

Rewriting microenvironment immunity in glioblastoma

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The most common primary brain tumor, glioblastoma (GBM), remains uniformly fatal despite surgical resection, radiotherapy, and chemotherapy.¹ A major barrier in treating this disease is the presence of myeloid-derived suppressor cells (MDSCs) in the glioma tumor microenvironment.² These cells are expanded in blood and glioma tissue from patients and are associated with poorer clinical outcomes.³ MDSCs have been well described to suppress T cell activation and proliferation.⁴ To overcome this disease and its associated immunosuppressive barrier, our group pioneered a novel platform called adoptive cellular therapy (ACT).^{5–7} This platform includes host conditioning, hematopoietic stem and progenitor cell transfer, and dendritic cell vaccine that is electroporated with total tumor ribonucleic acid (tRNA) and T cells that have been activated against tRNA.^{5–7} Our group's ACT significantly increased overall survival across multiple models of primary brain cancer including GBM while significantly altering the tumor microenvironment.^{5,8,9} In tumor-bearing mice treated with ACT, host-derived MDSCs and tumor-associated macrophages (TAMs) were significantly decreased in glioma tumor tissue and displaced by transferred hematopoietic stem cells (HSCs).⁵ While the mechanisms underlying the depletion of MDSCs after ACT are unknown, we hypothesized that this observation could be due to decreased MDSC recruitment alongside increased T cell migration and activation in the glioma microenvironment.

To address this hypothesis, we performed immunophenotypic analysis of the changes in the immune microenvironment in GBM after ACT using multimodal approaches. By leveraging flow cytometry and spatial genomics approaches, we characterized

cell frequencies and whole transcriptome changes across MDSCs, TAMs, and tumor infiltrating lymphocytes (TILs).

MDSCs: our publications have demonstrated that ACT is associated with significant decreases in relative frequencies of MDSCs in the glioma microenvironment relative to untreated or monotherapy-treated tumors.^{5,7,9} Further analysis employing spatial transcriptomics validated these findings and further revealed that relative to host conditioning radiation monotherapy, expression of multiple MDSC associated genes was significantly decreased after host conditioning followed by ACT. Notably, several of the downregulated MDSC-associated genes included extracellular markers like *Itgam* and *Csf1*, as well as enzymes like *Arg1* and transcription factors, *Stat3*.⁴

TAMs: in our recent article published in *Neuro-oncology Advances*, we showed that TAMs were significantly depleted in the tumor microenvironment after host conditioning alone.⁸ However, at the transcriptional level, we observed more nuanced alterations to macrophage polarization within the tumor microenvironment. In gliomas treated with ACT, we found the expression of genes associated with M1 macrophage polarization, including *Cxcl10*, *Cxcl9*, *H2-ab1*, and *Ccl5*, was significantly increased relative to host conditioning alone. Interestingly, we observed a significant downregulation of the expression of genes associated with M2 macrophage polarization, including *Tgfb β 2*, *F13a1*, *Mrc1*, and *Il4ra*.¹⁰ Taken as a whole, host conditioning may deplete immunosuppressive myeloid cells from the microenvironment, but the combined ACT platform alleviates immunosuppressive signaling while stimulating antitumor inflammatory signaling at the transcriptional level.

TILs: prior published work on our group's ACT identified extensive alterations to T lymphocytes within the microenvironment compartment of murine GBM. Notably, significant signaling reciprocity was observed between HSC and T lymphocytes in the tumor microenvironment. HSC interactions with T lymphocytes promoted T cell expansion and activation with increased IFN- γ secretion, which in turn polarized HSC differentiation toward antigen-presenting cell phenotypes.⁵ Additionally, clonal expansion and persistent of TCR V β subclones corresponds with efficacious responses after ACT in preclinical brain tumor models and in patient samples.⁶ Corroborating these results, observations in the present study found that ACT with host conditioning significantly increased TILs frequencies in the tumor microenvironment.⁸ Transcriptionally, ACT with host conditioning significantly amplified antitumor immune activation, with increased gene expression of *Cd3g*, *Cd28*, *Cd8a*, *Il2ra*, *Cd3e*, *Cd3d*, and *Pdcd1* among TILs compared to host conditioning alone. Overall, ACT with host conditioning tips the balance of microenvironment immunity away from immunosuppression toward immune activation and inflammatory responses.

Our observations related to cell and transcriptional changes prompted us to evaluate chemokine levels after ACT as a potential mechanism driving myeloid depletion in the tumor microenvironment. Following completion of ACT, we isolated glioma tissue and extracted proteins from the tumor microenvironment to quantify changes to specific chemokines after treatment. Interestingly, we noticed that CCL12 protein was significantly downregulated after ACT with host conditioning. We validated this finding using spatial transcriptomics

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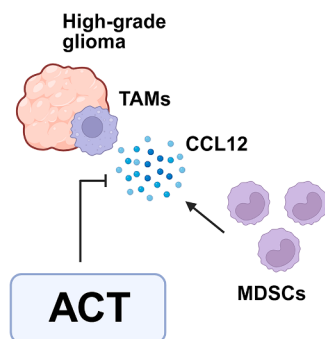


Figure 1. Proposed mechanism

Adoptive cellular therapy significantly depletes intra-tumoral MDSCs and CCL12 protein, a chemokine that is secreted by tumor-associated macrophages (TAMs) and promotes MDSC migration.

analysis and confirmed that ACT with host conditioning significantly decreased the number of CCL12 RNA transcripts. Given that CCL12 binds to CCR2, we suspected that it may have a role in cell migration.⁸ Next, we performed a series of experiments *in vitro* to confirm the impact of CCL12 on MDSC migration. Using a *trans*-well migration assay, we saw that recombinant CCL12 protein significantly increased MDSC migration in a dose-dependent manner. We then confirmed the presence of CCL12 in glioma conditioned media that was generated by exposing culture media to either our glioma cell line model or tumor mass excised from a mouse. Using this model, we observed that CCL12 neutralization significantly abrogated MDSC migration. These studies prompted further characterization of the cell source of CCL12 in the tumor microenvironment using single cell transcriptomics and enzyme-linked immunosorbent assays. This analysis identified that clusters annotated as M1 and M2 macrophages more significantly expressed CCL12 relative to other immune clusters. We then isolated murine gliomas and validated that CCL12 was sourced from TAMs with an enzyme-linked immunosorbent

assay. When we neutralized CCL12 in *trans*-well migration conditions containing TAMs from our glioma model, MDSC migration was significantly decreased relative to other cell type conditions. This suggests that TAM-derived CCL12 is a potent stimulus for MDSC migration and its loss due to ACT from the microenvironment may impact MDSC infiltration.

In sum, this work is significant in its identification of an ACT-regulated chemokine, CCL12, as an impetus of MDSC migration in a GBM context (Figure 1). Given that ACT is being evaluated in first-in-human trials at our institution (PI: Dr. Duane Mitchell, FDA IND #17298, 14058, 23881), this study's findings are highly translational to our understanding of ACT's impact on the host immune system and how it may be driving treatment efficacy. Our conclusions may offer treatment modifying approaches against chemokine signaling to potentially enhance immunotherapy responses, but this will need to be investigated in future studies. Ultimately, these observations connect with our long-term goal which is to accelerate the development of safer adoptive immunotherapies for clinical treatment of brain malignancies.

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DECLARATION OF INTERESTS

C.T.F. is a co-founder of iOncologi, an immunotherapeutic company.

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