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Unlocking CAR T cell potential: Inosine-induced stemness and enhanced potency

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Adenosine (Ado) drives immune suppression in the tumor microenvironment. In this issue of *Cancer Cell*, Klysz et al. investigate Ado-mediated immunosuppression. Overexpression of Ado deaminase (ADA-OE), metabolizing Ado to inosine (INO), induces stemness and improves CAR T cell functionality. Likewise, exposure to INO enhances CAR T cells' function and induces stemness features.

T cell exhaustion is a differentiation state that occurs when T cells are subjected to prolonged antigen stimulation. It is characterized by the progressive loss of effector function, stable expression of inhibitory surface receptors, distinct epigenetic profiles, defective cytokine production, impaired proliferation capacity, and suppressed metabolic activity.[1] This state of T cell exhaustion poses a significant challenge for immune checkpoint inhibitors and CAR T cell immunotherapies.[2] One of the primary contributors to T cell exhaustion is the immunosuppressive nature of tumor microenvironment (TME). Within this environment, various factors come into play, including immunosuppressive cytokines like IL-10 and transforming growth factor β (TGFβ), as well as inflammatory cytokines such as interferon gamma (IFNγ), IL-6, and so on.[1]

Among these factors, adenosine (Ado), a potent immunosuppressive metabolite, plays an important role in immune suppression. This has spurred extensive efforts to develop adenosine inhibition strategies, aiming to enhance antitumor immune responses.[3] Key players in this process are CD39 and CD73, working together in the conversion of ATP into Ado, which acts as a primary inducer of immunosuppression via A2aR signaling (Figure 1A).[3] In this issue of *Cancer Cell,* Klysz et al. provide compelling evidence that CAR T cells cultivated in inosine-containing media, and to a lesser extent, CAR T cells overexpressing Ado deaminase exhibit enhanced functionality driven by metabolic and epigenetic reprogramming that induces stemness features.[4]

At the outset of the paper, the authors explored the biology involving CD39 and Ado in the context of T cell exhaustion. They found that CD39 served as a marker for a profoundly dysfunctional subset of exhausted CD8$^+$ T cells. These cells frequently co-express CD73 and exhibit phenotypic traits associated with suppressive T cells. To tackle Ado-mediated immunosuppression, the authors utilized HA CAR T cells, whose tonic signaling induces T cell exhaustion *in vitro*,[5] and conducted genetic knockout (KO) experiments targeting CD39, CD73, or A2aR.

Notably, the authors introduced an innovative experimental group featuring overexpression of membrane-tethered Ado deaminase (ADA-OE) (Figure 1B). This strategy allowes the conversion of Ado into inosine (INO), offering an alternative approach to mitigate Ado-mediated immunosuppression. What's striking is that ADA-OE induced the most substantial transcriptional changes when compared to the KO and control groups. Furthermore, ADA-OE's ability to reduce progenitor exhausted and exhausted subsets is noteworthy. Moreover, through transcriptome analysis and protein-level assessment, the authors revealed that ADA-OE, and to a lesser extent CD39-KO or CD73-KO, but not A2aR-KO (Figure 1A), induced transcriptomic and phenotypic alterations linked to stemness. This suggests that ADA-OE may have broader effects beyond Ado signaling inhibition, possibly due to the production of INO (Figure 1B).

Continuing this investigation, the authors conducted a series of experiments to assess the impact of INO on the stemness of chronically activated CAR T cells (Figure 1C). The results revealed that INO inhibited proliferation, induced a stem-like phenotype, and significantly enhanced functionality in both non-exhausted and exhausted CAR T cells, surpassing the outcomes observed with ADA-OE. It is worth noting that previous studies have suggested that INO can act as an alternative carbon source to support CD8$^+$ T cell function under conditions of glucose restriction.[6] To investigate whether the observed enhancement of T cell function mediated by INO could be attributed to glucose deprivation, the authors designed control experiments and demonstrated that the beneficial effects of INO were not reliant on glucose deprivation, further highlighting the role of INO in improving CAR T cell potency.

In the assessment of INO's impact on CAR T cell function in an *in vivo* setting, the authors employed both exhaustion-prone HA-CAR T cells and non-exhaustion-prone CD19-CAR T cells in animal models. Interestingly, the exposure to INO during CAR T cell manufacturing substantially enhanced their antitumor effectiveness. Consequently, these cells exhibited heightened antitumor potency and improved CAR T cell persistence when challenged in a solid tumor model.

INO is a versatile purine compound with diverse roles in intracellular and extracellular contexts. Notably, INO derived from *Bifidobacterium pseudolongum* enhances the effectiveness of anti-CTLA4 therapy via A2aR and IFNγ-dependent induction of Th1 differentiation in both human and murine studies.[7] In murine models where tumors cannot utilize INO for bioenergetics, systemic INO administration enhances the efficacy of anti-CTLA4 and GD2-CAR therapy.[6] Beyond its effects in activating the immune system, the

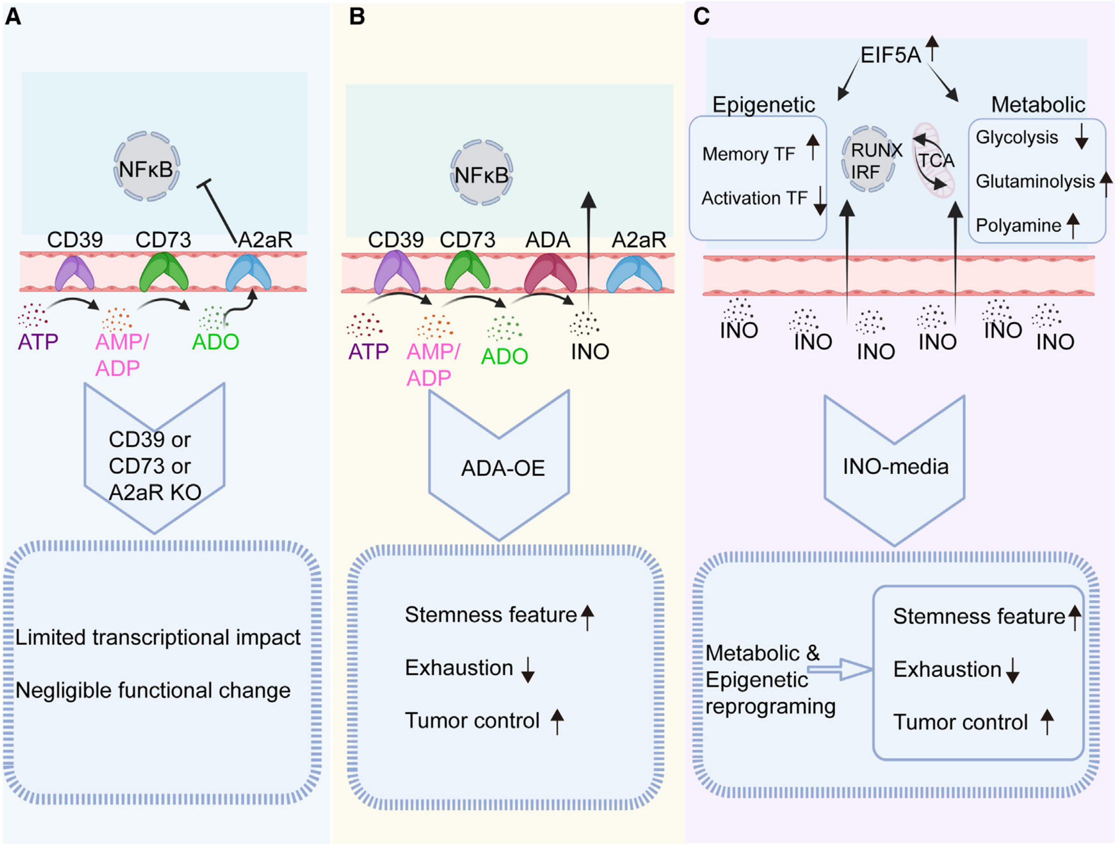

Figure 1. Strategies for inhibiting Ado generation or signaling
(A) Impact of CAR T cells with CD39, CD73, or A2aR knockout (KO).
(B) Effect of CAR T cells with Ado deaminase overexpression (ADA-OE).
(C) Influence of CAR T cells cultured in inosine-containing media.

production and release of INO by dendritic cells through a FAMIN-dependent pathway was found to inhibit autoimmunity and autoinflammation in mouse models.[8] In summary, INO exhibits immunomodulatory effects with mounting evidence, but its intricate mechanisms in T cells remain challenging to pinpoint due to its multifaceted nature.

To investigate the molecular mechanism of INO's impact on CAR T cells, the authors employed transcriptomic and proteomic profiling. The findings present a compelling picture: INO not only induces stemness programming but also significantly enhances functionality in these cells. What's even more intriguing is the profound metabolic reprogramming induced by INO exposure. This includes a reduction in glycolysis, an increase in glutaminolysis and polyamine synthesis, heightened activity in the TCA cycle and mitochondria, and an expanded glycolytic reserve (Figure 1C). These results shed light on the intricate mechanisms underlying the potent effects of INO on CAR T cells.

Furthermore, the authors conducted an ATAC-seq analysis on $CD8^+$ HA-CAR T cells and their INO-exposed counterpart HA-INO-CAR T cells to explore whether INO exposure leads to alterations in epigenetic patterns. The ensuing analysis uncovered that INO exposure resulted in a noteworthy increase in the accessibility of memory-associated TF binding motifs, particularly those linked to RUNT, IRF, and RHD family TFs. Simultaneously, it acted as a shield, reducing the susceptibility of cells to Ado-mediated suppression by decreasing the accessibility of CREB1 and ATF7 motifs. Furthermore, previous research had linked polyamines to the epigenetic regulation of Th1 differentiation through EIF5A hypusination.[9] It was intriguing to observe increased EIF5A expression in HA-INO-CAR T cells, hinting at a potential involvement of a similar pathway in INO-induced epigenetic programming. To put this hypothesis to the test, the authors utilized an inhibitor of EIF5A hypusination and resorted to EIF5A knockout using CRISPR-Cas9. These experiments demonstrated that INO induces stemness through the modulation of polyamine metabolism and EIF5A hypusination (Figure 1C).

Finally, the authors used GD2-CAR T cells as an example, which are currently in clinical trials for diffuse midline gliomas (DMGs),[10] to test the feasibility of incorporating INO into a GMP CAR T cell manufacturing process. The results illustrate that CAR T cells can be produced in INO-containing media through a GMP process, achieving clinically relevant doses that meet standard release criteria. Importantly, these cells exhibit enhanced functionality compared to those manufactured using standard glucose-based media.

In conclusion, while several strategies are under development to inhibit Ado generation or signaling,[3] the authors' findings endorse an approach that metabolizes Ado into INO within the tumor microenvironment. Furthermore, INO treatment improves CAR T cell potentcy in exhasuted or non-exhausted status and is compatible with GMP production. It is essential to acknowledge that this study primarily centered on CAR T cells. Future validation on other T cell types, such as tumor-infiltrating lymphocytes, holds the potential to expand the applicability of INO in the realm of tumor immunotherapy.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Envision the future of precision medicine in pediatric cancer

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Exploring the diversity within the tumor microenvironment (TME) can offer crucial insights to steer cancer therapy toward precision medicine. In this issue of *Cancer Cell*, Wienke et al. undertake a comprehensive single-cell analysis of neuroblastoma, unveiling its immune landscape and identifying NECTIN2-TIGIT as a promising target for immunotherapy.

Neuroblastoma (NB) stands as the second-most-common solid tumor among children. In 50% of the cases, the disease is high risk and requires an intensive treatment that combines chemotherapy, surgery, myeloablative autologous stem cell transplant, radiation, differentiating agents, and immunotherapy. Despite this treatment, less than 50% of children with high-risk NB achieve a long-term remission.[1] In contrast to several adult cancers like melanoma and lung and colorectal cancers with targetable driver mutations, NB has a low frequency of such mutations, with Anaplastic Lymphoma Kinase (ALK) mutations being a notable exception and observed in approximately 10% of NB tumors. Consequently, a genome analysis of this cancer is of limited insights for precise treatment strategies.

Single-cell RNA sequencing (scRNA-seq) analysis has revolutionized our way of studying the tumor microenvironment (TME), and it could also provide a much deeper understanding of the heterogeneity of the TME in NB. In this issue of *Cancer Cell*, Wienke et al. conduct an integrative scRNA-seq analysis of NB, unraveling the immune landscape of this cancer.[2] Authors demonstrate that such analysis at the single-cell level can provide valuable information to guide immunotherapy.

The NB tumor heterogeneity became first apparent by bulk RNA analysis using deconvolution methods like CYBERSORT or BASE. These methods were helpful in identifying, for example, up to 22 subpopulations of immune cells in 153 primary NB tumors from the TARGET database with a majority being M2 macrophages particularly dominant in tumors of poor prognosis. Other studies identified six