 80%. In recent unpublished work from our team, we identified that in subsets of pLGG and pHGG tumors, the environment of p53 positive tumor cells, exhibits high expression levels of PD1 and PDL1

in the tumor, macrophages and cytotoxic T cells, suggesting that a classical checkpoint inhibitor might represent an alternative treatment approach for non-resectrable tumors.

For the rational design of better and suitable therapeutic strategies that eradicate the tumor and critically also prevent relapse, a detailed understanding is required of 1) the mechanisms underlying shifting between tumoral cellular states that drive resistance, mostly in pHGG tumors, and 2) the reciprocal interactions between the different pHGG/pLGG tumor cell populations and their immune related immune environment, both at diagnosis and in response to standard of care or novel therapeutic options. For the latter, such insights will be key to prepare a clinical phase to evaluate the use of immunotherapy, certainly in unresectable pLGG.

Objectives

Based on a detailed characterization of pHGG and pLGG tumor cells and TME at diagnosis, we aim to identify suitable tumor- and/or immune-cell directed therapeutics that can drive novel approaches for pHGG and unresectable pLGG . The following objectives will be completed:

1. **Single cell mapping of pHGG and unresectable pLGG heterogeneity**
   Single nucleus RNA sequencing will be performed to study pHGG and non-resectable pLGG subpopulations and tumor-TME crosstalk. This approach will be applied on available samples collected through the PEDBRAIN multicentric study led by UZLeuven/KULeuven and in collaboration with 7 other Belgian clinical centers.

2. **Spatial mapping of pHGG and pLGG architecture**
   Spatial multiomics (combining state-of-the-art spatial transcriptomics and proteomics) will be performed to understand the spatial context in which the tumoral cellular states and predicted interactions occur, with a strong focus on p53 positive tumor cells. This approach will be applied on available samples collected through the PEDBRAIN multicentric study led by UZLeuven/KULeuven in collaboration with 7 other Belgian clinical centers.

3. **Advanced pHGG profiling**
   a. **Single-cell multiomics and CyTOF profiling of radiotherapy-treated *in vitro* pHGG models**
   As indicated above, radiotherapy remains the main treatment option of pHGG. We recently developed several patient-derived cell cultures models of H3 mutant pHGG tumors, which will be exposed to radiotherapy, following which we will profile the underlying drug response features associated with radio-responsiveness and -resistance, in addition to mechanisms of cellular plasticity.
   b. **Single cell CRISPR screening with CyTOF profiling and in vivo validation of radioresistance effectors in pHGG**
   Based on 1-3, effectors of radioresistance will be determined using single-cell crispr screening of the available patient-derived cell cultures and in xenograft models (once available also in the models developed by the Coosemans lab at KULeuven, who also applied for support by OHRF).

4. **pLGG profiling and preparation of a clinical trial setting**

a. **Prospective single-cell profiling of core-needle biopsies from non-resectable pLGG tumors**

Advanced profiling of prospective cases in preparation of a clinical trial to deploy immunotherapeutic approaches in non-resectable pLGG tumors

b. **Preparation of the clinical evaluation of immunotherapeutic options in non-resectable pLGG tumors**

Preparing a scientific, ethical and legal file, in addition to initiating discussions with the required industry and academic partners regarding a multicentric clinical trial to evaluate a novel immunotherapeutic strategy in p53 mutant, non-resectable pLGG cases.

Research project

Patient sample collection

Because obtaining tumor samples from pediatric cancer patients is challenging and the biopsy size is frequently limited, multicentric collaborations are necessary to obtain sufficiently large sample series. In 2020, a multicentric collaboration with several institutions in Belgium (Hopital Erasme, Brussel; Universitair Kinderziekenhuis Koningin Fabiola, Brussel; Cliniques universitaires Saint-Luc, Brussel; Universitair Ziekenhuis Brussel; Universitair Ziekenhuis Antwerpen; Citadelle Chateau Rouge Sainte Rosalie, Luik) was established by the UZLeuven/KUleuven team for the systematic retrospective as well as prospective collection of brain tumor patient samples. Ethical committee approval for the use of these samples for research purposes is available. So far, 100 samples have been collected covering the whole spectrum of pediatric brain tumors. Importantly, of several cases, apart from fresh frozen and FFPE biopsies, also matching fresh frozen material is available, allowing additional functional *in vitro* and *in vivo* studies.

Part I: single cell mapping of pHGG and non-resectable pLGG heterogeneity

In Part I of the project, single nucleus RNA sequencing will be performed to study the composition of pHGG and non-resectable pLGG patient biopsies and infer interactions occurring between the identified cell populations.

1.1 Tumor cell annotation

To gain insight in the composition of each tumor sample, single nucleus RNA sequencing (snRNA seq) will be performed on 10 diffuse midline glioma, 10 hemispheric glioblastoma, and 10 non-resectable pLGG samples for which frozen material is available will be performed. snRNA seq will be performed in collaboration with the KU Leuven Genomics Core (https://www.genomicscore.be) where the 10x genomics chromium Single Cell 3' platform is available, while protocols for snRNAseq on brain tissue have been established. Depending on the cell yield from each of the tissues, up to 8 sample will be pooled for sequencing to reduce batch effects.

To obtain larger sample series, we will pool our data with the few datasets of pediatric glioma that are currently publically available (ALSF: https://scpca.alexslemonade.org).

To demonstrate that the heterogeneity of pHGG can be captured by snRNA seq, a preliminary analysis was performed of an unpublished, public dataset of pediatric glioma (ALSF). Our data show for an integrated dataset of 16 pediatric glioblastoma (immune cells were removed) the heterogeneous expression of *PDGFRA*, frequently amplified in pHGG (***Figure 1***).

To reduce bias induced by manual annotation of the identified tumoral subpopulations, a common frame of reference for annotation of pHGG/pLGG tumor cell populations will be created. For this, we will use previously published datasets as well as annotated single cell data of normal fetal and adult brain. This will allow us to compare to composition of different tumors as well as assess the differentiation states of the tumor cells (more immature to more differentiated). Mapping of pediatric glioblastoma on annotated fetal germinal matrix data illustrates the extensive inter- as well as intraheterogeneity of the tumors (***Figure 2***).

1.2 pHGG microenvironment annotation

The pHGG and pLGG microenvironment consists of a complex network of cells and structures including blood vessels, neurons, astrocytes, microglia, oligodendrocytes and extracellular matrix that can interact with tumor cells either through direct physical connections or by more indirect mechanisms, such as cytokine secretion. In addition, several types of immune cells infiltrate the brain microenvironment, influencing tumor cell behaviour. However, the nature of these cells in pHGG and pLGG is vastly understudied compared with their adult counterparts. In an ongoing study (paper in final preparation), we investigated the cellular architecture of 8 pediatric brain tumor entities (ranging from pLGG to pHGG) through which we mapped the general cellular composition. This highlighted major differences between pLGG (which generally consist of a less immunosuppressive environment) and pHGG (which is highly immunospopressive), highlighting the need for more tailored approaches.
To assess the microenviroment in pHGG and pLGG, stromal and immune cells captured by snRNA seq will be studied. Tumoral and non-tumoral cells will be discriminated using a combination of gene expression signatures, fusions and copy number variation detection in the snRNA seq data.

For immune cell identification, several reference datasets and tools are already available. In a preliminary analysis, low resolution celltype identification was done using SingleR on CD45 positive cells subsetted from the ALSF dataset of 16 pediatric glioblastoma integrated with data of 8 pediatric low grade ganglioglioma. The largest fraction of cells was identified as myeloid cells (macrophages, monocytes and dendritic cells), similarly to what has been reported in adult brain tumors (***Figure 3A***). Also T cell and NK cells populations were present (***Figure 3B***), in particular at diagnosis (***Figure 3C***).

Notably, in glioblastoma, a different subset of myeloid cells was enriched compared to ganglioglioma. Further analysis of the subsetted myeloid cells showed (among others) high expression of LGALS1 (involved in immunosuppression) and S100A4 (upregulated in response to hypoxia and a regulator of immune suppressive T and myeloid cells in adult glioblastoma potentially contributing to the particularly aggressive behaviour of pediatric glioblastoma. (***Figure 4***).

Of note, at KU Leuven a therapeutic strategy was developed to target LGALS1 in glioblastoma using transnasal particles resulting in a shift from immune suppression to immune activation (by colleague Steven De Vleeschouwer). These findings support the high translational potential of our proposal.
In summary, our preliminary analyses point to the presence of potential anti-tumoral T-cells and NK cells and heterogeneity in the myeloid compartment in the TME of different pediatric brain tumor types. These findings support the utility of a systematic characterization of the TME of pediatric brain tumors.

1.3 Prediction of intercellular interactions

Several computational tools are available to infer cell–cell communication and will be used to predict interactions between different tumor subpopulations on the one hand and between tumor cells and non-tumoral stromal and immune cell subsets identified in 1.1 and 1.2 on the other hand. Recurring interactions will be prioritized for validation in spatial studies (Part II). A particular question we want to address is whether the immunosuppressive phenotype of immune cells in the TME of pHGG and pLGG (as demonstrated in our preliminary analysis in Figure 3) is actively induced by signals originating from the tumor cells.

Part II: spatial mapping of pHGG and pLGG tissue architecture

SnRNA seq offers detailed insight in the composition of a tumor sample and allows to infer cell-cell communication and interactions, however because the tissue is dissociated during processing, snRNA seq does not capture the spatial distribution and local environment within tissue. Given that most cell signaling events occur within a limited distance in tissues, incorporating spatial information into cell-cell communication analysis is critical for understanding pHGG and pLGG tissue organization and function. We are currently finalizing a first level analysis using multiplexed immunohistochemistry, and while highly relevant data have been uncovered (including a PD1/PDL1/TP53 ecosystem), a more extended and in depth analysis to confirm/validate these findings are required. Indeed, the glioma cellular states are influenced by the exact location of the tumor cells in the tissue. As such, spatial analyses provide critical insight into how the cellular architecture affects tumor phenotype and behaviour.
To understand the spatial context in which the tumor and TME cell populations reside and the predicted interactions occur, spatial transcriptomis (Vizgen MERSCOPE, or the 10X Xenium platform) and high-dimensional multiplex immunohistochemistry (MILAN) will be performed on biopsies of the patient series studied in Part I. For all pHGG and pLGG analysed in Part I, tissue microarrays will be generated including 16 cores per slide (available at the KU Leuven Instituut voor Single Cell Omics (LISCO, https://lisco.kuleuven.be).

Vizgen MERSCOPE and the 10X Xenium platforms for spatial transcriptomics and MILAN for spatial proteomics (offered as a service platform and optimized by the host lab) are both available at the KU Leuven Instituut voor Single Cell Omics (LISCO, https://lisco.kuleuven.be). Importantly, all methodologies can be applied to FFPE tissues, which is available for all patients included in the study.

2.1 spatial transcriptomics

Vizgen MERSCOPE or 10x Xenium enables the simultaneous detection of several hundreds of RNAs at the highest sensitivity of any spatial transcriptomics platform. We will design a panel of 300 RNA targets that most effectively capture sample heterogeneity in the snRNA seq data. The most important genes for these panels will be selected from the snRNAseq and available public data. Regarding data analysis, the Seurat package will be used (which is conveniently also used for snRNA seq analysis) as well as Giotto (www.spatialgiotto.com) to reduce potential bias introduced when using one single analysis pipeline.

2.2 spatial proteomics

The MILAN (Multiple Iterative Labeling by Antibody Neodeposition) pipeline for high-dimensional multiplex immunohistochemistry is fully operational in the host lab (***Figure 5A***) and has recently been applied to describe the infiltrating macrophages and resident microglial cells in glioblastoma brain tumors (***Figure 5B***). Briefly, multiplex immunohistochemistry is performed according to a previously published method. Following dewaxing and antigen retrieval immunofluorescence staining is performed using Bond RX Fully Automated Research Stainer (Leica Biosystems). A coverslip is placed onto the slides and the slides are scanned using a Zeiss Axio Scan Z.1 (Zeiss) at ×10 magnification. After completion of the staining procedure, the coverslips are removed and the antibodies are stripped. The staining procedure is repeated until all markers are stained and scanned on each of the slides.

The data analysis pipeline is optimized at the host lab and will be done in collaboration with an expert bioinformatician (Dr. Asier Antoranz). Final cellular annotation is done by an expert pathologist (prof. Raf Sciot).

Part III: Advanced profiling of pHGG tumors

a. Multiomics and CyTOF profiling of radiotherapy treated *in vitro* and *in vivo* pHGG models

Radiotherapy is the cornerstone of pHGG treatment, however provides a very limited survival benefit of only a few months. The lack of consecutive tissue samples, in particular for diffuse midline glioma where surgical intervention is rare because of the delicate location of the tumor hinders the exploration of the mechanisms that underlie radiotherapy resistance. To address this issue, the impact of radiotherapy on pHGG will be studied in *in vitro* and *in vivo* patient derived models.

3.1 Radiotherapy of patient derived cell cultures with snRNA seq and CyTOF readout

Glioma cell lines are not optimal models of pHGG because of longterm culturing. For this reason, the lab is establishing pHGG patient derived cell cultures (PDCL) derived from viable patient biopsies. For 2 pediatric diffuse midline glioma and 7 glioblastoma cases PDCL are currently readily available. We will perturb the PDCLs using a dose range of photon and carbon ion irradiation levels (0, 2, 4, 6, 8, 10 Gy), following which the models will be ranked according to their sensitivity to the applied therapeutic insults using classic cytotoxicity assays (*e.g.* using CellTiterGlo profiling). PDCL radiation will be performed at the MoSAIC facility (https://gbiomed.kuleuven.be/english/corefacilities/mosaic).

PDCL irradiated at IC50 will be collected for snRNA seq to determine the impact of radiotherapy on PDCL heterogeneity.
These results will be validated at single cell protein level using a previously optimized CyTOF antibody panel to measure glioma tumour cell plasticity at single-cell resolution (42-plex panel; paper under review at Nature Communications). CyTOF (Mass cytometry by time-of-flight) is a next-generation flow cytometry technology, enabling single cell analysis of millions of cells using metal-isotope labeled antibodies. A CyTOF instrument (Helios, Fluidigm) was introduced at KU Leuven and optimized by the host lab and is accessible through the KULeuven Flow and Mass Cytometry Core (https://gbiomed.kuleuven.be/english/corefacilities/facs).
In adult glioma a mesenchymal phenotype is associated with radioresistance[3], we hypothesize that also for pHGG tumor cells, changes of the cellular state are linked with response to radiation.
However, depending on the snRNA seq results, additional CyTOF antibody panels can be composed.

3.2 Radiotherapy of pHGG xenograft models with multiomics readout

For two pediatric diffuse midline glioma and two pediatric glioblastoma cases, GFP/luciferase transduced PDCL cells will be grafted orthotopically in the pons of six immune deficient mice for each case (this model will be further refined by the Coosemans lab).
The protocol as described by Monje *et al*. will be followed where injection of diffuse midline glioma tumor cells resulted in tumor infiltration throughout the brain, including the cortex, cerebellum, and pons. Briefly, 40.000 live tumor cells (2-µL volume of 20.000 cells/µL) will be transplanted into the lateral ventricles of anesthetized nonobese diabetic/SCID/γ-chain null–immunodeficient mouse pups by stereotactic injection through a 31-gauge burr hole (coordinates: 2 mm anterior to the bregma, 1 mm lateral to the sagittal suture, and 2 mm deep). Mice will be returned to the mother until weaned and monitored for disease symptoms (*e.g.* poor grooming and hemiparesis). Xenografting will be performed in collaboration with the Trace patient derived xenograft (PDX) platform at KU Leuven (https://gbiomed.kuleuven.be/english/research/50488876/54502087/Trace) specialized in the establishment and characterization of murine PDX models.

Tumor engraftment will be followed non-invasively biweekly via *in vivo* bioluminescence imaging (available at the Molecular Small Animal Imaging Center (MoSAIC) facility at KU Leuven). Based on previous experience, we expect tumor growth 3 to 4 weeks after injection. Upon evidence of engraftment, for each tumor case the six mice will be randomized into two groups. The treatment group will receive radiotherapy (2Gy for 5 days) whereas the control group will undergo the same procedure without radiation.
Mice will be sacrificed when showing disease symtomps (*e.g.* poor grooming and hemiparesis) and mouse brains will be partly snap frozen and partly FFPE embedded.

For every experiment, nuclei isolated from tumor samples of all six mice will be labelled and pooled for multiplex snRNA seq. Data analysis will focus on the identification and phenotyping of the tumor cells and resident brain stromal cells and interactions among these cells. For the four models, from each treatment group one representative tumor will be selected for spatial transcriptomics and MILAN multiplex IHC to validate the snRNA seq findings as described in Part II.

The data obtained will be integrated with Part I and II to assess whether cellular phenotypes and interactions associated with radiotherapy resistance are present in diagnostic patient samples.

b. Single cell CRISPR screening with CyTOF profiling and *in vivo* validation of radioresistance effectors

4.1 Crispr screening with CyTOF readout

Based on the data collected in Part I-III max. 300 genes will be selected for which a lentiviral library of CRISPR/sgRNAs will be designed. Genes associated with response to radiotherapy will be prioritized. Single-cell CRISPR screening will subsequently be performed using CyTOF profiling.

For CyTOF unique Pro-Code-barcoded sgRNAs will be combined with a previously optimized antibody panel to measure glioma cell plasticity at single-cell resolution (42-plex panel; paper under review at Nature Communications). By comparing control and conditions in several millions of single cells across the selected PDCLs (aim n = 6), this approach will allow us to identify genes/pathways that interfer with tumor cell survival and/or specific plastic behaviour. Because in adult glioma a mesenchymal phenotype is associated with radioresistance we hypothesize that also for pHGG tumor cells, changes in the cellular state are linked with response to radiation. However, depending on the data obtained in Part I-III additional CyTOF antibody panels can be composed.

4.2 *in vivo* validation

For the most promising targets, we will generate stable knockout clones via lentiviral CRISPR technology and puromycin selection (fully operational at the host lab), and transplant tumour cells intracranially to generate xenograft mouse models as described above. Mice xenografted with parental (control) lines and CRISPR edited lines will be subjected to radiation therapy, following which cells will be extracted and subjected to snRNA seq to monitor tumor cell plasticity. For the most interesting targets, spatial profiling will also be performed (Part II). This approach will allow us to validate the identified pathways in an *in vivo* context under therapeutic pressure.

Part IV: pLGG profiling and preparation of a clinical trial setting

a. Prospective single-cell profiling of core-needle biopsies from non-resectable pLGG tumors

Advanced profiling of prospective cases in preparation of a clinical trial to deploy immunotherapeutic approaches in non-resectable pLGG tumors. A similar approach as described in part 1 will be used to prospectively single-cell profile core-needle biopsies from non-resectable pLGG cases (either using dissociated or tissue based methods).

b. Preparation of the clinical evaluation of immunotherapeutic options in non-resectable pLGG tumors

In this last chapte, we will be preparing a scientific, ethical and legal file, in addition to initiating discussions with the required industry and academic partners regarding a multicentric clinical trial to evaluate a novel immunotherapeutic strategy in p53 mutant, non-resectable pLGG cases.

Requested funding

a. Personnel:

A dedicated Postdoctoral fellow (Dr. Julie Morscio) recently acquired a "Stichting tegen kanker" postdoctoral fellowship, and will be working full time on this project starting in October 2023. We are in addition searching for an additional PhD candidate with a clinical background (preferably a pediatric oncologist in training) who should join the team by fall. At this moment, we are not seeking for financial support regarding personnel from the Olivia Fund.

b. Consumables/working budget

Considering the high costs of consumables for the requested project, we are seeking for additional support from the Olivia Fund to support the practical work of this project. Below you can find a breakdown with the estimated costs:

topic	Estimated costs
Retro-and prospective sample collection across 7 Belgian centers	€7 500
snRNA seq	€18 000
Spatial transcriptomics	€28 000
Spatial proteomics	€12 500
Cell culture and scCRISPR screening	€14 500
scRNAseq screening	€18 000
Total	€98 500

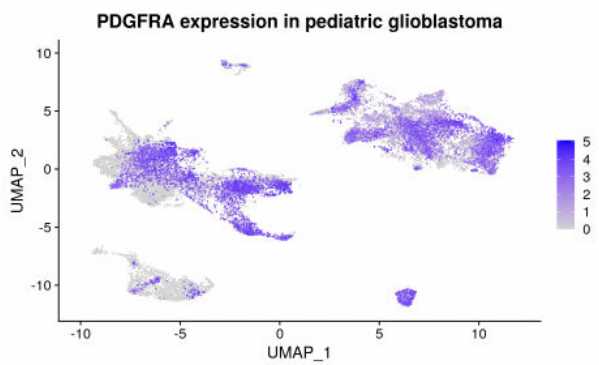

Figure 1. Heterogeneous PDGFRA expression in a dataset of 16 integrated pediatric glioblastoma cases (ASLF).

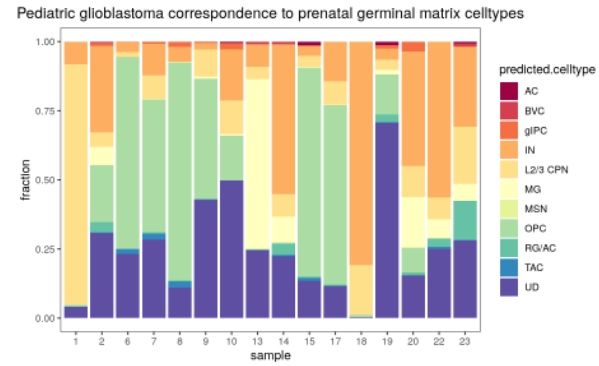

Figure 2. Sixteen glioblastoma cases (ALSF dataset) were annotated using fetal germinal matrix cell type signatures (Ramos S, *et al*. NatCommun (2022)). The plot illustrates the extensive inter- as well as intraheterogeneity of the tumors.
(AC, astrocytes; BVC, blood vessels; gIPC, glial intermediate progenitor cell ; IN, interneuron; L2/3 CPN, cortical projection neurons; MG, microglio; MSN, medium spiny neuron; OPC, oligodendrocyte precursor; RG/AC, radial glia/astrocyte; TAC, transit amplifying cell; UD, undetermined)

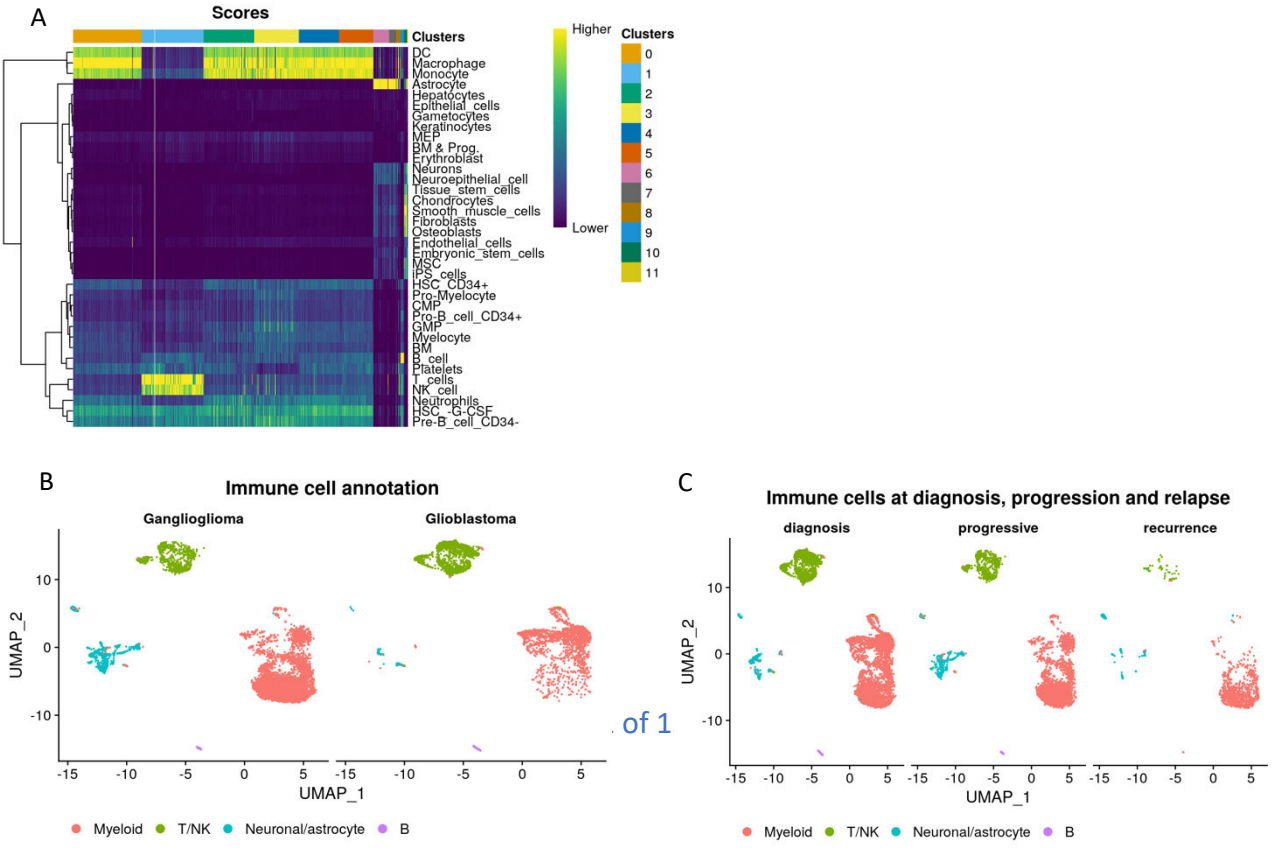

Figure 3. Immune cells were subsetted from pediatric ganglioglioma and glioblastoma and 11 clusters were identified (not shown). A) SingleR was used for immune cell annotation with high confidence annotation shown in yellow. Most clusters were identified as myeloid cells (macrophages, dendritic cells (DC) and monocytes. B) Comparison of immune cell types present in pediatric ganglioglioma and glioblastoma. Note the different myeloid cluster in both tumor subtypes. C) Comparison of immune cells present at diagnosis, progression and recurrence. Note the difference in T/NK cells at diagnosis and recurrence.

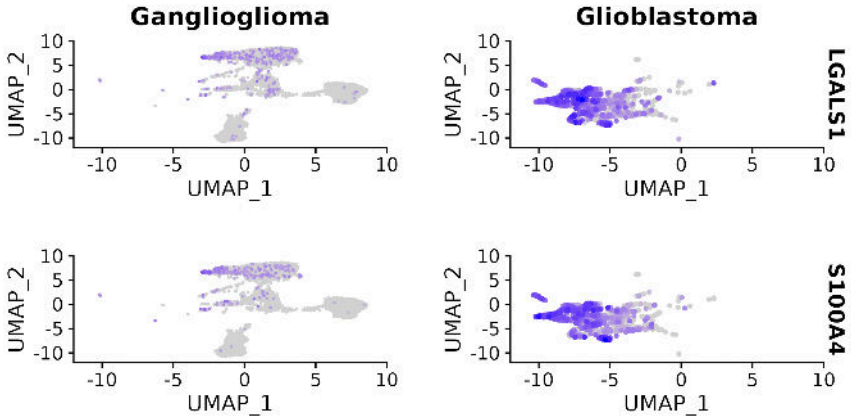

Figure 4. Myeloid cells identified in Figure 3 were subsetted for further analysis. The feature plots show higher expression of LGALS1 (top row) and S100A4 (bottom row) in myeloid cells of pediatric glioblastoma compared to ganglioglioma.

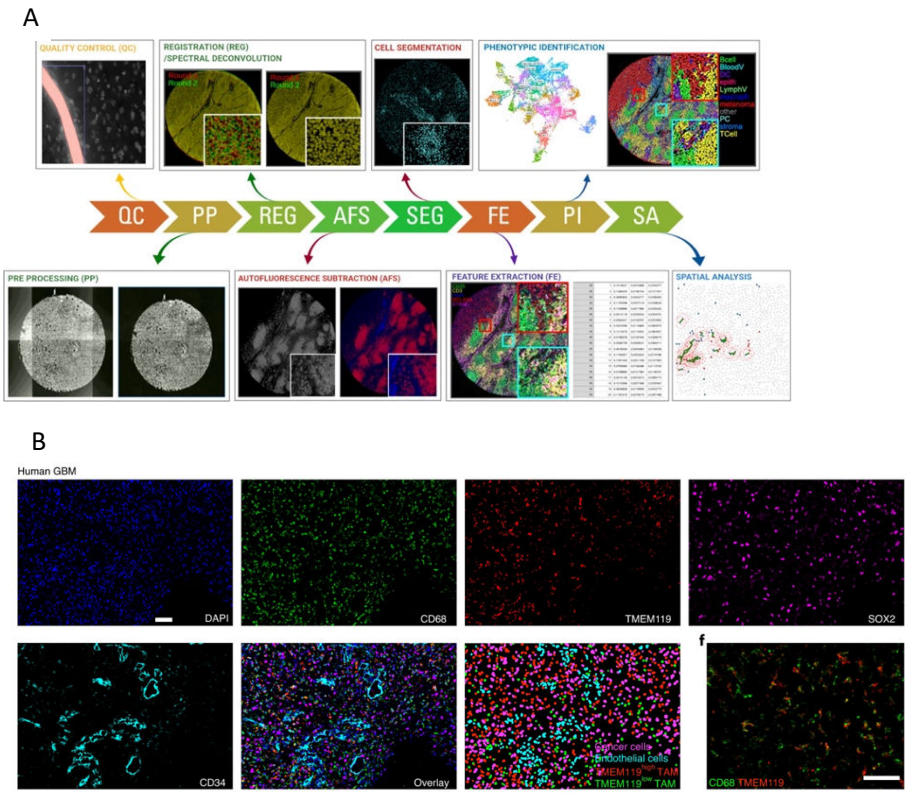

Figure 5. MILAN multiplex immunohistochemistry. A) Figure adapted from Bosisio F, *et al*. Front Oncol (2022) showing the MILAN workflow. B) Figure adapted from Antunes *et al*. Nat Neurosci (2021): MILAN

was applied on a section of adult glioblastoma to map cancer cells ($SOX2^{+}$), blood vessels and endothelial cells ($CD34^{+}$) and subsets of macrophages ($CD68^{+}$/$TMEM119^{high/low}$)