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In the Spotlight

Taking a swing at TIMP-1 armed immunosuppressive astrocytes unleashes T cell immunity against brain metastases

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Summary

Priego *et al*. identify a secreted glycoprotein TIMP-1, expressed downstream of the transcription factor STAT3, in a subpopulation of STAT3+ reactive astrocytes as a mediator of immunosuppression in late-stage brain metastases and demonstrate that the STAT3 inhibitor silibinin enhances the preclinical efficacy of the combined PD-1 / CTLA-4 immune checkpoint blockade, providing a rationale to translate the combination therapy into clinical use for this underserved patient group with poor prognosis.

Brain metastases (BrM) are the most common brain tumors. They mainly originate from melanoma, lung and breast cancer. BrM have a poor prognosis and limited treatment options, with recent advances in immune checkpoint blockade (ICB). While a combined ICB targeting PD-1 and CTLA-4 demonstrates intracranial response rates greater than 50% in asymptomatic melanoma BrM, a significantly lower response is observed in advanced/symptomatic BrM, including patients on corticosteroids (1).

The immunosuppressive tumor microenvironment, which progresses with time, is a major driver of resistance to ICB. In addition to immunosuppressive cell types commonly found in tumors, such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells, the brain tumor microenvironment also contains brain-resident stromal cells such as microglia and astrocytes. Astrocytes are the most abundant glial cells in the brain, with important supporting functions for neurons and the blood-brain barrier (2). However, recent studies implicate astrocytes in immunosuppression in BrM via various mechanisms, including CCL2-mediated MDSCs recruitment (3), MHC-I downregulation on cancer cells (4), and dampening of both innate and adaptive immunity (5). In this issue of *Cancer Discovery* Priego and colleagues (6) add an additional piece of the puzzle in our understanding of astrocyte-mediated immunosuppression by dissecting astrocyte heterogeneity in BrM and using elegant experimental approaches, preclinical models, and patient samples to identify STAT3/TIMP-1 axis as a novel mechanism of astrocyte-driven immunosuppression. They show that reactive astrocytes inhibit T cells within the peritumoral area, a hostile territory which T cells must cross to access BrM after exiting from the peritumoral venous vessels (7) (**Fig. 1**).

While heterogeneity of astrocytes at a single-cell level has been described for non-cancerous CNS disorders and glioma, astrocyte heterogeneity in BrM has been previously observed only by immunohistochemistry and flow cytometry (2). Thus, this is the first study using an unbiased approach to comprehensively characterize astrocytes in BrM by scRNAseq (6). Out of 9 astrocyte clusters identified, 3 significantly increased in experimental BrM as compared to non-tumor bearing brains. Two of these represented STAT3+ astrocytes, with different sets of STAT3-activating receptors, and enriched either in interferon pathway or in extracellular matrix, cytokines and interleukins. Corresponding STAT3+ astrocyte clusters were also identified in human BrM.

Building on their previous findings, showing that pSTAT3+ astrocytes secrete several immunosuppressive molecules, including glycoprotein TIMP-1 (8), and suppress T cell activation *in vitro* (5), Priego *et al*. here connect the dots between the transcription factor STAT3 (9), TIMP-1 and T cell inhibition. To demonstrate that astrocyte-specific STAT3 is driving the immunosuppression *in*

vivo, they deplete STAT3 in astrocytes in GFAP-Cre^{ERT2}; Stat3$^{loxP/loxP}$ transgenic mice, which increases the abundance of cytotoxic CD8+ T cells and reduces BrM burden in CD8+ T cell-dependent manner. *In vivo* pharmacological inhibition with the STAT3 inhibitor silibinin (9) mimics these observations.

Next, using a combination of *in vitro* assays, a transgenic mouse model, and analysis of patient BrM tissue, the authors demonstrate that pSTAT3+ astrocytes are the main source of TIMP-1 in patients and experimental BrM, and that TIMP-1 expression in astrocytes is STAT3-dependent.

The authors then combine T cell cultures stimulated with the conditioned medium from pSTAT3+ astrocytes with pharmacological and genetic approaches to directly implicate TIMP-1 in T cell suppression. TIMP-1 depletion in this assay leads to enriched T cell activation signature and phosphorylation of several kinase substrates, including ERK1/2, providing further insight into mechanisms underpinning TIMP-1 dependent T cell inhibition. These findings are confirmed *in vivo* following a conditional depletion of TIMP-1 in astrocytes in GFAP-Cre; Timp1$^{loxP/loxP}$ mice, which leads to reduced experimental BrM, as well as increased T cell abundance/activation, reduced exhaustion, and increased ERK1/2 phosphorylation in T cells.

The authors then drill into the molecular interactions downstream of TIMP-1 and demonstrate that the C-terminus of TIMP-1, known to interact with CD63, is involved in reduced BrM growth in organotypic brain cultures. CD63 is a tetraspanin partially localized at the cell surface and upregulated on activated T cells. Despite numerous CD63 interaction partners, its expression in a wide range of cell types and on exosomes (8), Priego and colleagues succeed in convincingly demonstrating the *in vitro* interaction between CD63 on T cells and astrocyte-secreted TIMP-1 by co-immunoprecipitation. They also confirm the existence of this interaction in patient BrM tissue using proximity ligation assay. In line with this, CD63 expression in CD8+ T cells is required for an efficient cancer cell killing in organotypic brain cultures, and TIMP-1 promotes effector memory phenotype in CD63^{high}, but not CD63^{low} CD8+ T cells sorted from experimental BrM. While the role of CD63 *in vivo* was not investigated, the presented evidence supports the scenario in which the interaction between CD63 on T cells and astrocyte-secreted TIMP-1 plays an important role in immunosuppression in BrM, although further TIMP-1 independent CD63 interactions may be involved *in vivo*. As only ~30% of CD8+ T cells in experimental BrM expressed CD63, a question arises whether CD63 expression is confined to a specific CD8+ T cell subset. It will be interesting to see whether TIMP-1 is targeting stem-like T cells, which is the main subset responding to ICB and trafficking to tumors (10), or more differentiated effector T cells.

A major appeal of this study comes from the evidence that links STAT3/TIMP1 to the immunosuppression in BrM in patients. *STAT3* and *TIMP1* gene expression levels in patient BrM positively correlated with the expression of immune cell-related genes and the extracted immune cell classifier (*CD8a*, *CD68*, *ITGAX*). Furthermore, TIMP-1 blockade in organotypic brain cultures of patient BrM from different cancer types reduced tumor burden to a higher degree in specimens with high CD8+ T cell infiltration and high *STAT3*/*TIMP1* expression. Collectively this suggests that the enhanced immune cell infiltration in BrM is counterbalanced by increased astrocyte-mediated immunosuppression via STAT3/TIMP-1 axis, effectively shielding BrM against the immune system independently of the cancer type, broadening the scope of a potential therapeutic translation of these findings.

So how can these findings be translated into a therapy? The first indication for the clinical efficacy of STAT3 inhibitor silibinin in BrM comes from a study using silibinin-based nutraceutical Legasil® in 2 patients with NSCLC BrM, and a subsequent study by Priego *et al*. (5) using Legasil in a cohort of 18 patients with NSCLC BrM, which demonstrated intracranial ORR of 75%, and significantly enhanced survival as compared to the control cohort (15.5 versus 4 months). Together with the preclinical evidence (5), this presented a compelling rationale for targeting STAT3 in BrM. Notably, STAT3 has emerged as a significant target in cancer due to its aberrant signaling within cancer cells and the tumor microenvironment across a range of tumor types (9). NIH ClinicalTrials.gov lists 26 trials of STAT3 therapies in cancer, with 15 completed across a range of tumor types, and 6 trials testing STAT3 inhibitor/ICB combinations, highlighting the increasing momentum in this field. While the clinical translation of STAT3 inhibitors has remained challenging, combination therapies with ICB in different cancer types show promising results in preclinical and early clinical studies (9). Compelling evidence presented in this study (6), demonstrating that T cells in BrM are restrained by STAT3-dependent astrocyte-mediated immunosuppression, together with an observed inverse correlation between the density of pSTAT3+ astrocytes and granzyme+ CD8+ T cells within the reach of astrocyte-secreted cytokines in patients (n=8) that responded to ICB systemically but later relapsed in the brain, lead authors to propose to combine silibinin with ICB in BrM.

Interestingly, in experimental BrM models STAT3+ astrocytes emerge in late-stage tumors, suggesting they are mediating immunosuppression specifically in advanced BrM, coinciding with reduced response rates to ICB in patients (1). This hypothesis is supported by observation that new BrM emerging in 4 patients while on Legasil remained below 1 cm in diameter (5). In line with this, the

current study showed that while ICB (combined PD-1/CTLA-4 blockade) and silibinin effected medium-size experimental BrM to a similar extent, silibinin was superior in controlling large BrM, and ICB/silibinin together further improved the overall BrM control. Furthermore, triple combination of radiotherapy with silibinin and ICB was superior to a dual combination of radiotherapy with either silibinin or ICB, demonstrating that in this clinically relevant context of local radiotherapy silibinin also enhances ICB efficacy. Overall, this provides a compelling rationale for combining STAT3 inhibitors with ICB in BrM.

As for extracranial metastases, not much effect by silibinin was observed in the current preclinical study, which was in line with the reported progression of extracranial metastases in most patients while on Legasil (5). However, silibinin and other STAT3 inhibitors previously demonstrated efficacy in extracranial tumor models (9), suggesting that extracranial effects of silibinin are model/cancer dependent.

Lastly, by demonstrating that TIMP-1 levels in the CSF significantly increase in the presence of experimental BrM and this is reversed through astrocyte-specific TIMP-1 deletion, this study shows the potential for TIMP1 as a liquid biopsy biomarker that could improve patient selection and even evaluate therapeutic benefit. Excitingly, the proposed combination therapy and biomarker could be particularly valuable for patients with advanced, symptomatic BrM, an understudied and immunotherapy-resistant population with significant clinical need (1).

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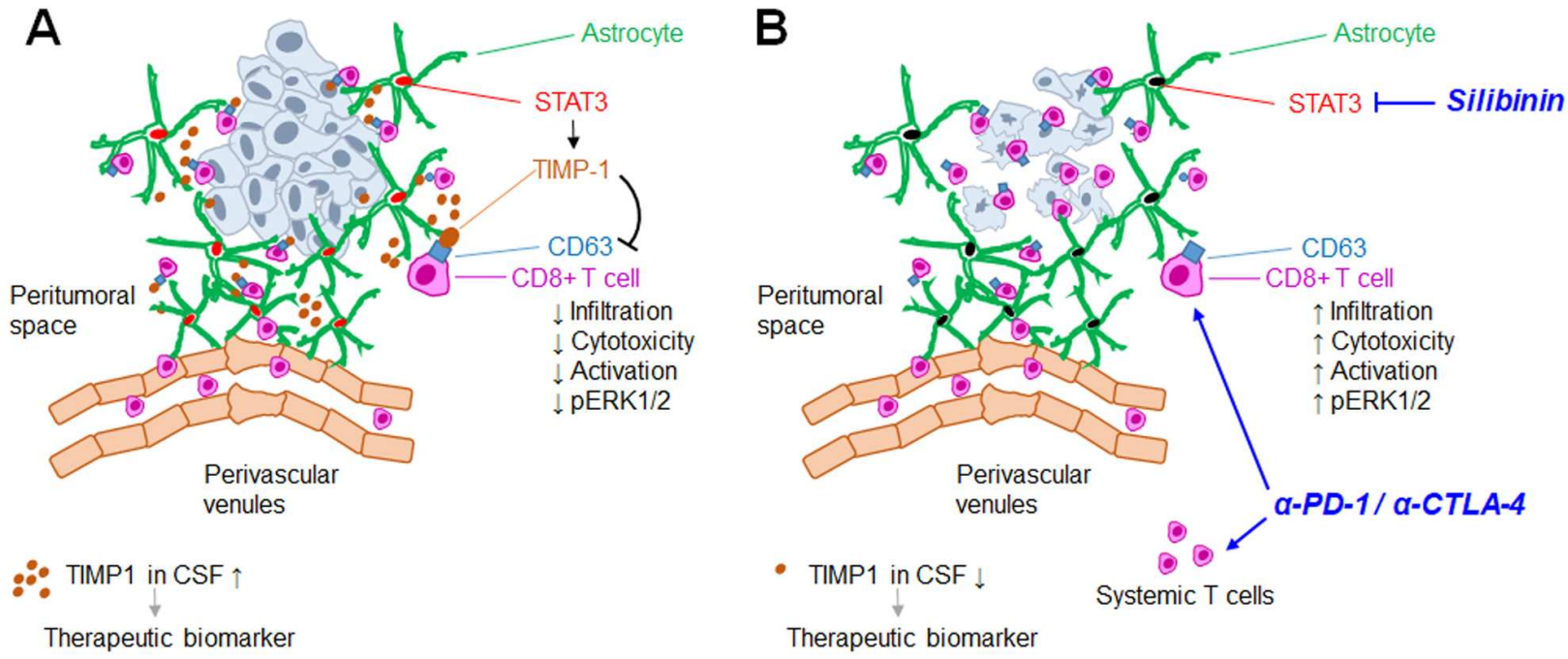
Figure 1. Dampening of TIMP1-dependent astrocyte immunosuppression unleashes T cells against brain metastases. (A) STAT3 is activated in reactive astrocytes surrounding late-stage BrM, leading to upregulation and secretion of TIMP-1, which binds to CD63 on T cells, causing their exhaustion, reduced activation and cytotoxicity, thereby hampering their ability to control BrM growth. **(B)** Targeting STAT3 with silibinin blocks TIMP-1 expression, promoting T cell activation and cytotoxicity, which synergizes with the blockade of immune checkpoints PD-1 and CTLA-4, acting on T cells systemically and within the tumor microenvironment to unleash anti-tumor responses.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Astrocyte
STAT3
TIMP-1
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CD8+ T cell
↓ Infiltration
↓ Cytotoxicity
↓ Activation
↓ pERK1/2
Peritumoral space
Perivascular venules
TIMP1 in CSF ↑
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Peritumoral space
Perivascular venules
α-PD-1 / α-CTLA-4
Systemic T cells
TIMP1 in CSF ↓
Therapeutic biomarker