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

Published version:

DOI:10.1158/1078-0432.CCR-16-1524

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

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

Loretta Gammaitoni[a], Lidia Giraudo[a], Marco Macagno[ab], Valeria Leuci[ab], Giulia Mesiano[a], Ramona Rotolo[ab]; Francesco Sassi[c], Martina Sanlorenzo[abf], Alessandro Zaccagna[d]; Alberto Pisacane[e]; Rebecca Senetta[e]; Michela Cangemi[b]; Giulia Cattaneo[ab]; Valentina Martin[a]; Valentina Coha[a], Susanna Gallo[a]; Ymera Pignochino[ac]; Anna Sapino[e]; Giovanni Grignani[a]; Fabrizio Carnevale-Schianca[a]; Massimo Aglietta[ab] and Dario Sangiolo[ab]

[a] Division of Medical Oncology - Experimental Cell Therapy, Candiolo Cancer Institute, FPO - IRCCS, Candiolo (Torino) Italy,

[b] Department of Oncology, University of Torino, Candiolo (Torino) Italy,

[c] Laboratory of Translational Cancer Medicine, Candiolo Cancer Institute, FPO- IRCCS, Candiolo (Torino), Italy

[d] Surgical Dermatology, Candiolo Cancer Institute, FPO - IRCCS, Candiolo (Torino) Italy,

[e] Pathology, Candiolo Cancer Institute, FPO - IRCCS, Candiolo (Torino) Italy,

[f] Section of Dermatology, Department of Medical Sciences, University of Torino, Torino, Italy

Key words: CIK cells, Adoptive Immunotherapy, Metastatic Melanoma.

Financial supports:

This study was financed in part by the "Associazione Italiana Ricerca sul Cancro" (AIRC) MFAG 2014 N.15731; IG. Grant. N.11515, FPRC ONLUS 5 × 1000, Ministero della Salute 2012; Ricerca Finalizzata-Giovani Ricercatori Ministero della Salute (GR-2011-02349197), University of Torino Fondo Ricerca Locale 2013.

Correspondence should be addressed to:

Dario Sangiolo, MD, PhD.
Division of Medical Oncology - Experimental Cell Therapy, Candiolo Cancer Institute,
FPO - IRCCS, 10060 Candiolo (Torino) Italy.

Phone: +390119933521
Fax: +390119933522
Email: dario.sangiolo@unito.it

Competing financial interests: The authors declare no competing financial interests.

Word and other counts:
Abstract: words 250
Text (introduction, materials and methods, results and discussion): 4884 words
Tables: 2 tables
Figures: 4 figures
References: 57 references.
Supplementary material: 2 supplementary tables, 6 supplementary figures, Supplementary figure legends, Supplementary methods

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 γδ 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-mm^3 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 1.5×10^6 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 phOCT4.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 SalI 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 allogenic 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 allogenic 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% CO_2. 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 (IC_{50}) 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 $5x10^5$ 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 ($1x10^7$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

($1x10^7$/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, whoever 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 µm) 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 um 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 mm^3-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 $1x10^7$ mature autologous CIK cells in 1X PBS (200 ul 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 µm), 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 um 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 Glycerato 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 $1x10^4$) 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 generate 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 allogenic 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 allogenic 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; $1x10^7$ 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 allogenic CIK cells for two weeks ($1x10^7$ 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 ($1x10^7$ every 4 days) following initial treatment with FM (600µg, 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 ($1x10^7$ 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 γδ 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 ($2.3x10^8$ 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.

ACKNOWLEDGMENTS

The phOCT4.EGFP1 vector was a kind gift from Dr. W. Cui (IRDB, Imperial College London). We are grateful to Dr. E. Vigna (University of Torino, Candiolo Cancer Institute, FPO - IRCCS, Candiolo, Turin, Italy) who provided the transfer vector pRRL.sin.PPT.hPGK.EGFP.Wpre (LV-PGK.EGFP). The authors sincerely thank Joan Leonard (Leonard Editorial Services, LLC - Miami, FL, USA) for the linguistic revision and editorial assistance. We thank P. Bernabei for sorting services.

This study was financed in part by the “Associazione Italiana Ricerca sul Cancro” (AIRC) MFAG 2014 N.15731; IG. Grant. N.11515, FPRC ONLUS 5 × 1000, Ministero della Salute 2012; Ricerca Finalizzata-Giovani Ricercatori Ministero della Salute (GR-2011-02349197), University of Torino Fondo Ricerca Locale 2013.

LG was sponsored by the ‘Associazione Italiana Ricerca sul Cancro–AIRC’; MM was sponsored by Ricerca Finalizzata-Giovani Ricercatori Ministero della Salute; VL and YP received a fellowship by MIUR (University of Turin); MS received support from L'Oréal-UNESCO For Women in Science Fellowship 2016.

BIBLIOGRAFIA

1. National Collaborating Centre for Cancer (UK). Melanoma: Assessment and Management. (UK) NCCfC, editor. London UK: National Institute for Health and Care Excellence (UK); 2015.
2. McArthur GA, Chapman PB, Robert C, Larkin J, Haanen JB, Dummer R, *et al.* Safety and efficacy of vemurafenib in BRAF(V600E) and BRAF(V600K) mutation-positive melanoma (BRIM-3): extended follow-up of a phase 3, randomised, open-label study. Lancet Oncol **2014**;15(3):323-32 doi 10.1016/S1470-2045(14)70012-9.
3. Hauschild A, Grob JJ, Demidov LV, Jouary T, Gutzmer R, Millward M, *et al.* Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. Lancet **2012**;380(9839):358-65 doi S0140-6736(12)60868-X 10.1016/S0140-6736(12)60868-X.
4. Flaherty KT, Robert C, Hersey P, Nathan P, Garbe C, Milhem M, *et al.* Improved survival with MEK inhibition in BRAF-mutated melanoma. N Engl J Med 2**012;**367(2):107-14 doi 10.1056/NEJMoa1203421.
5. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, *et al.* Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 2**010;**363(8):711-23 doi NEJMoa1003466 10.1056/NEJMoa1003466.
6. Weber JS, D'Angelo SP, Minor D, Hodi FS, Gutzmer R, Neyns B, *et al. N*ivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. Lancet Oncol 20**15;1**6(4):375-84 doi 10.1016/S1470-2045(15)70076-8.
7. Larkin J, Hodi FS, Wolchok JD. Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. N Engl J Med 20**15;3**73(13):1270-1 doi 10.1056/NEJMc1509660.
8. Watson I, Dominguez PP, Donegan E, Charles Z, Robertson J, Adam EJ. NICE guidance on pembrolizumab for advanced melanoma. Lancet Oncol 20**16;1**7(1):21-2 doi 10.1016/S1470-2045(15)00547-1.
9. Hall CJ, Doss S, Robertson J, Adam J. NICE guidance on ipilimumab for treating previously untreated advanced (unresectable or metastatic) melanoma. Lancet Oncol 20**14;1**5(10):1056-7.
10. Coit DG, Thompson JA, Algazi A, Andtbacka R, Bichakjian CK, Carson WE, *et al. M*elanoma, Version 2.2016, NCCN Clinical Practice Guidelines in Oncology. J Natl Compr Canc Netw 20**16;1**4(4):450-73.
11. Dummer R, Schadendorf D, Ascierto PA, Larkin J, Lebbé C, Hauschild A. Integrating first-line treatment options into clinical practice: what's new in advanced melanoma? Melanoma Res 20**15;2**5(6):461-9 doi 10.1097/CMR.0000000000000200.
12. Robbins PF, Kassim SH, Tran TL, Crystal JS, Morgan RA, Feldman SA, *et al. A* pilot trial using lymphocytes genetically engineered with an NY-ESO-1-reactive T-cell receptor: long-term follow-up and correlates with response. Clin Cancer Res 20**15;2**1(5):1019-27 doi 10.1158/1078-0432.CCR-14-2708.
13. Robbins PF, Morgan RA, Feldman SA, Yang JC, Sherry RM, Dudley ME, *et al. T*umor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. J Clin Oncol 20**11;2**9(7):917-24 doi JCO.2010.32.2537 10.1200/JCO.2010.32.2537.
14. Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, e*t al. Ca*ncer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells. Cancer Res 200**6;66**(19):9339-44 doi 10.1158/0008-5472.CAN-06-3126.
15. Croker AK, Allan AL. Cancer stem cells: implications for the progression and treatment of metastatic disease. J Cell Mol Med 200**8;12**(2):374-90 doi 10.1111/j.1582-4934.2007.00211.x.
16. Islam F, Gopalan V, Smith RA, Lam AK. Translational potential of cancer stem cells: A review of the detection of cancer stem cells and their roles in cancer recurrence and cancer treatment. Exp Cell Res 201**5;33**5(1):135-47 doi 10.1016/j.yexcr.2015.04.018.
17. Colak S, Medema JP. Cancer stem cells--important players in tumor therapy resistance. FEBS J 201**4;28**1(21):4779-91 doi 10.1111/febs.13023.
18. Cojoc M, Mäbert K, Muders MH, Dubrovska A. A role for cancer stem cells in therapy resistance: cellular and molecular mechanisms. Semin Cancer Biol 201**5;31**:16-27 doi 10.1016/j.semcancer.2014.06.004.
19. Ciurea ME, Georgescu AM, Purcaru SO, Artene SA, Emami GH, Boldeanu MV, e*t al. Ca*ncer stem cells: biological functions and therapeutically targeting. Int J Mol Sci 201**4;15**(5):8169-85 doi 10.3390/ijms15058169.
20. Rycaj K, Tang DG. Cancer stem cells and radioresistance. Int J Radiat Biol 201**4;90**(8):615-21 doi 10.3109/09553002.2014.892227.
21. Kaiser J. The cancer stem cell gamble. Science 201**5;34**7(6219):226-9 doi 10.1126/science.347.6219.226.
22. Gammaitoni L, Leuci V, Mesiano G, Giraudo L, Todorovic M, Carnevale-Schianca F, e*t al. Im*munotherapy of cancer stem cells in solid tumors: initial findings and future prospective. Expert Opin Biol Ther 201**4;14**(9):1259-70 doi 10.1517/14712598.2014.918099.
23. Li Y, Rogoff HA, Keates S, Gao Y, Murikipudi S, Mikule K, e*t al. Su*ppression of cancer relapse and metastasis by inhibiting cancer stemness. Proc Natl Acad Sci U S A 201**5;11**2(6):1839-44 doi 10.1073/pnas.1424171112.
24. Stuckey DW, Shah K. TRAIL on trial: preclinical advances in cancer therapy. Trends Mol Med 201**3;19**(11):685-94 doi 10.1016/j.molmed.2013.08.007.

25. Vik-Mo EO, Nyakas M, Mikkelsen BV, Moe MC, Due-Tønnesen P, Suso EM, *et al.* Therapeutic vaccination against autologous cancer stem cells with mRNA-transfected dendritic cells in patients with glioblastoma. Cancer Immunol Immunother 2013;**62**(9):1499-509 doi 10.1007/s00262-013-1453-3.
26. Cioffi M, Dorado J, Baeuerle PA, Heeschen C. EpCAM/CD3-Bispecific T-cell engaging antibody MT110 eliminates primary human pancreatic cancer stem cells. Clin Cancer Res 2012;**18**(2):465-74 doi 10.1158/1078-0432.CCR-11-1270.
27. Visus C, Wang Y, Lozano-Leon A, Ferris RL, Silver S, Szczepanski MJ, *et al.* Targeting ALDH(bright) human carcinoma-initiating cells with ALDH1A1-specific $CD8^+$ T cells. Clin Cancer Res 2011;**17**(19):6174-84 doi 10.1158/1078-0432.CCR-11-1111.
28. Sangiolo D, Martinuzzi E, Todorovic M, Vitaggio K, Vallario A, Jordaney N, *et al.* Alloreactivity and anti-tumor activity segregate within two distinct subsets of cytokine-induced killer (CIK) cells: implications for their infusion across major HLA barriers. Int Immunol 2008;**20**(7):841-8.
29. Todorovic M, Mesiano G, Gammaitoni L, Leuci V, Giraudo Diego L, Cammarata C, *et al.* *Ex* Vivo Allogeneic Stimulation Significantly Improves Expansion of Cytokine-Induced Killer Cells Without Increasing Their Alloreactivity Across HLA Barriers. J Immunother 2012;**35**(7):579-86 doi 10.1097/CJI.0b013e31826b1fd9.
30. Elia AR, Circosta P, Sangiolo D, Bonini C, Gammaitoni L, Mastaglio S, *et al.* Cytokine-induced killer cells engineered with exogenous T-cell receptors directed against melanoma antigens: enhanced efficacy of effector cells endowed with a double mechanism of tumor recognition. Hum Gene Ther 2015;**26**(4):220-31 doi 10.1089/hum.2014.112.
31. Gammaitoni L, Giraudo L, Leuci V, Todorovic M, Mesiano G, Picciotto F, *et al.* Effective Activity of Cytokine Induced Killer Cells against Autologous Metastatic Melanoma including Cells with Stemness Features. Clin Cancer Res 2013 **do**i 10.1158/1078-0432.CCR-13-0061.
32. Sangiolo D, Mesiano G, Gammaitoni L, Leuci V, Todorovic M, Giraudo L, *et al.* Cytokine-induced killer cells eradicate bone and soft-tissue sarcomas. Cancer Res 2014;**74**(1):119-29 doi 10.1158/0008-5472.CAN-13-1559.
33. Lu PH, Negrin RS. A novel population of expanded human CD3+CD56+ cells derived from T cells with potent in vivo antitumor activity in mice with severe combined immunodeficiency. JImmunol 1994;**15**3(4):1687-96.
34. Schmidt-Wolf IG, Lefterova P, Johnston V, Huhn D, Blume KG, Negrin RS. Propagation of large numbers of T cells with natural killer cell markers. BrJHaematol 1994;**87**(3):453-8.
35. Baker J, Verneris MR, Ito M, Shizuru JA, Negrin RS. Expansion of cytolytic CD8(+) natural killer T cells with limited capacity for graft-versus-host disease induction due to interferon gamma production. Blood 2001;**97**(10):2923-31.
36. Verneris MR, Karami M, Baker J, Jayaswal A, Negrin RS. Role of NKG2D signaling in the cytotoxicity of activated and expanded CD8+ T cells. Blood 2004;**10**3(8):3065-72.
37. Olioso P, Giancola R, Di Riti M, Contento A, Accorsi P, Iacone A. Immunotherapy with cytokine induced killer cells in solid and hematopoietic tumours: a pilot clinical trial. Hematol Oncol 2009.
38. Schmidt-Wolf IG, Finke S, Trojaneck B, Denkena A, Lefterova P, Schwella N, *et al.* Phase I clinical study applying autologous immunological effector cells transfected with the interleukin-2 gene in patients with metastatic renal cancer, colorectal cancer and lymphoma. Br J Cancer 1999;**81**(6):1009-16.
39. Verneris MR, Baker J, Edinger M, Negrin RS. Studies of ex vivo activated and expanded CD8+ NK-T cells in humans and mice. JClinImmunol 2002;**22**(3):131-6.
40. Pizzitola I, Anjos-Afonso F, Rouault-Pierre K, Lassailly F, Tettamanti S, Spinelli O, *et al.* Chimeric antigen receptors against CD33/CD123 antigens efficiently target primary acute myeloid leukemia cells in vivo. Leukemia 2014;**28**(8):1596-605 doi 10.1038/leu.2014.62.
41. Tettamanti S, Marin V, Pizzitola I, Magnani CF, Giordano Attianese GM, Cribioli E, *et al.* Targeting of acute myeloid leukaemia by cytokine-induced killer cells redirected with a novel CD123-specific chimeric antigen receptor. Br J Haematol 2013;**16**1(3):389-401 doi 10.1111/bjh.12282.
42. Pizzitola I, Agostoni V, Cribioli E, Pule M, Rousseau R, Finney H, *et al.* *In* vitro comparison of three different chimeric receptor-modified effector T-cell populations for leukemia cell therapy. J Immunother 2011;**34**(6):469-79 doi 10.1097/CJI.0b013e31821e763b.
43. Rettinger E, Huenecke S, Bonig H, Merker M, Jarisch A, Soerensen J, *et al.* Interleukin-15-activated cytokine-induced killer cells may sustain remission in leukemia patients after allogeneic stem cell transplantation: feasibility, safety and first insights on efficacy. Haematologica 2016;**10**1(4):e153-6 doi 10.3324/haematol.2015.138016.
44. Schmeel LC, Schmeel FC, Coch C, Schmidt-Wolf IG. Cytokine-induced killer (CIK) cells in cancer immunotherapy: report of the international registry on CIK cells (IRCC). J Cancer Res Clin Oncol 2014 **do**i 10.1007/s00432-014-1864-3.
45. Todaro M, Meraviglia S, Caccamo N, Stassi G, Dieli F. Combining conventional chemotherapy and γδ T cell-based immunotherapy to target cancer-initiating cells. Oncoimmunology 2013;**2(**9):e25821 doi 10.4161/onci.25821.

46. Todaro M, Orlando V, Cicero G, Caccamo N, Meraviglia S, Stassi G, *et al.* Chemotherapy sensitizes colon cancer initiating cells to Vγ9Vδ2 T cell-mediated cytotoxicity. PLoS One 2013;**8**(6):e65145 doi 10.1371/journal.pone.0065145.
47. Dieli F, Stassi G, Todaro M, Meraviglia S, Caccamo N, Cordova A. Distribution, function and predictive value of tumor-infiltrating γδ T lymphocytes. Oncoimmunology 2013;**2**(4):e23434 doi 10.4161/onci.23434.
48. Gammaitoni L, Giraudo L, Leuci V, Todorovic M, Mesiano G, Picciotto F, *et al.* Effective Activity of Cytokine-Induced Killer Cells against Autologous Metastatic Melanoma Including Cells with Stemness Features. Clin Cancer Res 2013;**19**(16):4347-58 doi 10.1158/1078-0432.CCR-13-0061.
49. Gerrard L, Zhao D, Clark AJ, Cui W. Stably transfected human embryonic stem cell clones express OCT4-specific green fluorescent protein and maintain self-renewal and pluripotency. Stem Cells 2005;**23**(1):124-33 doi 23/1/124 10.1634/stemcells.2004-0102.
50. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, et *al.* A third-generation lentivirus vector with a conditional packaging system. J Virol 1998;**72**(11):8463-71.
51. Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L, et *al.* Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J Virol 1998;**72**(12):9873-80.
52. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, et *al.* Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med 2013;**369**(2):122-33 doi 10.1056/NEJMoa1302369.
53. Lee J, Kefford R, Carlino M. PD-1 and PD-L1 inhibitors in melanoma treatment: past success, present application and future challenges. Immunotherapy 2016;**8(6**):733-46 doi 10.2217/imt-2016-0022.
54. Rosenberg SA, Yang JC, Sherry RM, Kammula US, Hughes MS, Phan GQ, et *al.* Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. Clin Cancer Res 2011;**17**(13):4550-7 doi 1078-0432.CCR-11-0116 10.1158/1078-0432.CCR-11-0116.
55. Sottile R, Pangigadde PN, Tan T, Anichini A, Sabbatino F, Trecroci F, et *al.* HLA class I downregulation is associated with enhanced NK-cell killing of melanoma cells with acquired drug resistance to BRAF inhibitors. Eur J Immunol 2016;**46(2**):409-19 doi 10.1002/eji.201445289.
56. Gargett T, Yu W, Dotti G, Yvon ES, Christo SN, Hayball JD, et *al.* GD2-specific CAR T Cells Undergo Potent Activation and Deletion Following Antigen Encounter but can be Protected From Activation-induced Cell Death by PD-1 Blockade. Mol Ther 2016 **doi** 10.1038/mt.2016.63.
57. Poh SL, Linn YC. Immune checkpoint inhibitors enhance cytotoxicity of cytokine-induced killer cells against human myeloid leukaemic blasts. Cancer Immunol Immunother 2016;**65(5**):525-36 doi 10.1007/s00262-016-1815-8.

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 (±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 ± 10.4% and 51.4 ± 10.9, respectively) and after FM treatment (48.0 ± 9.3% and 45.8 ± 10.2, respectively); (B) ULBP2-5-6 expression in $eGFP^{+}$mCSCs and $eGFP^{-}$ cells before (82.6 ± 5% and 82.9 ± 4, respectively) and after FM treatment (85.5 ± 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 $1x10^{7}$ 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±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 mm^3) from patient-derived melanoma biopsy (mMel2, n=20; mMel3 n=7). (B) Intravenous immunotherapy treatment (10^7 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±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

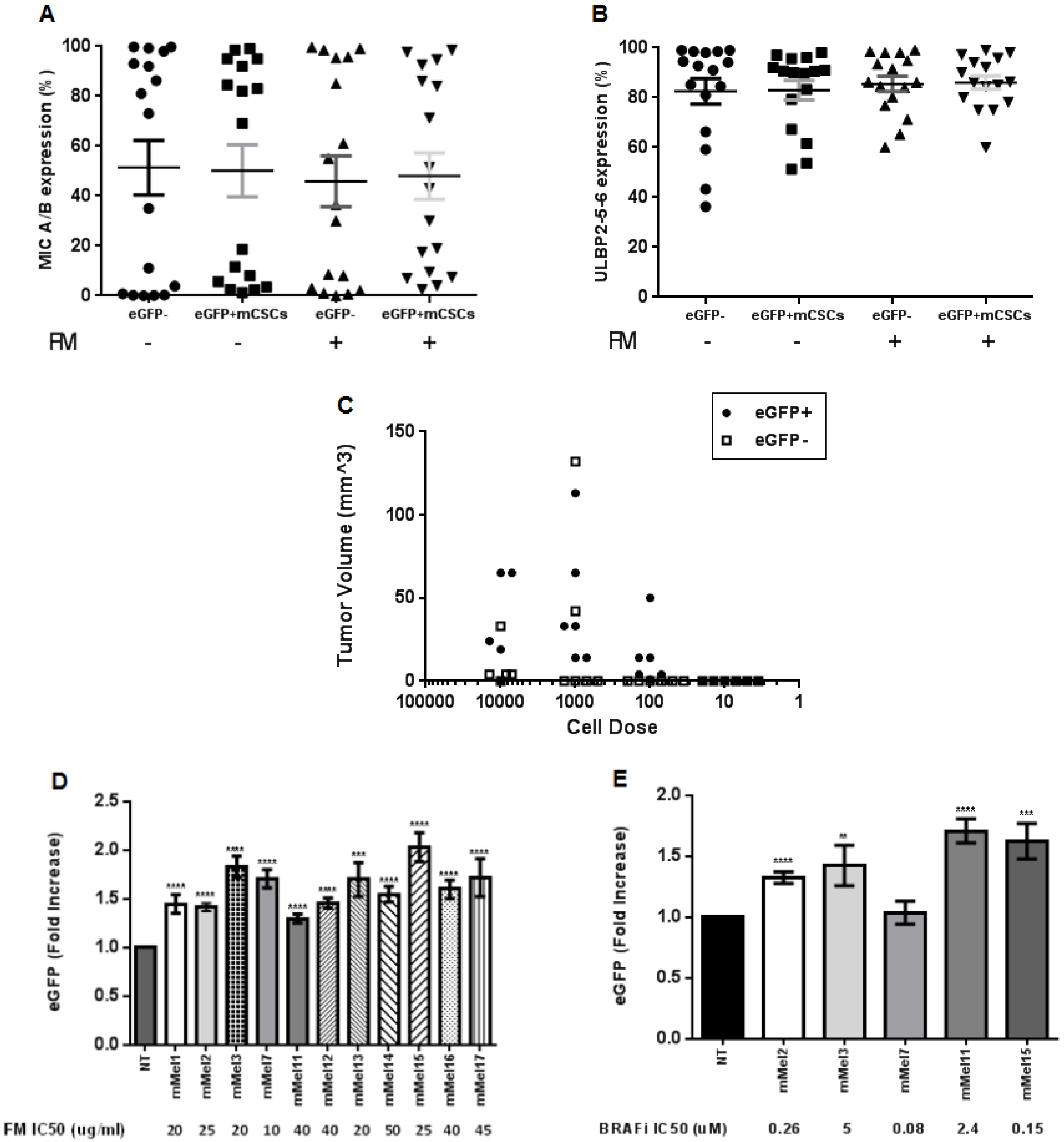
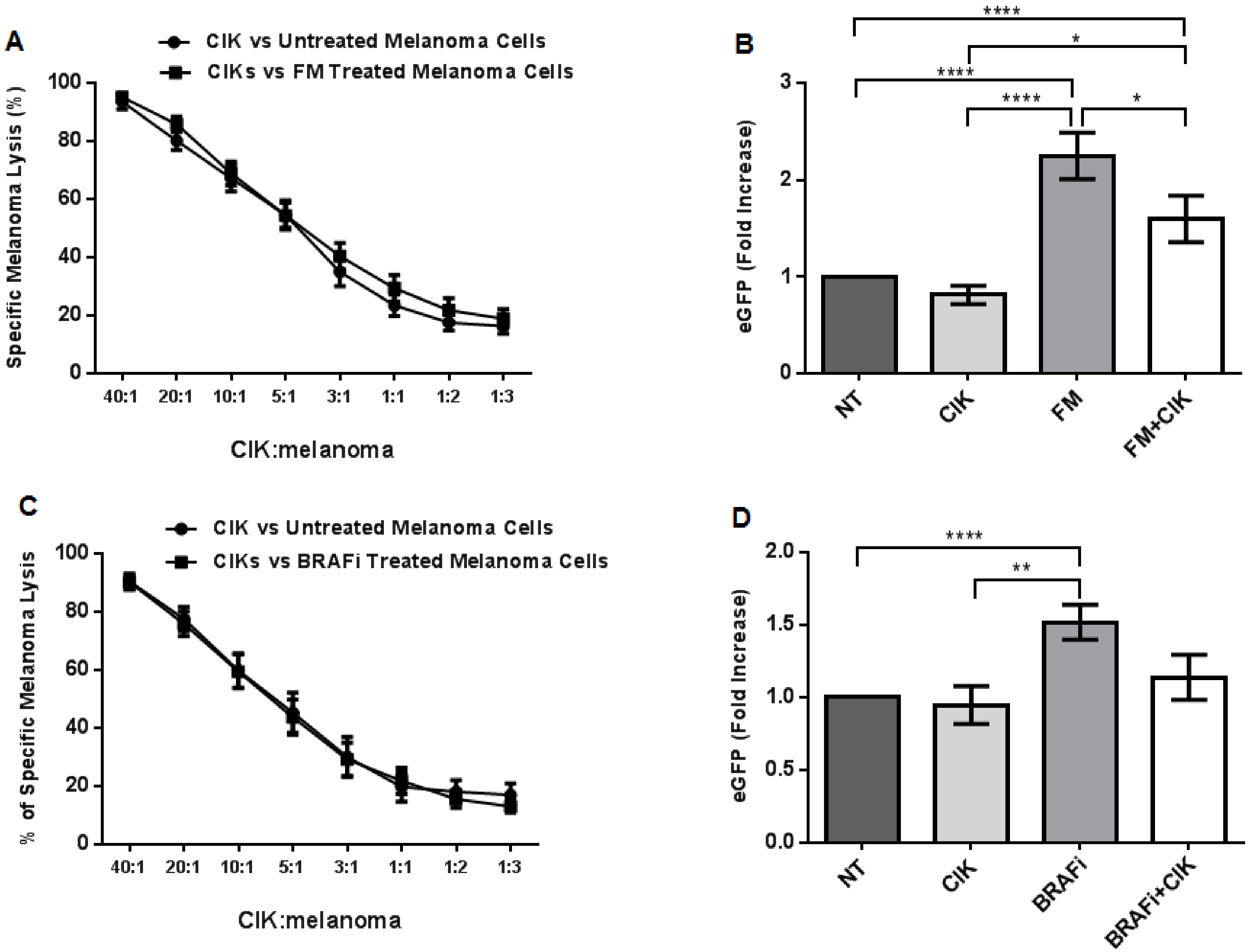

A
MIC A/B expression (%)
100
80
60
40
20
0
eGFP-
eGFP+mCSCs
eGFP-
eGFP+mCSCs
FM
-
-
+
+
B
ULBP2-5-6 expression (%)
100
80
60
40
20
0
eGFP-
eGFP+mCSCs
eGFP-
eGFP+mCSCs
FM
-
-
+
+
C
eGFP+
eGFP -
Tumor Volume (mm^3)
150
100
50
0
100000
10000
1000
100
10
1
Cell Dose
D
eGFP (Fold Increase)
2.5
2.0
1.5
1.0
0.5
0.0
NT
mMel1
mMel2
mMel3
mMel7
mMel11
mMel12
mMel13
mMel14
mMel15
mMel16
mMel17
FM IC50 (ug/ml)
20 25 20 10 40 40 20 50 25 40 45
E
eGFP (Fold Increase)
2.0
1.5
1.0
0.5
0.0
NT
mMel2
mMel3
mMel7
mMel11
mMel15
BRAFi IC50 (uM)
0.26 5 0.08 2.4 0.15

Figure 2



A
CIK vs Untreated Melanoma Cells
CIKs vs FM Treated Melanoma Cells
Specific Melanoma Lysis (%)
100
80
60
40
20
0
40:1
20:1
10:1
5:1
3:1
1:1
1:2
1:3
CIK:melanoma
B
eGFP (Fold Increase)
3
2
1
0
****
*
****
****
*
NT
CIK
FM
FM+CIK
C
CIK vs Untreated Melanoma Cells
CIKs vs BRAFi Treated Melanoma Cells
% of Specific Melanoma Lysis
100
80
60
40
20
0
40:1
20:1
10:1
5:1
3:1
1:1
1:2
1:3
CIK:melanoma
D
eGFP (Fold Increase)
2.0
1.5
1.0
0.5
0.0
****
**
NT
CIK
BRAFi
BRAFi+CIK

Figure 3

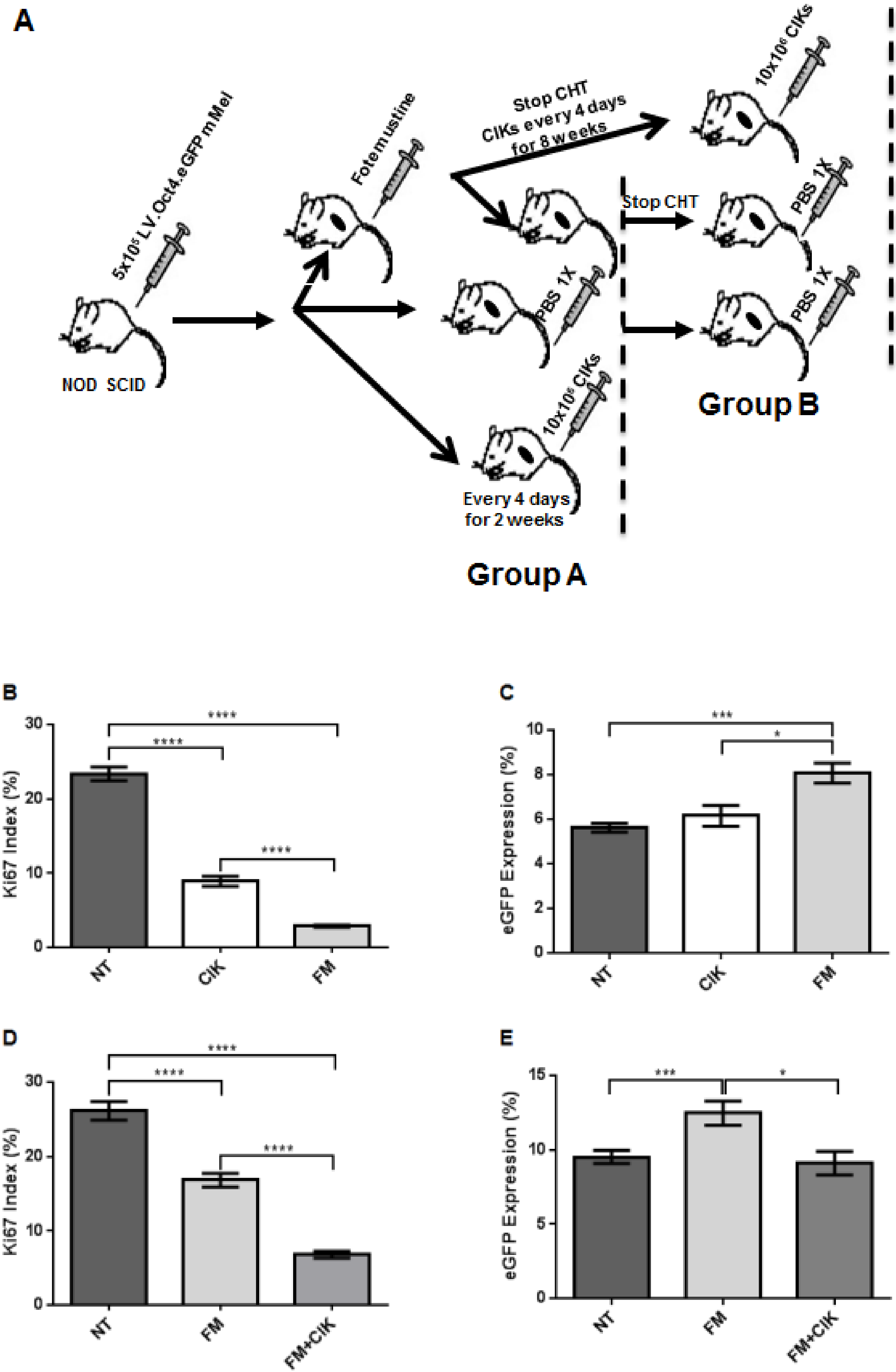
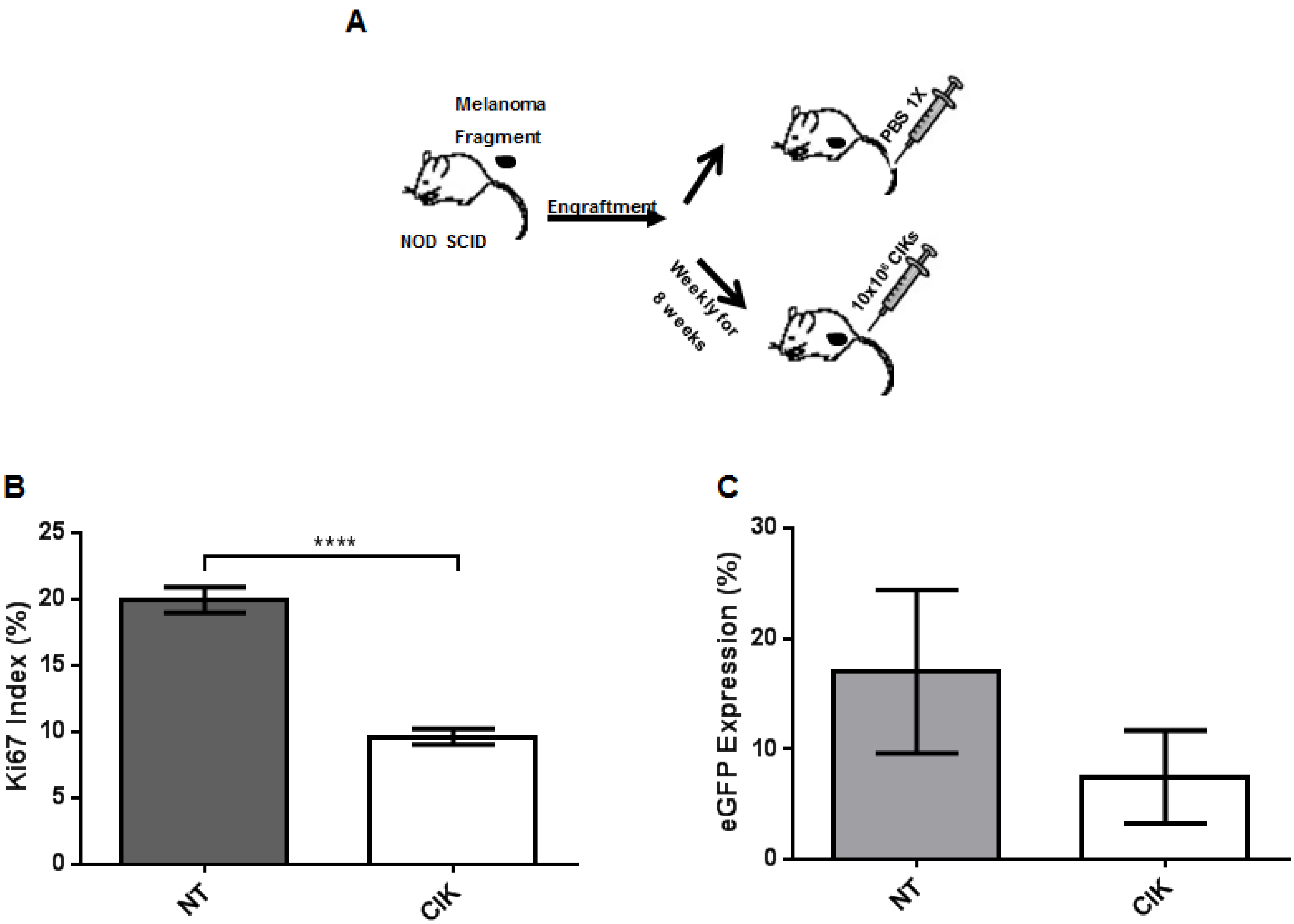

A
5x10^5 LV.Oct4.eGFP mMel
NOD SCID
Fotemustine
Stop CHT
CIKs every 4 days
for 8 weeks
10x10^6 CIKs
PBS 1X
Stop CHT
PBS 1X
PBS 1X
10x10^6 CIKs
Every 4 days
for 2 weeks
Group A
Group B
B
Ki67 Index (%)
NT
CIK
FM
C
eGFP Expression (%)
NT
CIK
FM
D
Ki67 Index (%)
NT
FM
FM+CIK
E
eGFP Expression (%)
NT
FM
FM+CIK

Figure 4


A
Melanoma
Fragment
NOD SCID
Engraftment
Weekly for
8 weeks
PBS 1X
10x10⁶ CIKs
B
Ki67 Index (%)
25
20
15
10
5
0
****
NT
CIK
C
eGFP Expression (%)
30
20
10
0
NT
CIK