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TITLE: Strategies to Counteract Resistance Mechanisms in CAR+ T-Cell-Based Immunotherapy for Triple-Negative Breast Cancer

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<b>14. ABSTRACT</b> This is the final report of our awarded proposal entitled "Strategies to Counteract Resistance Mechanisms in CAR T-Cell Based Immunotherapy for Triple-Negative Breast Cancer". The goal of this proposal is to test the in vitro and in vivo anti-tumor activity of the CSPG4-specific CAR T cell combinatorial immunotherapeutic strategy we have developed for the treatment of TNBC. Given the limited in vivo effectiveness of our strategy, we have tested a plethora of strategies aiming to enhance the ant-tumor activity of CSPG4 CAR T cells via distinct approaches and mechanisms..					
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**1. INTRODUCTION:** The goal of this proposal is to test the *in vitro* and *in vivo* anti-tumor activity of the CSPG4-specific CAR T cell combinatorial immunotherapeutic strategy we have developed for the treatment of TNBC. Given the limited *in vivo* effectiveness of our strategy, we have tested a plethora of strategies aiming to enhance the anti-tumor activity of CSPG4 CAR T cells via distinct approaches and mechanisms.

**2. KEYWORDS:** chimeric antigen receptor T cells; CSPG4; PD-1; triple-negative breast cancer

**3. ACCOMPLISHMENTS:**

- **What were the major goals of the project?**

1. CSPG4 up-regulation induced by hypoxia in TNBC tumors is associated with a poor clinical course of the disease;
2. T cells transduced with a CSPG4-specific CAR containing a PD-1 shRNA (CSPG4-specific CAR+ PD-1 shRNA-T cells) in combination with IL-2-anti-idiotypic (anti-id) mAb MK2-23 fusion protein and LDE225, an inhibitor of the SHH pathway, eradicate both differentiated TNBC cells and TNBC CICs incubated under hypoxic conditions *in vitro*;
3. CSPG4-specific CAR+ PD-1 shRNA-T cells in combination with IL-2-anti-id mAb MK2-23 fusion protein and LDE225 suppress in an adjuvant setting metastatic spread and disease recurrence and prolong survival of NSG mice which are orthotopically grafted with the TNBC MDA-MB-231-Luc- D3H1 cell line and then subjected to surgical removal of their primary tumor; and
4. The results obtained with the TNBC cell lines have clinical significance, as they are reproduced in NSG mice orthotopically grafted with patient derived TNBC xenografts (PDX).

- **What was accomplished under these goals?**

## Summary

The overarching goal of our study was to test the anti-tumor activity of our novel CSPG4-targeting CAR T cell-based combinatorial immunotherapeutic strategy *in vitro* and in mouse models. Although our strategy was highly effective *in vitro*, its *in vivo* anti-tumor activity was limited. Thus, we aimed to develop strategies that could enhance the *in vivo* activity of CSPG4 CAR T cells via distinct approaches. We focused on strategies which could:

i) upregulate CSPG4 expression on TNBC cells: we investigated whether CSPG4 is regulated by p63, STAT3 and STAT5. We also showed that the histone deacetylase inhibitor (HDACi) vorinostat (SAHA), as well as radiation, upregulate CSPG4 expression on TNBC cells;

ii) enhance the susceptibility of TNBC cells to CSPG4 CAR T cell-mediated lysis: we showed that the Sonic Hedgehog Homolog (SHH) pathway inhibitor sonidegib (LDE225) inhibits the proliferation of TNBC cells at doses lower than those required for CAR T cell elimination. In addition, we showed that treatment of TNBC with sonidegib increases the susceptibility of differentiated TNBC cells and TNBC cancer initiating cells (CICs). Although the results supported our hypothesis indicated in the original grant application, recent evidence suggests that sonidegib accelerates the progression of several malignancies. Therefore, because of the potential difficulty to translate the results to a clinical setting, we decided to abandon this strategy. Additionally, we found that radiation as well as vorinostat cause an imbalance between pro- and anti-apoptotic molecules, thus enhancing the susceptibility of TNBC cells;

iii) increase expression of CAR on T cells: in addition to its previously mentioned effects, vorinostat also enhances CAR expression on CAR T cells. In addition, we found that vorinostat increases the anti-tumor activity of CAR T cells and this effect may be mediated by downregulation of TET2, a methylcytosine dioxygenase. Of note, in mouse experiments, we tested both vorinostat administered to mice, and *ex vivo* vorinostat treatment of CAR T cells;

iv) enhance the anti-tumor activity of effector cells: we aimed to locally increase the level of IL-2 using an IL-2-anti-idiotypic monoclonal antibody (mAb) MK2-23 fusion protein, and to disrupt the PD-1/PD-L1 axis using an anti-PD-L1 mAb we were able to enhance the anti-tumor activity of CAR T cells. Furthermore, we aimed to counteract tonic signaling, a potential cause of reduced CAR T cell anti-tumor activity, by amino-acid

substitutions within the framework regions of the scFv used for the generation of the CAR. Additionally, because of the concerns that CARs derived from TA-specific mouse monoclonal antibodies (mAbs) may induce an immune response which interferes with the anti-tumor activity of CAR T cells, we developed the CSPG4-specific human scFv SK5 and used it to generate a CSPG4-specific human CAR. Lastly, aiming to increase the activity of the CAR approach, we have explored the use of cytokine-induced killer T-lymphocytes (CIK) instead of T cells as effector cells.

We would like to point out, that to screen the validity of the approaches we planned to test, we have utilized several tumor systems with the expectation to move to TNBC systems the ones which were effective. Our plan has been affected by the need to interrupt experimental work from March to July 2020 because of the mandatory research laboratory shutdown due to the COVID-19 pandemic at Massachusetts General Hospital. In July, research laboratories reopened partially, and we have been allowed to work with limited personnel and resources to the present day

**Specific Aim 1: CSPG4 up-regulation induced by hypoxia in TNBC tumors is associated with a poor clinical course of the disease.**

*Specific aim synopsis*

In this aim, we quantified CSPG4 expression frequency in a large number of TNBC tissue samples, we investigated the correlation between CSPG4 expression and HIF-1a expression in FFPE TNBC tissues, and evaluated the role of p63, STA3 and STAT5 in the regulation of CSPG4. We also showed that CSPG4 is upregulated on TNBC cells by vorinostat and by low dose radiation (this will be described in the following Specific Aim).

***Subtask 1: Immunohistochemical (IHC) staining of formalin fixed, paraffin embedded TNBC tumors for CSPG4 and hypoxia inducible factor expression.***

### CSPG4 expression frequency in TNBC

In our initial study we found CSPG4 expression in 32 (73%) of the 44 TNBC tumors tested. As described in the previous Progress Reports, in additional immunohistochemical studies performed during the first year of this grant utilizing the CSPG4-specific mAbs D2.8, 763.74 and TP41.2, 53/63 (87%) of TNBC tumors express high levels of CSPG4. Since information about the frequency of CSPG4 expression in TNBC tumors is important to determine the percentage of patients who might benefit from CSPG4 CAR T cell-based immunotherapy, we established a collaboration with Dr. Elda Tagliabue at the National Cancer Institute in Milan (Italy). Utilizing the pool of our CSPG4-specific mAb D2.8, 763.74 and TP41.2 (the same pool we have utilized in our laboratory), Dr. Tagliabue found CSPG4 expression in 13 of the 29 (45%) tested TNBC tumors. Please refer to previous Progress Reports for details.

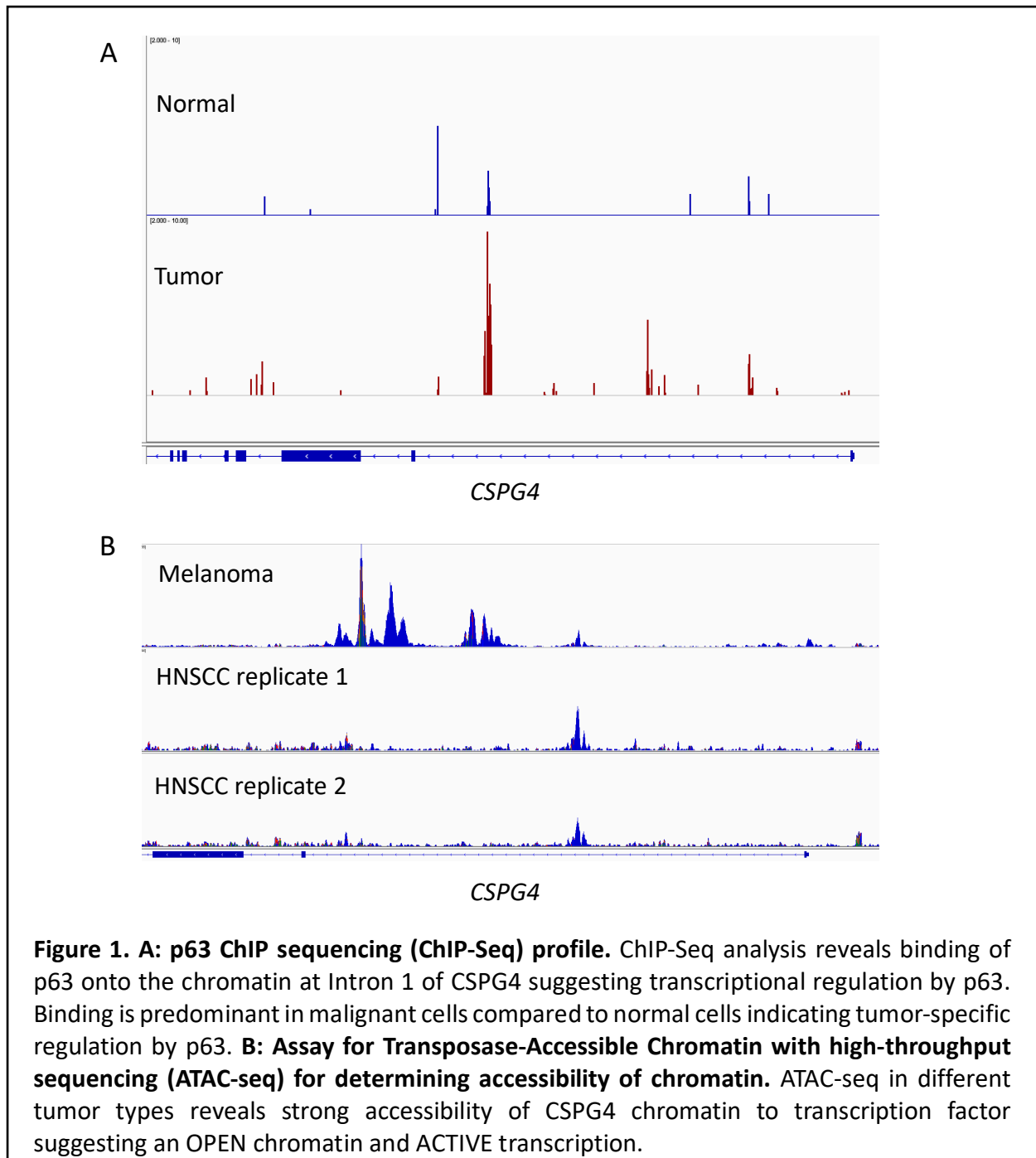
### CSPG4 expression regulation by hypoxia

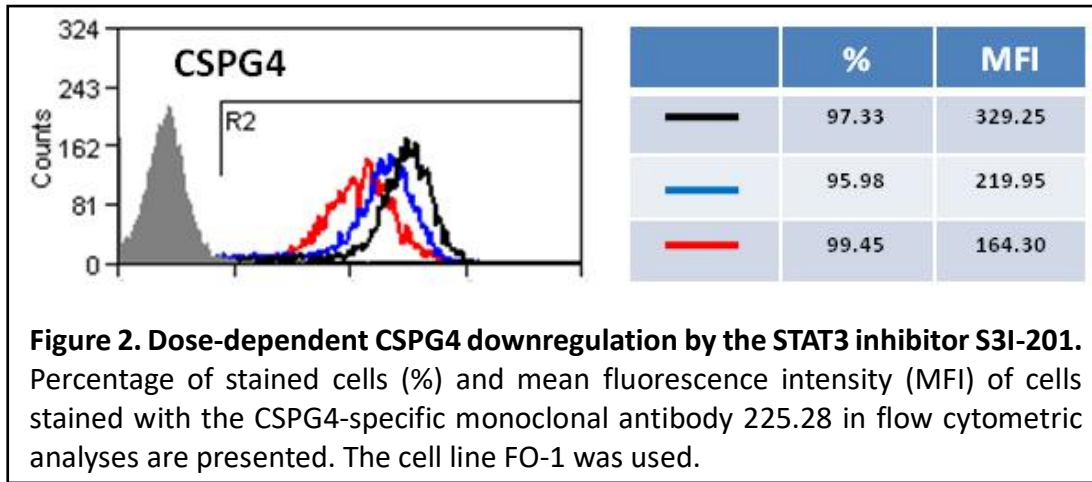
As we have previously reported, CSPG4 expression is upregulated on TNBC cells incubated for 72 hours under hypoxic conditions (1% O<sub>2</sub>) at 37<sup>o</sup> C. After identifying a commercially available HIF-1a-specific antibody which stains formalin fixed, paraffin embedded (FFPE) tissue sections in a reliable and reproducible way, we found no correlation between HIF-1a expression and CSPG4 expression in 62 FFPE TNBC samples. Therefore, our data suggest that HIF-1a does not regulate directly CSPG4 expression.

### CSPG4 regulation by p63, STA3 and STAT5

We have found that p63 (Fig. 1A), STA3 and STAT5 might be involved in the regulation of CSPG4, since they act as transcription factors that bind to the promoter region of CSPG4. Of note, STAT5a is activated in cells incubated under hypoxic conditions. Furthermore, in an Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-seq) in different tumor types we found strong accessibility of CSPG4 chromatin to transcription factors, suggesting an open chromatin and active transcription (Fig. 1B). Thus, we further analyzed their potential role with additional studies. As described in the previous Progress Report, following cisplatin-mediated p63 downregulation as well as p63 knock-out with shRNA, CSPG4 was still expressed on

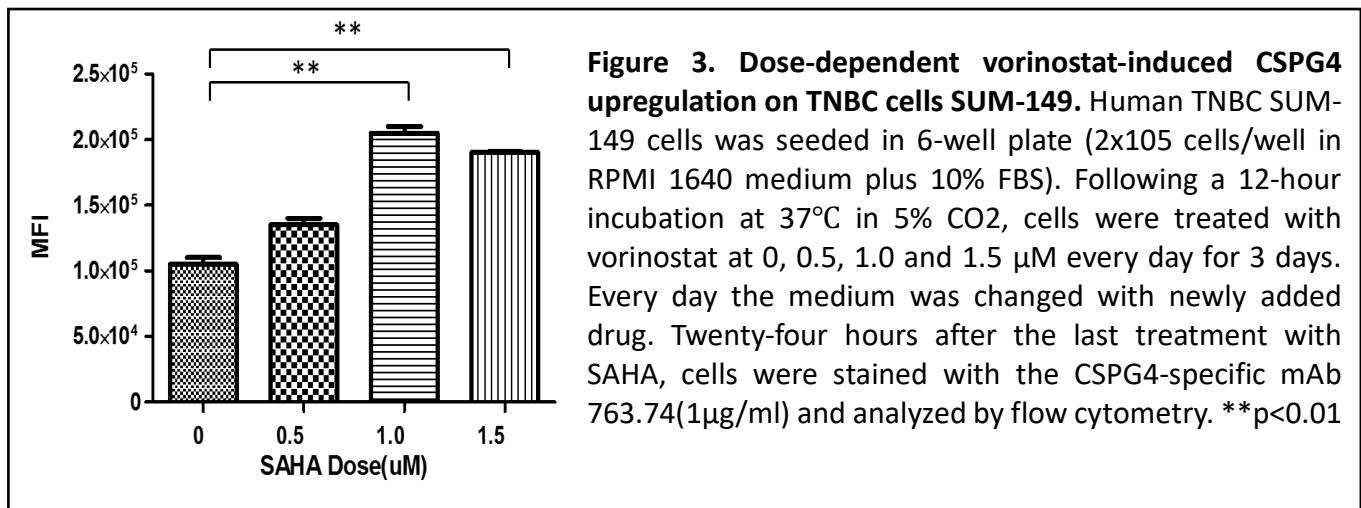
head and neck squamous cell carcinoma (HNSCC) cells. Furthermore, in early studies CSPG4 expression on TNBC and HNSCC cells did not change following STAT3 downregulation using the STAT3 inhibitor Static. However, in more recent experiments, we observed a dose-dependent CSPG4 downregulation using the STAT3 inhibitor S3I-201 (Fig. 2).





### CSGPG4 regulation by vorinostat

Human TNBC SUM-149 cells was seeded in 6-well plates. Following a 12-hour incubation at 37°C in 5% CO<sub>2</sub>, cells were treated with vorinostat for 3 days. Twenty-four hours after treatment with vorinostat, cells were stained with the CSPG4-specific mAb 763.74, and analyzed by flow cytometry. We found that vorinostat upregulated CSPG4 expression in a dose-dependent manner (Fig. 3)



### **Subtask 2: Statistical analysis of the IHC results and association with clinical data**

The number of samples analyzed was not sufficient to perform an informative statistical analysis of the association between IHC results and clinical parameters.

**Specific Aim 2: T cells transduced with a CSPG4-specific CAR containing a PD-1 shRNA (CSPG4-specific CAR+ PD-1 shRNA-T cells) in combination with IL-2-anti-idiotypic (anti-id) mAb MK2-23 fusion protein and LDE225, an inhibitor of the SHH pathway, eradicate both differentiated TNBC cells and TNBC CICs incubated under hypoxic conditions *in vitro*.**

*Specific aim synopsis*

In this aim we evaluated the enhancement of the *in vitro* anti-tumor activity of CSPG4-specific CAR T cells by other components of our strategy, namely sonidegib (LDE225), vorinostat, anti-PD-L1 mAb, IL-2-anti-id mAb MK2-23 fusion protein, radiation, counteracting tonic signaling, CSPG4-specific human scFv SK5 and cytokine-Induced killer T-lymphocytes (CIK) engineered with a CSPG4 CAR (CSPG4-CAR.CIK cells). In the original application we proposed the use of LDE225 to inhibit the activation of the Sonic Hedgehog Homolog (SHH) pathway which causes an increase of anti-apoptotic molecules and a reduced susceptibility of differentiated TNBC cells and leading to inhibition of TNBC proliferation, however, this component was replaced given that it has been shown to accelerate the progression of some malignant diseases. We found that the HDACi vorinostat enhances CAR expression on transduced T cells. We also found that vorinostat increases the anti-tumor activity of CAR T cells and this effect may be mediated by downregulation of TET2, a methylcytosine dioxygenase. Furthermore, we showed that the use of an anti-PD-L1 mAb enhances the anti-tumor activity of CSPG4 CAR T cells, and that radiation of tumor cells upregulates CSPG4, decreases the level of the major anti-apoptotic molecule bcl-2 and ultimately enhances the *in vitro* anti-tumor activity of CSPG4 CAR T cells. Additionally, we demonstrated that counteracting tonic signaling, a potential cause of poor CAR T cell anti-tumor activity, by amino acid substitutions in the framework regions of the CAR scFv and by humanization of the framework regions of the scFv, also enhances the *in vitro* anti-tumor activity of CSPG4 CAR T cells. Lastly, we tested whether the use of CIK cells transduced with CSPG4-CAR instead of T cells transduced with CSPG4-CAR could enhance the therapeutic efficacy of our strategy; we found that CSPG4-CAR.CIK cells exhibited superior *in vitro* tumor cell killing compared to CAR T cells

***Subtask 1: To generate CAR T cells and assess their function in normoxic (20% O<sub>2</sub> tension) and hypoxic (1% O<sub>2</sub> tension) conditions in vitro.***

***Functional activity of CSPG4 CAR T cells under hypoxic conditions***

CSPG4 CAR T cells co-cultured with the TNBC cell lines SUM149 and SUM159 were more efficient in recognizing the targets as determined by the IFN $\gamma$  release assay under hypoxic compared to normoxic conditions. Please refer to previous Progress Reports for details.

***Subtask 2: To optimize the mitogenic effect of LDE225 and IL-2-anti-id mAb MK2-23 fusion protein on CSPG4-specific CAR T cells.***

It is known that the Sonic Hedgehog Homolog (SHH) pathway is activated in differentiated TNBC cells and to an even greater extent in TNBC cancer initiating cells (CICs). This appears to modulate the expression of molecules which play a role in the apoptosis of tumor cells. As a result, they are less susceptible to recognition and elimination by CSPG4 CAR T cells. To counteract this potential escape mechanism, we have tested whether inhibition of the SHH pathway activation can restore the susceptibility of TNBC cells to recognition and elimination by CSPG4 CAR T cells. We have first tested the effect of sonidegib on the proliferation of TNBC cells and CAR T cells. We found that sonidegib inhibits the proliferation of both TNBC and T cells. The effect with both cells is dose dependent. For our experiment, we selected a dose sufficient to inhibit TNBC cell proliferation, but not CAR T cell proliferation.

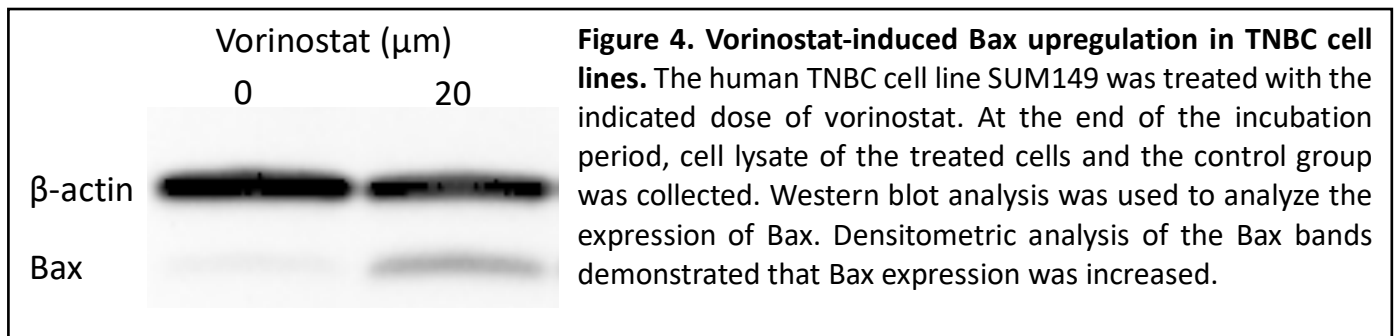
***Subtask 3: To test whether the LDE225 and IL-2-anti-id mAb MK2-23 fusion protein enhances the ability of CSPG4-specific CAR T cells to eliminate both TNBC differentiated cells and TNBC CICs in vitro.***

Recent clinical evidence indicates that sonidegib may accelerate the progression of some malignant diseases. Therefore, there is skepticism among clinicians about its use in the clinical setting. These results prompted us to replace LDE225 with other compounds (itraconazole, radiation, HDACi), which we anticipated would increase the susceptibility of tumor cells to recognition and elimination by CSPG4 CAR T cells by downregulating the expression of anti-apoptotic molecules in tumor cells, but would not inhibit the proliferation of T cells and would

not downregulate CSPG4 expression on target cells. Initially, we tested itraconazole, an anti-fungal agent, which inhibits the SHH pathway, however this compound inhibited the proliferation of CAR T cells and non-transduced T cells at a low dose.

**Vorinostat as a means to enhance TNBC cells' susceptibility to CSPG4-specific CAR T cell mediated lysis**

We have found that vorinostat increases the level of the major pro-apoptotic molecule Bax, leading to an imbalance between pro- and anti-apoptotic molecules, which can potentially increase TNBC cells' susceptibility to killing by CSPG4-specific CAR T cells (Fig. 4)



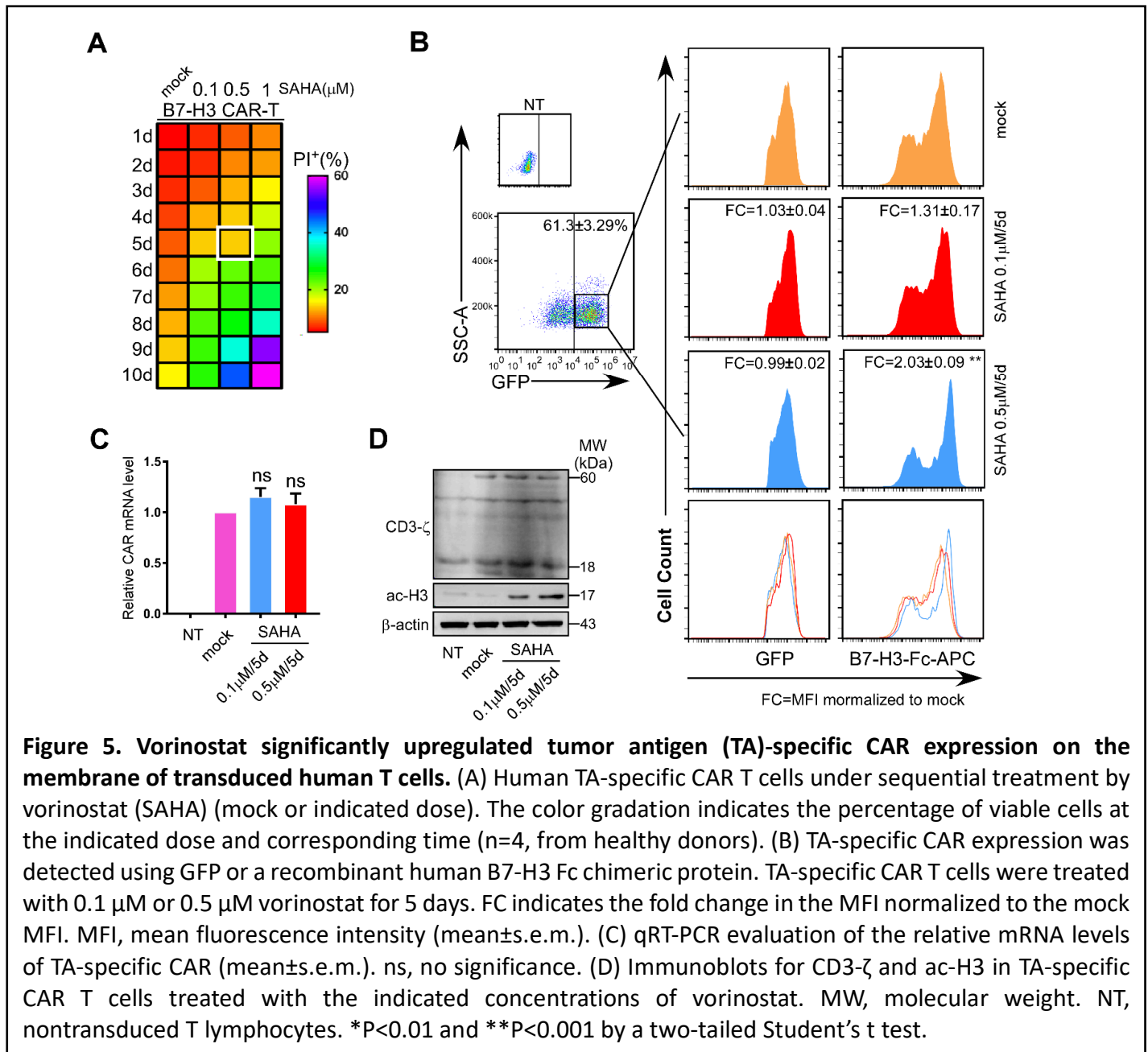
**Enhancement by vorinostat of the CSPG4 CAR T cell functional activity**

In the previous Progress Reports, we have indicated that *in vitro* treatment of CSPG4 CAR T cells with the HDAC inhibitor vorinostat enhances CAR expression, as indicated by the increase of the MFI; in contrast, there is no detectable change in the percentage of transduced T cells which express the tested CAR. In subsequent *in vitro* experiments, we found that vorinostat-treated CSPG4 CAR T cells display a significantly higher ability to specifically recognize CSPG4-bearing TNBC cells and display a significantly higher anti-tumor activity compared to untreated CSPG4 CAR T cells. Please refer to the previous Progress Reports for details.

**Mechanisms underlying effect of vorinostat on CAR T cells**

We further investigated the mechanisms underlying the beneficial effect of vorinostat on CAR T cell functional activity. We found that the effect of vorinostat is not limited to CSPG4, since similar findings were observed with B7-H3-specific CAR T cells. Specifically, we showed that vorinostat (0.5  $\mu\text{M}$ ) increased the surface expression

of CAR on T cells (Fig. 5B). Despite the ability of vorinostat to induce epigenetic changes, only a negligible increase in the CAR mRNA level was detected using quantitative real-time PCR (qRT-PCR) (Fig. 5C). Similarly, although the level of acetylated histone 3 (ac-H3) increased, the levels of the wild-type and chimeric CD3- $\zeta$  chain did not (Fig. 5D), consistent with the evaluation of GFP expression (Fig. 5B). Together, these results argue in favor of the possibility that the vorinostat-induced CAR upregulation reflected a posttranslational mechanism.



Additionally, we found that vorinostat inhibited the immunosuppressive signaling pathway and enhanced the protein transport signaling pathway in CAR T cells. To the best of our knowledge, the effect of epigenetic therapy

on CAR T cells has not been systematically studied. Therefore, we performed mRNA profiling to detect transcriptomic changes in CAR T cells under vorinostat treatment. As shown in Fig. 6A, the vorinostat treatment profoundly altered the transcriptome, with 6,172 significantly upregulated genes and 6,225 significantly downregulated genes. Then, we applied gene set enrichment analysis (GSEA) to identify the changed phenotype of the CAR T cells. Since we found upregulation of CAR expression at the posttranslational level, we focused on the protein transport and immunerelated signaling pathways. As expected, the GSEA revealed the upregulation of protein transport-related genes, while immunosuppression- and unfolded protein response (UPR)-related genes were downregulated (Fig. 6B). The corresponding associated genes included the upregulated genes GPC1-4 and ZG16 and downregulated genes CTLA-4 and TET2 (Fig. 6C). A detailed description of the gene sets, including upregulated Golgi lumen-related genes and downregulated UPR-related genes, PERK regulated genes, and T cell receptor signaling pathway genes, is provided in Fig. 6D. Interestingly, TET2, a methylcytosine dioxygenase, was recently discovered to be a potent regulator of CD19.CAR-T cell fate. Therefore, we detected the potential role of TET2 in the increased activity of CAR T cells following treatment with vorinostat. Knock-down of TET2 expression in CAR T cells increased the cytokine release (IFN- $\gamma$ , IL-2, TNF- $\alpha$ , and GM-CSF) and the cytotoxic activity of CAR T cells in the coculture system (Fig. 6E-H). Consistently, TET2 knockdown in CAR T cells increased perforin and granzyme B expression (Fig. 6I). Thus, vorinostat increases CAR expression on the T cell surface possibly through the upregulation of the protein transport signaling pathway.

#### Enhancement by *anti-PD-L1 mAb* of the anti-tumor activity of *CSPG4 CAR T cells*

CAR T cells express PD-1 which interacts with PD-L1. The latter is induced on tumor cells by hypoxia and/or IFN $\gamma$  on TNBC cells. The interaction of PD-L1 with PD1 “exhausts” CAR T cells, providing TNBC cells with an escape mechanism. To counteract this escape mechanism, we have added anti-PD-L1 mAb to the culture of CSPG4 CAR T cells with TNBC cells, in order to disrupt PD-1-PD-L1 interactions. This strategy was found to correct the dysfunction of CSPG4 CAR T cells and restore their anti-tumor activity with both differentiated TNBC cells and TNBC CICs. Please refer to the previous Progress Reports for details.

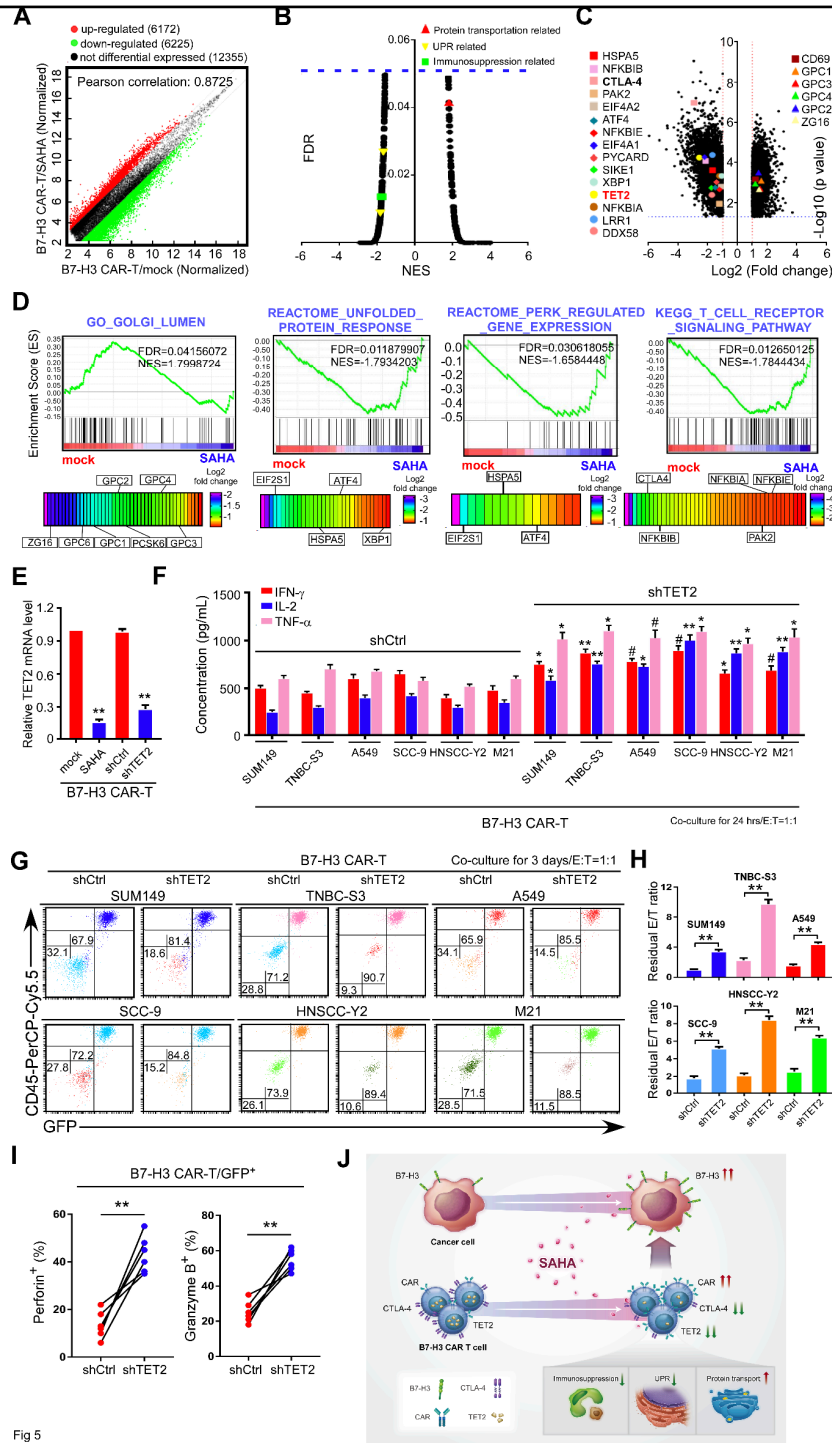


Fig 5

**Figure 6. Vorinostat upregulated the protein transport signaling pathway and reduced the expression of immunosuppressive factors, such as CTLA-4 and TET2.** (A) Scatterplot showing the variation in gene expression between B7-H3.CAR-Ts treated with 0.5  $\mu$ M vorinostat for 5 days and untreated CAR-Ts. The values on the X and Y axes are the average normalized signal values of the group (log2 scaled, n=3 per group). The green and red dots represent fold changes  $\geq 2.0$ . (B) GSEA (GO, REACTOME, and KEGG) pathway distribution of vorinostat-treated B7-H3.CAR-Ts versus untreated CAR-Ts. The vertical line denotes an FDR significance cutoff of 0.05. Protein transportation- (red), UPR- (yellow), and immunosuppression (green)-related gene sets are demarcated as indicated. (C) Volcano plot of the relative RNA expression in vorinostat-treated B7-H3.CAR-Ts compared to untreated CAR-Ts. Genes in the upper left and right quadrants were significantly differentially expressed. (D) Representative upregulated or downregulated GSEA plots with corresponding core-enriched genes in B7-H3.CAR-Ts (FDR < 0.05 and NES > 1.5) treated with vorinostat. The color gradation is based on GSEA NES (log2 scaled). (E) Efficiency of TET2 knockdown in B7-H3.CAR-Ts. Error bars denote the s.e.m. (F) Summary of the levels of IFN- $\gamma$ , IL-2 and TNF- $\alpha$  released by B7-H3.CAR-Ts with TET2 knockdown into the culture supernatant after 24 h of coculture with the indicated cell lines as measured by ELISA (n=5). (G) Evaluation of the coculture system by flow cytometry at 3 days. (H) Quantification of the effector cell:target cell ratio (n=5). Error bars denote the s.e.m. (I) Representative histograms illustrating the expression levels of perforin and granzyme B in B7-H3.CAR-Ts in the setting of TET2 knockdown (right) compared to the counterpart control setting (left) (n=5). (J) Schematic depicting our proposed model of the upregulation of B7-H3 expression on solid tumor cells and B7-H3.CAR expression on transduced T cells by vorinostat treatment accompanied by the downregulation of immunosuppression- and UPR-related pathways and upregulation of the protein transport signaling pathway in B7-H3.CAR-Ts. #P<0.05, \*P<0.01, and \*\*P<0.001 by a two-tailed Student's t test.

Enhancement by **IL-2-anti-id mAb MK2-23 fusion protein** of *in vitro* elimination of TNBC cells by CSPG4 CAR

T cells

We aimed to selectively increase the level of IL-2 in the tumor microenvironment to enhance the functional activity of CAR T cells while at the same time reducing the risk of systemic IL-2 adverse effects. In order to do the above, we generated a fusion protein by linking IL-2 to the anti-idiotypic mAb MK2-23. The latter recognizes an idiotype in the antigen combining site of the CSPG4-specific mAb 763.74 which was used to prepare the CSPG4-specific CAR. As indicated in previous Progress Reports, the fusion protein enhances the ability of CSPG4 CAR T cells to eliminate differentiated melanoma cells and melanoma CICs.

Generation of **IL-2-anti-id mAb MK2-23 fusion protein**

We have faced significant difficulties with the generation of large amounts of IL-2-anti-id mAb MK2-23 fusion protein, since the myeloma cells transfected with the IL-2-mAb MK2-23 construct produced small amounts of the fusion protein *in vitro* and when injected intraperitoneally in syngeneic mice. After extensive efforts we were able to isolate a clone of cells that is a good producer of the fusion protein. Cells were injected into the abdominal cavity of mice to generate ascites with the expectation to isolate the fusion protein from the ascitic fluid. We are currently purifying the fusion protein from the ascitic fluid. Given the aforementioned obstacle, no *in vitro* or *in vivo* experiments have been performed using the IL-2-anti-id mAb MK2-23 fusion protein. Please refer to previous Progress Reports for details.

**Radiation as a means to enhance TNBC cells' susceptibility to CSPG4-specific CAR T cell mediated lysis**

There is now increasing recognition of the complex interplay between radiation therapy and the immune system, with a greater appreciation of the ability of radiation therapy to influence systemic tumor responses. It can sensitize tumor cells to immunotherapy by promoting the expression of HLA class I molecules and other apoptosis-mediated proteins not only in the irradiated site but also in distant non-irradiated metastatic spots (abscopal effect). We have also shown for the first time that radiation therapy can upregulate the expression of various tumor antigens including CSPG4 on TNBC cells. As described in previous Progress Reports, we

performed serial titration experiments to identify the optimal dose of radiation that has minimal cytotoxic effect on TNBC cells and at the same time induces CSPG4 upregulation on tumor cells. We have found that the effect of radiation is both time- and dose- dependent. We have also shown that radiation decreases the level of the major anti-apoptotic molecule bcl-2, while Bax, a pro-apoptotic molecule, remains stable. Therefore, this imbalance between pro- and anti-apoptotic molecules induced by radiation can potentially increase TNBC cells' susceptibility to killing by CSPG4-specific CAR T cells. Please refer to previous Progress Reports for details.

*Enhancement by radiation of the ability of CSPG4-specific CAR T cells to eliminate in vitro TNBC cells*

As described in previous Progress Reports, in a co-culture experiment, pre-treatment of TNBC cells with radiation enhanced the *in vitro* anti-tumor activity of CSPG4 CAR T cells against them, thereby providing the rationale for utilizing this strategy in an *in vivo* setting.

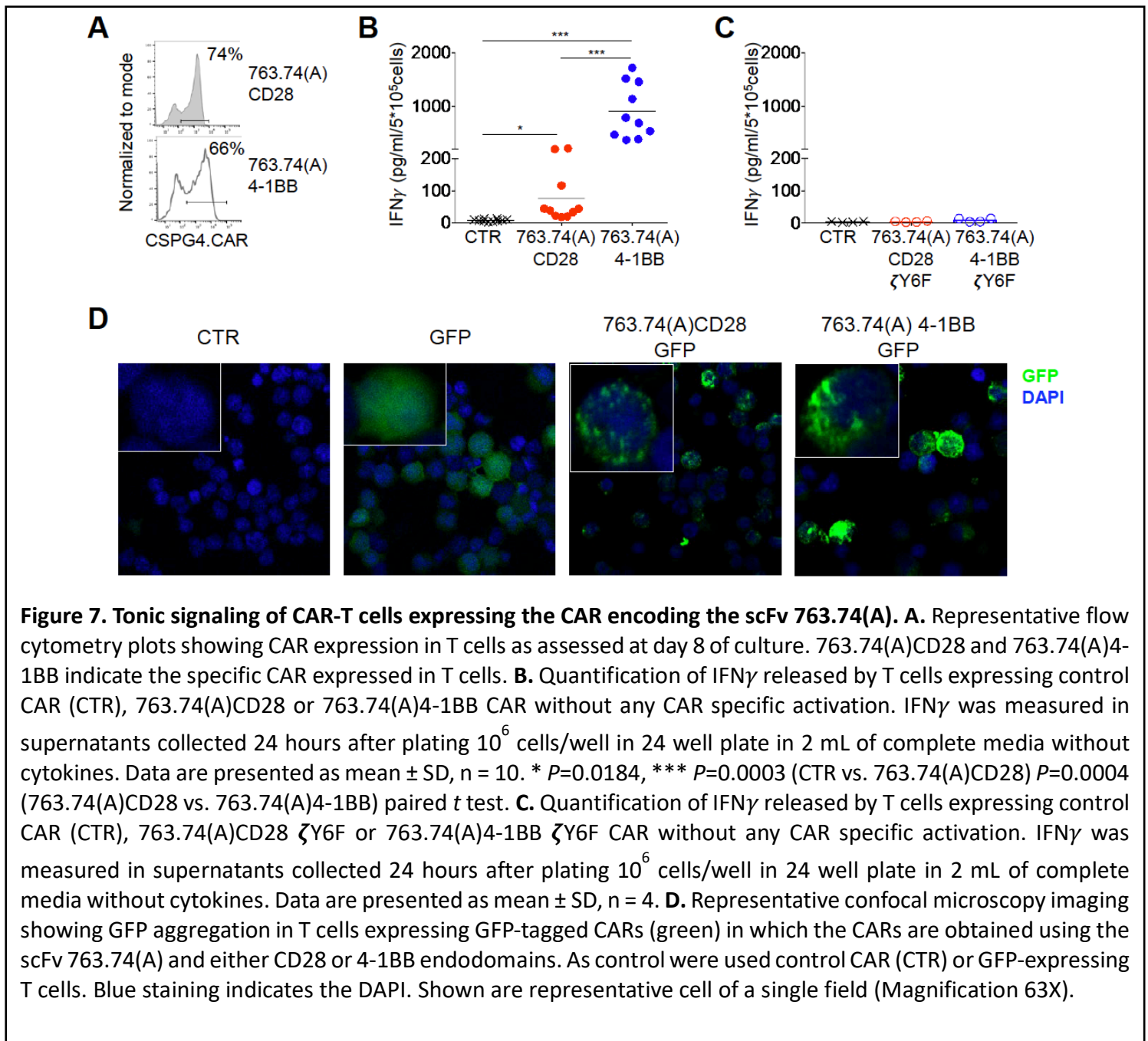
*Counteracting **tonic signaling**, a potential cause of poor in vivo CAR T cell anti-tumor activity*

In an effort to restore the limited *in vivo* anti-tumor activity of CAR T cells, we aimed to counteract mechanisms which detrimentally affect the functional activity of CAR T cells, such as tonic signaling. The latter denotes sustained, antigen independent activation leading to rapid T cell exhaustion and impaired anti-tumor activity. It has recently emerged that CARs can cause tonic signaling in T cells. The first report describing tonic signaling in CAR T cells attributed this effect to the propensity of certain scFvs to self-aggregate causing cell surface CAR clustering and consequent signaling. We found that amino acid substitutions in the framework regions (FWRs) of the antibody can stabilize the scFv and correct the tonic signaling of the CAR. We additionally found that substitution of the murine FWRs with stable human FWRs also prevents antigen-independent activation of CAR T cells.

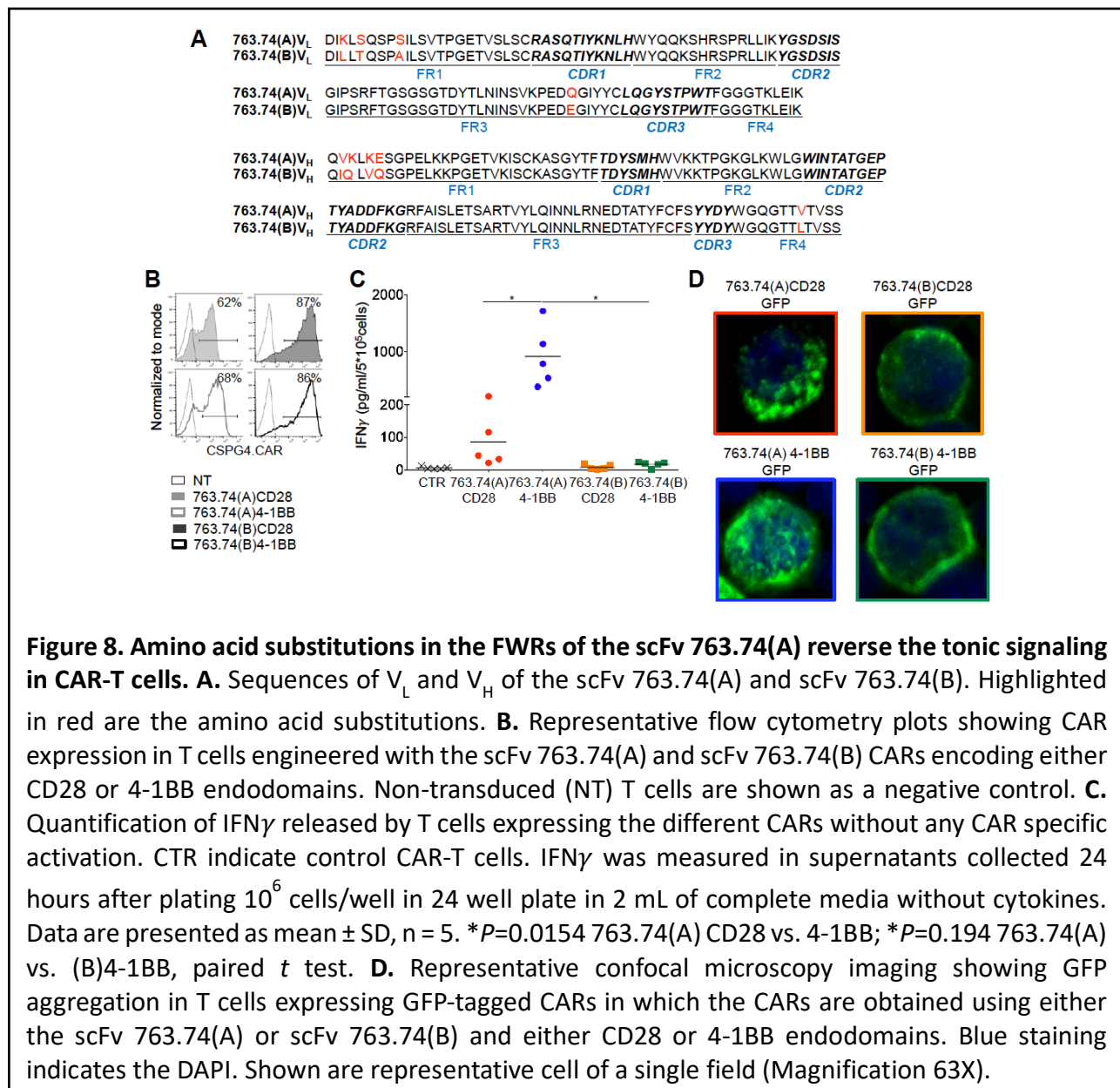
*Amino-acid substitutions within the FWRs of the scFv abrogate the tonic signaling*

In the CSPG4-specific CAR utilized in our strategy, the antigen binding moiety is the scFv 763.74(A) obtained from the 763.74 murine mAb. T cells expressing the scFv 763.74(A) CAR (Fig. 7A) encoding either CD28 or 4-

1BB costimulatory endodomains showed release of IFN $\gamma$  in the absence of antigen stimulation, a phenomenon defined as CAR tonic signaling (Fig. 7B). Spontaneous IFN $\gamma$  release by T cells expressing scFv 763.74(A) CARs was strictly dependent on CAR signaling, because mutations of conserved motifs of the CAR-CD3 $\zeta$  chain that prevent tyrosine phosphorylation completely abrogated the spontaneous IFN $\gamma$  release (Fig. 7C). To study the distribution of CAR molecules on the cell surface of T cells, we generated scFv 763.74(A) CARs in which the CD3 $\zeta$  chain is fused at COOH terminal with GFP. Using confocal microscopy imaging, we found that scFv 763.74(A) CARs form membrane clusters in the absence of CAR crosslinking, likely indicating self-aggregation



of CAR molecules (Fig. 7D). The sequence of the scFv 763.74(A) was obtained from an early passage of the hybridoma 763.74 secreting the murine IgG1 mAb, which recognizes a peptide epitope of the human CSPG4. It is well established that culture passages of hybridoma affect the growth rate of the hybridoma and the yield of the secreted antibody. It has also been described that upon culture passages amino acid substitutions may occur in both complementary diversity regions (CDRs) and FWRs in subclones derived from the hybridoma. In light of this possibility, we sequenced the  $V_L$  and  $V_H$  domains of a late passage of the 763.74 hybridoma. We obtained two  $V_L$  and  $V_H$  sequences in which amino acid substitutions were identified in the FRWs (FR1 and FR3) of both  $V_L$  and  $V_H$  (Fig. 8A). We assembled a new scFv called scFv 763.74(B), generated new scFv 763.74(B) CARs and

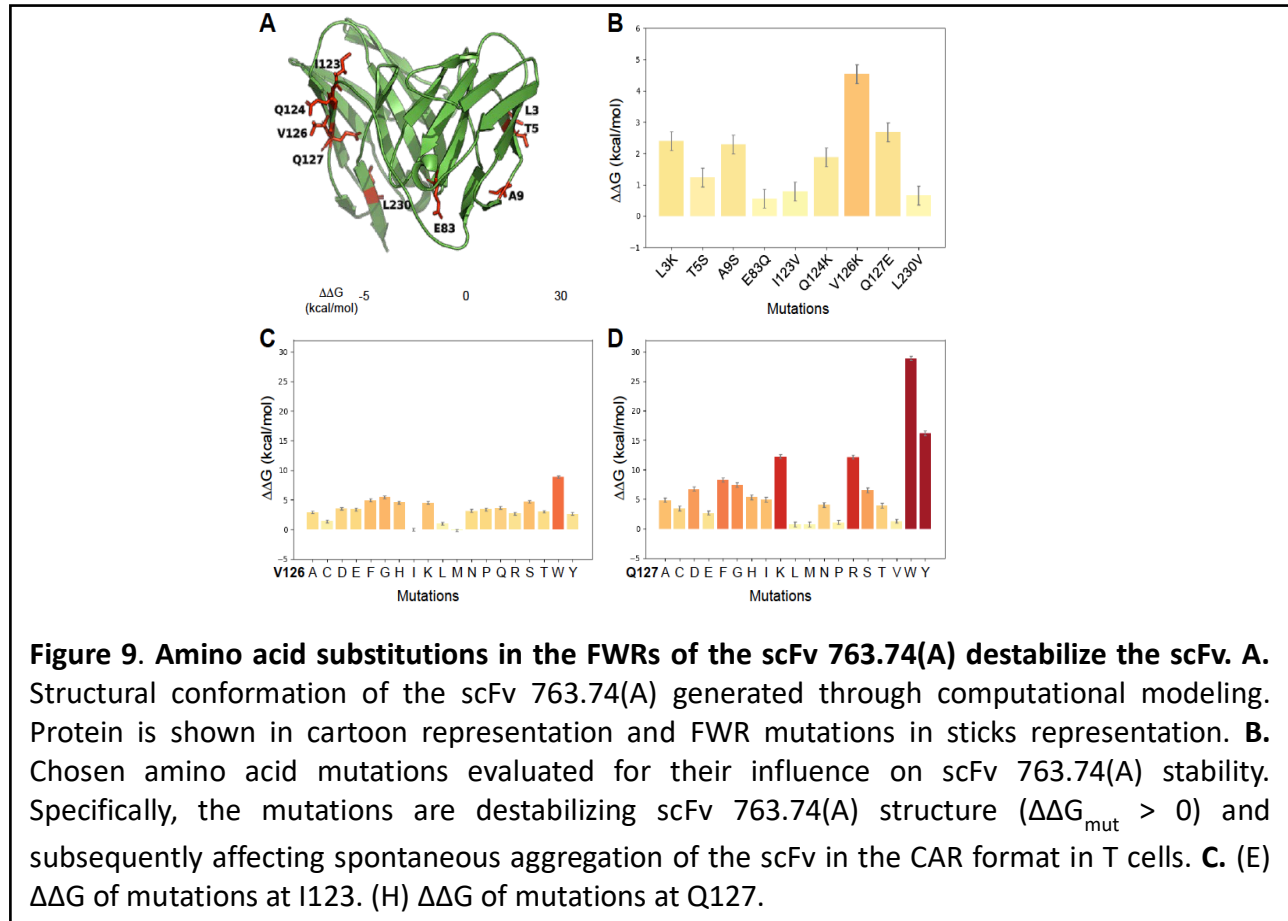


compared them side-by-side with scFv 763.74(A) CARs for evidence of tonic signaling. All CARs were equally expressed in T cells (Fig. 8B), and CAR-T cells equally expanded *in vitro*. However, T cells expressing scFv 763.74(B) CAR encoding either CD28 or 4-1BB costimulatory endodomains did not show spontaneous releases of IFN $\gamma$  (Fig. 8C). We also observed a more homogeneous distribution of the scFv 763.74(B) CARs on the membrane of T cells using confocal microscopy of GFP-tagged CARs (Fig. 8D). Of note, cross-linking of CARs expressed on T cells mediated by the anti-idiotypic mAb MK2-23 caused significant cluster formation of CAR molecules regardless the type of scFv expressed, further indicating that clusters identified by confocal microscopy truly reflect the formation of CAR aggregates. Phenotypic analysis of T cells expressing scFv 763.74(A) CARs or scFv 763.74(B) CARs did not show differences in the expression of memory and exhaustion markers indicating that tonic signaling may not induce an exhaustion phenotype during the 10-14 days of culture usually required to manufacture CAR T cells for clinical use. Overall, these data indicate that amino acid substitutions within the FRWs of a scFv antibody are sufficient in causing self-aggregation of the scFv in the CAR format and tonic signaling in T cells defined as basal release of IFN $\gamma$ .

#### *Amino-acid substitutions within the FWRs of the scFv cause protein destabilization*

To study if the differences in amino acids between the scFv 763.74(A) and scFv 763.74(B) cause destabilization of the scFv, we generated the 3D conformation of the scFv 763.74(A) through homology modeling and optimized the structure for *in silico* mutagenesis (Fig. 9A). We employed Eris tool to delineate the effect of FWR mutations, L3K, T5S, A9S, E83Q, I123V, Q124K, V126K, Q127E, and L230V on the structure of the scFv 763.74(A). Eris estimated the  $\Delta\Delta G_{mut}$  for the above-mentioned mutations as 2.41, 1.26, 2.29, 0.47, 0.79, 1.87, 4.58, 2.70, and 0.67 kcal/mol, respectively (Fig. 9B). Specifically, the mutations are destabilizing scFv 763.74(A) structure ( $\Delta\Delta G_{mut} > 0$ ) and subsequently affecting the CAR spontaneous aggregation. Further, to cross-validate the structural conformation of the scFv 763.74(A), we performed Eris analysis to identify stabilizing mutations ( $\Delta\Delta G_{mut} < 0$ ) at the FWR mutated sites. Our analysis indicated that mutations such as E83L, E83I, T5M, and Q124M result in negative  $\Delta\Delta G_{mut}$ , notifying their potential stabilizing capability. Furthermore, we identified that the residues such as I123 and Q127 are critical for the stability of the scFv 763.74(A) structure. The substitution of any of the other

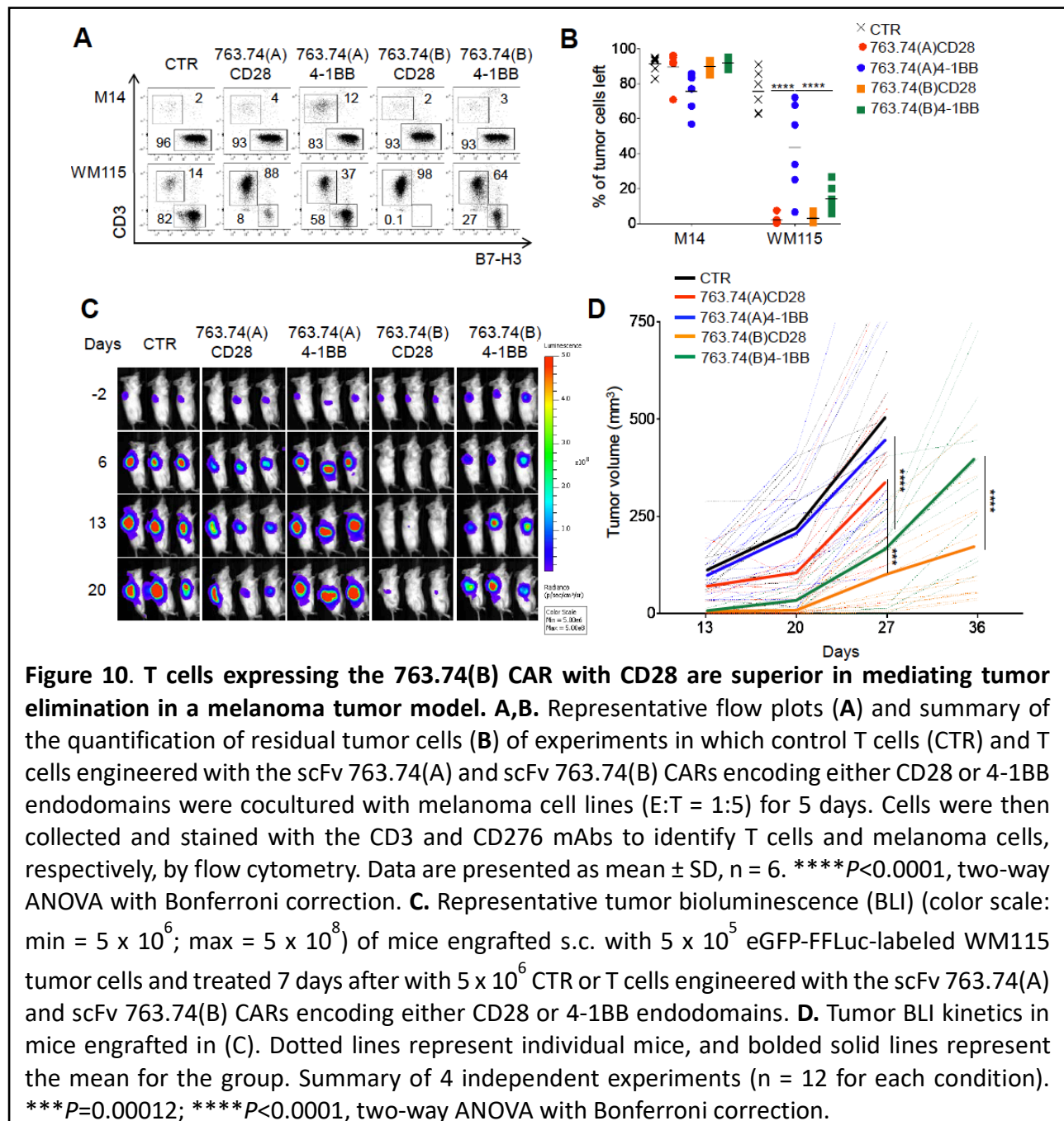
19 amino acids at I123 or Q127 positions highly destabilizes the scFv 763.74(A) structure ( $\Delta\Delta G > 0$ ) and thereby affects the protein aggregation (Fig. 9C). Overall, these data indicate that amino acid substitutions within the FRWs of a scFv mAb cause self-aggregation of the scFv by destabilizing the structure of the scFv and that computation guided analyses can be used to stabilize the protein structure.



Amino acid substitutions within the FWRs of the scFv enhance in vitro functions of CAR-T cells

To determine if the amino acid substitutions within the FWRs of the scFv affect the anti-tumor activity of CAR T cells, we used the melanoma cell lines WM115 (CSPG4<sup>+</sup>) and M14 (CSPG4<sup>-</sup>). Paralleling the spontaneous release of IFN $\gamma$ , T cells expressing the 763.74(A) CAR with 4-1BB showed the least capacity to eliminate tumor cells *in vitro* (residual tumor cells 43.6%  $\pm$  26.0%) at the end of a 4 day coculture when CAR T cells and tumor cells were plated at the 1 to 5 ratio (Fig. 10A,B). In contrast, CD28 costimulation seemed to allow complete tumor elimination for both 763.74(A) and 763.74(B) CARs (residual tumor cells 2.2%  $\pm$  2.7% and 2.9%  $\pm$  2.6%, respectively). Of note, T cells expressing the 763.74(B) CAR with 4-1BB costimulation, showed improved

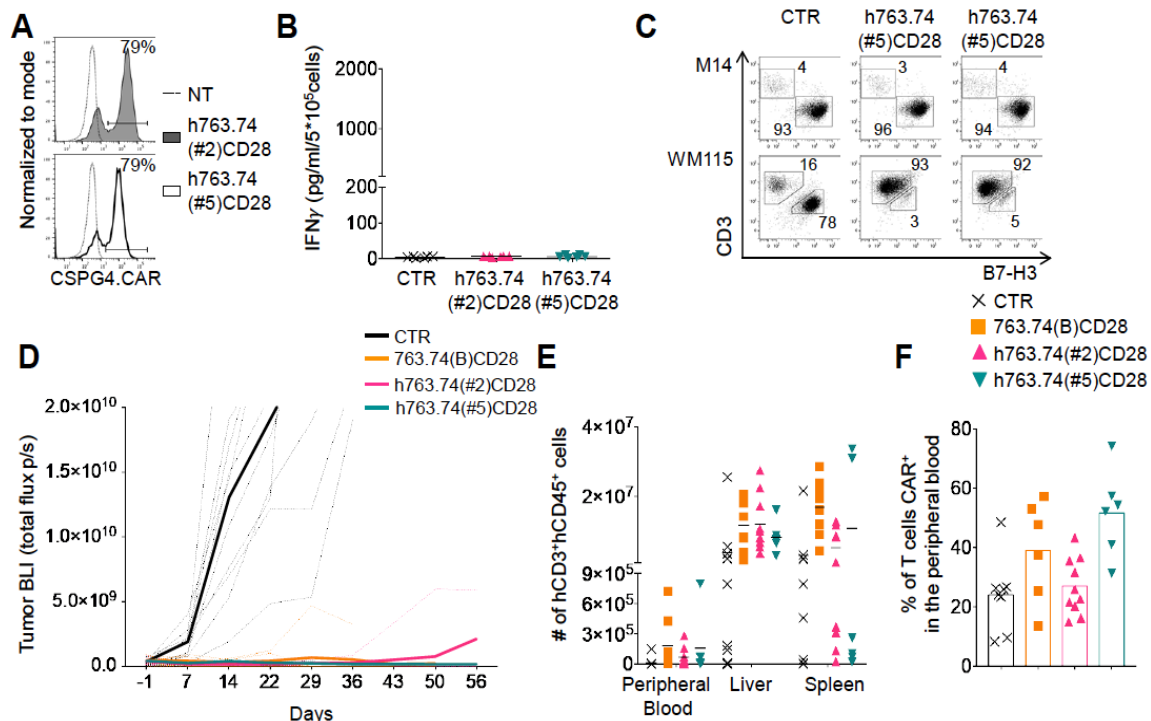
antitumor effects as compared to T cells expressing 763.74(A) CAR with 4-1BB, but did not completely eliminate the tumor cells (residual tumor cells  $14.1\% \pm 8.0\%$  and  $43.6\% \pm 26.0\%$ , respectively) (Fig. 10A,B). CAR T cells did not eliminate the melanoma cell line M14 that lacks CSPG4 expression indicating that antigen specificity was not affected by amino acid substitutions within the FWRs. Of note, only T cells expressing the 763.74(B) CARs consistently released detectable amounts of Th1 cytokines in the culture supernatant with WM115 tumor cells. Additional experiments *in vitro* further demonstrated the rapid antitumor effects of T cells expressing the 763.74(B) CAR with CD28.



### Humanization of the FWRs of the scFv abrogates CAR tonic signaling

Humanization of murine derived scFvs is a strategy proposed to prevent humoral and T cell responses to the CAR. We asked whether substituting the murine FWRs of the scFv with human FWRs could be used to abrogate the CAR tonic signaling. The sequence of 763.74(A) and rFW1.4 were aligned, and the critical amino acids were identified. One CDR graft with no mutations in the rFW1.4 sequence and seven variants with up to 24 mutations in the critical regions were designed in the first round of engineering. Humanized scFv variants alone (i.e. only extracellular domain of CARs) and the wild-type murine scFv were expressed in *E.coli*, refolded and purified by size exclusion chromatography (SEC). The wild-type murine scFv variant was not refoldable due to aggregation. Therefore, we were unable to purify it in a soluble form. This result further suggests that the murine 763.74(A) scFv is unstable. The humanized variant with no mutations in the rFW1.4 was expressed, but did not bind CSPG4. Nearly all other humanized scFv variants were successfully expressed and able to bind CSPG4<sup>+</sup> cells. In the next engineering rounds, humanized variants with the minimal number of 763.74(A) murine FW residues were further subjected to chain shuffling of the V<sub>H</sub> and V<sub>L</sub>. A total of 26 humanized scFvs were produced. Four humanized scFvs (h763.74 #2, h763.74 #3, h763.74 #4 and h763.74 #5) with the minimal number of murine FW residues and retained CSPG-4-binding activity were selected for further studies. We generated CARs with all four humanized scFv 763.74(A) with CD28 endodomain and performed *in vitro* coculture experiments with tumor cells. T cells expressing h763.74.CAR #2 and h763.74.CAR #5 showed a trend for better antitumor activity and higher production of IFN $\gamma$  and IL-2 *in vitro* and were selected for further studies. To characterize the two selected humanized scFvs h763.74.CAR #2 and h763.74.CAR #5, we performed storage stability studies with purified soluble scFvs. Proteins were prepared at 1 mg/ml and stored for a 48hr period at 4°C and 37°C. After incubation, samples were analyzed by SEC to estimate the percentage of monomeric proteins. Under tested conditions, no detectable protein loss was observed, and the percentage of monomers remained above 91% - 97%. This method demonstrated that all four selected humanized scFvs were monomeric under tested conditions and do not dimerize or aggregate upon storage at 4°C and 37°C. The expression of h763.74.CAR #2 and h763.74.CAR #5 in T cells was adequate (Fig. 11A), and T cells did not show spontaneous release of IFN $\gamma$  (Fig. 11B). T cells expressing the h763.74.CAR #2 and h763.74.CAR #5 successfully controlled the WM115 melanoma cell growth *in vitro*

(residual tumor cells  $11\% \pm 15\%$  and  $10\% \pm 17\%$  respectively), while they did not target the CSPG4<sup>+</sup> M14 melanoma cell line indicating that antigen specificity was maintained (Fig. 11C). The anti-tumor activity of h763.74.CAR #2 and h763.74.CAR #5 T cells was corroborated by specific production of IFN $\gamma$  and IL-2.



**Figure 11. Humanization of the FWRs of the scFv 763.74(A) abrogates CAR tonic signaling without affecting the anti-tumor activity.** **A.** Representative flow cytometry plots showing CAR expression in T cells engineered with h763.74 (#2) and h763.74 (#5) CARs encoding CD28 (h763.74(#2)CD28 and h763.74(#5)CD28) as assessed at day 8 of culture. Non-transduced (NT) T cells are shown as a negative control. **B.** Quantification of IFN $\gamma$  in supernatants collected 24 hours of resting control (CTR), h763.74(#2)CD28 and h763.74(#5)CD28 CAR-T cells after ( $5 \times 10^5$  cells/well) plated in 2 mL of medium without cytokines. Data are presented as mean  $\pm$  SD,  $n = 6$ . **C.** Representative flow plots of coculture experiments in which control (CTR) or T cells expressing either h763.74(#2)CD28 or h763.74(#5)CD28 CARs were plated with melanoma cell lines (E:T = 1:5) for 5 days. Cells were then collected and stained with the CD3 and CD276 mAbs to identify T cells and melanoma cells, respectively, by flow cytometry. **D.** Tumor BLI kinetics of mice engrafted s.c. with eGFP-FFLuc-labeled WM115 tumor cells ( $5 \times 10^5$  cells) and treated 7 days after with control CAR-T cells (CTR) or T cells engineered with 763.74(B)CD28 CAR or h763.74(#2)CD28 or h763.74(#5)CD28 CARs ( $5 \times 10^6$  cells). Dotted lines represent individual mice, and bolded solid lines represent the mean for the group. Summary of 2 independent experiments ( $n = 10$  for each group). **E.** Quantification of human CD3<sup>+</sup>CD45<sup>+</sup> cells in the peripheral blood, liver, and spleen at the time of euthanasia of WM115 tumor-bearing mice treated as in (D). Data are presented as mean  $\pm$  SD,  $n = 6$ . **F.** Percentage of CAR-T cells in the peripheral blood, gated on human CD3<sup>+</sup>CD45<sup>+</sup> cells, at sacrifice in WM115 tumor-bearing mice treated as in (D). Data are presented as mean  $\pm$  SD,  $n = 6$ .

Evaluation of the *in vitro* anti-tumor efficacy of T cells genetically engineered with a CAR generated with the

CSPG4-specific human scFv SK5

The use of a human CSPG4-specific mouse scFv to generate CSPG4 CAR T cells has generated concerns, since the immune response elicited in treated patients by mouse scFv may interfere with the persistence of CSPG4 CAR T cells in the treated patients. To overcome this potential limitation, we have developed and characterized a CSPG4-specific human scFv. The work performed has presented in detail in previous Progress Reports. Briefly, we have isolated the human CSPG4-specific human scFv SK5 from a phage display scFv library, and we have shown the specificity of SK5 for CSPG4. Subsequently, we characterized the scFv SK5-defined CSPG4 epitope, which is expressed on cancer differentiated cells and on cancer initiating cells. We then generated a human CSPG4 CAR with scFv SK5, and showed that T cells transduced with this CAR specifically recognize and eliminate CSPG4(+) TNBC cells *in vitro*.

Alternative CAR approach utilizing cytokine-induced killer T-lymphocytes (CIK) engineered with a CSPG4-specific CAR (CSPG4-CAR.CIK cells).

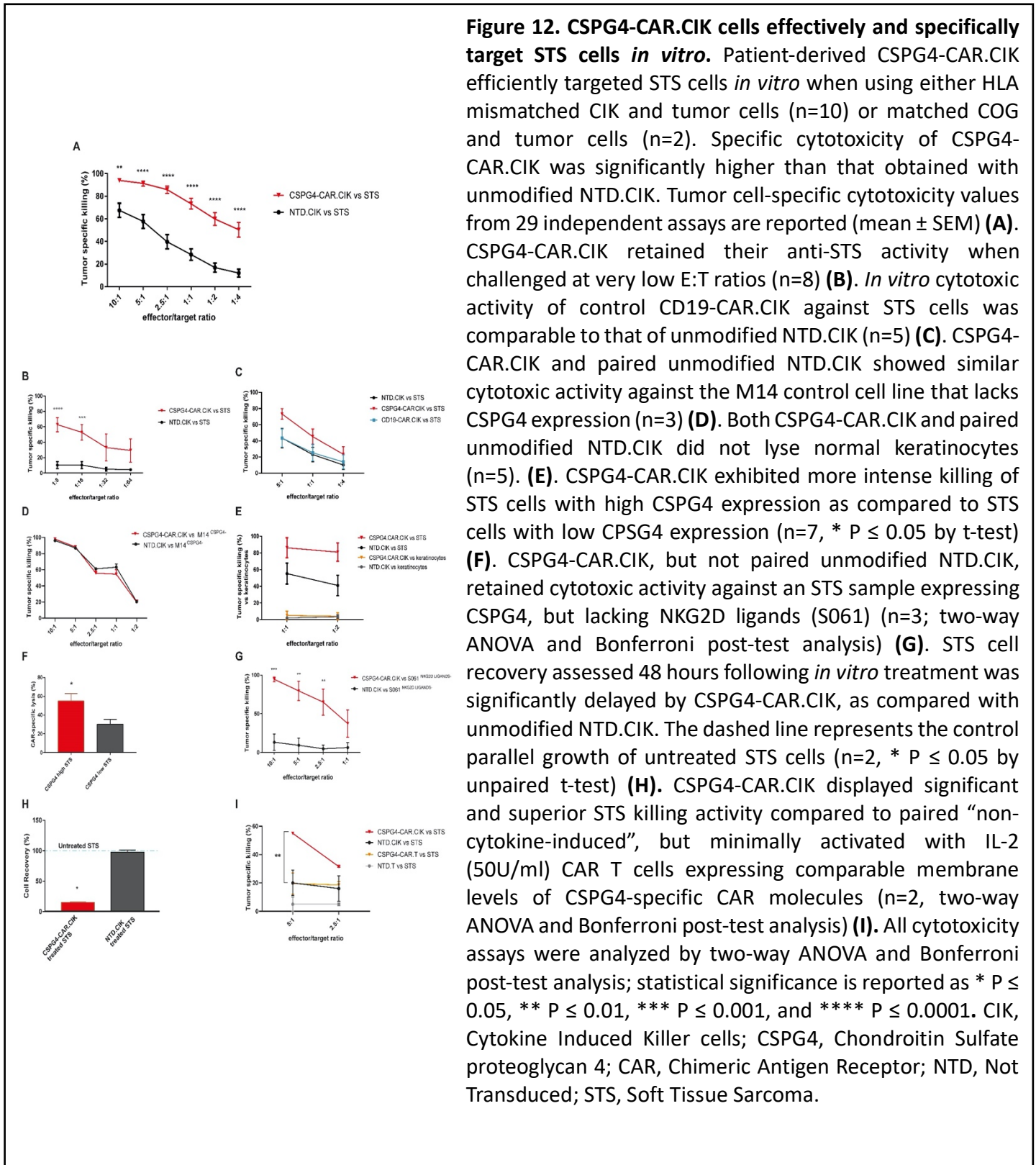
Aiming to increase the activity of the CAR approach, we have explored the use of cytokine-induced killer T-lymphocytes (CIK) instead of T cells. CIKs are patient-derived polyclonal T-NK lymphocytes endowed with HLA class I-independent antitumor activity, mediated mostly by the interaction of their NKG2D receptor with stress-inducible targets (MIC A/B; ULBPs 1-6) on tumor cells. To increase their tumor cell specificity, CIK cells were engineered with a CSPG4-specific CAR (CSPG4-CAR.CIK). We initially tested this approach in soft tissue sarcoma (STS).

Generation of CSPG4-CAR.CIK

Using CSPG4 CAR containing 4-1BB costimulatory endodomains we generated CSPG4-CAR.CIKs from peripheral blood mononuclear cells (PBMC) isolated from STS patients.

CSPG4-CAR.CIK cells effectively and specifically target STS cells *in vitro* and in 3D spheroids

We explored the *in vitro* anti-tumor activity of CSPG4-CAR.CIK against 12 STS cell lines. Briefly, CSPG4-CAR.CIK cells revealed superior *in vitro* CSPG4-specific cytotoxicity against STS compared to unmodified non-transduced CIK cells and control CD19-CAR.CIK cells. Furthermore, a significant and superior activity level of



STS killing was displayed by CSPG4-CAR.CIK cells compared to CAR T cells. Both effector cells were generated from PBMC collected from the same patient and expressed comparable levels of CSPG4-specific CAR molecules (Fig. 12). Along the same lines, CSPG4-CAR.CIK cells eliminated STS 3D spheroids more effectively than unmodified non-transduced CIK cells.

**Subtask 4: Repetition of some experiments in subtask 1 to 3 using T cells transduced with CARs (4-5 donors using buffy coat from the blood bank).**

Because of the restriction imposed by the COVID-19 pandemic, the results obtained could not be repeated with the planned number of donors.

**Specific Aim 3: CSPG4-specific CAR PD-1 shRNA-T cells in combination with IL-2-anti-id mAb MK2-23 fusion protein and LDE225 suppress in an adjuvant setting metastatic spread and disease recurrence and prolong survival of NSG mice which are orthotopically grafted with the TNBC MDA-MB-231-Luc-D3H1 cell line and then subjected to surgical removal of their primary tumor.**

*Specific Aim synopsis*

Based on the *in vitro* results obtained in Specific Aim 2, in Specific Aim 3 we tested the *in vivo* anti-tumor activity of the components of our combinatorial immunotherapeutic strategy. Specifically, we tested whether *in vivo* or *ex vivo* administration of vorinostat, alone or in combination with radiation, can enhance the *in vivo* anti-tumor activity of CAR T cells. Furthermore, we investigated whether counteracting tonic signaling would enhance the effectiveness of CAR T cells. Lastly, we investigated whether CSPG4-CAR.CIK cells can control tumor growth *in vivo*.

***Subtask 1: CSPG4-targeted CAR T cells-based immunotherapy in combination with IL-2-anti-id mAb MK2-23 fusion protein and LDE225 is effective in suppressing metastases and recurrence in NSG mice following***

### ***surgical removal of MDA-MB-231-Luc-derived xenografts.***

#### *CSPG4 expression by TNBC cells transfected with a GFP-luciferase lentivirus vector*

In order to assess the *in vivo* activity of CSPG4 CAR T cells with TNBC cells, TNBC cell line MDA-MB-231 has been transfected with GFP-luciferase, without affecting the expression of CSPG4. Cell sorting was performed in order to isolate luciferase transfected cells.

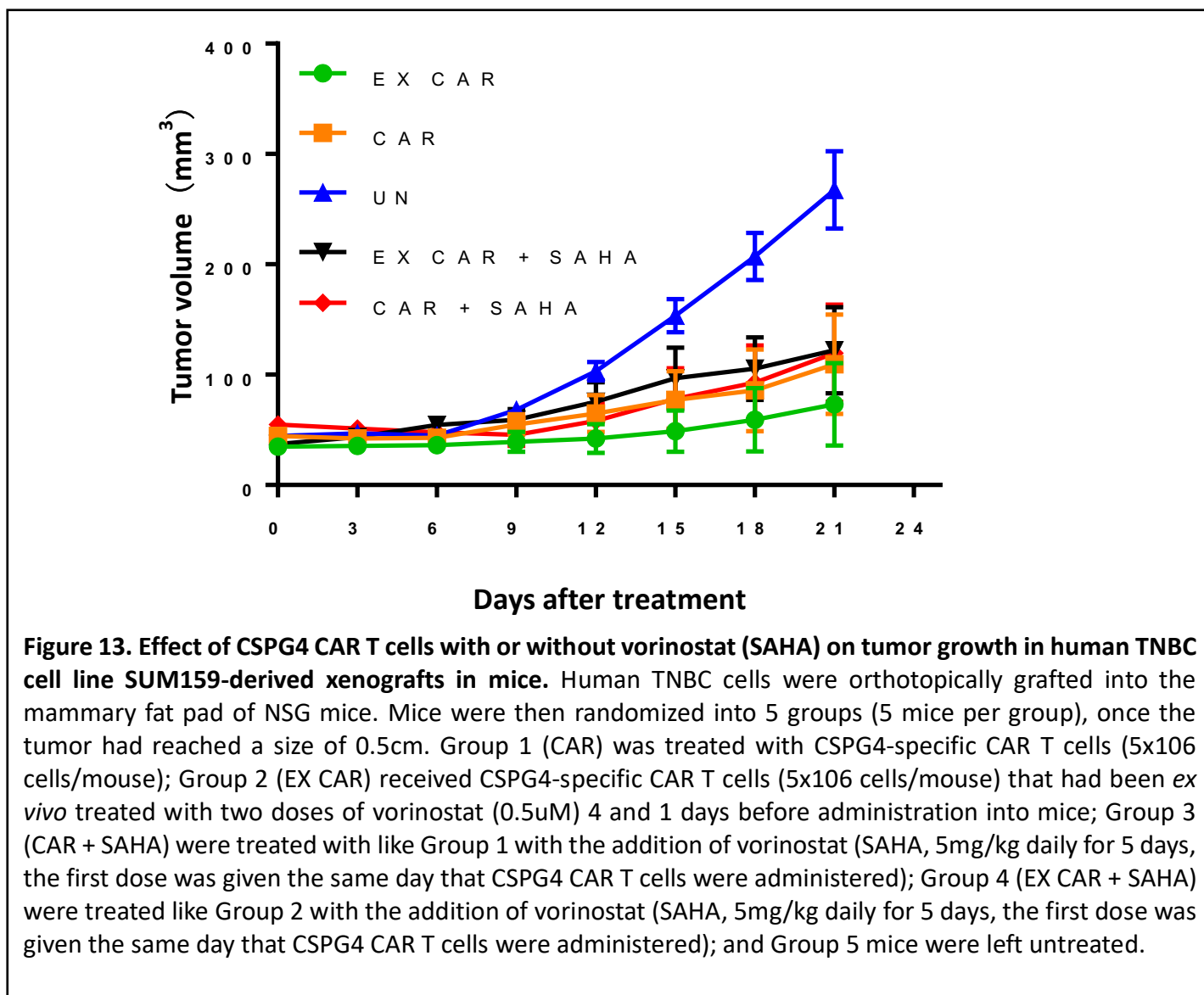
As already mentioned, LDE225 has accelerated disease progression in patients with pancreatic ductal adenocarcinoma. These unexpected clinical results have caused some apprehension among clinicians to use this small molecule in patients with malignant disease. These concerns have prompted us to seek alternative strategies. As a result, we have started to investigate whether the HDACi vorinostat can enhance the antitumor activity of CSPG4 CAR T cells in a mouse model system.

#### *Combinatorial treatment with CSPG4-specific CAR T cells and vorinostat*

As described above, vorinostat can enhance the *in vitro* anti-tumor activity of CSPG4 CAR T cells against TNBC cells. On the basis of these results, we performed an *in vivo* mouse study to assess the effect of the combinatorial therapy with vorinostat and CSPG4-specific CAR T cells. As mentioned in previous Progress Reports, mice treated with vorinostat and CSPG4-specific CAR T cell had a significantly better control of tumor growth compared to all the other control groups (non-transduced T cells, vorinostat + non-transduced T cells, CSPG4 CAR T cells).

In previous Progress Reports we described preliminary experiments investigating whether *ex vivo* treatment of CSPG4-specific CAR T cells with vorinostat can enhance their *in vivo* anti-tumor activity. We have now performed additional experiments with a larger sample size. Briefly, human TNBC cells were orthotopically grafted into the mammary fat pad of NSG mice. Mice were then randomized into 5 groups (5 mice per group), once the tumor had reached a size of 0.5cm. Group 1 was treated with CSPG4-specific CAR T cells ( $5 \times 10^6$  cells/mouse); Group 2 received CSPG4-specific CAR T cells ( $5 \times 10^6$  cells/mouse) that had been *ex vivo* treated with two doses of vorinostat (0.5uM) 4 and 1 days before administration into mice; Group 3 were treated with like Group 1 with the addition of vorinostat (5mg/kg daily for 5 days, the first dose was given the same day that

CSPG4 CAR T cells were administered); Group 4 were treated like Group 2 with the addition of vorinostat (5mg/kg daily for 5 days, the first dose was given the same day that CSPG4 CAR T cells were administered); and Group 5 mice were left untreated. There was no significant difference in terms of tumor volume among groups of treated mice. The tumor volume in all the groups treated with CAR T cells was lower than that in untreated controls (Fig. 13).

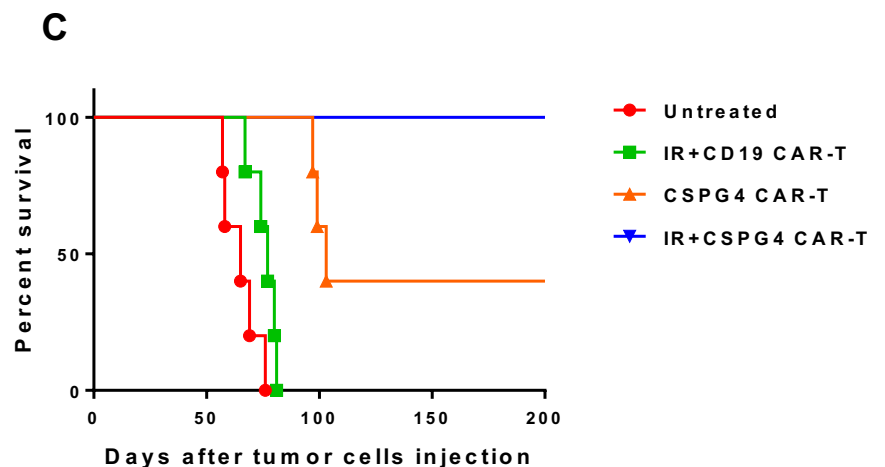
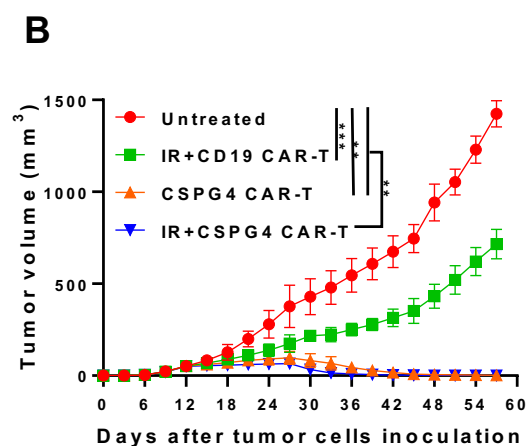
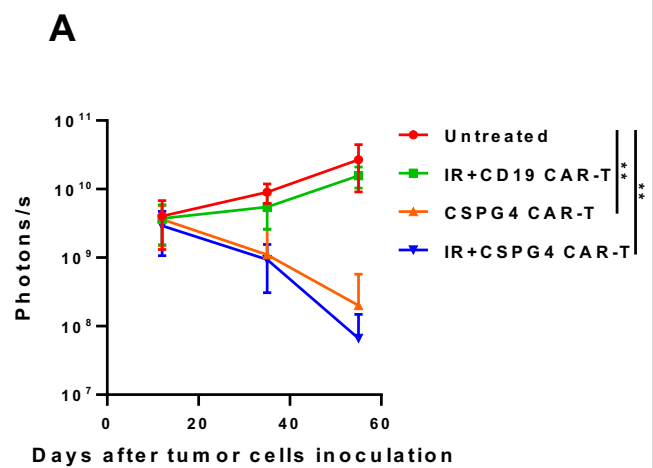


Enhancement by radiation of the ability of CSPG4-specific CAR T cells to eliminate tumor cells *in vivo*

Prompted by enhancement by radiation of the ability of CSPG4-specific CAR T cells to eliminate TNBC cells *in vivo*, as described in Specific Aim 2, we tested whether the same enhancement can be obtained *in vivo*. For our

initial experiments we used a chordoma mouse model. Human chordoma cells were injected subcutaneously in 20 NSG mice. Mice were assigned into 4 groups (5 mice per group): Group 1 was treated with CSPG4 CAR T cells ( $10^6$  CAR T cells/mouse,  $100\mu\text{l}$ , iv, on day 14 and 28), Group 2 was treated like Group 1 plus radiation (10Gy on day 14), Group 3 received CD19 CAR T cells plus radiation, and Group 4 mice were left untreated. In both groups treated with CSPG4 CAR T cells, tumor volume decreased to almost  $0\text{mm}^3$  40 days after tumor inoculation. While the experiment is still ongoing, all mice in Group 2 (with CSPG4 CAR T cells + radiation) remain alive 200 days after tumor inoculation (Fig. 14).

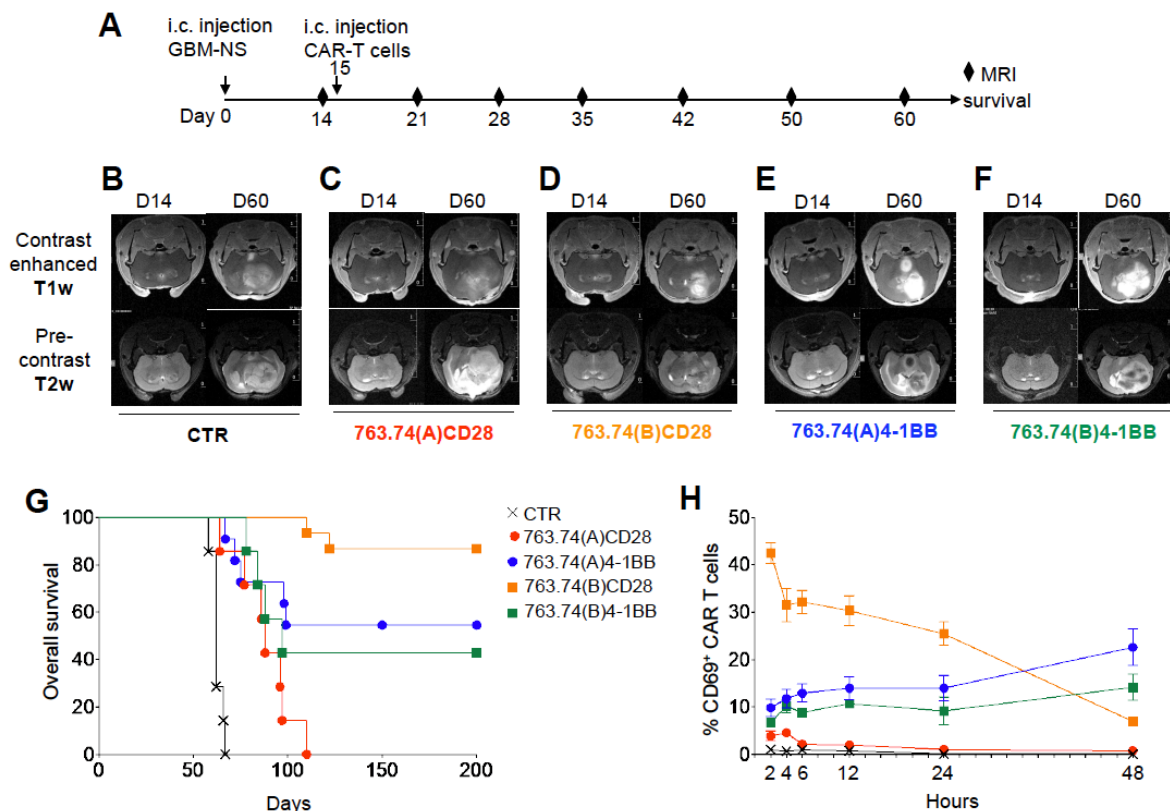
**Figure 14. Effect of combinatorial therapy with CSPG4 CAR T cells and radiation (IR) on growth of MUG-CC1 xenografts tumors in NSG mice.** Mcherry/Luc transfected human MUG-CC1 cells  $2 \times 10^6$  in  $100\text{ul}$  serum- and drug-free RPMI 1640 were injected subcutaneously into the right leg of 20 NSG mice. Mice were assigned in a stratified randomized manner to 4 groups ( $n=5/\text{group}$ ) according to the photons/second of each mouse and the treatments were initiated on day 14 and day 28 (IR=10Gy,  $2 \times 10^6$  CAR-T cells/mouse,  $100\text{ }\mu\text{l}$ , iv). Tumor growth was monitored by animal bioluminescent imaging (BLI) and photons/second of BLI are shown in (A), mean tumor volumes of each group  $\pm$  SD are shown in (B), and survival curve of each group are shown in (C).



**Counteracting tonic signaling: amino-acid substitutions within the FWRs of the scFv enhance in vivo functions of CAR-T cells**

Following the promising *in vitro* findings regarding counteracting tonic signaling described in Specific Aim 2,

we tested this strategy in a mouse model. The superior antitumor effects of 763.74(B) CAR T cells was more evident *in vivo* using the WM115 xenogeneic NSG mouse model. T cells expressing the 763.74(B) CAR with CD28 exhibited the most prominent antitumor effects measured as both tumor bioluminescence (Fig. 10C) and tumor size (Fig. 10D). Enhanced functions of CAR-T cells expressing the 763.74(B) CAR were confirmed in a glioblastoma (GBM) tumor model in which nude mice are engrafted in the brain with primary GBM-derived neurospheres (GBM-NS) and treated via intratumor inoculation of CAR-T cells (Fig.15A). In this model tumor engraftment and progression were monitored by magnetic resonance imaging (MRI). We observed rapid tumor progression in mice treated with control T cells or T cells expressing the 763.74(A) CAR encoding CD28, and in



**Figure 15. T cells expressing the 763.74(B) CAR with CD28 have rapid antitumor activity in a glioblastoma tumor**

**model.** **A.** Experimental schema of glioblastoma (GBM) xenograft model. GBM-NS ( $1 \times 10^5$  cells) were injected into the caudate nucleus and 15 days later mice were injected intratumorally with control T cells (CTR) or T cells engineered with the scFv 763.74(A) and scFv 763.74(B) CARs encoding either CD28 or 4-1BB endodomains ( $2 \times 10^6$  cells). Tumor growth was monitored with magnetic resonance imaging (MRI). **B-F.** Representative MRI performed with T1-weighted images (T1-wi) with contrast medium injection and T2-weighted images (T2-wi) showing the pattern of tumor progression and infiltration in mice treated as in (A). **G.** Kaplan-Meier survival curves of mice treated as in (A).  $n = 7$  mice/group for CTR, 763.74(A)CD28, 763.74(B)4-1BB.  $n = 15$  mice/group for 763.74(B)CD28 and 763.74(A)4-1BB. Overall survival statistical analysis was performed using the Mantel-Cox log rank test. **H.** Time course of CD69 expression in CAR-T cells isolated from the tumor at the indicated time points after intratumor delivery of the T cells. Data are presented as mean  $\pm$  SD,  $n = 2$ .

these animals tumor masses occupied the whole hemispheres and infiltrated the contralateral one (Fig. 15B,C). Mice treated with T cells expressing the 763.74(B) CAR encoding CD28 showed the most evident antitumor effects as indicated by smaller and more circumscribed lesions (Fig. 15D), but tumor control was also observed in mice treated with T cells expressing either 763.74(A) or 763.74(B) CAR encoding 4-1BB even if the antitumor effects were less dramatic (Fig. 15E,F). T cells expressing either 763.74(A) or 763.74(B) CARs encoding 4-1BB prolonged survival in 50% and 40% of the treated mice, respectively, as compared to mice treated with control T cells ( $p < 0.0001$ ). However, T cells expressing the 763.74(B) CAR encoding CD28 improved survival in 90% of mice ( $p < 0.0001$  vs. CTR;  $p = 0.04$  vs. 763.74(A) with 4-1BB,  $p = 0.01$  vs. 763.74(B) with 4-1BB). We also confirmed the modest activity of T cells expressing the 763.74(A) CAR encoding CD28 since no mice survived more than 110 days (Fig. 15G). To further characterize the remarkable antitumor effect of T cells expressing the 763.74(B) CAR encoding CD28 in this GBM model, we investigated the activation status of T cells immediately after intracranial infusion. We explanted the tumor masses 2, 4, 6, 12, 24 and 48 hours after CAR-T cell infusion and observed that T cells expressing the 763.74(B) CAR with CD28 upregulated CD69 within 2 hours after inoculation ( $42.5 \pm 2.1\%$  CD69<sup>+</sup> T cells), and maintained high CD69 levels for 24 hours ( $22.5 \pm 1.5\%$  CD69<sup>+</sup> T cells) (Fig. 15H).

#### Humanization of the FWRs of the scFv abrogates CAR tonic signaling

Finally, we compared T cells expressing the h763.74.CAR #2 and h763.74.CAR #5 with T cells expressing the 763.74(B) CAR encoding the CD28 endodomain in the xenogeneic WM115 melanoma mouse model. T cells expressing the h763.74.CAR #2 and h763.74.CAR #5 showed potent antitumor activity (Fig. 11D). Furthermore, T cells were detectable in the peripheral blood of treated mice at different time points, and in the liver and spleen at the time of euthanasia (Fig. 11E), and T cells retained the CAR expression (Fig. 11F). Of note, T cells expressing h763.74.CAR #2 and h763.74.CAR #5 did not show increased expression of PD-1 as compared to 763.74(B) CAR with CD28. Overall, these data show that the humanization of a scFv can be used to eliminate tonic signaling of CAR molecules maintaining specific antitumor effects.

*Lack of in vivo anti-tumor activity of T cells transduced with CSPG4 CAR generated with the human SK5 scFv*

As described in Specific Aim 2, we generated a human CSPG4 CAR with scFv SK5, and showed that T cells transduced with this CAR specifically recognize and eliminate CSPG4(+) TNBC cells *in vitro*. However, when tested *in vivo*, their anti-tumor activity against TNBC cells orthotopically grafted in mice was very poor. Similar results were obtained with human melanoma cells grafted subcutaneously in NSG mice. We hypothesize that the lack of therapeutic efficacy of CAR T cells *in vivo* may reflect changes in the conformation of the CAR because of low pH of the tumor microenvironment.

*CSPG4-CAR.CIK cells controlled tumor growth in vivo*

Following the promising *in vitro* results obtained in Specific Aim 2, we explored the *in vivo* antitumor activity of CSPG4-CAR.CIK utilizing three STS xenograft models. Briefly, we found that CSPG4-CAR.CIK cells, but not non-transduced CIK cells, caused a significant delay in tumor growth. This held true for all three models and for different initial tumor burdens. Of note, antitumor effects were obtained without any macroscopic toxicity. Tumor infiltration by CSPG4-CAR.CIK cells was confirmed in explanted tumors (Fig. 16).

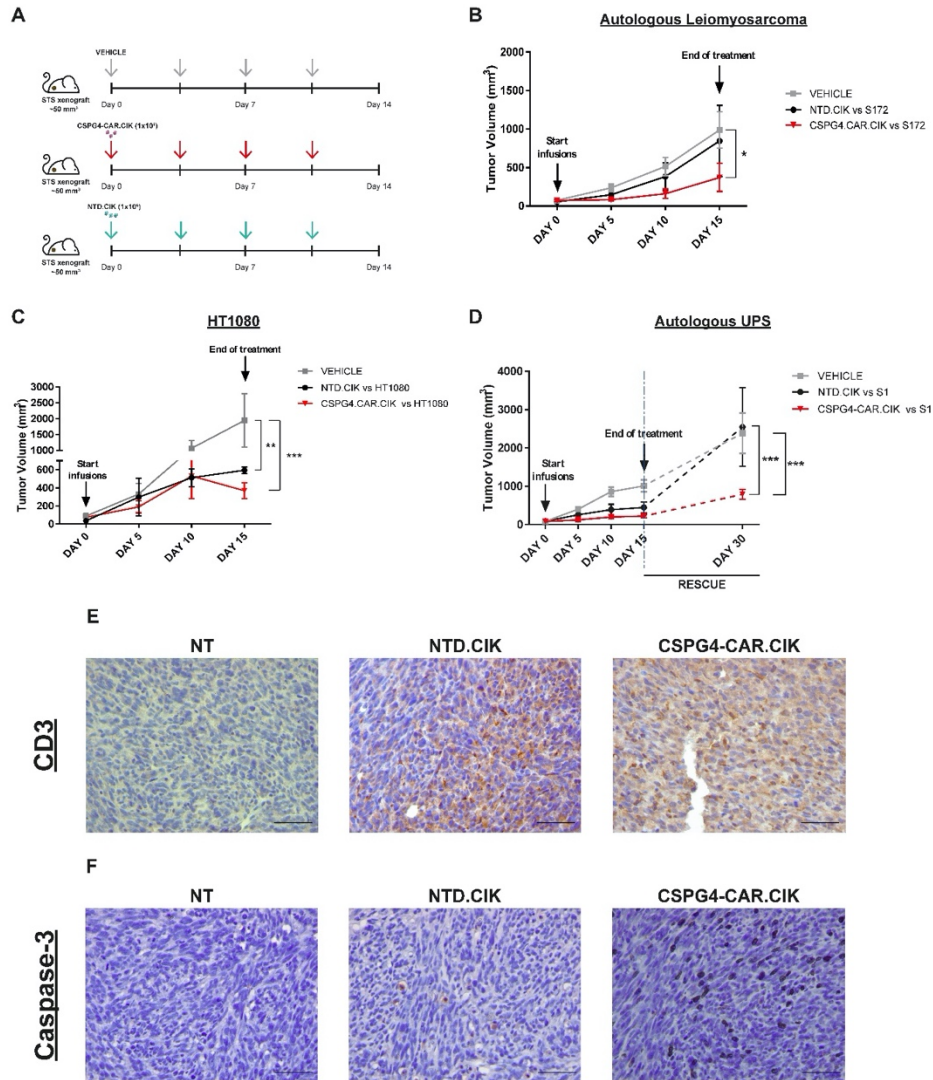
**Subtask 2: Characterization of metastases and recurrent tumors in mice treated with combinatorial therapy**

We omitted this subtask given that we did not perform the originally planned strategy of surgically removing primary tumors with subsequent treatment of metastases and recurrent tumors.

**Subtask 3: Assessment of side effects caused by the combinatorial therapy**

No side effects were observed as indicated by the lack of mouse weight changes and overall health condition.

**Subtask 4: Repetition of some experiments in subtask 1 to 3 using T cells transduced with CARs (4-5 donors using buffy coat from the blood bank).** Because of the restriction imposed by the COVID-19 pandemic, the results obtained could not be repeated with the planned number of donors.



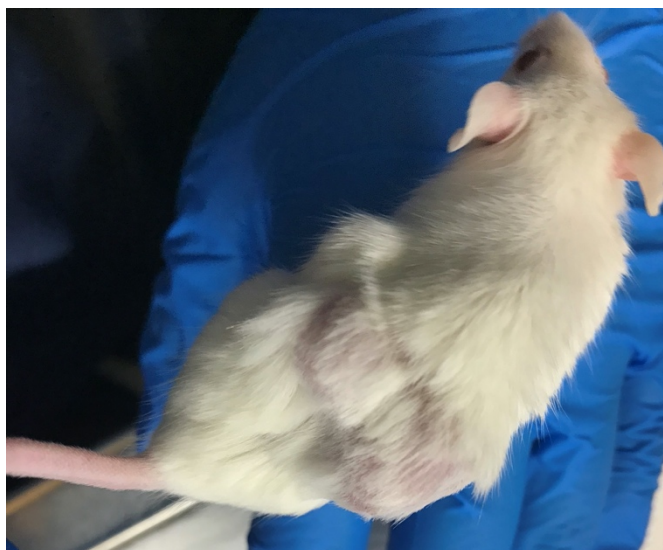
**Figure 16. CSPG4-CAR.CIK are active against STS in xenograft models.** Schematic representation of the STS xenografts and treatment with CSPG4-CAR.CIK (red arrows) and NTD.CIK (blue arrows). Vehicle-treated mice were infused with PBS (grey arrows). CIK were intravenously infused ( $1 \times 10^6$  cells/infusion) twice a week for 2 weeks **(A)**. Autologous CSPG4-CAR.CIK caused a significant delay of the growth of the S172 leiomyosarcoma (CSPG4=72% and CSPG4 density=262 molecule/cell) as compared to unmodified NTD.CIK or vehicle-treated mice ( $n=6$ ;  $p<0.05$ ) **(B)**. Autologous CSPG4-CAR.CIK caused a significant delay of the growth of the HT1080 fibrosarcoma (CSPG4=23% and CSPG4 density=521 molecule/cell) as compared to unmodified NTD.CIK or vehicle-treated mice ( $n=3$ ,  $p<0.001$ ). **(C)**. Autologous CSPG4-CAR.CIK effectively delayed the growth of S1 UPS (CSPG4=95% and CSPG4 density=499 molecule/cell) as compared with controls ( $n=3$ ,  $p<0.0001$ ). All results were analyzed by two-way ANOVA and the Bonferroni post-test; statistical significance is reported as \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ , and \*\*\*\*  $P \leq 0.0001$ . **(D)**. Tumor-homing of CSPG4-CAR.CIK and unmodified NTD.CIK was confirmed by IHC in explanted tumors using an anti-human CD3 antibody staining. Magnification: 40X; Scale bars: 50  $\mu\text{m}$  **(E)**. Apoptotic tumor cells were visualized by Cleaved caspase 3 IHC in explanted tumors **(F)**. CIK: Cytokine Induced Killer cells; CSPG4: Chondroitin Sulfate Proteoglycan 4; CAR: Chimeric Antigen Receptor; NTD: Not Transduced; NOD/SCID: Non-obese diabetic/severe combined immunodeficiency mice; NSG: NOD/SCID gamma mice.

**Specific Aim 4: The results obtained with the TNBC cell lines have clinical significance, as they are reproduced in NSG mice orthotopically grafted with patient derived TNBC xenografts (PDX).**

**Subtask 1: Characterization of patient derived orthotopic xenografts established in SCID mice from TNBC tumors**

As mentioned in the grant application we had succeeded in engrafting a small number of surgically removed TNBC tumors in NSG mice. Two of the 4 tumors that expressed CSPG4 metastasized. Xenografts derived from the expanded PDX resembled the original tumor in the histology, CSPG4 expression and ALDH<sup>bright</sup> CICs. The human nature of these tumors was confirmed by testing them for HLA class I antigen expression. However, when we attempted to expand the number of PDXs to be engrafted into mice for further PDX mouse experiments, we were not successful.

We have now expanded our PDX tumors and have banked enough PDX tumors to implant at least 100 mice for future experiments. We found that subcutaneous expansion of PDX is easier and faster. The histology of such expanded PDXs remained the same as the PDXs expanded orthotopically and the original patient tumor (Fig. 17). We observed similar findings with pancreatic ductal adenocarcinoma PDXs (Liu *et al.*, 2020, appended paper).



**Figure 17. Patient-derived xenograft (PDX) from a patient diagnosed with triple negative invasive ductal carcinoma (ER-, PR-and HER2-). PDX was successfully expanded subcutaneously (s.c) in female NSG mice.**

**Subtask 2: Suppression by CSPG4-targeted T cell- based immunotherapy in combination with IL-2-anti-id mAb MK2-23 fusion protein and LDE225 of recurrence and metastases in NSG mice following surgical removal of primary tumors established from patient derived TNBC tumors.**

**Subtask 3: Analysis of tumors and metastatic lesions.**

**Subtask 4: Prolongation by CSPG4-targeted T cell- based immunotherapy in combination with IL-2-anti-id mAb MK2-23 fusion protein and LDE225 of NSG mice following surgical removal of primary tumors established from patient derived TNBC tumors.**

**Subtask 5: Repetition of experiments in subtask 1 to 4 using T cells transduced with CARs (4-5 donors using buffy coat from the blood bank).**

The indicated subtasks in this Specific Aim could not be performed given the delayed generation of an adequate number of PDXs.

- **What opportunities for training and professional development has the project provided?**

Dr. Dotti, UNC group: Nothing to report

Dr. Ferrone, MGH group, has trained one graduate student and one post-doctoral research fellow.

- **How were the results disseminated to communities of interest?**

Dr. Dotti, UNC group: Nothing to report.

Dr. Ferrone, MGH group, has presented the data to patient advocate group at Dana Farber Cancer Center.

- **What do you plan to do during the next reporting period to accomplish the goals?**

Nothing to report.

#### 4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**
- **What was the impact on other disciplines?**

Dr. Dotti, UNC group: Nothing to Report

Dr. Ferrone, MGH group, is developing a strategy to improve the anti-tumor activity of tumor antigen-specific CAR T cells.

- **What was the impact on the technology transfer?**

Dr. Dotti, UNC group, and Dr. Ferrone, MGH group, have submitted a joint patent application.

- **What was the impact on society beyond science and technology?**

Dr. Dotti, UNC group: Nothing to report

Dr. Ferrone, MGH group: Nothing to report

#### 5. CHANGES/PROBLEMS:

Dr. Dotti, UNC group: Nothing to report.

Dr. Ferrone, MGH group:

- **Changes in approach and reasons for change:** Dr. Ferrone, MGH group has decided to replace sonidegib with a compound which downregulates anti-apoptotic molecules, since sonidegib has been recently shown to accelerate disease progression in some types of cancer. Therefore, it would be difficult to use in clinical trials in patients with TNBC.
- **Actual or anticipated problems or delays and actions or plans to resolve them**
- **Changes that had a significant impact on expenditures**

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
- **Significant changes in use or care of human subjects**
- **Significant changes in use or care of vertebrate animals.**
- **Significant changes in use of biohazards and/or select agents**

## 6. **PRODUCTS:**

Dr. Dotti, UNC group: Nothing to report

Dr. Ferrone, MGH group: Nothing to report

- **Publications, conference papers, and presentations**

- **Journal publications**

1. Piras LA, Riccardo F, Iussich S, Maniscalco L, Gattino F, Martano M, et al. Prolongation of survival of dogs with oral malignant melanoma treated by en bloc surgical resection and adjuvant CSPG4-antigen electrovaccination. *Vet Comp Oncol* 2017;15(3):996-1013 doi 10.1111/vco.12239.
2. Seliger B, Kloor M, Ferrone S. HLA class II antigen-processing pathway in tumors: Molecular defects and clinical relevance. *Oncoimmunology* 2017;6(2):e1171447 doi 10.1080/2162402X.2016.1171447.
3. Eng MS, Kaur J, Prasmickaite L, Engesaeter BO, Weyergang A, Skarpen E, et al. Enhanced targeting of triple-negative breast carcinoma and malignant melanoma by photochemical internalization of CSPG4-targeting immunotoxins. *Photochem Photobiol Sci* 2018;17(5):539-51 doi 10.1039/C7PP00358G.
4. Kasten BB, Oliver PG, Kim H, Fan J, Ferrone S, Zinn KR, et al. (212)Pb-Labeled Antibody 225.28 Targeted to Chondroitin Sulfate Proteoglycan 4 for Triple-Negative Breast Cancer Therapy in Mouse Models. *Int J Mol Sci* 2018;19(4) doi 10.3390/ijms19040925.
5. Maccalli C, Rasul KI, Elawad M, Ferrone S. The role of cancer stem cells in the modulation of anti-tumor immune responses. *Semin Cancer Biol* 2018;53:189-200 doi 10.1016/j.semcancer.2018.09.006.

6. Pellegatta S, Savoldo B, Di Ianni N, Corbetta C, Chen Y, Patane M, et al. Constitutive and TNF $\alpha$ -inducible expression of chondroitin sulfate proteoglycan 4 in glioblastoma and neurospheres: Implications for CAR-T cell therapy. *Sci Transl Med* 2018;10(430) doi 10.1126/scitranslmed.aao2731.
7. Pilla L, Ferrone S, Maccalli C. Methods for improving the immunogenicity and efficacy of cancer vaccines. *Expert Opin Biol Ther* 2018;18(7):765-84 doi 10.1080/14712598.2018.1485649.
8. Du H, Hirabayashi K, Ahn S, Kren NP, Montgomery SA, Wang X, et al. Antitumor Responses in the Absence of Toxicity in Solid Tumors by Targeting B7-H3 via Chimeric Antigen Receptor T Cells. *Cancer Cell* 2019;35(2):221-37 e8 doi 10.1016/j.ccell.2019.01.002.
9. Kasten BB, Ferrone S, Zinn KR, Buchsbaum DJ. B7-H3-targeted Radioimmunotherapy of Human Cancer. *Curr Med Chem* 2020;27(24):4016-38 doi 10.2174/0929867326666190228120908.
10. Leuci V, Donini C, Grignani G, Rotolo R, Mesiano G, Fiorino E, et al. CSPG4-specific CAR.CIK lymphocytes as a novel therapy for the treatment of multiple soft tissue sarcoma histotypes. *Clin Cancer Res* 2020 doi 10.1158/1078-0432.CCR-20-0357.
11. Liu Z, Ahn MH, Kurokawa T, Ly A, Zhang G, Wang F, et al. A fast, simple, and cost-effective method of expanding patient-derived xenograft mouse models of pancreatic ductal adenocarcinoma. *J Transl Med* 2020;18(1):255 doi 10.1186/s12967-020-02414-9.
12. Maggs L, Ferrone S. Improving the Clinical Significance of Preclinical Immunotherapy Studies through Incorporating Tumor Microenvironment-like Conditions. *Clin Cancer Res* 2020;26(17):4448-53 doi 10.1158/1078-0432.CCR-20-0358.
13. Hauth F, Ho A, Ferrone S, Duda D. Use of Radiotherapy to Enhance CAR-T Cell Therapeutic Efficacy in Solid Tumors: A Review. *JAMA Oncol*. Accepted pending major revisions.
14. Maggs L, Kontos F, Cattaneo G, Moghaddam A, Zhang Y, He L, Wang X, Ferrone S. Combinatorial strategies to increase CAR T cell activity in solid cancers. *Front. Immunol*. Accepted pending major revisions.

- **Books or other non-periodical, one-time publications.**

- **Other publications, conference papers, and presentations.**
- **Website(s) or other Internet site(s)**
- **Technologies or techniques**
- **Inventions, patent applications, and/or licenses**
- **Other Products**

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	<i>Dotti, Gianpietro</i>
Project Role:	<i>PI</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>0.97 month</i>
Contribution to Project:	<i>Dr. Dotti has supervised Dr. Sun and provided support for generation of the optimized CAR. He has also been discussing progress and updates with Dr Ferrone at MGH</i>
Funding Support:	

Has supervised the postdoctoral fellow:

Name:	<i>Savoldo, Barbara</i>
Project Role:	<i>Co-investigator</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>0.59 month</i>
Contribution to Project:	<i>Dr. Savoldo has supervised Dr. Sun in some of the functional assays described in Fig2</i>
Funding Support:	

Dr. Ferrone has supervised the collaborator

Name:	<i>Xinhui, Wang, MD, PhD</i>
Project Role:	<i>Assistant Professor</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>1.2 months</i>
Contribution to Project:	<i>Dr. Wang has purified the IL-2 fusion protein and has participated in the design of the experiments described in the progress report as well as in the interpretation of the results generated by the described experiments.</i>
Funding Support:	

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Dr Dotti, UNC group: Nothing to Report

Dr Ferrone, MGH group: Nothing to Report

- **What other organizations were involved as partners?**

None

## 8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:**
- **QUAD CHARTS:**

## 9. APPENDICES:

The published papers listed under “Journal publications” are appended.