

Cytokine Induced Killer cells kill chemo-surviving melanoma cancer stem cells

Running title: CIK cells kill autologous chemo-surviving melanoma CSCs

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Statement of Translational Relevance

This work reports the effective antitumor activity of patient-derived cytokine-induced killer (CIK) cells against autologous chemo-resistant melanoma Cancer Stem Cells (CSCs).

CSCs are clinical relevant targets, associated with disease relapse. We demonstrate that chemotherapy kills indeed proliferating melanoma cells but spares tumorigenic CSCs, *in vitro* and *in vivo*. The MHC-independent immunotherapy with CIK cells was proved successful in this challenging framework.

Consistent findings were obtained in selected cases of BRAF mutated melanoma treated with small molecule BRAFi. Our data, generated within an autologous system, support the exploration of CIK cells in clinical trials. Cost effectiveness, safety profile and ability to overcome tumor MHC-downregulation are favorable issues to be considered in clinical perspective. CIK cells may be integrated at different levels in the composite therapeutic scenario of metastatic melanoma, offering an additional weapon to control tumor spread and promote its eradication.

ABSTRACT

Purpose

The MHC-unrestricted activity of cytokine-induced killer (CIK) cells against chemo-surviving melanoma cancer stem cells (mCSCs) was explored, as CSCs are considered responsible for chemo-resistance and relapses.

Experimental design

Putative mCSCs were visualized by engineering patient-derived melanoma cells (MCs) with a lentiviral-vector encoding eGFP under expression control by stemness gene promoter *oct4*. Their stemness potential was confirmed *in vivo* by limiting dilution assays.

We explored the sensitivity of eGFP⁺mCSCs to chemotherapy (CHT), BRAF inhibitor (BRAFi) or CIK cells, as single agents or in sequence, *in vitro*. First, we treated MCs *in vitro* with fotemustine or dabrafenib (BRAF mutated cases); then surviving MCs, enriched in mCSCs, were challenged with autologous CIK cells.

CIK cell activity against chemoresistant mCSCs was confirmed *vivo* in two distinct immunodeficient murine models.

Results

We visualized eGFP⁺mCSCs (14±2.1%) in 11 MCs. The tumorigenic precursor rate *in vivo* was higher within eGFP-positive MCs (1/42) compared with the eGFP-negative counterpart (1/4870).

In vitro mCSCs were relatively resistant to CHT and BRAFi, but killed by CIK cells (n=11, 8/11 autologous), with specific lysis ranging from 95% (E/T 40:1) to 20% (E/T 1:3). *In vivo* infusion of autologous CIK cells into mice bearing xenografts from 3 distinct melanoma demonstrated significant tumor responses involving CHT-spared eGFP⁺mCSCs (p=0.001). Sequential CHT-immunotherapy treatment retained antitumor activity (n=12, p=0.001) reducing mCSC rates (p=0.01).

Conclusions

These findings are the first demonstration that immunotherapy with CIK cells is active against autologous mCSCs surviving chemotherapy or BRAFi. An experimental platform for mCSC study and rationale for CIK cells in melanoma clinical study is provided.

INTRODUCTION

Malignant melanoma is the most aggressive form of skin cancer. While localized tumors are curable with surgery, treatment possibilities for metastatic melanoma have long been limited due to its minimal response to conventional anticancer treatments (1). Recently, molecular targeted therapy and immunotherapy have greatly advanced metastatic melanoma treatment by employing respectively small molecules inhibiting mutated forms of *b-raf* (2-4) and immune checkpoint inhibitors ipilimumab (5), nivolumab (6,7), and pembrolizumab (8) to achieve such remarkable clinical trial results that they are now first-line options in international treatment guidelines (9-11). Despite these treatments a consistent portion of patients relapse or does not achieve disease control. In addition to checkpoint inhibitors, adoptive immunotherapy also appears highly promising, and after decades of preclinical relegation, is starting to find its way into clinical applications (12,13). While the two approaches may be complementary, tumors containing relatively few immunogenic mutations, or those with a “non-inflamed” tumor microenvironment, continue to represent an important immunotherapy challenge. Specifically, in some non-responsive or relapsing patient subsets, or when attempting to hit tumor-sustaining targets like cancer stem cells (CSCs), adoptive infusion of *ex vivo* expanded antitumor immune effectors is worth consideration.

Crucial to new therapeutic strategy planning is CSC analysis and targeting because this cell subpopulation is a key factor in chemotherapeutic agent and radiation therapy resistances, contributes to post-treatment relapse, and is involved in tumor metastasis (14-21). The fact that conventional chemotherapies preferentially target actively-cycling cells, as opposed to CSCs, may implicate these observed treatment failures. Thus, exploration of novel therapeutic strategies to target CSCs with immunotherapy holds great potential (21-27).

Among the various adoptive immunotherapy approaches we focused on an MHC-independent strategy based on cytokine-induced killer (CIK) cells (28-32), which are *ex vivo* expanded T-NK lymphocytes with MHC-independent antitumor activity (33-38). The principal mechanism of tumor killing is recognition of stress-inducible tumor-restricted molecules (e.g., MIC A/B; ULBPs 1-5) by the NKG2D receptor (35,36,39). Preclinical studies of intense CIK cell activity against several tumors have been reported, as has recent evidence of their successful re-direction with chimeric-antigen receptors (CAR) (40-42). Moreover, initial clinical trials demonstrated their safety profile, supporting their potential against solid tumors (37,38,43,44). We previously reported preclinical CIK cell activity against autologous melanoma and initial *in vitro* data against putative mCSCs. (31). MHC-independent immunotherapeutic approaches may present advantages over antigen-specific adaptive immune responses against CSCs. Indeed, effectors like CIK cells, or even natural killer and $\gamma\delta$ T cells, are unaffected by tumor-defensive downregulation of HLA molecules, and their activating targets (e.g., MIC A/B; ULBPs) are associated and expressed in undifferentiated tumor cells (22,31,32) (45-47).

This investigation builds on our previous findings. It explores the preclinical activity of CIK cells against autologous melanoma, focusing on mCSCs that may survive conventional chemo or molecular targeted therapies. To visualize putative CSCs, we used a strategy previously validated in our lab that relies on a

lentiviral vector encoding the enhanced Green Fluorescent Protein (eGFP) under expression control of the human *oct4* promoter (LV.Oct4.eGFP). The underlying idea is to reveal CSCs exploiting their selective ability to activate a well-characterized stemness promoter (31,32,48,49). Our central hypothesis is that chemotherapy kills proliferating tumor cells, and thereby reduces the tumor burden, but spares CSCs that support disease relapse. We simulated this scenario in an autologous preclinical model, both *in vitro* and *in vivo*, and then assessed the efficacy of immunotherapy with CIK cells.

MATERIALS AND METHODS

Establishment of patient-derived melanoma cell cultures

Human melanoma tissues were obtained from 11 surgical specimens (lymphnodal or cutaneous metastasis); patients with advanced stage melanoma provided consent under institutional review board approved protocols. Human melanoma tissues were cut into 3-mm³ pieces and processed for cell isolation. Tumor tissue was processed and melanoma cells were cultured as previously described (31).

Characterization of patient-derived melanoma cell cultures

Cell aliquots from patient-derived melanoma cell cultures were stained with FITC, PE, PE–Cyanin 7 (PC7), or APC-conjugated mouse monoclonal antibodies (mAbs) against extracellular and intracytoplasmic human antigens [anti-CD271-PE, anti-Oct4-PE, anti-Sox-2-APC, and anti-Nanog-PerCP-Cy5.5 (Becton Dickinson BD Biosciences Italy, Pharmingen); anti-MCSP-APC (Miltenyi Biotec Srl, Calderara di Reno, BO, Italy), anti-VEGFR1-APC and anti-ABCG2-PE (R&D Systems, Space Import Export); anti-MITF (Abcam, Prodotti Gianni Srl, Milan, Italy)], anti-HLA-ABC-FITC and anti-PD-L1-PE (BD Biosciences Pharmingen); CIK-target antigens [anti-MIC A/B (BD Biosciences Pharmingen);, anti-ULBPs, anti-CD112, and anti-CD155 (R&D System, Space Import Export, Milan, Italy)]. Intracellular expression of OCT4 and MITF was detected after fixation/permeabilization by the Cyoperm/Cytofix kit per manufacturer's instructions (BD Biosciences Pharmingen). To detect Mitf, we used a secondary goat antimouse PE-labeled mAb (Abcam, Prodotti Gianni Srl, Milan, Italy). Labeled cells were read on FACS Cyan (Cyan ADP, Beckman Coulter s.r.l., Cassina De' Pecchi – Milan, Italy) and analyzed using Summit Software. Gate criteria were set to isotype controls.

CIK culture, expansion and characterization

Human peripheral blood samples were obtained from subjects with histologically-confirmed advanced stage melanoma at the Candiolo Cancer Institute, Fondazione del Piemonte per l'Oncologia (FPO) - IRCCS. All individuals provided their informed consent.

Cultures were started with Peripheral Blood Mononuclear Cells (PBMCs) collected from 8/11 mMel patients, surgically treated and performed as previously described (31,32). Briefly, PBMCs, isolated by density gradient (Lymphosep, Aurogene s.r.l., Roma, Italy) centrifugation were cultured at a cell density of $1.5 \times 10^6 \text{ ml}^{-1}$ in RPMI (Gibco BRL Life Technologies Italia Monza Italy), supplemented with 10% fetal bovine serum (FBS) (Sigma) and with timed additions of 1000 U ml⁻¹ IFN- γ at day 0 (Miltenyi Biotec Srl,

Calderara di Reno, BO, Italy), 50 ng ml⁻¹ anti-CD3 at day+1 antibody (Miltenyi Biotec Srl, Calderara di Reno, BO, Italy), and 300 U ml⁻¹ recombinant human interleukin IL-2 (from day +1, refreshed every 3-4 days until the end of the expansion) (Miltenyi Biotec Srl, Calderara di Reno, BO, Italy).

Phenotypic analysis of CIK cells was performed weekly, using the following fluorescein isothiocyanate (FITC), phycoerythrin (PE), or allophycocyanin (APC)-conjugated mouse monoclonal antibodies (mAbs): CD3–FITC, CD8–PE, CD56–APC and CD314–APC (aka anti-NKG2D) (mAbs) (all from Miltenyi Biotec Srl, Calderara di Reno, BO, Italy) and anti-DNAM-1 (Becton Dickinson BD Biosciences Italy, *Pharmingen*).

Labeled cells were read on FACS Cyan (Cyan ADP, Beckman Coulter s.r.l., Cassina De' Pecchi – Milan, Italy) and analyzed using Summit Software. Gate criteria were set to isotype controls.

hOct4.eGFP Lentiviral Vector generation

VSV-G pseudotyped third-generation LVs were produced by transient four-plasmid co-transfection into 293T cells (50,51). Transfer vector pRRL.sin.PPT.hPGK.EGFP.Wpre (LV.PGK.EGFP), kindly provided by Dr. Elisa Vigna (Gene Transfer and Therapy, IRCCS Candiolo, Turin, Italy), has been described elsewhere.

The pHCT4.EGFP1 vector (49) was kindly provided by Dr. Wei Cui (IRDB, Imperial College, London). The pRRL.sin.PPT.hOct4.eGFP.Wpre (LV-Oct4.eGFP) was obtained by replacing expression cassette hPGK.eGFP in LV-PGK.eGFP with hOct4-eGFP1 cleaved from the pHOct4-eGFP1 vector through insertion into the Sall and XhoI restriction enzyme sites. Physical titers for lentiviral vector stocks were determined based on p24 antigen content (HIV-1 p24 ELISA kit; PerkinElmer, Milan, Italy).

Patient-derived Melanoma Cell Transduction

For each LV transduction, patient-derived melanoma cells were cultured in fresh KODMEM-F12 with 10% FBS. Virus-conditioned medium was added at a dose of 400ng P24/100,000 cells. After 16h, cells were washed twice and grown for a minimum of 10 days to reach steady state eGFP expression and to rule out pseudo-transduction prior to flow-cytometry analysis (31). As a transduction efficiency control, the same melanoma primary cells were transduced with LV.PGK.eGFP.

In vitro assessment of CSC sensitivity to fotemustine or dabrafenib

LV.Oct4.eGFP transduced melanoma cells were seeded into 6-well plates (120,000-180,000 cells/well). After overnight incubation, cells were treated with the IC50 dose of fotemustine (FM, Muphoran® ItalFarmaco, Milan, Italy) or dabrafenib (BRAFi, Sequoia Research Products, Pangbourne, UK) for 72 hours. LV.Oct4.eGFP transduced melanoma cells treated with an equal volume of drug diluent were utilized as the control. At the end of treatment, cells were harvested and counted. The cell viability was determined with Trypan Blue 0.1% exclusion dye and an automated cell counter Countess (Invitrogen) according to the manufacturer's instructions. The percentage of eGFP⁺ cells was determined by flow cytometry (Cyan ADP, Beckman Coulter s.r.l., Cassina De' Pecchi – Milan, Italy). The eGFP positivity was calculated on viable cell fraction, detected by DAPI permeability exclusion assay. Treatment effects were measured by conducting

four to six independent experiments, each of which included six replicates. The eGFP increment, expressed as fold increase, was separately calculated for each experiment to compare FM- or BRAFi-treated samples with their internal untreated control.

In vitro cytotoxicity assay with CIK cells against melanoma

CIK tumor-killing ability was assessed against 11 LV.Oct4.eGFP transduced patient-derived melanoma cells. Effector cells were assayed against autologous tumor targets when possible (8/11). In the absence of autologous PBMCs (3/11), CIK cells were generated from other melanoma patients and employed as allogeneic effectors. All melanoma cell cultures were assayed with allogeneic CIK cells as controls. Their immune-mediated killing was analyzed assessing target cell viability by flow cytometry (Cyan ADP Beckman Coulter s.r.l., Cassina De' Pecchi – Milan, Italy) by DAPI permeability. CIK cells were co-cultured with targets (either autologous or allogeneic LV.Oct4.eGFP transduced melanoma cells) previously treated for 72 hours with fotemustine or dabrafenib (IC₅₀ dose) or an equal diluent volume as a control. Essays were conducted at progressively decreasing effector/target (E/T) ratios, 40:1, 20:1, 10:1, 5:1, 3:1, 1:1, 2:1, and 1:3 for 72 hours in 200 µL of medium with IL2 at a concentration of 300 U/mL at 37°C 5% CO₂. A confirmatory method was tested in parallel to determine the number of viable, metabolically-active, target cells in culture, based on the quantitation of ATP present (CellTiter-Glo® Luminescent Cell Viability Assay, Promega Italia s.r.l, Milan, Italy).

Tumor cells, in the absence of CIK cells, were used as a control to assess spontaneous mortality. The percentage of tumor-specific lysis for each effector/target ratio was calculated as (Experimental-spontaneous Mortality/100-Spontaneous Mortality) x 100. The curve also allowed us to calculate the half-maximal inhibitory concentration (IC₅₀) value for each melanoma culture.

In vivo activity assay against mCSCs

Six-week-old Non-Obese Diabetic/LtSz-scid/scid (NOD/SCID) (Charles River, Calco, Italy) female mice were subcutaneously injected with 5x10⁵ LV.Oct4.eGFP transduced patient-derived melanoma cells (mMel7 n=34, mMel11 n=40), cultured in equal volumes of sterile PBS1X and BD Matrigel™ Basement Membrane Matrix (Becton Dickinson BD Biosciences Italy). Treatments started when tumors became palpable. Mice from CIK-immunotherapy group (mMel7 n=10; mMel11 n=12) received 4 intravenous infusions (1x10⁷ mouse every 3-4 days) of mature CIK cells (re-suspended in 200 ul of 1X PBS) without systemic administration of IL2. Mice from chemotherapy-group (n=34) received 2 intraperitoneal injections of fotemustine (600 ug/mouse days 1;15), while mice injected with PBS alone (n=18) represented untreated controls.

An early part of the experiment (group A: CIK-immunotherapy n=10, chemotherapy n=10, PBS n=8) was terminated and analyzed after 2 weeks (day+15) to assess the antitumor activity (Ki67 proliferative index) and residual rate of eGFP⁺mCSCs. A second branch (group B) of the experiment proceeded beyond day+15 to explore the effect of chemo-immunotherapy combination. Mice (n=12) from the initial chemotherapy cohort (treated with Fotemustine on days 1;15) started intravenous infusions with CIK cells

(1×10^7 /mouse every 4-5 days from a minimum of 2 weeks to a maximum of 10 weeks); remaining mice from all the initial cohorts (CIK-immunotherapy n=12, Chemotherapy n=12, PBS n=10) worked as control and continued to be infused with PBS alone up to the end of the experiment. In all cases the experiment was terminated and animals euthanized when tumor size reached 2 cm in its main diameter, unacceptable toxicity occurred or CIK infusions ended, whichever occurred first.

Tumor growth was monitored weekly with calipers and volume calculated according to the formula: $V = 4/3 \times \pi \times (l/2)^2 \times (L/2)$, where L is the length and l the width diameter of the tumor. The recovered tumors were aliquoted. A first aliquot was fixed overnight in 4% paraformaldehyde, then dehydrated, paraffin-embedded, and sectioned (5 μ m) and finally stained with Hematoxylin and Eosin (H&E) (Bio.Optica). To assess the antitumor activity, tumor sections were stained for Immunohistochemistry assay with Ki67 antibody (Dako-Agilent Technologies Italia S.p.A, Milan, Italy) (32). To assess CIK cell infiltrate, Immunohistochemistry assay was performed with human anti-CD3 antibodies (Novocastra, Leica Biosystem) and assessed by a pathologist. A second aliquot was processed by mechanical and enzymatic dissociation using the Tumor Dissociation kit, human and the gentleMACS. Dissociator, according to the manufacturer's instructions. Monocellular suspensions obtained after dissociation were filtered using 70 μ m CellStrainer and the percentage of eGFP⁺ cells was determined by flow cytometry (Cyan ADP, Beckman Coulter s.r.l., Cassina De' Pecchi – Milan, Italy) and analyzed using Summit Software.

Patient-Derived Xenografts (PDXs)

Six-week-old Non-Obese Diabetic/LtSz-scid/scid (NOD/SCID) (Charles River, Italy) female mice were subcutaneously injected with an 8 mm³-tumor fragment from patient-derived melanoma biopsies (mMel2 and mMel3). Starting one week after tumor implantation, mice (n=14) received 8 weekly intravenous infusions of 1×10^7 mature autologous CIK cells in 1X PBS (200 μ l total volume injected) without systemic administration of IL2. Mice (n=13) injected with PBS alone were used as the untreated control. Tumor growth was monitored weekly as described above. Animals were euthanized at experiment end or when tumor size reached 2 cm in its main diameter. The recovered tumors were fixed overnight in 4% paraformaldehyde, dehydrated, paraffin-embedded, sectioned (5 μ m), and finally stained for Immunohistochemistry assay with Ki67 antibody (Dako Italia Spa). In a selected experiment, the recovered tumors were mechanically and subsequently enzymatically dissociated (Collagenase Type I, Invitrogen) for 3 hours. Monocellular suspensions obtained after dissociation were filtered using 70 μ m CellStrainer (Becton Dickinson BD Biosciences Italy) and LV.Oct4.eGFP transduced as previously described. The percentage of eGFP⁺ cells was determined by flow cytometry (Cyan ADP Beckman Coulter s.r.l., Cassina De' Pecchi – Milan, Italy) 3 days after transduction and analyzed using Summit Software.

Statistical analysis

Statistical analysis was performed using software GraphPad Prism 6. A descriptive statistical analysis of CIK and melanoma cell median or mean values was used as appropriate. The relative increase of eGFP⁺mCSC in melanoma samples, treated either *in vitro* or *in vivo* with FM, BRAFi or CIK cells, were

compared with controls by unpaired t test. Comparison of Ki67 proliferative index between melanoma samples, treated *in vivo* with either FM or CIK cells, were compared with controls by unpaired t test. The mixed model analysis of variance (ANOVA) was employed to assess CIK cytotoxic activity curves *in vitro*. Statistical significance has been expressed as P value, and all values less than 0.05 were considered statistically significant. The CSC frequency was calculated with L-Calc T software program (Stem Cell Technologies Company, Voden Medical Instruments spa, Peschiera Borromeo, Milan, Italy), which uses Poisson statistics and the method of maximum likelihood.

RESULTS

Putative melanoma cancer stem cells (mCSCs) survive chemotherapy *in vitro*.

Melanoma cell cultures and visualization of putative mCSCs

We established 11 melanoma cell cultures from metastatic tissue biopsied from 11 patients with advanced stage melanoma (Supplementary Table S1). *Braf* mutational analysis revealed that 5/11 of the patient-derived cell cultures were *braf*-mutated (4 V600E: mMel3, mMel7, mMel11, mMel15, 1 V600K: mMel2).

Each culture was assessed for expression of the main melanoma surface antigens: Melanoma-associated Chondroitin Sulfate Proteoglycan (MCSP), Nervous Growth Factor Receptor (NGFR, aka CD271), and Vascular Endothelial Growth Factor Receptor 1 (VEGFR1) (Table 1). All tumors retained membrane expression of HLA class I molecules (>99% HLA-ABC⁺) (data not shown).

We visualized putative mCSCs by a gene transfer strategy (27,28) based on stable transduction of patient-derived melanoma cells with a lentiviral vector encoding eGFP under the control of the *oct4* gene promoter regulatory element (LV.Oct4.eGFP) (Supplementary Figure S1A-D, Supplementary Table S2). Using this approach, the average rate of eGFP⁺mCSC within the 11 cultures was 12 ± 2.1% (Table 1). As parallel control, we confirmed that melanoma cells could be transduced efficiently (>95%) when the strong ubiquitous promoter (Phospho Glycerate Kinase, PGK, regulatory element) was utilized in place of the Oct4 promoter to control eGFP expression (Supplementary Figure S1E, S1F, Supplementary Table S2). Furthermore, the integration of LV.Oct4.eGFP was confirmed by PCR in both eGFP⁺ and eGFP⁻ melanoma cell subsets (Supplementary Figure S1G).

Each melanoma culture was assessed for expression of the principal ligands recognized by CIK cell receptors NKG2D (MIC A/B, ULBP1, ULBP2-5-6, and ULBP3) and DNAM-1 (CD112 and CD155). As Table 1 indicates, MIC A/B and ULBP2-5-6 were expressed in all melanomas. Although sample values generally varied highly, MIC A/B and ULBP2-5-6 were comparable in eGFP⁺mCSC and eGFP⁻ melanoma cells (Figure 1A and 1B). The expression of ULBP1, ULBP 3, CD112, and CD155 was negligible (data not shown). Also the expression of Programmed death-ligand 1 (PD-L1) was negligible (Table 1).

Additional molecules reported in the literature as mCSC phenotype-associated were also evaluated. OCT4, NANOG, SOX2, ATP Binding Cassette G2 (ABCG2), and aldehyde dehydrogenase (ALDH) were each detected in all samples at expressions averaging 14 ± 1.3%, 12 ± 1.7%, 18 ± 2.1%, 6 ± 1.3%, and 10 ± 1.9%, respectively (Table 2). Melanoma cells negative for MITF expression averaged 16 ± 4.4% (Table 2).

Tumorigenicity of putative mCSCs

To assess the tumorigenic potential of putative eGFP⁺mCSCs, we subcutaneously transplanted NOD/SCID mice with scalar dilutions (from 10 to 1x10⁴) of eGFP⁺ and eGFP⁻ melanoma cells separated by Fluorescence-Activated Cell Sorting. Nine weeks after transplant, palpable tumors were evident in 4/6 mice injected with the highest dose of eGFP⁺ cells compared with 1/6 in mice transplanted with the corresponding eGFP⁻ cell dose. Limiting dilution analysis performed with L-Calc software indicated that the average frequency of tumorigenic melanoma cells, 12 weeks post-transplant, was 1/42 for eGFP⁺ melanoma cells and 1/4,870 for eGFP⁻ melanoma cells (Figure 1C).

Sensitivity of putative mCSCs to chemotherapy or molecular targeted therapy in vitro

We explored putative mCSC sensitivity to chemotherapy treatment. Each of the 11 melanoma cell cultures was treated *in vitro* for 72 hours with a culture-specific dose (IC50) of fotemustine (FM) to attain a 50% melanoma kill. FM sensitivity differed among the melanomas, such that IC50 ranged between 10 and 50 ug/ml (Figure 1D). Activity against mCSCs was calculated by the rate of eGFP positivity among viable cells at treatment end. The rate of viable eGFP⁺mCSCs significantly increased after chemotherapy (mean fold increase 1.61 ± 0.04) as compared to the untreated controls (n=62 p<0.0001), which confirmed their reduced sensitivity to conventional chemotherapy (Figure 1D). Comparable results were obtained treating braf-mutated melanomas (n=5) with BRAFi dabrafenib (culture-specific IC50 dose, ranging between 0.08 and 5 uM). Even in this case, the reduced sensitivity of mCSCs was assumed by their increased rate (1.5 ± 0.11 fold, n=20 p<0.0001) following treatment with BRAFi dabrafenib (Figure 1E).

Immunotherapy with CIK cells against mCSC that survived chemo- or targeted therapy *in vitro*.

Generation of CIK cells from patients

CIK cell activity against the mCSCs that survived FM or BRAFi was assessed *in vitro*. CIK cells were generated from 8 of our 11 patients (described above), as PBMCs were unavailable for three patients in the study cohort. Within three to four weeks, cells from fresh or cryopreserved PBMCs were successfully expanded *ex vivo*, per a standard protocol with timed additions of IFN-gamma, Ab-anti-CD3, and IL2 (29-32). The median expansion of CIK cells was 29-fold (range 16 to 125-fold).

The mature CIK cell subset co-expressing CD3 and CD56 molecules (CD3⁺CD56⁺) was detected at a rate of 39% (range: 25-58%), and 77% (69-89%) of CD3⁺ cells concurrently expressed CD8⁺ (Supplementary Figure S2A). Our results were comparable to previously published data (28, 29).

Pure Natural Killer (CD3⁻CD56⁺) cell presence was negligible (< 5%, data not shown). The median membrane expression of NKG2D and DNAM-1 receptors was 84% (range: 69-89%) and 97% (range: 89-100%), respectively (Supplementary Figure S2A).

In vitro killing of mCSCs surviving chemo- or targeted therapy by CIK cells

CIK cells efficiently killed melanoma cells that survived chemotherapy and were enriched in putative eGFP⁺mCSCs *in vitro*. Mean tumor-specific killing values were determined at decreasing effector/target (E/T) ratios. They resulted as 95 ± 2% (40/1), 85 ± 3% (20/1), 68 ± 4% (10/1), 54 ± 4% (5/1), 40 ± 4% (3:1), 29 ± 5% (1:1), 22 ± 4% (1/2), and 19 ± 3% (1/3), which agreed with results obtained against untreated controls (Figure 2A).

CIK cells were autologous-matched to melanoma targets in 8/11 cases; the three remaining melanomas were targeted with CIK cells from allogeneic patients only.

Comparable results were obtained when CIK cells were challenged against melanoma cells that survived BRAFi dabrafenib (Figure 2C).

Our findings confirmed that the killing activity of CIK cells involved eGFP⁺mCSCs. Immunotherapy killing by CIK cells resulted in no relative increase of eGFP⁺mCSCs in the viable cell population ($p=0.87$); instead, they were enriched after treatments with chemo or targeted therapy of the same melanoma ($p<0.0001$). The activity of CIK cells against eGFP⁺mCSCs is summarized in Figures 2B and 2D.

In selected experiments ($n=5$) we confirmed that the expression levels of NKG2D ligands (MIC A/B, ULBP2-5-6) and PDL-1 were comparable in eGFP⁺mCSCs before and after treatments (Supplementary Figure S3A-C).

We compared the killing ability of autologous CIK cells with that of allogeneic CIK cells against identical melanoma targets. The killing curves of autologous and allogeneic CIK cells trended similarly; the specific values for melanoma killing by autologous and allogeneic CIK cells are reported in Supplementary Figure S2B.

Immunotherapy activity of CIK cells against mCSCs in vivo

To verify that putative mCSCs can survive chemotherapy, yet retain sensitivity to immunotherapy with CIK cells, we replicated our *in vitro* findings *in vivo*. The experiments utilized NOD/SCID mice bearing tumors generated by subcutaneously-implanted LV.Oct4.eGFP-engineered melanoma cells from two different patients (mMel7 and mMel11). We explored the activity of chemotherapy and immunotherapy with CIK cells, alone and in sequence.

The experimental design is detailed in Figure 3A.

For group A, infusion of autologous CIK cells for two weeks ($n=6$; 1×10^7 every 4 days) and chemotherapy ($n=6$; 600 ug/mouse, days 1;15) yielded significant tumor (mMel11) response, indicated by significant decrease of Ki67 proliferation index ($p<0.0001$, Figure 3B).

Chemotherapy spared eGFP⁺mCSCs that were instead killed by immunotherapy. The rate of residual viable eGFP⁺mCSCs, compared to untreated controls ($n=6$) was in fact significantly increased by fotemustine ($p=0.0005$) but not by CIK cells ($p=0.3250$, Figures 3C).

Like results were obtained with intravenous infusion of allogeneic CIK cells for two weeks (1×10^7 every 5 days) (mMel7, Supplementary Figure S4A and B).

In addition, we assessed the effect of sequential treatment with chemotherapy and immunotherapy (group B, Figure 3A). In two separate experiments, CIK cells were infused intravenously (1×10^7 every 4 days) following initial treatment with FM (600 μ g, day 1;15). Our results indicated that CIK cells (n=12) not only retained significant antitumor activity ($p < 0.0001$), but that they also involved putative eGFP⁺mCSCs. The rate of viable eGFP⁺mCSCs assessed in residual tumors explanted at the end of the experiments was comparable to untreated controls (n=10); on the contrary, a significant increment was observed and also maintained over time in mice treated with chemotherapy alone (n=12, $p < 0.05$) (Figure 3D and E; Supplementary Figure S4C and D).

The ability of CIK cells to localize and infiltrate tumor sites was confirmed by immunohistochemistry detection of CD3⁺ cells (Supplementary Figure 5A-C).

Finally, CIK cell activity against putative mCSCs was confirmed in PDX models that were generated by direct subcutaneous implantation of fresh tumor samples from two patients in NOD/SCID mice (see Figure 4A and Materials and Methods). Autologous CIK cell infusion for eight weeks (1×10^7 every 7 days) exerted significant antitumor activity (n=14) as compared to untreated controls (n=13) (Figure 4B). The antitumor activity was shown to also involve putative mCSCs, as evidenced by no observed significant enrichment of viable eGFP⁺mCSCs in residual tumors ($p = 0.3$) (Figure 4C).

DISCUSSION

This work represents the first report of the immunotherapy activity with CIK cells against autologous chemo-surviving mCSCs *in vitro* and *in vivo*.

Previously, we provided proof of concept that CIK cells could kill autologous melanoma *in vitro*, including a subset of cells with stemness features (31,32). Here, we build on those findings and demonstrate that putative mCSCs are, indeed, relatively resistant to conventional CHT, yet sensitive to MHC-independent immune attack by autologous CIK cells. Furthermore, our observations *in vivo* confirmed the activity potential of CIK cells against mCSCs in a sequential treatment strategy. In selected cases of melanoma harboring BRAF mutations we confirmed a similar effect *in vitro* with BRAFi dabrafenib instead than chemotherapy. The ability to target mCSCs gives new rationale for considering CIK cells among adoptive immunotherapy approaches against melanoma. Currently, checkpoint inhibitors dominate center stage in melanoma immunotherapy. However, adoptive immunotherapy may also have an important role for prospective applications in dedicated settings, such as for the proportion of patients who fail to respond to upfront treatment with either anti-CTLA4 or anti-PD1 antibodies, or who experience relapses after initial response (52,53). Similar scenarios may include relapses following molecular targeted therapy in patients with BRAF-mutated melanoma.

Expansion and reinfusion of tumor-infiltrating lymphocytes (TIL) or genetically redirected T cells have already demonstrated great potential and provided proof of clinical activity (54). Their activity induces or forces an adaptive immune response targeting precise HLA-restricted tumor-associated antigens (TAA). CIK cells represent an alternate approach that exploits the killing mechanisms of the innate immune system (*e.g.*, natural killer cells or $\gamma\delta$ T lymphocytes). Such an approach may be advantageous against not

infrequent mechanisms of melanoma immune escape, such as abnormalities or downregulation of HLA molecules that impair strategies based on TAA-specific lymphocytes or the effector phase of checkpoint inhibitors. Furthermore, such tumor-defense mechanisms may be more pronounced in quiescent mCSCs that might not share or properly present TAAs (55). Treatment with CIK cells might be offered to virtually all patients, without restriction based on HLA-haplotype. Clinical application of adoptive immunotherapy, however, is highly limited by logistic issues regarding cell preparation and production costs balanced against safe manufacturing procedures (GMP). The intense *ex-vivo* expansibility of CIK cells and their cost-effectiveness can be shown to compare favorably with other approaches based on TAA-specific or genetically redirected lymphocytes. Based on our data, we tried to calculate the theoretical dose of CIK cells (per Kg) each patient would have received. If we assume 50 ml of peripheral blood (day 0) is collected initially, then the final average cumulative dose of mature CIK cells per patient would have been clinical relevant (2.3×10^8 CIK cells/kg, sem ± 0.53).

Support for the possibility of positive synergism between adoptive immunotherapy and checkpoint inhibitor antibodies in melanoma comes from preclinical evidence (56) that may also have a rationale with CIK cells. Patients would double-benefit from the MHC-unrestricted approach plus stimulation of antitumor adaptive immune response. CIK cells, as T-activated lymphocytes, do express PD-1 molecules but the functional role and potential benefit of modulation with checkpoint inhibitors remains undefined, despite its suggestion in selected settings (57).

CHT for the treatment of metastatic melanoma is less common due to the availability of more effective therapeutic approaches. Nevertheless, CHT may still be considered for patients relapsing after immunotherapy or molecular targeted treatments. In our model, the point of using FM was to functionally characterize and define the “clinical relevance” of putative mCSCs that might survive such treatment. Furthermore, we found consistent results even in the case of BRAF-mutated melanoma treated *in vitro* with BRAFi. Our findings support the concept that mCSCs may be, at least partially, resistant also to molecular targeted approaches. On these bases, there may be rationale to explore synergisms with immunotherapy strategies that demonstrated activity against mCSCs.

In recent years, various CSC markers have been reported in several cancer types; however, no conclusive consensus exists. Our strategy was designed specifically to visualize a subset of melanoma cells capable of activating *oct4*, the main stem-related gene endowed with peculiar intrinsic biologic features. CSCs can be defined by their relative dormancy or by their ability to form spheres or to initiate tumors *in vivo*. Our previous study showed that putative eGFP⁺mCSCs displayed a slow-growing phenotype compared to their eGFP⁻ counterparts, and we demonstrated that only putative eGFP⁺mCSCs were able to generate spheroids (31). However, for conclusive evidence, the reliability of any CSC marker, even OCT4, should be tested experimentally *via* the *in vivo* tumor-initiating assay that is currently considered the most reliable standard test for CSC analysis. In the present study we demonstrated that eGFP⁺mCSCs possess higher tumorigenic potential *in vivo* compared to their eGFP⁻ counterparts, resulting in potentially enriched cells endowed with stemness features.

We are aware that our system may have limits and cannot ensure that all mCSCs are detected. Nevertheless we aimed to demonstrate that the eGFP⁺mCSCs, even if not all of them are visualized, may survive conventional treatments but are killed by CIK cells.

Such a melanoma cell subset is less sensitive to conventional CHT compared to other melanoma cells, at least enough to be considered a relevant target from a clinical perspective.

We set up two distinct *in vivo* models, the first with melanoma cell cultures and the second with PDX. We observed consistent findings of chemo- and immune-sensitivity in mCSCs. When working with patient-derived samples, the possibility for experimental replicates is limited. Nevertheless, the results obtained in all our models were consistent with *in vitro* findings. We acknowledge that our experimental design and related endpoints were conceived to assess the effect of treatments on mCSCs. Even with these considerations, mice treated with sequential chemo-immunotherapy showed a trend of improved survival compared with controls (Supplementary Figure S6). Overall the therapeutic meaningfulness of our findings may be indirectly derived by the clinical relevance attributed to CSCs.

Our work demonstrates that immunotherapy with CIK cells is active against a “relevant” target like melanoma cells surviving chemo or molecular targeted therapy and enriched in mCSCs. Adoptive immunotherapy approaches with CIK cells could be developed and integrated with ongoing treatment strategies for selected subsets of melanoma patients.

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FIGURE LEGENDS

Figure 1. Putative mCSCs express ligands for CIK cells, are tumorigenic and relatively resistant to chemotherapy.

Putative mCSCs were visualized as eGFP⁺ following lentiviral transduction with the lentiviral CSC-detector (LV.Oct4.eGFP). (A-B) Mean (\pm sem) membrane expression of MIC A/B (n=17) and ULBP2-5-6 (n=16) were comparable between eGFP⁺mCSCs and eGFP⁻ melanoma cells before and after *in vitro* treatment with fotemustine (FM). MIC A/B expression in eGFP⁺mCSCs and eGFP⁻ cells before ($50.1 \pm 10.4\%$ and 51.4 ± 10.9 , respectively) and after FM treatment ($48.0 \pm 9.3\%$ and 45.8 ± 10.2 , respectively); (B) ULBP2-5-6 expression in eGFP⁺mCSCs and eGFP⁻ cells before ($82.6 \pm 5\%$ and 82.9 ± 4 , respectively) and after FM treatment ($85.5 \pm 3\%$ and 86 ± 3 , respectively). **(C) Tumorigenic cell frequency evaluation by LDA.** Summary of tumor volume (y-axis) in mice subcutaneously inoculated with decreasing doses of eGFP⁺ or eGFP⁻ melanoma cells (x-axis) in LDAs as described in Materials and Methods. Each symbol represents a mouse. Tumorigenic cell frequency in eGFP⁺ fraction was 1:42 (Lower Frequency: 1 in 103; Upper Frequency: 1 in 17; X² (Pearson): 2,226, p-value: 0,5269); tumorigenic cell frequency in eGFP⁻ fraction was 1:4,870 (Lower Frequency: 1 in 12.467; Upper Frequency: 1 in 1.902; X² (Pearson): 1,233, p-value: 0,7452).

Viable eGFP⁺mCSCs enrichment after CHT (D) or Targeted Therapy (E). LV.Oct4.eGFP transduced melanoma cells were treated with the IC₅₀ dose of FM or BRAFi for 72 h. The eGFP enrichment for each melanoma culture (n=11 e n=5), expressed as fold increase, was calculated for each experiment separately to compare FM or BRAFi treated samples with their internal untreated control. In all cases, viable eGFP⁺ cells resulted significantly enriched after CHT ($p \leq 0.0004$) and after BRAFi ($p \leq 0.0018$ except for mMel7, $p = 0.6781$).

Figure 2: Killing activity of chemo or targeted therapy surviving mCSCs by patient-derived CIK cells. (A and C) Patient-derived CIK cells efficiently killed *in vitro* melanoma targets that survived 72 h treatment with fotemustine (FM+CIK) (n=11) or with dabrafenib (BRAFi+CIK) (n=5); results were comparable to those observed in melanomas treated with CIK immunotherapy alone. Tumor killing was performed after co-culturing mature CIK cells with melanoma targets for 72 h to and evaluated by both flow cytometry assay and by CellTiter-Glo® Luminescent Cell Viability Assay. Mean values of tumor specific killing are reported at decreasing CIK:Melanoma ratios. **(B and D)** The activity against mCSCs was explored by tracking the rate of viable eGFP⁺mCSCs among surviving melanoma targets at the IC₅₀ (E/T between 10:1 and 2:1) point of the killing curve. eGFP⁺mCSCs were spared by chemotherapy (n=11) or targeted therapy (n=5), but efficiently killed by patient-derived CIK cells.

Figure 3. *In vivo* activity of CIK cells against autologous mCSCs.

(A) Experimental design: NOD/SCID mice (n=40) were subcutaneously inoculated with melanoma cells (from patient mMel11) engineered with the CSC-detector (LV.Oct4.eGFP). Mice with palpable tumors were divided into three treatment cohorts. CIK-immunotherapy cohort (n=12) received 4 intravenous infusions, each with 1×10^7 autologous mature CIK cells, chemotherapy cohort (n=18) was treated with 2 intraperitoneal injections (days 1;15) of FM (600 ug/mouse). Mice injected (n=10) with PBS alone were used as the untreated control. (B) An early part of the experiment (Group A) was terminated and tumors analyzed at day +15; both chemotherapy (n=6) and CIK cells (n=6) exerted significant antitumor activity, assessed by reduction of tumor Ki67 proliferative index compared to the controls (n=6) ($p < 0.0001$). (C) The rate of residual viable eGFP⁺mCSCs was significantly increased by chemotherapy ($p = 0.0005$) but not by immunotherapy with CIK cells. (D) A second branch of the experiment (Group B) proceeded

beyond day+15 to explore the effect of the chemo-immunotherapy combination. Mice (n=6) from initial chemotherapy cohort (treated with fotemustine on days 1;15) started intravenous infusions with CIK cells. Remaining mice from all initial cohorts [CIK-immunotherapy (n=6), chemotherapy (n=6), and PBS cohorts (n=4)] acted as controls and continued to be infused with PBS alone. The sequential chemo-immunotherapy treatment resulted in significant antitumor activity (reduction of tumor Ki67 proliferative index compared to untreated controls ($p < 0.0001$)). (E) Activity against mCSCs was indirectly assumed as the rate of residual viable eGFP⁺mCSCs, after sequential chemo-immunotherapy, resulted significantly decreased compared to mice treated with fotemustine only ($p = 0.0229$). All the results were expressed as mean \pm sem and analyzed by the unpaired t-test.

Figure 4. *In vivo* activity of autologous CIK cells in PDX models.

(A) Experimental design: NOD/SCID mice were subcutaneously implanted with a tumor fragment (8 mm³) from patient-derived melanoma biopsy (mMel2, n=20; mMel3 n=7). (B) Intravenous immunotherapy treatment (10⁷ CIK cells/mouse, weekly for 8 total infusions) started 1 week after tumor implantation (n=14) and resulted in significant antitumor activity (assessed by Ki67 tumor proliferative index) compared to controls ($p < 0.0001$). (C) Activity against mCSCs was indirectly assumed as the rate of residual viable eGFP⁺mCSCs after immunotherapy treatment, assessed in explanted tumors at experiment end, was comparable with untreated controls ($p = 0.3071$). All the results were expressed by mean \pm sem and analyzed by the unpaired t-test.

Table 1: Immunophenotype of melanoma cell cultures

n. Melanoma	^{ab} eGFP	^a MCSP	^a MIC A/B	^a ULBP2,5,6	^a NGFR	^a VEGFR1	^a PD-L1
mMel1	21	65	34	63	74	89	2
mMel2	8	71	15	41	58	95	0
mMel3	2	81	90	98	93	98	0
mMel7	17	78	21	65	39	89	2
mMel11	24	95	2	44	67	94	0
mMel12	16	94	86	60	14	89	6
mMel13	14	80	2	40	9	95	9
mMel14	13	79	10	59	93	95	2
mMel15	4	92	2	58	58	97	1
mMel16	11	33	6	52	10	85	6
mMel17	5	70	0	15	52	58	2
Average	12	76	24	54	52	89	3
sem	2.1	5.2	10.0	6.2	9.2	3.4	0,9

^a Value expressed as percentage of viable positive cells

^b eGFP analyzed on viable cells ≥ 10 days after transduction with LV.Oct4.eGFP vector

Table 2: Immunophenotype of melanoma cell cultures: stemness markers

n. Melanoma	^a OCT4	^a SOX-2	^a NANOG	^b ALDH high	^a MITF ^{minus}	^b ABCG2
mMel1	11	16	22	9	16	12
mMel2	14	22	3	9	17	12
mMel3	15	18	11	29	17	12
mMel7	22	19	16	9	10	3
mMel11	9	14	18	10	6	2
mMel12	18	18	15	11	11	8
mMel13	15	21	9	10	15	6
mMel14	14	29	12	6	6	2
mMel15	9	0	7	6	8	1
mMel16	19	20	8	7	58	2
mMel17	11	20	7	9	12	7
Average	14	18	12	10	16	6
SEM	1	2	2	2	4	1

^a Value is expressed as percentage of positive cells

^b Value is expressed as percentage of viable positive cells

Figure 1

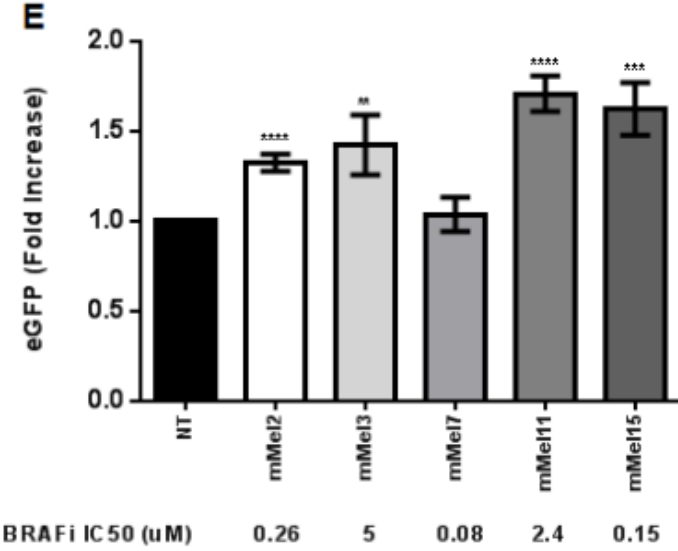
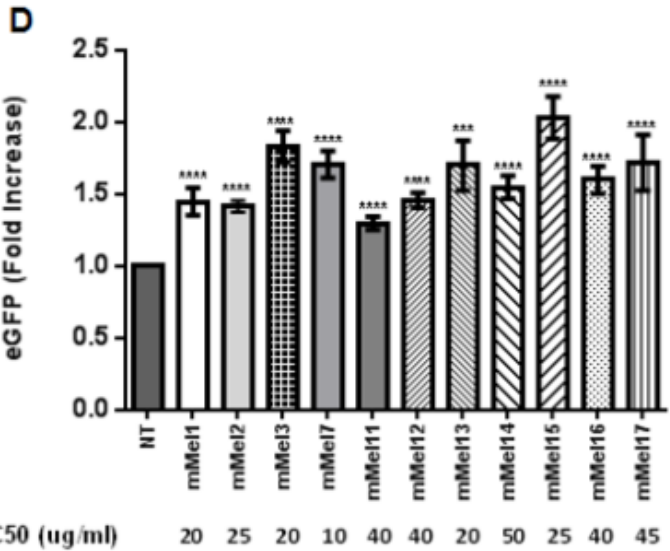
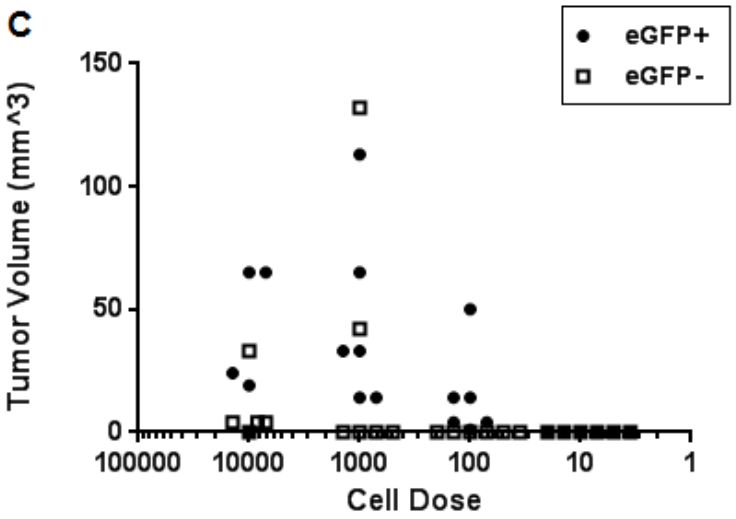
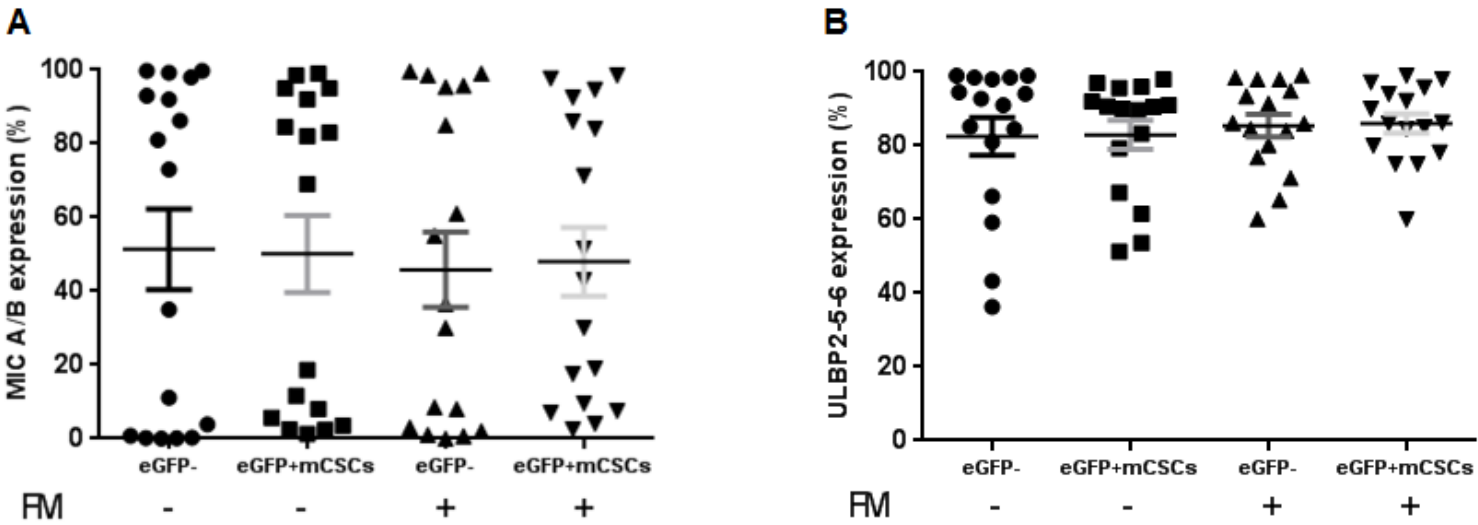


Figure 2

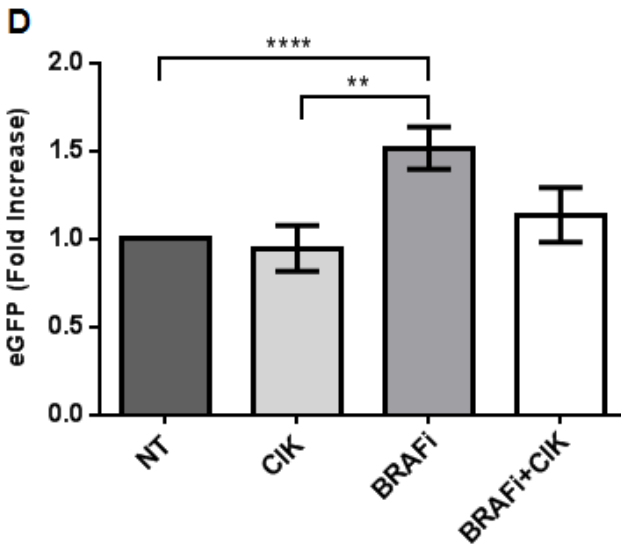
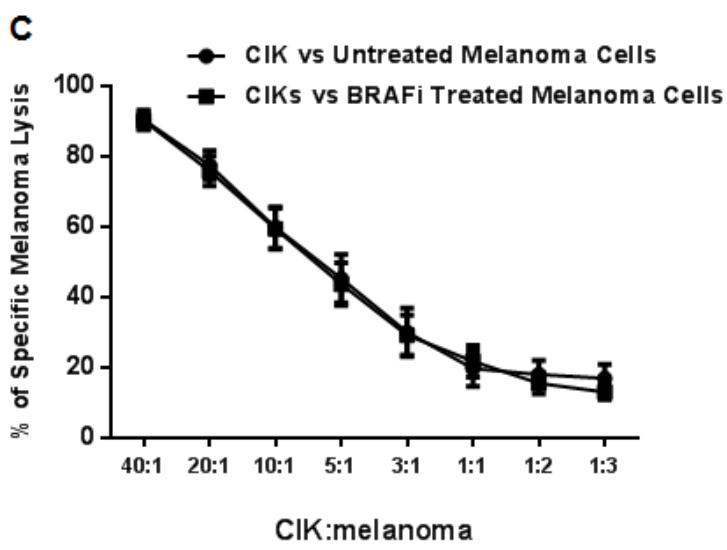
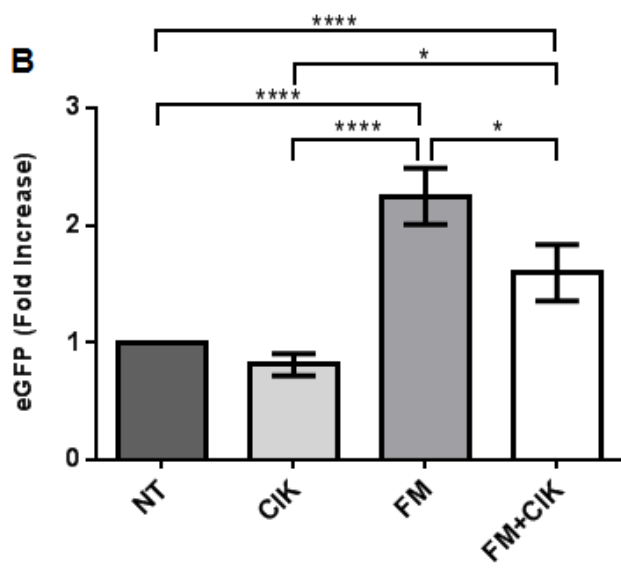
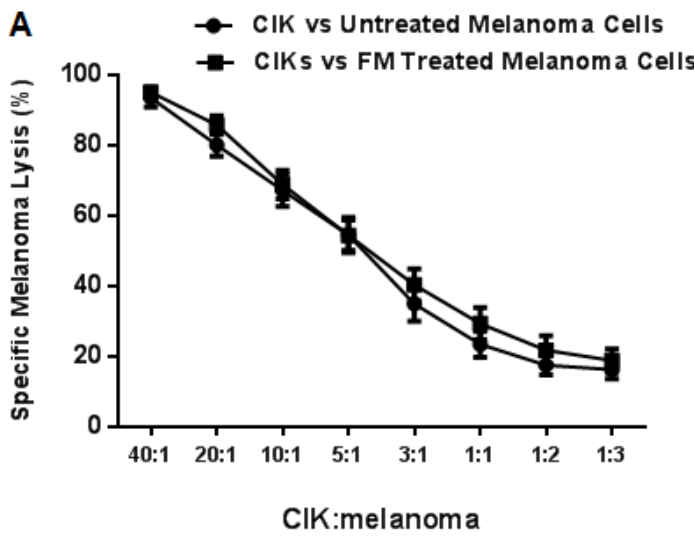
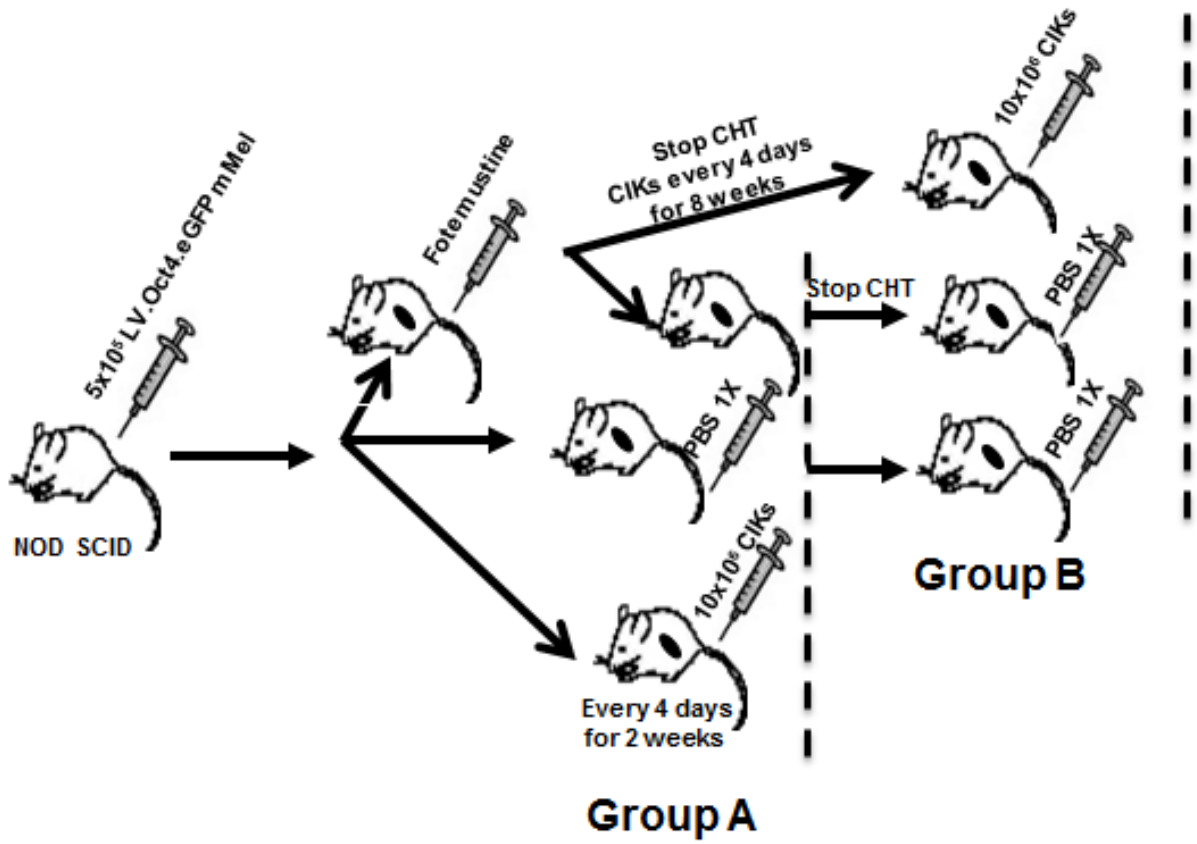
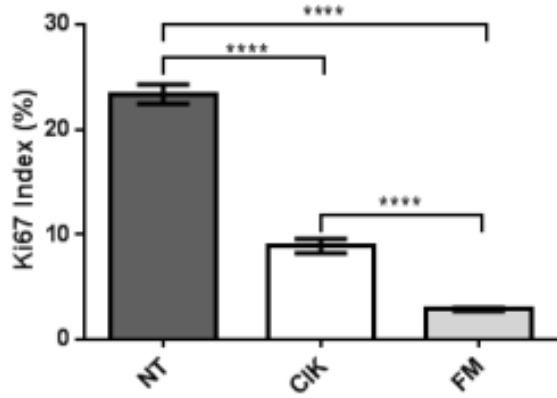


Figure 3

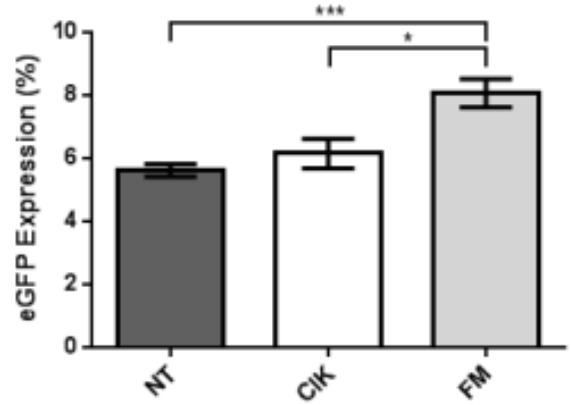
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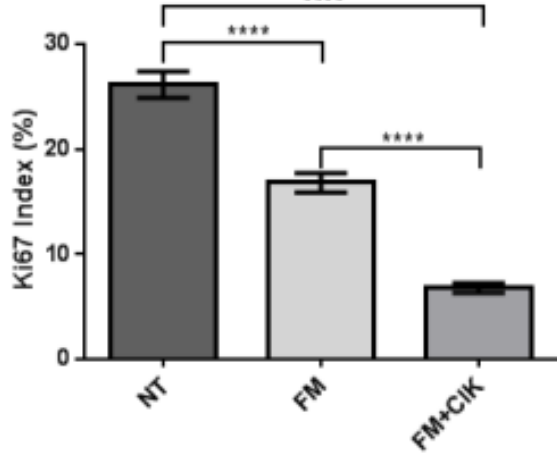
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C



D



E

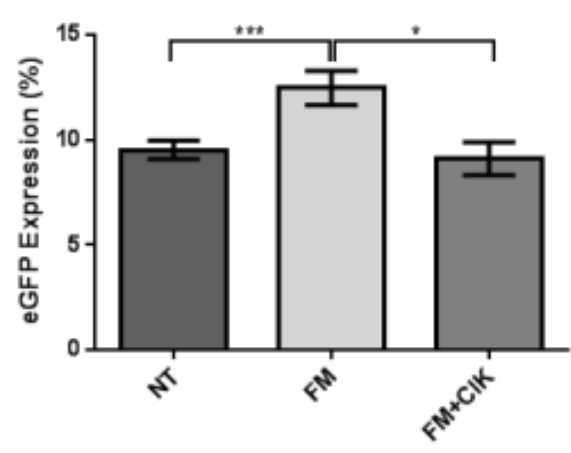


Figure 4

