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

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Abstract

Purpose: We investigate the unknown tumor-killing activity of cytokine-induced killer (CIK) cells against autologous metastatic melanoma and the elusive subset of putative cancer stem cells (mCSC).

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 patients with metastatic melanoma 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 metastatic melanoma (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 metastatic melanoma cultures presented an average of 11.5% /C6 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 with results reported against differentiated metastatic melanoma cells (P = 0.8).

Conclusions: For the first time, the intense killing activity of CIK cells against autologous metastatic melanoma, including mCSCs, has been shown. These findings move clinical investigation of a new immunotherapy for metastatic melanoma, 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 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 metastatic melanoma treatment. Despite some survival advantage, results have almost always been short-lived; it seems that as hypothesized for other tumors, metastatic melanoma 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 the ones that are able to target melanoma cancer stem cells (mCSC).

Much promise lies in adoptive immunotherapy for the treatment of metastatic melanoma as supported by results reported with ex vivo expanded tumor infiltrating lymphocytes (TIL; ref. 10) or T cells engineered with melanoma antigen-specific T-cell receptors (TCR; refs. 11, 12). The extended clinical application of TIL is, however, limited by available, suitably sized resectable tumor lesions, whereas specific human leukocyte antigen (HLA) restriction, complex expansion conditions, and stringent regulatory requirements confound the TCR transfer approach.
Cytokine-induced killer (CIK) cells are ex vivo expanded T-natural killer (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-γ, Ab-anti-CD3, and interleukin (IL)-2 through simple standardized culture conditions (15–18). CIK cell activity is not dependent on HLA restriction; it is mediated mostly by the interaction of its NKG2D receptor with stress-inducible molecules [MHC class I-related chain A (MIC A/B) and UL-16–binding proteins (ULBPs)] on tumor targets (19, 20). Initial clinical trial resulting in various tumor settings are encouraging, but data are generally absent on CIK cell potential activity against autologous metastatic melanoma (13, 14, 21, 22), let alone 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.

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 (CSC). The translational relevance of this work is born 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, chemoresistant- 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.

Materials and Methods

CIK 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; Candidolo, Torino, Italy). All individuals provided their informed consent.

Cultures were started with PBMCs collected from same patients with metastatic melanoma and isolated by density gradient centrifugation using cell separation media Lymphoprep (Sentinel Diagnostic). 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 × 10^6/mL RPMI (Gibco BRL) supplemented with 10% FBS (Sigma) in 1,000 U/mL IFN-γ (PeproTech). After 18 to 24 hours in culture at 37°C and 5% CO₂, 50 ng/mL anti-CD3 antibody (OKT3, PharMingen) and 300 U/mL recombinant human IL-2 (Proleukin, Aldesleukin, Chiron Corporation) 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 mm³ pieces and processed for cell isolation. Tumor tissue was processed by mechanical and enzymatic dissociation (Collagenase Type I, Invitrogen) for 3 hours and then subsequently for an additional 12 hours. Cells were then resuspended in KnockOut Dulbecco’s modified Eagle medium: 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), and 300 U/mL recombinant human IL-2 (Proleukin, Aldesleukin, Chiron Corporation) were added. Fresh medium with IL-2 was added as needed.
**In vivo tumorigenesis**

Four-week-old nonobese diabetic/LtSz-scid/scid [nonobese diabetic/severe combined immunodeficiency (NOD/SCID)] (Charles River) female mice were injected subcutaneously with 10⁶ cells from melanoma primary cultures resuspended in sterile PBS1X and BD Matrigel Basement Membrane Matrix (Becton Dickinson). Tumor growth was monitored weekly with calipers; volume was calculated using formula, \( V = \frac{4}{3} \times \pi \times (\frac{L}{2})^2 \times (L/2) \), where \( L \) is the length and \( W \) the width diameter of the tumor. When the tumor volume reached 2 cm in 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 lentiviral vectors were produced by transient four-plasmid cotransfection into 293T cells as described by Follenzi and colleagues (29). The transfer vector pRRL.sin.PPT.hPGK.eGFP.Wpre (LV-PGK.eGFP) was kindly provided by Dr. Elisa Vigna (Gene Transfer and Therapy, IRCC Candiolo, Torino, Italy) and has been described elsewhere (29).

The phOCT4.EGFP1 vector (30) was provided by Wei Cui (Gene Transfer and Therapy, IRCC Candiolo, Torino, Italy), derived from metastases of malignant melanomas as described elsewhere (31). The allogeneic melanoma cell line (DettMel) and melanoma primary tumor cells. The allogeneic melanoma cell line DettMel was a kind gift from Dr V. Russo (Cancer Gene Therapy Unit, Scientific Institute S. Raffaele, Milan, Italy), derived from metastases of malignant melanomas as described elsewhere (31).

**Melanoma primary cell transduction**

For each lentivector transduction, melanoma primary cells were resuspended in fresh KDMEM-F12 with 10% FBS. Virus-conditioned medium was added at a dose of 400 ng P24/100,000 cells. After 16 hours, cells were washed twice and grown for a minimum of 10 days to reach steady-state eGFP expression and to rule out pseudotransduction before flow cytometry analysis. As a transduction efficiency control, the same melanoma primary cells were transduced with LV-PGK.eGFP. Murine embryonic cells and PBMCs 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 PCR-based amplification of the expression cassette Oct4.eGFP. Genomic DNA was extracted separately from eGFP⁺ and eGFP⁻ cells using a commercial kit (Qiagen). PCR was conducted 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 CCCCAGGAA-TAGAAGA-3’ and LV reverse primer 5’-CCACATAGCC-TAAAAGGAGCA -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 lipophilic 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 labeling vehicle was added to the previously washed cell pellet (es. 1 × 10⁶ cells/0.5 mL) to obtain a 2 × single-cell suspension. A 2 × (4 μmol/L) PKH26 dye solution was prepared and added to 2 × 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 added with 10% heat-inactivated serum (5 minutes at 400 × g). An aliquot of labeled and counted cells was 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 (Cancer Gene Therapy Unit, Scientific Institute S. Raffaele, 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 nonradioactive stain of melanoma target cells was used from PKH26 kit (Sigma-Aldrich) to conduct 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 cocultured with either autologous or allogeneic melanoma primary cells with a 40:1, 20:1, 10:1, and 5:1 effector:target ratio for 2 to 6 hours in 200 μL of medium with IL-2 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) × 100.

**In vivo activity assay**

NOD/SCID (Charles River) 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 1 × 10⁷ mature autologous CIK...
cells resuspended in 1 × 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: \[ V = \frac{4}{3} \pi \times \left(\frac{L}{2}\right)^2 \times \left(\frac{L}{2}\right) \], 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 H&E (Bio.Optica). Immunohistochemical assay was conducted with human anti-CD5 and anti-CD56 (NCMA) antibodies (Novocastra, Leica Biosystem). A certified pathologist evaluated the necrotic areas.

### Statistical analysis

Statistical analysis was conducted using software GraphPad Prism 5. A descriptive statistical analysis of CIK and melanoma primary cell culture median values and ranges, or mean ± SEM, was used as required. Subgroup phenotype and necrotic area extension were compared with the unpaired, two-tailed \( t \) test. The mixed model ANOVA was 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.

### Table 1. Main characteristics of melanoma patients and corresponding samples

<table>
<thead>
<tr>
<th>Subject number</th>
<th>Age/sex</th>
<th>Status</th>
<th>Lesion site</th>
<th>Primary cell culture</th>
<th>CIK cell expansion</th>
<th>Autologous cytotoxicity assay</th>
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<td>mMel1</td>
<td>67/M</td>
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<td>SC</td>
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<td>Y</td>
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Abbreviations: Y, yes; mMel, metastatic melanoma.

*b*Biopsy was conducted at different metastatic sites including subcutaneous (SC) or lymph node (LN) sites.

**in vitro** primary cell cultures derived from tumor biopsies.

*c*CD5~/CD56~ cell number-fold increase after 3 weeks of expansion.

*d*Cytotoxicity assay with CIK cells versus autologous tumor target cells.

*e*mMel samples assessed for tumorigenic capacity in vivo in NOD/SCID mice. The same mMel samples were transduced with LV-Oct4.

eGFP to visualize mCSC and subsequently were sorted on the basis of eGFP expression.

*m*mMel samples used for cytotoxicity assay with CIK cells versus LV-Oct4-eGFP-transduced target cells sorted on the basis of eGFP expression.
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 Fig. S2C and S2D).

All melanoma cell cultures, except 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 show their tumorigenicity. All mice inoculated with 6 different melanoma primary cell cultures produced tumor growth starting between 1 and 8 weeks after injection (Fig. 1).

**Expansion and phenotype of CIK cells**

The in vitro 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-γ, Ab-anti-CD3, and IL-2. CIK cells from all patients were successfully expanded within 3 to 4 weeks of culture.

<table>
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<tr>
<th>Subject number</th>
<th>% MIC</th>
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<th>% ULBP3</th>
<th>% NGFR</th>
<th>% MCSP</th>
<th>% Oct4</th>
<th>% ALDH bright</th>
<th>% ABCG2</th>
<th>% MitF bright</th>
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</table>

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

To test the antitumor 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 showed 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, Fig. 2A and B).

**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 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 Fig. 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 (1 × 107/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 with untreated controls (Fig. 2B; P = 0.0255), and we could confirm the presence of CIK cells infiltrating the autologous tumor (Fig. 2C). Moreover, a significant delay in the tumor growth curve was observed in treated mice compared with untreated controls (P = 0.0305) after two-way ANOVA analysis (Fig. 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; Fig. 3A and B). The average eGFP expression, 7 days after 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 (Fig. 3B), whereas no eGFP expression was detected on differentiated PBMCs from healthy donors transduced with the same vector (LV-Oct4.eGFP). As an additional control, we confirmed that both primary melanoma cell cultures (Fig. 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 used in place of the Oct4 promoter to control eGFP expression (Fig. 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 Fig. S4A–S4D). 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 Fig. S4E).

The integration of LV-Oct4.eGFP was confirmed by PCR in both eGFP⁺ and eGFP⁻ melanoma cell subsets (Fig. 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 coexpressed eGFP (Supplementary Figs. S5 and S6A).

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 Fig. S6).

Figure 1. Primary melanoma cultures successfully generate tumor xenografts in vivo. A, primary metastatic melanoma cell cultures were successfully proved for their tumorigenic activity in vivo. Representative H&E-stained paraffin-embedded sections from tumor generated after subcutaneous injection in NOD/SCID mice (1 × 10⁶ metastatic melanoma 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.
CIK Cells Kill Autologous Melanoma and mCSCs

Table 3. Expansion rate and phenotype characterization of CIK cells

<table>
<thead>
<tr>
<th>Subject number</th>
<th>FI cells</th>
<th>% iCD3</th>
<th>% iCD5</th>
<th>% fCD3</th>
<th>% fCD8</th>
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4Fold increase [FI = (cell number T - week 3)/(cell number T = 0)] of total cell number after 3 weeks of expansion.
5Percentage of cells expressing different surface antigens at the basal time (T = 0).
6Percentage of cells expressing different surface antigens after 3-week expansion.

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 Fig. 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 conducted on total tumor cells without preemptive sorting of putative eGFP+ mCSCs. A summary of cytotoxicity against autologous tumor targets is reported in Fig. 3D.

Discussion

The present work addresses two main issues regarding the activity of immunotherapy in preclinical models: first, to show tumor-killing activity towards autologous solid tumor cells; second, to show 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 metastatic melanoma, with insight on their potential to target putative mCSCs. CIK cells represent promise for cancer adoptive immunotherapy and carry biologic 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 patients with metastatic melanoma were expanded at clinically relevant levels. The simplicity and relative low expense of the expansion protocol, already validated in good manufacturing practice–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 antitumor activity against autologous metastatic targets; indeed, no data are currently available for metastatic melanoma.

For 10 different patients, CIK cells efficiently killed autologous metastatic melanoma cells. Such findings are both novel and potentially clinically relevant. 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 metastatic melanoma.

Our in vitro cytotoxicity assays were evaluated conservatively, within a 6-hour experimental timeframe, to favor 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 (n = 12; left) was comparable with those obtained with allogeneic CIK cells assessed in parallel versus the same tumor cells (n = 8; right). Tumor killing was evaluated by flow cytometry assays conducted after coculturing mature CIK cells with PKH-26–stained targets for 4 hours. B, NOD/SCID mice (n = 12) were subcutaneously implanted with an 8 mm³ tumor fragment of patient-derived melanoma biopsy (mMel2). One week after tumor implantation, 10⁷ 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 histologic sections. The results were expressed by mean ± SEM and the extension of necrotic areas was analyzed by an unpaired, two-tailed t-test (*, P = 0.0259). C, at the end of the infusions, infiltration of CIK cells at tumor sites were shown by immunohistochemistry 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 with the controls (n = 4). Tumor volume increments were expressed as mean ± SEM and CIK activity was analyzed by two-way ANOVA (P = 0.0308).

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); results were comparable with those obtained with allogeneic CIK cells assessed in parallel versus the same tumor cells (n = 20; right). Tumor killing was evaluated by flow cytometry assay conducted after coculturing mature CIK cells with PKH-26–stained targets for 4 hours. B, NOD/SCID mice (n = 12) were subcutaneously implanted with an 8 mm³ tumor fragment of patient-derived melanoma biopsy (mMel2). One week after tumor implantation, 10⁷ 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 histologic sections. The results were expressed by mean ± SEM and the extension of necrotic areas was analyzed by an unpaired, two-tailed t-test (*, P = 0.0259). C, at the end of the infusions, infiltration of CIK cells at tumor sites were shown by immunohistochemistry 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 with the controls (n = 4). Tumor volume increments were expressed as mean ± SEM and CIK activity was analyzed by two-way ANOVA (P = 0.0308).
tumor characteristics and displayed great immunophenotypic heterogeneity among samples. Most differentiation antigens detected on metastatic melanoma cells showed variable levels of expression, as others have described (23, 24). In 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, reacquisition 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 metastatic melanoma 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 coexpress the stemness marker ABCG2 relative to its eGFP− 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, whereas eGFP+ cells encountered a maximum of 2 cell divisions in the same elapsed time. Moreover, as CSCs can withstand anoikis, they proliferate/differentiate in anchorage-independent and serum-free conditions. 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

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 (100 ng 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 fluorescent dye fluorescence intensity decrement over time. A representative experiment is reported in Figure S1. D, the antitumor activity of patient-derived CIK cells was equally intense against autologous Oct4.eGFP+ mCSC (n = 4); results were comparable with those observed against Oct4.eGFP− bulk melanoma cells.
grow, and spheroids were generated exclusively from eGFP<sup>+</sup> cells that maintained the fluorescence even when heterogeneously distributed within the spheres. CIK cells intensively killed the autologous eGFP<sup>+</sup>-sorted fraction at a lysis rate comparable with that observed against eGFP<sup>-</sup> 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 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<sup>+</sup> mCSCs. Nevertheless, our findings provide new and additional weight to the potential of cancer immunotherapy with CIK cells. Data from Kumar and colleagues confirmed that the Oct4 expression correlates with putative CSC features and that Oct4-expressing cells display a significantly higher chemotherapy agent resistance (28). 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 increased 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 regrowth is speculated to start.

The MHC-unrestricted tumor killing mechanism of CIK cells showed 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 NK2G2 ligands is maintained on putative mCSCs. Vice versa, it is still unknown 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, shown here, for the first time, is the intense tumor killing activity of CIK cells against autologous metastatic melanoma, including putative mCSCs. These data point to CIK cells as favorable candidates for clinical trials in patients with melanoma. 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.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Gammaitoni, L. Giraudo, V. Leuci, M. Todorovic, G. Mesiano, F. Picciotto, A. Zaccagna, E. Giacone, G. Grignani, F. Carnevale-Schianca, M. Aglietta, D. Sangiolo

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Gammaitoni

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**References**
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