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Cellular Immunotherapy with CAR-redirected Cytokine-Induced Killer Lymphocytes (CIK) against HLA class I-defective Melanoma

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Abstract

The purpose of this study was to explore the preclinical efficacy of Chondroitin Sulfate Proteoglycan 4 (CSPG4)-specific CAR-engineered Cytokine-Induced Killer lymphocytes (CIK) in the context of HLA-defective metastatic melanoma. Abnormalities in the expression of HLA molecules play a major role in clinical resistance to Immune Checkpoint Inhibitors (ICI). Therefore, there is a strong rationale for the study of an HLA-independent therapy.

CSPG4 was selected as the CAR target because of its high expression on melanoma and its restricted distribution in normal tissues. CIK lymphocytes are *ex vivo* expanded T-NK lymphocytes that we chose as immune effectors for our strategy since they are endowed with an intrinsic HLA-independent antitumor activity.

Our first aim was to demonstrate the feasibility of this immunotherapeutic approach. We therefore generated CAR CIK lymphocytes from 4 melanoma patients. As second aim, we characterized different patient-derived melanoma cell lines for the expression of HLA class I molecules and Antigen Processing Machinery components to explore the presence of defect in the expression of these molecules. We found that some melanoma showed a deficient expression of such molecules; in particular, one melanoma was HLA-negative. We also confirmed that CSPG4 was intensely expressed by all the analyzed melanoma with no correlation to HLA-I levels.

Finally, we wanted to assess the antitumor potential of CSPG4 CAR CIK against HLA defective melanoma *in vitro* and *in vivo*. We showed that CSPG4 CAR CIK lymphocytes efficiently killed melanoma *in vitro,* including the HLA-negative melanoma and those melanoma with a low expression of HLA class I molecules. We confirmed the preclinical efficacy of CSPG4 CAR CIK lymphocytes in a xenograft model of an HLA-negative melanoma.

In conclusion, we reported the activity of CSPG4 CAR CIK lymphocytes against melanoma, including those with low or defective HLA-I expression. CIK lymphocytes may provide a valid platform for CAR-based strategies against melanoma and solid tumors in general. Our data provide the rationale to implement clinical studies exploring the proposed strategy in melanoma patients not responding or relapsing after immunotherapy with ICI.

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INTRODUCTION

An overview of metastatic melanoma and the currently available treatments

Melanoma is the most common type of cutaneous malignancy after basal cell and squamous cell carcinoma; even though it counts only 5% of cases, it remains the most aggressive skin cancer and it is associated with the large majority of skin cancer-related mortality, with an overall mortality excess of 10%. (1) Melanoma originates from the malignant transformation of melanocytes, pigment-producing cells derived from pluripotent neural crest stem cells. After the development these cells can be found predominantly in the basal layer of the epidermidis but also in extra-cutaneous sites, such as the eyes, meninges, esophagus, and mucous membranes. Therefore, a melanoma can be identified as cutaneous, mucosal, or uveal, arising respectively from melanocytes located in the epidermidis, in the mucous membranes and in the ocular stroma. Among these subtypes, cutaneous melanoma is the most common one, accounting for 90% of the cases. (2)

Melanocytes are characterized by the production of melanin, a dark pigment responsible of the color of the skin. Various etiological factors can induce the conversion of melanin from an antioxidant to a pro-oxidant agent, which causes an increase in the level of intracellular oxygen radicals. This phenomenon turns in multiple DNA damages and a consequent activation of several cell signaling pathways, such as uncontrolled proliferation, dedifferentiation, and immortalization of melanocytes. Some of the potential etiological factors are external agents and include ultraviolet radiation, use of pesticides, geographical location; other factors depend on intrinsic features related to the subject, like skin phototype, the presence of pigmented nevi, and immunosuppressive conditions. (3,4) Genetic factors can also play a role in the disease outbreak; approximately 5-12% of melanoma, in fact, are hereditary. The mutational profile of hereditary melanoma is different from the non-hereditary ones; alterations commonly found in familial melanoma are CDKN2A mutations, which lead to defects in tumor suppressor proteins ($p14^{ARF}$, $p16^{INK4A}$). (5)

According to the 2020 Melanoma Skin Cancer Report the incidence of melanoma rose by 44% between 2008 and 2018, when 287,723 cases were globally diagnosed, and 60,712 deaths were reported. Even more concerning are the latest data from the World Health Organization (WHO), which predict that, by 2025, the number of deaths caused by melanoma will increase by 20%, rising to 74% by 2040. The incidence rate varies between countries, with Australia having the highest rate of melanoma in 2018, followed by New Zeland and Norway (age-standardized rate per 100,000 of 33.6, 33.3 and 29.6 respectively) (World Cancer Research Fund data). Also, the lifetime risk of getting melanoma is higher for whites than African Americans and Hispanics

(2.6%-1 in 38, 0.1%-1 in 1,000 and 0.6%-1 in 167 respectively). The incidence of melanoma differs also between men and women: men are 10% more likely to develop the disease and 4% more likely to die from melanoma than women. Even though it is more common in men overall, before the age of 50 the rates are higher in women (2020 Melanoma Skin Cancer Report). The American Cancer Society estimated that in 2021 about 106,110 new cases (about 62,260 in men and 43,850 in women) would have been diagnosed and that about 7,180 people (about 4,600 men and 2,580 women) were expected to die of melanoma in the United States. The incidence rates increase with age: on average melanoma is diagnosed when people are 65 but it is also one of the most common cancers in young adults. Incidence of melanoma continued to increase over the years both in male and female; cases have been arising on average 1.4% each year over 2009 and 2018. **Figure 1**

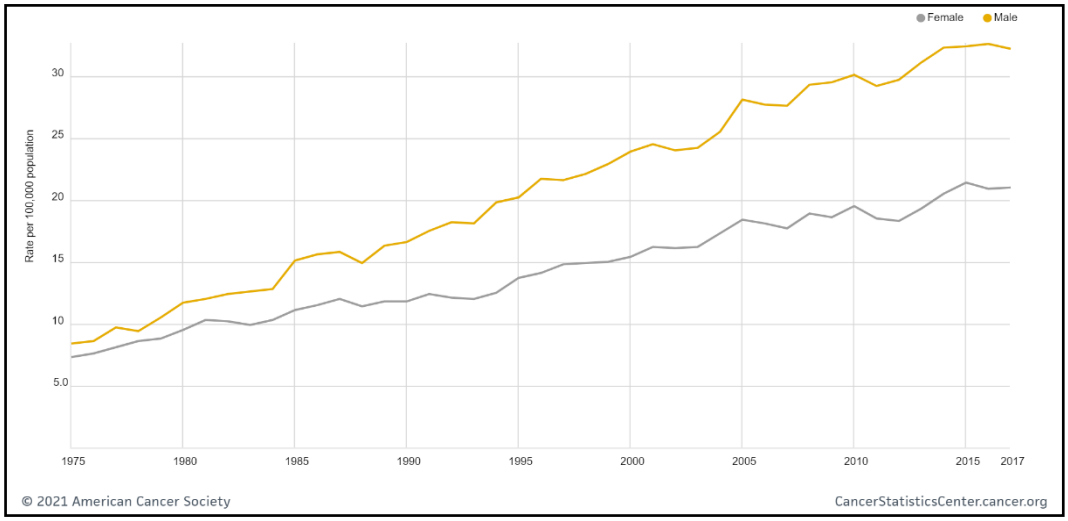

Figure 1. Trends in incidence rates between 1975 and 2017 by sex, for melanoma of the skin

The overall 5-year survival depends on the thickness of the primary tumor, whether the lymph nodes are involved and if the disease is localized or has spread to distant sites. For localized and 'thin' melanomas (defined as being less than 1 millimeter in maximal thickness) discovered in an early stage and without any lymph node involved, the 5-year survival is 99%. This rate goes down to 80% for people with thicker melanoma and to 66% if the disease affects the lymph node(s). For distant melanomas, meaning that the tumor has metastasized to distant organs, such as lungs and liver, the survival rate is lower, about 27% (American Cancer Society, Cancer.Net-Doctor-Approved Patient Information from ASCO).

The dermatologist Wallace Clark was the first to revolutionize the way melanoma was diagnosed. His classification was based on the identification of three histological variants of the malignancy: superficial spreading melanoma (SSM), lentigo maligna melanoma (LMM) and nodular melanoma

(NM). Clark also introduced the depth of invasion of melanoma cells into the dermis and subcutaneous fat, by dividing the skin into anatomic compartments, known as 'Clark levels': the risk of distant spread increases as tumor cells migrated into each compartment. The three histological variants and the 'Clarks level' are still recognized today and remain a standard way to acquire information about the aggressiveness the disease. (6) A more accurate classification was provided by Alexander Breslow, and it was based on measuring the depth of invasion in millimiter rather than anatomic compartments; this classification, known as 'Breslow's depth', divided melanoma into five stages based on the thickness of the tumor. Despite these systems initially helped physicians to assess the risk of spreading and the consequent treatment plan, it soon became evident that new guidelines were needed. The staging of melanoma follows the TNM (tumor, node, metastasis) system created by the America Joint Committee on Cancer (AJCC), which combines histological features of the primary tumor, the involvement of lymph nodes, the presence of distant metastasis and, in addition, ulceration, mitotic rate, tumor-associated inflammation and regression of the primary site. (5) The 8th edition AJCC staging system was implemented in 2018 with the introduction of key changes in the TNM staging system, to improve staging, prognosis, risk stratification and selection for patients for clinical trials. The most important changes include: measurement of the tumor thickness to the nearest 0.1mm, instead of 0.01mm, a revised definition of T1a and T1b, the inclusion of T1b N0 M0 (formerly pathologic stage IB) in stage IA, a new definition of the N category descriptions for regional node metastasis, changed from 'microscopic' and 'macroscopic' to 'clinically occult' and 'clinical apparent', prognostic stage III groupings increased from 3 to 4 subgroups, definitions of N categories are revised, a new M1d designation is added for central nervous system metastasis. (7) Recently imaging techniques have been developed to facilitate the diagnosis of melanoma; in particular, two devices (MelaFind and SIAscope) use visible and near infrared light to visualize lesions and help clinicians to decide whether the observed lesion need to be biopsied. A study showed that the use of MelaFind increased biopsy sensitivity from 76% (clinical
evaluation alone) to 92% , specificity from 52% to 79% and overall biopsy accuracy from 64% to 86%. (8) Regardless the detection method, after the lesion is biopsied, immunohistochemistry (IHC) analysis is performed to confirm the diagnosis of melanoma. Two types of markers are mainly used: melanocytic markers (MelanA/MART-1, gp100, S100, micropthalmia transcription factor (MITF)), utilized to confirm the origin of the lesion, and proliferative markers, which are indicator of the proliferative activity of the tumor and have prognostic implication. Other useful biomarkers performed on surgical biopsies include BRCA1-associated protein 1 (BAP-1), P16,

the preferentially expressed antigen of melanoma (PRAME), the endothelial markers D2-40 or CD31, KIT and the telomerase reverse transcriptase (TERT). (9)
The information derived by the analysis of these biomarkers is a powerful instrument for helping clinicians in the decision of the treatment plan.

Current treatment of metastatic melanoma

The treatment of metastatic melanoma is challenging. This malignancy is considered a relatively radioresistant tumor and the benefits derived from the surgical resection of distant metastases are limited. Once melanoma cells have metastasized to distant sites, chemotherapy or surgery are not curative options. Currently new approaches are available. In particular, in the scenario of metastatic melanoma, systemic target therapies and immunotherapies appear very promising. (10)

- ***Target therapy***

Treating melanoma with target therapies is a potentially successful approach, since, compared to other tumors this malignancy has a significantly higher mutational burden, caused by the extensive exposure to carcinogenic ultraviolet radiation. (11) The majority of these mutations are found in the mitogen-activated protein (MAP) kinase pathway; these mutational events are thought to be early oncogenic events. The activation of this pathway begins with the binding of a growth factor to the extracellular domain of its receptor tyrosine kinase (RTK) expressed on the cell surface. The intracellular signaling is mediated by a cascade composed of the G-protein RAS, followed by the protein kinases BRAF, MEK and ERK. The migration of activated ERK to the nucleus induces the activation of different transcription factors involved in several biological processes, like the growth, proliferation, and survival of the cell. Mutations lead to a constitutive activation of the MAPK pathway and to a consequent uncontrolled proliferation of melanocytes. (12) In 66% of melanomas the BRAF oncogene is mutated; another 15-20% of melanomas have NRAS mutations, 2% have CKIT mutations (especially mucosal melanoma). (5)
The presence of alteration in the MAPK pathway is essential to determine the treatment plan of patients with metastatic melanoma. The most common alteration in the BRAF gene is the missense mutation V600E, where a Valine is replaced by a Glutamate, followed by V600K and V600E. The evidence that BRAF V600 point mutations are the most common oncogenic drivers in melanoma led to the development of a series of kinase inhibitors that selectively bind to the active form of the mutant protein: this binding prevents the interaction with ATP molecules and inhibits the enzymatic activity of the kinase. (13) The first agent targeting BRAF, Vemurafenib, was approved

in 2011, followed by Dabrafenib and Encorafenib. (14) Multiple trials demonstrated the superiority in terms of survival of BRAF inhibitors (BRAFi), compared to standard chemotherapy in the setting of metastatic melanoma. In a randomized phase III study including patients with previously untreated unresectable stage III or IV BRAF V600E mutated melanoma, Vemurafenib has been associated with a better median progression-free survival (PFS) and overall survival (OS) compared to dacarbazine. (15,16) An open-label phase III clinical trial conducted on patients with the same characteristics as the previous study, showed that Dabrafenib significantly improved the PFS compared to dacarbazine. (17) Preclinical studies suggesting that Encorafenib is more potent and has a longer pharmacodynamic activity compared to the already approved BRAFi, lead to the design of a Phase I study including treatment-naïve or pretreated patients with metastatic melanoma; Encorafenib induced a longer PFS compared to the one reported for Vemurafenib and Dabrafenib. (18)

Despite these encouraging results, a subset of patients does not benefit from this therapeutic approach and the majority of responders rapidly develops resistance and disease relapse occurs after BRAFi monotherapy. Resistance can be induced by several mechanisms, including mutational and non-mutational events and changes in the tumor microenvironment. (19) Furthermore, BRAF inhibitors can lead to a paradoxical activation of the MAPK pathways in non BRAF-mutated tissues; in fact, these drugs disrupt the constitutively activation of BRAF V600E monomer, but they are ineffective in inhibiting the signaling of wild-type BRAF homo- or hetero-dimerized. As a result, the use of BRAFi as monotherapy is associated with the development of cutaneous squamous cell carcinoma and keratoacanthoma; this mechanism is more frequent with Vemurafenib (20%), rather than Dabrafenib (6%) and Encorafenib (3%). (14) In order to overcome these resistance mechanisms and avoid the paradoxical downstream activation of the MAPK pathway, BRAFi have been combined with MEK inhibitors (MEKi). Multiple clinical trials demonstrated the superiority of the combination compared to the single agents used as monotherapy in terms of PFS and OS for the treatment of patients with metastatic melanoma harboring BRAF mutations. (20–23) Currently the standard of care for patients with locally advanced or metastatic BRAF mutant melanoma, is based on the combination treatment with with BRAF inhibitors and MEK inhibitors.

- ***Immune Checkpoint inhibitors (ICI)***

Even though the introduction of target therapy improved the clinical outcome of melanoma, there is still a high percentage of patients with BRAF wild-type melanoma that can't benefit from this approach. In this setting immunotherapy plays an important role, especially after the advent of the Immune Checkpoint Inhibitors (ICI). Furthermore, patients with BRAF mutated melanoma, often encounter resistance to the treatment with BRAF inhibitors. For these patients ICI represent an effective therapeutic strategy.

Immune Checkpoints are regulatory molecules which activate or downregulate the activation of

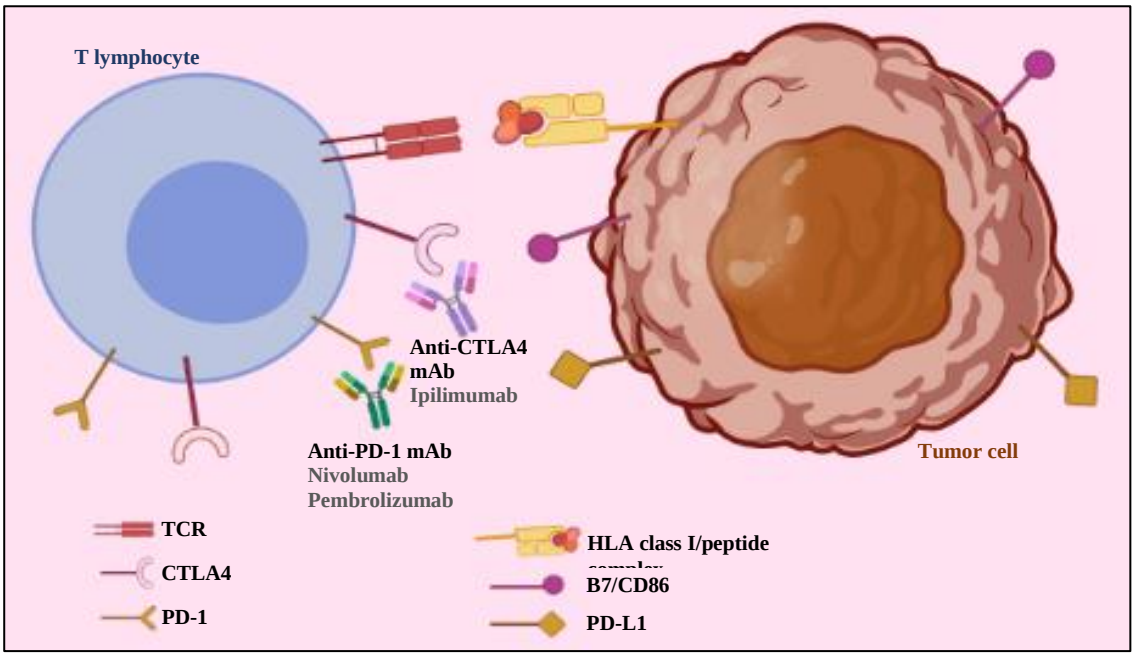

Figure 2. Immune Checkpoint Inhibitors as strategy to counteract exhaustion of T lymphocytes, in the treatment of metastatic melanoma

immune cells. In physiological conditions these proteins are essential to avoid an abnormal response of the immune system against healthy cells and ensure its proper functioning without damaging normal tissues. However, one of the mechanisms of tumor escape from the immune surveillance involves some of these immune checkpoints. The overexpression of inhibitory Immune Checkpoints on immune cells and their interaction with the respective ligands on tumor cells, allows tumor cells to escape from the antitumor immune response. The effort to restore the endogenous antitumor activity by using ICI is mainly focused on T cells, because of their mechanism of recognition and elimination of tumor cells. (24,25) The most targeted checkpoints in the setting of melanoma are Cytotoxic T-Lymphocyte-Associated protein 4 (CTLA-4) and Programmed cell Death protein 1 (PD-1). These two proteins are both essential to prevent autoimmunity but operate at different stages of T cell activation. The CTLA-4 pathway is involved in the early steps of the immune response and stops potentially autoreactive T cells when they are still in the lymph nodes, at the initial stage of naïve T-cell activation; the PD-1 pathway, instead,

regulates the later stages of an immune response, interfering with the activity of previously activated T cells which have reached peripheral tissues. (26) **Figure 2**

CTLA-4 is a receptor of the B7 family expressed on both CD4+ and CD8+ T lymphocytes. CTLA-4 and CD28 are homologous receptors with, respectively, inhibitory, and co-stimulatory function in T cells; they share CD80 and CD86 ligands, which are expressed on the surface of antigen presenting cells (APCs). CTLA-4 is induced on activated T cells and compete with CD28 for ligand binding at the immune synapse between T cells and APCs; it has been shown that CTLA-4 can recruit CD80 with higher affinity compared to CD28, thereby limiting its interaction with the ligand, and inhibiting T cell response. (27,28)

PD-1 is a membrane receptor involved in the inhibition of both adaptive and innate immune response; it is expressed on activated T, natural killer (NK) and B lymphocytes, macrophages, dendritic cells (DCs) and monocytes. PD-L1 and PD-L2 (programmed cell death ligand 1/2), also known as CD279 or B7-H1 and CD273 or B7-DC respectively, are PD-1 ligands; they are members of the B7 family and share similar expression pattern and function. PD-L1 is constitutively expressed on several types of cell populations, such as macrophages, in part on activated T and B lymphocytes, DCs and it is induced under inflammatory conditions on nonhematopoietic cells, such as vascular endothelial cells, pancreas, islets cells, placenta and testicles. Furthermore, it has been demonstrated that inflammatory cytokines produced by immune cells infiltrated in the tumor, including IFNγ, IL-4, IL-10, tumor necrosis factor-α (TNF-α), can upregulate PD-L1 expression in different types of cancer. The interaction of PD-1 with its ligands leads to the downregulation of T cell response through several mechanisms, including the inhibition of proliferation and the downmodulation of the TCR signaling. The latter is caused by different signals induced by the recruitment of the protein SHP-2, which mediates the inhibition of the phosphoinositide-3-kinase/Akt (PI3K/AKT) and the Ras/MEK/ERK pathways, blocks the phosphorylation of ZAP70 and the activity of the leukocyte-specific tyrosine kinase (LCK) and inhibits the function of CK2 protein, leading to the constitutive activation of the phosphatase and tensin homolog (PTEN) pathway. (29–31)

The anti-CTLA-4 antibody Ipilimumab was the first ICI to be approved by the U.S. Food and Drug Administration (FDA) in 2011 after showing/inducing an improved OS in patients with metastatic melanoma. (32) Following studies showed that the two monoclonal antibodies targeting PD-1, Nivolumab and Pembrolizumab, were associated with a significant improvement in PFS and OS with less high-grade toxicity compared to Ipilimumab. (33,34) Moreover, further clinical trials demonstrated that better OS and PFS were observed in patients treated with the combination of Ipilimumab and Nivolumab compared to patients who received nivolumab or ipilimumab alone.

(35) Several ongoing trials are also evaluating the association between target therapy and ICI as first-line treatment of BRAF V600E mutant melanoma patients, also exploring the optimal sequence strategy. The phase II KEYNOTE-022 study showed that the addition of Pembrolizumab to the combination of Dabrafenib and the MEKi Trametinib as first-line treatment, prolonged the PFS of patients from 10.3 to 16 months. (36)

Currently, the combination of Ipilimumab plus Nivolumab has been approved as first line treatment, regardless BRAF mutational status. (37–39)

Mechanisms of resistance to Immune Checkpoint Inhibitors

Despite ICIs are currently the standard care for melanoma and despite the improved clinical outcome of melanoma patients treated with anti-PD-1 and anti-CTLA4 mAb alone or in combination regimen, a significant part of patients does not benefit from this immunotherapeutic approach. Non-responder patients can be divided in two populations: those that fail to respond from the beginning of the treatment, therefore characterized by an innate or primary resistance, and those that initially respond but eventually develop disease progression, meaning that mechanisms of acquired or secondary resistance occur. (40) Different factors, both known and unknown, can negatively impact the efficacy of ICI-based therapies (41), including a defective expression of HLA class I molecules.

Defective expression of HLA class I molecules

One of the causes that can induce a deficient immune response and therefore a lack of response to ICI, is the mechanism of immune escape adopted by tumor cells based on a defective presentation of tumors antigens mediated by the HLA complex. The immune system plays a fundamental role in cancer immunosurveillance, a mechanism based on the concept that immune cells can recognize and, in most cases, destroy precursors of cancer before the development of the tumor. (42) Melanoma is characterized by a high genomic instability and a high mutational burden; this leads to the expression of multiple specific antigens, known as tumor associated antigens, which are associated to tumor cells and not expressed in normal tissues. (43) The innate immune antitumor response is mainly mediated by cytotoxic T lymphocytes (CTL), that exert their antitumor activity through the T Cell Receptor (TCR) which is able to recognize the tumor associated tumor associated antigens. In order to be presented and recognized by CTLs, the peptides must be produced by proteolysis, transported and assembled in the HLA class I -loading complex. Several

proteins and multimeric structures are involved in this process, and all together form the complex known as MHC class I antigen processing and presentation machinery (APM). The first step involves the activity of the proteasome, a multimeric protein complex characterized by a cylindrical shape and composed by catalytic core and regulatory particles. Under inflammatory conditions or when a particularly intense immune response occurs, three of the proteasome's catalytic core subunits can be replaced by functionally different counterparts named LMP2, LMP7 and LMP10 (low molecular weight protein 2,7,10); in this case, the proteasome is called immunoproteasome. After the passage through the proteasome, peptides ranging from 2 to 25 residues are generated and released into the cytosol. From this point, the peptide loading complex (PLC) coordinates the peptide translocation and editing within the ER and its loading onto the HLA class I complex. The passage from the cytosol to the ER is mediated by the transporter associated with antigen processing (TAP), which is composed by two subunits (TAP1 and TAP2), which are members of the ATP-binding cassette transporter family. TAP transports more efficiently peptides of 8-12 residues; longer peptides are transported with reduces efficiency and once transferred in the ER, are further trimmed by ER aminopeptidase enzymes (ERAP1, ERAP2) to create peptides able to fit within the HLA class I complex. The loading process takes place with the aid of multiple chaperone proteins: calnexin, ERp57, calreticulin and tapasin. Specifically, calnexin facilitates the complete folding of a newly synthetized MHC class I heavy chain and, along with ERp57, ensures its correct oxidation. After these modifications, the heavy chain has the right conformation to be recognized by β2 microglobulin, which is essential for the final presentation of the antigen. The heavy chain/ β2 heterodimers is characterized by an 'open' conformation that interacts with calreticulin. Finally, tapasin interacts with TAP and helps to recruit the HLA I-β2 complex, stabilizes the heavy chain/ β2 heterodimers and optimizes the peptide loading. After the binding of the peptide, the chaperone proteins are released and the peptide-HLA class I complex leaves the ER inside vescicles that migrates to the cell membrane and fuse with it. (44) At this point the membrane complex can be recognized by the TCR. The interaction between the TCR and the HLA class I molecule loaded with the tumor antigen induces

the activation of T cells that are now able to eliminate tumor cells by releasing perforins and granzyme. (45) **Figure 2**

However, several mechanisms of resistance can occur and contribute to the escape from immunosurveillance. (46) One of these mechanisms is the downregulation, alteration, or complete loss of HLA class I molecules. In melanoma several alterations are responsible for an abnormal expression of HLA class I molecules, including microsatellite instability, genomic haplotype loss, locus specific downregulation, selective loss of single HLA class I allospecificities. (47) Some HLA alterations lead to cancer immune escape and therefore correlate with metastatic progression and a worst clinical outcome of melanoma patients. For example, down-regulation of TAP and HLA class I has been associated to an increased thickness of primary lesions, tumor progression and metastasis in melanoma patients; in addition, the frequency of this defect is higher in metastasis than in the primary lesions. (48,49) The loss or presence of a mutated β2m, protein which is essential for the functioning of the HLA class I complex and its correct translocation to the cell membrane, is another frequent alteration in melanoma. (50,51) The downregulation or loss

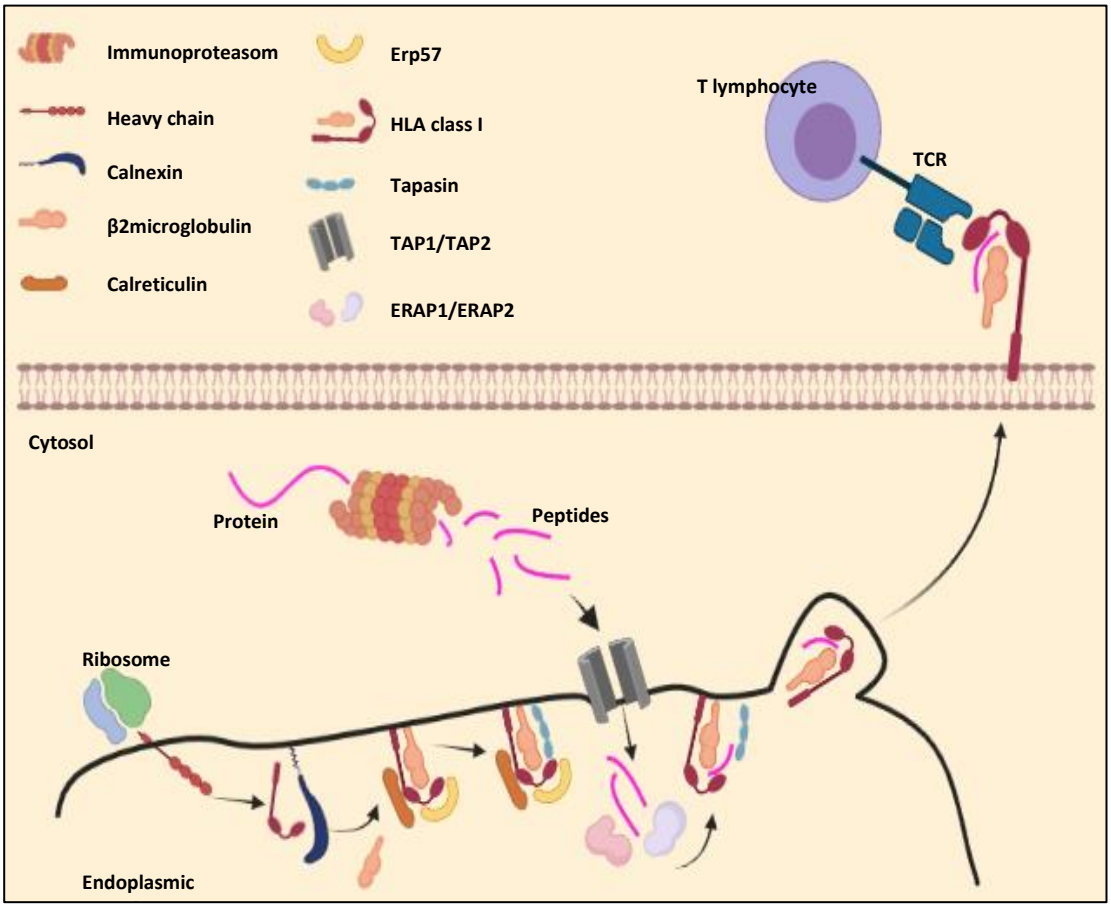

Figure 2. Schematic representation of the tumor antigen processing, mediated by the Antigen Processing Machinery (APM), and presentation, mediated by the HLA class I complex, on the surface of tumor cells.

of HLA class I molecules is one of the mechanisms responsible for failure of ICI-based therapy and in particular resistance to PD-1 inhibitors. Genetic alterations in antigen presentation, in fact,

have been associated to limited T cell infiltration in the tumor: it has been found that CTLs were present and abundant in melanoma lesions at the time of the relapse, but their presence was restricted to the tumor margin, meaning that CD8 T cells are no longer able to exert their cytotoxic activity because of the lack of tumor antigen recognition and consequent activation. (52,53)

Adoptive cellular immunotherapy (ACT) for the treatment of metastatic melanoma

Adoptive cell transfer (ACT) therapy is a very promising approach that could help to overcome the problem of resistance to target therapy and ICIs. This immunotherapeutic strategy is based on the infusion of immune cells into cancer patients to mediate an antitumor response. The adoptive transfer of *ex vivo* expanded immune effectors has several advantages compared to other immunotherapies, such as the *in vitro* activation and stimulation which allows to avoid .the inhibitory factors that exist *in vivo* and the chance to manipulate the host to create a favorable microenvironment and the possibility to genetically modify the isolated immune cells. (54) There are several ACT strategies, which differ for the type of the utilized immune effectors and for the additional genetic manipulation.

Tumor infiltrating lymphocytes (TILs)

A first type of ACT is based on the use of tumor-infiltrating lymphocytes (TILs); it is a personalized treatment that relies on the natural ability of T lymphocytes to recognize and eliminate the tumor. TILs are CD4+ and CD8+ T lymphocytes that naturally migrate into the tumor and are specific for tumor antigens (TAs) presented by the MHC class I complex. Melanoma reactive TILs are autologous T cells isolated from surgically resected tumor samples and expanded *ex vivo* in the presence of IL-2 alone or in combination with IL-7, IL-15, and/or IL-21. The fraction of T cells reactive against the tumor are selected, further expanded, and then re-infused into the patient. It has been demonstrated that a preparative non-myeloablative lymphodepleting chemotherapy regimen before the administration of *ex vivo* expanded TILs improves the efficacy of the therapy, by increasing the availability of homeostatic cytokines as well as by depleting T regulatory cells (Tregs). (55,56) Melanoma is an ideal target for this type of therapy because it is considered one of the most immunogenic types of tumors; in addition, it has been shown that there is a strong correlation between the presence of TILs and the clinical outcome. (57) Melanoma is characterized by a high tumor mutational burden and this feature leads to the generation of multiple

tumor associated antigens, which are a consequence of unique mutations in the tumor tissue and, therefore, are highly tumor specific. T cells reactive against tumor associated antigens recognize and eliminate tumor cells in a selective way and play a major role in the development of personalized TIL ACT. (58,59) Several clinical trials have shown a significant tumor regression in melanoma patients treated with TIL ACT, pointing out the potential benefit of this approach for patients with few treatment options. (55) However, several obstacles are related to the use of TILs therapy, including high rate of grade 3 or 4 toxicity and conditions of anemia, thrombocytopenia and sustained neutropenia caused by the lymphodepleting regimen. Furthermore, the administration of high-dose IL-2 post TILs' infusion to support expansion and survival of transferred TILs, is associated with significant toxicity. To overcome this effect, in a recent Phase II clinical trial, patients with metastatic melanoma were treated with autologous TILs in combination with a low dose of IL-2, to avoid toxicities. Despite the toxicity attributable to IL-2 was acceptable, no patient achieved a complete response and none of the partial responses were durable. (60) Another limitation of this approach is the limited availability of resectable tumors.

Genetic engineering of immune effectors with a T Cell Receptor (TCR)

An attempt to improve the efficacy of ACT and improve the specificity of tumor cell recognition is the genetic engineering of lymphocytes with antitumor receptors. One approach is based on the *ex vivo* transduction of T cells with a viral vector encoding for a TCR specific for a selected tumor antigen. In addition to viral vectors, a variety of non-viral gene transfer methods have been developed, such as the 'Sleeping Beauty' transposon system. (60) The TCR is a heterodimer composed of two transmembrane polypeptide chains (α and β), each consisting of a constant region, which anchors the chain to the cell membrane, and a variable region, which recognizes and

binds to the antigen presented by the HLA class I complex. The TCR is associated with the CD3 complex, which is essential for T cell activation. (61) In the setting of melanoma, some clinical trials demonstrated the efficacy in inducing a durable response, of autologous T cells transduced with a TCR reactive against MART-1, gp100 melanocyte differentiation antigen or NY-ESO-1 cancer-testis antigen. (62–64) Some limitations restrained the enthusiasm raised by this innovative approach. Firstly, a suboptimal surface expression of the transferred TCR might result from the competition between endogenous and exogenous TCRs for assembly with CD3 subunits. A second issue is the TCR mispairing: α and β chains from exogenous and endogenous TCRs could mis-pair, therefore introducing new specificities and potentially leading to undesired reactivities. Many strategies have been explored to address this safety issue and to increase the expression level of

the transferred TCR, including the replacement of the human TCR constant region with a murine-derived sequence, the introduction of cysteine residues to stabilize the pairing of TCR chains, the generation of a human TCR inclusion of the CD3ζ chain and the incorporation in the viral vector of small interfering RNA sequences able to reduce the expression of the endogenous TCR. (65)

Genetic engineering of immune effectors with a Chimeric Antigen Receptor (CAR)

A major limitation of ACT based on the use of TCR-engineered lymphocytes is that it requires the presentation of tumor associated antigen by the HLA class I complex and its interaction with the TCR. As previously mentioned, the downmodulation of HLA class I molecules is one of the several mechanisms of tumor immune escape; without the tumor antigen presentation the transferred T lymphocytes are not able to recognize and eliminate tumor cells. A very promising approach developed to overcome this mechanism of resistance involves the use of immune effectors engineered to express a Chimeric Antigen Receptor (CAR). CAR is a recombinant protein composed by an extracellular domain for tumor antigen recognition, linked to an intracellular signaling domain by a transmembrane portion. The extracellular domain (ED) mediates the specific recognition of the target antigen, and it is composed by the single-chain variable fragment (scFv) derived from the antigen-binding fragment (Fab) of a mouse monoclonal antibody (mAb). The interaction between the ED and the target antigen expressed on tumor cells determines the killing of the latter and the proliferation of CAR-engineered effectors. (66) The antigen binding domain is linked to a peptide fragment, referred as hinge or spacer, of approximatively 15 residues in length. This portion separates the binding unit from the transmembrane domain (TM), providing stability for efficient CAR expression and activity and it confers the adequate flexibility for the correct orientation of the CAR and binding to the antigen. It has been shown that the spacer length affects CAR function. Long spacers provide more flexibility and allow for better access to membrane-proximal epitopes or complex glycosylated antigens; by contrast, CARs with short spacers are more effective in binding membrane-distal epitopes. (67) This extracellular complex/structure is connected, by the TM, to an intracellular signaling domain comprised of the TCR CD3ζ chain alone (first generation CARs) or with the addition of one (second generation CARs) or more (third generation CARs) tandem costimulatory domains. First generation CARs, endowed only with the ITAM (immune receptor tyrosine-based activation motif) segment of CD3 showed lack of persistence and low proliferation capacity; the introduction of the 4-1BB or CD28 costimulatory domains allows the generation of CARs

characterized by a stronger antitumor activity, a longer persistence and weaker tonic signaling, which means that exhaustion occurs more slowly. However, a single costimulatory domain was not sufficient to solve persistence and relapses problems associated with CAR-based therapy and this leads to the generation of CARs with both costimulatory domains, showing better persistence, proliferation, and antitumor activity. (68) Several efforts have been made to improve the efficacy of CAR immune effectors, by genetically modifying the CAR construct. Therefore, fourth generation CARs, also known as TRUCKs (T cells redirected for antigen-unrestricted cytokine-initiated killing) have been developed by introducing in the construct transgenes for cytokine secretion, such as IL-12, IL-18, IL-15. (69) Fifth generation CARs comprise a further costimulatory region, consisting of the intracellular domain of a cytokine receptor (e.g., the JAK-STAT activation domain derived from IL2RB chain fragment) binding STAT3 and incorporated between CD28 and CD247. This domain stimulates cell proliferation, prevents terminal differentiation, and shows better persistence. (70) **Figure 3**

CAR-engineered immune effectors recognize tumor cells through the interaction with the target

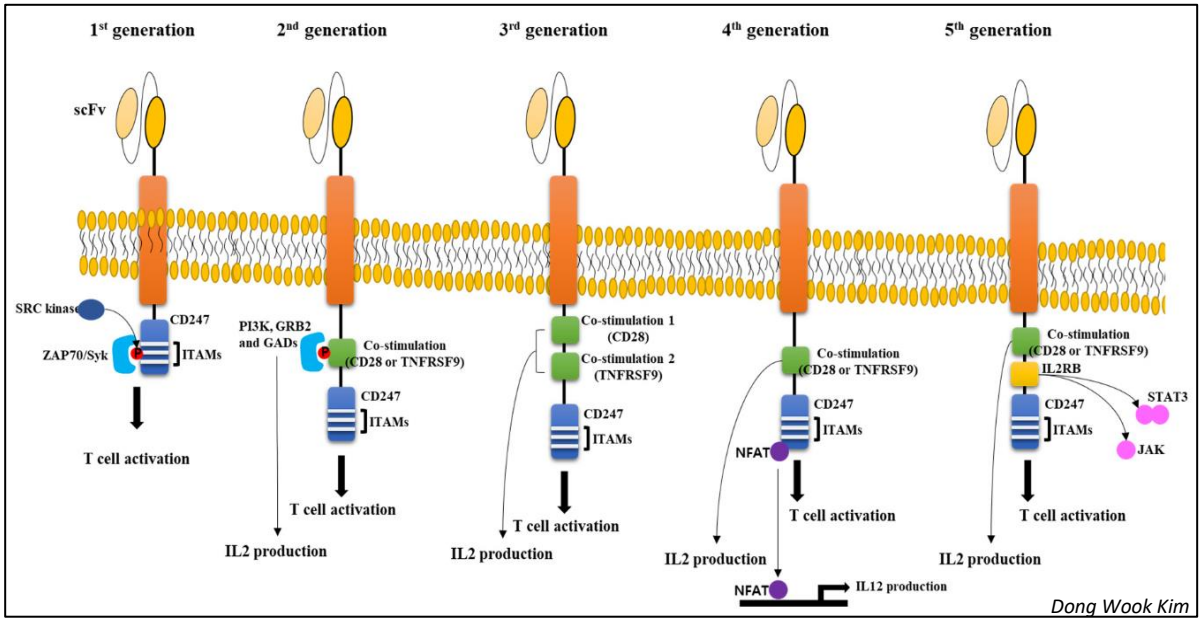

Figure 3. Structure of the CAR constructs from the first to the fifth generation. (62)

antigen, in an HLA-unrestricted manner. Upon the antigen recognition, the lysis of tumor cells is mediated by the release of various cytotoxic molecules, such as granzyme and perforin as well as cytokines, by the CAR lymphocytes. (66)

CAR-based immunotherapy in melanoma

Adoptive cell therapy based on the use of CAR-engineered immune effectors has revealed impressive results in the field of hematological malignancies. This has led to the recent approval

by the U.S Food and Drug Administration (FDA) of two CD19 CAR T cell products, Kymriah from the Novartis and Yescarta from the Kite Pharma, for the treatment of B cell acute lymphoblastic leukemia (B-ALL) and diffuse large B-cell lymphoma (DLBCL) respectively. (71,72) Adoptive cell therapy with CAR-engineered immune effectors has been investigated also in the field of solid malignancies, including melanoma. Several preclinical studies are focused on the use of T lymphocytes genetically modified to express CARs specific for different target antigens. These antigens include surface molecules involved in the process of melanoma metastasis, such as the angiogenic factor VEGFR2 (vascular endothelial growth factor 2) (73,74), the integrin αvβ3 (75) and the cluster of differentiation 20 (CD20) expressed on a subset of melanoma cells known as cancer stem cells. (76) Further studies showed encouraging results in preclinical *in vitro* and *in vivo* models of melanoma, involving CAR T cells targeting CD16, CD126, GD2, GD3, HER2, gp100, CSPG4, B7-H3 and CD70 molecules. The obtained results demonstrated that CAR T lymphocytes can efficiently eliminate melanoma cells expressing these target antigens *in vitro*, mediate tumor regression and enhanced survival in different mouse models of melanoma. (77) Ten early Phase clinical trials have been conducted to assess the safety and effectiveness of CAR T cells for the treatment of melanoma patients, with the earliest study started in 2010. Six out of these ten studies are still recruiting and include CAR T cells targeting IL13R-α2, CD20, NY-ESO-1, gp100, GD2 and B7-H3 together with CD19; one trial has been suspended for halt in funding and only two are completed as of now. The latter were based on the use of CAR T cells specific for VEGFR2 and GD2: the first one has been terminated because no objective response was observed in patients, whilst no published data are available for the second one. (77) Important evidence emerging from the study involving anti-VEGFR2 CAR T cells, is the high frequency of toxicity induced by this type of immune effectors: 23/24 patients (95.8%) had serious (5/24, 20.8%) or non-serious adverse event. This study also underlines that the treatment of metastatic melanoma with CAR T cells is still a challenging approach: in the same study, only one patient (4.1%) had a stable disease, whilst 23/24 patients (95.8%) experienced disease progression. These results demonstrate that the approach based on the genetic manipulation of immune effectors has the potential to improve the treatment of metastatic melanoma, but the optimal setting has yet to be defined. Our research group made an effort to explore the use of a novel type of immune effectors as alternatives to CAR T-based strategies. Our approach is mainly based on the evidence that the currently available immunotherapies for the treatment of melanoma need the HLA class I mediated antigen presentation to be effective. The high frequency of defects in the expression of HLA class I molecules in the setting of melanoma, led us to focus our studies on an HLA-unrestricted approach, based on the use of CAR-redirected Cytokine Induced Killer cells

(CIKs). This promising ACT strategy is independent from HLA class I expression both for the intrinsic cytotoxic activity of CIK lymphocytes and for the antitumor potential mediated by the CAR specific recognition of malignant cells.

Cytokine Induced Killer lymphocytes (CIKs)

Cytokine Induced Killer cells are a heterogeneous population of *ex vivo* expanded T lymphocytes characterized by a mixed T-NK phenotype, endowed with an HLA-unrestricted antitumor activity. Results previously obtained by our research group and the information present in the literature confirm that these immune effectors are promising candidate for the design of a CAR-based ACT.

Generation and immunophenotype of CIK lymphocytes

CIKs lymphocytes can be obtained from Peripheral Blood Mononuclear Cells (PBMCs), bone marrow and umbilical cord and can easily be expanded *in vitro*, by culturing them with the time addition of IFNγ, anti-CD3 and IL-2. (78) IFNγ, added on day 0, induces the activation of macrophages present in the culture, which provide both contact-dependent and cytokine-mediated (IL-12) signals favoring the acquisition of a Th1 phenotype. An initial mitogenic signal is provided by the anti-CD3 and then sustained by the continuous presence of IL-2. (79) After 3-4 weeks of expansion, it is possible to obtain a high number of CIK lymphocytes; the final expansion levels, in fact, are usually in the range of a hundredfold. This is a feature that makes such immune effectors very attractive candidates; the opportunity to obtain a sufficient number of immune effectors is indeed a key issue for a successful clinical translation. To further improve the expansion rate of CIK lymphocytes, several groups have been testing different protocols, including the addition of cytokines to the culture medium, such as IL-7 and IL-15. (80,81) This is particularly important for those patients who may be ‘poor expanders’ and consequently could not fully benefit from this approach.

At the end of the expansion period, CIK lymphocytes appear as a heterogeneous population composed by two major subsets: the first one is positive for CD3 and negative for CD56 molecules (CD3+ CD56-), the second one is positive for both CD3 and CD56 (CD3+ CD56+). The latter is considered the main responsible for the intrinsic cytotoxic activity. As previously mentioned, CIK lymphocytes have a mixed T-NK phenotype. In fact, they express some receptors typical of NK cells, such as NKG2D (Natural Killer Group 2-member D) and DNAM-1 (DNAX accessory molecule-1). However, CIK lymphocytes are negative or express at low levels NK-specific activating (NKp30, NKp44, NKp46) and inhibitory (KIR2DL1, NKG2A) receptors. The circulating precursors of CIK lymphocytes are naïve T lymphocyte, mainly characterized by a

CD4 and CD8 double negative phenotype; mature CIK lymphocytes are mainly CD8+, with a terminally differentiated late effector phenotype (CD45RO+, CD27low, CD28low, CD62L-, CCR7-). (82) In addition to the described markers, CIK lymphocytes also express the TCR, CD45RA, CD2 and Fas ligand. (83)

Antitumor activity mediated by CIK lymphocytes

One of the most promising features of Cytokine Induced Killers is that they are endowed with an HLA-unrestricted antitumor potential, meaning that they can bypass the HLA restrictions of the clinical setting an offer the attractive opportunity to widen the number of patients that can benefit from this cell transfer-based immunotherapy. Furthermore, the HLA-independent mechanism makes this strategy effective against those tumors that use the downregulation of HLA class I molecules as a mechanism of immune escape.

Cytotoxic activity of CIK lymphocytes relies on multiple mechanisms and molecular pathways which are activated after the interaction with tumor cells. The first one is based on the binding between the adhesion molecule Leukocyte Function-associated Antigen-1 (LFA-1) expressed on CIK lymphocytes and its ligands on tumor target cells. A second mechanism is the induction of tumor cell apoptosis by Fas ligand via the Fas signaling pathway. Also, the receptor DNAM-1 takes part in the recognition and elimination of tumor cells, by the interaction with CD122 and CD155 ligands. However, the main contribution to the antitumor potential of CIK lymphocytes is given by the NKG2D receptor, a member of the c-type lectin activating receptor family. (84) The *in vitro* inhibition of NKG2D with blocking antibodies or small interfering RNA (siRNA) experiments confirmed that the CIK-induced lysis of tumor cells is mediated by NKG2D rather than TCR. (85,86) The main known NKG2D ligands are stress-inducible molecules, including MHC class I-like molecules A and B (MICA, MICB) and members of the UL16-Binding Proteins family (ULBP1-6), which are highly and almost exclusively expressed on malignant cells. (87,88) The release of perforin and granzyme by CIK lymphocytes mediates the final lysis of tumor cells. (86) Beside their cytotoxic activity, CIK lymphocytes release several cytokines, including IFNγ and Tumor Necrosis Factor-α (TNF-α), that activate antitumor responses and are involved in regulate innate and adaptive immunities in patients. (89) The antitumor activity of CIK lymphocytes has been described *in vitro* and confirmed *in vivo* in preclinical models of several types of hematologic (90) and solid malignancies, including renal cell carcinoma (91), colorectal cancer (92), metastatic melanoma (93,94), ovarian cancer (95), bone and soft tissue sarcoma. (96,97)

Clinical translation of CIK-based therapy for the treatment of solid and hematologic tumors

Overall, CIK lymphocytes can be considered a promising candidate for the development of novel immunotherapy strategies. Their functional properties, in fact, might address some of the limitations that are currently preventing the successful clinical translation of the many adoptive cell transfer therapies, including the possibility to obtain clinically adequate numbers of immune effectors and the HLA-dependent mechanism of tumor recognition and killing. Another attractive feature of CIK lymphocytes is that they present a reduced alloreactivity across HLA-barriers, meaning that they can be used as alternative to conventional Donor Lymphocyte Infusions after allogeneic hematopoietic cell transplant with a reduced risk of Graft versus Host Disease (GVHD), which represents a severe complication related to the transfusion of allogeneic lymphocytes. (98) The low alloreactive capacity of CIK lymphocytes can be explained with their terminally differentiated late effector phenotype, the limited proliferative potential, and the release of cytokines protective against GVHD, such as IFNγ. (99,100) Between 1999 and 2019, a total of 16 clinical trials have been registered; these studies have been focusing mainly on the treatment of patients with lung cancer (26.4%), hepatocellular carcinoma (18.9%), gastric cancer (16%), renal cell carcinoma (15.1%) and lymphoma (15.1%). The information obtained with these clinical trials show that CIK cell-based therapy has a significant short-term antitumor efficiency and improves the long-term benefits; an increased 5-year survival rate, an improved PFS and OS were reported in multiple studies. (101) Based on these encouraging results, several Phase III and Phase IV studies have been designed to better assess the efficacy and safety of CIK lymphocytes-based therapy. (102,103) However, the heterogeneity in the design of these studies makes challenging to interpret the results and compare them to draw comprehensive conclusions. Therefore, in 2010, an international online platform called International Registry on CIK lymphocytes (IRCC) has been created, with the goal to collect information about clinical trials involving CIK lymphocytes for the treatment of patients with advanced malignant tumors and standardize the CIK cell-based treatment approach, as well as their generation protocol.

Several attempts have been made to improve the efficacy of CIK lymphocytes, including the improvement of the culture method and the combination with other strategies, including ICI, target therapies and Dendritic Cell (DC) vaccination. (104)

Our approach aims to enhance the antitumor potential of CIK lymphocytes by genetic manipulation with CARs. Our group recently published a study, which demonstrated, in a preclinical model of soft tissue sarcoma, the superior antitumor activity of CAR CIK lymphocytes

engineered with a lentiviral vector encoding for a CD44v6-specific CAR, compared to unmodified CIK lymphocytes. (105) In the present study, we investigated the preclinical efficacy of a CAR CIK-based immunotherapy targeting the Chondroitin Sulfate Proteoglycan 4 (CSPG4), a tumor antigen particularly relevant in the setting of metastatic melanoma, with a particular focus on targeting HLA-defective melanoma.

Chondroitin Sulfate Proteoglycan 4 (CSPG4) as promising target of CAR-engineered CIK lymphocytes

The Chondroitin Sulfate Proteoglycan 4 (CSPG4), also known as high molecular weight-melanoma associated antigen (HMW-MAA) and melanoma chondroitin sulfate proteoglycan (MCSP), is a tumor antigen characterized for the first time on human melanoma cells more than 30 years ago. (106) CSPG4 is a cell surface type I transmembrane protein, characterized by a complex structure, that can be expressed in two forms: a N-linked glycoprotein of ~250 kDa or a ~450 kDa form, composed by a N-linked glycoprotein associated to a proteoglycan component. (107) The extracellular region of CSPG4 is characterized by three domains (D1, D2, D3), which contain binding sites for extracellular matrix proteins, growth factors, integrins, matrix metalloproteinasis and lectins. The D1 N-terminal globular domain is enriched in disulfide bonds, and it is critical for a correct tertiary structure. The D2 domain is composed of 15 ‘CSPG repeat’ motifs, whereas the D3 region is a membrane proximal globular domain with binding sites for integrins and lectins. The extracellular region is linked to an intracellular domain by a transmembrane sequence. The cytoplasmic region contains a binding domain for scaffold proteins, such as MUPP1, syntenin and GRIP1, two sites that can be phosphorylated by PKCα and ERK 1-2 and a proline-rich region which mediates other protein interactions. (108,109) **Figure 4**

Two main signaling pathways are activated via the intracellular part of CSPG4. The first is the MAPK pathway, activated by ERK 1-2 or triggered by tyrosine kinase receptors (RTKs) stimulated by growth factors, such as platelet-derived growth factor (PDGF), binding to the extracellular domain of CSPG4. (110,111) The interaction with integrins activates the focal adhesion kinase (FAK) pathway, which establishes a connection with the extracellular matrix. (112) The activation of these pathways implicates that CSPG4 is involved in multiple cellular functions, including cell adhesion, motility, and migration; therefore, it is associated with tumor progression and invasion. (113)

CSPG4 expression has been detected on normal tissues such as mesenchymal stromal cells, which lose its expression during terminal differentiation, nevi, epidermidis and hair follicles. However, this molecule is not expressed in the brain, thyroid, thymus, lung, liver, ureter, testis, spleen, ovary, or peripheral nerves. (114) Although CSPG4 expression at the RNA level has been reported in several normal tissues (skin, trachea, veins, lung, heart, muscle, diaphragm, adipose tissue, uterus, prostate, thymus, spleen, bone marrow, gastrointestinal tissue), such level il 6.6 times lower than the level detected in tumors. (115) On the contrary, CSPG4 is overexpressed in different types of solid tumors, including melanoma (113), glioma (116), breast cancer (117), head and neck carcinoma (118) and mesenchymal tumors. (119)

CSPG4 is expressed on 70% of melanoma at different stages and it has been detected on the primary lesion as well as on metastatic sites. Several preclinical studies in vitro and in vivo have demonstrated the importance of CSPG4 for the survival, growth, and motility of melanoma cells. (114,120) As previously mentioned, melanoma harboring BRAF mutation are characterized by a constitutive activation of the MAPK pathway; in vitro studies reported that in these cases CSPG4 further contributes to this effect and showed that small molecules inhibitors for mutant BRAF can synergize with anti-CSPG4 monoclonal antibodies (mAb) in the control of tumor growth, and that CSPG4 inhibition can decrease the activation of ERK signaling. (121,122)

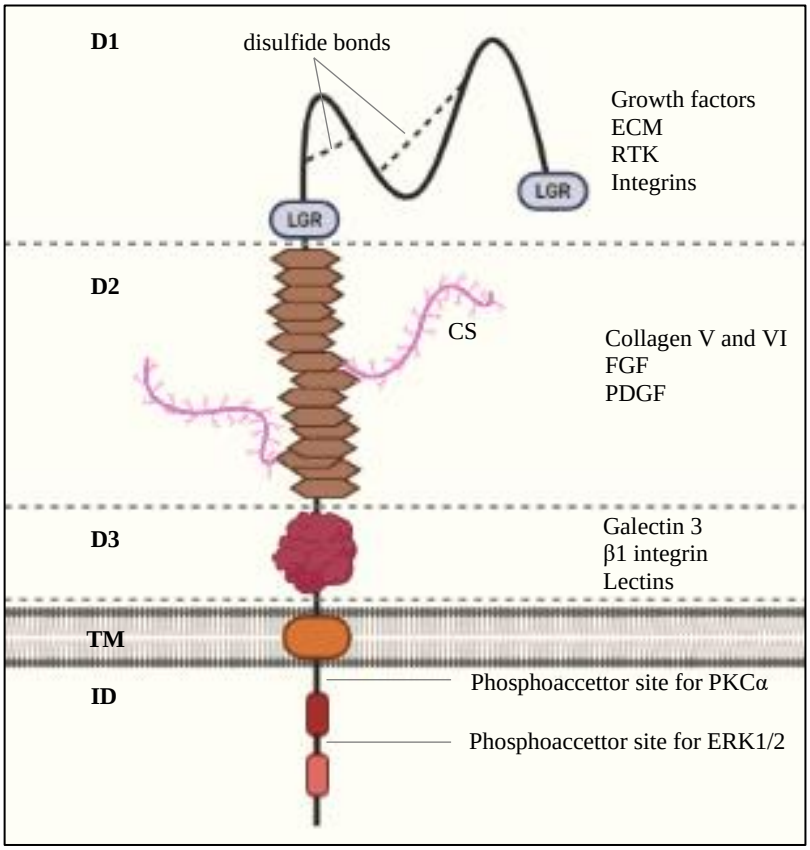

Figure 4. Structure of the Chondroitin Sulfate Proteoglycan 4 (CSPG4), characterized by the three extracellular domains (D1, D2, D3), a transmembrane region (TM) and an intracellular domain (ID). LGR laminin G-type regions, CS chondroitin sulfate, ECM extracellular matrix, RTK receptor tyrosine kinase, FGF fibroblast growth factor, PDGF platelet -derived growth factor, PKC protein kinase C, ERK extracellular signal-regulated kinase 1/2.

Due to its overexpression in different types of cancer, including melanoma, its restricted distribution in normal tissues, alongside with the crucial role in tumor growth, survival, and invasion, CSPG4 is an attractive therapeutic target. Strategies based on the use of CSPG4-specific monoclonal antibodies (mAb) alone or conjugated to

radioisotopes or toxins have shown promising results. (114,123) Anti-CSPG4 bispecific T cell engagers (BiTEs) represent a novel therapeutic approach which is based on the fusion of two scFv: in addition to the recognition and binding of the target antigen, like classic mAb, BiTEs are designed to bind also to T cells via CD3 and induce the cytotoxic activity of these lymphocytes specifically against tumor cells. The relevance of CSPG4 as therapeutic target has also been confirmed *in vitro* and murine models by using T cells engineered with a CSPG4-specific CAR against different types of tumors, including melanoma. A recent study showed that CSPG4 CAR T cells efficiently eliminate melanoma cells *in vitro* and limit tumor growth of a xenograft mouse model of melanoma. (124)

An additional relevant feature of CSPG4 is that it is expressed not only on differentiated tumor cells but also on cancer stem cells (CSCs), a subpopulation of tumor cells characterized by self-renewing ability, and which is associated with high tumorigenicity and resistance to chemotherapy and radiotherapy. (125) It is crucial to target and eliminate this subset of cancer cells, since they are considered responsible for tumor recurrence and metastatic spreading. CSPG4 has been shown to be expressed by CSCs in melanoma, breast cancer and squamous cell carcinoma. (126)

To summarize, we selected CSPG4 as target antigen for our CAR-based immunotherapy strategy since i) it is overexpressed on melanoma and barely detectable or absent on normal tissues, ii) it is involved in tumor growth, progression, and metastasis, iii) it is expressed on both differentiated melanoma cells and the subpopulation of melanoma CSCs. All these features make CSPG4 an 'ideal' target antigen for our CAR CIK lymphocytes immunotherapy.

Radiotherapy as strategy to improve the efficacy of CAR-redirected immune effectors against melanoma

Despite the promising results and advances in the development of CAR-based adoptive immunotherapies, especially in the field of hematological malignancies, the treatment of solid with this approach is still challenging. This therapeutic approach is currently not effective in completely eradicate solid tumor, including melanoma, for several reasons, such as the hostile conditions of the tumor microenvironment (TME) and the multiple mechanisms of immune escape adopted by tumor cells. (127–129)

To overcome this issue, several efforts have been made to combine CAR-redirected immune effectors with other therapeutic approaches. To this end, a first potential strategy is to manipulate the effectors and improve or render more potent the CAR construct or combine them with ICI to counteract their exhaustion. (130,131) A second option is to act on the tumor and utilize strategies that create a tumor microenvironment more 'friendly' and less hostile to the infiltration of CAR-

immune effectors and make tumor cells more susceptible to the recognition and elimination. A discrepancy between the *in vitro* and *in vivo* results has been observed in studies with CARs recognizing tumor antigens expressed in different types of tumors. (132) This effect is likely to be mediated by the hostile conditions of the TME, such as hypoxia, acidic pH and adenosine (129,133), factors that have been shown to reduce CAR T cell expansion and differentiation, as well as cytotoxicity via reduction of cytokine and granzyme B production. (134) TME conditions can reduce the susceptibility of tumor cells by upregulating the expression of anti-apoptotic molecules and by inducing or increasing the level of PD-L1 expression. (135,136)

In our project we identified radiotherapy as potential strategy to combine with CAR-immune effectors, in particular of T lymphocytes transduced with a CSPG4 CAR. The rationale for the use of radiation relies on its potential to make tumor cells more sensitive to the CAR-mediated lysis. It has been shown that low dose radiation induces an upregulation of the tumor antigen recognized by the CAR, in a preclinical model of pancreatic adenocarcinoma with an heterogeneous expression of the target. (137) Since antigen density on target cells correlates with CAR T cell recognition and elimination (138,139), the upregulation of the target antigen induced by radiation is expected to contribute to increase CAR-mediated recognition and elimination of tumor cells. Furthermore, radiation can modulate the expression of pro- and anti- apoptotic molecules. (140) It has also been shown that radiation facilitates the trafficking and infiltration of CAR T cells into the tumor by enhancing T cell adhesion, chemotaxis and by remodeling blood vessels within the TME. (141)

AIM OF THE STUDY

The aim of this study is to explore the efficacy of an adoptive immunotherapy strategy based on the use of immune effectors engineered with a Chimeric Antigen Receptor (CAR) specifically redirected against the tumor antigen Chondroitin Sulfate Protecoglycan 4 (CSPG4), in a preclinical setting of metastatic melanoma, in particular focusing on HLA defective melanoma. The evidence that defects in the expression of HLA class I molecules are highly frequent in melanoma and can cause resistance to checkpoint inhibitors or other immunotherapy strategies prompted us to choose Cytokine Induced Killer (CIK) cells as immune effectors, since they are endowed with an HLA-independent cytotoxic potential. In fact, their antitumor activity is mainly mediated by the NKG2D receptor, which recognizes stress inducible molecules selectively expressed on tumor cells. In addition, we plan to 'arm' CIK lymphocytes with a second HLA-independent mechanism of tumor recognition and elimination by the genetically engineering with a CAR that specifically recognize CSPG4. This tumor antigen is a surface type I transmembrane proteoglycan, involved in malignant progression and metastatic dissemination. It is considered an attractive target for a CAR-based approach because of its high expression on tumor cells and limited presence on normal tissues.
A parallel and secondary study will be focused on studying the role of radiotherapy as strategy to increase the activity of CAR-engineered immune effectors, in particular CAR T cells. The rationale for this study relies on the evidence that CAR T cells have shown a limited antitumor activity in multiple preclinical models *in vivo*. In this secondary study we explored a strategy based on the use of T lymphocytes redirected with a CSPG4-specific CAR combined with radiotherapy against melanoma. The goal was to show that the pretreatment of tumor cells with radiation can increase the sensitivity of melanoma cells to the CAR T cell mediated lysis.

Specific Aims

Generation and characterization of patient derived melanoma cell lines

As preclinical tumor model we will focus on metastatic melanoma. We will generate primary cell lines generated *in vitro* starting from tumor biopsies of patients with metastatic melanoma. All the cell lines will be characterized for the expression of HLA class I molecules (HLA-ABC, β2microglobulin) and of some relevant components of the Antigen Processing Machinery (APM), including the Immunopreoteasome, composed by LMP2, LMP7, LMP10 and the Protein Loading Complex, composed by TAP1, TAP2, Erp57 and Tapasin. We will evaluate the effect of IFNα and IFNγ in upregulating the expression of HLA class I and APM molecules on the generated melanoma cell lines. Secondly, we will check the expression of CSPG4 on the generated cell lines,

to confirm that it is broadly expressed on melanoma and therefore a good target for our strategy. Finally, we will analyze the expression of the NKG2D ligands (MIC A/B, ULBPs).
These results will allow us to confirm that melanoma is a good preclinical model to test the efficacy of our immunotherapeutic approach and that melanoma patients can potentially benefit from this therapy.

Generation and characterization of CSPG4 CAR CIK lymphocytes

CIK lymphocytes will be generated starting from PBMCs of melanoma patients. PBMC will be genetically engineered with a retroviral vector encoding for a CSPG4-specific CAR. As a control of the CAR specificity, in parallel, we will generate CIK lymphocytes expressing a CD19-specific CAR. We will assess the transduction efficiency by measuring the percentage of cells expressing the CAR construct. Furthermore, we will characterize the generated CSPG4 CAR CIK for the expression of the markers typical of the known immunophenotype (CD3, CD8, CD56, NKG2D, CXCR3) and compare the phenotype of the engineered CIK lymphocytes with their non-transduced controls, to confirm that the genetic manipulation will not affect the features of these immune effectors.
With our results we will be able to confirm that CSPG4 CAR CIK can be easily generated and therefore they are an attractive candidate for a feasible clinical translation of a CAR- based adoptive cell therapy.

Assessment of CSPG4 CAR CIK lymphocytes antitumor activity against HLA defective melanoma in vitro

The antimelanoma activity of CSPG4 CAR CIK will be evaluated against the previously generated melanoma cell lines, with a particular focus on those melanoma characterized by a low or absent expression of HLA molecules. We will set up co-culture experiments *in vitro* using different effector to target (E:T) ratios, from 10:1 to 1:4. The specific tumor lysis will be measured after a 48hour incubation period and CD19 CAR CIK will be utilized as control of the CAR specificity.
With these experiments we will test the hypothesis that CIK lymphocytes armed with a CSPG4-specific CAR are more efficient in eliminating melanoma cells compared to unmodified CIK lymphocytes that relay only on the natural cytotoxic activity mediated by the NKG2D receptor. In this way we will demonstrate *in vitro* the efficacy and feasibility of our CAR-based immunotherapy strategy.

Evaluation of CSPG4 CAR CIK lymphocytes antitumor activity in vivo against an HLA-defective melanoma

After the *in vitro* assessment of the antitumor activity of CSPG4 CAR CIK lymphocytes, we will confirm our results in a preclinical setting *in vivo*. For this specific aim we will utilized a melanoma that is negative for the expression of HLA class I molecules. Immunodeficient mice will be subcutaneously injected in the flank with tumor cells derived from the HLA-defective melanoma cell line M017. Mice will be treated with CSPG4 CAR CIK by tail vain injection. We will assess their ability to control the tumor growth melanoma by measuring the tumor volume. In parallel, a group of mice will be treated with NTD CAR CIK lymphocytes as control and another group will be left untreated as control of the natural course of the tumor growth.
The obtained results will confirm the *in vitro* finding that CSPG4 CAR CIK lymphocytes can efficiently eliminate melanoma. The preclinical *in vivo* model will help us to assess the ability of CSPG4 CAR CIK to reach and infiltrate the tumor site, providing relevant information for the potential translation in patients.

Study of a combinatorial strategy based on the use of radiation to increase tumor cell sensitivity to CSPG4 CAR T lymphocytes

In this last part of the project, the aim is to generate preliminary data that will be presented as proof of concept for the design of a future and more detailed study. We will explore the potential ability of radiation to increase the efficacy of CSPG4 CAR T cells against melanoma. T cells will be isolated from PBMCs of healthy donors, transduced with a retroviral vector encoding for the CSPG4-specific CAR and expanded *in vitro*.
We will test the hypothesis that radiation can increase tumor cells susceptibility to the killing activity of CSPG4 CAR T lymphocytes. Tumor cells will be treated with a non-lethal dose of radiation and then CSPG4 CAR T cells will be added to the co-culture at different E:T ratios (1:5 and 1:10).
The obtained results will give us more information about the potential advantages of combining radiation with CSPG4 CAR T cells against melanoma. If our hypothesis is correct, this combinatorial strategy could help to improve the efficacy of CAR-based immunotherapies against solid tumors.

MATERIAL AND METHODS

Generation of patient-derived melanoma cell lines

Human melanoma tissues were obtained from surgical specimens (lymph nodal or cutaneous metastasis) from patients with advanced stage melanoma. All patients provided consent under Institutional Review Board-approved protocols. Human melanoma tissues were cut into $3mm^3$ pieces and then dissociated by using the gentleMACS Dissociator instrument to obtain a single cell suspension. Tumor cells were seeded into plates and expanded with Knock Out DMEM F12 culture medium supplemented with 10% Fetal Bovine Serum (FBS) heat inactivated, 1% PenStrep (100U/ml Penicillin, 100 μg/ml Streptomycin).

Characterization of patient-derived melanoma cell lines

The expression of HLA class I surface molecules (HLA-ABC, β2microglobulin) and the intracellular APM components (LMP2, LMP7, LMP10, TAP1, TAP2, Erp57) was evaluated by flow cytometry by utilizing mouse monoclonal primary antibodies at the concentration of 10ug/ml, kindly provided by Soldano Ferrone (MD, PhD at Massachusetts General Hospital, Boston, USA), who has an open collaboration with our group. Samples were stained with a phycoerythrin (PE)-conjugated Goat anti-Mouse secondary antibody (BD Pharmigen). For the analysis of intracytoplasmic molecules, pd-mel cells were fixated with BD Cytofix™ Fixation Buffer for 20 minutes at 4°C, washed twice with BD Stain Buffer and permeabilized with BD Phosflow™ Perm Buffer III (BD Pharmingen™). The presence of CSPG4 (PE or APC- conjugated mAb, BD Pharmingen™) and the expression of NKG2D's ligands MIC A/B (Anti-Human MiC A/B PE Mouse, BD Pharmingen™), ULBP-2 (anti-hULBP-2-5-6-APC, R&D Systems®), was determined by flow cytometry. In order to quantify the number of single-type molecules harbored by the cell, we used a set of microspheres, Quantum™ R-PE MESF labeled with increasing amounts of PE (Bangs Laboratories, Inc.).
Labeled cells were analyzed by flow cytometry (CyAn ADP, Beckman Coulter s.r.l.) and analyzed using Summit Software. Gate criteria were set to isotype controls.

Gene analysis of HLA-defective melanoma

Genomic DNA was subjected to deep sequencing using the Illumina TruSight Oncology 500 panel (TSO500, Illumina, San Diego, California, USA). HLA-I negative melanoma DNA was sonicated using the Covaris Focused-ultrasonicator (Covaris, Inc. Woburn, Massachusetts, USA) according

to the manufacturer protocol. The targeted sequence was enriched with two rounds of hybrid-capture and the libraries were sequenced on the Illumina-NovaSeq 6000 instrument (Illumina, San Diego, California, USA). Raw data were processed by the Illumina local app associated with the TSO500 panel (TruSight Oncology 500 v2.2 Local App) to produce.fastq files through the alignment of the sequence to the human reference sequence GRCh37 (hg19). The Local App also performed sequencing QCs and somatic variant calling with a tumor-only pipeline.

Treatment of melanoma cell lines with Interferons (IFNs)

To evaluate the potential beneficial effect of IFNs on the expression of HLA class I molecules and APM components on melanoma with a deficient expression, tumor cells were seeded in 6 well plates with Knock Out DMEM F12 culture with the addition of 16 ng/mL of IFNα (Human IFNα2b, research grade Milteny Biotech), 29 ng/mL of IFNγ (Human IFN-γ1b, premium grade, Miltenyi Biotec), or left untreated as control. After an incubation period of 48 hours, tumor cells were detached with Accutase Cell Dissociation Reagent (ThermoFisher Scientific) and analyzed by flow cytometry. The primary mouse mAb kindly provided by Soldano Ferrone, MD, PhD were used.

Generation and characterization of CSPG4 and CD19 CAR CIK lymphocytes

CIK lymphocytes were generated from Peripheral Blood Mononuclear Cells (PBMC) of patients with metastatic melanoma at the Candiolo Cancer Institute FPO-IRCCS. Patients released informed consent under Institutional Review Board-approved protocols.

PBMC were isolated by density gradient centrifugation (Lymphosep, Aurogene) and seeded in cell culture flasks at a concentration of 2 x 10^6 cells/mL in RPMI-1640 medium (Gibco BRL), supplemented with 10% fetal bovine serum (FBS, Sigma) 1% PenStrep (100 U/mL Penicillin and 100 μg/mL Streptomycin, Gibco BRL). IFNγ (Miltenyi Biotec; 1,000 U/mL) was added on day 0 and after 24 hours (day 1) we activated PBMC using Anti-Biotin MACSiBead Particles loaded with CD2, CD3, and CD28 antibodies (Miltenyi Biotec) and human interleukin (IL)-2 IS (Miltenyi Biotec, 300 U/mL). On day +2, stimulated PBMC were transduced with a retroviral vector MoMLV-RVCSPG4.CAR coding for an anti-CSPG4 CAR and containing the costimulatory domain 4-1BB. The vector has been provided by Dt. Gianpietro Dotti (University of North Carolina, U.S.A.) in the context of an active collaboration with our research group. In parallel, the PBMCs were transduced with the retroviral vector MoMLV-RVCD19.CAR coding for an anti-

CD19 CAR containing costimulatory domain 4-1BB, to have a control of the CAR specificity. PBMCs were transduced with 0.5 ml of retroviral supernatant in 24 wells plates not-treated for cell cultures (Corning), previously coated with recombinant fibronectin fragments (25 µg/well; FN CH-296, Retronectin, Takara Shuzo, Otsu, Japan). Subsequently, after a centrifuge (1000 g) of 2 hours at 32 ° C, the viral supernatant was removed, $1x10^6$ PBMCs were plated in each well and the plates were incubated at 37 ° C and 5% CO_2.

After an overnight incubation, the medium containing the virus was replaced with fresh medium and new IL-2 (300 U / ml). Cells were expanded over 3 weeks and fresh medium was replaced every 2–3 days with the addition of IL-2 (300 U/mL) as needed: cell concentration was maintained at 1.5 x 10^6 cells/mL. Non-transduced CIK (NTD CIK) were expanded with the same protocol and used as paired controls.

The immunophenotype of CSPG4 CAR/CD19 CAR/NTD CIK lymphocytes was assessed at the third week of expansion. The following mAbs were used: CD3 (anti-human CD3 FITC Mouse, BD Pharmingen), CD8 (anti-human CD8 PE, MACS Miltenyi Biotec), CD56 (anti-human CD56 PE, MACS Miltenyi Biotec), NKG2D (anti-human CD314 APC, MACS Miltenyi Biotec), CD45RA (anti-human CD45RA FITC, Miltenyi Biotec), CD45RO (anti-human CD45RO PECy5, Miltenyi Biotec), CD62L (anti-human CD62L PE, Miltenyi Biotec), CXCR3 (anti-human CXCR3 PE, BD Pharmingen™),

CAR expression was detected by flow cytometry with an APC-conjugated mAb specific for the IgG1/ CH2CH3 spacer (The Jackson Laboratory), 3 weeks after the infection.

Labeled cells were analyzed by flow cytometry and analyzed using Summit Software. Gate criteria were set to isotype controls.

Assessment of antitumor activity of CSPG4 CAR CIK against patient-derived melanoma cell lines in vitro

The antitumor activity of CSPG4 CAR CIK lymphocytes was assessed in vitro against melanoma cells with flow cytometry. Target cells were stained with the vital dye CFSE (5,6-carboxyfluorescin diacetate succinimidyl ester, Molecular Probes) according to the manufacturer's protocol. After the staining, melanoma cells were seeded in 48 wells plates (20.000 cells/well), CIK lymphocytes were added at different effector:target (E:T) ratios (10:1, 5:1, 2:1, 1:1, 1:2, 1:4, 1:8) and co-cultured for 48 hours. In parallel, stained melanoma cells were cultured without the addition of effector cells as a control of the spontaneous death of the target. In detail, 400 µL of RPMI were added to each well with the addition of IL-2 (300 U/mL) and the coculture was maintained at 37°C, 5% CO_2. The rate CIK lymphocytes-mediated killing was determined by flow

cytometry, assessing the percentage of viable melanoma cells, population identified by gating strategy as DAPI-negative. The same experimental procedure was adopted with CSPG4 CAR/CD19 CAR/NTD CIK lymphocytes against the different melanoma cell lines. Experiments were repeated at least two independent time for each cell line.
The data obtained after flow cytometry analysis were examined by Summit Software and the percentage of specific tumor lysis was calculated with the formula: (experimental death - spontaneous death) / (100 - spontaneous death) x 100.

Treatment of melanoma cell lines with CSPG4 CAR CIK supernatant

In selected experiments we evaluated the ability of CAR CIK lymphocytes-supernatant in increasing the expression of HLA Class I molecules on melanoma cells. This effect is likely mediated by several factors released by CAR CIK lymphocytes, including perforins, cytokines. Our hypothesis is that the effect is mainly mediated by the release of IFNγ. Target cells were seeded in 6 wells plates in the presence of ul of supernatant previously collected from a flask of CSPG4 CAR CIK lymphocytes. Melanoma cells incubated with non-conditioned medium were used as control. After 48 hours of incubation, the expression of HLA-ABC and β2microglobulin molecules was assessed by flow cytometry, by using the previously described specific mAbs. The obtained data were analyzed using Summit Software.

Assessment of antitumor activity of CSPG4 CAR CIK in a xenograft model of melanoma

To test the antitumor activity of CSPG4 CAR CIK lymphocytes in vivo, we utilized the patient-derived melanoma cell line M017 to establish a murine model of melanoma. We selected this cell line because it is characterized by a complete loss of HLA class I expression and it is partially resistant to CIK cells *in vitro.* Twenty-four NOD/SCID female mice were subcutaneously injected on the right flank with melanoma cells (850.000 cells/mouse) resuspended in PBS and BD Matrigel Basement Membrane Matrix (Becton Dickinson, 1:1) and divided in three groups (6 mice/group). Two days after the injection of tumor cells, the first group received the first injection of CSPG4 CAR CIK lymphocytes, the second group received first injection of NTD CIK lymphocytes, and the last group was left untreated as control of the spontaneous growth of the tumor. In detail, a total $3x10^6$ CSPG4 CAR or NTD CIK lymphocytes resuspended in PBS (200ul), were injected by tail vein to each mouse of the respective groups every three days for a total of

five infusions. Tumor growth was monitored by caliper measurement be-weekly, and the volume was calculated according to the formula: $V=(width^2 \cdot length)/2$. The experiment ended 35 days after the injection of tumor cells, when some of the tumor reached the 1.5cm in the main diameter; all the mice were sacrificed, and tumors harvested for further analysis.
The day before the end of the in vivo experiment, mice were intravenously infused with the fluorescent probe XenoLight RediJect 2-DeoxyGlucosone (DG)-750 (PerkinElmer) at the dose of 5nmol/mouse. When mice were sacrificed, tumor were harvested and acquired with Living Image Software and the IVIS Spectrum CT (Caliper Corporation, PerkinElmer Company).

Generation of CSPG4 CAR T lymphocytes

For the transduction of T cells, PBMCs of healthy donors were used. As first step, CD3 (Miltenyi) and CD8 (Pharmigen) antibodies were diluted in sterile water to a final concentration o 1ug/ml. The antibody solution containing both antibodies was added to a non-tissue culture treated 24 well plate and incubated at 37°C for 4 hours. After this incubation, the antibody solution was removed and complete CAR T cells medium (50% RPMI 1640, 50% Clicks media supplemented with 10% FBS, 1% Glutamine, 1% Pen/Strep) was added for a 15-30 min incubation at 37°C. Then media was removed and $1x10^6$ PBMCs/well were added and stimulated with IL-15 (5ng/ml) and IL-7 (10ng/ml). The activated PBMCs were transduced with 0.5ml of retroviral supernatant (the same described for the generation of CSPG4 CAR CIK lymphocytes) in 24 wells plates not-treated for cell cultures (Corning), previously coated with recombinant fibronectin fragments (25 µg/well; FN CH-296, Retronectin, Takara Shuzo, Otsu, Japan). Subsequently, after a centrifuge (1000 g) of 2 hours at 32 ° C, the viral supernatant was removed, $1x10^6$ PBMCs were plated in each well and the plates were incubated at 37 ° C and 5% CO_2; fresh medium with IL-15 (5ng/ml) and IL-7 (10ng/ml) was added.
On day 6 the transduction efficiency was assessed by flow cytometry by using an anti-idiotype monoclonal antibody CSPG4 CAR.

Evaluation of antitumor activity of CSPG4 CAR T lymphocytes on melanoma cells treated with radiation in vitro

For this experiment, the M14 melanoma cell line was stably transduced with a retroviral vector encoding for the Green Fluorescent Protein (GFP) and seeded at 40% confluency in t25 flasks. Tumor cells were then treated with radiation using an X-RAD 320 Biological Irradiator, at a dose

rate of approximatively 0.76Gy/min. Two days after the last dose of radiation, melanoma cells were harvested, counted, and seeded in 24 wells plate (40.000 cells/well) in the presence of CSPG4 CAR T cells at different E:T ratios (1:5, 1:10). The coculture plate was incubated for 5 days in an Incucyte Zoom live cell analysis system. The total green fluorescence object area was selected as parameter to determine the cell viability of melanoma cells in each group of treatment. Data were analyzed with Excel and then showed as percentage of tumor cell viability.

RESULTS

1. *Evaluation of HLA class I molecules on patient-derived melanoma cell lines*

Our first step was to characterize the patient-derived melanoma cell lines generated in our laboratory and utilized as experimental platform to evaluate the efficacy of our CAR-based immunotherapy strategy. As previously mentioned, one of the potential mechanisms of resistance to immunotherapy based on the use of Checkpoint Inhibitors is the lack or altered presentation of tumor antigens by tumor cells to Cytotoxic T Lymphocytes. This phenomenon is likely due to an abnormal expression of the HLA class I and APM molecules in tumor cells. To explore this aspect in our preclinical model and point out the significance of an HLA-independent therapeutic approach, we started by evaluating the expression of HLA class I and APM molecules in our patient-derived melanoma cell lines.

Over the years, in our laboratory, we have established primary cell lines derived from patients with metastatic melanoma, to create a preclinical experimental platform for our projects. In this study, part of the effort has been focused on contributing to the generation and enrichment of this platform. Primary melanoma cell lines have been established starting from tumor tissues obtained after resection or surgical biopsy of patients with metastatic melanoma. As described in Material and Methods, tumor samples were processed, and tumor cells were expanded *in vitro* to establish human melanoma cell lines.

For our first aim, we focused on 24 patient-derived melanoma cell lines generated in our laboratory during the course of the years, identified as M005, M006, M008, M011, M016, M017, M026, M029, M030, M036, M038, M039, M040, M041, M044, M054, M056, M058, M060, M063, M067, M069, M071, and M072.

We characterized each cell line by flow cytometry for the presence of HLA class I components, including

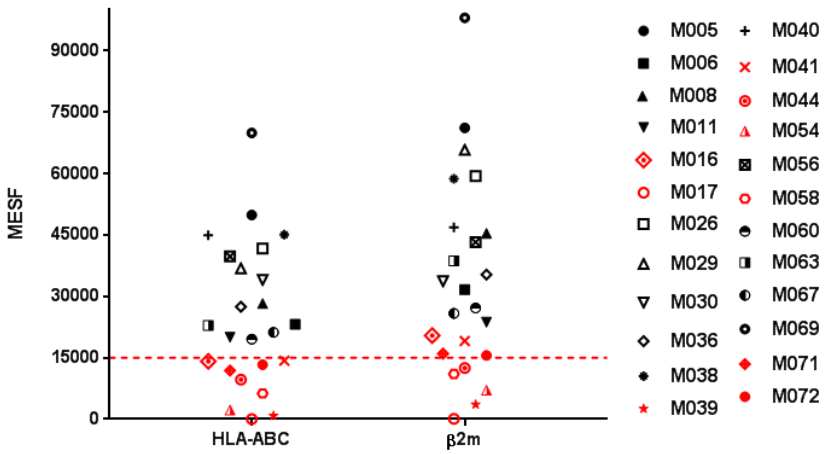

Figure 1. Expression rate of HLA class I molecules on patient-derived melanoma cell lines. We assessed by flow cytometry the expression level of the surface molecules HLA-ABC and β2 microglobulin (β2m) on 24 primary melanoma cell lines The expression level of HLA-ABC and β2m molecules was quantified by flow cytometry utilizing Molecules of Equivalent Soluble Fluorochrome (MESF). Nine out of 24 melanoma (red symbols) showed an expression rate of both HLA ABC and β2m, lower than 15000 molecules. The expression level of HLA-ABC and β2m was equal to or higher than 15000 molecules in 15/24 melanoma (black symbols).

surface molecules (HLA-ABC and β2 microglobulin) and the intracellular Antigen Processing Machinery (APM).

Membrane expression of HLA-ABC and β2 microglobulin (β2m) was detected in 23/24 melanoma cell lines, including some melanoma with a defective expression (9/24) and one melanoma that did not express HLA-ABC and β2m molecules (M017). The latter can be identified as an example of HLA-negative melanoma since our analysis showed a complete loss of both HLA-ABC and β2m expression on the surface of tumor cells. Therefore, we focused our interest on M017 to better understand the mechanism responsible for the lack of expression of the HLA class I complex on the surface of tumor cells. We performed a genome sequencing analysis on the DNA extracted from M017 tumor cells and we identified a single nucleotide variant (SNV) mutation in the transcript's AUG codon of the β2m gene. Such mutation, identified as p.Met1Ile, results in the reduction or, like in the case of M017, complete elimination of the protein.

Beside the analysis of the percentage of tumor cells, another way to describe the expression of a certain molecule, is to utilize the Mean Fluorescence Intensity (MFI). This parameter becomes particularly relevant when, like in our model, the percentage of expression is high in all the analyzed samples. Measuring the MFI allowed us to discriminate different expression level of HLA class I molecules between melanoma cell lines in which we detected a population of positive cells higher than 90%.

For a more accurate analysis of the MFI, we utilized fluorochrome-labeled beads to quantify the number of HLA-ABC and β2m molecules expressed on the cell surface. The number of molecules, in fact, is directly correlated with the Mean Fluorescence Intensity (MFI). We observed a heterogeneous distribution of the 24 melanoma, based on the expression levels of HLA-ABC and β2m (median density of molecules per cell: 21232, with a range of 784-49871). In detail, we identified 9/24 defective melanoma, characterized by a particular low expression rate of both HLA-ABC and β2m (less than 15000 molecules), compared to the other analyzed cell lines. We confirmed M017 as HLA-negative melanoma since no molecules were detected for both HLA-ABC and β2m. The remaining melanoma (14/24) showed an expression level of HLA-ABC and β2m molecules equal to or higher than 15.000 molecules. (**Figure 1**)

Furthermore, in the same 24 melanoma, we assessed by flow cytometry the expression profile of some key APM molecules that are crucial for the correct processing and presentation of the tumor antigens. We focused our analysis on the subunits of the immunoproteasome (LMP2, LMP7, LMP10) and on the components of the Protein Loading Complex (TAP1, TAP2, Tapasin, Erp57). The rate of cells expressing these proteins appeared highly heterogeneous in the analyzed

melanoma, and it did not appear to be correlated with the previously measured level of HLA-ABC and β2m molecules. As shown in **Figure 2** a particularly low expression level was detected for Tapasin.

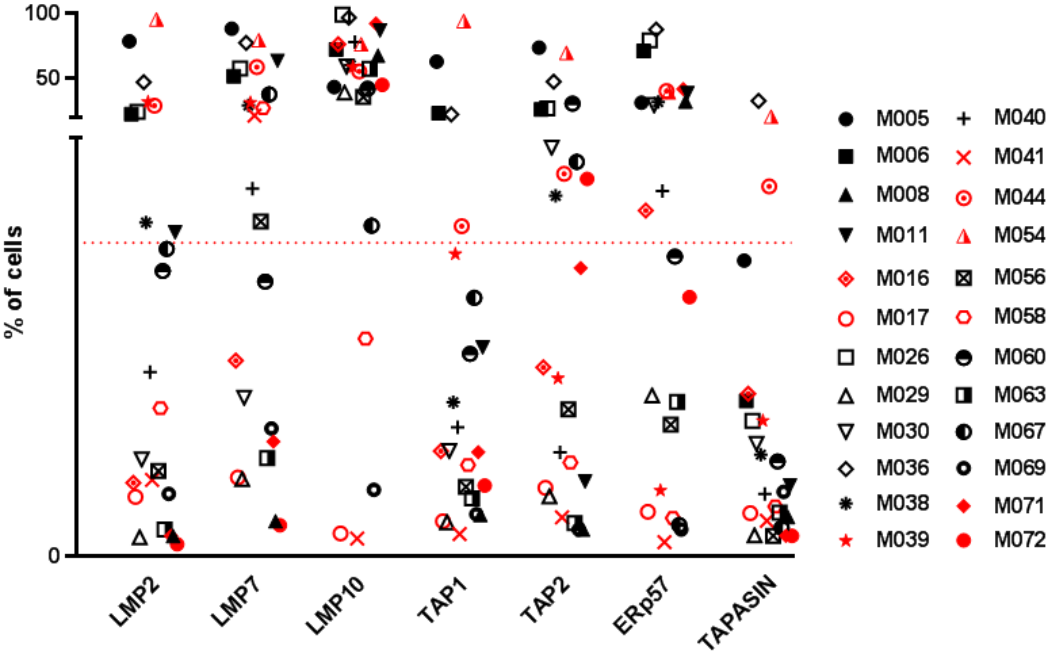

Figure 2. Expression rate of APM components on patient-derived melanoma cell lines. We assessed by flow cytometry the expression of some key molecules of the Antigen Processing Machinery (APM), crucial for the antigen processing and presentation on 24 melanoma cell lines. The distribution of the expression levels of the Immunoproteasome (composed by LMP2, LMP7, LMP10) and of the Protein Loading complex (composed by TAP1, TAP2, Erp57, Tapasin) appeared heterogeneous in the 23 analyzed melanoma cell lines. Results are shown as percentage (%) of tumor cells expressing the molecule of interest.

Our results confirmed that defects in the expression of HLA molecules can be observed on melanoma cells at different levels. In some cases, such alterations are more evident and occur on the surface of tumor cells that show a low expression or even complete loss of HLA class I molecules.

On the other hand, some defects can be more insidious and are characterized by an absent or suboptimal expression of intracellular APM components. This latter scenario appears to be more heterogeneous, and it is not clear if and how it may have an impact on the correct processing and presentation of tumor antigens. An interesting information could derive from the study of how these alterations can be correlated with the clinical outcome of the patient; such data could help in the identification of melanoma patients who would benefit more from a treatment with a Checkpoint Inhibitors based therapy. Further studies are needed; however, these findings are potentially clinically relevant, and they may play a role in better understanding the mechanisms underlying resistance to immune checkpoint inhibitors-based therapy.

The potential significance of our findings prompted us to focus on an HLA-independent therapeutic approach that could be successful in the treatment of a challenging target, such as melanoma, a tumor in which, as we showed, alterations in HLA expression can occur at multiple levels.

In particular, our strategy is based on the use of Cytokine Induced Killer (CIK) cells genetically engineered to express a Chimeric Antigen Receptor (CAR). This cell therapy is HLA-independent both for the nature of the selected immune effectors and for the CAR-mediated mechanism of recognition and elimination of tumor cells.

2. ***Interferons (IFNs) upregulate the expression of HLA class I molecules and APM components on melanoma cell lines***

Interferons (IFNs) are cytokines involved in the regulation of the expression of HLA class I molecules. (142,143) To investigate the potential role of IFNs in the regulation of HLA class I molecules and APM components in melanoma, we performed *in vitro* experiments to investigate whether IFNs, in particular IFNα and IFNγ, could upregulate the expression level of HLA-ABC, β2m, LMP2/7/10, TAP1/2, Tapasin and Erp57 molecules on the previously described patient-derived melanoma cell lines. Melanoma cells were cultured with medium in which we added either IFNα (16 ng/mL) or IFNγ (29 ng/mL). Melanoma cells cultured with normal medium were kept

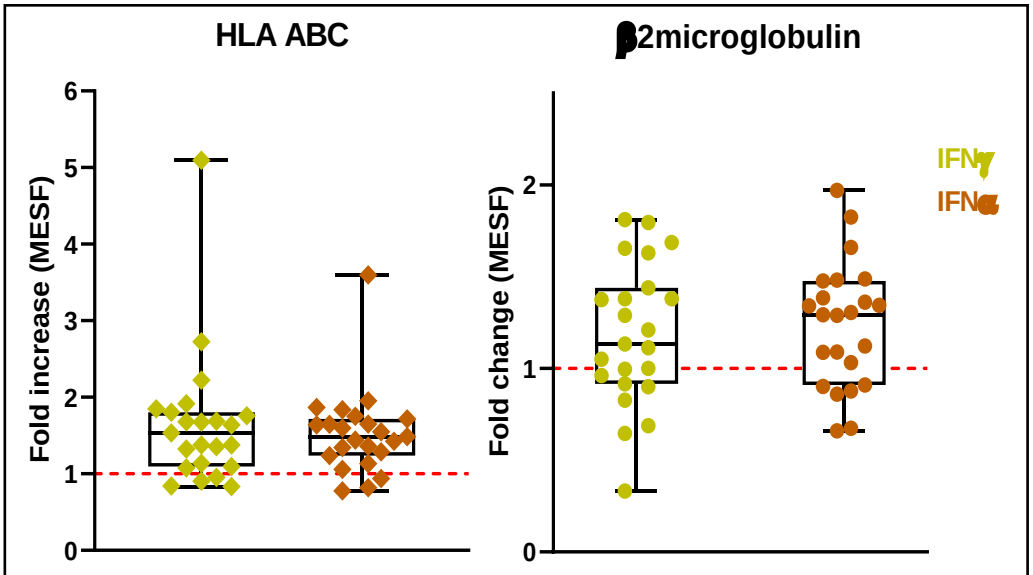

Figure 3.a Modulation of the expression level of HLA class I molecules on melanoma cells after treatment with Interferons (IFNs). A total of 23 melanoma cell lines were treated with IFNα (16 ng/mL) or IFNγ (29 ng/mL) and kept in culture for 48 hours. The expression of HLA-ABC and β2m was then analyzed by flow cytometry. A trend of upregulation in the expression of HLA-ABC and β2m molecules was observed on the majority of the analyzed melanoma cell lines after the treatment with IFNs. Results are shown as median fold change of the Mean Fluorescence Intensity, quantified utilizing Molecules of Equivalent Soluble Fluorochrome (MESF); any fold change > 1 is considered as an increase of the expression of the molecule of interest.

in culture and used as control of the basal expression of the analyzed molecules. After an incubation period of 48 hours, we assessed the expression level of HLA-ABC and β2m molecules on melanoma cells by flow cytometry, utilizing fluorochrome-labeled beads (Quantum MESF beads). We compared the expression level of HLA-ABC and β2m molecules on tumor cells cultured in the presence of either IFNα or IFNγ, with their respective basal expression on untreated cells. We observed that the addition of IFNα to the cell culture medium induced an upregulation of HLA-ABC expression in 19/23 melanoma (median fold change: 1,48) and of β2m expression in 17/23 melanoma cell lines (median fold change: 1.29).

The addition of IFNγ to the cell culture medium induced an upregulation of HLA-ABC expression in 19/23 melanoma (median fold change: 1.53) and of β2m expression in 14/23 melanoma cell lines (median fold change: 1.13). (**Figure 3.a**) In detail, in 5 of the 10 melanoma characterized by a low expression (less than 15000 molecules) of HLA-ABC and β2m, both IFNα and IFNγ (4/5: M016, M041, M044, M067) or only IFNα (1/5: M072) raised the expression level above the level of 15000 molecules, the arbitrary cut off line previously mentioned.

Following the same experimental designed, we measured by flow cytometry the expression level of the APM components on 19 melanoma cell lines treated with IFNα or IFNγ. We observed a trend of upregulation induced by both IFNs on melanoma cells, in the expression of APM components, that we grouped in Immunoproteasome (LMP2, LMP7, LMP10) and Protein Loading Complex (TAP1, TAP2, Erp57, Tapasin). IFNα upregulated the expression of the IP (Immunoproteasome) components in 13/19 melanoma (median fold increase: 1.21) and of the PLC (Protein Loading Complex) molecules in 12/19 melanoma (median fold increase: 1.4). The median folds increase induced by IFNγ were 2.32, observed in 14/19 melanoma, and 1.96 in 15/19 melanoma, for the IP and PLC components respectively. (**Figure 3.b**)

These results show that IFNs (IFNα and IFNγ) can upregulate the expression level of HLA-ABC, β2m molecules and of the APM components on melanoma cells.

This effect is especially relevant in our experimental context, since i) we showed that alterations in the expression of HLA molecules are frequent in melanoma and could contribute to induce resistance to Checkpoint Inhibitors and ii) in our previous studies we reported that CIK lymphocytes release high levels of IFNγ and therefore they can potentially increase the expression level of HLA molecules on melanoma cells. In particular, we showed that CAR-engineered CIK lymphocytes produced higher levels of IFNγ compared to non-transduced CIK used as control.

(105) For this reason, we hypothesized that CSPG4 CAR CIK lymphocytes have the potential to induce an upregulation of HLA class I molecules on tumor cells.

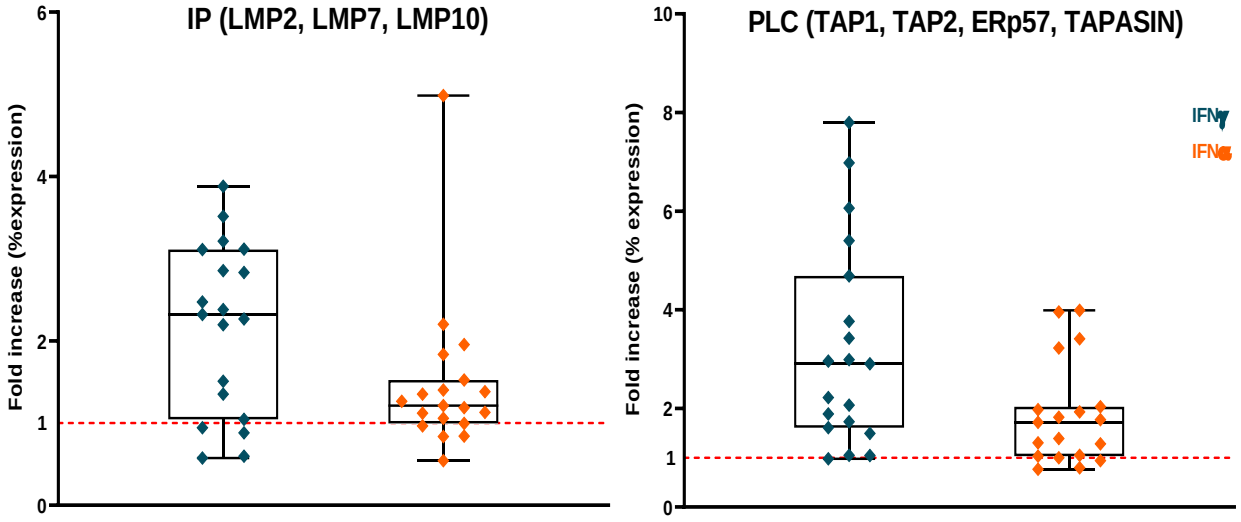

Figure 3. Modulation of the expression level of APM components on melanoma cells after treatment with Interferons (IFNs). A total of 19 melanoma cell lines were treated with IFNα (16 ng/mL) or IFNγ (29 ng/mL) and kept in culture for 48 hours. A trend of upregulation in the expression of APM components after the treatment with IFNs was observed on the analyzed melanoma cell lines. Fold increase of the Immunoproteasome's subunits is shown in the left panel; the upregulation of the Protein Loading Complex' components is shown in the right panel. Results are shown as median fold change of the percentage of positive cells; any fold change > 1 is considered as an increase of the expression of the molecule of interest.

3. *CSPG4 CAR CIK lymphocytes as an HLA-unrestricted therapeutic approach for the treatment of melanoma*

3.a Generation and characterization of CSPG4 CAR CIK lymphocytes

Cytokine Induced Killer (CIK) cells were generated from Peripheral Blood Mononuclear Cells (PBMCs) obtained from 4 melanoma patients and successfully expanded *ex vivo*. The standard expansion protocol includes the addition of IFN-γ on day 1, anti-CD3 antibody and Interleukin-2 (IL-2) on day 2 and the replacement of fresh medium supplemented with IL-2 for 3 to 4 weeks. On day 4 we transduced PBMCs with the retroviral vector CSPG4-470, encoding for a CSPG4-specific CAR. The CAR construct includes an extracellular domain composed by a scFv derived from the CSPG4-specific mAb 763.74 and a transmembrane domain linked to an intracellular region that contains the costimulatory domain 4-1BB and the CD3ζ signaling domain. (**Figure 5.a**) Three weeks after the transduction, we checked by flow cytometry the CSPG4 CAR expression on the transduced CIK lymphocytes to determine the transduction efficiency. In parallel, we transduced part of the PBMCs with a retroviral vector encoding for a CD19-specific

CAR. CD19 CAR CIK lymphocytes were used as control of the CAR specificity, since CD19 is expressed only on B cell malignancies but not on melanoma cells. (144) The mean transduction efficiency of CSPG4 CAR and CD19 CAR CIK lymphocytes was 48% ± 8.6 and 26% ± 4.8 respectively (mean ± SD) (**Figure 5.b**)

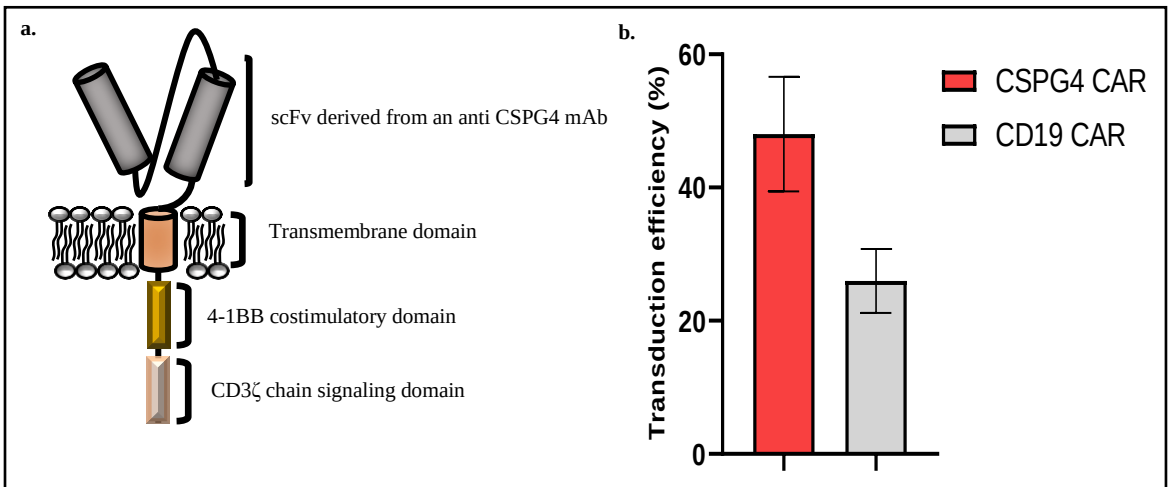

Figure 5. Engineering of CIK lymphocytes with a retroviral vector encoding for a CSPG4-specific CAR. PBMCs derived from *n=4* melanoma patients were transduced with a retroviral vector encoding for a Chimeric Antigen Receptor (CAR) that specifically recognizes CSPG4. **a.** Schematic representation of the CAR construct, composed by an extracellular domain responsible of the specific binding to CSPG4, a transmembrane domain and an intracellular region which includes the co-stimulatory domain 4-1BB and the CD3ζ chain signaling domain. **b.** The transduction efficiency of CSPG4 CIK and CD19 CIK lymphocytes was 48% ± 8.6 and 26% ± 4.8 respectively. CD19-specific CAR was used as control. Results as shown as mean percentage ± SD.

We further characterized the immunophenotype of the generated genetically modified immune effectors by flow cytometry. Mature CSPG4 CAR CIK lymphocytes were positive for the CD3 molecule, with the majority of them showing a cytotoxic T lymphocytes phenotype by co-expressing the CD8 molecule (CD3+ CD8+ 71% ± 13.5, mean ± SD). A relevant subset of the analyzed population was positive for both CD3 and CD56 molecules (CD3+ CD56+ 22.5% ± 13.1, mean ± SD), confirming the heterogeneous nature of CIK lymphocytes. We also observed a high membrane expression of the NKG2D receptor (68% ±32.3, mean ± SD), which is the main responsible for the intrinsic cytotoxic potential of CIK lymphocytes, as we previously mentioned. In addition, we found that CXCR3 is highly expressed on CSPG4 CAR CIK lymphocytes (71.7% ± 20.5, mean ± SD). This data may be relevant since CXCR3 is an inflammatory chemokine receptor known to be expressed on activated CD8 T lymphocytes and involved in T cell migration to inflammation sites. (**Figure 6.a**)

In general, the effector memory (EM) subset was the most represented (CD45RA+ CD45RO+ CD62L-, 47.8% ± 16.7, mean ± SD), followed by naive (N) (CD45RA+ CD45RO- CD62L+,

27.6% ± 12.4, mean ± SD) and central memory (CM) phenotype (CD45RA- CD45RO+ CD62L+, 33.4% ± 22.7, mean ± SD). (**Figure 6.b**)

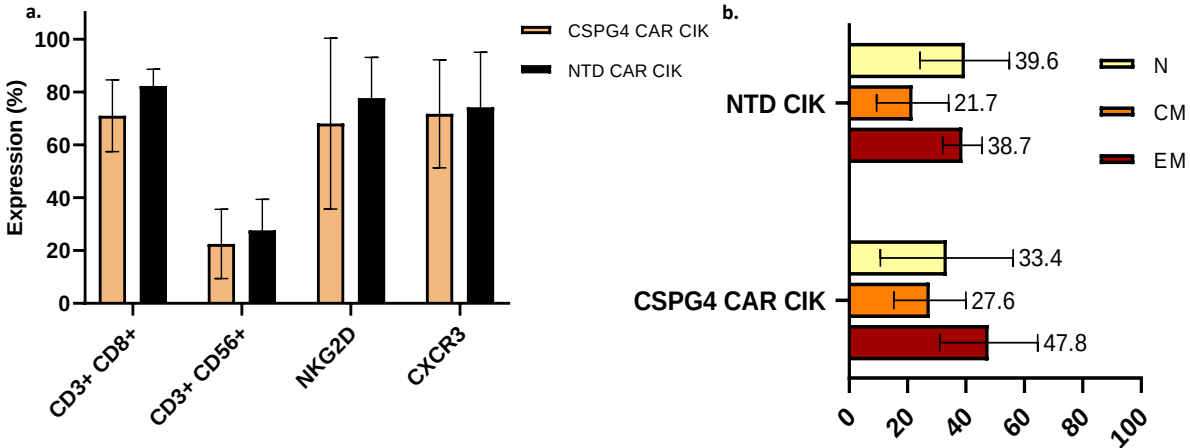

Figure 6. Characterization of CSPG4 CAR CIK lymphocytes. After their generation, CSPG4 CAR CIK lymphocytes were characterized by flow cytometry for the expression of the markers typical of CIK lymphocytes. Non-transduced (NTD) CIK were used as paired control. **a.** The majority of CSPG4 CAR CIK lymphocytes was CD3+ CD8+ (71% ± 13.5), with a subset expressing CD56 molecule (22.5% ± 13); the NKG2D receptor and the chemokine receptor CXCR3 were expressed at high levels (68% ± 32.3 and 71.7% ± 20 respectively). **b.** The most represented subpopulation in CSPG4 CAR CIK lymphocytes was the effector memory (EM) lymphocyte subtype, followed by naïve (N) and central memory (CM). Results were obtained from *n=4* expansions/experiments and shown as mean percentage ± SD.

Based on the obtained results we can confirm that the phenotype of mature CSPG4 CAR CIK lymphocytes was comparable to that of non-transduced (NTD) CIK lymphocytes, used as paired control. Since any statistically significant difference was observed, we can state that the transduction process and the CAR expression did not alter the phenotype or the differentiation status of our immune effectors. These results, together with the relatively high rate of the obtained transduction efficiency, confirm that CIK lymphocytes represent an attractive experimental platform for the clinical translation of a CAR-based adoptive immunotherapy for the treatment of metastatic melanoma.

3.b Melanoma as a suitable target for CSPG4 CAR CIK lymphocytes

To confirm that melanoma is a suitable target for a therapy based on the use of CSPG4 CAR CIK lymphocytes, we characterized our patient-derived melanoma cell lines for the expression of CSPG4, the tumor antigen specifically targeted by the CAR construct, and for the presence of MIC A/B, ULBP2,5,6, as the main ligands recognized by the NKG2D receptor.

We selected the Chondroitin Sulfate Proteoglycan 4 (CSPG4) as target of our engineered immune effectors since it is known to be highly expressed on melanoma and it has a restricted distribution on normal tissues. To support the rationale of a CSPG4 CAR-based strategy, we assessed by flow cytometry the expression of the CSPG4 on 10 patient-derived melanoma cell lines (M005, M017,

M026, M030, M038, M060, M067, M069, M071, M072). We confirmed that all the analyzed cell lines showed a high expression of the target antigen (78±5%). (**Figure 7**)

Besides the CAR-mediated killing mechanism, CIK lymphocytes are endowed with an intrinsic HLA-independent cytotoxic activity, mainly mediated by the NKG2D receptor. Therefore, we assessed by flow cytometry the presence of the main ligands recognized by this receptor on the previously listed melanoma (MIC A/B, ULBP2,5,6). All the analyzed cell lines expressed at least one of the NKG2D ligands, with a higher rate of cells expressing ULBP2,5,6 compared to MIC A/B (ULBP2-5-6: 56.6% ± 18.17, MIC A/B: 11.6 ± 16.1, mean ± SD). (**Figure 8**)

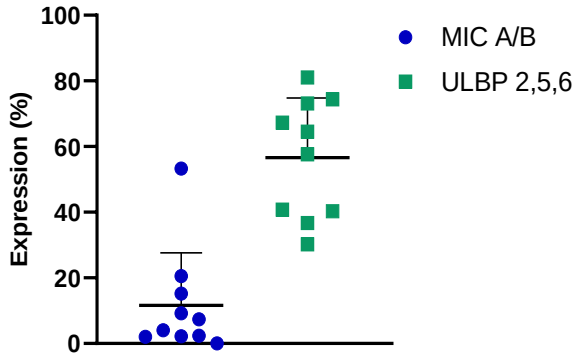

Figure 8. Expression rate of NKG2D ligands on patient-derived melanoma cell lines. The expression level of the main ligands of NKG2C (MIC A/B and ULPB 2,5,6) was assessed by flow cytometry on *n=10* primary melanoma cell lines. The graph shows that the rate of cells expressing these molecules was heterogeneous, but all the analyzed cell lines expressed at least one of the two ligands. Results are shown as mean ± SEM.

With these results we confirmed that melanoma is a good target for an ACT approach based on the use of CAR CIK lymphocytes specifically redirected against CSPG4, because i) CSPG4 is a tumor antigen highly and frequently expressed on melanoma, ii) the presence of NKG2D ligands on melanoma cells allows CIK lymphocytes to exert their intrinsic cytotoxic activity along with the CAR-mediated killing mechanism. An additional feature that makes CSPG4 a promising antigen to target in the setting of melanoma is that its expression is independent from the level of HLA molecules. In fact, its presence was detectable in all the analyzed cell lines, including M017, the HLA-defective melanoma.

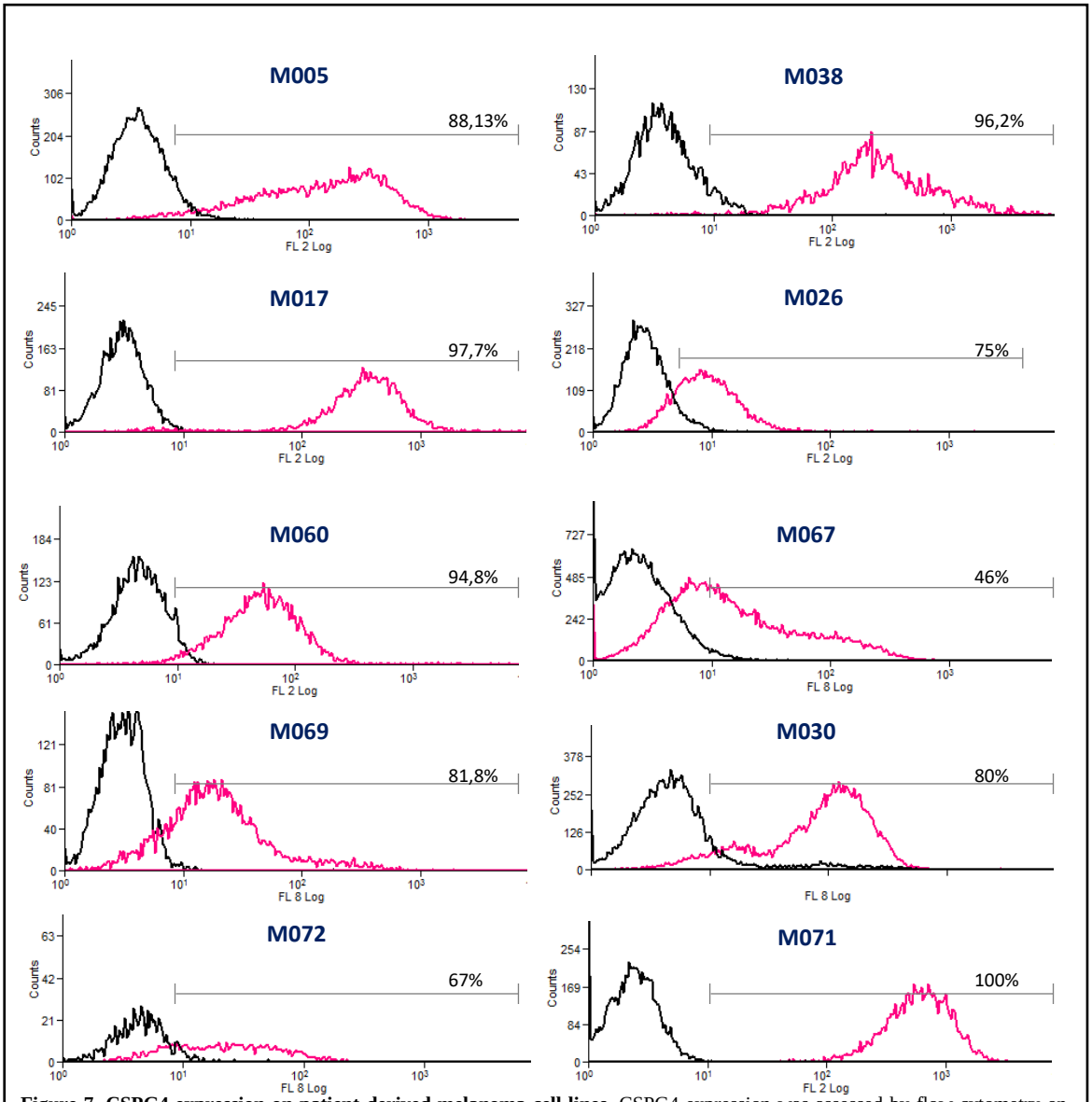

Figure 7. CSPG4 expression on patient-derived melanoma cell lines. CSPG4 expression was assessed by flow cytometry on *n=10* primary melanoma cell lines. All the analyzed cell lines expressed the antigen recognized by our CAR construct, confirming that melanoma is a good target for a CSPG4 CAR CIK- based therapy. Representative histograms of melanoma cells expressing CSPG4 (pink line) are shown for each cell line; unstained cells (black line) were used as negative control.

3.c CSPG4 CAR CIK lymphocytes efficiently kill melanoma cells in vitro and indirectly mediate an upregulation of HLA-ABC and β2microglobulin molecules on melanoma cells

To assess the antitumor activity of CSPG4 CAR CIK *in vitro* we tested their efficiency in eliminating melanoma cells, by setting up co-culture experiments. We selected 10 of the patient-derived melanoma cell lines generated in our laboratory, in order to have an experimental platform including melanoma with a high (M005, M026, M038, M030, M067, M069) and low (M017,

M071, M072) expression of HLA class I molecules. The concept of 'high' and 'low' expression was defined based on the observed number of HLA-ABC and β2m molecules on tumor cells (higher or lower than the cut-off line set at 15000).

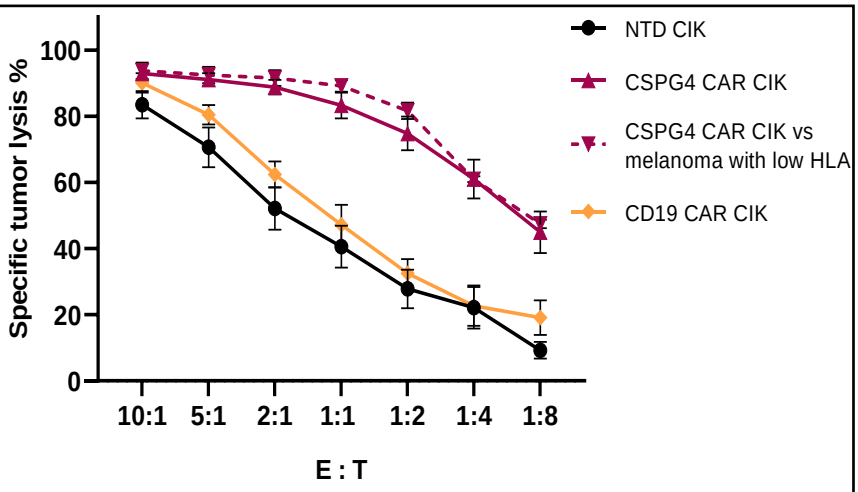

Figure 9. Antimelanoma activity of CSPG4 CAR CIK lymphocytes *in vitro*. A total of *n=10* melanoma cell lines were cultured in the presence CSPG4 CAR CIK or NTD CIK lymphocytes for 48 hours at different E:T ratios. The graph shows that CSPG4 CAR CIK lymphocytes efficiently killed *n=7* melanoma cell lines (purple line) and eliminated in a similar extent *n=3* melanoma cell lines characterized by a low expression of HLA molecules (discontinuous purple line), as previously defined (Figure 1). In fact, no significant difference was observed between the two curves. The cytotoxic activity of NTD CIK lymphocytes (black line) was significantly lower compared to CSPG4 CAR CIK lymphocytes (p=0.0098). CD19 CAR CIK lymphocytes (orange line) were used as control. Results are shown as percentage of specific lysis of tumor cells, assessed by flow cytometry (mean ± SEM).

CSPG4 CAR CIK lymphocytes generated from *n=4* melanoma patients were co-cultured with target cells at different effector to target (E:T) ratios (10:1, 5:1, 2:1, 1:1, 1:2, 1:4, 1:8). After an incubation of 48 hours, we determined by flow cytometry the percentage of residual melanoma cells and calculate the specific tumor lysis. In the case of melanoma with a high HLA expression, the percentage of CSPG4 CAR CIK mediated lysis at different E:T ratios (respectively from 10:1 to 1:8) was 93.1 ± 1.9, 91.3 ± 2.2, 88.7 ± 2.6, 82.8 ± 4.6, 73.7 ± 5.9, 59.8 ± 6.7, 44.9 ± 6.3 (mean ± SEM); the percentage of CSPG4 CAR CIK mediated lysis against melanoma with a low HLA expression at different E:T ratios (respectively from 10:1 to 1:8), was 93.2 ± 1.8, 91.7 ± 1.9, 91 ± 1.8, 88.4 ± 1.4, 81.5 ± 1.7, 62.8 ± 2, 47.4 ± 1.3 (mean ± SEM). (**Figure 9**) Non transduced (NTD) CIK lymphocytes were used as control to discriminate the tumor lysis induced by the intrinsic cytotoxic activity of CIK lymphocytes, from the CAR-mediated killing. In selected experiments (n melanoma=5), we included CD19 CAR CIK lymphocytes as control of the CAR specificity, since CD19 is a molecule expressed on B lymphocytes but absent on melanoma cells.

Our results demonstrated that CSPG4 CAR CIK efficiently eliminated melanoma cells *in vitro* and their cytotoxic activity was significantly higher compared to NTD CIK lymphocytes (p = 0.0098). Furthermore, the antitumor effect of CD19 CAR CIK was similar to NTD CIK lymphocytes, showing that the elimination of melanoma cells was due to the specific recognition of CSPG4 and not to a non-specific activity of the CAR. Importantly, CSPG4 CAR CIK cytotoxic activity

remained high at low E:T ratios; this aspect is particularly relevant for the translation in the clinical setting, where the number of immune effectors is always lower compared to the number of tumor cells. Finally, all the tested melanoma cell lines were efficiently eliminated by CSPG4 CAR CIK lymphocytes, independently from their HLA expression profile (no statistically significant difference was observed between the two groups characterized by high and low HLA expression levels).

These data confirm that our strategy could help overcome the issue resistance to Checkpoint Inhibitors due to alterations or loss of HLA class I molecules expression on tumor cells.

In parallel with their cytotoxic activity, we hypothesized that CSPG4 CAR CIK lymphocytes could modulate the expression of HLA class I molecules on tumor cells, through their release of IFNγ. To confirm this hypothesis, we cultured two melanoma cell lines (the patient derived M005 and the commercial A375 melanoma cell lines) in the presence of conditioned medium derived from cultured CSPG4 CAR CIK lymphocytes. After a 48 hours incubation period, we assessed by flow cytometry the expression level of HLA-ABC and β2m molecules on tumor cells. We observed an increase in the MFI of both molecules compared to the respective control groups of melanoma cells cultured with non-conditioned medium (HLA-ABC median fold: 1.48, β2m median fold: 2.24). (**Figure 10**)

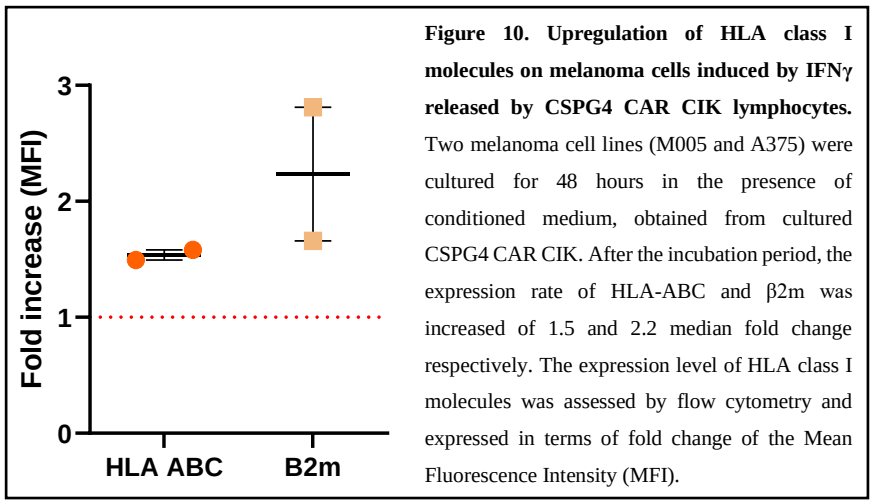

Figure 10. Upregulation of HLA class I molecules on melanoma cells induced by IFNγ released by CSPG4 CAR CIK lymphocytes. Two melanoma cell lines (M005 and A375) were cultured for 48 hours in the presence of conditioned medium, obtained from cultured CSPG4 CAR CIK. After the incubation period, the expression rate of HLA-ABC and β2m was increased of 1.5 and 2.2 median fold change respectively. The expression level of HLA class I molecules was assessed by flow cytometry and expressed in terms of fold change of the Mean Fluorescence Intensity (MFI).

We observed that the factors present in the culture medium of CSPG4 CAR CIK lymphocytes mediates a modulation of HLA class I molecules expression in a similar way to the treatment with exogenous IFNs (**Figure 3.a**); this effect is likely to be due to the IFNγ, which is produced in high amount by the engineered immune effectors. This evidence implies that CSPG4 CAR CIK have the potential to indirectly stimulate the innate immune response by improving the antigen presentation process in the setting of HLA-defective tumors, including melanoma. This is one of the features that, beside their potent antitumor activity, makes CSPG4 CAR CIK cell-based immunotherapy an attractive strategy for the treatment of melanoma patients.

3.d CSPG4 CAR CIK lymphocytes are effective against melanoma tumors engrafted in immunodeficient mice

To confirm the results obtained with our *in vitro* experiment, we planned to test the antitumor activity of CSPG4 CAR CIK lymphocytes in a xenograft xenograft murine model of melanoma. For this purpose, we selected the patient-derived melanoma cell line M017 for two main reasons: first, it is an HLA defective tumor, since it is negative for the expression of HLA-ABC and β2m, and therefore it is representative of the challenging clinical setting and second, we observed that the *in vitro* cytotoxic activity of CIK lymphocytes against M017 was lower compared to the other tested melanoma cell lines. (**Figure 11**)

This evidence underlines that some tumors are more challenging than others to treat and it strengthens the rationale for the engineering of CIK lymphocytes with a CAR construct to increase their efficiency.

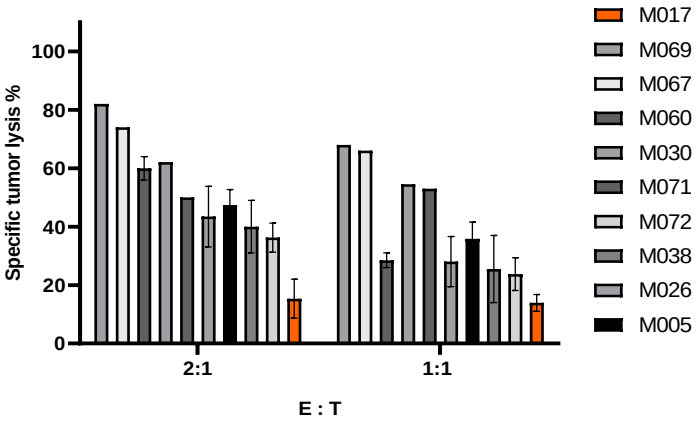

Figure 11. Antitumor activity of CIK lymphocytes against patient-derived melanoma cell lines. The graph summarizes the percentage of specific tumor lysis induced by CIK lymphocytes *in vitro* against *n=10* different melanoma cell lines, at two representative E:T ratios (2:1 and 1:1). The killing efficiency appeared to be heterogeneous and the HLA-defective melanoma M017 (orange bars) is eliminated in a lower extent compared to the other targets. Results are showed as mean percentage of tumor specific lysis from independent experiments, assessed by flow cytometry (mean ± SEM).

We generated the xenograft murine model by subcutaneously injecting M017 melanoma cells n=23 NSG female mice (850.000 cells/mouse). After two days mice were randomized in 3 groups and the same day of the randomization, **Group 1** (n=8) was treated with CSPG4 CAR CIK lymphocytes, **Group 2** (n=8) was treated with NTD CIK lymphocytes, **Group 3** (n=8) was left untreated as control of the spontaneous course of the tumor. All the mice developed a tumor, confirming an engrafment rate of 100% and therefore the reliability of our model. CSPG4 CAR and NTD CIK lymphocytes were administered by tail vein injection every three days for a total of five times ($3x10^6$ cells/mouse each infusion). Tumor growth was monitored twice a week by caliper measurement. Five weeks after the injection of M017 cells, when the untreated tumors reached the maximum consented size of 1.5cm in diameter, mice were euthanized, and tumors harvested. As shown in **Figure 12.a**, tumor growth was efficiently controlled by CSPG4 CAR CIK lymphocytes compared to NTD CIK

control (p value < 0.0001). These data was confirmed by the measurement of tumor weight, which was significantly lower in mice treated with CSPG4 CAR compared to NTD CIK lymphocytes (p value < 0.0001). (**Figure 12.b**)

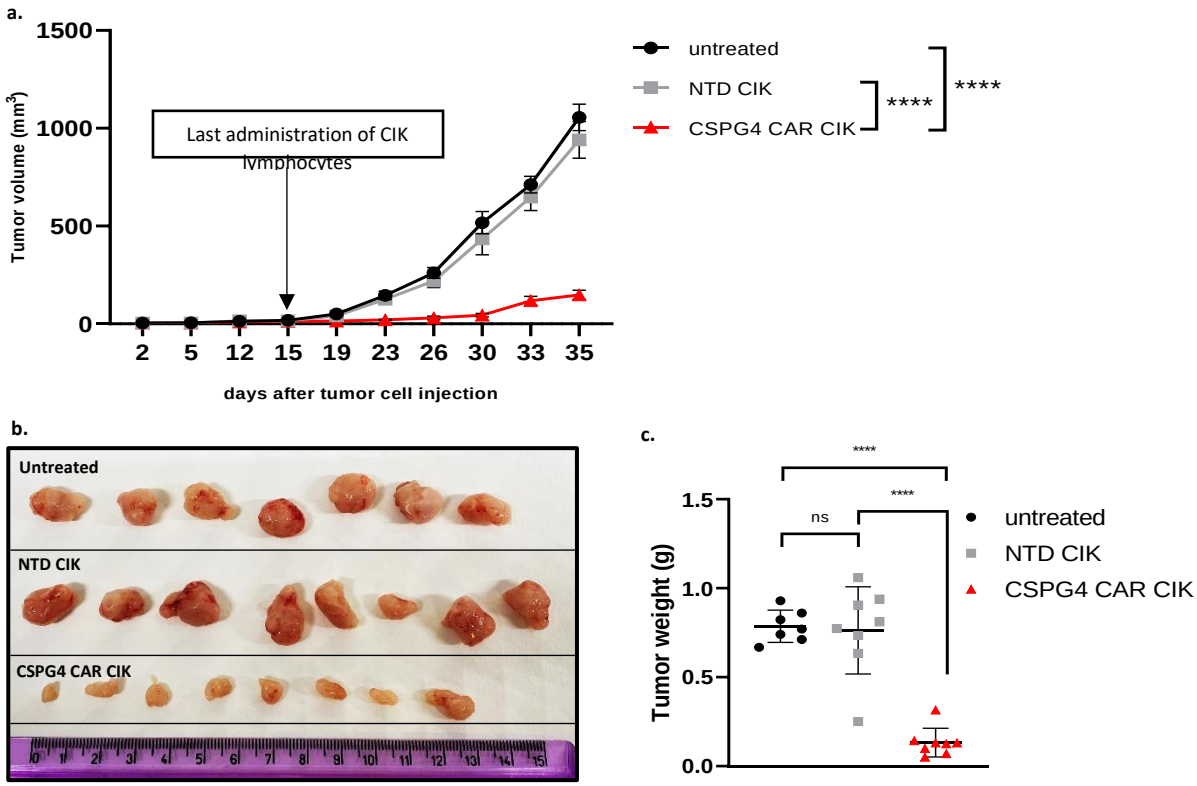

Figure 12. Antimelanoma activity of CSPG4 CAR CIK lymphocytes *in vivo*. Melanoma cells derived from the M017 cell line were engrafted in *n=24* NSG mice (850.000 cells/mouse). Group 1 (red line) was treated with CSPG4 CAR CIK lymphocytes ($3x10^6$ cells/mouse for 5 times), Group 2 (grey line) was treated with NTD CIK lymphocytes ($3x10^6$ cells/mouse for 5 times), and Group 3 (black line) was left untreated. a. Tumor growth was monitored biweekly by caliper measurement. CSPG4 CAR CIK lymphocytes efficiently controlled tumor growth up to 2 weeks after the last injection compared to NTD CIK lymphocytes *(p*<0.0001*)*. b. Visual comparison between the tumors harvested from the different groups. c. Tumor weight measured on the tumors harvested from the mice of the three different groups at the endpoint of the experiment. Data are shown as mean ± SEM of the measurements of the mice included in the three groups (8 mice each group, except the control group that included 7 mice, since one died of natural causes).

Furthermore, we quantified *ex vivo* the metabolic activity of the collected tumors by measuring the emission signal of the fluorescent glucose probe administered to the mice by tail vein injection 24 hours before the end of the experiment. This fluorescent imaging technique, based on the use of a fluorescent-labeled deoxyglucose analog, allowed us to monitor and quantify the glucose uptake of tumor cells; the acquired signal is proportional to the metabolic activity of the tumor.

We observed that melanoma treated with CSPG4 CAR T cells had a significantly lower metabolic rate compared to tumors treated with NTD CIK lymphocytes ($5.5x10^8 \pm 4.4x10^8$ and $1.1x10^9 \pm 3.5x10^8$ photons/sec, respectively, mean ± SD, p value=0.028). (**Figure 13**)

Our results proved that CSPG4 CAR CIK lymphocytes successfully control the growth of melanoma *in vivo*.

Nevertheless, this appears evident two weeks after the last administration of CSPG4 CAR CIK lymphocytes and it may be due to their limited half-life and persistence *in vivo*. Therefore, we hypothesize that CSPG4 CAR CIK lymphocytes are able to keep under control the tumor growth if continuously administered. These results i) show that our therapeutic approach has the potential to control the tumor growth in patients with melanoma, and ii) provide relevant information for the optimization of the administration protocol in a clinical setting.

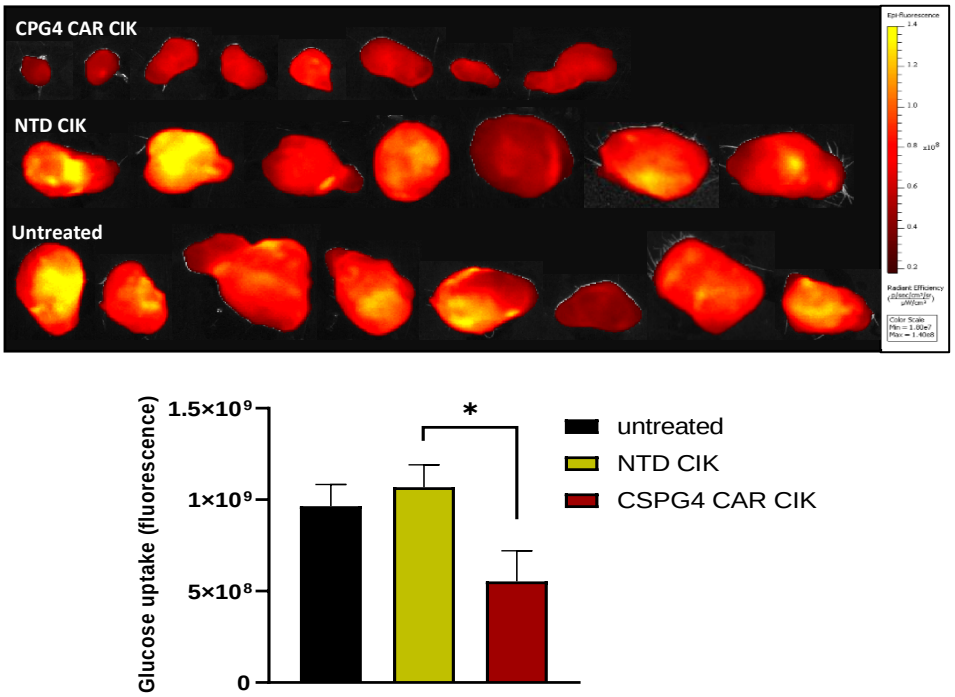

Figure 13. Reduced metabolic activity of melanoma treated with CSPG4 CIK lymphocytes. The uptake of fluorescent glucose, which is an indicator of the metabolic activity of the tumor, was measured in the tumors harvested at the endpoint of the experiment. The images of the harvested tumors after the injection of the fluorescent glucose are shown on the upper part of the panel; the quantification of the measured fluorescent signal is showed in the graph. The glucose uptake of the tumors treated with CSPG4 CAR CIK lymphocytes was significantly lower compared to the ones treated with NTD CIK lymphocytes or the untreated control *(p*=0.0284*)*.

4. ***Increased susceptibility of irradiated tumor cells to the killing activity of CSPG4 CAR T lymphocytes***

A crucial issue related to the use of CAR-based adoptive immunotherapy is that, whereas this strategy appears to be effective for the treatment of hematological malignancies, the complete eradication of solid tumors remains challenging. Furthermore, in previous studies, we observed some discrepancies between the results obtained *in vitro*, where CAR T cells efficiently eliminate tumor cells, and the results obtained *in vivo*, where tumors grafted in mice were not completely eradicated by the engineered immune effectors. In the last part of our study, we focused our interest on a parallel project that aims to overcome this obstacle.

Therefore, we explored the possibility to improve the efficacy of CAR-engineered immune effectors by exploring a combinatorial approach based on the treatment of tumor cells with a non-lethal dose of radiation prior the administration of the CAR-based therapy.

To assess the efficacy of this strategy, we tested T lymphocytes as immune effectors and engineered them with the same CSPG4-specific CAR that we utilized to generate the previously described CSPG4 CAR CIK lymphocytes.

T cells were isolated from PBMCs obtained from healthy donors, expanded *in vitro* with the addition of IL-15 (5ng-ml) and IL-7 (10ng/ml) and transduced with the retroviral vector CSPG4-470. The percentage of T cells expressing the CAR was assessed by flow cytometry two weeks after the transduction. We confirmed a successful transduction efficiency, which was around 45.5%.

As target we utilized the M14 melanoma cell line, that we had previously transduced with a retroviral vector encoding for the Green Fluorescent Protein (GFP). Tumor cells were treated with different doses of radiation (6Gy and 12Gy), administered in three fractions of 2 and 4Gy respectively, for three consecutive days. Forty-eight hours after the last dose of radiation, tumor cells were incubated with CSPG4 CAR T lymphocytes at different E:T ratios (1:5, 1:10) and kept in culture for five days. Non-irradiated tumor cells were cultured in parallel and used as control.

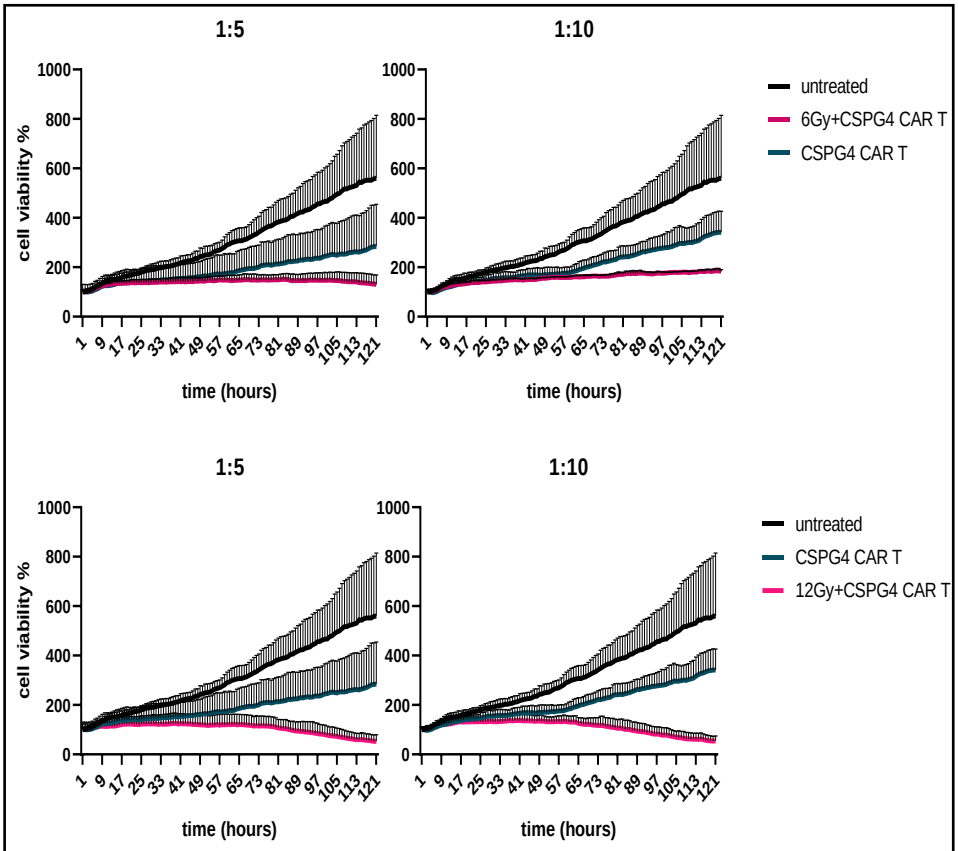

Figure 14. Increased susceptibility of irradiated melanoma cells to CSPG4 CAR T lymphocytes. The melanoma cell line M14 was irradiated with a total dose of 6 or 12Gy, administered in fractions of 2 or 4Gy respectively, for three consecutive days. Forty-eight hours following the radiation CSPG4 CAR T cells were added to the culture at 1:5 and 1:10 E:T ratios. Cells were then incubated for 5 days in an Incucyte Zoom live cell analysis system. Total green fluorescence object area which directly correlates to the number of viable tumor cells was monitored and expressed as percentage of cell viability in the graphs. Irradiated melanoma cells resulted more sensitive to the CAR T-mediated lysis *($p<0.0001$)* compared to the untreated control.

The rate of viable tumor cells was monitored using the Incucyte Live-cell Analysis System; the total green fluorescence object area, which correlates with the quantity of viable M14.GFP tumor cells, was recorded every hour and utilized as output to determine the tumor cell viability. The obtained results showed that tumor cells pre-treated with radiation were significantly more

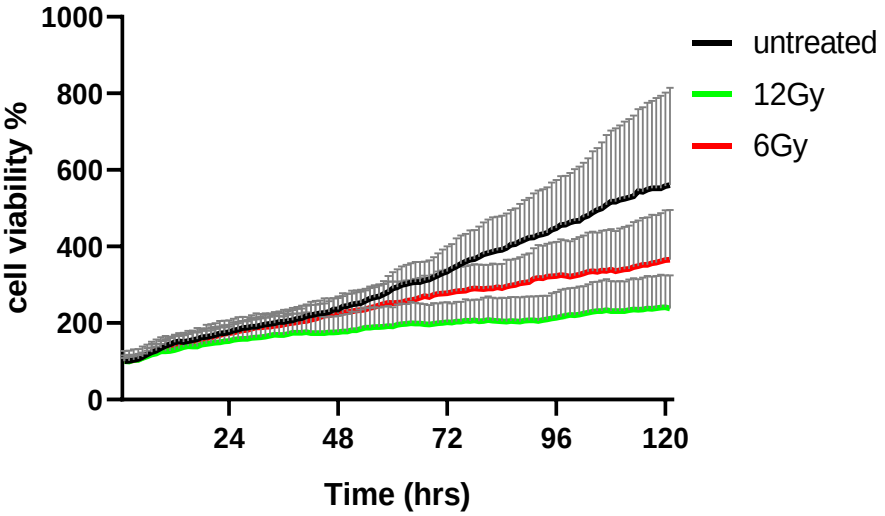

Figure 15. Radiation inhibits melanoma cell proliferation in a dose dependent way. The melanoma cell line M14 was irradiated with a total dose of 6 or 12Gy, administered in fractions of 2 or 4Gy respectively, for three consecutive days. Cells were then incubated for 5 days in an Incucyte Zoom live cell analysis system. Total green fluorescence object area which directly correlates to the number of viable tumor cells was monitored and expressed as percentage of cell viability in the graphs. Irradiated melanoma cells showed a reduced proliferation ability *($p<0.0001$)* compared to the untreated control.

sensitive to the antitumor activity of CSPG4 CAR T lymphocytes compared to non-irradiated control (6Gy: 1:5 $p<0.0001$, 1:10 $p<0.0001$; 12Gy: 1:5 $p<0.0001$, 1:10 $p<0.0001$). (**Figure 14**) Multiple mechanisms are potentially responsible for this increased susceptibility to the CAR T cell mediated lysis. We hypothesized that one of those is the reduced proliferation ability of irradiated tumor cells. To confirm this hypothesis, M14.GFP cells were treated with different doses of radiation (6Gy and 12Gy), administered in three fractions of 2 and 4Gy respectively, for three consecutive days and kept in culture for five days. The cell viability was monitored using the Incucyte Live-cell Analysis System, as previously described. **Figure 15** shows that melanoma cells treated with radiation have a significantly lower proliferation rate compared to the non-irradiated control and this effect is dose-dependent (6Gy: $p<0.0001$, 12Gy: $p<0.0001$).

The obtained results show that radiation has the potential to make tumor cells more susceptible to the antitumor activity of CAR-engineered immune effectors. These are preliminary data that could provide interesting information for further study this combinatorial approach.

DISCUSSION

In this study we explored the therapeutic potential of an HLA-independent adoptive cell therapy based on the use of lymphocytes redirected with a Chimeric Antigen Receptor (CAR) that specifically recognizes the Chondroitin Sulfate Proteoglycan 4 (CSPG4), in a preclinical setting of metastatic melanoma.

The rationale underlying our study is that there are no effective therapies for patients with metastatic melanoma, who are often resistant to the currently available treatments or relapse after an initial response. This is the case of Immune Checkpoint Inhibitors (ICI) and Target therapies, which, despite the improvement in PFS and OS, are still not completely effective. Therefore, there is an urgent need of new therapeutic options to improve the clinical outcome of these patients. In this scenario, a CAR-based adoptive cell therapy could represent a promising alternative or integrative approach to the current available treatments. Currently two CD19 CAR products have been approved for the treatment of hematological malignancies and, despite solid tumors appear more challenging to treat with a CAR-based approach, successful and promising preclinical results have been achieved also in this field. In fact, several CAR constructs targeting a broad panel of tumor antigens have been developed and tested against different types of solid malignancies in a preclinical setting *in vitro* and in murine models and clinical studies have been designed for the treatment of patients with solid tumors. (132)

One of the reasons that cause resistance to ICI is the presence of defects in the expression of HLA class I molecules or the Antigen Processing Machinery (APM) components in tumor cells; such deficiencies compromise the correct presentation of tumor antigens and allow the tumor to escape from the T cell-mediated immune response. In our study, using a representative sample of 23 melanoma, we found that the expression of HLA class I molecules or the Antigen Processing Machinery (APM) components is particularly heterogeneous, with some case of low expression or even total loss; this finding may be representative of what is the situation in patients and an explanation for the phenomenon of resistance to ICI. This evidence prompted us to develop and assess the efficacy of a therapy that does not rely on the tumor antigen processing and presentation. Therefore, as immune effectors we selected Cytokine Induced Killer cells (CIKs), *ex vivo* expanded lymphocytes that are endowed with an intrinsic HLA-independent cytotoxic activity, and transduced them with a CAR, which is also characterized by an HLA-independent mechanism of tumor cell recognition and elimination.

Our group has previously demonstrated the antitumor activity of CIK lymphocytes against different types of tumors, including melanoma. (94) We have also showed in a recently published study, that the killing potential of CIK lymphocytes can be increased by their engineering with a CAR specific for the tumor antigen CD44v6 in a preclinical model of sarcoma. (105) This genetic

manipulation provides CIK lymphocytes with a higher antitumor potential, especially in challenging contexts, such as low effector to target ratios, a condition representative of the real clinical scenario, and tumors that are more resistant and difficult to eliminate.

Beside the HLA-independent antitumor activity, CIK lymphocytes, and therefore CAR CIK lymphocytes, have other multiple attractive features for a feasible future clinical translation. The expansion protocol is relatively simple, short timed, cost-effective and leads to the generation of a large number of effectors, all essential requirements for a successful adoptive cell therapy. CAR CIK can be cryopreserved without losing their efficiency and this is particularly relevant since they have a limited *in vivo* persistence (approximately 10-20 days), and multiple infusions may be needed. This apparent limitation can actually be seen as an advantage from a safety perspective: the limited lifespan of CAR CIK lymphocytes after the infusion in patients and therefore their relatively rapid disappearing, could limit the potential side effects that may occur and address the concerns about the risk of insertional mutagenesis consequent to the engineering with a viral vector. We demonstrated that the genetic manipulation of CIK lymphocytes did not affect their phenotype, that was fully comparable with the non-transduced (NTD).

An additional attractive feature of out therapeutic approach is the choice/selection of CSPG4 as the tumor antigen recognized by our CAR construct. CSPG4 appears as an ideal target since i) it is broadly expressed on melanoma, meaning that all the patients can benefit from this therapy, ii) its expression is high on tumor cells but limited or absent on normal tissues, a requirement which is particularly important to limit the toxicity related to the "on target-off tumor" effect, iii) it is present on both melanoma differentiated cells and melanoma CSCs: this condition implies that CSPG4 CAR CIK lymphocytes are able to target and eliminate melanoma CSCs, a subpopulation of tumor cells which is considered responsible for tumor progression and recurrence. In this regard, we have previously demonstrated that CIK lymphocytes equally eliminate both differentiated cells and melanoma CSCs.

In our study we tested this CSPG4 CAR CIK cell-based therapy in a preclinical setting of melanoma characterized by an heterogenous expression of the HLA class I molecules and the Antigen Processing Machinery (APM) components, to further confirm the relevance of our strategy for the treatment of patients that may only partially benefit from an immunotherapy based on the use of ICI. First, we showed, in this experimental platform, that CSPG4 expression is not affected by the presence of HLA defects, since all the melanoma we included tested positive for the expression of the antigen, including M017, a melanoma characterized by a complete loss of HLA class I molecules expression.

Second, we demonstrated that our CSPG4 CAR CIK lymphocytes eliminate *in vitro* melanoma cells in a specific way and with a significantly higher extent compared to NTD CIK importantly our CAR-modified immune effectors are equally effective against the different melanoma we tested, independently from the expression of HLA class I molecules and the APM components. Beside their antitumor activity, we also showed that CSPG4 CAR CIK are able to induce an upregulation of HLA class I molecule on melanoma cells without the need of a direct contact with the target. This effect is likely to be mediated by IFNγ and, in part, IFNα, released by CAR CIK lymphocytes. This hypothesis is confirmed by the evidence that the *in vitro* treatment of melanoma with IFNγ or IFNα increased the expression level of HLA class I molecules on tumor cells and by our previous studies where we showed that CSPG4 CAR CIK lymphocytes produce large amounts of IFNγ. Furthermore, we found that the trend of upregulation following the treatment with IFNs was true also for the APM components. With this parallel indirect effect, CSPG4 CAR CIK lymphocytes have the potential to restore or at least contribute to the adaptive response mediated by T cells, that requires a functioning antigen processing and presentation machinery.

Finally, we generated a preclinical *in vivo* model, by grafting melanoma cells in NSG mice, to confirm our *in vitro* results. For this experiment we selected M017 as a target because it represents a challenging case of melanoma, since i) we identified it as an HLA-defective tumor, with no detectable expression of the HLA ABC and β2microglobulin on the surface of tumor cells and ii) we observed that its elimination *in vitro* by CIK lymphocytes was lower/reduced compared to other melanoma. The obtained results showed that, in a challenging context were the antitumor activity of CIK lymphocytes is not sufficient, the introduction of a CSPG4 CAR allows to efficiently control the growth of the engrafted tumors.

A parallel project was focused on the emerging issue of the challenging treatment of solid tumors with CAR-engineered immune effectors. Contrary to hematological malignancies, solid tumors are surrounded by a complex and immunosuppressive microenvironment that impairs the infiltration and the consequent antitumor potential of CAR-based therapies. Several studies aim to find strategies to combine with CAR-engineered immune effectors to improve their efficacy and counteract the immunosuppressive microenvironment. We selected radiation as approach to make tumor cells more sensitive and susceptible to the CAR-mediated lysis. It is known that treating tumor cells with radiation causes an unbalance between the pro- and anti-apoptotic molecules in favor of the pro-apoptotic ones (140), upregulates the expression level of tumor antigens (137), augments the immunogenicity of tumor cells (145), and increases the level of T cell infiltration within the tumor. (141) We investigated our hypothesis by treating a melanoma cell line with a non-lethal dose of radiation, with the purpose to limit the damage to normal tissues in a patient, in

combination with CSPG4 CAR T lymphocytes. Our results showed that irradiated melanoma cells are more susceptible to be eliminated by our immune effectors compared to the non-irradiated control. These preliminary data provide the basis for a future study and further explore the use of radiation as strategy to improve the efficacy of CAR-based immunotherapy. Further studies are needed to optimize the radiation dose and administration and to understand the molecular mechanisms underlying this increased sensitivity. However, our preliminary data collected on melanoma and other types of tumors, including anaplastic thyroid cancer and triple negative breast cancer, together with the evidence present in the literature, confirm the therapeutic potential of a strategy that combines CAR-engineered immune effectors with radiation.

Overall, our findings support the rationale to propose the clinical exploration of our HLA-independent adoptive cell therapy based on the use of CSPG4 CAR CIK lymphocytes for the treatment of patients with metastatic melanoma, relapsed or refractory to conventional treatments with ICI.

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