Cytokine-Induced Killer Cells Eradicate Bone and Soft-Tissue Sarcomas

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Abstract

Unresectable metastatic bone sarcoma and soft-tissue sarcomas (STS) are incurable due to the inability to eradicate chemo-resistant cancer stem-like cells (sCSC) that are likely responsible for relapses and drug resistance. In this study, we investigated the preclinical activity of patient-derived cytokine-induced killer (CIK) cells against autologous bone sarcoma and STS, including against putative sCSCs. Tumor killing was evaluated both in vitro and within an immunodeficient mouse model of autologous sarcoma. To identify putative sCSCs, autologous bone sarcoma and STS cells were engineered with a CSC detector vector encoding eGFP under the control of the human promoter for OCT4, a stem cell gene activated in putative sCSCs. Using CIK cells expanded from 21 patients, we found that CIK cells efficiently killed allogeneic and autologous sarcoma cells in vitro. Intravenous infusion of CIK cells delayed autologous tumor growth in immunodeficient mice. Further in vivo analyses established that CIK cells could infiltrate tumors and that tumor growth inhibition occurred without an enrichment of sCSCs relative to control-treated animals. These results provide preclinical proof-of-concept for an effective strategy to attack autologous sarcomas, including putative sCSCs, supporting the clinical development of CIK cells as a novel class of immunotherapy for use in settings of untreatable metastatic disease. Cancer Res 74(1): 119–29. ©2013 AACR.

Introduction

Bone and soft-tissue sarcomas (STS) are a heterogeneous group of mesenchymal tumors. Regardless their heterogeneity, advanced, not surgically amenable, bone sarcoma and STS are linked by a common dismal clinical prognosis (1–4).

Although new hope has been brought by molecular targeted therapies, results are still insufficient. Indeed, metastatic and unresectable diseases remain incurable with less than 10% of the patients alive after 5 years (3, 5). As new approaches are urgently needed, adoptive immunotherapy is considered a promising option to be explored (6–8). In this scenario, reliable patient-specific preclinical models are crucial to provide basis for an effective clinical translation of this strategy.

Key and open issues faced by cancer immunotherapy research models are (i) the possibility to dispose of autologous tumor targets, (ii) expansion of “clinical relevant” numbers of immune effectors, (iii) restrictions to specific human leukocyte antigen (HLA) haplotypes when targeting tumor-associated antigens (TAA), and (iv) the possibility to kill putative cancer stem cells (CSC) considered responsible for tumor relapses and chemoresistance. The frequent use of commercially available allogeneic tumor cell lines is a useful tool to generate proof of concept but cannot account for the individual biologic and immunogenic variability properties of each patient and his/her tumor. On the basis of these considerations, we set up a preclinical HLA-unrestricted autologous immunotherapy model for the treatment of bone sarcoma and STS, based on cytokine-induced killer (CIK) cells, and dedicated specific insights to the ability of such immunotherapy to kill putative sarcoma CSCs (sCSC).

CIK cells are heterogeneous ex vivo expanded T lymphocytes generated from peripheral blood precursors (9, 10). They present a mixed T/NK phenotype and are endowed with MHC-unrestricted antitumor activity (11). The great ex vivo expansibility and the absence of specific MHC restrictions are crucial characteristics that favor CIK cells over conventional cytotoxic T lymphocytes (12–15).

CIK cells have been reported to exert high in vitro antitumor activity against several solid tumor cell lines (14, 16–21), and initial encouraging data were translated in recent clinical trials (22–33). Tumor-killing activity of CIK cells is mainly mediated...
by the interaction of their membrane receptor NKG2D with stress-inducible molecules, MHC class I-related chain A and B (MIC A/B) and UL16–binding proteins (ULBP), on target cells (34, 35). The expression of MIC A/B and ULBP has been described in several epithelial tumors, but data on mesenchymal tumors of various histotype are missing (36–39). The antitumor activity of CIK cells against autologous bone sarcoma and STS is currently not explored, nor is their ability to kill putative sCSCs.

The phenotype and precise definition of CSCs is currently an object of intense research and final consensus has not been reached (40–44). Besides the presence of various membrane markers, alternatively associated with stemness features, peculiar genetic signatures have been reported to characterize putative CSCs with the re-expression of stem genes like Oct3/4, NANOG, SOX typically activated within normal stem cells (45–47). Recent experimental evidences confirmed that Oct4 expression able to induce dedifferentiation, stemness phenotype, and tumor-initiating features in cancer cells, including sarcomas (47, 48).

We developed a gene transfer strategy to visualize and track putative sCSCs exploiting the selective ability of stem cells to activate the stemness gene Oct4. In our study, we report for the first time the effective preclinical antitumor activity of patient-derived CIK cells against autologous bone sarcoma and STS of various histotype, including evidence of killing putative sCSCs.

Materials and Methods

Ex vivo expansion and phenotype of CIK cells

CIK cells were expanded from peripheral blood collected from patients with histologic confirmed bone sarcoma and STS at our Center. All individuals provided informed consent for blood donation according to a protocol approved by the internal review board and ethic committee.

Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation (Lymphoprep, Sentinel Diagnostic) and seeded in cell culture flasks at a concentration of 1.5 × 10^6 cells/mL in RPMI-1640 medium (Gibco BRL) consisting of 10% FBS (Sigma), 100 U/mL penicillin, and 100 U/mL streptomycin (Gibco BRL). IFN-γ (PeproTech; 1,000 U/mL) was added on day 0, after 24 hours of interleukin (IL)-2 (Proleukin, Aldesleukin, Chiron Corporation) and anti-CD3 antibody (OKT3, Pharmingen) were added at a concentration of 300 U/mL and 50 ng/mL, respectively. Cells were expanded over 3 weeks of time period. Fresh medium and IL-2 (300 U/mL) were added weekly (every 3 days) during culture, and the cell concentration was maintained at 1.5 × 10^6 cells/mL. Phenotype of CIK cells was weekly analyzed by standard flow cytometric assays. The following monoclonal antibodies (mAb) were used: CD3–FITC, CD4–PE, CD56–APC, CD8—PE, and CD314–APC (anti-NKG2D) (mAbs were Miltenyi Biotec).

The projected dose of CIK cells/kg for each patient was obtained assuming a theoretic basal concentration of 3 × 10^8 mononuclear cells per patient according to the formula: (3 × 10^8 × observed fold increase)kg of body weight.

Primary cell cultures of bone sarcoma and STS

Human tumor samples were obtained from surgical specimens; patients provided consent under institutional review board–approved protocols and investigations were conducted after approval by a local Human Investigations Committee. Approximately 10 mm^3 of each tissue sample was mechanically dissociated with surgical blade, digested in collagenase I (200 U/mL, Invitrogen), and incubated for 3 hours at 37°C. At the end of this first step of digestion, single-cell suspension was recovered and seeded in culture both in attachment and ultralow attachment. The debris were submitted to an additional collagenase digestion at 37°C overnight. At the end of enzymatic digestion, single-cell suspension was recovered. Finally, cells were resuspended in KO DMEM F12 (KO Out Dulbecco’s Modified Eagle Medium, Gibco BRL) medium with 10% FBS and plated at clonal density (10^5 cells per cm^2) in 6-well plates (Corning). An additional aliquot of cells were seeded at 10,000 cells per cm^2 in ultralow attachment 24-well plates (Corning) in KO DMEM/F12 medium with 10% FBS.

Cell aliquots were stained with fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE–Cy5an 7 (PC7), or allophycocyanin (APC)-conjugated mouse mAbs against HLA-ABC (anti-HLA-ABC–FITC, BD Pharmingen) and CIK target antigens (anti-MIC A/B, BD Pharmingen; anti-ULBP, R&D System, Space Import Export). Intracellular expression of Oct4 was detected after fixation/permeabilization by the Cytoperm/Cytofix Kit according to the manufacturer’s instructions (BD Biosciences Pharmingen). ALDH activity was evaluated by ALDEFLUOR assay kit (Aldagen, Stemcell Technologies), according to manufacturer’s instructions. Stro-1 expression was detected by flow cytometric staining tumor cells with APC-conjugated anti-Stro-1 monoclonal antibody (Biolegend). Labeled cells were read on a FACS Cyan (Cyan ADP, Dako) and analyzed using Summit Software. Gate criteria were set according to isotype controls.

For histology analysis, mesenchymal primary cell cultures were washed in PBS 1× and cytopsins were prepared onto slides (100,000 cells per slide in 100 μL of PBS 1×) at 1,500 rpm for 5 minutes using a Shandon Cytocentrifuge Cytospin 2. Following air drying, slides were fixed in methanol for 30 minutes. Cytopsins were stained using May Grunwald–Giemsa stain (Merck) stained for 5 minutes in May-Grunwald and 20 minutes in Giemsa.

Allogeneic tumor cell lines

All cell lines [MNNG-HOS and Sjsa 1 osteosarcoma cell lines and MES-SA leiomyosarcoma cell lines, American Type Culture Collection (ATCC)] used in this study were grown in RPMI-1640 supplemented with 10% FBS (Sigma), 25 mmol/L HEPES, 100 U/mL penicillin, and 100 U/mL streptomycin (Gibco BRL) in a humidified 5% CO₂ incubator at 37°C. To authenticate sarcoma cell lines, genotype analysis of MNNG-HOS, Sjsa 1, and MES-SA cell lines was conducted and confirmed by Cell ID system (Promega) comparing their profile with those published in the DMSZ database.

Cell aliquots were stained with FITC, PE, PC7, or APC-conjugated mouse mAbs against CIK-target antigens (anti-MIC A/B, BD Pharmingen; anti-ULBP, R&D System, Space Import Export).
In vivo tumorigenesis of patient-derived primary cell cultures

Non-obese diabetic/LtSz-scid/scid (NOD/SCID; Charles River) female mice were subcutaneously injected with 1 × 10^6 primary bone sarcoma and STS cells (n = 5), resuspended in sterile PBS and BD Matrigel Basement Membrane Matrix (Becton Dickinson) 1:1. Mice were housed in filtered cages under specific pathogen-free conditions and permitted unlimited access to food and water. Tumor growth was monitored weekly with a caliper, and the volume was calculated using the following formula: \( V = \frac{4}{3} \pi \left(\frac{a}{2}\right)^2 \left(\frac{b}{2}\right) \), where \( a \) is the length and \( b \) is the width diameter of the tumor. When the tumor reached 2 cm in the main diameter, the animal was euthanized and the tumor was recovered and fixed overnight in 4% paraformaldehyde, dehydrated, paraffin-embedded, sectioned (5 μm), and stained with hematoxylin and eosin (H&E.; Bio Optica).

Generation of hOct4.eGFP lentiviral vector and tumor cell transduction

VSV-G pseudotyped third-generation lentiviral vectors were produced by transient 4-plasmid co-transfection into 293T cells as described (Follenzi 2000). The transfer vector pRRL.sin.PPT.hPGK.eGFP.Wpre (LV-PGK.eGFP) was kindly provided by Dr Elisa Vigna and described elsewhere (49).

The phOct4.EGFP1 vector (50) was kindly provided by Wei Cui (IRDB, Imperial College London). The pRRL.sin.PPT.hOct4.eGFP.Wpre (LV-Oct4.eGFP1) was obtained by replacing the expression cassette hPGK.eGFP into LV-PGK.eGFP with the hOct4-eGFPPL, cleaved from phOct4-eGFP1 vector, by insertion into Sall and Xhol restriction enzyme sites.

Physical titers for lentiviral vector stocks were determined on the basis of p24 antigen content (HIV-1 p24 ELISA kit; PerkinElmer).

For each lentiviral vector transduction, bone sarcoma and STS primary cells were resuspended in fresh medium with 10% FBS. Virus-conditioned medium was added at dose of 400 ng P24/100,000 cells. After 16 hours, cells were washed twice and further incubated with 4% paraformaldehyde, dehydrated, paraffin-embedded, sectioned (5 μm), and stained with hematoxylin and eosin (H&E.; Bio Optica).

In vitro proliferation assay

To evaluate the proliferation rate of eGFP^+ versus eGFP^- fractions, cells were labeled with lipophilic dye PKH26 according to manufacturer's instructions (PKH26GL kit, Sigma). Baseline fluorescence level was analyzed by flow cytometry. Labeled cells were seeded in culture, and after 7 and 14 days, reduction in fluorescence intensity was quantified by flow cytometry.

Analysis of LV-Oct4.eGFP in both eGFP-positive and -negative cell fractions

Detection of LV-Oct4.eGFP in freshly sorted cells was verified by a PCR-based amplification of the expression cassette Oct4.eGFP. gDNA was extracted separately from eGFP^+ and eGFP^- cells using a commercial kit (Qiagen). PCR reaction was conducted using 100 ng of gDNA for each sample and Phusion High-Fidelity DNA Polymerase (Thermo Scientific) according to the manufacturer's protocol.

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

Cytotoxic activity of patient-derived CIK cells

The tumor-killing ability of patient-derived CIK cells was assessed in vitro against primary bone sarcoma and STS cells obtained from patient tumor samples, whenever available, or against allogeneic tumor cell lines (Sjas 1, MNNG HOS, MES-SA) obtained from ATCC.

Target cells were stained with PKH26 Red Fluorescent Cell Linker kit ( Sigma) or with the vital dye CFSE (5-b-carboxylfluorescein diacetate succinimidyl ester; Molecular Probes) in accordance with the manufacturer's protocol. The immunemediated killing was determined evaluating cell viability by flow cytometry (Cyan ADP, Dako), after 6-hour incubation with expanded CIK cells at various effectors/target ratios (40:1, 20:1, 10:1, and 5:1 for 6 hours in 200 μL of culture medium with 300 U/mL IL2 at 37°C, 5% CO2), according to the formula: experimental – spontaneous mortality/(100 – spontaneous mortality) × 100. Killing against sCSCs was similarly evaluated against primary tumor target cells preventively engineered with LV-Oct4.eGFP to visualize putative sCSC. Cytotoxicity was selectively calculated evaluating the decrease of viable eGFP^+ target cells, following the addition of CIK cells, compared with untreated controls. In selected experiments, we preincubated CIK cells with 20 μg/mL of inhibitory anti-NKG2D neutralizing antibody (Clone #149810, R&D Systems) that was maintained during the cytotoxicity assay against autologous targets.

In vivo activity of patient-derived CIK cells

NOD/SCID (Charles River) female mice were subcutaneously injected with 10^6 primary cells of patient-derived pleomorphic sarcoma, resuspended in sterile PBS and BD Matrigel Basement Membrane Matrix (Becton Dickinson) 1:1. Starting 4 days after tumor implantation, mice received 9 weekly intravenous infusions with 1 × 10^7 mature autologous CIK cells resuspended in PBS (200 μL). Mice injected with PBS only were used as control. Tumor growth was weekly monitored with a caliper and volume calculated according to the formula: \( V = \frac{4}{3} \pi \left(\frac{a}{2}\right)^2 \left(\frac{b}{2}\right) \), where \( a \) is the length and \( b \) is the width diameter of the tumor. Animals were euthanized when tumor reached 2 cm in the main diameter. Recovered tumor was fixed overnight in 4% paraformaldehyde, dehydrated, paraffin-embedded, sectioned (5 μm), and stained with hematoxylin and eosin (H&E.; Bio Optica). Immunohistochemical assay was conducted with human anti-CD5 antibody. Animal experiments were approved by internal review board.
Evaluation of Ki-67 expression was conducted on all 12 tumor samples explanted from treated and untreated mice. Tumor slides were incubated with monoclonal mouse anti-human Ki-67 (Dako, Agilent Technologies Company; 1:100) overnight at 4°C. After washings in TBS, anti-mouse secondary antibody (Dako Envision+ System-herosherald peroxidase-labeled polymer, Dako) was added for 1 hour. Immunoreactivities were revealed by DAB chromogen (Dako Cytomation Liquid DAB Substrate Chromogen System, Dako).

To evaluate the in vivo activity of CIK cells against autologous putative sSCCs, we transduced sarcoma cells derived from all 12 explanted tumors, cryopreserved at the end of the experiment described above, with the CSC detector vector LV-OCT4.eGFP. Lentiviral transduction was conducted in parallel in all samples from treated and control mice with the same modalities described above. The percentage of residual EGFP+ putative sSCC was analyzed by flow cytometry 7 days after transduction.

Statistical analysis
As descriptive statistical analysis, medians and ranges, mean ± SEM were used as appropriate. The mixed model ANOVA was used to compare antitumor activity curves in vitro and in vivo. Mean eGFP values and Ki-67 expression between tumors from treated and untreated mice were compared by unpaired t test. Mean tumor-specific lysis of CIK cells with and without neutralizing anti-NKG2D was compared by paired t test.

Statistical significance has been expressed as true P value. All P <0.05 were considered statistically significant. Statistical analysis was conducted using the software GraphPad Prism 5.

Results
Expansion and phenotype of CIK cells
We evaluated the ex vivo expansion of CIK cells from 21 patients with a diagnosis of advanced or metastatic bone sarcoma or STS [osteosarcoma, n = 7; leiomyosarcoma, n = 2; rhabdomyosarcoma, n = 2; liposarcoma, n = 2; gastrointestinal stromal tumor (GIST), n = 2; undifferentiated pleomorphic sarcomas, n = 6]; CIK cells were expanded from fresh or cryopreserved PBMCs cultured with the timed addition of IFN-γ, Ab-anti-CD3, and IL-2. CIK cells were successfully expanded from all patients within 4 weeks of culture; the median expansion of bulk CIK cells, calculated on the total CD3+ fraction, was 52-fold (range, 3–924), whereas 179-fold expansion (18–3968) was obtained for the CD3+CD56+ cell fraction. The presence of pure NK (CD3+CD56+) cells was negligible, median 0.74% (0.1%–3%) at the end of the expansion. The subset of mature CIK cells co-expressing CD3 and CD56 molecules (CD3+CD56+) was present with a median of 97% (range, 40%–99%) of total bulk CIK cells were CD8+. The median membrane expression of the NKGD2 receptor, main responsible for tumor recognition, on expanded CIK cells was 89% (55%–97%). To simulate a real clinical scenario, we evaluated the theoretic dose of CIK cells/Kg that our patients would realistically receive, based on their individual ex vivo expansion rate. Considering a realistic but conservative dose of 3 × 10^8 PBMCs collected at day 0, the average dose per patient at the end of the ex vivo expansion would have been of 4.24 × 10^8 CIK cells/kg (SEM: 1.9 × 10^8). This dose of CIK cells is compatible with values so far adopted in phase I and II clinical studies with CIK cells (31).

A summary of patients’ characteristics and relative CIK expansion data is reported in Table 1.

Patient-derived primary cell cultures of bone sarcoma and STS
In 8 cases, we successfully generated primary tumor cell cultures (osteosarcoma, n = 2; liposarcoma, n = 1; undifferentiated pleomorphic sarcomas, n = 3; GIST, n = 1; leiomyosarcoma, n = 1) from sample biopsies of metastatic (n = 5) or primary tumor (n = 3) sites that served as targets to assess the antitumor activity of autologous CIK cells. All cell cultures displayed morphologic features consistent with the corresponding original tumors as confirmed by pathologic evaluation. A representative picture of primary tumor cell cultures is shown in Fig. 1A–F. MIC A/B, main known ligands recognized by CIK cells, were highly expressed only on osteosarcoma cells whereas less present or practically absent on STS. Analysis for ULBPs 1, 2, and 3 displayed ubiquitous and predominant expression of ULBP2 compared with all the other molecules (P <0.05). Overall, the median expression of MIC A/B was 38% (range, 3%–99%), whereas median values for ULBPs 1, 2, and 3 were 1% (0%–10%), 97% (31%–99%), and 8% (0%–77%), respectively. Detailed expression values of MIC A/B and ULBPs for each histotype are reported in Table 1.

All tumor cell cultures were confirmed to retain the expression of HLA class-I.

Primary metastatic bone sarcoma and STS cells from in vitro cultures were proved able to generate tumor xenografts when inoculated subcutaneously into immunocompromised (NOD/SCID) mice (n = 5). Tumor xenografts developed within 3 to 6 weeks, displaying morphologic and architectural features typical of the original tumor as confirmed by pathologic review. For example, in the xenograft derived from metastatic osteosarcoma cells, the production of abundant osteoid matrix was observed as typical feature of this histology. A representative picture of primary tumor cell cultures and osteosarcoma xenograft is shown in Fig. 1G.

In vitro and in vivo tumor-killing activity of CIK cells against bone sarcoma and STS cells
To test the antitumor activity of CIK cells expanded from all our patients, we started evaluating their ability to kill in vitro allogeneic bone sarcoma and STS cell lines (MNNG-HOS; SJSA 1; MES-SA). The cytotoxicity test was conducted at the end of the ex vivo expansion and showed an efficient killing even if variable among patients. The mean specific tumor killing was 81% (76%–61%), and 44% at 40:1, 20:1, 10:1, and 5:1 effector/target ratio, respectively (n = 24, Fig. 2).

Next, we assessed the ability of patient-derived CIK cells to kill autologous targets from all the 8 primary tumor cell cultures. Results showed mean specific killing of 83%, 79%, 74%, and 62% at a 40:1, 20:1, 10:1, and 5:1 effector/target ratio, respectively (n = 15). The intensity of killing against autologous targets was comparable among different histotype and was not...
CIKs Kill Autologous Sarcomas and Putative Cancer Stem Cells

### Table 1. Characteristics of patients, CIK cells, and primary tumor cell cultures

<table>
<thead>
<tr>
<th>Pts. (UPN)</th>
<th>Diagnosis</th>
<th>Age, y</th>
<th>Availability of autologous tumor samples</th>
<th>MIC A/B (%)</th>
<th>ULBP2 (%)</th>
<th>ULBP3 (%)</th>
<th>Final rate of CD3+CD56+ CIK cells (%)</th>
<th>Ex vivo expansion of CIK cells (fold)</th>
<th>Projected dose of CIK cells/kg</th>
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<td>12</td>
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NOTE: "y" indicates patients for which tumor samples were available and primary tumor cell cultures generated in vitro. The expression of NKG2D ligands (MIC A/B, ULBPs 1–3) was evaluated on early established primary tumor cell cultures. Abbreviation: N/A, not available.

*Fold expansion is calculated for bulk CIK cells, intended as proliferation of CD3+ cells.

Table 1. Characteristics of patients, CIK cells, and primary tumor cell cultures.

Inferior to that observed with allogeneic CIK cells assessed in parallel versus the same primary tumor cells (n = 8, Fig. 2), P > 0.05. In selected experiments (n = 5), we observed that addition of anti-NKG2D neutralizing antibody (20 μg/mL) partially inhibited tumor killing, 47% and 42% average inhibition (P = 0.003) at 40:1 and 5:1 effector/target ratio, respectively (P = 0.003), compared with controls (Fig. 3).

To more closely simulate the real clinical situation, we tested the activity of patient-derived CIK cells in vivo against autologous tumor xenografts in NOD/SCID mice.

Mice were implanted with primary cells of metastatic pleomorphic sarcoma and received 9 weekly intravenous infusions of mature autologous CIK cells (107 per week). A significant reduction of tumor growth was observed in treated mice (n = 6) compared with untreated controls (n = 6) P = 0.017, mean volumes at the end of experiment were in treated mice 807 mm3 (SEM, 138) versus 1,702 mm3 (SEM, 441) of controls (P < 0.0001, Fig. 4A). Evaluation of residual proliferative index, by Ki-67 analysis, on residual tumor samples explanted at the end of the experiment revealed a significant reduction in treated mice compared with controls (mean, 4.5; SEM, 1.2 vs. 9.3; SEM, 0.5; P = 0.009, Fig. 4B). These last data are consistent with recent observation from immunotherapy clinical trials, requiring additional metabolic or histologic data beyond the conventional Response Evaluation Criteria in Solid Tumors (RECIST) to evaluate clinical responses.

At the end of the experiment, we confirmed the presence of CIKs infiltrating the autologous tumor (Fig. 4C).

**CIK cells effectively kill putative sarcoma cancer stem cells**

To identify putative sCSCs, we transduced bulk primary bone sarcoma and STS cells with a "CSC detector" made by a lentiviral vector encoding the eGFP regulated by the human Oct4 promoter (Fig. 5A). The underlying idea is that sCSCs can be visualized on the basis of their exclusive ability, property of both normal and cancer stem cells, to activate the Oct4 promoter and consequently express eGFP. The average presence of eGFP+ putative sCSC, within the bulk metastatic cell assessed 7 days after transduction, was 14.3% (SEM, 2.5; n = 7; Fig. 5B); data were consistent with mean levels of Oct4 protein expression (8.2%; SEM, 4.2). As other putative stemness markers, we observed 4% (SEM, 1) average expression of Stro-1 and 8.4% (SEM, 1) of ALDH activity; representative plots of Oct4, Stro-1, and ALDH activity are reported in Fig. 5C. As positive control, a murine embryonic cell line expressing Oct4 (mES) was successfully transduced with LV-Oct4-eGFP up to 90.5%, whereas no eGFP expression...
was detected on differentiated PBMCs from healthy donors transduced with the same vector. In a selected experiment, it was possible to separate by laser sorting the eGFP\(^+\) and eGFP\(^/-\) cell fractions confirming the integration of LV-Oct4-eGFP in both cell subsets. As additional control, we confirmed that primary bone sarcoma and STS cells could efficiently be transduced (>90% of eGFP expression) when the strong ubiquitous promoter (Phospho Glycerato Kinase, PGK, regulatory element) was used in place of the Oct4 promoter in controlling eGFP expression (Fig. 5B).

Putative sCSCs displayed a proliferative potential \textit{in vitro} that was on average 3 times lower compared with their eGFP\(^/-\) counterpart after 14 days of culture (\(n = 6\)), showing a slow-growing phenotype typical of CSCs; a representative histogram is reported in Fig. 5D.

CIK cells efficiently killed sCSCs; the average specific killing was 79%, 73%, 68%, and 67% (\(n = 7\)) at 40:1, 20:1, 10:1, and 5:1 effectors/target ratio, respectively. The specific killing against sCSCs overlaid that observed against differentiated eGFP\(^/-\) metastatic cells (\(P = 0.88\); Fig. 6A). As initial evidence of \textit{in vivo} activity of CIK cells against putative sCSCs, we evaluated the presence of residual putative eGFP\(^+\) sCSCs from all tumor samples explanted at the end of the \textit{in vivo} experiment described above. We did not observe an enrichment of

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**Figure 1.** Primary bone sarcoma and STS cell cultures. Primary tumor cell cultures were obtained from biopsies of metastatic bone sarcoma and STS. Representative pictures from cultures of pleomorphic undifferentiated sarcomas (A–C), leiomyosarcoma (D), GIST (E), osteosarcoma (F) are shown. Common features were high cellularity, elevated pleomorphism, abundant cytoplasm, voluminous nuclei with clumped chromatin and evident nucleolus. Numerous mitotic figures are observed. Primary tumor cell cultures were confirmed to be able to generate tumors \textit{in vivo} when implanted subcutaneously into NOD/SCID mice (\(n = 4\)). G, a representative picture of osteosarcoma generated \textit{in vivo}.

**Figure 2.** CIK cells efficiently kill autologous metastatic bone sarcoma and STS cells. Patient-derived CIK cells efficiently killed autologous metastatic bone sarcoma and STS cells generated from fresh biopsies. The specific killing against autologous tumors was at least as effective as that observed against allogeneic targets. Mean ± SEM of tumor-specific killing from all experiments are reported.

**Figure 3.** Blocking of NKG2D receptor reduces tumor killing activity. Blocking of NKG2D receptor with neutralizing monoclonal antibody resulted in significant reduction of tumor killing (\(n = 5\)). Mean ± SEM of tumor-specific killing with (gray bars) and without (black bars) addition of anti-NKG2D Ab is reported.
CIKs Kill Autologous Sarcomas and Putative Cancer Stem Cells

Discussion

Our study reports for the first time the intense preclinical activity of CIK cells against autologous metastatic bone sarcoma and STS, including killing of putative sCSCs. All our data are generated within a patient-specific autologous model, with the intent to account for the intrinsic biologic variability property of each tumor and single patient.

Overall the study reflects the potentials of this adoptive immunotherapy strategy, providing elements to discuss the realistic prospective and limitations of future clinical applications.

In our work, CIK cells were efficiently ex vivo expanded directly from PBMCs of patients with various histotype of bone sarcoma and STS. Previous or even concomitant conventional chemotherapy treatments did not affect the expansion rates, phenotype or functionality of CIK cells; values were consistent and comparable with those previously published by our and other groups (10, 14, 31). Simulating a projected dose of CIK cells/kg for each of our patients, calculated on our experimental data, we obtained average values compatible with those so far used in clinical trials (31). Furthermore, the simplicity and relative cost effectiveness of the procedure would allow patients with lower expansion rates to undergo repeated blood collections for multiple ex vivo expansion cycles. Recent clinical trials are supporting the potential of CIK cells in the treatment of advanced solid tumors; however, data of activity against bone sarcoma and STS are missing.

In general, preclinical studies have a key role in providing reliable biologic basis for clinical applications. The complex interaction between immune effectors and tumor cells is regulated by biologic and immunologic elements, partially unknown, that are specific and unique for each patient. A first report from Kuci and colleagues nicely provided evidences of CIK cells activity against rhabdomyosarcoma (20). In that work, allogeneic cell lines were used as targets. We provided activity data within a new-generation, patient-specific model where patient-derived CIK cells intensely killed autologous metastatic tumor cells. The killing was not different among various histotype of bone sarcoma and STS.

Furthermore, we posed a dedicated and innovative question whether CIK cells might be able to kill a peculiar subset of putative sCSCs. To this aim, we developed a new methodology to visualize putative sCSCs based on a lentiviral "CSC detector" vector encoding the eGFP protein controlled by the promoter of stem gene Oct4. With this strategy, we visualized a small fraction of putative sCSCs, endowed with a low-proliferating phenotype and could provide a formal demonstration of killing by autologous CIK cells in vitro. Within our in vivo model, we did not detect sCSC enrichment in tumor samples explanted from treated mice compared with untreated controls suggesting that in vivo activity of CIK cells is capable to involve putative sCSCs. We observed a reduction of eGFP+ sCSCs in treated mice compared with controls, but a relative reduction was detected instead (mean, 5.04%; SEM, 4.19; Fig. 6B). A representative plot of residual eGFP+ sCSCs in treated mice compared with untreated controls is shown in Fig. 6C.

eGFP+ sCSCs in treated mice compared with controls, but a relative reduction was detected instead (mean, 5.04%; SEM, 4.19; P = 0.004; Fig. 6B). A representative plot of residual eGFP+ sCSCs in treated mice compared with untreated controls is shown in Fig. 6C.
concluding for a preferential sCSC killing \textit{in vivo}. More dedicated studies are required in this direction. Of course we cannot claim a definitive identification of sCSCs. This was not the goal of our study and the issue is still debated despite ongoing and numerous efforts by many research groups. Our data however may have prospective clinical relevance, based

\begin{figure}[ht]
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\caption{Visualization of putative sCSCs. We engineered a “CSC detector” lentiviral vector (LV-Oct4-GFP) where eGFP is encoded under control of Oct4 promoter (A). The average presence of eGFP$^+$ CSC within OS and STS cells was 14.3\% SEM 2.5 ($n = 7$). B, a representative microscopy picture and flow cytometric plot. The average flow cytometric expression of Stro-1 and ALDH activity was 4\% (SEM, 1) and 8.4\% (SEM, 1), respectively. C, a representative plot is reported with correspondent negative controls shown on the left. eGFP$^+$ sCSCs displayed a slow-growing phenotype, average 3 times less compared with eGFP$^-$/counterpart after 14 days of culture ($n = 6$). D, a representative experiment of proliferation assay.}
\end{figure}

\begin{figure}[ht]
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\caption{CIK cells are active against putative sCSCs. Patient-derived CIK cells efficiently killed eGFP$^+$ sCSCs ($n = 7$) \textit{in vitro}. The specific killing overlaid that observed against differentiated GFP$^+$ target cells. Results were reproducible against autologous ($n = 3$) and allogeneic ($n = 4$) samples. Means of tumor-specific killing ± SEM are reported (A). \textit{In vivo}, we evaluated the residual presence of sCSC by transduction of all explanted tumors, at the end of the experiment, with the CSC detector vector (LV-OCT4.eGFP). We did not observe any relative enrichment of eGFP$^+$ sCSCs in treated mice compared with controls but a relative reduction was detected instead (B). C, a representative plot of residual eGFP$^+$ sCSCs in treated mice compared with untreated controls.}
\end{figure}
on the rationale that CSC are implicated in tumor relapse and drug resistance. Their potential targeting should now since be considered when evaluating the power of a given experimental antitumor strategy.

A general issue to improve the quality of preclinical models is the choice of targets that could be as representative as possible of real clinical situations. Metastases may display important biologic and immunogenic differences compared with the primitive tumors. In the hypothesis of a clinical application of CIK cells, patients will certainly have advanced metastatic diseases and our model, based on targets that mainly included metastatic samples, may provide additional valuable information in this perspective.

The membrane expression of main ligands recognized by CIK cells have not been yet fully described on metastatic mesenchymal tumors. MIC A/B molecules have been reported to be present in almost all types of epithelial tumors, we observed minimal values in STS and only osteosarcoma cells displayed high expression of these molecules.

Interestingly, all types of STS expressed very high levels of ULBP2, justifying, at least in part, the intense killing by CIK cells. MIC A/B and ULBPs are the main, but not exclusive, ligands recognized by CIK cells; other molecules may be implicated, accounting at various levels for the observed tumoricidal effect. This could explain the significant reduction but not abrogation of cytotoxicity observed blocking the NK2D receptor on CIK cells in our study. It was not the aim of this study to investigate in detail the mechanisms underlying the tumoricidal effect of CIK cells; the issue is however of potential clinical relevance deserving dedicated investigations, a more complete definition of all tumor ligands, their setting of expression and different role in mediating the cytotoxicity of CIK cells may help the identification of subsets of patients that could better benefit from CIK-based immunotherapy approaches.

The activity of patient-derived CIK cells was confirmed in vivo against autologous STS xenografts, displaying the ability of CIK cells to localize at tumor site. The reported curves of tumor growth may however appear somehow discouraging, without a real tumor regression, and raise concerns about the real efficacy of clinical applications of this approach. To this regard, at least 2 considerations may be done. First, initial clinical trials with immunotherapy strategies showed that dimensional parameters, like RECIST, may not be the optimal method to appreciate clinical responses and exploration of additional metabolic and/or histologic criteria are warranted and under investigation. Supporting this evidence, we observed a significant reduction of Ki6-7 expression in tumor samples from treated mice compared with untreated controls. Second, our preclinical model is representative of a bulky disease, a realistic clinical setting for many experimental trials but not the most suitable to detect the real efficacy of immunotherapy strategies. Treatments in limited stages or even without evident disease (e.g., adjuvant treatments after surgical resection) would reasonably give the best results, and future trials, including those with CIK cells, as well experimental models should evolve in such direction. The decision to conduct 9 adoptive infusions was only based on the availability of patient-derived CIK cells that limited the set up of dose–response curves. In clinical prospective, considering CIK cell’s safety profile, it seems reasonable that multiple infusions should be pursued to provide a better effect. We acknowledge that the experimental size may appear limited. Using autologous biologic samples, we had to respect a limited size imposed by the restrict quantity of blood donated upfront from patients who were no more available over time for further blood withdrawals. Endpoints related to in vivo safety, kinetic, and tumor trafficking of CIK cells confirm what is already shown by other groups but it is the first report within an autologous tumor setting.

In the composite scenario of cancer immunotherapy, many strategies are potentially of interest and to be explored; however, patient-specific preclinical models are utmost necessary to orient the clinical translation. We believe that CIK cells showed reliable activity against challenging and currently incurable mesenchymal tumors, including killing a subset of putative sCSC with clinical relevant implications. In vivo data highlight the need to explore the efficacy of adoptive immunotherapy approaches outside contests of bulk diseases.

These data support further scientific investigations and picture CIK cells as promising candidates for immunotherapy clinical trials, especially considering settings with limited or surgically resected metastatic disease.

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

Authors' Contributions
Conception and design: D. Sangiolo, G. Mesiano, L. Gammaitoni
Development of methodology: D. Sangiolo, L. Gammaitoni
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Mesiano, L. Gammaitoni, V. Leuci, M. Todorovic, L. Giraudo
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Sangiolo, G. Mesiano, V. Leuci, M. Todorovic, L. Giraudo
Writing, review, and/or revision of the manuscript: D. Sangiolo, G. Mesiano, L. Gammaitoni
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