Molecular Therapy
Beyond Molecular Therapy

Tumor-targeted cytokine delivery by engineered myeloid cells unlocks CAR T cell efficacy and endogenous T cell responses in glioblastoma

Federico Rossari,[1,2] Giorgia Alvisi,[1] Nadia Coltella,[1] and Luigi Naldini[1,2]

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Chimeric antigen receptor (CAR) T cell therapies have revolutionized the treatment of hematological malignancies, but their translation into solid tumors has remained elusive.[1] This is commonly ascribed to heterogeneous expression of tumor-associated antigens (TAAs) and an immunosuppressive, myeloid-rich tumor microenvironment (TME), which antagonizes T cell penetration and function. A paradigmatic example is glioblastoma (GBM), the most common and aggressive adult brain tumor, which remains a highly unmet medical need.[2] While T cells can be engineered with CAR to recognize some GBM-associated antigens, they fail to provide durable anti-tumor responses, likely due to limited persistence, premature exhaustion, and the adverse tumor milieu.[1] In our recent study,[3] we report a gene therapy strategy to reprogram the TME and revive the therapeutic potential of CAR T cells by leveraging tumor-infiltrating macrophages as vehicles for tumor-targeted gene-based delivery of immune-activating cytokines (Figure 1).

We built on previous work exploiting TIE2-expressing monocytes/macrophages (TEMs) as tumor-targeted delivery systems.[4] TEMs are a pro-angiogenic macrophage subpopulation commonly recruited to tumors, where they are viciously captured to support their growth.[5] By transplanting hematopoietic stem cells (HSCs) transduced with lentiviral vectors expressing their payload under a reconstituted *Tie2* promoter, we turned their TEM progeny into smart vehicles that, upon recruitment to the TME, release immune-activating cytokines such as interferon alpha (IFN-α) and trigger a pleiotropic immune activation that effectively fights tumors "from within."[4,6] A careful design of the vector, which also included negative post-transcriptional regulation by microRNA binding sites, guarantees expression stringently confined to the TME, preventing potentially detrimental systemic and off-target IFN exposure.[7] This strategy is currently undergoing its first-in-human testing in a phase 1/2a trial on GBM (Temferon, ClinicalTrials.gov: NCT03866109) led by Genenta Science.

In the new study, we evaluated the efficacy of our targeted delivery system in combination with CAR T cells targeting B7-H3, a clinically relevant antigen expressed in GBM, generated through an optimized protocol for efficient lentiviral transduction of murine T cells. The model used—named mGB2[8]—is a syngeneic mouse cell line orthotopically grown in immunocompetent mice, which better recapitulates the growth pattern and immune dynamics of human GBM, including the recruitment of TEMs, compared to more permissive xenograft systems or other, less infiltrative syngeneic models.[6] We engineered TEMs to secrete either IFN-α for broad immune cell activation throughout the TME and/or an interleukin-2 mutein (called orthogonal IL-2 [oIL-2][9]) that preferentially binds an orthogonal cognate receptor (oIL-2Rβ) co-delivered with the CAR to T cells to establish a private crosstalk between the two engineered myeloid and lymphoid cell populations within the TME. The targeted cytokines rescued the activity of CAR T cells that, given alone intravenously and/or intratumorally, were ineffective—as mostly seen in clinical trials.[1] In turn, the rescued CAR T cells synergized with cytokine delivery, significantly enhancing their effect on delaying tumor growth and extending mouse survival.

Single-cell surface and transcriptome profiling showed that local release of IFN-α in the tumor prevented early CAR T cell exhaustion and led to a shift from terminal exhaustion toward effector and effector memory phenotypes associated with successful tumor killing. Importantly, this strategy not only enhanced the functionality of adoptively transferred CAR T cells targeting B7-H3 but also engaged the host's endogenous T cells to react against TAAs beyond the CAR target. This was shown by phenotypic and gene expression changes typical of antigen-driven activation in endogenous circulating and tumor-infiltrating cells, induction of their *ex vivo* reactivity against B7-H3-negative cells, and resistance of a long-term surviving mouse to rechallenge with B7-H3-negative mGB2 cells. Overall, these findings support the contention that our combined strategy led to antigenic spreading of the anti-tumor immune response, a highly sought-after outcome of immunotherapy and likely a requirement for durable and protective CAR T effect in solid tumors. Mechanistically, we envision that antigen-presenting cells such as macrophages, dendritic cells (DCs), and inflammatory monocytes in the TME can capture and effectively present TAAs to endogenous T cells through the up-regulated major histocompatibility complex

[1]San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), IRCCS San Raffaele Scientific Institute, 20132 Milan, Italy; [2]Vita-Salute San Raffaele University, 20132 Milan, Italy

Correspondence: Luigi Naldini, San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), IRCCS San Raffaele Scientific Institute, 20132 Milan, Italy.
E-mail: naldini.luigi@hsr.it

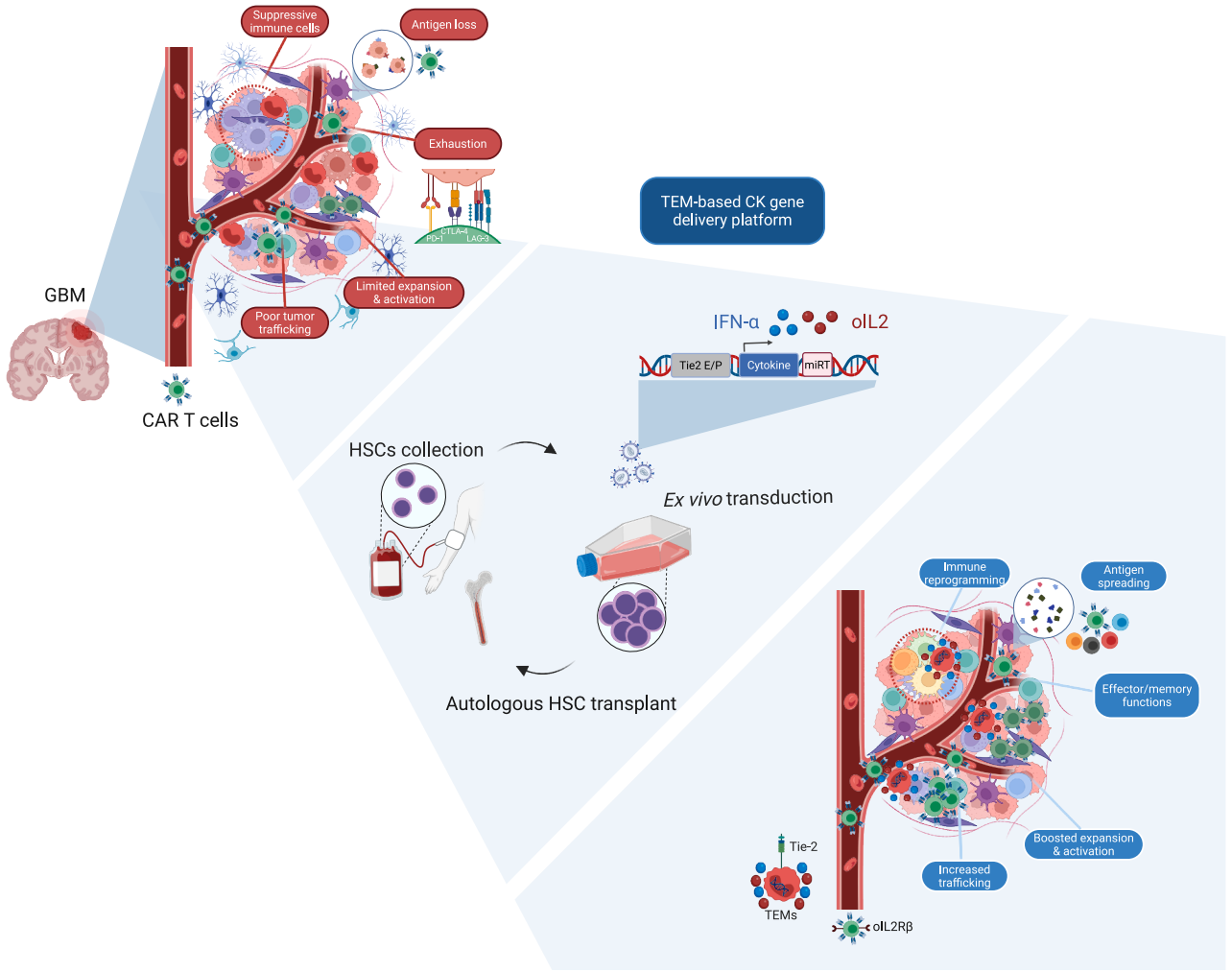

Figure 1. Schematics showing the functioning of TEM-based cytokine gene delivery in combination with CAR T cells against GBM

Top left: biological hurdles to CAR T cell function in GBM. Middle: TEM-based cytokine gene delivery platform. Bottom right: reshaping of the tumor microenvironment by TEM-based cytokine gene delivery to rescue CAR T cell function. CAR, chimeric antigen receptor; CK, cytokine; GBM, glioblastoma; HSCs, hematopoietic stem cells; IFN-α, interferon-α, miRT, microRNA target sequence; oIL-2, orthogonal interleukin-2; oIL-2Rβ, orthogonal IL-2 receptor β chain; TEMs, Tie2-expressing monocytes/macrophages; Tie2 E/P, Tie2 enhancer/promoter. Designed in BioRender.

(MHC) machinery and key co-stimulatory signals by IFN-α. While we previously reported that this can occur by IFN gene therapy alone,[6] CAR T-mediated tumor cell killing can strongly increase the efficiency of the process, not only by contributing to tumor cell killing but also by promptly providing a robust source of TAAs from the dying cells.

The orthogonal IL-2/IL-2Rβ system[9] increased the intratumor abundance and proportion of effector phenotypes only of CAR T cells, indicating the establishment of a selective crosstalk between both populations of engineered immune cells in the TME, although it conferred a more transient and less substantial therapeutic benefit compared to IFN-α delivery. However, oIL-2 combination with IFN-α delivery further and significantly boosted therapeutic efficacy compared to single-cytokine treatment. The dual-cytokine approach reduced terminal exhaustion, preserved stem-like and memory T cell populations, and promoted an even more robust immune response from circulating and tumor-infiltrating endogenous T cells that was able to delay tumor progression even when only a fraction of tumor cells expressed B7-H3.

In the study, we applied an innovative analysis named MultiNicheNet, a computational framework designed to analyze cell-cell communication across multi-condition single-cell RNA sequencing data.[10] A distinctive feature of MultiNicheNet is its direct use of downstream differentially expressed genes as targets of ligand-receptor interactions. This links ligand-receptor activity to gene expression changes in receiver cells,

offering a clearer and more biologically relevant view of intercellular signaling across varied treatments. In this way, we were able to demonstrate that IFN-α, particularly when combined with oIL-2, reprograms the TME by promoting inflammatory macrophages ($Cxcl9^+$ tumor-associated macrophages [TAMs]) and enhancing conventional (c)DC1-derived IL-12 signaling to CAR T cells. The combination also revealed an IL-18-mediated interaction between cDC1s and CAR T cells, potentially expanding effectors while preserving memory cells. In contrast, oIL-2 alone induced limited changes and was associated with interactions involving MHC and KLRG (killer cell lecitin-like receptor G family) molecules, suggestive of a dysfunctional, natural killer (NK)-like T cell state driven by persistent stimulation,[11] likely explaining the minor benefit. These insights highlight the dynamic landscape of immune cell communication and suggest new strategies to optimize CAR T cell therapies by more precisely modulating the TME.

The use of multiple orthogonal and tumor-localized signals in a coordinated fashion showcases a sophisticated and potentially clinically relevant immune engineering approach, which can be further expanded to build multiplex circuitry in the TME that engages distinct actors, establishes self-sustaining feedbacks, and ultimately drives powerful therapeutic responses.

Our study repositions the TME as a modifiable barrier rather than an absolute hurdle. By delivering pleiotropic cytokines like IFN-α directly to the TME, we showed that it is possible to convert a hostile environment into a supportive one toward T cell activation, expansion, and memory formation. In solid tumors, especially those as heterogeneous as GBM, reliance on a single target antigen poses a major limitation due to antigen loss. The finding that IFN-α promotes antigenic spreading should favor the deployment of durable anti-tumor immunity. By providing a sustained and spatially confined source of immune stimulation, the pitfalls of systemic cytokine therapy—such as toxicity and lack of efficacy—can be effectively circumvented.[12]

Despite the compelling results, some limitations warrant consideration. The study relies on a single GBM model that, while immunocompetent and representative of key human features, may not fully capture the complexity and heterogeneity of the human counterpart. The use of only one murine CAR construct, targeting B7-H3 and incorporating a CD28 co-stimulatory domain, limits the generalizability of the findings. Expanding these observations across diverse tumor models, CAR designs, and target antigens will be important to validate the platform's robustness. Additionally, the therapeutic efficacy observed in this study, while significant, did not lead to complete tumor eradication in a substantial fraction of animals. Residual disease may reflect intrinsic resistance mechanisms, limitations in CAR T cell persistence, or the aggressive kinetics of orthotopic tumor growth that outpaces immune remodeling.

In recent years, several strategies have been explored to selectively deliver cytokines to the tumor, including T cell armoring.[12] While T cell armoring offers a promising and convenient "all-in-one" delivery solution, it strictly depends on the persistence and fitness of the T cells within the TME, which are commonly compromised by suppressive signals and rapidly exhausted. Moreover, the mass of secreting cells may substantially expand early after *in vivo* administration and trigger cytokine-dependent toxicity, as observed in some recent trials exploiting IL-12 armoring.[13] The TEM platform circumvents these issues by enabling stable cytokine production directly at the tumor site and reprogramming the TME before the CAR T challenge. However, it entails genetic modification and administration of an additional cell type, i.e., HSCs, increasing the complexity of the procedure and constraining its application to patients that may not tolerate the sub-myeloablative conditioning required for HSC engraftment. This hurdle may be alleviated by exploiting emerging biological conditioning strategies, which should mitigate systemic toxicity while easily meeting the low target chimerism required by our strategy. In the future, alternative cell types—such as engineered macrophages[14] and myeloid progenitors—or direct *in vivo* engineering approaches[15,16] may reach similar benefits with more accessible manufacturing pipelines.

Nonetheless, as both Temferon (the clinical TEM-mediated IFN-α gene therapy platform) and B7-H3-directed CAR T cells are undergoing clinical testing for several tumor types, including GBM, our study offers a mechanistically grounded rationale for their future combination to enhance the benefit of each treatment and broaden their efficacy to more tumor types and a larger fraction of patients.

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DECLARATION OF INTERESTS

F.R., G.A., N.C., and L.N. are inventors on patents on miRNA-regulated LV technology and targeted gene delivery into the TME, filed by the San Raffaele Scientific Institute and the Telethon Foundation. L.N. owns equity and is founder and advisor of Genenta Science, a biotechnology company that licensed TEM-mediated IFN-α gene therapy and develops its clinical applications.

REFERENCES

1. Uslu, U., and June, C.H. (2025). Beyond the blood: expanding CAR T cell therapy to solid tumors. Nat. Biotechnol. *43*, 506–515. https://doi.org/10.1038/s41587-024-02446-2.

2. Woroniecka, K., Chongsathidkiet, P., Rhodin, K., Kemeny, H., Dechant, C., Farber, S.H., Elsamadicy, A.A., Cui, X., Koyama, S., Jackson, C., et al. (2018). T-Cell Exhaustion Signatures Vary with Tumor Type and Are Severe in Glioblastoma. Clin. Cancer Res. *24*, 4175–4186. https://doi.org/10.1158/1078-0432.CCR-17-1846.

3. Rossari, F., Alvisi, G., Cusimano, M., Beretta, S., Birocchi, F., Ambrosecchia, D.I., Vitaloni, O., Brombin, C., Rancoita, P.M.V., Canu, T., et al. (2025). A cross-talk established by tumor-targeted cytokines rescues CAR T cell activity and engages host T cells against glioblastoma in mice. Sci. Transl. Med. *17*, eado9511. https://doi.org/10.1126/scitranslmed.ado9511.

4. De Palma, M., Mazzieri, R., Politi, L.S., Pucci, F., Zonari, E., Sitia, G., Mazzoleni, S., Moi, D., Venneri, M.A., Indraccolo, S., et al. (2008). Tumor-Targeted Interferon-α Delivery by Tie2-Expressing Monocytes Inhibits Tumor Growth and Metastasis. Cancer Cell *14*, 299–311. https://doi.org/10.1016/j.ccr.2008.09.004.

5. Venneri, M.A., De Palma, M., Ponzoni, M., Pucci, F., Scielzo, C., Zonari, E., Mazzieri, R., Doglioni, C., and

Naldini, L. (2007). Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. Blood *109*, 5276–5285. https://doi.org/10.1182/blood-2006-10-053504.

6. Birocchi, F., Cusimano, M., Rossari, F., Beretta, S., Rancoita, P.M.V., Ranghetti, A., Colombo, S., Costa, B., Angel, P., Sanvito, F., et al. (2022). Targeted inducible delivery of immunoactivating cytokines reprograms glioblastoma microenvironment and inhibits growth in mouse models. Sci. Transl. Med. *14*, eabl4106. https://doi.org/10.1126/SCITRANSLMED.ABL4106.

7. Gentner, B., Visigalli, I., Hiramatsu, H., Lechman, E., Ungari, S., Giustacchini, A., Schira, G., Amendola, M., Quattrini, A., Martino, S., et al. (2010). Identification of Hematopoietic Stem Cell-Specific miRNAs Enables Gene Therapy of Globoid Cell Leukodystrophy. Sci. Transl. Med. *2*, 58ra84. https://doi.org/10.1126/scitranslmed.3001522.

8. Costa, B., Fletcher, M.N.C., Boskovic, P., Ivanova, E.L., Eisemann, T., Lohr, S., Bunse, L., Löwer, M., Burchard, S., Korshunov, A., et al. (2021). A set of cell lines derived from a genetic murine glioblastoma model recapitulates molecular and morphological characteristics of human tumors. Cancers (Basel) *13*, 230. https://doi.org/10.3390/cancers13020230.

9. Sockolosky, J.T., Trotta, E., Parisi, G., Picton, L., Su, L.L., Le, A.C., Chhabra, A., Silveria, S.L., George, B.M., King, I.C., et al. (2018). Selective targeting of engineered T cells using orthogonal IL-2 cytokine-receptor complexes. Science *359*, 1037–1042. https://doi.org/10.1126/science.aar3246.

10. Browaeys, R., Gilis, J., Sang-Aram, C., De Bleser, P., Hoste, L., Tavernier, S., Lambrechts, D., Seurinck, R., and Saeys, Y. (2023). MultiNicheNet: a flexible framework for differential cell-cell communication analysis from multi-sample multi-condition single-cell transcriptomics data. Preprint at bioRxiv. https://doi.org/10.1101/2023.06.13.544751.

11. Good, C.R., Aznar, M.A., Kuramitsu, S., Samareh, P., Agarwal, S., Donahue, G., Ishiyama, K., Wellhausen, N., Rennels, A.K., Ma, Y., et al. (2021). An NK-like CAR T cell transition in CAR T cell dysfunction. Cell *184*, 6081–6100.e26. https://doi.org/10.1016/j.cell.2021.11.016.

12. Rossari, F., Birocchi, F., Naldini, L., and Coltella, N. (2023). Gene-based delivery of immune-activating cytokines for cancer treatment. Trends Mol. Med. *29*, 329–342. https://doi.org/10.1016/J.MOLMED.2023.01.006.

13. O'Cearbhaill, R.E., Park, J.H., Halton, E.F., Diamonte, C.R., Mead, E., Lakhman, Y., Kane, P., Riviere, I.C., and Brentjens, R.J. (2020). A phase I clinical trial of autologous chimeric antigen receptor (CAR) T cells genetically engineered to secrete IL-12 and to target the MUC16ecto antigen in patients (pts) with MUC16ecto+ recurrent high-grade serous ovarian cancer (HGSOC). Gynecol. Oncol. *159*, 42. https://doi.org/10.1016/j.ygyno.2020.06.089.

14. Jiang, R., Yang, L., Liu, X., Xu, Y., Han, L., Chen, Y., Gao, G., Wang, M., Su, T., Li, H., et al. (2025). Genetically engineered macrophages reverse the immunosuppressive tumor microenvironment and improve immunotherapeutic efficacy in TNBC. Mol. Ther. *33*, 3339–3359. https://doi.org/10.1016/j.ymthe.2025.03.024.

15. Milani, M., Fabiano, A., Perez-Rodriguez, M., Hernandez, R.J., Zecchillo, A., Zonari, E., Ottonello, S., Basso-Ricci, L., Canepari, C., Volpin, M., et al. (2025). In vivo haemopoietic stem cell gene therapy enabled by postnatal trafficking. Nature *643*, 1097–1106. https://doi.org/10.1038/s41586-025-09070-3.

16. Kerzel, T., Giacca, G., Beretta, S., Bresesti, C., Notaro, M., Scotti, G.M., Balestrieri, C., Canu, T., Redegalli, M., Pedica, F., et al. (2023). In vivo macrophage engineering reshapes the tumor microenvironment leading to eradication of liver metastases. Cancer Cell *41*, 1892–1910.e10. https://doi.org/10.1016/j.ccell.2023.09.014.