



RNA silencing of GM-CSF in CAR-T cells reduces the secretion of multiple inflammatory cytokines

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Abstract

Chimeric antigen receptor T (CAR-T) cell therapy has become a research hotspot in the field of hematological malignancies. However, CAR-T cell therapy can lead to immunotherapy-associated side effects including cytokine release syndrome and neurotoxicity. Gene depletion of GM-CSF in CAR-T cells was found preventive against adverse effects, but additional transfections were required to produce CAR-T cells. In this study, we interrupted GM-CSF expression in CAR-T cells by inserting the GM-CSF shRNA-expression cassette in the CAR vector. Reduction of GM-CSF in CAR-T cells could decrease the level of several proinflammatory cytokines without hampering the killing capacity. The manufacture of GM-CSF knockdown CAR-T cells does not require complicated transfections, which makes it more practical and feasible for clinical application.

Keywords Chimeric antigen receptor T cells · RNA silencing · Cytokine release syndrome · GM-CSF

Introduction

Chimeric antigen receptor T (CAR-T) cell therapy has demonstrated extraordinary potentials in the treatment of acute lymphocytic leukemia and B-cell lymphoma [1, 2]. However, immunotherapy-associated side effects strongly hampered the development of CAR-T cell therapy, as severe complications are life-threatening for patients [3, 4]. Cytokine release syndrome (CRS; manifested by fever, hypotension, hypoxia, and target-organ damage) and neurotoxicity (characterized by headaches, confusion, seizure, and other

neurologic manifestations) are the most representative [3, 4]. Various inflammatory factors were found elevated in serum samples after infusing CAR-T cells; these factors include interleukin-6 (IL-6), interferon- γ (IFN- γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), soluble interleukin-6 receptor (sIL-6R), macrophage inflammatory protein-1 (MCP-1), interleukin-1 (IL-1), etc. [5, 6]. Activated CAR-T cells release various inflammatory cytokines including GM-CSF, which subsequently plays a major role in the stimulation and differentiation of innate monocytes-macrophages lineage [5, 7, 8]. Stimulated monocytes-macrophages massively produce cytokines including IL-6 and IL-1. Inflammatory cascade is thus initiated and leads to CRS and neurotoxicity [6, 8, 9]. Previous studies reported that gene editing of GM-CSF in CAR-T cells could prevent against adverse effects in vitro and in xenograft mouse models without damaging the cytotoxicity of CAR-T cells [8, 10]. However, these studies were conducted using CRISPR/Cas9 or TALENs, which require either a dual transduction of CAR-expressing vector and GM-CSF-gRNA-lentiCRISPRv2 lentiviruses [10] or electroporation of mRNA encoding TRAC TALEN arms and subsequent AAV transfection [8] to prepare GM-CSF^{KO} CAR-T cells. These additional manufacturing procedures might hamper the viability of CAR-T cells. Meanwhile, the knockout efficiency of GM-CSF through double transduction could be unpredictable,

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and additional examinations would be needed. In the present study, we inserted a short hairpin RNA (shRNA)-expression cassette in the CAR vector to reduce GM-CSF secreted by CAR-T cells. Regular manufacturing procedures would suffice to produce GM-CSF^{KD} CAR-T cells and achieve comparable gene silencing efficiency to gene knockout, thereby promoting the clinical application of CAR-T cell therapy.

Materials and methods

Cells and reagents

K562 (ATCC, #CCL-243) and HEK-293T (ATCC, #ACS4500) cells were obtained from ATCC. Cryopreserved human adult purified PBMCs (MT-BIO, #PB010C) were purchased from MT-BIO. The cryopreserved PBMCs were thawed and cultivated in PRIME-XV T cell CDM (Irvine SCIENTIFIC, #91,154) at 1×10^6 /ml supplemented with interleukin-2 (100 IU/ml) to prepare CAR-T cells. Anti-CD3/CD28 Dynabeads (Gibco, #1132D) were added at a bead-to-cell ratio of 1:1 for 24 h to stimulate and expand T cells. The amplified T cells were resuspended at 3×10^5 /ml and transfected with lentiviruses (MOI = 3×10^5 vg/cell) on RetroNectin (Takara, #T100B) pre-coated plates for 72 h. Transfection efficiency was assessed by detecting GFP expression through flow cytometry and the final transfection efficiencies were approximately at 30–50%.

Generation of GM-CSF knockdown CAR-T cells

Four sets of shRNA sequences (Supplementary Table. S1) for GM-CSF silencing were modified to be constructed on the pLVX-shRNA2 plasmid. The knockdown efficiency in K562 cells was compared through Western blot assay before application to CAR-T cells. The shRNA-expressing fraction was then amplified through polymerase chain reaction and inserted into the anti-human CD19 CAR (CAR19) vector. The CAR19 involved in this research contains an anti-human CD19 single-chain variable fragment (scFv), hinge and transmembrane (TM) regions, and intracellular signaling domains including coactivator 4-1BB as well as CD3 zeta. Lentivirus was produced in 293T cells with Lipofectamine 3000 (Thermo Fisher Scientific, #L3000015) and purified using ultrafiltration kit (Millipore, #UFC910096). Transfection was conducted as described above.

Flow cytometry analysis

For CAR19 expression assay, the transfected 293T cells were incubated with diluted biotinylated human CD19 (Acrobiosystems, #CD9-H8259) and then with BV605

Streptavidin (Biolegend, #405,229) following the manufacturer's instruction. Flow cytometry was performed on BD LSR Fortessa X-20 Cell Analyzer. As for other cell surface markers, cells were harvested, washed in Dulbecco's phosphate-buffered saline, and incubated with Human TruStain FcX (Biolegend, #422,302) to block the Fc receptor. Anti-CD3 (Gibco, #555,335, #555,340), anti-CD4 (Gibco, #555,349), anti-CD19 (BD, #562,440), anti-CD14 (Biolegend, #301,804), and anti-CD11b (Biolegend, #101,208) antibodies and Zombie NIR Fixable Viability Kit were added as needed for flow cytometry analyses. All flow cytometric data was analyzed on FlowJo X10.0.7r2.

Enzyme-linked immunosorbent assay

Nalm6 cells were co-cultured with CAR-T cells or mock T cells at 1:1 ratio for 16 h. Cell supernatant was collected for cytokine assays. ELISA kits of human IL6 (Biolegend, #430,504) and GM-CSF (Biolegend, 432,004) were used to detect the concentration of inflammatory cytokines according to manufacturer's instructions.

Cell killing assay

Nalm6 cells were transduced with lentivirus for co-expression of luciferase-zsGreen (Hanbio Biotechnology) beforehand. Nalm6^{Luc+} cells were later co-incubated with CAR-T cells or mock T cells at effector to target (E: T) ratios of 0.25:1, 0.5:1, and 1:1 for 12 h. D-Luciferin (Thermo Scientific, #8829) was added simultaneously to the cell culture. Residual live cells were measured by detecting bioluminescence on a Thermo Scientific Microplate Reader.

Monocyte cocultivation and endotheliocyte activation assay

Total monocytes were isolated from cryopreserved human PBMCs of the same donor by using the MojoSortTM Human Pan Monocyte Isolation Kit (Biolegend, #480,059) immediately after thawing. CD14⁺ monocytes were harvested, and about 2×10^5 was placed at the upper chamber of a 24-well transwell plate (pore size:3.0 μ m), with equal numbers of CAR-T cells and Nalm6 cells cultured in the lower chamber. After 16 h, supernatant was collected for cytokine measurement.

Multi-analyte flow assay

Beads-based multi-analyte flow cytometry analysis was conducted using LEGENDplexTM Human Inflammation Panel 1 (13-plex, Biolegend, #740,809), which included IL-1 β , IFN- α 2, IFN- γ , TNF- α , MCP-1, IL-6, IL-8, IL-10,

IL-12p70, IL-17 A, IL-18, IL-23, and IL-33, to quantify cytokines and chemokines in the supernatant of cocultured cells. Data were analyzed via Legendplex Version 8.

Statistical analysis

Statistical significance was calculated using Student's *t* test on GraphPad Prism. The results were expressed as mean (SD). Two-sided *p*-values < 0.05 were considered statistically significant.

Results

To obtain GM-CSF^{KD}-shRNA-CAR vector, we first inserted the shRNA sequences for GM-CSF knockdown on the pLVX-shRNA2 plasmid (Supplementary Table. S1). We chose four sets of shRNA and compared their knockdown efficiency in K562 cells by Western blot assays. Compared with the scramble shRNA group, GM-CSF^{KD}-shRNA-2 (shGM2) exhibited moderate GM-CSF silencing capacity, while GM-CSF^{KD}-shRNA-4 (shGM4) was the most effective (Supplementary Fig. S1). The shRNA expression cassette of shGM2/shGM4 was inserted into the anti-human CD19 CAR vector (Fig. 1a). The expression levels of FMC63 and GFP were not hampered in the modified CAR vector (Supplementary Fig. S2).

Cryopreserved human adult purified PBMCs were used to produce activated and amplified T cells. The transfected T cells were divided into five groups: mock T cells (GFP⁺), CAR19 T cells (CAR19⁺, GFP⁺), shGM2-CAR19 T cells, shGM4-CAR19 T cells, and scramble-CAR19 T cells (Supplementary Fig. S3). We used Nalm6 cells (a cell line of CD19⁺ B cell acute lymphoblastic leukemia) as target cells to coculture with CAR-T cells or mock T cells for 16 h. Cell supernatant was collected, and enzyme-linked immunosorbent assay (ELISA) experiments were conducted to detect the levels of GM-CSF. The average GM-CSF concentration in the regular CAR19 T supernatant was 25,086.21 ng/ml. The GM-CSF concentrations were 2,815.42 ng/ml in the shGM2-CAR19 T group and 284.22 ng/ml in the shGM4-CAR19 T group (Fig. 1b). The average KD ratio in shGM2-CAR19 T cells and shGM4-CAR19 T cells reached 89% and 98.9%, respectively. Co-expressing GM-CSF^{KD} shRNA in CAR-T cells proved to be an efficient method to reduce GM-CSF secretion.

To evaluate the killing efficacy of GM-CSF^{KD} CAR-T cells, we used Nalm6 cells marked with luciferase-zsGreen (Nalm6^{Luc+} cells) as target. In luciferase-based killing assays, CAR-T cells or mock T cells were added at effector to target (E: T) ratios of 0.25:1, 0.5:1, and 1:1 for 12 h. The killing efficiency was significantly higher in all CAR-T groups than

in mock T cells (Fig. 1c). CAR-T cells with different levels of GM-CSF secretion exhibited comparable killing capacity. We indicated that the cytotoxicity of CAR19 T cells was not affected by the reduction of GM-CSF.

To mimic the complicated interplay of immunocytes and inflammatory factors, we used a transwell co-culture system containing mock T/regular/GM-CSF^{KD} CAR-T cells, Nalm6 cells, and monocytes (Fig. 1d). The concentration of GM-CSF in the supernatant of the mock T group was significantly lower than that of CAR19 T cells, regardless of monocyte cocultivation (Fig. 1e). CAR-T cells were proved to be the main source of elevated GM-CSF in CAR-T cell therapy. IL-6 was evidently elevated in the cocultivation of CAR19 T cells, Nalm6 cells, and monocytes (Fig. 1e). GM-CSF KD in CAR19 T cells could alleviate IL-6 approximately to basic secretion (Fig. 1e). We then performed multi-analyte flow assay to assess the profile of inflammatory factors. By comparing secreted cytokines in activated CAR-T cells without participations of CD14⁺ cells, we noticed that IFN- γ , TNF- α , IL-17 A, MCP-1 and IL-8 were reduced simultaneously with the knockdown of GM-CSF (Fig. 1f). Although a slight raise of IL-6 secretion from GM-CSF^{KD} CAR19 T cells was observed, the majority of IL-6 was secreted upon myeloid cells mediation. The overall production of IL-6 was lower in GM-CSF^{KD} CAR19 T cells. Besides, IFN- γ , TNF- α , IL-17 A, MCP-1, IL-8 and IL-10 were firstly produced by activated CAR-T cells, and elevated when cocultured with monocytes (Fig. 1f). We confirmed that the inflammatory cytokine cascade was initiated at the encounter of CAR-T cells and target cells, while the outburst was mediated by monocytes and macrophages. The supernatant of GM-CSF^{KD} CAR19 T cells cocultured with Nalm6 cells and monocytes exhibited lower levels of IL-6, IL-8, IL-10 MCP-1, IFN- γ , TNF- α , and IL-17 A (Fig. 1f). GM-CSF^{KD} CAR19 T cells have the potential to reduce the extent of CRS/CRES by alleviating the release of pro-inflammatory factors.

Discussion

Antagonizing CRS biomarkers have been the main strategy in the management of immune-related syndromes. IL-6 receptor blockage with tocilizumab is widely employed in patients with CRS [4, 11]. Our group previously found that the blockage of TNF- α and IL-1 β signaling could ameliorate endothelial activation in CAR-T cell therapy [12]. The IL-1 receptor antagonist anakinra managed to protect from CRS and neurotoxicity in vivo [6, 13]. Compared with blocking downstream cytokines, the prevention of immune-related adverse effects is also of great importance. Recent studies set monocytes–macrophages as key mediators and

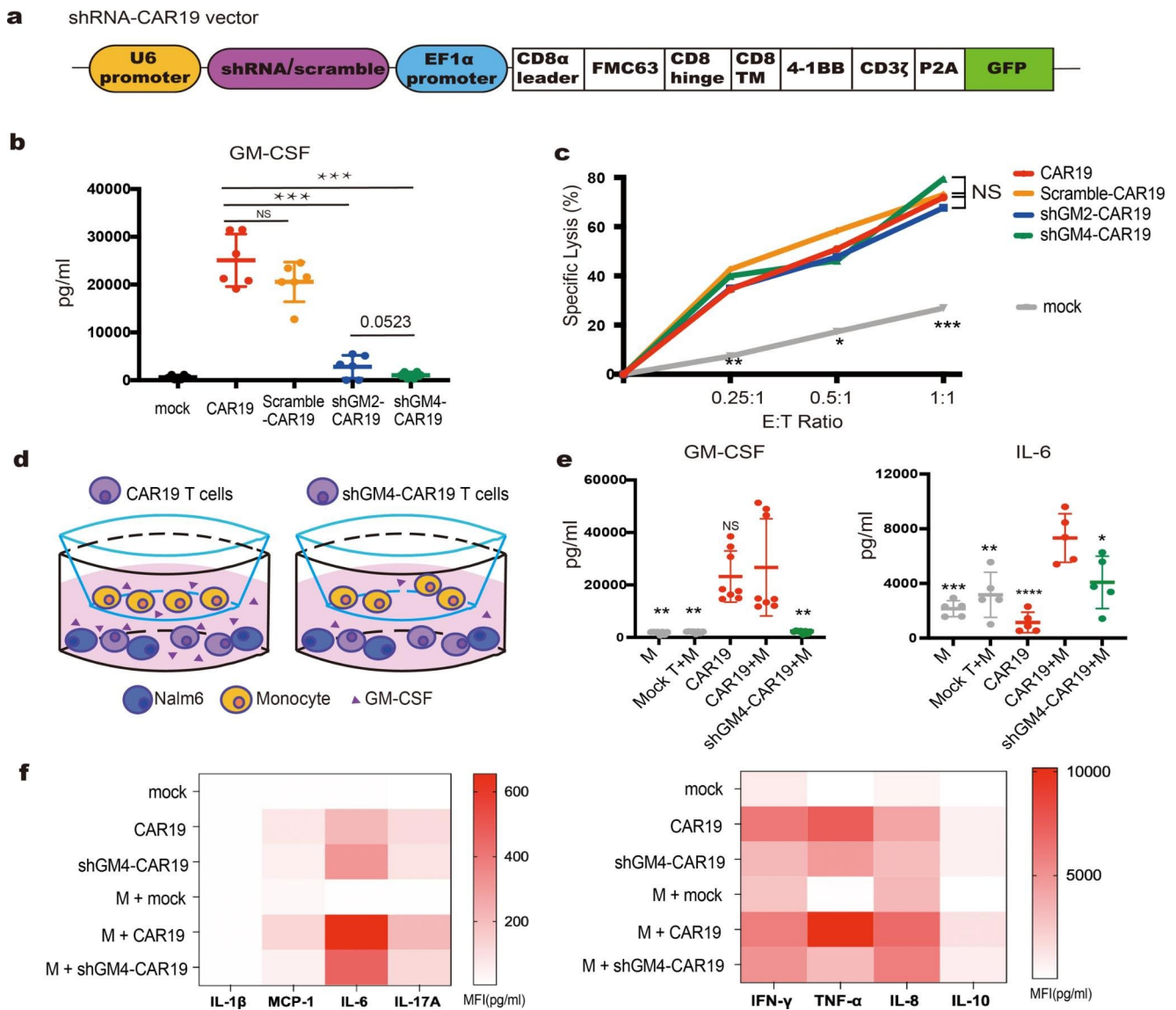


Fig. 1 Characterization and effects of GM-CSF^{KD} CAR-T cells. (a) Schematic presentation of the GM-CSF^{KD}-shRNA-CAR19 construct used in this study. (b) CAR-T cells (or mock T cells) were cocultured with CD19⁺ Nalm6 cells for 16 h. Concentration of GM-CSF in the supernatant was analyzed by ELISA. (c) Cytotoxicity of conventional CAR19 T cells and GM-CSF^{KD} CAR19 T cells were compared 12 h after cocultivation with Nalm6^{Luc+} cells. (d) Diagram of the space division of CAR-T, Nalm6 and CD14⁺ cells cocultured in transwell (pore size=0.3 μ m). (e) Concentration of GM-CSF (left) and IL-6 (right)

in the supernatant collected after coculturing CAR-T/Nalm6/ CD14⁺ cells for 16 h assessed by ELISA. (f) Cytokine profiles of co-cultured supernatants were measured by multi-analyte flow assay. The concentrations of IFN- α 2, IL-12p70, IL-18, IL-23, and IL-33 were below the minimum value and not shown in this figure. (Each experiment was repeated for at least three times, and the results were expressed as mean (SD)). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns: not significant)

GM-CSF as messenger in CRS and neurotoxicity [6, 8]. Monocyte depletion contributed to prevention from CRS, but it would also impede leukemia clearance in xenograft models [6]. Inhibiting GM-CSF and hence weakening the circuit between CAR-T cells and monocytes have been inspiring. GM-CSF neutralization with lenzilumab and GM-CSF knockout in CAR-T cells did not hamper their anti-tumor effects [8, 10, 14]. Recent study revealed that GM-CSF knockout with CRISPR/Cas9 could ameliorate

early activation, contribute to proliferation, and exhibit anti-tumor functions in CAR-T cells [15]. Here, we demonstrated that GM-CSF^{KD} CAR-T cells possess the potential to protect from immune-related adverse effects by reducing the secretion of multiple inflammatory cytokines including not only GM-CSF, but also IL-6, IFN- γ , TNF- α , IL-17 A, MCP-1, IL-8 and IL-10. Among them, moderate decrease of IFN- γ and TNF- α doesn't affect the overall cytotoxicity of GM-CSF^{KD} CAR-T cells. IFN- γ and TNF- α were found

associated with endothelial damage caused by CAR-T cells [16] and held responsible for a lethal cytokine shock in a COVID-19 mouse model [17]. Neutralization of TNF- α with adalimumab could ameliorate endothelial activation without hampering killing capacity in CAR-T cell therapy [12]. GM-CSF^{KD} CAR-T cells possess great potential in developing CRS free CAR-T cells. At the same time, the efficacy and toxicities of the CAR-T cells in vivo are susceptible to the interplay with the innate immune system. We still need to perform further experiments on suitable animal models for verifying the advantages of GM-CSF^{KD} CAR-T cells in the future studies.

In conclusion, we introduced a new method to reduce GM-CSF secretion from CAR-T cells by inserting the GM-CSF shRNA-expression cassette in the CAR vector. This approach can be applied on any newly-developed CAR construct. The manufacture of GM-CSF^{KD} CAR-T cells does not require repeated transfection, and the knockdown efficiency is more predictable and controllable. Hence, the method is practical and feasible for clinical application.

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Author contributions All authors contributed to the study conception and design. Siqi Shang performed experiments, analyzed data, and wrote the first draft of the manuscript. Yunshuo Chen, Xuejiao Yang and Ying Yang assisted with experiments and contributed to statistical analysis. Yueying Wang and Wenbo Wang supervised the study. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

Ethics approval This study was conducted in vitro without involving any human participants or animal subjects. Hence no ethical approval is required.

Consent to participate This study was conducted in vitro without involving any human participants or animal subjects. Hence informed consent is not required.

Consent to publish This study was conducted in vitro without involv-

ing any human participants or animal subjects. Hence consent from participants is not required.

References

1. Yan Z, Cao J, Cheng H, Qiao J, Zhang H, Wang Y et al (2019) A combination of humanised anti-CD19 and anti-BCMA CAR T cells in patients with relapsed or refractory multiple myeloma: a single-arm, phase 2 trial. [https://doi.org/10.1016/s2352-3026\(19\)30115-2](https://doi.org/10.1016/s2352-3026(19)30115-2). *The Lancet Haematology*
2. Locke FL, Miklos DB, Jacobson CA, Perales MA, Kersten MJ, Oluwole OO et al (2022) Axicabtagene Ciloleucel as Second-Line therapy for large B-Cell lymphoma. *N Engl J Med* 386(7):640–654. <https://doi.org/10.1056/NEJMoa2116133>
3. Karschnia P, Jordan JT, Forst DA, Arrillaga-Romany IC, Batchelor TT, Bachring JM et al (2019) Clinical presentation, management, and biomarkers of neurotoxicity after adoptive immunotherapy with CAR T cells. *Blood* 133(20):2212–2221. <https://doi.org/10.1182/blood-2018-12-893396>
4. Brudno JN, Kochenderfer JN (2016) Toxicities of chimeric antigen receptor T cells: recognition and management. *Blood* 127(26):3321–3330. <https://doi.org/10.1182/blood-2016-04-703751>
5. Teachey DT, Lacey SF, Shaw PA, Melenhorst JJ, Maude SL, Frey N et al (2016) Identification of predictive biomarkers for Cytokine Release Syndrome after chimeric Antigen receptor T-cell therapy for Acute Lymphoblastic Leukemia. *Cancer Discov* 6(6):664–679. <https://doi.org/10.1158/2159-8290.CD-16-0040>
6. Norelli M, Camisa B, Barbiera G, Falcone L, Purevdorj A, Genua M et al (2018) Monocyte-derived IL-1 and IL-6 are differentially required for cytokine-release syndrome and neurotoxicity due to CAR T cells. *Nat Med* 24(6):739–748. <https://doi.org/10.1038/s41591-018-0036-4>
7. Vico T, Youssif C, Zare F, Comalada M, Sebastian C, Lloberas J et al (2022) GM-CSF protects macrophages from DNA damage by inducing differentiation. *Cells* 11(6). <https://doi.org/10.3390/cells11060935>
8. Sachdeva M, Duchateau P, Depil S, Poirot L, Valton J (2019) Granulocyte-macrophage colony-stimulating factor inactivation in CAR T-cells prevents monocyte-dependent release of key cytokine release syndrome mediators. *J Biol Chem* 294(14):5430–5437. <https://doi.org/10.1074/jbc.AC119.007558>
9. Giavridis T, van der Stegen SJC, Eyquem J, Hamieh M, Piersigilli A, Sadelain M (2018) CAR T cell-induced cytokine release syndrome is mediated by macrophages and abated by IL-1 blockade. *Nat Med* 24(6):731–738. <https://doi.org/10.1038/s41591-018-0041-7>
10. Sterner RM, Sakemura R, Cox MJ, Yang N, Khadka RH, Forsman CL et al (2019) GM-CSF inhibition reduces cytokine release syndrome and neuroinflammation but enhances CAR-T cell function in xenografts. *Blood* 133(7):697–709. <https://doi.org/10.1182/blood-2018-10-881722>
11. Santomasso BD, Nastoupil LJ, Adkins S, Lacchetti C, Schneider BJ, Anadkat M et al (2021) Management of Immune-Related adverse events in patients treated with chimeric Antigen receptor T-Cell therapy: ASCO Guideline. *J Clin Oncol* 39(35):3978–3992. <https://doi.org/10.1200/JCO.21.01992>
12. Chen Y, Li R, Shang S, Yang X, Li L, Wang W et al (2021) Therapeutic potential of TNF α and IL1 β Blockade for CRS/ICANS in CAR-T therapy via ameliorating endothelial activation. *Front Immunol* 12:623610. <https://doi.org/10.3389/fimmu.2021.623610>
13. Wehrli M, Gallagher K, Chen YB, Leick MB, McAfee SL, El-Jawahri AR et al (2022) Single-center experience using anakinra

- for steroid-refractory immune effector cell-associated neurotoxicity syndrome (ICANS). *J Immunother Cancer* 10(1). <https://doi.org/10.1136/jitc-2021-003847>
14. Yi Y, Chai X, Zheng L, Zhang Y, Shen J, Hu B et al (2021) CRISPR-edited CART with GM-CSF knockout and auto secretion of IL6 and IL1 blockers in patients with hematologic malignancy. *Cell discovery* 7(1):27. <https://doi.org/10.1038/s41421-021-00255-4>
 15. Cox MJ, Manriquez Roman C, Tapper EE, Siegler EL, Chappell D, Durrant C et al (2022) GM-CSF disruption in CART cells modulates T cell activation and enhances CART cell anti-tumor activity. *Leukemia* 36(6):1635–1645. <https://doi.org/10.1038/s41375-022-01572-7>
 16. Sun Y, Wang S, Zhao L, Zhang B, Chen H (2019) IFN-gamma and TNF-alpha aggravate endothelial damage caused by CD123-targeted CAR T cell. *Onco Targets Ther* 12:4907–4925. <https://doi.org/10.2147/OTT.S205678>
 17. Karki R, Sharma BR, Tuladhar S, Williams EP, Zalduondo L, Samir P et al (2021) Synergism of TNF-alpha and IFN-gamma triggers inflammatory cell death, tissue damage, and Mortality in SARS-CoV-2 infection and cytokine shock syndromes. *Cell* 184(1):149–168e17. <https://doi.org/10.1016/j.cell.2020.11.025>

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