

## REPROGRAMING OF TUMOR-ASSOCIATED MYELOID CELLS BY TLR9-TARGETED STAT3 ANTISENSE OLIGONUCLEOTIDES SENSITIZES MALIGNANT GLIOMA TO PD1-SPECIFIC IMMUNOTHERAPY

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**Background** Malignant gliomas (MG) are rapidly fatal despite multimodal treatments including radiation therapy, used to treat nearly all MG patients, or even the emerging cellular immunotherapies. Therapeutic resistance in glioma is at least partly related to tolerogenic STAT3 activity in both glioma cancer stem cells (GSCs) and in the tumor-associated myeloid immune cells, such as macrophages and microglia, which dominate MG microenvironment. We previously demonstrated that STAT3 activity in GSCs and tumor-associated myeloid cells can be targeted using Toll-like Receptor-9 (TLR9)-targeted oligonucleotide therapeutics such as siRNA or antisense oligonucleotides (ASO).<sup>1-3</sup>

**Methods** Here, we describe development of a new TLR9-targeted and double-stranded STAT3 antisense oligonucleotide (CpG-STAT3dsASO) with optimized efficacy and tolerability for glioma immunotherapy.

**Results** Compared to our benchmark ASO oligonucleotides, the locked nucleic acid (LNA)-modified CpG-STAT3dsASO showed enhanced *STAT3* knockdown in human and in mouse glioma cells and also in TLR9<sup>+</sup> immune cells, such as macrophages and microglia. When tested against orthotopic model of human U251 glioma, intracranial injections of CpG-STAT3dsASO (1 mg/kg/q2w) inhibited tumor growth and significantly extended survival of immunodeficient NSG mice compared to benchmark oligonucleotide. Next, we tested the efficacy of CpG-STAT3dsASO against syngeneic GL261 or QPP8 glioma in immunocompetent mice.<sup>4</sup> Our initial results demonstrated that CpG-STAT3dsASO was more effective and significantly better tolerated than single-stranded CpG-STAT3ASO when injected intracranially. All tested CpG-STAT3ASO variants induced activation of intratumoral DCs, macrophages and microglia, while reducing numbers of tumor-associated macrophages (TAMs), resting microglia and regulatory T cells as assessed using flow cytometry. However, the intratumoral recruitment of effector CD8 T cells was limited. To improve CD8 T cell activation, we next combined intracranial CpG-STAT3dsASO administration at low 0.25 mg/kg dosing with systemic PD1 blockade. The anti-PD1/CpG-STAT3dsASO combination triggered complete rejection of both orthotopic GL261 and the PD1-refractory QPP8 tumors in the majority of treated mice, while neither treatment was curative alone. Importantly, Our single-cell transcriptomic analysis and spatial profiling of the brain sections from all treatment groups confirmed the complementary effect of CpG-STAT3dsASO and PD1 blockade on the glioma microenvironment. CpG-STAT3dsASO reprogrammed glioma-associated myeloid cells into antigen-presenting cells and phagocytes, expanded Th1 CD4 lymphocytes while reducing Treg percentages. These conditions unlocked the potential of PD1 blockade to recruit effector CD8 T cells into glioma without indication of lymphocyte exhaustion.

**Conclusions** Our results underscore the potential of using myeloid cell-targeted CpG-STAT3dsASO to overcome glioma immune evasion and thereby to sensitize tumors to PD1

immune checkpoint blockade and potentially other T-cell based cancer immunotherapies.

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