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Modulation of both tumor & T cell apoptosis to enhance CAR-T immunotherapy

Marco Ruella

CAR-T cell immunotherapy is leading to outstanding clinical results, but only one-third of patients have long-term responses in mature lymphomas. Resistance to apoptosis in cancer cells is a key feature of CAR-T immunotherapy failure, and strategies to enhance tumor apoptosis during CAR-T therapy lead to better tumor control. This article will highlight the importance of apoptosis in both cancer cells and CAR-T cells in driving response to CAR-T immunotherapy, and describes potential strategies to overcome resistance.

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BUILDING UPON THE SUCCESS OF CAR-T THERAPY

CAR-T cell therapy has now seen tremendous success in the clinic for certain cancers. Anti-CD19 CAR-Ts have been particularly successful against B cell leukemia and lymphoma, with promising results leading to the approval of the first CART19 product, tisagenlecleucel, by the FDA. Registrational trials for tisagenlecleucel show that the outcomes are excellent for many patients with relapsed/refractory disease [1,2]. However, many patients do not see long-term benefit due to either relapse or lack of response. An important focus for researchers is therefore to understand CAR-T cell treatment pitfalls and to build upon current therapies via novel approaches.

Broadly, etiologies of CAR-T cell therapeutic failures can be broken down into pre-infusion barriers (e.g. lymphocyte collection failure, manufacturing failure, early-disease

progression, access, and cost) and post-infusion factors (e.g. toxicity or CAR T-cell biological failure). Biological factors contributing to CAR-T cell failure consist of three main categories: so-called CAR dysfunction, immunosuppressive tumor microenvironment, other host factors, and tumor intrinsic mechanisms.

RATIONALE FOR TARGETING APOPTOSIS TO IMPROVE RESPONSE

One strategy to identify key tumor genes involved in CART19 resistance is to utilize a functional genomics screen. To do this, the Brunello CRISPR library was used to engineer the $CD19^+$ B cell acute lymphoblastic leukemia (B-ALL) cell line, NALM6, allowing for single gene knockout (KO) per discrete leukemia cell. This Brunello lentiviral library transduced cell line was enriched using puromycin, purifying the leukemic pool to ensure each cell represented one of ~18,000 gene KOs. The gene-edited cells were then incubated with CART19 for short- and long-term co-cultures. The surviving leukemic cells were harvested to define the significantly enriched or depleted gene KOs.

Enriched gene KOs in leukemic cells were universally involved in the extrinsic apoptotic pathway, including CASP8, BID, FADD, and TNFRSF10B. Depleted gene KOs were of interest as they are negative regulators of apoptosis – so if depleted, would lead to CAR-T-sensitized apoptosis.

These results suggested that extrinsic apoptosis may be a key mechanism for resistance to CAR-T cells. The next step was to prove this in the laboratory and correlate in the clinical setting.

IN VIVO & *IN VITRO* EXPERIMENTS

In vivo and *in vitro* experiments were carried out, with a focus on BID and FADD KO. As shown in Figure 1, FADD KO in leukemia resulted in greater CART19 apoptosis resistance with earlier tumor progression compared to controls. Similarly, using a BID KO, tumor progression again occurs much faster than controls, and in both cases there is a clear difference in overall survival – the KO mice die earlier than the controls.

However, it is not clear why other mechanisms available to the T cells, that in theory should still induce tumor apoptosis, fail to overcome these defects in extrinsic apoptosis. For example, the perforin and granzyme axis does not rely on extrinsic pathway apoptosis signaling, but rather on an intrinsic mediated apoptosis.

Understanding how T cells function while interacting with tumor cells that are intrinsically apoptosis resistant is important for characterizing CAR T-cell dysfunction. CAR-T cells co-cultured with tumor cells that fail to die become progressively dysfunctional, and no longer proliferate (Figure 2). At the same time, they also ceased production of perforin and show limited production of granzyme. When RNA sequencing analysis of these T cells was carried out, it was observed that a number of exhaustion and dysfunction factors were enriched such as BTLA, TIGIT, and CTLA4.

CLINICAL VALIDATION

Following promising results in the laboratory, clinical validation was sought via studying the RNA expression in leukemic blasts of CART19 treated patients enrolled in ELIANA, a registrational trial for pediatric B-ALL. As seen in Figure 3A, there is a clear trend – patients without complete response at day 28 have diminished expression of the pro-apoptotic factors in the extrinsic pathway. This is similar to what was observed in the model discussed above.

Utilizing a scoring system based on RNA expression of the extrinsic pathway signaling (death receptor signature), the patients who have low expression (low score) of pro-apoptotic factors have a very poor prognosis (Figure 3B). As previously noted, CAR-T cells tend to develop exhaustion when they are

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FIGURE 1

Loss of death receptor signaling enables resistance to CART19.

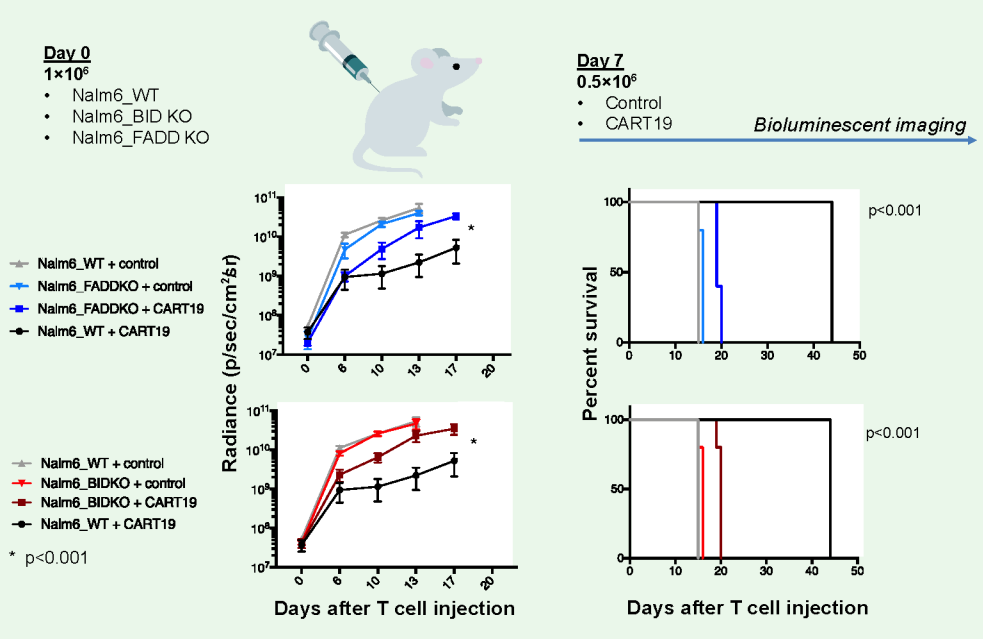

Figure reproduced from [3].

exposed to tumor cells that cannot undergo apoptosis. This was demonstrated in the clinic; CAR-T cell expansion in patients with a low score was much lower as compared to controls. A similar result was seen for persistence, indicating that these CAR-T cells do not perform as well as ones encountering a tumor that can be killed. To confirm this,

FIGURE 2

BID or FADD KO leukemia leads to CAR-T dysfunction.

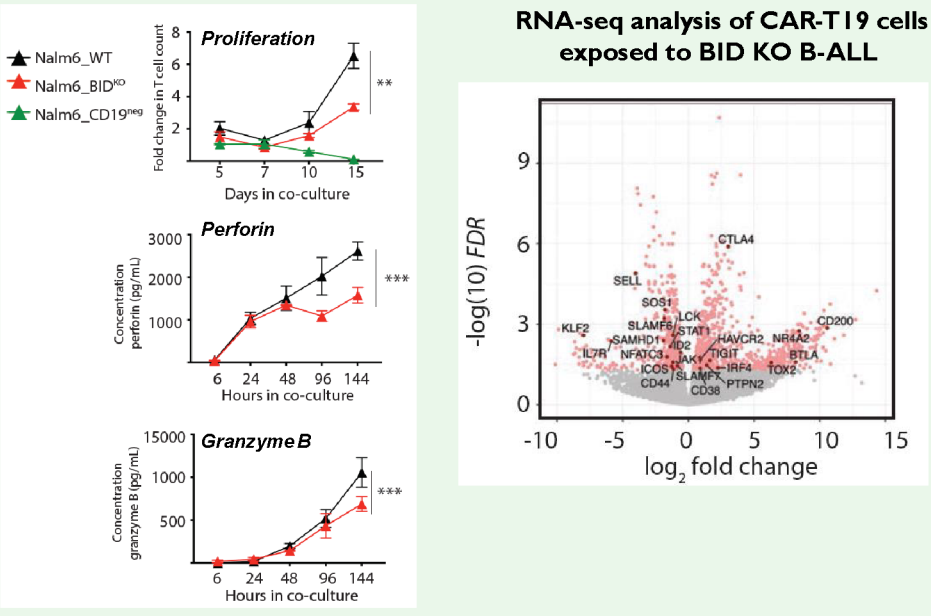

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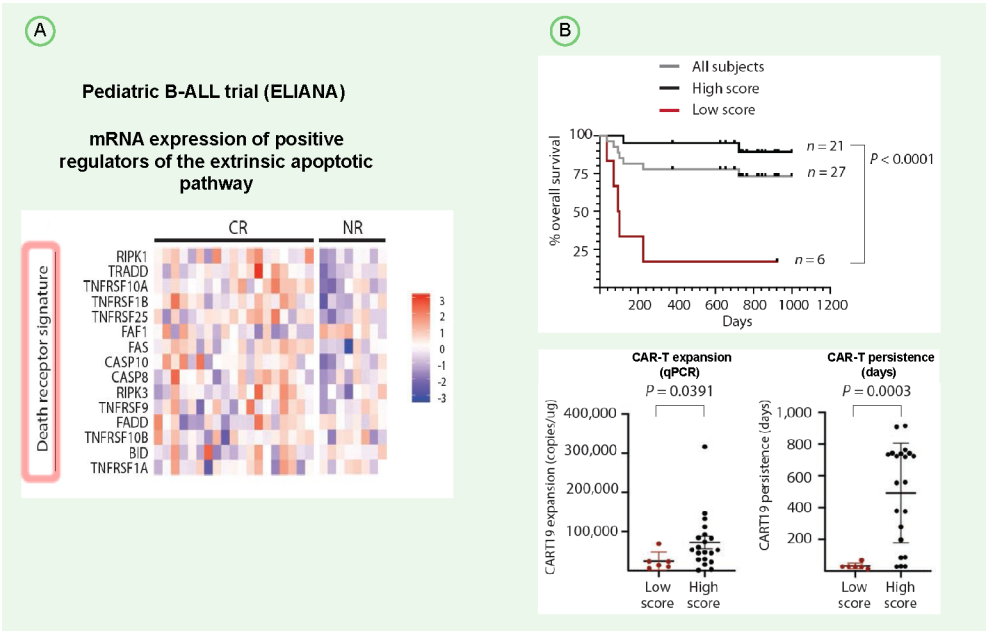
FIGURE 3

Extrinsic apoptosis and CAR-T outcomes in B-ALL.



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single-cell RNA sequencing was performed and a scoring system used to quantify dysfunction of the T cell. In patients who were non-responders, this revealed high numbers of exhausted T cells [Data not shown].

Taken together, these findings led to the hypothesis that these issues can be overcome using small molecules that can stimulate or sensitize apoptosis in cancer cells.

SMALL MOLECULE SCREENING TO ENHANCE CAR-T IMMUNOTHERAPY

To identify small molecules that could lower the apoptotic threshold during a T-cell-tumor interaction, a library of pro-apoptotic small molecules was screened with co-cultured CART19 and NALM6. Inhibitors of apoptosis proteins (IAP) inhibitors and BCL-2 inhibitors are two categories of small molecules found to enhance apoptosis in tumor cells under CART attack. IAP inhibitors, also termed SMAC mimetics due to SMAC's natural inhibition of IAPs, demonstrated the highest degree of synergy with CART. The lead compound, birinapant, has been tested in the clinical setting as an independent standalone therapy, and in combination. The lead BCL-2 antagonist, which is FDA-approved, is venetoclax. Both compounds were investigated to explore their potential for enhancing CAR-T immunotherapy.

SMAC mimetics

When tested *in vitro*, birinipant significantly enhanced CAR-T killing of cancer cells from 20% (vehicle control) to 60% (Figure 4). In a solid tumor ovarian cancer model using a HER2 CAR-T a similar trend is observed.

However when tested *in vivo*, despite an early trend of improved efficacy, the combination of birinapant and CAR-T showed progression compared to tumor controls treated with the CAR-T cell alone. To understand this disappointing result, CAR-T cell expansion was analyzed in peripheral blood, identifying a significant decrease in CAR-T cells when treated with birinapant compared to vehicle, indicating toxicity to the T cells. Further work is being carried out with the aim of overcoming this issue.

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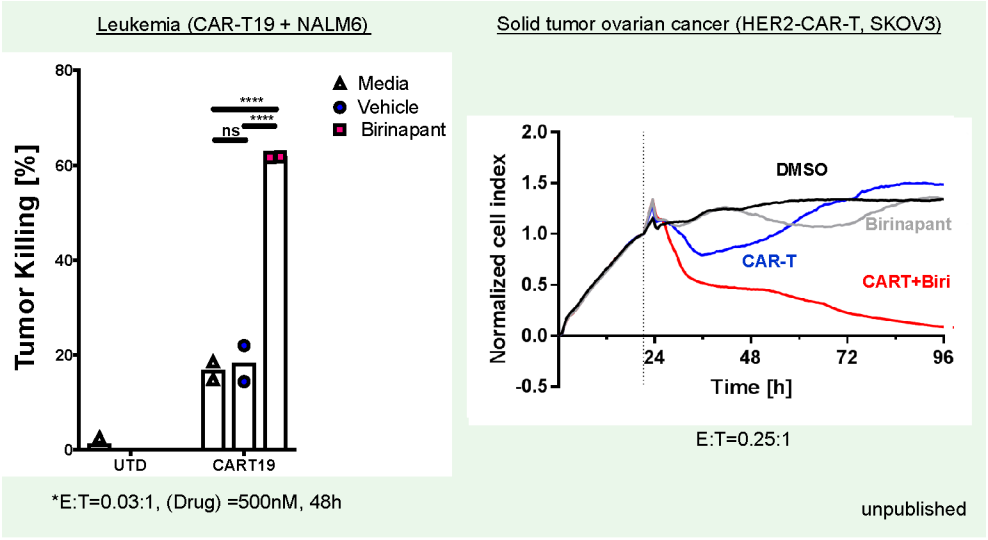
FIGURE 4

SMAC mimetics enhance CAR-T cell killing *in vitro*.



BCL-2 inhibition

Venetoclax is an FDA-approved agent used for a variety of indications in the clinic including leukemias and lymphomas. Immune deficient mice engrafted with three human cell lines (OCI-Ly18, MINO and NALM6) were treated with CART19 in combination with either a vehicle or venetoclax, administered five times a week via oral gavage. An untransduced T cell (UTD) control was also performed.

Starting with the venetoclax-sensitive model (OCI-Ly18), results were promising with the combination of CART19 and venetoclax

FIGURE 5

BCL-2 inhibition and CAR-T in venetoclax-sensitive lymphomas.

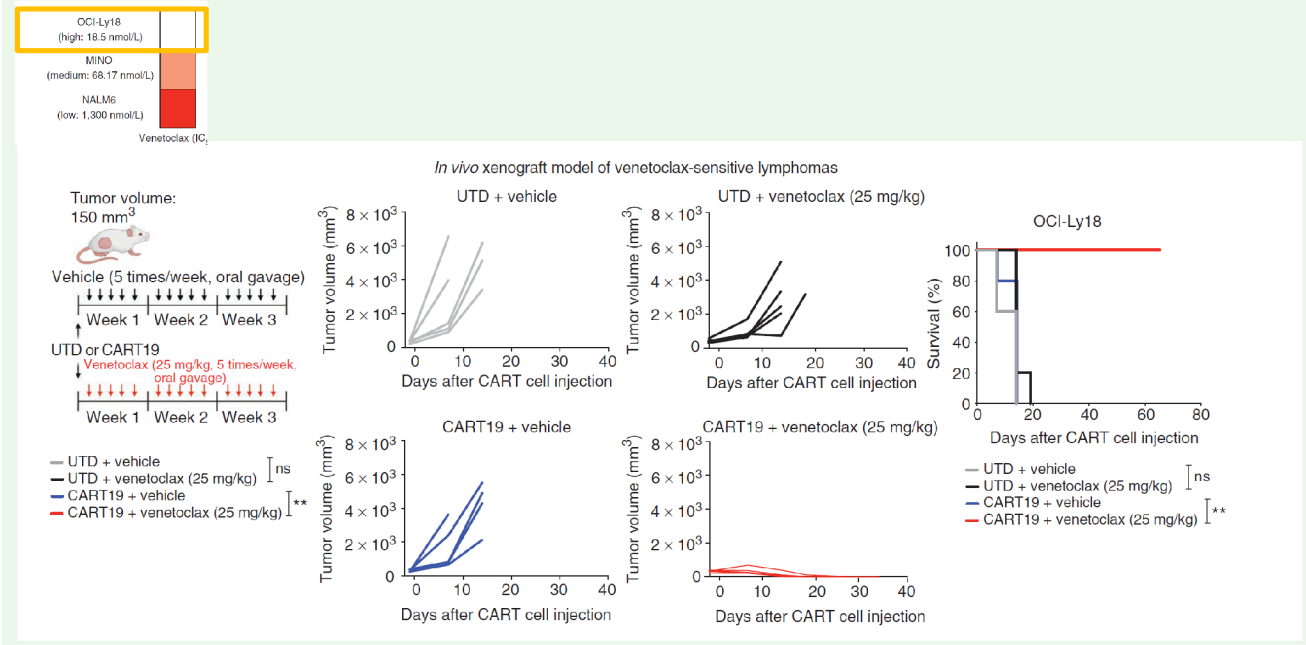


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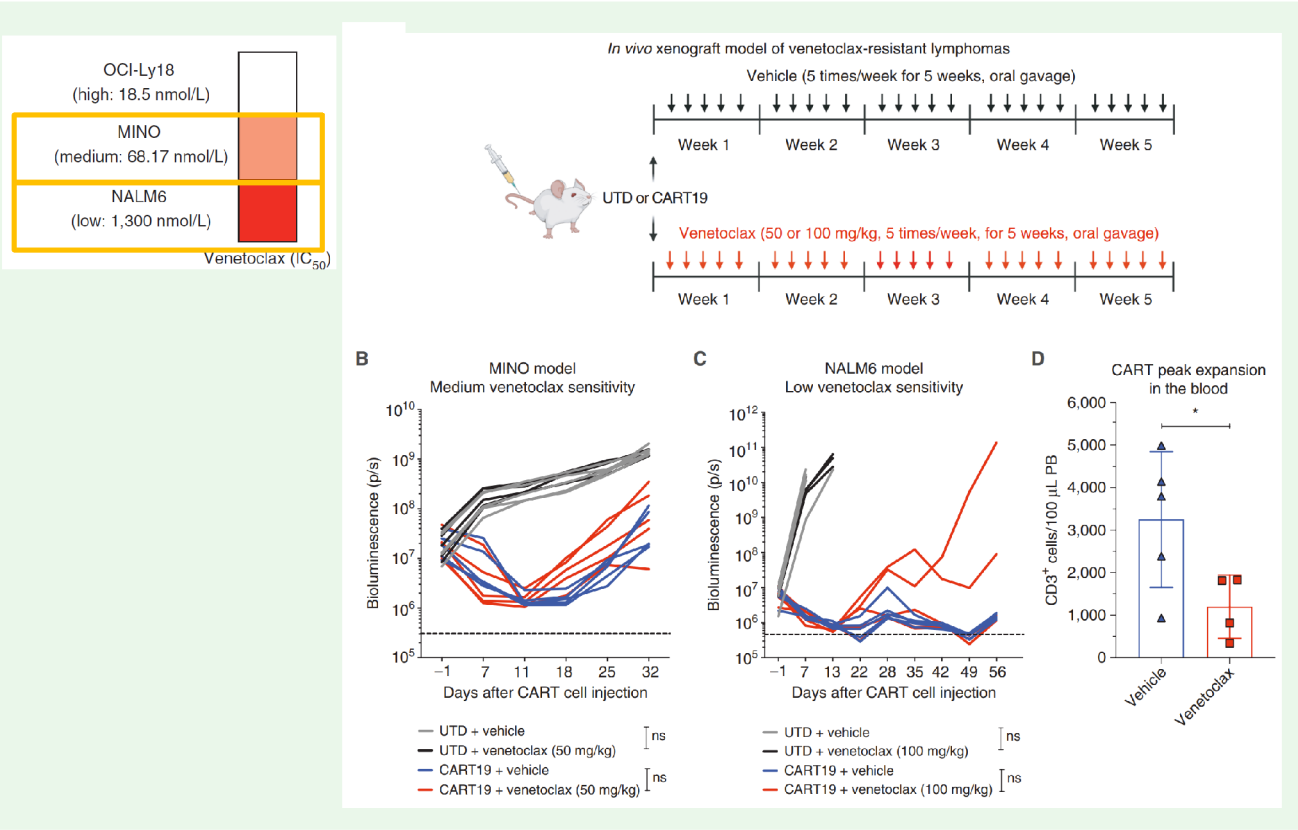
FIGURE 6

BCL-2 inhibition and CAR-T in venetoclax-resistant lymphomas.



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at 25 mg/kg – all mice were in complete remission and this translated to an advantage in order of survival (Figure 5).

For the models that are more resistant to venetoclax (MINO and NALM6), a similar experimental design was used, with higher doses of venetoclax administered to account for this resistance. When using a higher dose, toxicity was observed, and single agent CART19 performed better than

FIGURE 7

Potent synergy of CAR-T19-BCL2F104L with venetoclax.

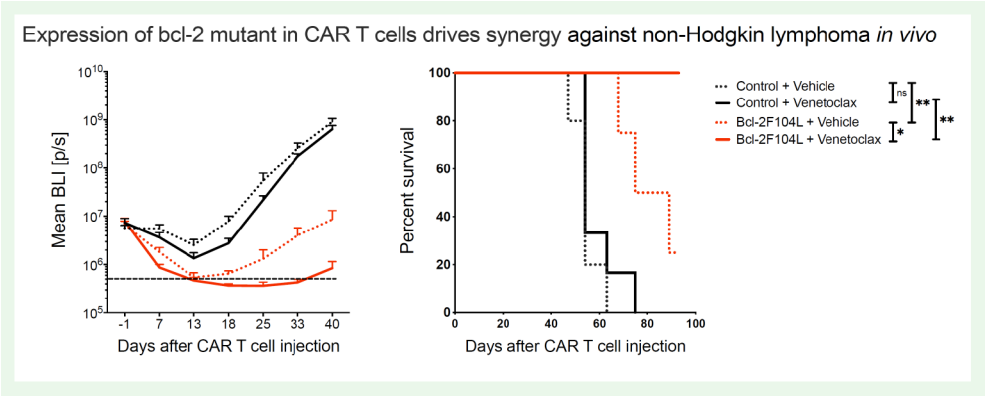

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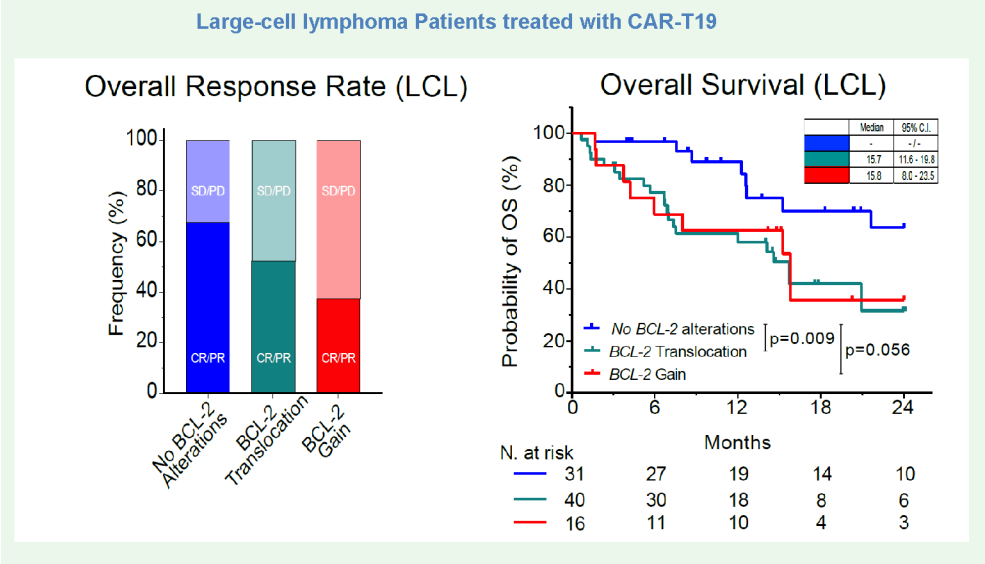
FIGURE 8

BCL-2 inhibition and CAR-T in lymphoma.



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the combination (Figure 6). As with birinapant, mice that were treated with CART19 plus a pro-apoptotic agent showed defects in CART19 expansion assessed in peripheral blood.

Based on these results, our aim was to widen an otherwise narrow therapeutic window in which these two agents could be combined efficaciously. It was hypothesized that apoptotic-resistant CAR-T cells could be developed in order to combine them with pro-apoptotic agents.

To resist venetoclax, cancers acquire and enrich for certain mutations, such as the mutant form of BCL-2 (F104L), that might also allow for resistance in CAR-T cells. This concept was then moved to *in vivo* models and a synergistic effect of CART19 combined with venetoclax was observed, along with a significant effect on overall survival (Figure 7).

Finally, in a large cohort of patients with large cell lymphoma with translocation or gain of BCL-2 showed reduced complete responses as compared to patients with non-BCL-2 alteration, and these differences in overall response rate correlate with a very clear difference in overall survival (Figure 8).

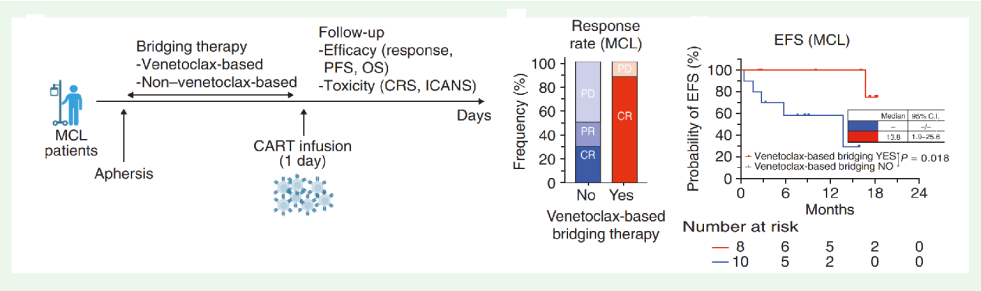
VENETOCLAX AS A BRIDGING THERAPY & OVER-EXPRESSION OF BCL-2

To find further evidence supporting the potential synergy of venetoclax and CAR-T, a good clinical scenario is mantle cell lymphoma patients who are treated with venetoclax as a bridging therapy prior to CAR-T cell therapy. Venetoclax-based bridging therapy was compared to non-venetoclax-based bridging therapy to explore if priming the tumor with venetoclax might lead to a differential effect. Results from a small group of 18 patients showed that patients receiving venetoclax-based bridging therapy had a very high rate of complete response as compared to those who did not receive venetoclax. These differences in response rates also translated to a difference in event-free survival (Figure 9).

Wild-type over-expression of BCL-2 in CAR-T cells also led to an improvement of CAR-T cell function, albeit not as significantly as with the mutation in combination with venetoclax [Data not shown]. This beneficial effect was explored further via a proliferation assay *in vitro* that demonstrated a significant increase in the persistence of CAR-T cells over-expressing wilde-type BCL-2.

FIGURE 9

Venetoclax bridging, response and event-free survival.



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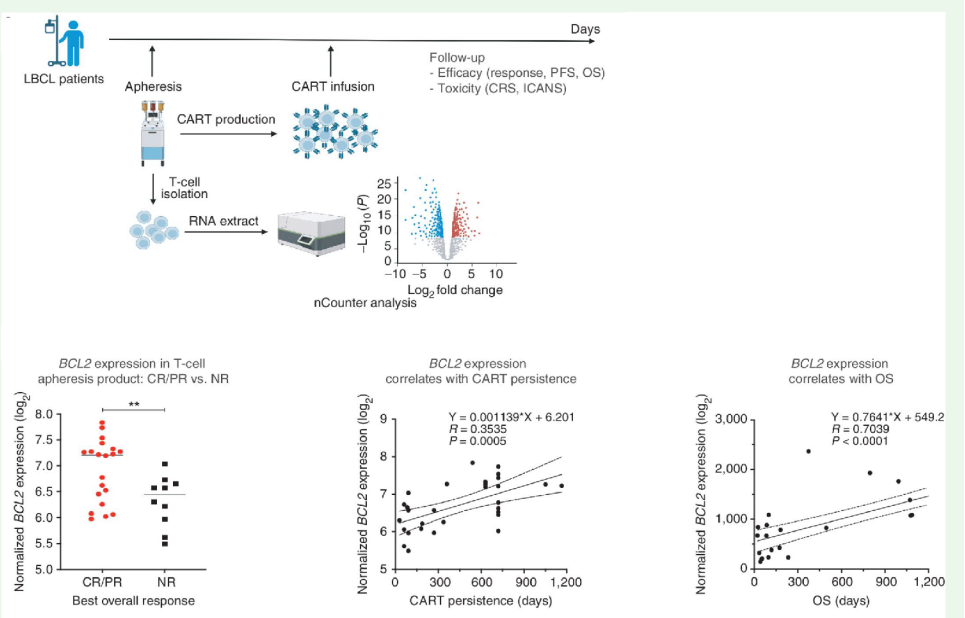
To validate this observation with a clinical-correlate, we utilized a biobank of CART19 apheresis products to ask the question "can we see a differential expression of BCL-2 in these products, and do these differences correlate with outcomes?" This was done via a collaboration with Nanostring in which T cells were isolated from the apheresis product, RNA was extracted, and then studied using the nCounter® CAR-T Characterization Panel run on the NanoString nCounter® Analysis System. One of the top genes that was expressed in patients with complete response as compared to lack of response was BCL-2. BCL-2 expression in complete responders and partial responders as compared to non-responders, with higher expression in the complete and partial responses can be seen in Figure 10. In addition, BCL-2 expression correlated with CAR-T cell persistence, showing a clear and direct correlation between the levels of BCL-2 in the T-cells and both their persistence in patients, and overall survival.

FIGURE 10

BCL-2 expression in CART19 apheresis and outcomes.



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TRANSLATION INSIGHT

To summarize:

- Using unbiased screening assays, apoptosis was identified as a key mechanism for cancer resistance to CAR-T immunotherapy
- In particular, reduction of pro-apoptotic factors in the extrinsic pathway leads to resistance in B-ALL, while in NHL the BCL-2 pathway plays a major role
- Small molecules against IAPs or BCL-2 lead to enhanced killing at short term but drive CAR-T cell apoptosis over time
- A strategy was devised to make CAR-T resistant to BCL-2 inhibition and lead to synergy when combined with venetoclax

In terms of future directions, we plan to extend this approach to solid cancer and further test its safety.

Q&A

Róisin McGuigan, Editor, BioInsights speaks to (pictured) **Marco Ruella, Assistant Professor of Medicine, Scientific Director Lymphoma Program, Division of Hematology and Oncology and Center for Cellular Immunotherapies, University of Pennsylvania**

Q What is the most promising combination of pro-apoptotic small molecule and CAR-T?

MR: The challenge with combining small molecules with CAR-T cell therapy is that the small molecule can be toxic to the CAR-T cells. We showed that with both SMAC, mimetics such as birinapant, and BCL-2 antagonists, such as venetoclax. Based on our data, a safe way to combine apoptotic small molecules with CAR-T cells would have to include a modification of the CAR-T cell to make them resistant to the toxicity of the small molecule. In that regard, I do think the combination with venetoclax is the most promising one, as venetoclax is an FDA-approved agent with clear activity in both lymphoid and myeloid malignancies. But again we need protection for the CAR-T cells to ensure they don't die when they are administered with small molecules.

Q Can you expand on the possible clinical translation of the findings you outlined?

MR: There are two things we are investigating. Firstly, I showed the results in eighteen patients who were treated with Venetoclax as a bridging therapy before CAR-T cell

therapy. In this case you don't have overlap between the administration of the small molecule and the CAR-T cells. There were some signals that the bridging therapy as a way of priming the tumor with venetoclax would be beneficial for those patients. We would like to expand this retrospective core to see if the pre-exposure before CAR-T of patients with a small molecule can prime the tumor for better anti-tumor effect of the CAR-T cell. That is a possible clinical translation that is essentially short, because CAR-T cells are available and venetoclax is available, it's just about using it as a bridging strategy.

The second option would be more of an experimental one where we would need to use a product that would express the construct that we describe – BCL-2 mutation – and I've shown that the BCL-2 over-expression gives a stronger function to the T-cells. We could get a dual effect, the first one would be that the CAR-T cell *per se* would be better because they over-expressed BCL-2, it doesn't matter whether mutated or un-mutated, and the second advantage would be that you could combine that with venetoclax. This is something that we are working on, and we want to get more data on the safety of such an approach before moving forward.

Q Have you tested these combinations in solid cancers, and if not, what would be the challenges?

MR: We think that apoptosis is obviously also very relevant or the killing in solid cancer. We are still trying to figure out what pathway is the predominant one. We know that second mitochondrial activator of caspases (SMAC) mimetics do work in solid cancers. There are some initial clinical signals, and in our hands SMAC mimetics can enhance the killing of solid cancer with CAR-T cells, but again we would need to define a strategy that allows the CAR-T cell to be protected from these drugs. We are testing several ones, and it is somewhat complicated.

With the venetoclax approach we are a little bit behind from the point of view in solid cancer. I think there could be room for it. Although venetoclax doesn't have a single agent activity in solid cancer, it might be able to enhance the killing of CAR-T cells. So this is to be determined, but I think this is an option that will be effective in solid cancer. It's just a case of identifying the right small molecule for the right type of cancer.

Q What is the possible clinical toxicity of this approach?

MR: One issue could be that you are now using a small molecule with a CAR-T cell, so you can have the toxicity of the CAR-T cell, which we are very aware of, and the toxicity of the small molecule. The main toxicity of venetoclax is the cytopenias, and so cytopenias are a possibility. This should be fine if it's limited in time, and then venetoclax is stopped.

With this type of approach venetoclax doesn't need to be given for too long because you want to have the presence of venetoclax during the main action of the CAR-T cell as an anti-tumor effect. However, if you are thinking about using our construct of over-expressing

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BCL-2 in a T cell, that definitely changes the ability of the CAR-T cell to survive. We might have some increased cytokine release syndrome (CRS), we might have some increased neurotoxicity, but again, these possible side-effects need to be weighed against the benefits we might get with a stronger product.

The last comment is more about the role of using a construct that leads to over-expression of a mutant BCL-2. BCL-2 is often over-expressed in lymphomas, and so obviously the risk of transformation of the B cells is something that patients would need to be closely monitored. That's something I would be probably thinking about that as a possible toxicity resulting from the gene editing.

Q Can this approach be translated to other combinations?

MR: I believe it can. We described for the first time this idea of modifying the T cells to allow them to be combined with small molecules that would be otherwise toxic to them. Thinking of any small molecule that also has an activity against T cells, for example BRAF inhibitors with vemurafenib, if you want to combine that with an adoptive T cell therapy we will need to come up with a strategy to make the T cell persistent. So I do think that this is applicable to other combinations in the future.

Q Can you comment a bit more on the challenges when using CAR-T for solid tumors?'

MR: This is an important topic, because of course there are strong efforts from the scientific community in both academia and pharma to develop CAR-T cells for solid tumors.

There are several challenges related to this. We don't have optimal targets for solid cancers as most of the targets available are also expressed in healthy normal tissues that cannot really be spared. In addition the expression of the target we see in the tumor is always heterogenous, it's really rare to have 100% of the cells being highly positive for the antigen in question. The antigen issue is a major one.

Then there is the issue that the tumor microenvironment in solid cancer is particularly immunosuppressive, so any T cell or CAR-T cell that is able to get to the tumor site will need to overcome the strong immunosuppression that we see in our patients. Lastly there is an intrinsic lack of co-stimulation with solid cancer cells, where the interaction between T cells and the solid cancer cell might be more challenging as compared to interaction between a CAR-T cell in a lymphoma or leukemic cell.

However, there are a few publications, especially with brain tumors and localized regional administration of CARs, that show some responses. The field is progressing well and there will be better results in this setting, potentially using strategies that also take advantage of the stimulation of the native immune system, or some adaptive responses including tumor infiltrating lymphocytes, and so on. There is more progress to come in the next few years.

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AUTHORSHIP & CONFLICT OF INTEREST

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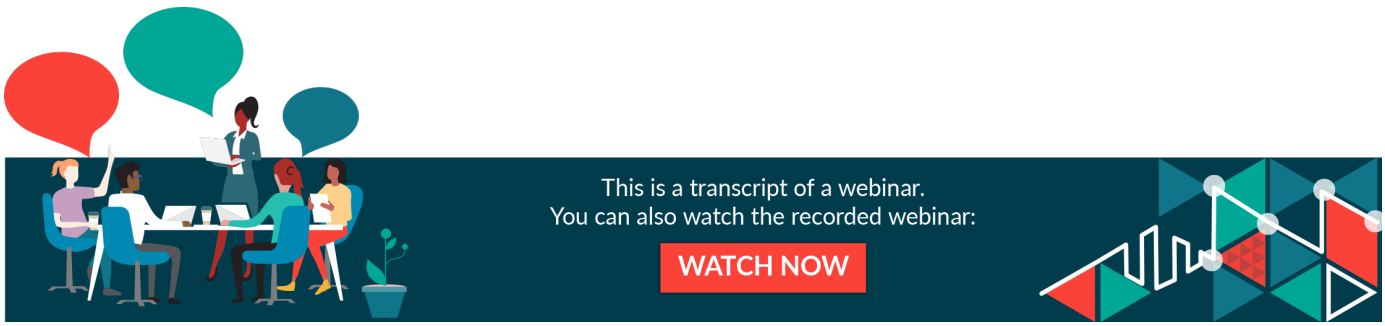

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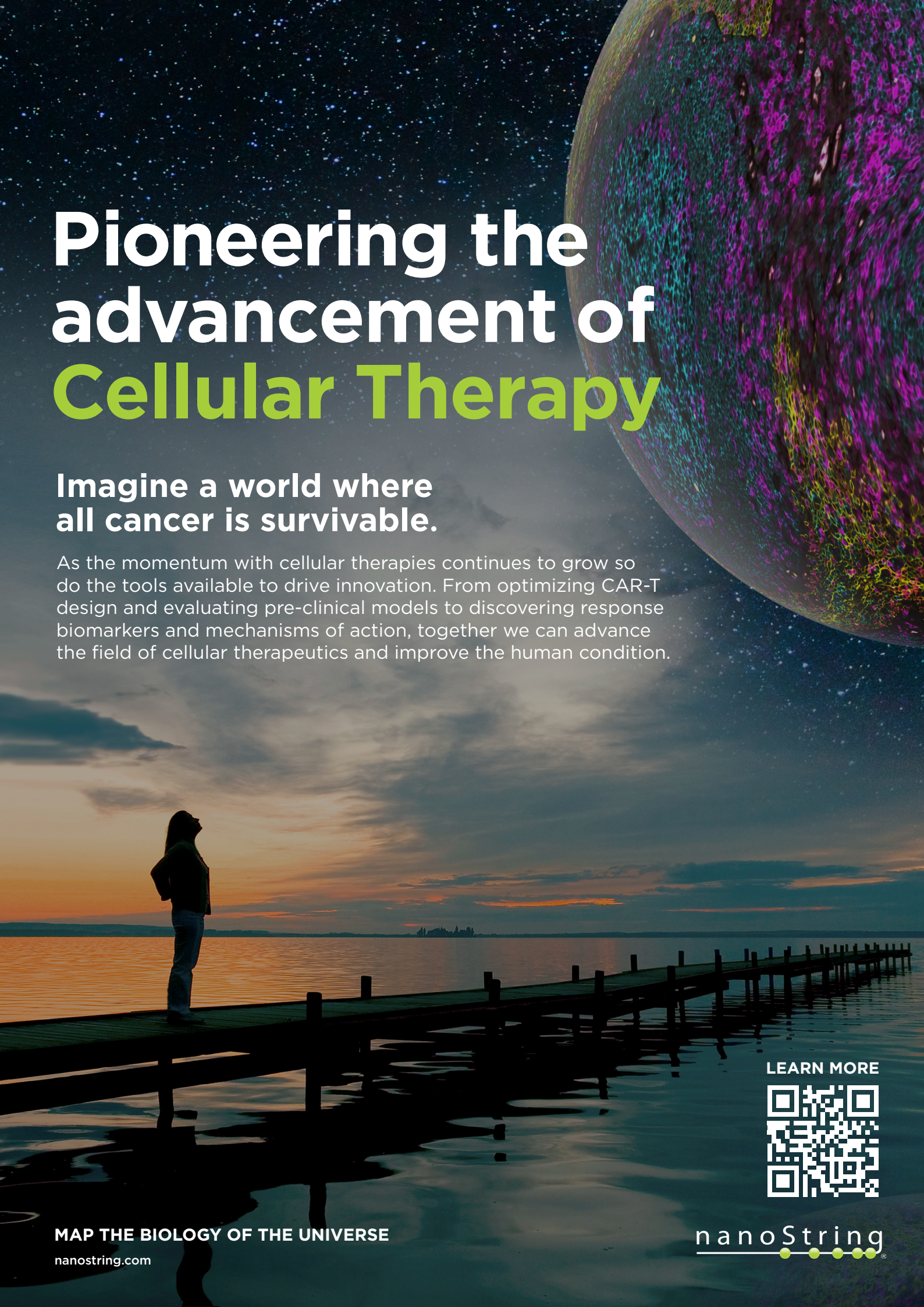
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