Differentiating tumor-associated-macrophages from microglia in human brain tumors - an effective new marker

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Introduction

Glioblastoma (GBM) is the most common and lethal primary brain tumor. The major non-neoplastic cell population within the GBM microenvironment includes cells of the innate immune system called glioma-associated microglia and macrophages. Tumor-associated macrophages (TAMs) and microglia are known to promote tumor cell invasion and angiogenesis. It is however a challenge to differentiate between these two cell subsets as no adequate marker has been found in human to differentiate between them. Such differentiation may aid our understand of the different roles of these two cell types within the tumor microenvironment.
Previous research in our lab has identified such potential differentiating marker expressed on TAMs but not on microglia. Interestingly, this protein is also known to play multiple roles in tumor progression in non-brain tumors. In this project, we aimed to test the efficacy of this marker in separating myeloid/innate cells within the tumor microenvironment using multi-parametric flow cytometry. We also in-vitro differentiated monocytes to M1 and M2 macrophages (Mf) to assess the efficacy of various inhibitors of this molecule (an antibody and a clinically tested small molecule inhibitor (SMI)) for their capacity to reduce the invasive enzymatic activity of the queried protein. Future development of this project may enable a to generate the scientific infrastructure for a clinical trial testing the inhibition of this molecule in brain-tumor patients.

Methods

Flow cytometry
PBMC or dissociated human tumor cells (from GBM and brain metastases) were stained using our innate panel and analyzed using 3 laser FACS CANTO-II. Common microglia and TAM markers were compared to the novel markers to assess marker efficacy

Macrophage polarization
Monocytes were enriched from PBMC through plastic adhesion and then cultured with GM-CSF or M-CSF for M1 or M2 polarization, respectively. On day 5, LPS and IFNɣ were added to the M1 Mf and IL-4 was added to the M2 macrophages.

Enzymatic activity assay
Mfs were incubated with treatments (SMI or antibody) for 1 hour, then a substrate was added to the cells. The enzymatic activity was assessed by spectrophotometer.

Invasion assay
Macrophages were FBS-starved for 24H prior to experiment. Cells were then placed on top of matrigel-coated 8μm transwells which were then placed in chambers containing medium + FBS (or without FBS for control). An SMI or antibody was then added to the transwells. After 24H, cells that have invade to the lower chamber were counted using trypan blue.

Results

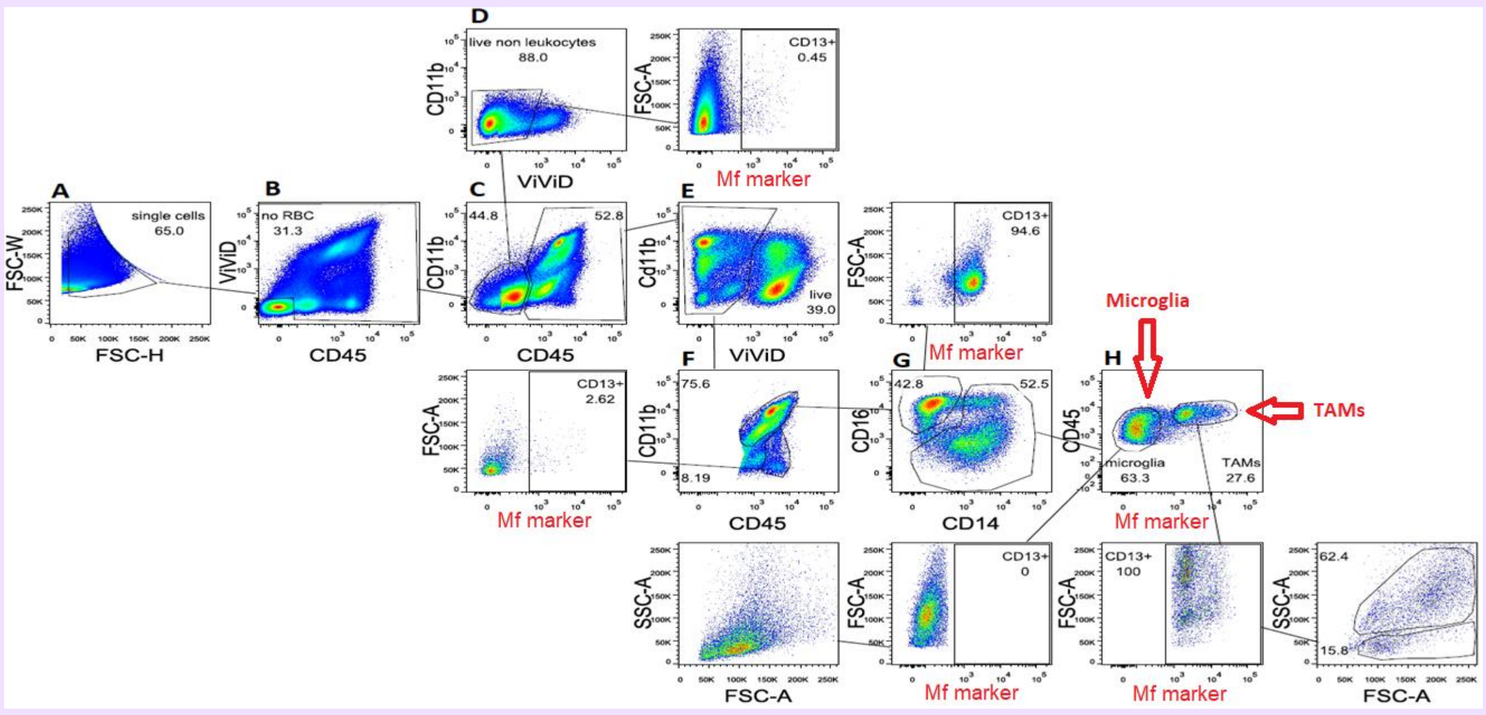

Figure 1: Gating strategy of a representative human brain tumor sample. Single cells dissociated from a GBM sample were stained using the innate panel and analyzed by flow cytometry. Cells were serially gated in the following way: A- Non-doublet/clumped single cells . B- Non red blood cells (RBC) . C- Leukocytes and non-leukocytes (CD45-) which are each gated for. D-E live cells on ViViD. F- Lymphocytic and non-lymphocytic (innate) cells . G- Non lymphocytic cells were gated for neutrophils ($CD16^{high}CD14^{low\text{-}medlow}$) and other non-granulocytic myeloid cells ($CD14^{Med\text{-}High}$). H- cells were then gated on CD45/Mf marker to separate microglia ($Mf\ marker^{Low}\ CD45^{Low\text{-}High}$) from TAMs($Mf\ marker^{High}\ CD45^{High}$). Each final population was then gated for their Mf marker expression (Mf marker/FSC-A). TAMs and microglia were also gated on their physical parameters (FSC/SSC) exhibiting expected size and granularity parameters.

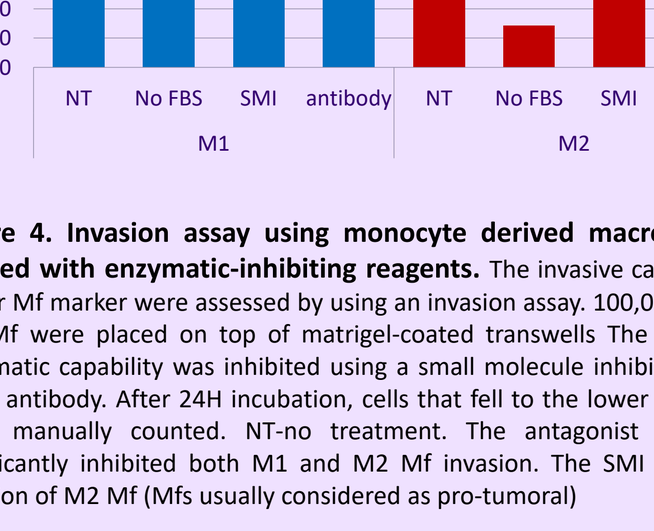

Figure 2. Evaluation of Mf marker expression levels in monocytes and monocytes-derived macrophages. Monocytes from peripheral blood (PB) were cultured to M1 or M2 phenotype These Mf and fresh blood derived monocytes were then stained with the innate panel including the Mf marker (or a fluorescent minus one control and analyzed by flow cytometry. The marker is strongly expressed on both M1 and M2 Mf and more weakly expressed on blood derived monocytes

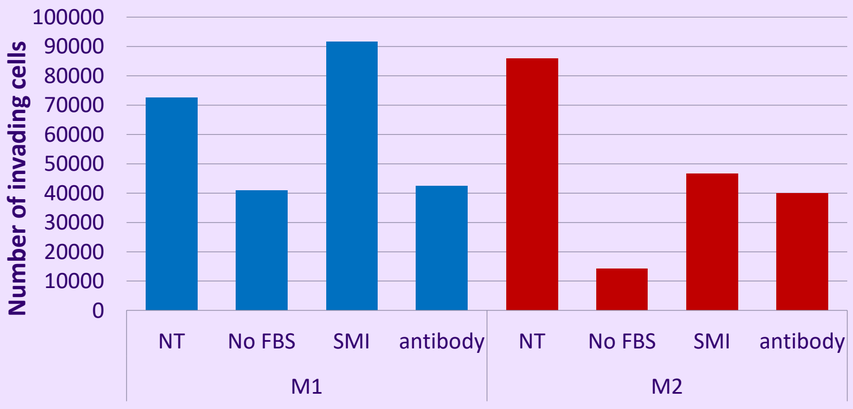

Figure 4. Invasion assay using monocyte derived macrophages treated with enzymatic-inhibiting reagents. The invasive capabilities of our Mf marker were assessed by using an invasion assay. 100,000 M1 or M2 Mf were placed on top of matrigel-coated transwells The marker's enzymatic capability was inhibited using a small molecule inhibitor (SMI) or an antibody. After 24H incubation, cells that fell to the lower chamber were manually counted. NT-no treatment. The antagonist antibody significantly inhibited both M1 and M2 Mf invasion. The SMI inhibited invasion of M2 Mf (Mfs usually considered as pro-tumoral)

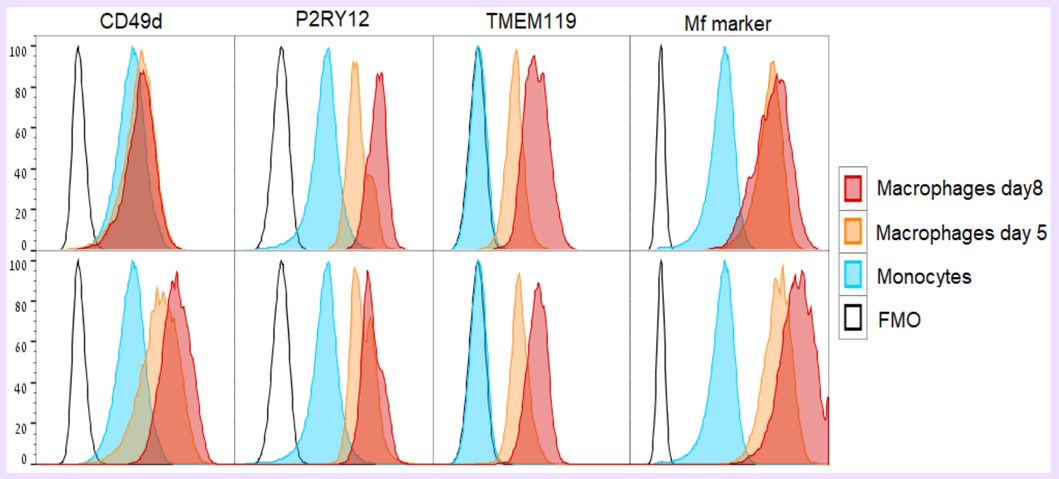

Figure 3. Comparison of our marker with known Mf or microglial markers Monocyte-derived Mf and monocytes were stained with our marker as well as **CD49d** (which is considered a Mf marker) and **P2RY12** and **TMEM119** (which are both considered microglial markers.)
Cells were stained on day 5 (before adding the last differentiating factors) and on day 8 (at the end of their differentiation).
Surprisingly both microglial markers were found to be expressed also on macrophages. CD49d, a Mf marker, differentiated between monocytes and macrophages, yet not as potently as our marker

Figure 5. Enzymatic inhibition of our Mf marker in monocyte-derived macrophages. The enzymatic activity of the Mf marker in M1 and M2 Mf was inhibited by an antagonistic antibody and a small molecule inhibitor. The enzymatic activity was assessed after 30min, 1 hour and 2 hours through spectrophotometry using a colorimetric substrate. Both inhibitors specifically inhibited enzymatic activity

Conclusions

- Our Mf marker is expressed by TAMs and tumor associated neutrophils TANs but not by microglia or lymphocytes
- Both M1 and M2 Mf express our marker, yet M2 Mfs, considered pro-tumoral more strongly express it
- Our Mf marker better differentiates macrophages from microglia than any marker currently used in literature (e.g. CD49d, TMEM119, P2RY12) Microglial markers TMEM119, P2RY12 are also expressed by Mfs
- A small molecule inhibitor and an antagonistic antibody are effective enzymatic inhibitors that could functionally inhibit the markers' enzymatic activity
- Future work will test the SMI in animal models, as proof of concept for possible future clinical trials for GBM