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Ménage à trois

Sustained therapeutic anti-tumor immunity requires multiple partners in malignant glioma

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Glioblastoma is an aggressive primary brain cancer. Given our interest in novel immunotherapies, we have recently shown that inhibiting CTLA-4, PD-L1 and IDO results in a dramatic survival advantage in mice with brain tumors. Our preclinical study supports the rapid translation of this approach into phase I clinical trial.

Glioblastoma multiforme (GBM) is the most common primary brain tumor in adults and has a very poor prognosis.[1] Part of the challenge in treating GBM is related to its localization within the naturally immunosuppressive central nervous system (CNS), which is anatomically-protected by a blood brain barrier and devoid of the traditional lymphatic structure. Interestingly, even with these specialized immunological restrictions, leukocytes are known to traverse the CNS and invade GBMs.[2]

Of the various T cell subsets, regulatory T cells (Treg; $CD3^+CD4^+Foxp3^+CD25^+$) are selectively enriched in GBM-infiltrating $CD4^+$ T cell pool[3] and their neutralization and/or depletion has been correlated with an increase in overall survival.[4,5] Given the potently immunosuppressive effector function of Treg, therapeutic strategies that diminish their activity, while simultaneously (re-)activating the cytotoxic lymphocyte response, have been an intense area of study.

We have recently shown that inhibiting cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed cell death ligand 1 (PD-L1) and indoleamine 2,3-dioxygenase (IDO) (**Fig. 1**) via seemingly non-redundant pathways in mice intracranially-injected with GL261, a glioma cell line utilized to study malignant glioma, leads to the significant reduction in Treg and ~100% survival.[6] This is an exciting development, since clinical-grade analogs for CTLA-4 and PD-L1 monoclonal antibodies exist, in addition to the clinical-grade IDO inhibitor (D1-MT), all of which were used in our studies. In addition, Wolchok et al. (2013) recently demonstrated that a similar strategy utilizing humanized CTLA-4 and PD-1 mAb in melanoma patients mediated tumor regression in > 50% of patients.[7] Collectively, these pre-clinical data provide promising evidence that combinatorial blockade utilizing CTLA-4, PD-(L)1 and/or IDO inhibition in patients with GBM is worthy of a phase 1 clinical trial.

It was interesting to find that depleting $CD4^+$- and/or $CD8^+$-T cells abrogated the therapeutic effect of the CTLA-4/PD-L1/IDO blockade. This is in contrast to many tumor models localized outside of the brain, suggesting that CTLs, alone, are the primary T cell subtype required to carry out tumor rejection,[8] although this is not a universal trend.[9] Moreover, it was surprising to find that, when administered at 14 d post-intracranial injection, CTLA-4/PD-L1 blockade achieved an identical survival rate (~80%), as mice treated with CTLA-4/PD-L1/IDO, since only the latter treatment regimen achieved a significant decrease in brain tumor-infiltrating Treg (5.3 ± 1%), when compared with untreated control brain tumor-bearing mice (38 ± 2%) ($P < 0.001$). One notable caveat to our studies was the lack of measurement related to Treg suppressor function after double vs. triple immunotherapeutic blockade, which may explain the discrepancy between the survival and Treg levels.

Notably, we found that the triple immunotherapeutic approach was ineffective in generating long-term survival in mice with intracranial B16/F10 melanoma-based tumors. While this likely reflects the overall aggressiveness of melanoma, it may also reflect a selective permissiveness that melanoma cells gain after accessing the CNS. We demonstrated that there was a significant decrease in Treg levels between intracranial GL261 and B16-F10 tumors [27 ± 7% and 2 ± 0.3% Treg ($P < 0.01$), respectively] when analyzed at 12 d post-injection. Although the mechanism causing Treg recruitment was not explored in our study, it is interesting to hypothesize that the triple immunotherapeutic approach is more effective against intracranial tumors that utilize Tregs as a dominant immunosuppressive mechanism. Future studies will be necessary to further elucidate this point.

As the title of this review suggests, more may mean better when it comes to

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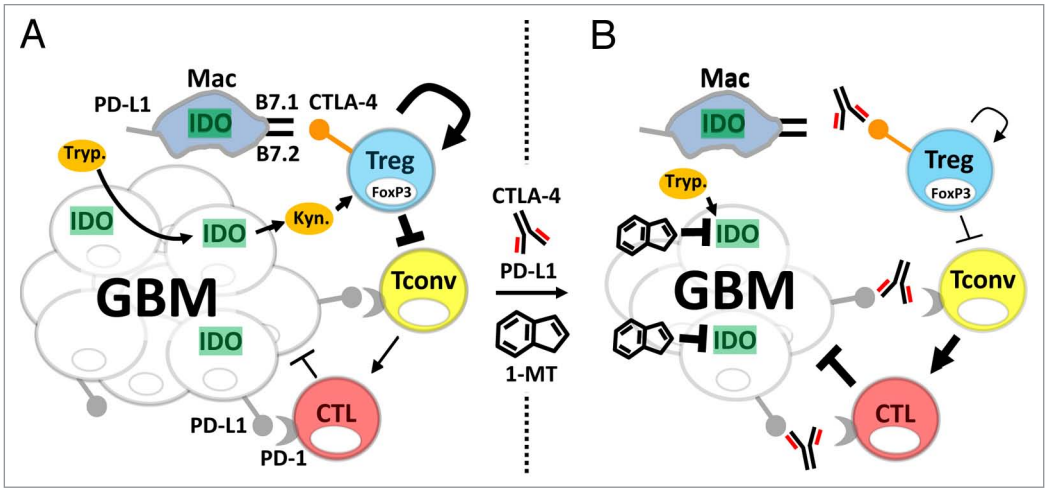

Figure 1. Simultaneously targeting CTLA-4, PD-L1 and IDO decreases Treg and increases survival. (**A**) Under normal conditions, primary brain tumors express IDO, a cytoplasmic enzyme that catabolizes the essential amino acid, tryptophan (Tryp.), to the downstream metabolite, kynurenine (Kyn). Based on recent studies showing that kynurenine interacts with the aryl hydrocarbon receptor in $CD4^+$ T cells to increase FoxP3 expression, this may be a primary mechanism contributing to Treg expansion in the brain tumor. Coincidently, CTLA-4 is constitutively and highly expressed on the tumor-resident Treg population that can both directly inhibiting conventional $CD4^+$ T (Tconv) function, as well as interact with surrounding macrophages, microglia and/or dendritic cells (not shown) via B7.1 and B7.2 to induce IDO expression. Finally, PD-L1 is highly expressed by both tumor cells, as well as tumor-infiltrating macrophages, both of which contribute to the highly immunosuppressive tumor microenvironment. When CTLA-4 and PD-L1 mAbs, in addition to the IDO chemical inhibitor, 1-methyl tryptophan (1-MT), is combinatorially administered to mice bearing brain tumors, (**B**) Treg levels decline, presumably by the decreased kynurenine availability, contributing to the Tconv-mediated assistance to $CD8^+$ cytotoxic T lymphocytes (CTL), which produce higher interferon-gamma levels (not shown) that contribute to brain tumor clearance and increased survival. It should be noted that, although the PD-L1 and CTLA-4 mAbs, as well as 1-MT, are schematically represented here to be acting directly within the tumor microenvironment, we cannot rule out that their primary mechanism of action is limited to the central nervous system compartment. Cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed cell death ligand 1 (PD-L1) and indoleamine 2,3-dioxygenase (IDO)

modulating the immune response against tumors. This concept may be somewhat problematic from the standpoint of clinical trial development, since the majority of early phase trials focus on evaluating the safety and potential efficacy of single agents. However, such an approach, in the opinion of the authors, represents a missed opportunity since it assumes that cancer immunotherapy can be achieved via modulation of a single pathway. In fact, as we propose here and is shown in our published data, modulation of the immunosuppressive tumor microenvironment is optimal when several pathways are concomitantly inhibited at the same time. Reversing immunosuppression that is associated with cancer requires multiple partners and in many ways, complementary approaches.

The argument in favor of using multiple agents does not imply a free-for-all. In fact, as shown in our work, temozolomide -the standard of care for GBM- does not offer any synergy with anti-CLTA-4/PD-L1/IDO blockade. In fact, concurrent addition of temozolomide to our therapeutic regimen decreased survival, suggesting that active chemotherapy in the context of productive antitumor immunity is an undesirable approach. Temozolomide is a known agent to cause lymphopenia. Therefore, repeated and frequent dosing with this agent may abrogate an ongoing and/or productive immune response. Many immunotherapy-based clinical protocols, however, continue to include temozolomide as part of the standard-of-care, often without preclinical evidence to suggest its benefit or harm in the proposed therapy. The neuro-oncology community must therefore carefully evaluate the context in which clinical protocols are developed, paying attention to strong preclinical data which should drive the development of clinical trials.

In summary, our work provides a proof-of-concept for reversing glioma-induced immunosuppression via the incorporation of multiple partners in the clinical setting. As shown in our published work in *Clinical Cancer Research*, simultaneously administered triple therapy of mice with established brain tumors led to 100% overall survival. Whether we can achieve similar results in patients remains to be seen. However, the design and execution of a clinical trial focused on CTLA-4/PD-L1/IDO inhibition in malignant glioma represents a most reasonable and timely approach, given the availability of all three reagents for use in the clinical setting.

Disclosure of Potential Conflicts of Interest

The authors declare that no competing interests exist.

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