AWARD NUMBER: W81XWH-16-1-0500

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

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REPORT DATE: September 2018

TYPE OF REPORT: ANNUAL

PREPARED FOR: U.S Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
September 2018	Annual	1 Sep 2017 - 31 AUG 2018

4. TITLE AND SUBTITLE

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

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-1-0500

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Massachusetts General Hospital
55 Fruit Street, Boston, MA 02114
AND ADDRESS(ES)

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)
DOD

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The lack of effective therapies for the treatment of metastatic triple negative breast cancer (TNBC) has prompted us to develop a combinatorial strategy for the treatment of this highly malignant type of breast cancer. In this strategy, the tumor antigen chondroitin sulphate proteoglycan 4 (CSPG4) is used as a target, since it is expressed on both differentiated TNBC cells and TNBC cancer initiating cells (CICs) and has a restricted distribution in normal tissues. Therefore, immunotargeting of CSPG4 is not expected to cause major side effects because of targeting of normal tissues and is expected to eliminate not only differentiated TNBC cells, but also TNBC CICs. According to the cancer stem cell theory, CICs play a major role in disease recurrence and in metastatic spreading. The effector mechanism is represented by T cells transduced with CSPG4-specific chimeric antigen receptor (CAR), since i) this strategy allows rapid generation of polyclonal T cells with tumor antigen (TA)-specificity and ii) the recognition of tumor cells by CAR T cells does not depend on HLA class I antigen expression by target cells. CAR T cells are combined with strategies which counteract the escape mechanisms utilized by TNBC cells to avoid recognition and destruction by CSPG4 CAR T cells. The escape mechanisms, which are triggered by the changes induced by hypoxia in the tumor microenvironment, include i) reduced susceptibility of TNBC cells to the lytic activity of CAR T cells because of the upregulation of antiapoptotic molecules. The latter is caused by the activation of the Sonic Hedgehog Homolog (SHH) pathway triggered by hypoxia, a hallmark of tumor microenvironment; ii) the dysfunction of CAR T cells caused by the interaction of PD-1 they express with PD-L1 induced by hypoxia on tumor cells and on non malignant cells present in the tumor microenvironment and iii) the reduced viability of CAR T cells because of the unbalanced level of cytokines in the tumor microenvironment. Our combinatorial strategy includes i) sonidegib, an inhibitor of SHH pathway, ii) anti-PD-L1 monoclonal antibodies and iii) fusion proteins which target cytokines to the tumor microenvironment.

The therapeutic efficacy of our combinatorial immunotherapy is tested both *in vitro* and in mouse models. TNBC cell lines and TNBC PDXs are used as targets.

15. SUBJECT TERMS

CSPG4, TNBC, PD1, Chimeric antigen receptors, T cell immunotherapy

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	22	

TABLE OF CONTENTS

1. **INTRODUCTION:** The goal of this proposal is to test the anti-tumor activity of the combinatorial strategy which utilizes the individual approaches we have developed and shown to be effective in counteracting the corresponding escape mechanism triggered by hypoxia.

2. **KEYWORDS:** CSPG4 TNBC
PD1
Chimeric antigen receptor T cell immunotherapy

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
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?**

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

Subtask 1: Immunohistochemical (IHC) staining of formalin fixed paraffin embedded TNBC tumors for CSPG4

In our initial study we found CSPG4 expression in 32 (73%) of the 44 TNBC tumor tested. In an additional study performed during the first year of this grant we found CSPG4 expression in 53 (87%) of the 63 TNBC tumors analyzed. 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 have 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 has tested 29 TNBC tumors. They have found CSPG4 expression in 13 tumors.
We have continued the analysis of the mechanisms which regulate CSPG4 expression on TNBC cells. Preliminary studies suggest that CSPG4 is not expressed on TNBC cells from African American women. This

phenotype appears to be caused by the silencing of the CSPG4 gene because of methylation of its promoter. Preliminary results suggest that CSPG4 expression can be restored by treating TNBC cells with demethylating agents.

As we have previously reported, CSPG4 expression is upregulated on TNBC cells incubated for 72 hours under hypoxic conditions at 37^0 C. This effect is not unique of TNBC cells, since CSPG4 expression is upregulated also on head and neck cancer cell lines incubated for 72 hours under hypoxic conditions at 37^0 C. In addition, the effect of hypoxia is not unique of CSPG4, since other antigens such as ICAM1 and HLA-A antigens are upregulated on TNBC cell lines incubated under hypoxic conditions at 37^0 C. However, the extent of upregulation of the latter antigens varies among the TNBC cell lines tested.

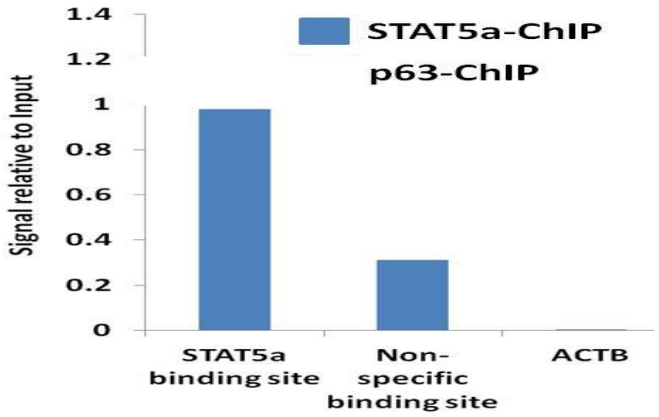


Figure 1. Binding of STAT5a to CSPG4 promoter region in ChIP assays. STAT5a-ChIP and p63-ChIP assays were performed on SUM149 cells with anti-STAT5a antibodies and on SCCHN JHU029 cells with anti-p63 antibodies, respectively. Binding of STAT5a and p63 to CSPG4 promoter sequences was demonstrated by real-time PCRs.

We have continued to analyze the mechanisms which underlie CSPG4 upregulation on TNBC cells incubated under hypoxic conditions at 37^o C. We have found that STAT5a is a transcription factor for CSPG4, since in a chip assay it binds to its promotor region **(Fig. 1)**. STAT5a is activated in cells incubated under hypoxic conditions.

We have been able to perform the analysis of the association of CSPG4 and HIF-1a expression, since we have finally identified a commercially available HIF-1a-specific antibody which stains formalin fixed, paraffin embedded (FFPE) tissue sections in a reliable and reproducible way. At variance with the monoclonal and polyclonal rabbit antibody purchased from Abcam, the mouse mAb from Santa Cruz yields reliable and reproducible results. Representative examples of staining patterns obtained by staining

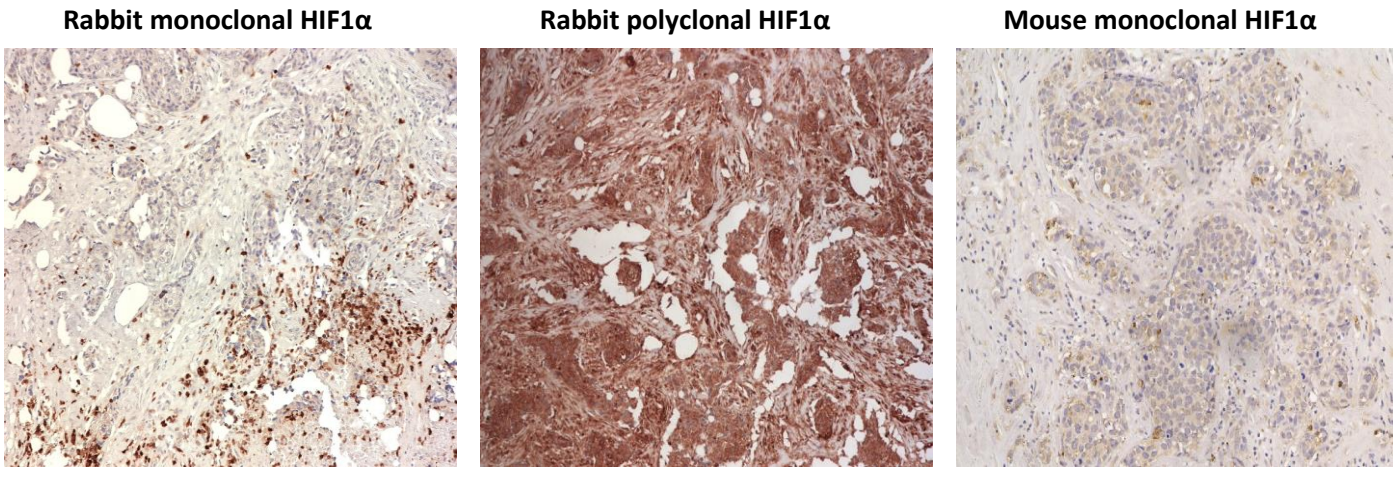


Figure 2. Specific staining of TNBC tumors by HIF-1a-specific Abs. TNBC sections were IHC stained with HIF1a-specific Abs (Rabbit monoclonal(ab51608), Rabbit polyclonal(ab2185) and mouse monoclonal(sc-53546)).

TNBC cancer tissue sections with the antibodies we have tested are shown in **Fig. 2. Fig. 3** shows representative staining patterns of 62 FFPE TNBC sections with the Santa Cruz HIF-1a mAb. Thirty-six (58%) TNBC sections

stained positive. Correlation of HIF-1a expression with CSPG4 expression in TNBC samples did not reveal any association between the expression level of the two molecules **(Fig.4)**.

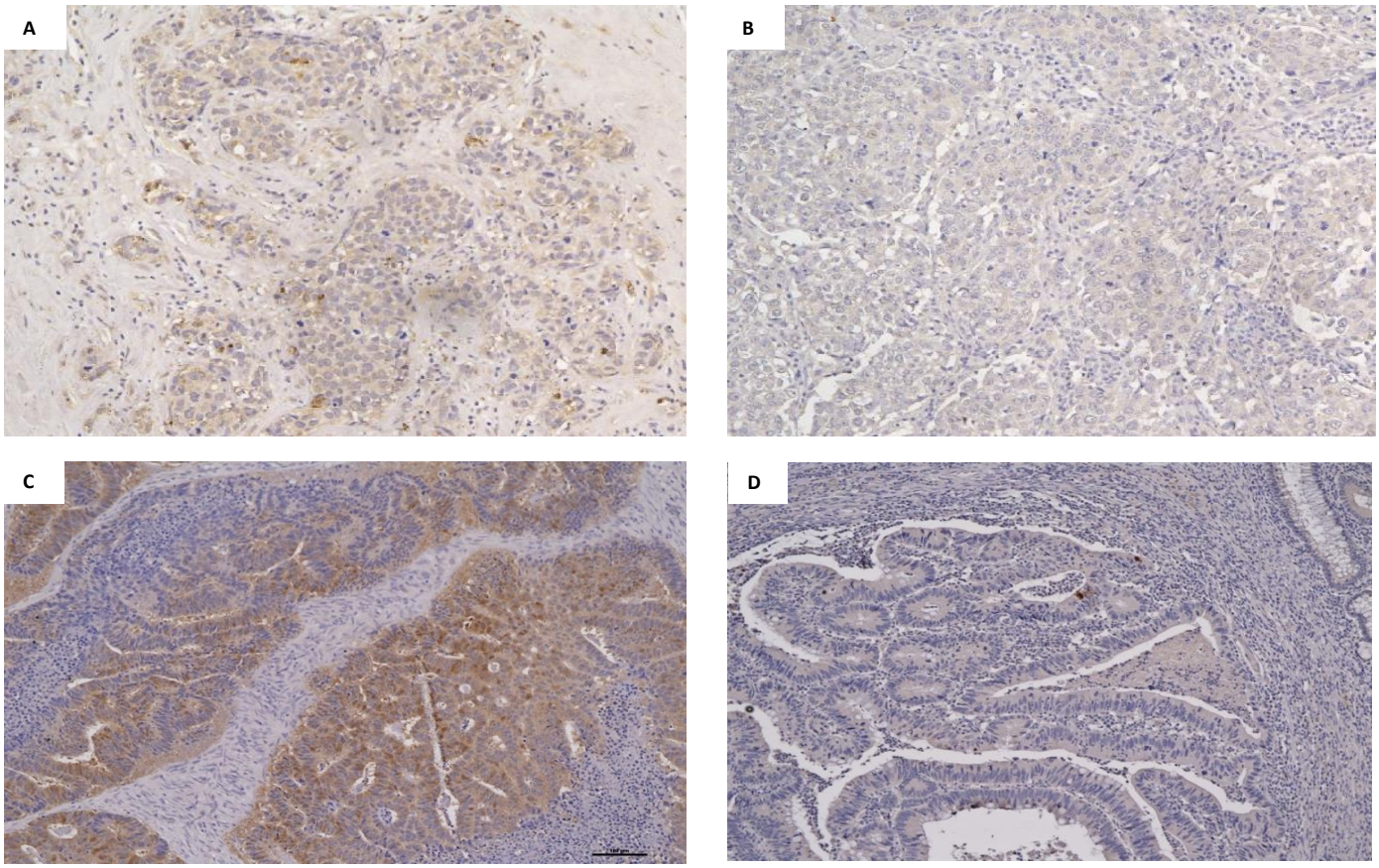
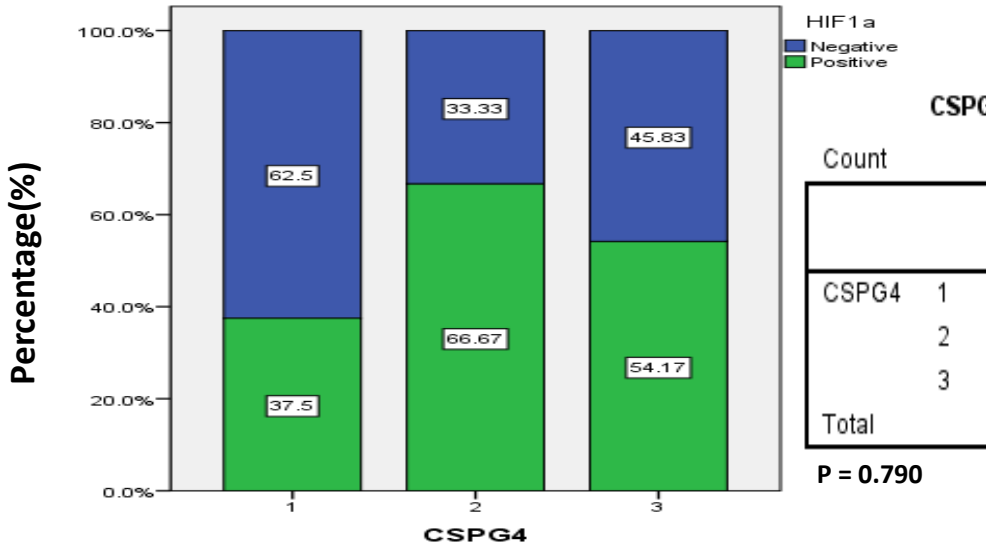


Figure 3. Representative immunohistochemical staining patterns of TNBC tumors stained with the HIF-1a-specific mAb purchased from Santa Cruz. Panels A and B show a positive and negative staining pattern of TNBC tumors, respectively. Panels C and D show positive and negative controls with colon cancer tumors. (original magnification 200X)



CSPG4 * HIF1a Crosstabulation

Count

		HIF1a		
		0	1	Total
CSPG4	1	5	3	8
	2	10	20	30
	3	11	13	24
Total		26	36	62

P = 0.790

Figure 4. Lack of significant association between CSPG4 and HIF-1a expression in TNBC tumors stained with the CSPG4-specific mAb 763.74 and the HIF-1a-specific mAb (Santa Cruz).

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*.

Subtask 1: To generate CAR T cells and assess their function in normoxic (20%O_2 tension) and hypoxic (1%O_2 tension) conditions in vitro.

Functional activity of CSPG4 CAR T cells under hypoxic conditions. In these experiments the TNBC cell lines SUM149 and SUM159 were used as targets. As a first step we measured the expression level on the cell lines incubated under hypoxia. As shown in **Fig. 5**, the CSPG4 expression level was significantly increased on both cell lines. In addition, CSPG4 CAR T cells co-cultured with the two TNBC cell lines were more efficient in recognizing the targets as determined by the IFNγ release assay **(Fig.6)** and in eliminating the target cells **(Fig.6)**.

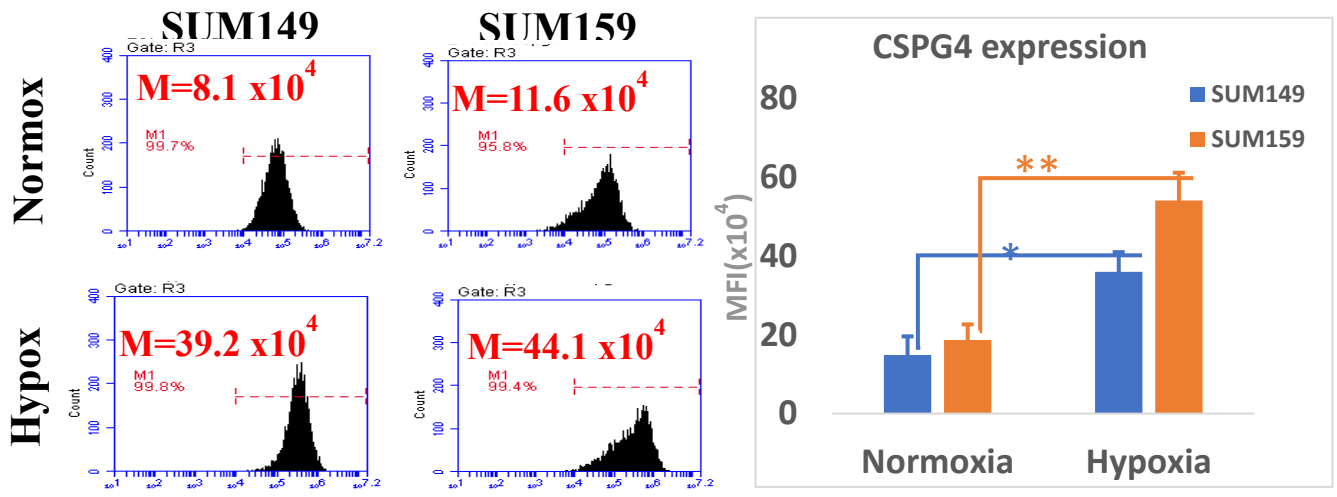


Figure 5. Up-regulation of CSPG4 expression on TNBC cells induced by hypoxia. TNBC cell lines SUM149 and SUM159 were incubated under normoxic or hypoxic conditions (1% O_2) for 72 hours. Cells were then stained with the CSPG4 mAb 763.74. The % and MFIs(M) of stained cells were analyzed by flow cytometry. *p<0.05, **p<0.01

Enhancement by SAHA of the CSPG4 CAR T cell functional activity. In the previous Progress Report, we have indicated that *in vitro* treatment of CSPG4 CAR T cells with the HDAC inhibitor SAHA 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. The effect of SAHA is both dose and time dependent. To assess the functional significance of these changes, we have tested the *in vitro* anti-tumor activity of SAHA-treated CSPG4 CAR T cells in co-culture experiments with TNBC cell lines as targets. The results are summarized in **Fig. 7 and 8**. SAHA treated CSPG4 CAR T cells display a significantly higher ability to specifically recognize CSPG4 bearing TNBC cells than untreated CSPG4 CAR T cells, as indicated by the amount of IFNγ released following a 24-hour incubation at 37^o C with TNBC cells. Furthermore, SAHA treated CSPG4 CAR T cells display a significantly higher anti-tumor activity than untreated CAR T cells, when incubated for 72 hours with TNBC cell lines at 37^0 C. The effect of SAHA appears to be mediated by CAR since it was not detectable with

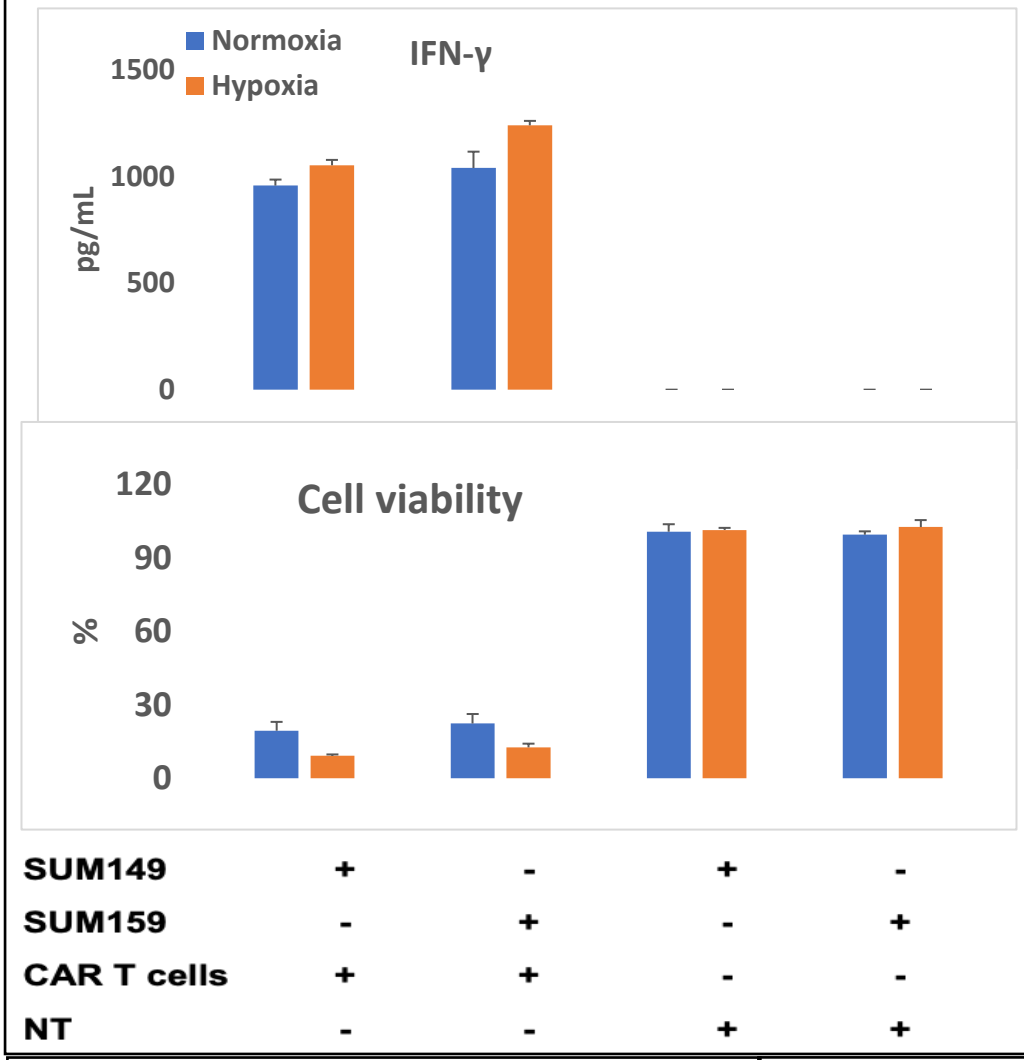


Figure 6. *In vitro* specific recognition *and* elimination of TNBC cancer cells by CSPG4 specific CAR T cells under normoxic and hypoxic conditions. TNBC cell lines SUM149 and SUM159 cells were co-cultured with CSPG4 specific CAR T cells at tumor cell:effector ratio=1:1 at 37℃ under normoxic and hypoxic conditions. At the end of a 24 hours incubation, the medium was harvested and IFN-γ content was measured utilizing a human IFN gamma ELISA kit (Thermo Scientific). At the end of a 72 hours incubation, tumor cell viability was analyzed using a MTT assay. Non-transduced T cells (NT) were used as a specificity control.

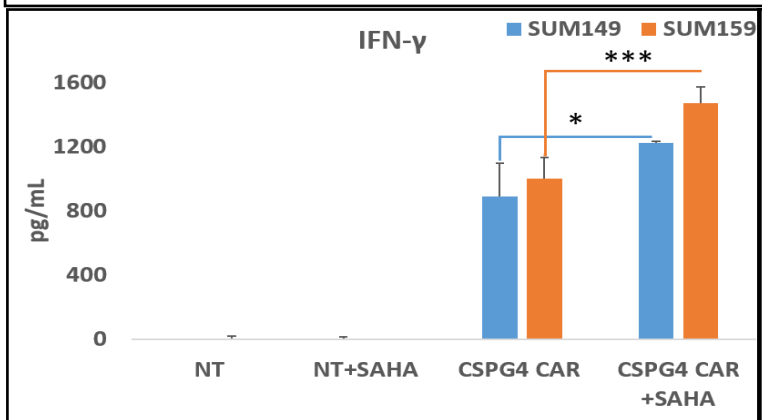


Figure 7. Enhancement by SAHA of *in vitro* specific recognition of human TNBC cell lines by CSPG4-specific CAR T cells. CSPG4-specific CAR T cells were first incubated with SAHA(0.5 μM) for 72hours at 37℃. Then CAR T cells were co-cultured with SUM149 and SUM159 TNBC cells at tumor:effector cell ratio=1:1 in fresh medium. At the end of a 24 hour incubation at 37℃, the medium was harvested and IFN-γ content was measured utilizing a human IFN gamma ELISA kit (Thermo Scientific). Non-transduced T cells (NT) and were used as a specificity control.
*p<0.05, ***p<0.001

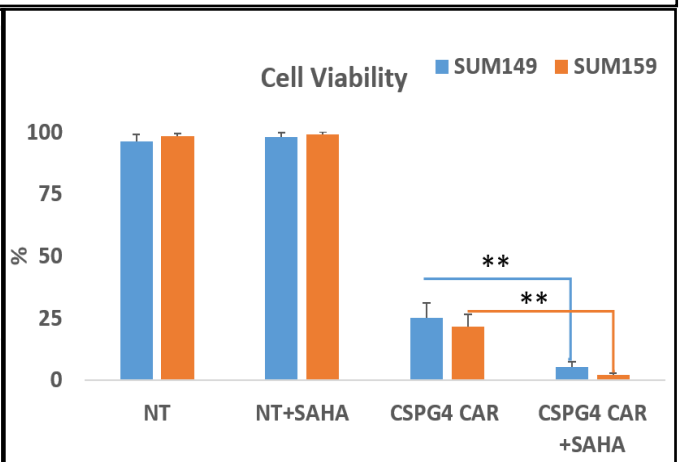


Figure 8. Enhancement by SAHA of *in vitro* elimination of human TNBC cells by CSPG4-specific CAR T cells. CSPG4-specific CAR T cells were first incubated with SAHA(0.5 μM) for 72hours at 37℃. Then CAR T cells were co-cultured with SUM149 and SUM159 cells at tumor cell:effector ratio=1:1 at 37℃ in fresh medium. At the end of a 120 hour incubation at 37℃, tumor cell viability was analyzed using a MTT assay. Non-transduced T cells (NT) were used as a specificity control.
**p<0.01

T cells not transduced with CAR. The effect of SAHA appears to be not restricted to CSPG4 CAR T cells and to

TNBC, but appears to be a general phenomenon. As shown in **Fig. 9 and 10**, SAHA treatment increases also the ability of B7-H3 CAR T cells to recognize and eliminate TNBC cells which express B7-H3. Furthermore, the effects of SAHA have been observed also when head and neck cancer cell lines and chondrosarcoma cell lines have been used as targets.

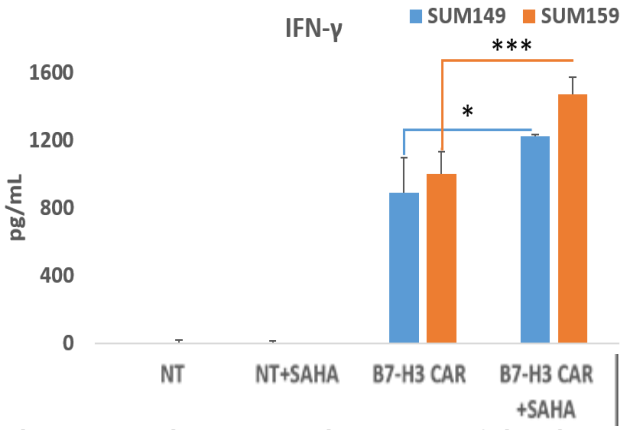


Figure 9. Enhancement by SAHA of *in vitro* specific recognition of human TNBC cell lines by B7-H3-specific CAR T cells. B7-H3 specific CAR T cells were firstly incubated with SAHA (0.5 μM) for 72hours, then CAR T cells were co-cultured with SUM149, SUM159 cells at tumor cell:effector ratio=1:1 at 37℃ in fresh medium. At the end of a 24 hour incubation, the medium was harvested and IFN-γ content was measured utilizing a human IFN gamma ELISA kit (Thermo Scientific). Non-transduced T cells (NT) were used as a specificity control. *p<0.05, **p<0.01

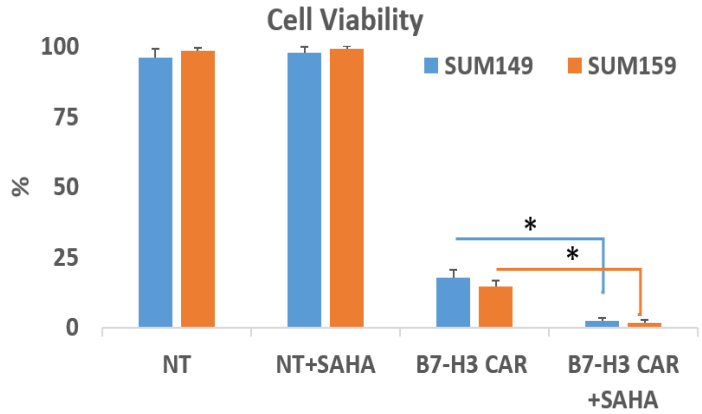


Figure 10. Enhancement by SAHA of *in vitro* elimination of human TNBC cell lines by B7-H3-specific CAR T cells. B7-H3 specific CAR T cells were firstly incubated with SAHA(0.5 μM) for 72hours, then CAR T cells were co-cultured with SUM149, SUM159 cells at tumor cell:effector ratio=1:1 at 37℃ in fresh medium. At the end of a 120 hour incubation, tumor cell viability was analyzed using a MTT assay. Non-transduced T cells (NT) were used as a specificity control.
*p<0.05

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 a 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. Smo is activated by the binding of SHH to Ptch. In turn, the activated Smo activates Gli, a transcription factor, which binds to the promotor of its target genes. We have first tested the effect of sonidegib on the proliferation of TNBC cells and CAR T cells. As shown in **Fig. 11 and 12**, sonidegib inhibits the proliferation of both TNBC and T cells. The effect

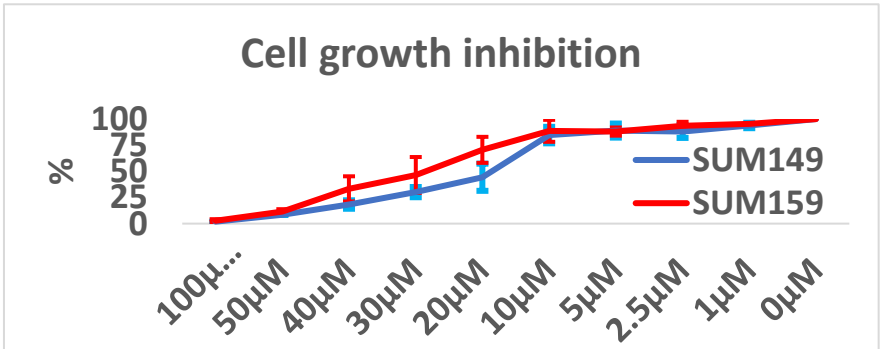


Figure 11. Dose dependent inhibition by sonidegib of TNBC cell growth. SUM149 and SUM159 cells (3000 /well) were incubated with sonidegib at indicated concentration for 48hrs at 37°C. The viability of cells was analyzed by MTT assay. The percentages of growth inhibition of TNBC cells are shown.

with both cells is dose dependent. Twenty uM of sonidegib are required to inhibit the proliferation of both target cells. Therefore, sonidegib has been used at the concentration of 10uM.

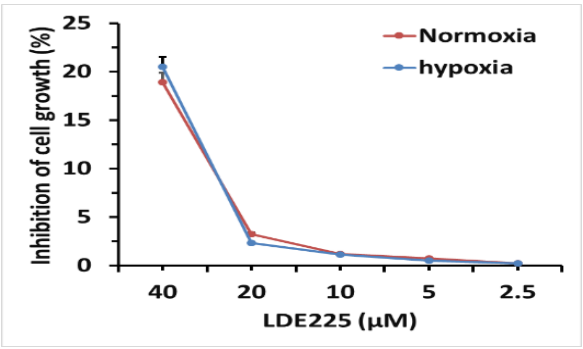


Figure 12. Dose dependent inhibition by sonidegib of CAR T cell proliferation. CAR T cells ($2.5x10^5$ /well) were incubated under hypoxic conditions (1% O_2) or normoxia with sonidegib at 40, 20, 10, 5, 2.5µM for 72 hrs at 37 °C in RPMI 1640 medium + 10% FCS with IL-15. The viability of cells was analyzed by MTT assay. The percentages of growth inhibition of CAR T cells are shown.

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 have prompted us to replace in our strategy sonidegib with other compounds. These compounds should 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 do not inhibit the proliferation of T cells and do not downregulate CSPG4 expression on target cells. We have tested itraconazole, an anti-fungal agent, which inhibits the SHH pathway by antagonizing Smo. However, this compound was excluded since it inhibits the proliferation of CAR T cells and non-transduced T cells at a low dose **(Fig. 13)**. Another inhibitor of SHH pathway which is being tested is arsenic trioxide, which inhibits Gli. We have shown that this compound upregulates the expression of Fas on TNBC cells. Lastly, we have tested erlotinib, an EGFR inhibitor. We have found that erlotinib at low dose induces apoptosis of TNBC cells. Erlotinib is presently being tested for its effect on the level of anti-apoptotic molecules and on the proliferation of CAR T cells.

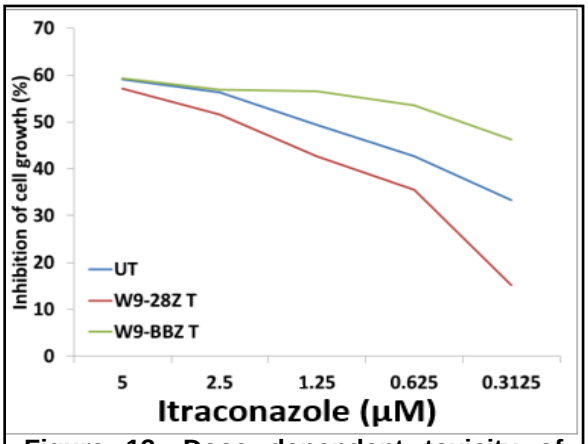


Figure 13. Dose dependent toxicity of itraconazole to CAR T cells. CAR T cells ($2.5x10^5$/well) were incubated with itraconazole at 5, 2.5, 1.25, 0.625, 0.3125 µM for 72 hrs at 37° C in RPMI 1640 medium + 10% FCS . The viability of cells was analyzed by MTT assay. The percentages of growth inhibition of CAR T cells and untransduced (UT) T cells are shown.

Enhancement by anti-PD-L1 mAb of the anti-tumor activity of CSPG4 CAR T cells. CAR T cells express PD-1. A representative example is shown in **Fig. 14**. PD-1 interacts with PD-L1 which is induced on tumor cells by hypoxia and/or IFNγ on TNBC cells. A representative example of the induction of PD-L1 on TNBC cells incubated under hypoxic conditions is shown in **Fig. 15**. 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. As shown in **Fig. 16, 17 and 18**, this strategy corrects the dysfunction of CSPG4 CAR T cells and restores their anti-tumor activity with both differentiated TNBC cells and TNBC CICs.

Enhancement by IL2 fusion protein of in vitro elimination of TNBC cells by CSPG4 CAR T cells. The IL-2 fusion protein was generated by linking IL-2 to the anti-idiotypic mAb MK2-23. The latter recognizes an idiotope in the antigen combining site of the CSPG4-specific mAb 763.74. The latter was used to prepare CSPG4-specific CAR. In initial experiments we have used melanoma cells as targets, since they express higher levels of CSPG4. As shown in **Fig. 19,** the fusion protein enhances the ability of CSPG4 CAR T cells to eliminate differentiated melanoma cells and melanoma CICs. With this background information, we have purified IL-2 fusion protein and

assessed its mitogenic activity with mouse splenocytes **(Fig. 20)** The fusion protein will be tested for its ability to enhance the antitumor activity of CSPG4 CAR T cells with differentiated TNBC cells and TNBC CICs.

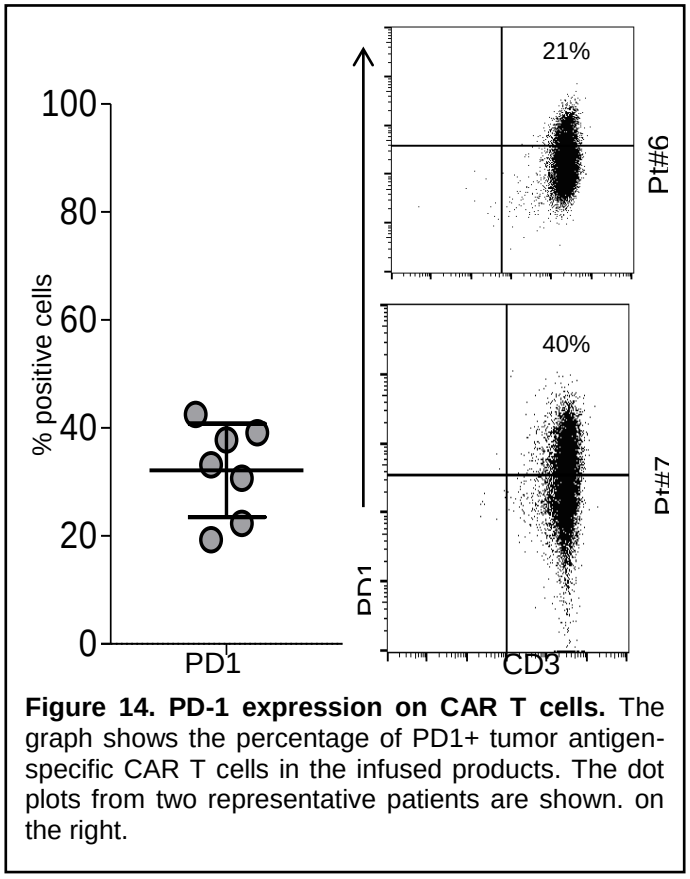


Figure 14. PD-1 expression on CAR T cells. The graph shows the percentage of PD1+ tumor antigen-specific CAR T cells in the infused products. The dot plots from two representative patients are shown. on the right.

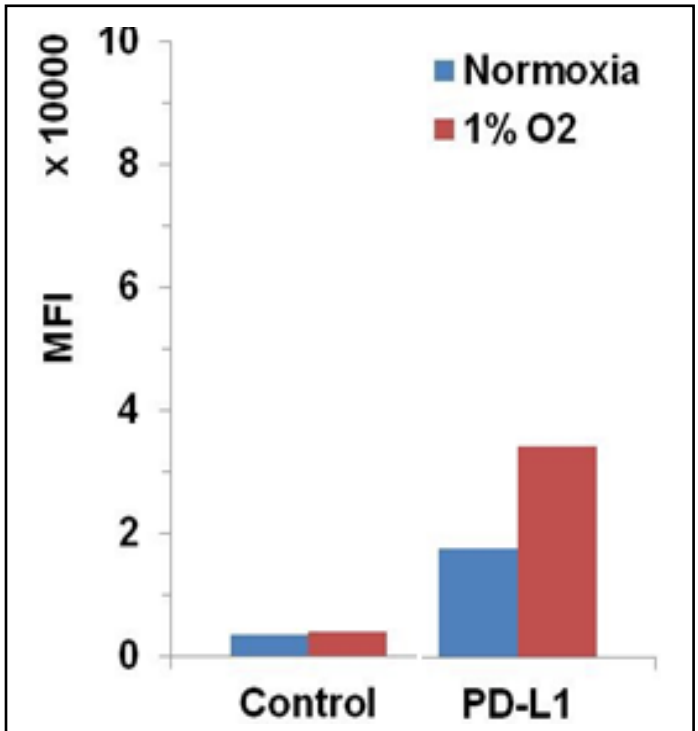


Figure 15. PD-L1 up-regulation on human TNBC cells incubated under hypoxic conditions. Human TNBC SUM149 cells were incubated under hypoxic conditions (1% O_2) for 72 hours. Cells were then stained with PD-L1-specific mAb. The stained cells were analyzed by flow cytometry. The MFIs of cells in each condition are shown. Isotype matched irrelevant mAbs were used as controls.

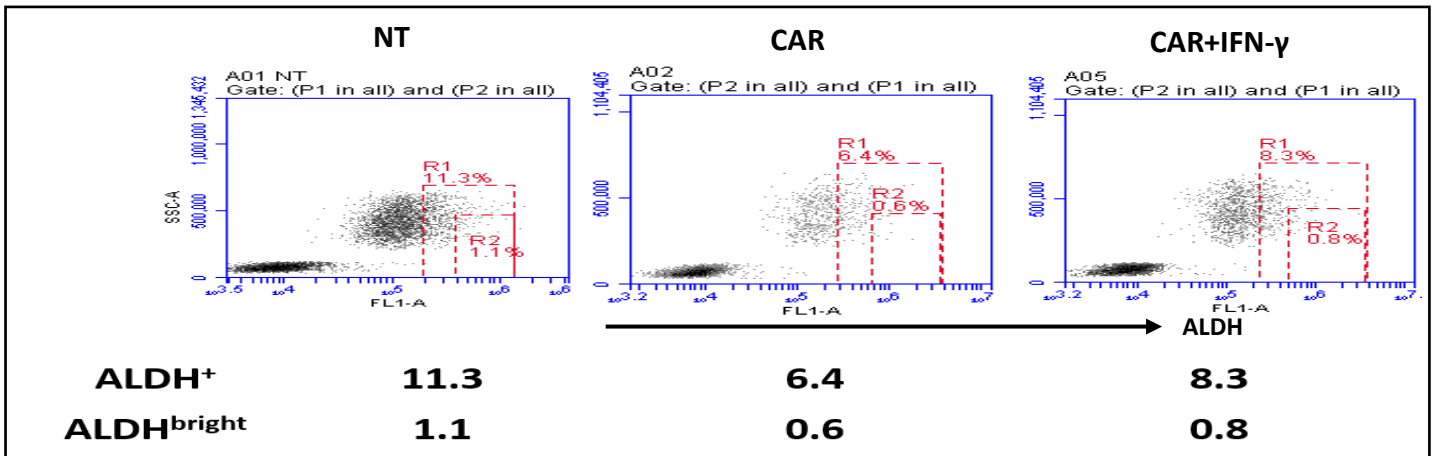


Figure 16. IFN-γ reduces the ability of CSPG4-specific CAR T cells to eliminate TNBC CICs. Human SUM149 cells were co-cultured with CSPG4-specific CAR T cells at E:T=1:1 ratio in a medium supplemented with IFNγ (100IU/ml). At the end of a 72 hour incubation at 37℃, cells were harvested and stained using ALDEFLUOR with and without DEAB inhibitor to identify CICs. Percentage of $ALDH^+$ cells is gated in R1. Percentage of total $ALDH^{bright}$ cells is gated in R2. Non-transduced T cells(NT) were used as a control.

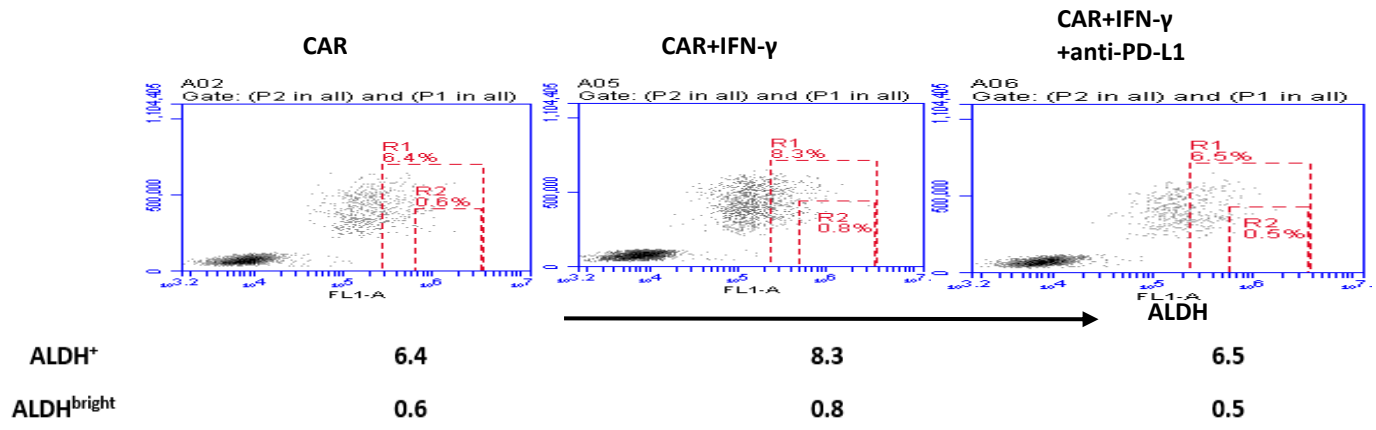


Figure 17. Anti-PD-L1 mAb counteracts the IFNγ induced reduction of the ability of CSPG4-specific CAR T cells to eliminate *in vitro* TNBC CICs. Human SUM149 cells were co-cultured with CSPG4-specific CAR T cells at E:T=1:1 ratio in a medium supplemented with IFNγ and/or anti-PD-L1 mAb. At the end of a 72 hour incubation at 37℃, cells were harvested and stained using ALDEFLUOR with and without DEAB inhibitor to identify CICs. Percentage of $ALDH^{+}$ cells is gated in R1. Percentage of total $ALDH^{bright}$ cells is gated in R2. Non-transduced T cells(NT) were used as a control.

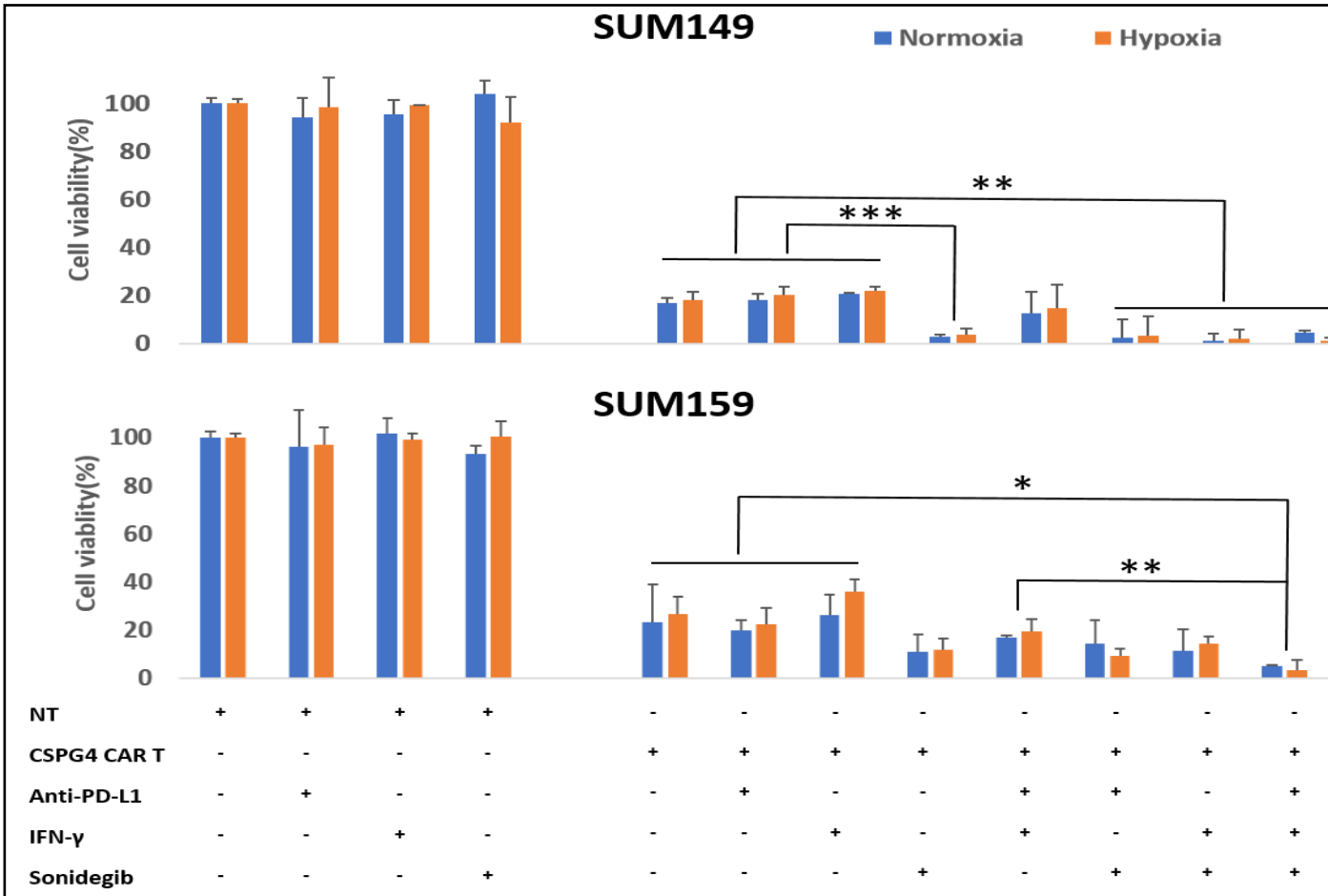


Figure 18. Enhancement by anti-PD-L1 mAb and sonidegib of the *in vitro* elimination of TNBC cells by CSPG4 specific CAR T cells. SUM149 and SUM159 cells were co-cultured with CSPG4 specific CAR T cells at the tumor:effector cell ratio of 1:1 in the presence of the indicated reagents. At the end of a 72 hour incubation at 37^{0} C, tumor cell viability was analyzed using a MTT assay. Non-transduced T cells (NT) were used as a control.
*p<0.05, **p<0.01, ***p<0.001

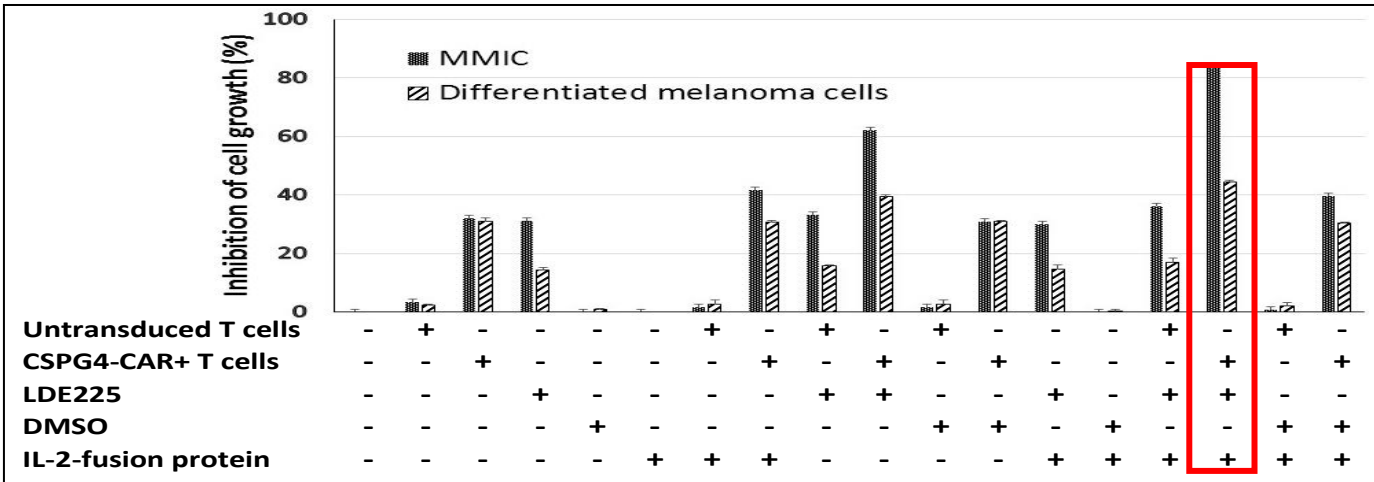


Figure 19. Enhancement by LDE225 (sonidegib) and/or IL-2-fusion protein of the ability of CSPG4-specific CAR+ T cells to eliminate i*n vitro* differentiated melanoma cells and malignant melanoma initiating cells (MMICs) in the human melanoma cell line M21. M21 cells (2.5x105 /well) were incubated with LDE225 (10 uM) and/or IL-2-fusion protein (0.2ug/mL) for 72 hrs at 37°C in RPMI 1640 medium + 10% FCS. Then CSPG4-specific CAR+ T cells were added to tumor cells (E:T=20:1) and incubation was continued for an additional 24 hrs with LDE225 and/or IL-2-fusion protein present in the culture. M21 cells co-cultured with non-transduced T cells and/or DMSO (0.1%) were used as a control. M21 cells were tested for content of MMICs, defined as ALDHbright/ABCB5+ cells, with ALDEFLUOR with or without the DEAB inhibitor and ABCB5-specific mAb RK1. Tumor cells were gated according to size (forward scatter) and granularity (side scatter). These results indicate that LDE225 and IL-2-fusion protein enhance the ability of CSPG4-specific CAR+ T cells to eliminate MMICs in the M21 cell line.

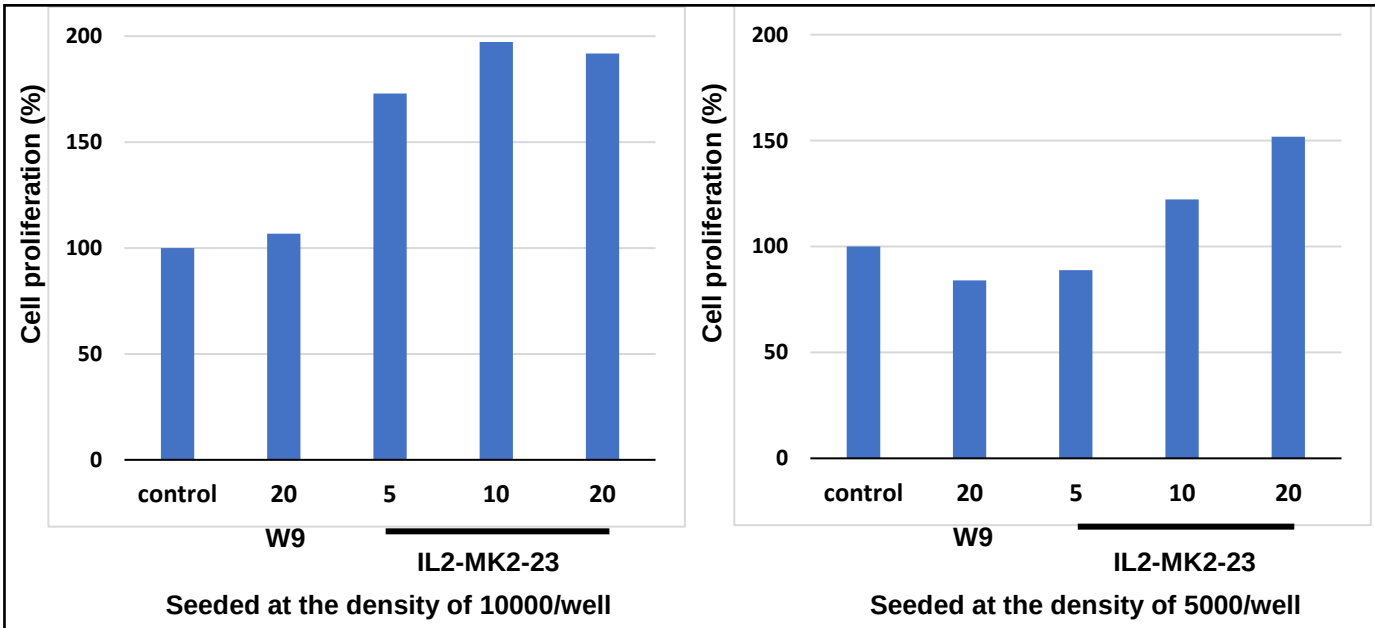


Figure 20. Mitogenic activity of IL-2-anti-id mAb MK2-23 fusion protein with mouse splenocytes. Splenocytes were seeded at the indicated density. Following a 3-day incubation at 37° C, cell count was determined using the CCK8 assay. mAb W9 was used as a specificity control.

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.

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. Staining assays have been performed to show that the transfection does not affect the expression of CSPG4 and B7-H3 **(Fig.21)**. However, a subpopulation of cells was not transfected with luciferase **(Fig.22)**. Sorting of the cell line is being performed in order to isolate luciferase transfected cells. These cells will be used in the *in vivo* experiments.

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 for alternative strategies. As a result, we have started to investigate whether the HDAC inhibitor vorinostat (SAHA) can enhance the antitumor activity of CSPG4 CAR T cells in a mouse model system. As a first step we have tested the effect of the administration of SAHA to tumor bearing mice on CSPG4 expression by tumor cells. Tumors have been surgically removed and will be tested for CSPG4 expression level by immunohistochemical staining with CSPG4-specific mAb 763.74.

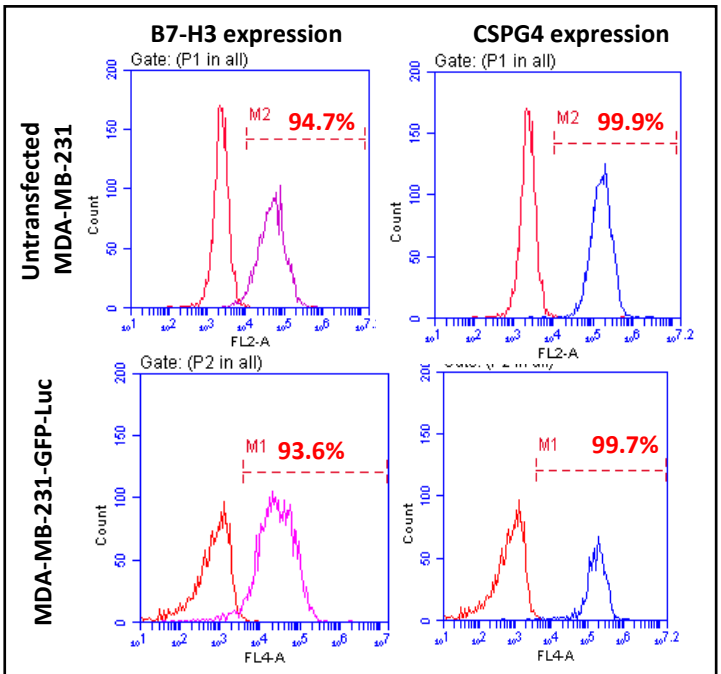


Figure 21. Expression of the mAb 376.96-defined B7-H3 epitope and 763.74-defined CSPG4 epitope on MDA-MB-231-GFP-Luc. Cells were stained with the B7-H3-specific mAb 376.96（pink curve） or CSPG4-specific mAb 763.74 （blue curve）. The unrelated mAb F3-C25 （red curve） was used as a control. Stained cells were subjected to flow cytometry analysis. The percentage of cells stained with the B7-H3-specific mAb 376.96 and CSPG4-specific mAb 763.74 are shown in each histogram.

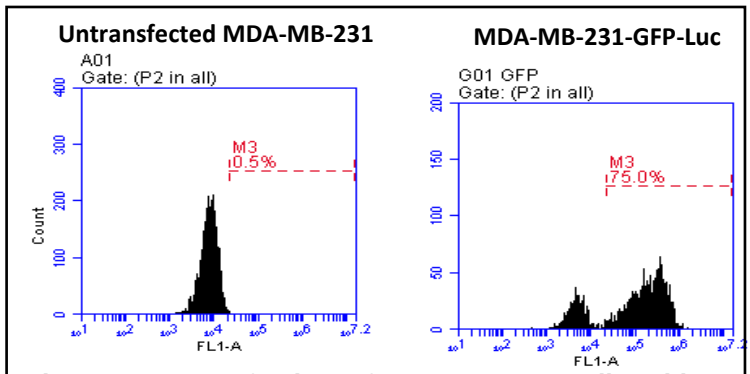


Figure 22. Transfection of MDA-MB-231 cells with a GFP-Luc lentivirus vector. MDA-MB-231-GFP-Luc cells were collected and GFP expression was analyzed by flow cytometry. % of GFP positive cells were shown. Non-transfected MDA-MB-231 cells were used as a control.

*Evaluation of the therapeutic 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 is summarized as follows:

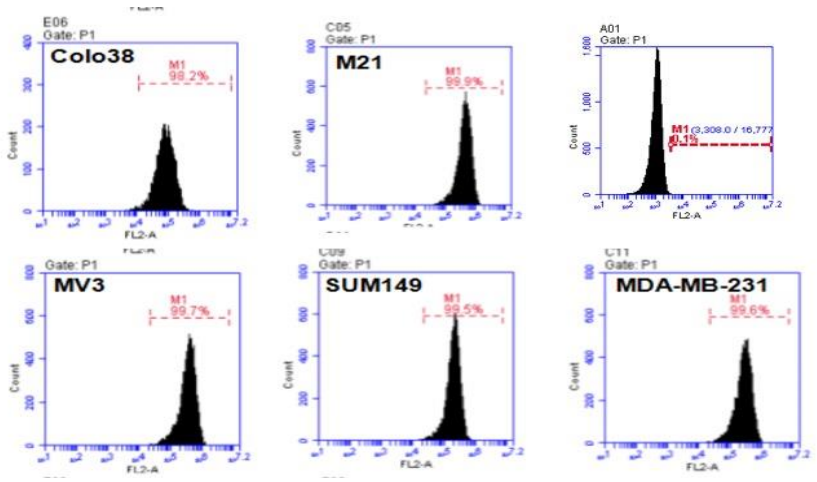


Figure 23. Expression of the scFv SK5 defined CSPG4 epitope on cultured human cell lines of different histotypes. The human melanoma Colo38, MV3, and M21, TNBC MDA-MB-231 and SUM149 were stained with the scFv SK5 and anti-c-Myc mouse mAb 9E10 and analyzed by FACS. Raji cells were used as a negative control.

i) *Isolation and characterization of the human CSPG4-specific human scFv SK5*. The human scFv SK5 was isolated from a phage display scFv library with human CSPG4(+) melanoma cells SK-MEL-28. The specificity of SK5 for CSPG4 is shown by a) the selective reactivity in binding assays with CSPG4(+) cultured human cells **(Fig. 23)**; b) immunoprecipitation of the molecules with the characteristic SDS-PAGE profile of CSPG4 **(Fig. 24)**; c) restoration of the reactivity of scFv SK5 with the CSPG4(-) melanoma cell line M14 following transfection with human CSPG4 cDNA which restores CSPG4 expression **(Fig. 25)**; and d)loss of reactivity of scFv SK5 with the human melanoma cell line in which CSPG4 expression has been knocked down by CSPG4- specific shRNA **(Fig. 26)**.

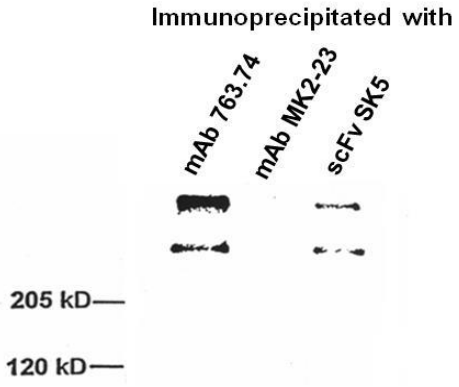


Figure 24. SDS-PAGE analysis of molecules immunoprecipitated from 125I-labeled human melanoma cells WM1158 by scFv SK5. A 1% NP-40 extract of 125I-labeled human melanoma cells WM1158 was immunoprecipitated with scFv SK5. Antigens were eluted and analyzed by SDS-PAGE in a 6-15% gradient polyacrylamide gel. The gel was fixed, dried and autoradiographed for 1 day at -70°C. CSPG4-specific mAb 763.74 and anti-idiotypic (anti-id) mAb MK2-23 were used as postive and negative controls, respectively.

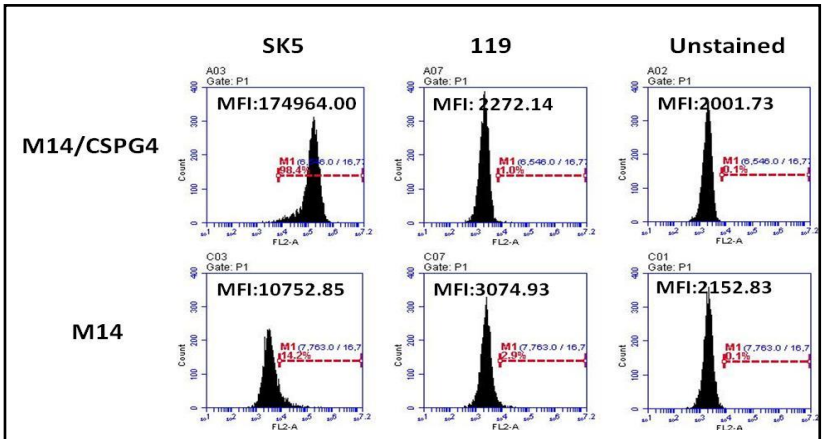


Figure 25. Differential reactivity of scFv SK5 with M14/CSPG4 melanoma cells which acquire CSPG4 expression following transfection with CSPG4 cDNA and with CSPG4- parental M14 melanoma cells. Cells were incubated on ice with scFv SK5 and mAb 9E10 and control scFv 119 and mAb 9E10. Binding of antibodies was detected using RPE-labeled F(ab')2 fragments of goat anti-mouse Ig antibodies. Cells were analyzed with a flow cytometer.

ii) *Characterization of the scFv SK5 defined CSPG4 epitope*. This epitope appears to be carbohydrate in nature, since it is not detectable on cultured human melanoma cells MV3 treated for 24hrs at 37° C with PNGase F+ O-Glycosidase+ α-2(3,6,8,9)-Neuraminidase+ β-1→ 4-Galactosidase+ β-N-Acetylglucosaminidase **(Fig. 27)**. This epitope is expressed on cancer differentiated cells and on cancer initiating cells in the cancer types in which it has been tested **(Fig. 28)**.

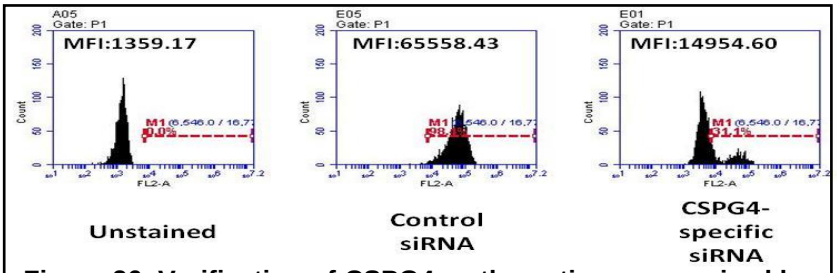


Figure 26. Verification of CSPG4 as the antigen recognized by scFv SK5. scFv SK5 loses its reactivity with the human melanoma M21 cells in which CSPG4 expression was knocked down by transduction with lentivirus carrying CSPG4-specific siRNA. Control siRNA was used as a control.

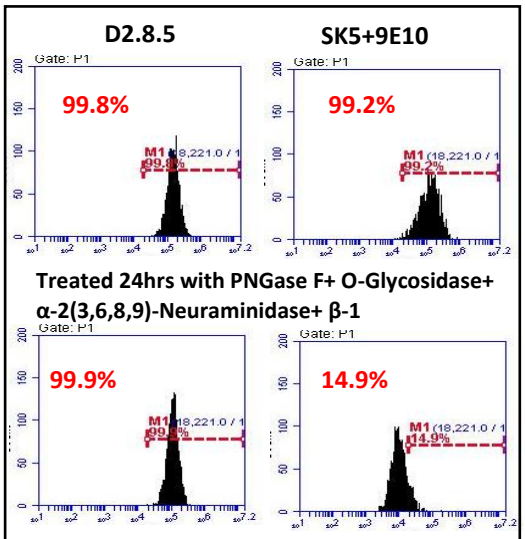


Figure 27. The epitope of CSPG4 recognized by SK5 seems to be carbohydrate in nature. The epitope recognized by SK5 is not detectable on cultured human melanoma cells MV3 treated for 24hrs at 37° C with PNGase F+ O-Glycosidase+ α-2(3,6,8,9)-Neuraminidase+ β-1→4-Galactosidase+ β-N-Acetylglucosaminidase

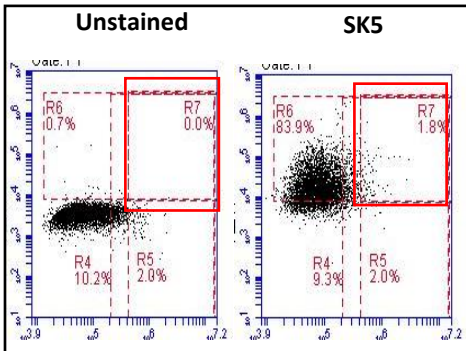


Figure 28. The epitope recognized by SK5 in not only expressed on TNBC differentiated but also on TNBC cancer initiating cells. The TNBC cell line SUM149 was incubated with ALDEFLUOR to detect ALDH activity and stained with the CSPG-specific Ab SK5. DEAB, a specific inhibitor of ALDH, was used to establish the baseline fluorescence of these cells. ALDH^{bright} cells were identified as those ALDH+ cells with twice the mean fluorescence intensity(MFI) of the ALDH+ cell population. The % of cells stained by ALDEFLUOR and/or by mAbs are shown.

iii) *Generation of human CSPG4 CAR with scFv SK5 and in vitro assessment of its specificity and activity*. A CSPG4-specific CAR was generated utilizing the scFv SK5. The structure of the CAR is indicated in **Fig. 29**. T-cells transduced with this CAR specifically recognize and eliminate in vitro CSPG4(+) malignant cells, as tested by the release of IFNg and by the assessment of the elimination of cancer cells in co-culture experiments **(Fig. 30)**, respectively. It is noteworthy that the scFv orientation VH-VL is better than the VL-VH orientation, as determined by the extent of specific IFNg release and specific elimination of CSPG4(+) tumor cells.

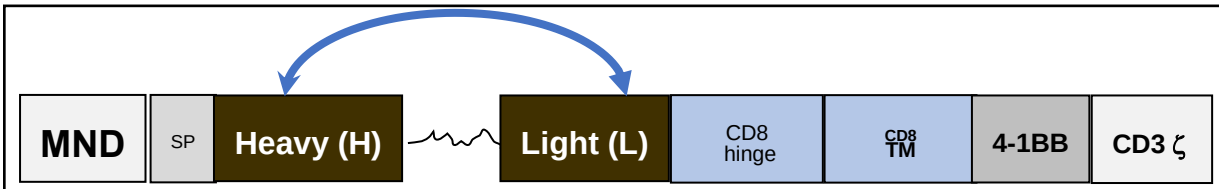


Figure 29. Structure of the CAR construct utilized to generate CSPG4-specific CAR T cells with SK5 scFv.

iv) *Lack of in vivo anti-tumor activity of T cells transduced with CSPG4 CAR generated with the human SK5 scFv.* T cells transduced with the CAR generated with the human scFv SK5 eliminated CSPG4(+) tumor cells **(Fig. 31)**. In contrast, the CAR T cells had no detectable effect on the growth of human CSPG4(+) tumor cells grafted in NSG mice **(Fig. 32)**. This negative result does not reflect the lack of CSPG4 expression in the tumor, since immunohistochemical staining with CSPG4-specific mAb 763.74 detected the antigen in surgically removed tumors **(Fig. 33)**. It is our working hypothesis 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. We are exploring the possibility to mutagenize the scFv in order to change its conformation and hopefully increase its anti-tumor activity in the tumor microenvironment.

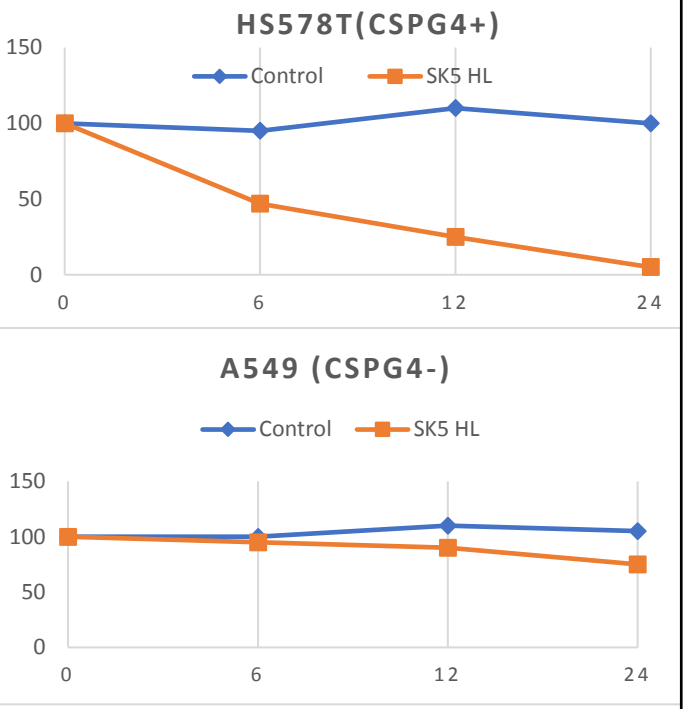


Figure 30. Specific elimination after 24hrs of co-culture of the human TNBC cell line HS578T, which expresses CSPG4 at high levels, by CSPG4-specific CAR T cells. The cell line A549, which does not express CPSG4 was used as a negative control.

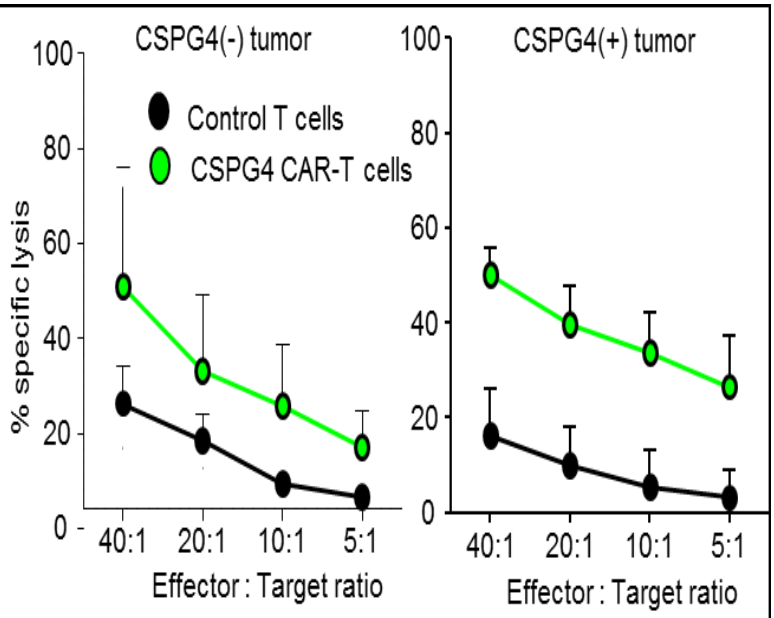


Figure 31. *In vitro* specific elimination of CSPG4(+) tumor cells by CSPG4 CAR T cells generated by with the human SK5 scFv. CSPG4(+) and CSPG4(-) tumor cells were co-cultured with CSPG4 CAR T cells generated with the human SK5 scFv at different tumor cell:effector ratios at 37°C. At the end of a 72 hours incubation, tumor cell viability was analyzed using a MTT assay. Control T cells were used as a specificity control.

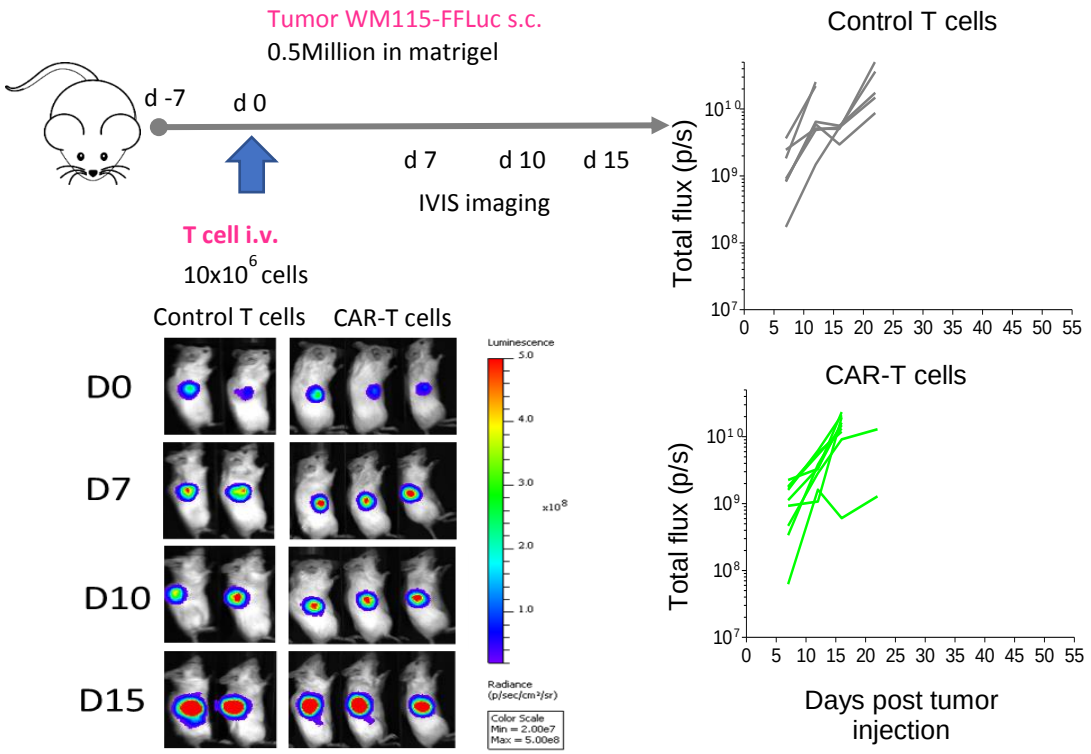


Figure 32. Lack of *in vivo* anti-tumor activity of T cells transduced with CSPG4 CAR generated with the human SK5 scFv. 0.5x10^6 WM115-FFLuc tumor cells were grafted subcutaneously in NSG mice. 7 days post-tumor engraftment, CSPG4-specific CAR T cells or control CAR T cells were administered i.v. in mice. Tumor growth was followed by bioluminescence imaging (BLI).

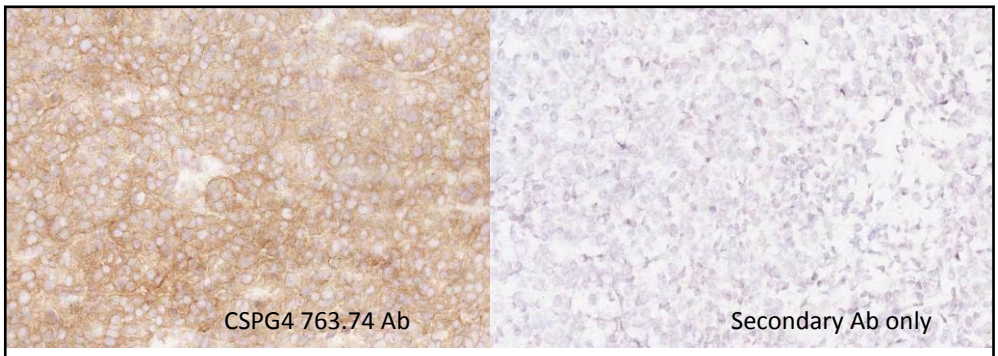


Figure 33. IHC staining with CSPG4-specific mAb 763.74 of surgically removed tumors from NSG mice. The negative results of the *in vivo* experiments do not reflect the lack of CSPG4 expression in the tumors, since IHC staining with the CSPG4-specific mAb 763.74 detected the antigen in surgically removed tumors. Staining with the secondary Ab only was used as specificity control.

- **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?**
  Dr. Dotti, UNC group: Complete subtask 1 and 4 for Aim 2 and initiate work on subtask 4 for Aim 3 as described in the original application.
  Dr. Ferrone, MGH group, will focus on the work described in Aims 3 and 4 of the original application.

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. Ferrrone, 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. Seliger B, Kloor M, **Ferrone S**. HLA class II antigen-processing pathway in tumors: molecular defects and clinical relevance. Oncoimmunology 6:2, 2017. PMID 28344859
2. Pellegatta S, Savoldo B, Di Ianni N, Corbetta C, Chen Y, Patané M, Sun C, Pollo B, **Ferrone S**, DiMeco F, Finocchiaro G, Dotti G. Constitutive and TNFα-inducible expression of chondroitin sulfate proteoglycan 4 in glioblastoma and neurospheres: Implications for CAR-T cell therapy. Sci Transl Med. 10(430), 2018. PMID: 29491184
3. Pilla L, **Ferrone S**, Maccalli C. Methods for improving the immunogenicity and efficacy of cancer vaccines. Expert Opin Biol Ther. 17:1-20, 2018. PMID: 29874943
4. 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 pii: S1044-579X(18)30044-0. doi: 10.1016/j.semcancer.2018.09.006. [Epub ahead of print] Review. PMID: 30261276

- **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:	*Dotti, Gianpietro*
Project Role:	*PI*

Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*0.97 month*
Contribution to Project:	*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
Funding Support:	

Has supervised the postdoctoral fellow:

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

Dr. Ferrone has supervised the collaborator

Name:	*Xinhui, Wang, MD, PhD*
Project Role:	*Assistant Professor*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.2 months*
Contribution to Project:	*Dr. Wang has purified the IL2 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.*
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 N/A

- **COLLABORATIVE AWARDS:** N/A
- **QUAD CHARTS:** N/A

9. APPENDICES: N/A