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Cytokine-Induced Killer Cells Effectively Kill Autologous Bone and Soft Tissue Sarcomas including Putative Cancer Stem Cells.

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Abstract

Unresectable metastatic bone (BS) and soft tissue sarcomas (STS) are currently incurable. To overcome present results the challenge is to eradicate chemoresistant sarcoma cancer stem cells (sCSC), responsible for relapses and drug resistance. We investigated the preclinical activity of patient-derived Cytokine-Induced Killer (CIK) cells against autologous BS and STS, including the potential killing of putative sCSC.

We set an autologous immunotherapy model. Tumor killing activity was evaluated *in vitro* and *in vivo* in immunodeficient mice. To identify putative sCSC, autologous BS and STS cells were engineered with a "CSC-detector" vector encoding eGFP under control of the human promoter for the stem-gene Oct4, selectively activated by putative sCSC.

We expanded CIK cells from 21 patients (BS=7; STS=14). *In vitro* CIK cells efficiently killed allogeneic and autologous (n=8) BS and STS cells. The mean specific killing against autologous targets ranged between 83% and 62%, comparable with that against allogeneic targets (p=0.2). The average presence of $eGFP^+$ sCSC in primary BS and STS was 14.6±7.9% (n=7). CIK cells killed *in vitro* up to 79% of $eGFP^+$ sCSC.

Intravenous infusion of CIK cells significantly ($p<0.017$) delayed autologous tumor growth into immunodeficient mice (n=6). Significant inhibition of tumor Ki-67 expression (p=0.0002) was observed in treated mice, without relative enrichment of sCSC compared to controls. CIK cells were able to infiltrate tumor sites. The reported activity against autologous sarcomas, including putative sCSC, is the first of its kind supporting CIK cells as candidates for immunotherapy trials especially in settings with limited or surgically resected metastatic disease.

Introduction

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

Though 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: 1) the possibility to dispose of autologous tumor targets, 2) expansion of "clinical relevant" numbers of immune effectors, 3) restrictions to specific HLA-haplotypes when targeting tumor-associated antigens (TAA), 4) the possibility to kill putative cancer stem cells (CSC) considered responsible for tumor relapses and chemo-resistance. The frequent use of commercially available allogeneic tumor cell lines is a useful tool to generate proof of concept but can not account for the individual biological and immunogenic variability proper of each patient and her/his tumor. Based on these considerations we set up a preclinical HLA-unrestricted autologous immunotherapy model for the treatment of BS and STS, based on Cytokine-Induced Killer (CIK) cells, and dedicated specific insights to the ability of such immunotherapy to kill putative sarcoma CSC (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 UL-16 binding proteins (ULBPs), on target cells (34, 35). The expression of MIC A/B and ULBPs 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 BS and STS is currently not explored, nor is their ability to kill putative sCSC.

The phenotype and precise definition of CSC is currently 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 CSC 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 sCSC 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 BS and STS of various histotype, including evidence of killing putative sCSC.

Methods and Materials

Ex-vivo expansion and phenotype of CIK cells.

CIK cells were expanded from peripheral blood collected from patients with histological confirmed BS 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.

PBMC were separated by density gradient centrifugation (Lymphoprep, Sentinel Diagnostic, Milan, Italy) and seeded in cell culture flasks at a concentration of 1,5 x 10^6 cells/mL in RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA), consisting of 10% FBS (Sigma), 100 U/ml penicillin, 100 U/ml streptomycin (Gibco BRL, Grand Island, NY, USA). IFN-γ (PeproTech, London, UK) 1000 U/ml was added on day 0, after 24 hours IL-2 (Proleukin, Aldesleukin, Chiron Corporation, Emeryville, CA, USA) and anti-CD3 antibody (OKT3, PharMingen, San Diego, CA, USA) were added at a concentration of 300 U/mL and 50 ng/mL respectively. Cells were expanded over 3 weeks 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 x 10^6 cells/mL. Phenotype of CIK cells was weekly analyzed by standard flow cytometry essays. The following mAbs 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 theoric basal collection of $3x10^8$ mononuclear cells per patient according to the formula: ($3x10^8$ x observed fold increase) / Kg of body weight.

Primary cell cultures of BS and STS

Human tumor samples were obtained from surgical specimens; patients provided consent under institutional review board-approved protocols and investigations were performed 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 (200U/ml, Invitrogen), and incubated for 3h 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 I 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's Medium, Gibco BRL, Grand Island, NY, USA) medium with 10% FBS and plated at clonal density (10^5 cells per cm^2) in six-well plates (Corning). An additional aliquot of cells were seeded at 10000 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–Cyanin 7 (PC7) or allophycocyanin (APC)-conjugated mouse monoclonal antibodies (mAbs) against HLA-ABC (anti-HLA-ABC-FITC – BD-Pharmingen) and CIK-target antigens (anti-MIC A/B – BD Pharmingen; anti-ULBPs – R&D System, Space Import Export). Intracellular expression of Oct4 was detected after fixation/permeabilization by the Cyoperm/Cytofix kit according to the manufacturer's instructions (BD Biosciences Pharmingen). ALDH activity was evaluated by ALDEFLUOR assay kit (Aldagen, Stemcell Technologies), according to manufactures instructions. Stro-1 expression was detected by flow cytometry 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 1X and cytospins were prepared onto slides (100,000 cells per slide in 100uL of PBS1X)

at 1500 rpm for 5 minutes using a Shandon Cytocentrifuge Cytospin 2. Following air-drying, slides were fixed in methanol for 30 minutes. Cytospins were stained using May Grunwald – Giemsa stain (Merk) stained 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 mM HEPES, 100 U/ml penicillin and 100 U/ml streptomycin (Gibco BRL, Grand Island, NY, USA) in a humidified 5% CO^2 incubator at 37°C. To authenticate sarcoma cell lines, genotype analysis of MNNG-HOS, Sjsa 1 and MESS-SA cell lines was performed and confirmed by using Cell_ID system (Promega, Milan, Italy) comparing their profile with those published on the DMSZ database.

Cell aliquots were stained with fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE–Cyanin 7 (PC7) or allophycocyanin (APC)-conjugated mouse monoclonal antibodies (mAbs) against CIK-target antigens (anti-MIC A/B – BD_Pharmingen; anti-ULBPs – 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 Italy) female mice were subcutaneously injected with $1x10^6$ primary BS and STS cells (n=4), 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 $V = 4/3 \times \pi \times (a/2)^2 \times (b/2)$, where "*a*" is the length and "*b*" 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 LVs were produced by transient four-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.eGFP) was obtained by replacing the expression cassette hPGK.eGFP into LV-PGK.eGFP with the hOct4-eGFP1, cleaved from phOct4-eGFP1 vector, by insertion into 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).

For each LV transduction, BS and STS primary cells were resuspended in fresh medium with FBS 10%. Virus-conditioned medium was added at dose of 400ng P24/100,000 cells. After 16h cells were washed twice and grown for at least 10 days before flow-cytometry analysis to reach steady state eGFP expression and to rule out pseudo-transduction. As transduction efficiency control the same primary tumor 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 control.

***In vitro* proliferation assay**

To evaluate the proliferation rate of eGFP$^+$ *versus* eGFP$^-$ fractions, cells were labeled with lipophylic dye PKH26 according to manufactures instructions (PKH26GL kit, Sigma). Baseline fluorescence level was analysed by Flow Cytometry. Labelled 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 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 reaction was performed using 100 ng of gDNA for each sample and Phusion High-Fidelity DNA Polymerase (Thermo Scientific) according to the manufacturer's protocol.

The following primers, annealing on the lentiviral vector backbone sequences upstream and downstream the expression cassette, were used: LV forward primer 5'-aggcccgaaggaatagaaga-3'; LV reverse primer, 5'-ccacatagcgtaaaaggagca -3'.

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

Cytotoxic activity of patients-derived CIK cells

The tumor killing ability of patients-derived CIK cells was assessed in-vitro against primary BS and STS cells obtained from patient tumor samples, whenever available, or against allogeneic tumor cell lines (Sjsa 1, MNNG HOS, MES-SA) obtained from American Type Culture Collection (ATCC).

Target cells were stained with PKH26 Red Fluorescent Cell Linker kit (Sigma) or with the vital dye CFSE (5,6-carboxyfluorescein diacetate succinimidyl ester) (Molecular Probes, Eugene, OR, USA) in accordance with the manufacturer's protocol. The immune-mediated killing was determined evaluating cell viability by flow cytometry

(Cyan ADP, Dako), after 6 hours 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% CO_2), according to the formula: experimental-spontaneous mortality/(100-spontaneous mortality) x100. Killing against sCSC 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 to untreated controls. In selected experiments we pre-incubated CIK cells with 20 µg/ml of inhibitory anti-NKG2D neutralizing antibody (Clone #149810, R&D Systems) that was maintained during the cytotoxicity essay against autologous targets.

In vivo activity of patients derived CIK cells

Non-Obese Diabetic/LtSz-scid/scid (NOD/SCID) (Charles River Italy) 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 four days after tumor implantation mice received 9 weekly intravenous infusions with $1x10^7$ 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: $V = 4/3 \times \pi \times (a/2)^2 \times (b/2)$, where "*a*" is the length and *b* 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). Immunohistochemistry essay was performed with human anti-CD5 antibody. Animal experiments were approved by internal review board.

Evaluation of Ki-67 expression was performed 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, Denmark) (1:100) overnight at 4°C. After washings in TBS, anti-mouse secondary antibody (Dako Envision+System-horseradish peroxidase–labeled polymer, Dako) was added. Incubations were carried out for 1 hour at room temperature. Immunoreactivities were revealed by incubation in DAB chromogen (Dako Cytomation Liquid DAB Substrate Chromogen System, Dako) for 10 minutes. A negative control slide was processed with secondary antibody only.

To evaluate the *in vivo* activity of CIK cells against autologous putative sCSC, 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 performed in parallel in all samples from treated and control mice with the same modalities described above. The percentage of residual $eGFP^+$ putative sCSC was analysed by flow cytometry 7 days after transduction.

Statistical analysis

As descriptive statistical analysis of CIK and primary tumor cell cultures, median values and ranges or mean ± standard error measurement (SEM) were used as appropriate. The mixed model analysis of variance (ANOVA) test was used to compare cytotoxic activity curves of CIK cells among the different treatments *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 values of less than 0.05 were considered statistically significant. Statistical analysis was performed 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 BS or STS (Ostesarcoma n=7, Leiomyosarcoma n=2; Rhabdomyosarcoma n=2; Liposarcoma n=2; GIST n=2 Undifferentiated pleomorphic sarcomas n=6). CIK cells were expanded from fresh or cryopreserved peripheral blood mononuclear cells (PBMC) cultured with the timed addition of IFN-γ, Ab-anti-CD3 and IL2. 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) while 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 35% (range 15-90) while 78% (40-99) of total bulk CIK cells were $CD8^+$. The median membrane expression of the NKG2D receptor, main responsible for tumor recognition, on expanded CIK cells was 89% (55-97). To simulate a real clinical scenario we evaluated the theoric 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 $3x10^8$ PBMC collected at day 0, the average dose per patient at the end of the *ex-vivo* expansion would have been of $4.24x10^8$ CIK cells/kg (SEM$1.9x10^8$). 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 BS and STS.

In eight 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 primitive 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 Figure 1 (A-F). MIC A/B, main known ligands recognized by CIK cells, were highly expressed only on osteosarcoma cells while less present or practically absent on STS. Analysis for ULBPs 1, 2 and 3, displayed ubiquitous and predominant expression of ULBP2 compared to all the other molecules ($p < 0.05$). Overall, the median expression of MIC A/B was 38% (range 3-99), while 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 is reported in table 1.

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

Primary metastatic BS 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 3-6 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 Figure 1G.

In vitro and in vivo tumor-killing activity of CIK cells against BS and STS cells.

In order to test the antitumor activity of CIK cells expanded from all our patients, we started evaluating their ability to kill *in vitro* allogeneic BS and STS cell lines (MNNG-HOS; SJSA 1; MES-SA). The cytotoxicity test was conducted at the end of the ex-

vivo expansion and demonstrated 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 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 explored the inhibitory activity of anti-NKG2D neutralizing antibody when added (20 µg/ml) to the cytotoxicity essay against autologous targets. We observed 47% and 42% average inhibition (p=0.003) of tumor killing at 40:1 and 5:1 effector/target ratio respectively, compared to controls.

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 (10^7/week). A significant reduction of tumor growth was observed in treated mice (n=6) compared to untreated controls (n=6) p=0.017, mean volumes at the end of experiment were in treated mice *807 mm^3* (SEM 138) *vs.* 1702 mm^3 (SEM 441) of controls ($p < 0.0001$, Fig. 3A). 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 to controls (mean 4.58 SEM 0.72 vs 9.35 SEM 1.06, p=0.0002) (Fig. 3B). These last data are consistent with recent observation from immunotherapy clinical trials, requiring additional metabolic or histological data beyond the conventional RECIST to evaluate clinical responses.

At the end of the experiment we confirmed the presence of CIK cells infiltrating the autologous tumor (Fig. 3C).

CIK cells effectively kill putative sarcoma Cancer Stem Cells.

To identify putative sCSC, we transduced bulk primary BS and STS cells with a "CSC-detector" made by a lentiviral vector encoding the eGFP regulated by the human Oct4 promoter (Fig.4A). The underlying idea is that sCSC can be visualized based on their exclusive ability, proper 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.4B); 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 is reported in figure 4C. As positive control, a murine embryonic cell line expressing Oct4 (mES) was successfully transduced with LV-Oct4-GFP up to 90.5% while no eGFP expression was detected on differentiated PBMC 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-GFP in both cell subsets. As additional control, we confirmed that primary BS and STS cells could efficiently be transduced (>90% of eGFP expression) when the strong ubiquitous promoter (Phospho Glycerato Kinase, PGK, regulatory element) was utilized in place of the Oct4 promoter in controlling eGFP expression (Fig. 4B).

Putative sCSC displayed a proliferative potential *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 CSC; a representative histogram is reported in figure 4D.

CIK cells efficiently killed sCSC; 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 sCSC overlaid that observed against differentiated eGFP negative metastatic cells p=0.88 (Fig.5A). As initial evidence of *in vivo* activity of CIK cells against putative sCSC, we evaluated the presence of residual putative $eGFP^+$sCSC from all tumor samples explanted at the end of the *in vivo* experiment described above. We did not observe an enrichment of $eGFP^+$sCSC in treated mice compared to controls but a relative reduction was detected instead (mean 5.04% SEM 1.14 vs 20,73% SEM 4.19, p=0.004) (Fig. 5B). A representative plot of residual $eGFP^+$sCSC in treated mice compared to untreated controls is shown in Figure 5C.

Discussion

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

Overall the study reflects the potentialities 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 BS 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 BS and STS are missing.

In general, preclinical studies have a key role in providing reliable biological 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 et al. 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 BS and STS.

Furthermore, we posed a dedicated and innovative question if CIK cells might be able to kill a peculiar subset of putative sCSC. To this aim we developed a new methodology to visualize putative sCSC 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 sCSC, 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 to untreated controls suggesting that *in vivo* activity of CIK cells is capable to involve putative sCSC subsets. We observed a reduction of eGFP+ sCSC in treated mice compared to controls however, while encouraging, the experimental design and small size impose caution before concluding for a preferential sCSC killing *in vivo*. More dedicated studies are required in this direction. Of course we cannot claim a definitive identification of sCSC. 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 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 NKG2D 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 regarding the real efficacy of clinical applications of this approach. To this regard however, at least two considerations may be done. First, initial clinical trials with immunotherapy strategies demonstrated that dimensional parameters, like RECIST criteria, may not be the optimal method to appreciate clinical responses and exploration of additional metabolic and/or histological 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 to 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 reasonable give the best results and future trials, including those with CIK cells, as well experimental models should evolve in such direction. The decision to perform nine adoptive infusions was only based on the availability of patient-derived CIK cells that limited the set up of dose-response curves. 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 already showed 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 demonstrated 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.

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Legends to Figures

Figure 1. Primary BS and STS cell cultures.

Primary tumor cell cultures were obtained from biopsies of metastatic BS and STS. Tumor cells reproduced morphologic features typical of the original tumor and were validated by pathology review. In the figure are shown representative pictures from cultures of Pleomorphic undifferentiated sarcomas (A-C), Leiomyosarcoma (D); GIST (E), Osteosarcoma (F). 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 able to generate tumors *in vivo*, when implanted subcutaneously into NOD/SCID mice (n=4), retaining morphologic features typical of the original tumor. A representative picture of osteosarcoma generated *in vivo* is shown the figure (G).

Figure 2. CIK cells efficiently kill autologous metastatic BS and STS cells.

Patient-derived CIK cells efficiently killed autologous metastatic BS and STS cells generated from fresh biopsies. The specific killing against autologous tumors was at least as effective as that observed against allogeneic targets. Six hours cytotoxicity essays were performed by staining target cells with PKH26 and analyzing results by flow-cytometry. Means + SEM of tumor specific killing from all experiments are reported in the figure.

Figure 3. In vivo activity of CIK cells against BS and STS.

NOD/SCID mice (n=12) were subcutaneously implanted with $1x10^6$ primary cells from pleomorphic undifferentiated sarcoma. Four days after tumor implantation $1x10^7$ autologous CIK cells were weekly infused by tail vein injection (n=6). After 9 weeks of treatment a significant delay in tumor growth was observed in treated mice compared to controls (n=6), p= 0.017.

Tumor volume at each week of treatment is reported in the figure (A). A significant reduction of K-i67 expression was observed in tumors from treated mice compared to controls (p=0.0002) (B). Representative pictures of the different Ki-67 expression are shown in the figure (C). Infiltration of CIK cells at tumor sites were demonstrated by IHC (Ab anti CD5) at the end of experiment (D).

Figure 4. Visualization of putative sCSC.

We engineered a "CSC-detector" lentiviral vector (LV-Oct4-GFP) where eGFP is encoded under control of Oct4 promoter (A). Putative sCSC in BS and STS primary cell cultures were visualized exploiting their exclusive ability to activate the promoter of stem-gene Oct4. The average presence of $eGFP^{+}$CSC within OS and STS cells was 14.3% SEM 2.5 (n=7). A representative microscopy picture and flow cytometry plot are reported (B). The average flow-cytometry expression of Stro-1 and ALDH activity, evaluated on primary BS and STS cultures as putative stemness markers, were 4% (SEM 1) and 8.4% (SEM 1) respectively. A representative plot is reported in the figure with correspondent negative controls shown on the left side (C). $eGFP^{+}$sCSC displayed a slow-growing phenotype, average 3 times less compared to $eGFP^{-}$ counterpart after 14 days of culture (n=6). Proliferation essay *in vitro* was evaluated staining LV-Oct4-GFP-transduced tumor cells with the vital dye PKH26 and assessing the fluorescence intensity decrement over time. A representative experiment is reported (D).

Figure 5. CIK cells are active against putative sarcoma cancer stem cells (sCSC).

Patient-derived CIK cells efficiently killed sCSC visualized with our "CSC-detector" vector LV- Oct4-GFP (n=7).

The specific killing against $eGFP^{+}$sCSC overlaid that observed against differentiated GFP^{-} metastatic cells. Results were reproducible against autologous (n=3) and allogeneic (n=4) samples

Six hours cytotoxicity essays were performed staining target cells with the vital dye PKH26 and analysing results by flow-cytometry. Means of tumor-specific killing + SEM from all the experiments are reported in the figure.

As evidence of CIK cells activity *in vivo* against putative sCSC 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 $eGFP^+$sCSC in treated mice compared to controls but a relative reduction was detected instead (Fig. 5B). A representative plot of residual $eGFP^+$sCSC in treated mice compared to untreated controls is shown (C).

Figure Legends

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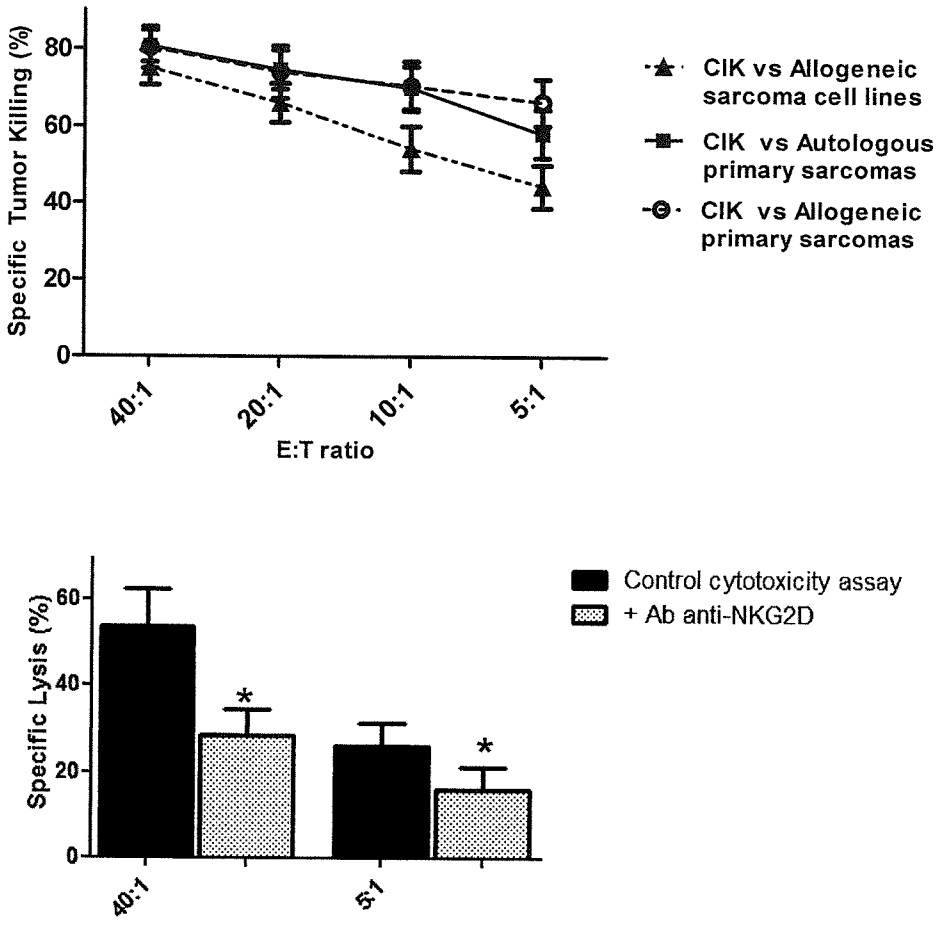
Pts. (UPN)	Diagnosis	Age	Availability of autologous tumor samples	MIC A/B (%)	ULBP2 (%)	ULBP3 (%)	Final rate of CD3+CD56+ CIK cells (%)	Ex vivo Expansion of CIK cells (fold)*	Projected dose of CIK cells/kg
S001	Pleomorphic S.	67	y	36	99	20	44	14	6,18E+07
S002	Pleomorphic S.	43	n	N/A	N/A	N/A	45	52	2,48E+08
S003	Pleomorphic S.	86	y	8	99	12	30	96	5,43E+08
S004	Pleomorphic S.	64	n	N/A	N/A	N/A	25	61	1,99E+08
S005	Pleomorphic S.	72	y	3	99	68	25	57	1,63E+08
S006	Pleomorphic S.	82	n	N/A	N/A	N/A	16	30	1,25E+08
S007	Rhabdomyosarcoma	20	n	N/A	N/A	N/A	66	95	5,59E+08
S008	Rhabdomyosarcoma	20	n	N/A	N/A	N/A	25	17	7,08E+07
S009	Liposarcoma	64	n	N/A	N/A	N/A	89	100	4,62E+08
S010	Liposarcoma	72	y	60	94	0	39	99	3,71E+08
S011	Leiomyosarcoma	83	n	N/A	N/A	N/A	34	924	4,14E+09
S012	Leiomyosarcoma	68	y	40	88	3	45	37	1,10E+08
S013	GIST	45	n	N/A	N/A	N/A	50	17	6,00E+07
S014	GIST	45	y	16	99	1	52	86	4,30E+08
S015	Osteosarcoma	18	y	75	90	3	31	20	1,00E+08
S016	Osteosarcoma	58	y	99	99	77	35	38	1,14E+08
S017	Osteosarcoma	34	n	N/A	N/A	N/A	31	3	1,84E+07
S018	Osteosarcoma	18	n	N/A	N/A	N/A	38	99	4,95E+08
S019	Osteosarcoma	33	n	N/A	N/A	N/A	30	28	1,42E+08
S020	Osteosarcoma	24	n	N/A	N/A	N/A	32	54	2,25E+08
S021	Osteosarcoma	22	n	N/A	N/A	N/A	40	48	2,62E+08

Table 1. Characteristics of patients, CIK cells and primary tumor cell cultures.
"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) were evaluated on early established primary tumor cell cultures.
* Fold expansion is calculated for bulk CIK cells, intended as proliferation of CD3+ cells.

Figure 2. CIK cells efficiently kill autologous metastatic BS and STS cells.



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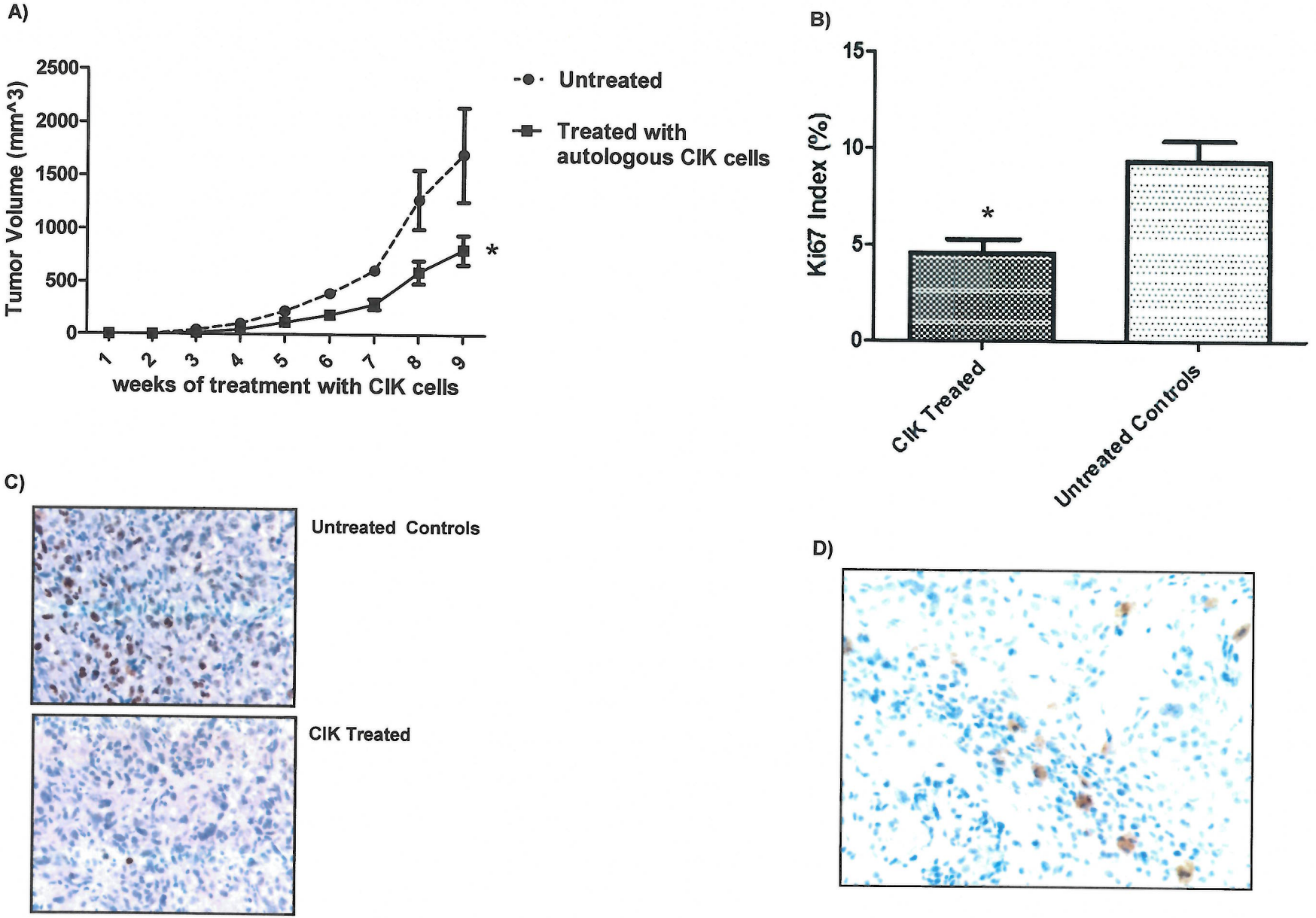
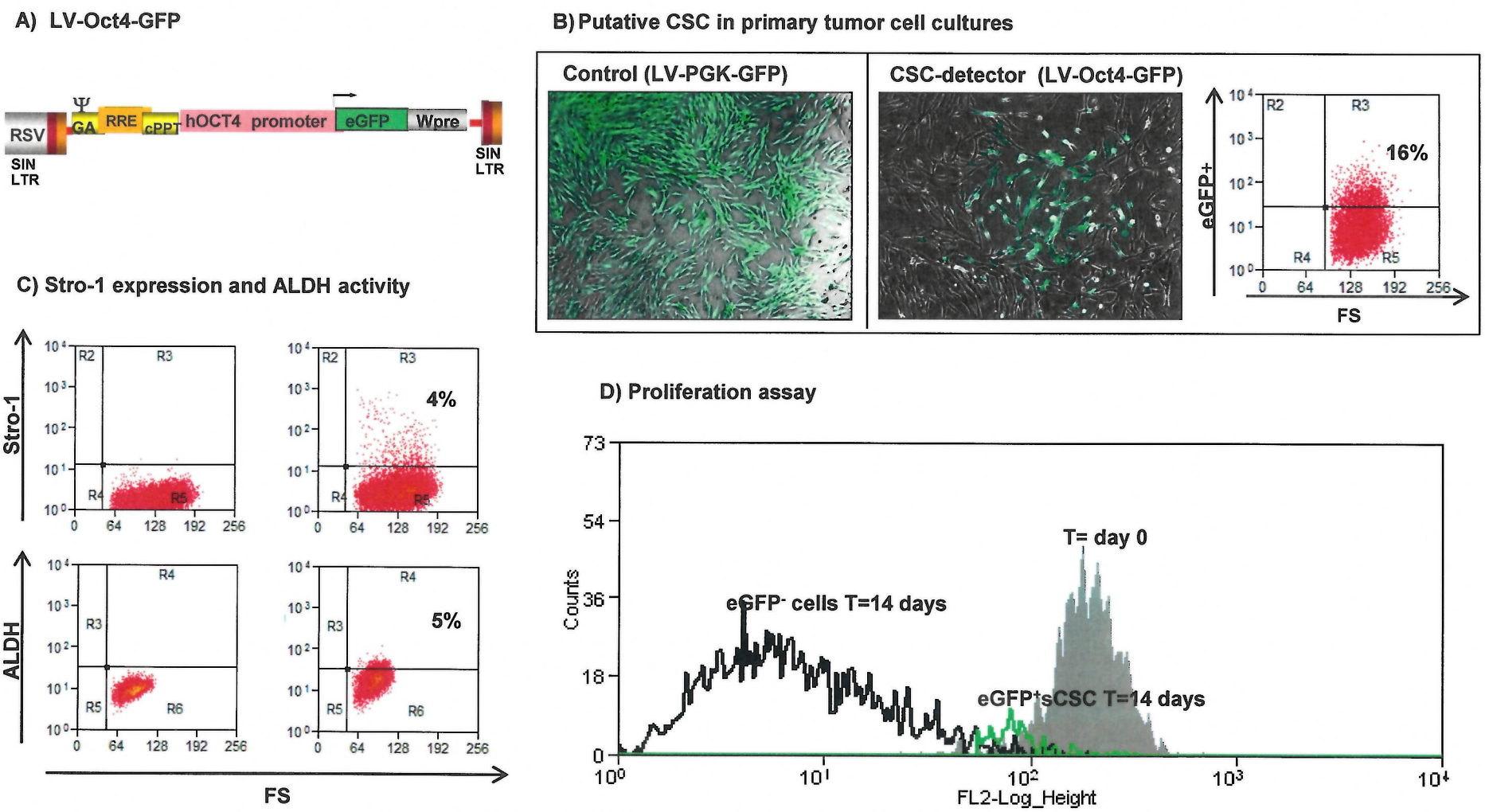


Figure 4. Visualization of putative sCSC.



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