Effective Activity of Cytokine Induced Killer Cells against Autologous Metastatic Melanoma including Cells with Stemness Features

Running head: CIK cells kill autologous melanoma and mCSCs

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

Word and other counts:
Abstract: 248 words
Text (introduction, materials and methods, results and discussion): 4,870 words
Tables: 3 tables
Figures: 3 figures
References: 56 references.
Supplementary material: 6 supplementary figures, Supplementary figure legends, Supplementary methods
Key words: CIK cells, Adoptive Immunotherapy, Metastatic Melanoma.
Translational Relevance

This work is the first report of effective antitumor activity of patient-derived Cytokine-Induced Killer (CIK) cells against autologous metastatic melanoma, including putative cancer stem cells (CSCs). The translational relevance of this work is borne out in two ways. First, the autologous model (same patient CIK cells and tumor targets) uniquely considers the intrinsic biology of each patient—tumor, and second, chemo-resistant- and relapse-associated putative melanoma CSCs are identified and targeted. The ability of CIK cells to kill both differentiated melanoma cells and CSCs may promote them into clinical trials, either alone or in association with emerging molecular targeted therapies.

Abstract

**Purpose:** We investigate the unknown tumor-killing activity of Cytokine-Induced Killer (CIK) cells against autologous metastatic melanoma (mMel) and the elusive subset of putative cancer stem cells (mCSCs).

**Experimental Design:** We developed a preclinical autologous model using same patient-generated CIK cells and tumor targets to consider the unique biology of each patient/tumor pairing. In primary tumor cell cultures we visualized and immunophenotypically defined a putative mCSC subset using a novel gene-transfer strategy that exploited their exclusive ability to activate the promoter of stemness gene Oct4.

**Results:** The CIK cells from 10 mMel patients were successfully expanded (median: 23-fold, range: 11-117). Primary tumor cell cultures established and characterized from the same patients were used as autologous targets. Patient-derived CIK cells efficiently killed autologous mMel (up to 71% specific killing (n=26)). CIK cells were active *in vivo* against autologous melanoma, resulting in delayed tumor growth, increased necrotic areas and lymphocyte infiltration at tumor sites. The mMel cultures presented an average of 11.5 ±2.5% putative mCSCs, which was assessed by Oct4 promoter activity and stemness marker expression (Oct4, ABCG2, ALDH, MITF). Expression was confirmed on mCSC target...
molecules recognized by CIK cells (MIC A/B; ULBPs). CIK tumor killing activity against mCSCs was intense (up to 71%, n=4) and comparable to results reported against differentiated mMel cells (p=0.8).

Conclusions: For the first time, the intense killing activity of CIK cells against autologous mMel, including mCSCs, has been demonstrated. These findings move clinical investigation of a new immunotherapy for mMel including mCSCs closer.

Introduction
The incidence of malignant melanoma in western populations has increased in recent decades. Although surgical resection of primary lesions has high cure rates, metastatic melanoma (mMel) remains largely refractory to conventional therapies with a dismal prognosis (1, 2). Targeted strategies against key molecules, such as B-RAF and MEK(3-5), and immune system antitumor activity restoration to block the CTLA4 (6, 7) or PD-1 molecules (8) are two recent breakthroughs that have positively impacted mMel treatment. Despite some survival advantage, results have almost always been short-lived; it seems that as hypothesized for other tumors, mMel includes a small cell subpopulation endowed with the stemness features that sustain drug-resistance and disease relapse (9). This challenging clinical scenario demands new therapeutic approaches, ideally ones able to target melanoma cancer stem cells (mCSCs).

Much promise lies in adoptive immunotherapy for the treatment of mMel as supported by results reported with ex-vivo expanded tumor infiltrating lymphocytes (TIL) (10) or T cells engineered with melanoma antigen-specific T cell receptors (TCRs) (11, 12). The extended clinical application of TIL is, however, limited by available, suitably-sized resectable tumor lesions, while specific HLA restriction, complex expansion conditions, and stringent regulatory requirements confound the TCR-transfer approach.

Cytokine-Induced Killer (CIK) cells are ex-vivo expanded T-NK lymphocytes potentially able to address some of the issues currently limiting clinical application of other immunotherapies (13, 14). CIK cells can be massively expanded from peripheral blood mononuclear cells (PBMC) cultured with the timed addition of IFN-gamma, Ab anti CD3, and IL2 through simple standardized culture conditions (15-18). CIK cell activity is not dependent on HLA-restriction; it
is mediated most by the interaction of its NKG2D receptor with stress-inducible molecules (MHC class I-related chain A and B (MIC A/B) and UL-16 binding proteins (ULBPs)) on tumor targets (19, 20). Initial clinical trial results in various tumor settings are encouraging, but data is generally absent on CIK cell potential activity against autologous mMel (13, 14, 21, 22), let alone work that might take into account the unique biologic and immunogenic features of a specific tumor in a specific patient.

Furthermore, completely unexplored is whether or not the tumor-killing ability of CIK cells affects subpopulation mCSCs. Currently, clear identification of mCSCs is intensely debated. Several membrane molecules or genes have been proposed as putative markers, but agreement remains elusive. Quintana et al recently failed to identify any marker that robustly distinguished mCSCs despite examining 85 markers, including ATP-Binding Cassette B5 (ABCB5), CD271 (aka Nervous Growth Factor Receptor, NGFR), and CD133 (23, 24).

Several facts germane to this topic are commonly accepted. First, expression of human embryonic stem cell pluripotency markers SOX2, Klf4, and Oct4 indicate a high plasticity (25, 26). Second, inhibition of microphthalmia-associated transcription factor (Mitf), the master regulator of melanocyte differentiation, increases the tumorigenic potential of melanoma cells and upregulates stem cell markers Oct4 and Nanog as shown by Ballotti et al (27). Third and recently reposted is that forced expression of the Oct4 gene promoted dedifferentiation of melanoma cells toward mCSCs with decreased expression of melanocytic markers, acquisition of multipotent differentiation capacity, membrane expression of ABCB5 and CD271, resistance to chemotherapy, hypoxia, and increased tumorigenic capacity (28).

Here, we first report preclinical activity of patient-derived CIK cells against autologous mMel including putative mCSCs. To highlight the mCSCs, we introduced a gene transfer strategy whereby bulk melanoma cell cultures were transduced with a lentiviral vector encoding the enhanced Green Fluorescence Protein (eGFP) under expression control of the Oct4 promoter. We visualized mCSCs exploiting their exclusive ability to activate the Oct4 promoter and sorted them on the basis of eGFP expression. CIK cells efficiently killed autologous melanoma targets regardless of their stemness or differentiated phenotype.
Methods

Clk culture and expansion

Human peripheral blood samples were obtained from subjects with histologically-confirmed stage IV melanoma at the Fondazione del Piemonte per l’Oncologia – Institute for Cancer Research and Treatment (FPO-IRCC). All individuals provided their informed consent. Cultures were started with Peripheral Blood Mononuclear Cells (PBMCs) collected from mMel same patients and isolated by density gradient centrifugation using cell separation media Lymphoprep (Sentinel Diagnostic, Milan, Italy). For some experiments, fresh PBMCs collected from volunteer donors were used. PBMCs were cultured overnight in cell culture flasks at a cell density of $1.5 \times 10^6$ ml$^{-1}$ RPMI (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma) in 1000 U ml$^{-1}$ IFN-γ (PeproTech, London, UK). After 18–24 h in culture at 37°C and 5% CO$_2$, 50 ng ml$^{-1}$ anti-CD3 antibody (OKT3, PharMingen, San Diego, CA, USA) and 300 U ml$^{-1}$ recombinant human interleukin IL-2 (Proleukin, Aldesleukin, Chiron Corporation, Emeryville, CA, USA) were added. Fresh medium with IL-2 was added as needed.

Establishment of primary melanoma cell cultures

Human melanoma tissues were obtained from surgical specimens; patients 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 by mechanical and enzymatic dissociation (Collagenase Type I, Invitrogen) for 3 hours, then subsequently for an additional 12 hours. Cells were then resuspended in KnockOut Dulbecco’s Modified Eagle’s: Nutrient Mixture F-12 Medium (KO DMEM:F12 medium Gibco BRL) with the addition of penicillin (50 U/ml), streptomycin (50 μg/ml), Glutamax 100X (all from Gibco BRL); cells could be seeded either in serum-free conditions with the addition of N2 supplement (Gibco BRL) or in 10% heat-inactivated Fetal Bovine Serum (FBS, Euroclone). Cells were plated at clonal density ($10^4$ - $10^5$ cells per cm$^2$) either in ultralow attachment multi-well plates for suspension cell culture applications or in multi-well plates treated for anchorage-dependent cultures (Corning/Costar).
**In vivo tumorigenesis**

Four-week-old Non-Obese Diabetic/LtSz-scid/scid (NOD/SCID) (Charles River, Italy) female mice were injected subcutaneously with $10^6$ cells from melanoma primary cultures resuspended in sterile PBS1X and BD Matrigel™ Basement Membrane Matrix (Becton Dickinson) 1:1. Tumor growth was monitored weekly with calipers; volume was calculated using formula, 

$$V = \frac{4}{3} \times \pi \times (\frac{L}{2})^2 \times (\frac{l}{2})$$

where $L$ is the length and $l$ the width diameter of the tumor. When the tumor volume reached 2 cm of diameter, the animal was euthanized, the tumor was recovered and fixed overnight in 4% paraformaldehyde, then dehydrated, paraffin-embedded, sectioned (5 µm), and stained with Hematoxylin and Eosin (H&E) (Bio.Optica).

**hOct4.eGFP Lentiviral Vector generation**

VSV-G pseudotyped third-generation LVs were produced by transient four-plasmid co-transfection into 293T cells as described by Follenzi et al (29). The transfer vector pRRL.sin.PPT.hPGK.EGFP.Wpre (LV-PGK.EGFP) was kindly by Dr Elisa Vigna (Gene Transfer and Therapy, IRCC Candiolo, Turin, Italy) and has been described elsewhere (29). The phOCT4.EGFP1 vector(30) was provided by Wei Cui (IRDB, Imperial College, London). The pRRL.sin.PPT.hOct4.eGFP.Wpre (LV-Oct4.eGFP) was obtained by replacing the expression cassette hPGK.eGFP into LV-PGK.eGFP with the hOct4-eGFP1 cleaved from the phOct4-eGFP1 vector by 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, Milano, Italy).

**Melanoma Primary Cell Transduction**

For each LV transduction, melanoma primary cells were resuspended in fresh KODMEM-F12 with FBS 10%. 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. As a transduction efficiency control, the same melanoma primary cells were transduced with LV-PGK.eGFP. Murine Embryonic Cells (ECs) and Peripheral Blood Mononuclear Cells (PBMC) were transduced with LV-Oct4.eGFP as positive and negative expression controls.
Analysis of LV-Oct4.eGFP presence in eGFP positive and negative cell fractions

Detection of the presence of LV-Oct4.eGFP in both fractions of freshly sorted cells was verified by polymerase chain reaction (PCR)-based amplification of the expression cassette Oct4.eGFP. Genomic DNA was extracted separately from eGFP+ and eGFP- cells using a commercial kit (Qiagen, Hilden, Germany). PCR was performed using 100 ng of gDNA per sample and Phusion High-Fidelity DNA Polymerase (Thermo Scientific) according to the manufacturer’s protocol.

Annealed on the lentiviral vector backbone sequences both upstream and downstream the expression cassette were primers LV forward primer 5’-AGG CCCGAAGGAATAGAAGA-3’ and LV reverse primer 5’-CCACATAGCGTAAAAGGAGCA -3’.

The PCR products were separated by electrophoresis on 1% agarose gel.

In vitro proliferation assay and PKH26 Staining

To evaluate the proliferation rate of eGFP+ versus eGFP- cell sorted fractions, cells had been labeled with lipophylic dye PKH26, for which fluorescence intensity decreased by half at each cell division per kit protocol (PKH26GL kit, Sigma). Briefly, an adequate quantity of Diluent C labelling vehicle was added to the previously-washed cell pellet (es. 1x10⁶ cells/0.5 ml) to obtain a 2x single cell suspension. A 2x (4 μM) PKH26 dye solution was prepared and added to 2x single cell suspension. Cell membrane dye uptake was stopped by adding an equal volume of heat-inactivated serum. PKH26-stained cells were then washed twice with culture medium additioned with 10% heat-inactivated serum (5 minutes at 400 x g). An aliquot of labeled and counted cells has been read on a Flow Cytometry Cyan (Cyan ADP, Dako) and analyzed using Summit Software to set the baseline fluorescence level. The remaining cells were seeded in culture under optimal conditions as previously described. After 7, 14, and 21 days, the reduction in fluorescence was quantified by flow cytometry.

Cytotoxicity assay

CIK tumor-killing ability was assessed against an allogeneic cell line (DettMel) and melanoma primary tumor cells. The allogeneic melanoma cell line DettMel was a kind gift from Dr V. Russo (Milan, Italy), derived from metastases of malignant melanomas as described
elsewhere (31). The effector cells were assayed against both autologous and allogeneic tumor targets (32). A non-radioactive stain of melanoma target cells was used from PKH26 kit (Sigma-Aldrich) to perform in-vitro assays. Their immune-mediated killing was analyzed by flow cytometry (Cyan ADP, Dako) by Propidium Iodide permeability of target cells (PKH26+ gate). CIK cells were co-cultured with either autologous or allogenic melanoma primary cells with a 40:1, 20:1, 10:1, and 5:1 effector:target ratio for 2-6 hours in 200 µL of medium with IL2 at a concentration of 300 U/mL at 37°C 5% CO2. 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 according to the following formula: (experimental-spontaneous mortality/100-spontaneous mortality) x 100.

**In vivo Activity Assay**

Non-Obese Diabetic/LtSz-scid/scid (NOD/SCID) (Charles River, Italy) female mice were subcutaneously injected with an 8 mm³ tumor fragment from a patient-derived melanoma biopsy (mMel2). Starting 1 week after tumor implantation, mice received 8 weekly intravenous infusions with 1x10⁷ mature autologous CIK cells resuspended in 1X PBS (200 µl total volume injected). Mice injected with PBS alone were used as the untreated control. Tumor growth was monitored weekly with calipers and volume calculated according to the formula: \( V = \frac{4}{3} \times \pi \times \left(\frac{l}{2}\right)^2 \times \left(\frac{L}{2}\right) \), where \( L \) is the length and \( l \) the width diameter of the tumor. Animals were euthanized when the tumor size reached 2 cm in its main diameter. The recovered tumor was fixed overnight in 4% paraformaldehyde, dehydrated, paraffin-embedded, sectioned (5 µm), and finally stained with Hematoxylin and Eosin (H&E) (Bio.Optica). Immunohistochemistry assay was performed with human anti-CD5 and anti-CD56(NCMA) antibodies (Novocastra, Leica Biosystem). A certified pathologist evaluated the necrotic areas.

**Statistical analysis**

Statistical analysis was performed using software GraphPad Prism 5. A descriptive statistical analysis of CIK and melanoma primary cell culture median values and ranges, or mean ± s.e.m, was used as required. Subgroup phenotype and necrotic area extension were compared with the unpaired, two-tailed t-test. The mixed model analysis of variance (ANOVA) was employed to assess CIK cytotoxic activity curves in vitro and to compare tumor volumes
in vivo. Statistical significance has been expressed as true P value, and all less than 0.05 were considered statistically significant.

Results

Establishment and characterization of autologous melanoma primary cell cultures

Autologous melanoma cell lines were successfully established from 10 patients with stage IV melanoma. The characteristics of the 10 patients are shown in Table 1.

Primary cell cultures were generated from tumor tissue biopsies on metastatic sites in 4 to 12 weeks. All cell cultures displayed morphologic features consistent with the pathology evaluation of the corresponding tumor; a representative picture of the primary tumor cell cultures is shown in Supplementary Figure 1.

We analyzed each cell culture for expression of previously described main melanoma surface antigens: CD271, Melanoma-associated Chondroitin Sulfate Proteoglycan (MCSP), CD34, and Vascular Endothelial Growth Factor Receptor 1 (VEGFR1). All tested tumors (Table 2) showed heterogeneous expression of CD271 and MCSP at medians of 55% (range: 15 – 93%) and 78% (range: 2 – 99%), respectively (Supplementary Figures 2a and 2b). CD34+ was expressed at a median of 7% (range: 2-25%) and VEGFR1bright at 10% (range: 2-19%) while CD34 and VEGFR1 were co-expressed in 4% of the cells (range: 2-21%) (Supplementary Figure 2e). mMel6 and mMel10 were the only two melanoma cell cultures found to express CD117 (aka c-kit) at 89% and 70%, respectively (Supplementary Figure 2k).

Some of the principal molecules reported as associated with mCSC phenotype were also evaluated. Oct4, ATP Binding Cassette G2 (ABCG2), and aldehyde dehydrogenase (ALDH) were detected in all samples that averaged expression of 10.9 ± 0.9%, 11.1 ± 1.4%, and 9.7 ± 2.3%, respectively (Supplementary Figures 2f-h). Melanoma cells negative for Mitf expression were 10.8 ± 1.5% (Supplementary Figure 2i). All data are expressed as mean ± sem.

Each melanoma cell culture was analyzed for expression of the principal known ligands recognized by the NKG2D receptor on CIK cells (MIC A/B, ULBP1, ULBP2, and ULBP3). MIC A/B and ULBP2 were found expressed in every tumor tested to varying levels with medians of
24% (range: 7-90%) and 61% (range: 40-98%), respectively. ULBP 1 and 3 expression was negligible except in mMel10 (16.57% ULBP3+ cells) (Supplementary Figures 2c and 2d).

All melanoma cell cultures save for two (mMel9 and mMel10) retained membrane expression of HLA class I molecules (>99% HLA-ABC+). Melanoma cell cultures generated from selected representative patients (n=6) were subcutaneously inoculated in NOD/SCID mice to demonstrate their tumorigenicity. All mice inoculated with 6 different melanoma primary cell cultures produced tumor growth starting between 1 and 8 weeks after injection (Figure 1).

**Expansion and phenotype of CIK cells**

The ex-vivo expansion of CIK cells was evaluated in the same 10 patients from whom we had generated melanoma primary cultures. CIK cells were classically expanded from fresh or cryopreserved PBMCs cultured with the timed addition of IFN-gamma, Ab-anti-CD3, and IL2. CIK cells from all patients were successfully expanded within 3-4 weeks of culture.

Median expansion of bulk CIK cells was 23-fold (range 11 to 117-fold) after 3 weeks of culture while 252-fold expansion was obtained for the CD3+CD56+ fraction (range 49 to 1870-fold).

The presence of pure NK (CD3+CD56+) cells was negligible at less then 5% in all cases at the end of expansion. The subset of mature CIK cells co-expressing CD3 and CD56 molecules (CD3+CD56+) was present with a median of 49% (range 23-80%), while 78% (59-91%) of CD3+ cells were also CD8+ (Supplementary Figure 3).

The median membrane expression of the NKG2D receptor, which is the main molecule responsible for tumor recognition, on expanded CD3+ CIK cells was 84% (range: 57-93%).

A summary of patient characteristics and relative CIK expansion data is reported in Table 3.

**Killing activity of CIK cells against melanoma cell line**

To test the anti-tumor activity of CIK cells expanded from the 10 patients, we evaluated their ability to kill in vitro a melanoma cell line (DettMel). The cytotoxicity test was conducted at the end of ex-vivo expansion and demonstrated efficient killing that varied among patients. The
average specific tumor killing was 63 ± 4%, 52 ± 5%, 39 ± 5%, and 28 ± 5% (mean ± sem) at 40:1, 20:1, 10:1, and 5:1 effector/target ratio, respectively (n=18, Figures 2a and 2b).

**In vitro and In vivo Killing activity of CIK cells against autologous metastatic melanoma cells**

Patient-derived CIK cells efficiently killed *in vitro* autologous metastatic melanoma targets with an average specific killing of 71 ± 2%, 61 ± 3%, 49 ± 3%, and 37 ± 3% (mean ± sem) at a 40:1, 20:1, 10:1 and 5:1 effector/target ratio, respectively (n=26). The intensity of killing against autologous targets was comparable (p=0.9991) with that observed with allogeneic CIK cells assessed in parallel versus the same tumor cells with an average specific killing of 70 ± 4%, 61 ± 4%, 49 ± 5%, and 35 ± 4% (mean ± sem) at a 40:1, 20:1, 10:1, and 5:1 effector/target ratio, respectively (n=20). A summary of cytotoxicity *in vitro* against autologous or allogeneic tumor targets is reported in Figure 2a.

We evaluated also the activity of patient-derived CIK cells *in vivo* against autologous metastatic melanoma. NOD/SCID mice (n=12) were subcutaneously implanted with an 8 mm³ tumor fragment from a patient-derived melanoma biopsy (mMel2). One week after tumor implantation, a group of implanted mice (n=8) were infused weekly by tail vein injection with mature autologous CIK cells (1x10⁷/week for 6 weeks). When tumor growth in untreated mice (n=4) was more than 2 cm in at least one dimension, all animals were euthanized. Tumors were excised and analyzed for the presence of lymphocytic infiltration and the extension of necrotic tissue areas. At the end of the experiment, tumors from animals treated with CIK cells had significantly larger necrotic areas compared to untreated controls (Figure 2b, p=0.0255), and we could confirm the presence of CIK cells infiltrating the autologous tumor (Figure 2c). Moreover, a significant delay in the tumor growth curve was observed in treated mice compared to untreated controls (p=0.0305) after 2-way ANOVA analysis (Figure 2d).
Activity of CIK cells against autologous putative mCSCs.

Visualization of putative mCSCs was accomplished by stably transducing the, primary melanoma cell cultures using a lentiviral vector that carried eGFP controlled by the promoter regulatory element of the Oct4 gene (LV-Oct4.eGFP) (Figures 3a and 3b). The average eGFP expression, 7 days post transduction, was 11.5 ± 2.5%. As a positive control, a murine embryonic cell line expressing Oct4 (mES) was successfully transduced with LV-Oct4.eGFP up to 90.5% of eGFP expression (Figure 3b) while no eGFP expression was detected on differentiated PBMC from healthy donors transduced with the same vector (LV-Oct4.eGFP). As an additional control, we confirmed that both primary melanoma cell cultures (Figure 3b) and mES could be transduced efficiently (>90% of eGFP expression) (data not shown) when the strong ubiquitous promoter (Phospho Glycerato Kinase, PGK, regulatory element) was utilized in place of the Oct4 promoter to control eGFP expression (Figure 3b).

On the basis of eGFP expression, transduced melanoma cells were sorted into two fractions (eGFP+ and eGFP−) that served as targets to assess separately the antitumor activity of patient-derived CIK cells against their own putative mCSCs (eGFP+) and bulk eGFP− melanoma cells (Supplementary Figure 4 a-d). As further evidence of stem cell enrichment within the eGFP+ fraction, we transduced bulk primary melanoma cells with LV-Oct4.eGFP vector, and cultured them in anchorage-independent and serum-free conditions at low cell concentration (10⁴ cells/cm²) to observe the formation of mainly fluorescent (eGFP+) spheroids (Supplementary Figure 4e).

The integration of LV-Oct4.eGFP was confirmed by PCR in both eGFP+ and eGFP− melanoma cell subsets (Figure 3a). Additional evidence that the eGFP+ melanoma cell fraction was enriched in putative mCSCs, LV-Oct4.eGFP transduced cells were evaluated on the basis of ABCG2 expression. The average cell percentage expressing ABCG2 was 10.6 ± 2.3%, 72.4% of which co-expressed eGFP (Supplementary Figures 5 and 6a).

We then measured the distribution of the main NKG2D ligands (MIC A/B, ULBP2) expressed in the target cells for eGFP expression. The percentage of MIC A/B+ or ULBP2+ cells were equally represented in eGFP+ and eGFP− fractions without a statistically significant difference (p=0.5181 and 1.000, respectively) (Supplementary Figure 6).
Through a functional assay we evaluated the proliferation ability of putative mCSCs. Melanoma cells transduced with LV-Oct4.eGFP were stained with PKH26 dye to distinguish the rare quiescent/slowly-dividing cells of putative CSCs from the more differentiated fast-growing population. Cell fluorescence was acquired initially and weekly for 3 weeks. Putative eGFP⁺ mCSCs displayed a proliferative potential in vitro that was, on average, three times less than their eGFP⁻ counterparts after 21 days of culture (n=5), showing a slow-growing phenotype typical of CSCs; a representative histogram is reported in Figure 3c.

Patient-derived CIK cells efficiently killed in vitro autologous eGFP⁺ melanoma cells with an average specific killing of 71 ± 5%, 56 ± 7%, 44 ± 7%, and 40 ± 6% (mean ± sem) at a 40:1, 20:1, 10:1, and 5:1 effector/target ratio, respectively (n=4). Comparable (p=0.8224) tumor killing intensity was reported against autologous eGFP⁻ targets with an average specific killing of 66 ± 6%, 54 ± 8%, 44 ± 10%, and 36 ± 8% at a 40:1, 20:1, 10:1, and 5:1 effector/target ratio, respectively (n=4). The killing activity of CIK cells remained equally effective against both eGFP⁺ and eGFP⁻ autologous melanoma cells (n=5, p=0.6286) even when the assay was performed on total tumor cells without preemptive sorting of putative eGFP⁺ mCSCs. A summary of cytotoxicity against autologous tumor targets is reported in Figure 3d.

Discussion

The present work addresses two main issues regarding the activity of immunotherapy in preclinical models: to show tumor-killing activity towards autologous solid tumor cells; secondly, to demonstrate effective killing of autologous putative cancer stem cells hitting one of the reservoirs responsible for tumor resistance to standard treatments.

For the first time we report the strong preclinical activity of patient-derived CIK cells against autologous mMEL, with insight on their potential to target putative mCSCs. CIK cells represent promise for cancer adoptive immunotherapy, and carry biological features that compare positively with other immunotherapies for reliable and effective clinical translation.

The intense expansibility is the first of such features (13). Our data confirmed that CIK cells from metastatic melanoma patients were expanded at clinically-relevant levels. The simplicity and relative low expense of the expansion protocol, already validated in GMP controlled
conditions (33, 34), may positively impact the clinical perspective. Recent clinical trials with CIK cells have reported encouraging observations in challenging settings like metastatic lung, renal, and gastrointestinal tumors (13, 21, 35-37). However, such trials lack formal demonstration of CIK cell anti-tumor activity against autologous metastatic targets; indeed, no data are currently available for metastatic melanoma.

For 10 different patients, CIK cells efficiently killed autologous mMel cells. Such findings are both novel and potentially relevant clinically. They overcome important limitations linked to the use of commercially available allogeneic tumor cell lines, avoiding confounding results based on alloreactive HLA-mismatches, and allowed full appreciation of the unique biology of each tumor. Furthermore, within each patient, it is possible to hypothesize the existence of additional and important biologic differences between metastases and the primitive tumor, supporting the importance of the observed killing of CIK cells against autologous mMel.

Our in vitro cytotoxicity assays were evaluated conservatively, within a 6 hours experimental timeframe, to favour killing specificity of the extremely delicate autologous tumor targets. Results are indicative of CIK killing capacity but a linear projection and quantification of prospective clinical efficacy is difficult to be predicted. In vivo persistence of patient-infused CIK cells is expected to be about 2 weeks and multiple infusions will be possible based on their intense ex-vivo expansion and production simplicity.

A selected experiment to assess the in vivo activity of patient-derived CIK cells against autologous melanoma targets engrafted into NOD/SCID mice has demonstrated a delayed tumor growth, along with increased extension of necrotic areas and infiltration of CIK cells at tumor sites. This additional work was intended to provide proof of in vivo activity of CIK cells; however, a deeper and more definitive in vivo analysis will require a dedicated study.

The second important feature of CIK cells is their HLA-unrestricted tumor killing, which extends to virtually all patients the possibility to benefit from this approach, regardless of the expression of specific tumor associated antigen (TAA) restricted by precise HLA haplotypes. The mechanistic investigation of CIK cell tumor killing was not the aim of our study; however, we showed the expression of target molecules, recognized by NKG2D receptor, on all mMel primary cell cultures from our patients. The ULPB2 molecule was most consistently represented while more variability among patients was observed for MIC A/B.
Direct expression of MIC A/B molecules has been described on both primary and metastatic melanomas (38, 39). The possible upregulation of NKG2D-ligands in various types of treatment (e.g. chemotherapy, statins, doxycyclin), with a consequent increased susceptibility to MHC-independent immune-mediated lysis, has been described in various experimental models (40-42) and may provide intriguing prospective synergies with immunotherapy.

Downregulation of MHC expression is one of the main immune-escape mechanisms developed by tumor cells. In our study, CIK cells were effective against two MHC class I negative melanoma samples, which confirmed their potential in melanomas with immunogenicity alterations.

Melanoma primary cell cultures were derived from patient tumor biopsies. These cultures retained original tumor characteristics and displayed great immunophenotypic heterogeneity among samples. Most differentiation antigens detected on mMel cells showed variable levels of expression, as others have described (23, 24). By contrast, the expression and average levels of putative stemness markers, Oct4 and ABCG2, as well as the lack of Mitf expression, were quite comparable among different melanoma samples (27, 43-45). Together these data suggest that the cell fraction endowed with stemness features is stably detectable and retained in different samples. This is consistent with the decade-old CSC theory that tumors contain a subset of cells that both self-renew and generate differentiated progeny (9, 46, 47). CSCs are, therefore, the driving force of the tumor.

Truthfully, the identification of molecular and phenotypic markers for CSCs still remains partially unsolved. In fact, CSCs seem to have a dynamic phenotype, more likely as expression of a functional state rather than a precise cellular entity (48, 49). The expression of the Oct4 gene seems able to be reliably associated with cancer cells of various histotypes endowed with stemness features (50-55). Recently, Oct4 expression was correlated to dedifferentiation of melanoma cells, re-acquisition of stem phenotype, increased tumorigenic capacity and resistance to chemotherapy (28). We exploited these observations by designing a gene-transfer strategy to detect mCSCs and assess their susceptibility to CIK-mediated killing. Bulk, patient-derived mMel cells were transduced with a lentiviral vector that encodes eGFP under control of the human Oct4 regulatory element, with the idea that only mCSCs are
able to activate the Oct4 promoter to express eGFP, which allows their specific killing by CIK cells to be tracked and evaluated.

This approach uncovered a small subpopulation of eGFP⁺ putative mCSCs, consistent with the expected rate of mCSCs given detected Oct4 protein. This small fraction appeared to preferentially co-express the stemness marker ABCG2 relative to its eGFP negative counterpart. To identify functionally rare quiescent/slowly dividing CSCs, a lipophilic fluorescent dye, PKH26, was used to visualize relatively quiescent cells within a proliferating population (56). Indeed, eGFP⁺ cells encountered up to 5 cell divisions during a 3-week culture period, while eGFP⁺ cells encountered a maximum of 2 cell divisions in the same elapsed time. Moreover, since CSCs can withstand anoikis, they proliferate/differentiate in anchorage-independent conditions, and give rise to clonal spheroids. Melanoma primary cells after LV-Oct4.eGFP transduction were then cultured in anchorage-independent and serum-free conditions. Only a small fraction of cells retained the ability to grow, and spheroids were generated exclusively from eGFP⁺ cells that maintained the fluorescence even when heterogeneously distributed within the spheres. CIK cells intensely killed the autologous eGFP⁺-sorted fraction at a lysis rate comparable to that observed against eGFP negative tumor targets.

Killing the true melanoma stem cells currently remains an ideal concept. Yet, our data suggest that CIK cells kill a subset of autologous metastatic melanoma cells able to activate Oct4 that, based on current knowledge, reliably defines a subpopulation of tumor cells with stemness features (28). Dedicated studies are required and currently ongoing to investigate deeply the functional and tumorigenic characteristics of eGFP⁺ mCSCs. Nevertheless, our findings provide new and additional weight to the potential of cancer immunotherapy with CIK cells. Data from Kumar et al confirmed that the Oct4 expression correlates with putative CSC features, and that Oct4-expressing cells display a significantly higher chemotherapy agent resistance. Indeed, the observed killing ability of CIK cells against putative mCSCs may reveal other valuable perspectives on the potential of this immunotherapy strategy. Moreover, the elevated safety profile of CIK cells does not preclude their use in association with other approaches. One appealing possibility would be to explore their potential synergism with conventional chemotherapies or even molecular targeted treatments. This strategy could
reduce resistance occurrence by improving the odds of targeting the crucial CSC subset from which tumor re-growth is speculated to start.

The MHC-unrestricted tumor killing mechanism of CIK cells demonstrated in this study may advantage it over other immunotherapy approaches because it addresses the difficult quest of targeting mCSCs and also HLA negative tumors. We showed that the membrane expression of NKG2D ligands is maintained on putative mCSCs. Vice versa, still unknown is whether or not such a peculiar tumor subpopulation retains the same antigenic features (specific TAA or MHC molecule expression), as do other tumor cells.

Overall, demonstrated here, for the first time, is the intense tumor killing activity of CIK cells against autologous mMEL, including putative mCSCs. These data point to CIK cells as favorable candidates for clinical trials in melanoma patients. The biologic basis is set for further preclinical and clinical investigations on the prospective potential of targeting mCSCs with CIK cells, either independently or in synergism with other therapeutic strategies.

Acknowledgements
We are grateful to Dr. W. Cui (IRDB, Imperial College London) who provided the phOCT4.EGFP1 vector and to Dr. E. Vigna (IRCC 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 E. Lantelme for sorting services. The fellowships of L.Gi, M.T., PhD, and Y.P. are sponsored by MIUR (University of Turin) and the fellowship of G.M., PhD is sponsored by an “Associazione Italiana Ricerca sul Cancro–AIRC I.G. grant. N. 11515. This work was supported by grants from "Progetti di Ricerca Rete Oncologia Piemonte-Valle d'Aosta," “Associazione Italiana Ricerca sul Cancro–AIRC I.G. grant. N. 11515, “Associazione Italiana Ricerca sul Cancro–AIRC 5X1000,” and University of Torino-Progetti di Ateneo 2011 grant RETHE-ORTO 11RKTW.

Contributions
L.G. coordinated the experiments, generated primary tumor cell cultures, phenotyped putative mCSCs, participated in study design, and co-wrote this paper. L.Gi ex-vivo expanded CIK
cells, performed the phenotype analysis, and the tumor killing experiments. V.L. generated the lentiviral vector LV-Oct4.eGFP and performed transduction experiments. M.T. and G.M. performed the killing experiments with eGFP⁺-sorted cells and contributed to ex-vivo expansion and phenotyping of CIK cells from patients. M.G.V. and Y.P contributed to generation, culturing, and phenotyping of primary tumor cell cultures. A.P., T.V., and A.B. performed the pathology analysis on patient-collected tumor samples, primary tumor cell cultures, and tumor samples from murine xenografts. F.P., A.Z., and E.G. performed melanoma sample surgical resections, provided clinical data, and contributed to manuscript revision. G.G., F.C.S., D.C., and S.G provided clinical data, participated in study design and contributed to manuscript revision. M.A participated in study design and manuscript revision. D.S. designed the study, participated in experiment coordination, and co-wrote this paper.

References


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$^a$ Biopsy was performed at different metastatic sites including subcutaneous (SC) or lymph node (LN) sites.

$^b$ In vitro Primary Cell Cultures derived from tumor biopsies.

$^c$ CD3$^+$/CD56$^+$ cell number-fold increase after 3 weeks of expansion.

$^d$ Cytotoxicity assay with CIK cells vs autologous tumor target cells.

$^Y$ = yes

$mMel$ samples assessed for tumorigenic capacity in vivo in NOD/SCID mice. The same mMel samples were transduced with LV-Oct4.eGFP to visualize mCSCs, and subsequently were sorted based on eGFP expression.

$^#$ mMel samples used for cytotoxicity assay with CIK cells vs LV-Oct4-eGFP transduced target cells sorted based on eGFP expression.
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*a* Fold Increase (Fl, cell n. T=week 3/cell n. T=0) of total cell number after 3 weeks of expansion.

*b* % of cells expressing different surface antigens at the basal time (T=0).

*c* % of cells expressing different surface antigens after 3 week expansion.

*d* Fold Increase (Fl, cell n. T=week 3/cell n. T=0) of absolute cell number after 3 weeks of expansion calculated for every subpopulation of cells expressing different surface antigens.
Figure Legends

Figure 1. Primary melanoma cultures successfully generate tumor xenografts in-vivo.

a) Primary mMel cell cultures were successfully proved for their tumorigenic activity in-vivo. Representative Hematoxylin and Eosin stained paraffin embedded sections from tumor generated after subcutaneous injection in NOD/SCID mice (1x10^6 mMel cells): mMel1, mMel2, mMel3, mMel4, mMel5, mMel6. Tumor xenograft morphology was consistent with the original human tumor as confirmed by a pathology review. Scale bars, 12 µm.

b) Tumor growth curves of the melanoma cell lines transplanted into the NOD/SCID mice.

Figure 2. In vitro and in vivo activity of CIK cells against autologous melanoma.

a) Patient-derived CIK cells efficiently killed in vitro all bulk autologous melanoma targets (n=35) (left panel); results were comparable with those obtained with allogeneic CIK cells assessed in parallel versus the same tumor cells (n=20) (right panel). Tumor killing was evaluated by flow cytometry assay performed after co-culturing mature CIK cells with PKH-26 stained targets for 4 hours.

b) NOD/SCID mice (n=12) were subcutaneously implanted with an 8 mm^3 tumor fragment of patient-derived melanoma biopsy (mMel2). One week after tumor implantation, 10^7 CIK cells were infused weekly by tail vein injection (n=8). Percentage of tissue necrosis on tumor growth was calculated at the end of the experiment on paraffin-embedded histological sections. The results were expressed by mean±sem and the extension of necrotic areas was analyzed by an unpaired, two-tailed t-test (p=0.0255). (c) At the end of the infusions, infiltration of CIK cells at tumor sites were demonstrated by IHC using antibodies against CD5 and CD56. Scale bars, 12 µm. (d) A significant delay in tumor growth was observed in NOD/SCID treated mice compared to the controls (n=4). Tumor volume increments were expressed as mean±sem and CIK activity was analyzed by the 2-way ANOVA (p=0.0308).

Figure 3. CIK cells efficiently killed autologous melanoma putative mCSC.

(a) Schematic representation of lentiviral vector LV-Oct4.eGFP used to visualize putative mCSC. The presence of integrated LV-Oct4.eGFP was confirmed by PCR in both eGFP+ and eGFP- fractions of freshly sorted cells. Picture shows representative PCR electrophoresis gel (100ng gDNA/each sample) with primers annealing on the lentiviral vector backbone upstream and downstream the Oct4.eGFP expression cassette. (b) Representative eGFP expression in melanoma primary cell cultures transduced with LV-Oct4.eGFP or LV-PGK.eGFP; as positive transduction control, mES cells transduced with LV-Oct4-eGFP (Scale bars, 15 µm). (c) Proliferation assay in vitro was evaluated by staining LV-Oct4.eGFP-transduced tumor cells with the vital dye PKH26, and assessing the fluorescence intensity decrement over time. A representative experiment is reported in figure. (d) The antitumor activity of patient-derived CIK cells was equally intense against autologous Oct4.eGFP+ m-
CSC (n=4); results were comparable to those observed against Oct4.eGFP bulk melanoma cells.
Figure 1

a

mMel1  mMel2  mMel3

mMel4  mMel5  mMel6

b  Melanoma Primary Cell Growth Curves into the NOD/SCID mice

Melanoma Primary Cell Growth Curves into the NOD/SCID mice

- mMel1
- mMel2
- mMel3
- mMel4
- mMel5
- mMel6

Weeks After Tumor Cell Inoculation (1x10^6)

Tumor Volume (cm^3)
Figure 2

CIK in vitro tumor killing activity

(a) Graphs showing specific lysis of CIKs vs autologous melanoma and CIKs vs DettMel at different effector:target ratios.

(b) Bar graph comparing area of tissue necrosis between treated (Autologous CIK) and untreated groups.

(c) Images of Anti-CD5 and Anti-CD56 staining.

(d) Line graph comparing tumor volume over weeks of treatment between CIK treated (1x10^7 CIK/mouse) and untreated groups.
Figure 3

(a) LV-Oct4.eGFP expression cassette

(b) LV-Oct4.eGFP transduced melanoma cells, LV-PGK.eGFP transduced melanoma cells, LV-Oct4.eGFP transduced mES cells

(c) Proliferation Assay

(d) CIK in vitro tumor killing activity

PKH26 Dye Fluorescence
Clinical Cancer Research

Effective Activity of Cytokine Induced Killer Cells against Autologous Metastatic Melanoma including Cells with Stemness Features

Loretta Gammaitoni, Lidia Giraudo, Valeria Leuci, et al.

Clin Cancer Res  Published OnlineFirst June 21, 2013.

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doi:10.1158/1078-0432.CCR-13-0061

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