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29 March 2023

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ORIGINAL RESEARCH

Cytokine Induced Killer cells are effective against sarcoma cancer stem cells spared by chemotherapy and target therapy.

Giulia Mesiano[a], Giovanni Grignani[a], Erika Fiorino[b], Valeria Leuci[a,b], Ramona Rotolo[b], Lorenzo D'Ambrosio [a,b], Chiara Salfi[b], Loretta Gammaitoni[a], Lidia Giraudo[a], Alberto Pisacane[c], Sara Butera[d], Ymera Pignochino[a,b], Marco Basiricó[a], Federica Capozzi[a], Anna Sapino[c,e], Massimo Aglietta[a,b], and Dario Sangiolo[a,b]

[a]Medical Oncology Division, Candiolo Cancer Institute, FPO–IRCCS. Str. Prov. 142, km 3.95, I-10060, Candiolo (To), Italy; [b]Department of Oncology, University of Torino, Candiolo (Torino) Italy; [c]Pathology Division, Candiolo Cancer Institute, FPO-IRCCS, Str. Prov. 142, km 3.95, I-10060, Candiolo (To), Italy; [d]Department of Molecular Biotechnologies and Healthy Sciences, Haematology Division 1, University of Torino, Italy; [e]Department of Medical Sciences, University of Torino, Italy

ABSTRACT

Metastatic bone and soft tissue sarcomas often relapse after chemotherapy (CHT) and molecular targeted therapy (mTT), maintaining a severe prognosis. A subset of sarcoma cancer stem cells (sCSC) is hypothesized to resist conventional drugs and sustain disease relapses. We investigated the immunotherapy activity of cytokine induced killer cells (CIK) against autologous sCSC that survived CHT and mTT. The experimental platform included two aggressive bone and soft tissue sarcoma models: osteosarcoma (OS) and undifferentiated-pleomorphic sarcoma (UPS).

To visualize putative sCSC we engineered patient-derived sarcoma cultures (2 OS and 3 UPS) with a lentiviral sCSC-detector wherein the promoter of stem-gene Oct4 controls the expression of eGFP.

We visualized a fraction of sCSC (mean 24.2 ± 5.2%) and confirmed their tumorigenicity in vivo. sCSC resulted relatively resistant to both CHT and mTT in vitro. Therapeutic doses of doxorubicin significantly enriched viable eGFP$^+$sCSC in both OS (2.6 fold, n = 16) and UPS (2.3 fold, n = 29) compared to untreated controls. Treatment with sorafenib (for OS) and pazopanib (for UPS) also determined enrichment (1.3 fold) of viable eGFP$^+$sCSC, even if less intense than what observed after CHT.

Sarcoma cells surviving CHT and mTT were efficiently killed in vitro by autologous CIK even at minimal effector/target ratios (40:1 = 82%, 1:4 = 29%, n = 13). CIK immunotherapy did not spare sCSC that were killed as efficiently as whole sarcoma cell population. The relative chemo-resistance of sCSC and sensitivity to CIK immunotherapy was confirmed in vivo. Our findings support CIK as an innovative, clinically explorable, approach to eradicate chemo-resistant sCSC implicated in tumor relapse.

Introduction

Bone and soft tissue sarcomas (STS) are an heterogeneous group of solid tumors of mesenchymal origin. Undifferentiated pleomorphic sarcoma (UPS) is among the most frequent STS in adults (10-20%),[1,2] osteosarcoma (OS) is the most common primary bone tumor occurring predominantly in adolescents and young adults.[3]

Multidisciplinary treatment significantly improved the outcome of patients with early stage UPS and OS.[4-7] Nonetheless, the prognosis of patients with relapsing and metastatic disease still remains poor, with 5-year overall survival approximately ranging between 10 and 30% regardless the use surgery, chemotherapy, molecular targeted therapy and radiotherapy.[8-14] This clinical scenario is in high need of research efforts for new therapeutic strategies, including the exploration of immunotherapy that has significantly improved the prognosis of patients with melanoma and is currently being explored in other solid tumors. The clinical successes recently observed with checkpoint inhibitors, however have not been replicated in the initial sarcoma trials. This is likely due to their relatively minor neo-antigen load and the presence of important immunosuppressive elements in the tumor microenvironment.[15,16] In this field, a promising immunotherapy approach is based on the adoptive infusion of antitumor immune-effectors, with important results recently reported against selected cases of synovial sarcoma treated with anti-NY-ESO1 engineered T lymphocytes.[17-21]

Whatever may be the new strategy explored, a crucial emerging and still unsolved issue is the ability to target cancer stem cells (CSC). CSC represent a sub-type of tumor cells involved in tumor propagation, therapy resistance, recurrence and metastatisation.[22-29]

We recently reported that putative sarcoma CSC (sCSC), in patient-derived STS and OS cultures, could be visualized by their selective activation of the stemness gene promoter OCT4.[30] We also provided proof of concept that sCSC could be killed in vitro by a MHC-independent immunotherapy approach based on Cytokine-Induced Killer cells (CIK).[30,31] We now asked whether

sCSC were resistant to chemo and targeted therapies currently used in clinical practice, exploring if a sequential immunotherapy with CIK might be effective against chemo- and target- therapy resistant sCSC.

CIK are *ex vivo* expanded T lymphocytes, endowed with T-NK phenotype and intense MHC-independent antitumor ability[32-36] mainly mediated by the NKG2D receptor that binds stress inducible ligands (MIC A/B; ULBPs) selectively expressed on various tumor histotypes including sarcomas.[35-37]

CIK may be an intriguing therapeutic option as they would be applicable to all patients,[38-40] regardless their HLA haplotype, and would not be affected by HLA-downregulation, a common tumor immune-escape mechanism[41-43] recently associated also with CSC.[44,45]

We set an autologous experimental platform with UPS and OS patient-derived cultures. We assessed their sCSC relative resistance to both chemotherapy (doxorubicin) and molecular targeted drugs (sorafenib or pazopanib) along with the sequential activity of autologous CIK cells against the resistant sCSC.

Results

Putative sCSC survive chemotherapy and molecular targeted therapy

Visualization of putative sCSC

We successfully detected putative sCSC in 5 patient-derived sarcoma cell cultures (OS, n = 2; UPS, n = 3) generated from biopsies of advanced sarcomas.

Visualization of putative sCSC was performed by a gene transfer strategy, previously validated in our lab, based on stable transduction of sarcoma cells with a lentiviral vector encoding eGFP under control of the promoter regulatory element of the stemness gene Oct4 (LV-Oct4.eGFP). With this approach the average rate of eGFP$^+$sCSC within the 5 sarcoma cultures was 24.2 ± 5.2% (mean ± SEM).

Oct4, Sox2 and Aldehyde Dehydrogenase (ALDH), reported in literature as molecules associated with CSC phenotype, were assessed in all sarcoma samples with average expression of 18 ± 3.5%, 28 ± 6.8%, and 3.5 ± 1.3% (mean ± SEM), respectively. A complete phenotype description of sarcoma cultures, including the main ligands recognized by CIK cells is summarized in Table 1.

All sarcoma cultures were confirmed to retain membrane expression of HLA class-I molecules (> 99% HLA-ABC$^+$).

The expression of NKG2D ligands MICA/B, ULBP 2-5-6 and ULBP3 was comparable in both eGFP$^+$sCSC and eGFP$^-$ sarcoma cells (Fig. 1).

In vivo tumorigenicity of putative sCSC

To assess the tumorigenic potential of putative eGFP$^+$sCSC, we subcutaneously transplanted NOD/SCID mice (n = 4) with eGFP$^+$ sorted sCSC from 2 different UPS (S1 and S3). In both cases tumors grew in all mice starting 4 weeks after transplant. We confirmed the persistence of eGFP$^+$sCSC in tumors explanted (8 – 13 weeks after tumor growth) at the end of the experiment (Fig. 2).

In a selected experiment we performed a limiting dilution assay to explore the different tumorigenic potential of eGFP$^+$ and eGFP$^-$ sarcoma cells. We assessed the rate of S3 primary culture (UPS) growth in NOD/SCID mice, subcutaneously implanted with progressively scalar doses (from 7×10^4 to 0.7) of both eGFP$^+$ and eGFP$^-$ sorted tumor cells. At the dose of 7×10^3 tumor cells 67% (n = 4/6) of tumors grew from eGFP$^+$ sarcoma cells, while no tumor growth was observed (n = 0/6) from the eGFP$^-$ group (p = 0.03).

Sensitivity of putative sCSC to chemotherapy and molecular targeted therapy in vitro

We explored the sensitivity of putative sCSC to conventional chemotherapy and molecular targeted therapy. We used doxorubicin as chemotherapy for all 5 sarcomas, while we used pazopanib and sorafenib as targeted therapy for UPS and OS, respectively. We evaluated the percentage of tumor lysis and the rate of residual sCSC after each treatment. Putative sCSC displayed a relative resistance to both chemotherapy and molecular targeted therapy. Doxorubicin used at therapeutic doses (range IC50 – IC75) determined a significant enrichment of viable eGFP$^+$sCSC (UPS: mean 2.3 ± 0.2 fold, n = 29; OS: mean 2.6 ± 0.3 fold, n = 16; p < 0.0001, Fig. 3 and Table 2) compared to untreated controls. Similarly, treatment with sorafenib and pazopanib also determined an enrichment of viable eGFP$^+$sCSC even if it was less intense than what observed after chemotherapy (UPS: mean 1.3 ± 0.03 fold, n = 24 p < 0.0001; OS: mean 1.3 ± 0.1 fold, n = 15, p = 0.009; Fig. 3 and Table 2).

CIK are effective against sCSC that survive chemotherapy or molecular targeted therapy

Generation of CIK from patients

We explored the activity of CIK against sCSC that survived chemotherapy (doxorubicin) or molecular targeted therapy (pazopanib and sorafenib, for UPS and OS, respectively).

Table 1. Patients' characteristics and corresponding sarcoma cell cultures.

Pts.	Diagnosis	Age	MICA/B (%)	ULBP1 (%)	ULBP2-5-6 (%)	ULBP3 (%)	CD155 (%)	CD112 (%)	OCT4 (%)	Sox-2 (%)	ALDH high (%)
S1	UPS	67	17	1	99	19	2	1	24	14	3
S3	UPS	86	61	1	100	64	72	3	29	35	4
S5	UPS	72	1	2	97	50	7	2	15	20	8
S16	OS	58	85	7	100	75	20	14	7	44	3
S22	OS	18	49	1	99	60	8	6	10	10	1

Main ligands recognized by CIK (MICA/B, ULBP 1, 3, 2-5-6, CD112 and CD155) were expressed at variable levels by all sarcomas. Average expression of CSC markers Oct4, Sox2 and Aldehyde Dehydrogenase (ALDH) are also reported in the table.
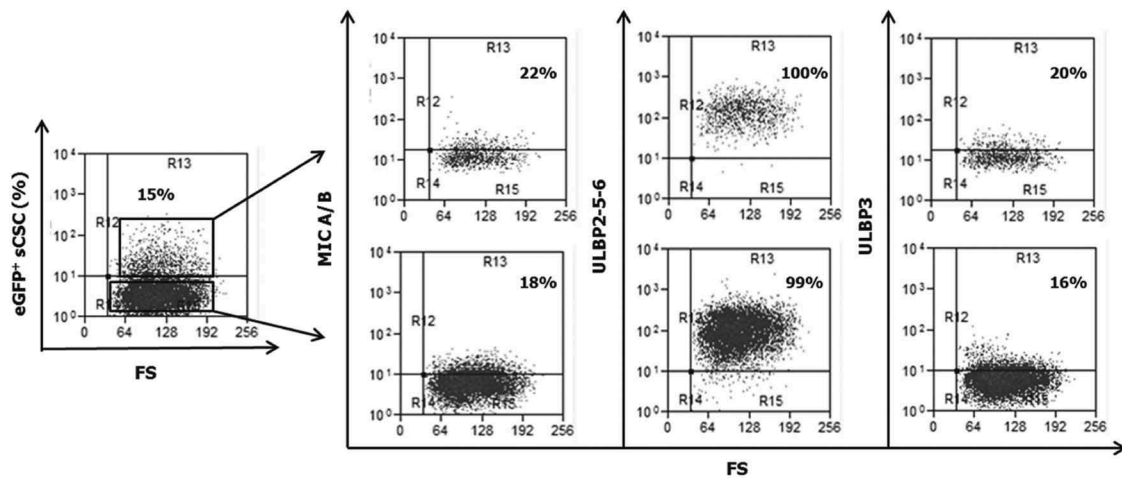Abbreviations: UPS, Undifferentiated Pleomorphic Sarcoma; OS, Osteosarcoma

Figure 1. Expression of NKG2D ligands in putative sCSC. Representative flow-cytometry dot plots reporting the comparable membrane expression of NKG2D ligands in eGFP⁺sCSC and the eGFP⁻ counterpart (S16). Quadrants were set based on negative controls, separately assessed for eGFP⁺ and eGFP⁻ fraction.
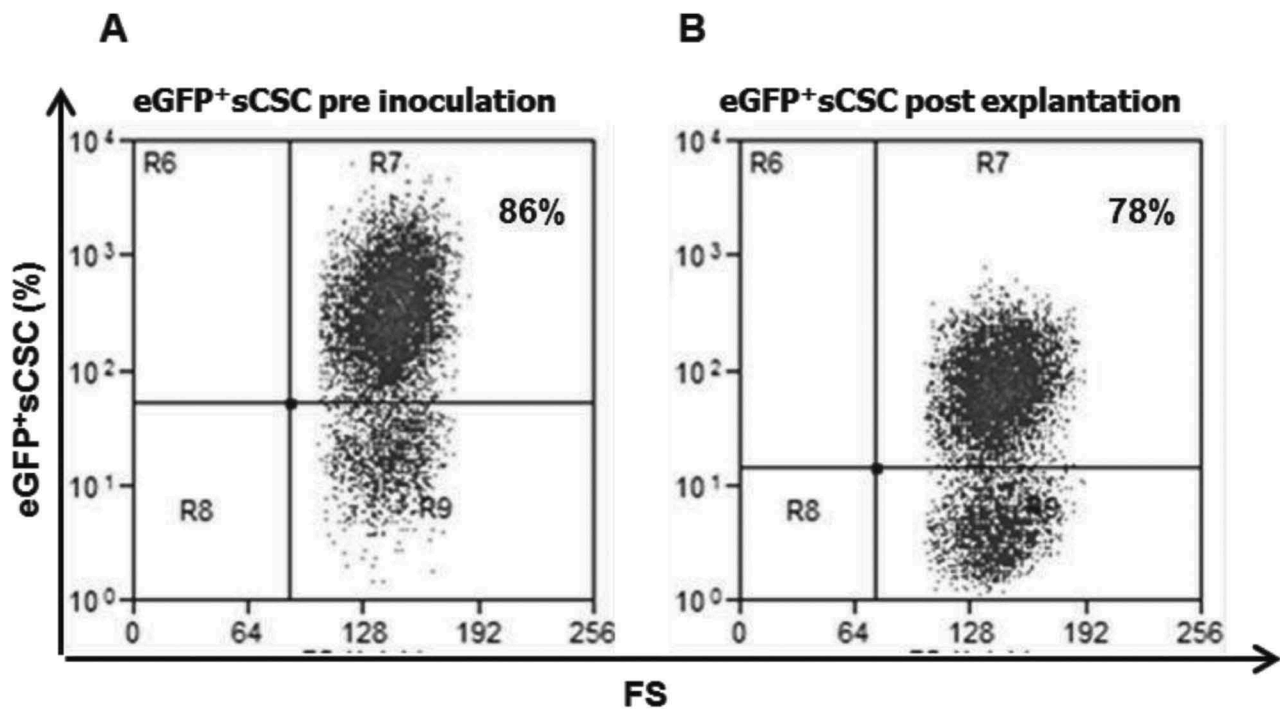

Figure 2. *In vivo* persistence of tumorigenic sCSC. Subcutaneous implantation of eGFP⁺sCSC (S3) generated tumors in NOD/SCID mice (n = 4). eGFP⁺sCSC persisted *in vivo* and were recovered in explanted tumors at the end of the experiment.

CIK were generated from patients with UPS (n = 3) and OS (n = 2). In 4 out of 5 cases we could reproduce an autologous setting as CIK were expanded from the same patients from whom we had generated the sarcoma cell cultures described above. In 1 case (patient S22) PBMC were not available and CIK were obtained from a third party (allogeneic) metastatic OS patient.

CIK were successfully *ex vivo* expanded within 3 – 4 weeks of cultures from fresh or cryopreserved PBMC according to the standard protocol that includes timed addition of IFN-Ɣ, Ab anti-CD3, and IL2.[30,31]

The median expansion of bulk CIK, calculated on the total CD3⁺ fraction, was 40 fold (range, 24 – 90). The subset of mature CIK co-expressing CD3 and CD56 molecules (CD3⁺CD56⁺) was present with a median of 35% (range 28 – 60%), while 85% (range 58 – 95%) of CD3⁺ cells were also CD8⁺ (Fig. 4). The median membrane expression of the NKG2D receptor, main responsible for tumor recognition, was always > 80% (Fig. 4).

The presence of pure NK (CD3⁻CD56⁺) cells within mature CIK was negligible, median 0.5% (range 0.2 – 1.2%).

The main characteristics of patients and relative CIK expansion data are reported in Table 3.
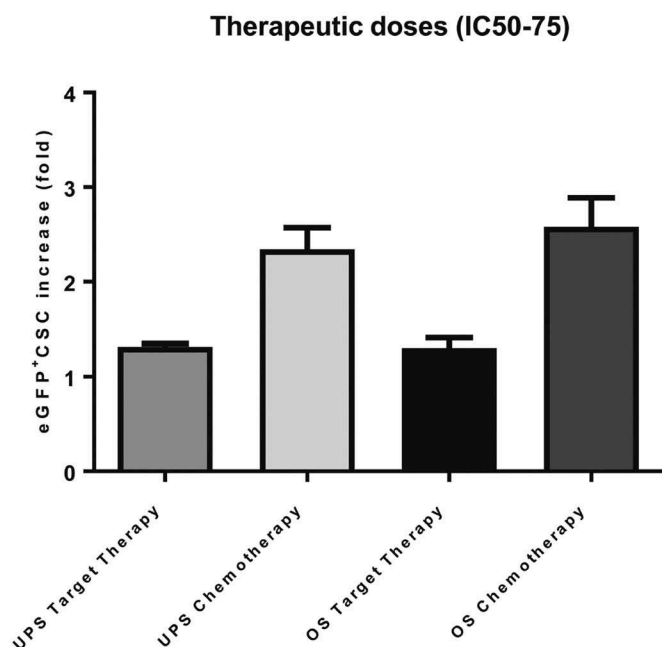
Therapeutic doses (IC50-75)

Figure 3. Sarcoma CSC are relatively resistant to chemotherapy and molecular targeted drugs. Therapeutic doses of doxorubicin significantly enhanced the residual rates of sCSC (visualized as eGFP+) in both UPS (n = 29) and OS (n = 16), supporting the hypothesis of their relative chemo-resistance. Similar result, but less intense were observed following treatment with targeted drugs pazopanib (UPS, n = 24) and sorafenib (OS, n = 15). Cumulative data (mean fold ± SEM) of eGFP+sCSC enrichment in UPS and OS following treatments are reported in the figure.

Table 2. Dose dependence of sCSC enrichment by chemo and target therapy.

| | eGFP$^+$CSC Increase (fold) | | | |
| | Chemotherapy | | Targeted therapy | |
Pts.	IC50	IC75	IC50	IC75
S1 (UPS)	2,5	3,3	1,3	1,3
S3 (UPS)	1,5	2,2	1,3	N/A*
S5 (UPS)	1,9	4	1,3	1,1
S16 (OS)	2,1	4,3	1,1	2,1
S22 (OS)	2,1	3,5	1,2	1,4

*Not Available: for UPS S3 the target therapy IC75 was not found in the therapeutic dose range considered (pazopanib: from 30 to 5 µM).

CIK kill sCSC surviving chemotherapy and molecular targeted therapy

Patient-derived CIK efficiently killed *in vitro* sarcomas enriched in putative sCSC after treatment with IC50 doses of chemotherapy (doxorubicin 0.1 µM) and molecular targeted therapy (pazopanib 30 µM for UPS; sorafenib 5 µM for OS). We could reproduce the autologous CIK/sarcoma target matching in 4/5 cases. In the single case (S22) where autologous PBMC were not available, allogeneic CIK from a patient with OS were used.

Mean values of tumor specific killing against chemo-surviving sarcomas at progressively decreasing effector/target (E/T) ratios were 87 ± 2% (40:1), 80 ± 4% (20:1), 70 ± 3% (10:1), 68 ± 3% (5:1), 59 ± 5% (2.5:1), 49 ± 3% (1:1), 45 ± 3% (1:2) and 35 ± 4% (1:4) (n = 7, Fig. 5A). The observed killing activity was comparable with that obtained against untreated controls (n = 9, p = 0.89) (Fig. 5A).

CIK were equally effective against sarcomas that survived treatment with molecular targeted therapy. Mean values of

tumor specific killing at progressively decreasing effector/target (E/T) ratios were 83 ± 3% (40:1), 77 ± 5% (20:1), 72 ± 7% (10:1), 66 ± 9% (5:1), 59 ± 9% (2.5:1), 48 ± 10% (1:1), 48 ± 15% (1:2) and 40 ± 12% (1:4) (n = 6, Fig. 5B). The observed killing activity was comparable with that obtained against untreated controls (n = 8, p = 0.99) (Fig. 5B).

We confirmed by flow cytometry that the antitumor activity of CIK involved sCSC. We did not observe enrichment of eGFP$^+$sCSC in any point of the immunotherapy CIK killing curve, against targets recovered after either chemotherapy or molecular targeted therapy, compared to controls (Fig. 5). The absence of relative increase of eGFP$^+$sCSC indirectly confirms their susceptibility to immunotherapy with CIK.

In a selected *in vivo* experiment (S1 UPS) we confirmed that sCSC are relatively chemo-resistant while sensitive to autologous CIK immunotherapy.

Both doxorubicin (5mg/Kg, day 1, n = 5) and CIK immunotherapy (days 1, 3 and 5, n = 4) delayed tumor growth (mean volume fold increase 1.4 ± 0.2 and 2.7 ± 0.4 respectively) compared to untreated controls (mean volume fold increase 4.9 ± 1.4, n = 3) but, while a relative enhancement of residual sCSC (mean 62 ± 6.8% vs 29 ± 0.3%, Fig. 6) was observed in tumors explanted following chemotherapy, the rate of sCSC after CIK immunotherapy remained comparable with untreated controls (mean 30 ± 1.7% vs 29 ± 0.3%, Fig. 6).

A sequential chemo-immunotherapy treatment with Doxorubicin (5mg/Kg, day 1) and CIK (day 5) reestablished the rates of eGFP$^+$sCSC back to levels comparable with untreated controls (mean 37 ± 4.7% vs 29 ± 0.3%, Fig. 6), while maintaining the delay of tumor growth (mean volume fold increase 2.5 ± 0.4, n = 6).

Discussion

In this work we reported the preclinical activity of immunotherapy with CIK against sCSC that survived treatment with chemotherapy and molecular targeted therapies, commonly used in the clinical practice. Results are generated in an autologous-matched patient-derived preclinical setting, in the effort to enhance data reliability in clinical perspective.

We previously provided preclinical proof of concept that CIK were active against OS and STS, including a subset of cells with stemness features.[30] Here, we advanced to the next experimental level, closer to a pragmatic clinical scenario. We demonstrated that sCSC are relatively resistant to chemotherapy and target therapies but susceptible to MHC-independent immunotherapy with autologous CIK cells.

The approach for visualization of CSC is based on the selective ability of putative sCSC to activate the promoter of stemness gene Oct4. This was previously described and validated by our group.[30,31,46] We provide a new functional angle of analysis, exploring the sensitivity of putative sCSC to conventional chemotherapy. We acknowledge that the proposed methodology to visualize sCSC has objective limitations and cannot guarantee to capture all the "true" sCSC. This would be however beyond the scope of our research work. What this system allows to highlight is that tumor cells with stemness features, like the ability to activate Oct4 and enhanced
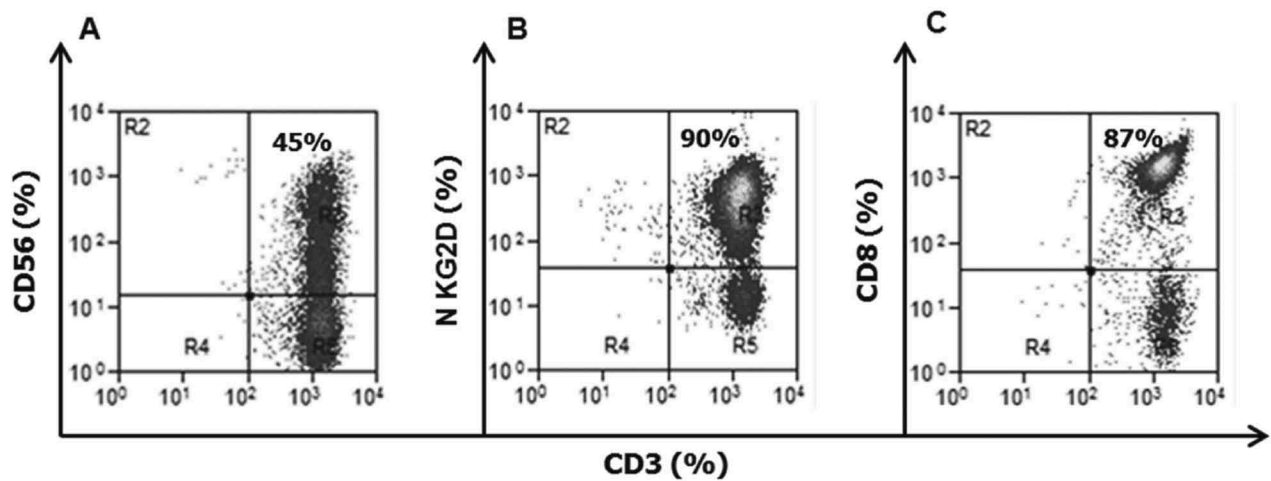
Figure 4. Phenotype of mature CIK. Representative cytofluorimetric dot plots show the typical phenotype of mature CIK. At the end of the 3–4 weeks of culture, within mature CIK it is possible to distinguish two main T-cell subsets positive (CD3$^+$CD56$^+$) and negative (CD3$^+$CD56$^-$) for the co-expression of the CD56 molecule (A), respectively. A high percentage of expanded CD3$^+$ CIK expresses membrane NKG2D receptor (B) responsible of tumor target recognition. Moreover, the majority of CIK are CD8$^+$ (C).

Table 3. Characteristics of patients and CIK.

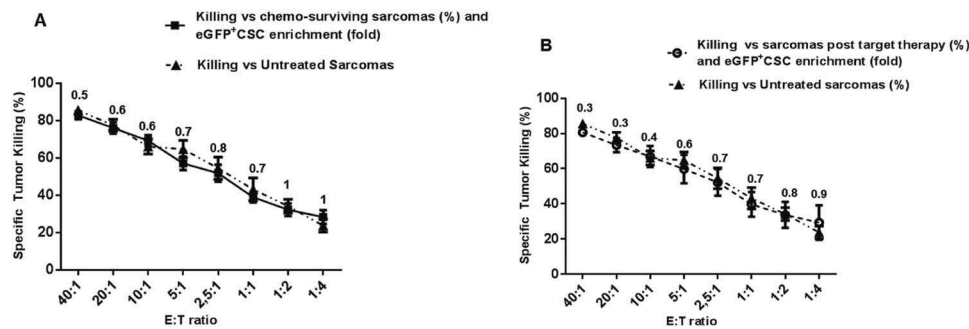| Pts.	Age	Treatments (n)	Tumor site	Basal CD3$^+$CD56$^+$ (%)	CD3$^+$CD56$^+$ (%)	CD3$^+$CD8$^+$ (%)	CD3$^+$NKG2D$^+$ (%)	Expansion (fold)
S1 (UPS)	67	2	Local Relapse	3	51	58	80	35
S3 (UPS)	86	0	Metastasis	1	30	60	91	96
S5 (UPS)	72	0	Primitive	3	28	95	89	41
S16 (OS)	58	4	Metastasis	15	35	85	90	38
S22 (OS)	18	4	Metastasis	4	60	87	88	575

Figure 5. CIK activity against sCSC recovered after chemo and molecular targeted therapy. Patient-derived CIK efficiently killed eGFP$^+$sCSC surviving to chemotherapy (doxorubicin) (5A, n = 7) or target therapy (pazopanib and sorafenib) (5B, n = 8). The ratio between viable sCSC post and pre CIK-immunotherapy are reported for each point of the killing curves. Means of tumor specific killing ± SEM are reported.

tumorigenic potential in vivo, are indeed resistant to conventional treatments making them a clinically relevant target.

With the experimental use of doxorubicin, sorafenib (for OS) and pazopanib (for UPS) we tried to represent realistic chemotherapy and target drugs of well known clinical activity in advanced STS and OS. Despite these treatments showed responses and prolonged progression-free survival of sarcoma patients, invariably relapses/progressions and chemo-resistance/refractoriness appeared after a relatively short interval. Our data support the notion that even when more than 50% of the tumor mass is debulked, sCSC may escape this "first line therapy" with relevant implications in clinical perspective.

Even if a statistical significant enrichment of sCSC (1.3 fold) was observed with targeted drugs, in our models, the

effect of CSC-sparing is much more evident with doxorubicin compared to sorafenib or pazopanib. Indeed, the mechanisms of resistance are likely different. In the case of doxorubicin ATP-binding cassette membrane proteins that are more represented in CSC are certainly involved in the so-called multidrug resistance phenotype. TKIs are not known to be susceptible to the same superfamily of transporters. An alternative hypothesis is that sCSC are commonly in more quiescent state and therefore less dependent on activated molecular pathways targeted by sorafenib or pazopanib. This difference is consistent with the absence of a direct dose-effect correlation with molecular targeted drugs that is instead present with doxorubicin in all the sarcomas explored. Even if the underlying mechanism may remain at a speculative level, from a
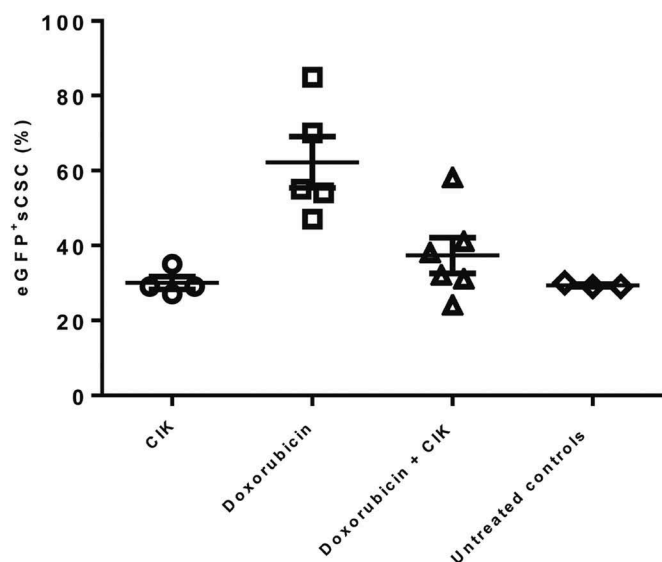
Figure 6. Autologous CIK are active against chemo-surviving eGFP⁺sCSC *in vivo*. Chemotherapy treatment with doxorubicin (5 mg/Kg, day 1) determined a relative enhancement of residual sCSC (62 ± 6.8%, n = 5) while the rate of sCSC after CIK immunotherapy (30 ± 1.7%, n = 4) remained comparable with untreated controls (29 ± 0.3%, n = 3). The sequential chemo-immunotherapy treatment (doxorubicin 5mg/Kg, day 1 and CIK 1×10^7 at day 5) resulted in a relative decrease of residual viable eGFP⁺sCSC (37 ± 4.7%, n = 6), confirming *in vivo* that CIK are equally effective against sarcomas that survived chemotherapy treatment.

pragmatic point of view this observation is in favor of a more relevant anti-CSC activity of molecular targeted therapies compared to conventional chemotherapy.

Recently published findings from other groups reported similar results in Ewing sarcoma, liposarcoma and synovial sarcoma cell lines treated with sorafenib, while contrasting results were reported for pazopanib.[47]

We confirmed that stress-inducible ligands (MIC A/B and ULBPs) are expressed by sCSC, at comparable levels with those found on the rest of tumor cells, supporting the observed susceptibility to immunotherapy with CIK. Furthermore, we demonstrated that sCSC retain membrane expression of HLA molecules. This observation may suggest the conclusion that also adaptive immunotherapy approaches may be effective. It is however important to note that we detected only extracellular HLA chains. Tumor-associated defects in the HLA-antigen presentation may occur at various levels of the intracellular HLA machinery affecting antigen processing/loading molecules, HLA-heavy chains or beta2microglobulin.[41-45,48,49]

Our data, regarding chemosensitivity and immunotherapy, are obtained without physical separation of putative sCSC that are visualized within the bulk sarcoma cell cultures. This may be a relevant aspect of our model as the biologic behavior and differentiation status of putative CSC may potentially change when extracted and separated from the surrounding tumor progeny.

Thinking to the hypothesis of clinical translation, the best setting for an experimental trial with CIK adoptive immunotherapy is likely that one of minimal residual disease following conventional chemo or target therapies. Based on our preclinical findings, we could imagine that CIK may contribute to the elimination of residual sCSC spared by other therapies, contributing to reduce the risk of later relapse. Our in vivo data support the chemoresistance of sCSC and their potential susceptibility to CIK immunotherapy. Even if CSC are considered to sustain disease relapse, from our experimental design we can only speculate, without drawing conclusions, about the potential "clinical" relevance of such activity. For this purpose dedicated investigations, with appropriate endpoints are required. For istance, at this point a contribution of CIK-mediated inflammation to tumor volume cannot be excluded, as we noted that at the end of the experiment the tumor volume of mice treated with chemotherapy-immunotherapy sequence was slightly higher than mice treated with chemotherapy only.

CIK cells immunotherapy may be somehow easier to explore in the field of sarcomas, where immune-checkpoint inhibitor antibodies have not been able to replicate so far the clinical successes obtained against melanoma and other solid tumors. The adoptive infusion of CIK does not have to be considered necessarily as alternative to other immunotherapies. Their favorable safety profile allows envisioning a possible integration with other approaches including checkpoint inhibitors themselves. In fact, initial preclinical data suggest a potential synergism as CIK express PD1 on their membrane at variable rates. Preclinical data in this direction were recently reported in the setting of hematologic malignancies and are likely to be confirmed also in the field of solid tumors.[15,16,50-52] The cytotoxic effect of CIK may promote a "positive" inflammation at tumor sites, with release of tumor antigenic fragments and Th1 cytokines that might in turn promote an adaptive immune response favored by checkpoint inhibitors.

Overall our findings support the existence of a clinical relevant subset of UPS and OS cells with stemness features that are relatively resistant to chemotherapy, less sensitive to molecular targeted approaches and susceptible to MHC-independent immunotherapy with CIK. Clinical studies are warranted to explore the activity of CIK within the multistep and composite therapeutic strategy of advanced sarcomas relapsing following conventional treatments.

Materials and Methods

Generation of primary sarcoma cell cultures

We successfully generated 5 primary sarcoma cell cultures, 2 OS and 3 UPS, starting from biopsies or thoracentesis of metastatic (n = 4) or primitive (n = 1) tumor sites. All individuals provided informed consent according to a protocol approved by the Internal Review Board and Ethic Committee.

Four sarcoma cultures (S1, S3, S5 and S16) were previously obtained from surgical biopsy and characterized by our group.[30] S22 osteosarcoma culture was generated starting from freshly isolated thoracentesis, obtained in a sterile vacuum bottle. Tumoral cells were isolated from fluid by centrifugation and resuspended in KnockOut Dulbecco's Modified Eagle's: Nutrient Mixture F-12 Medium (KO DMEM:F12 medium, Gibco BRL, Life Technologies Italia)

with the addition of penicillin (50 U/ml), streptomycin (50 µg/ml), Glutamax 100X (all from Gibco BRL, Life Technologies Italia). Cells were seeded in 10% heat-inactivated Fetal Bovine Serum (FBS, Euroclone Spa), in multi-well plates treated for anchorage-dependent cultures (Corning/Costar, VWR International PBI S.r.l.) at clonal density (10^4-10^5 cells per cm^2).

Characterization of sarcoma cell cultures

Cell aliquots were stained with fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE–Cyanin 7 (PC7), or allophyco-cyanin (APC)-conjugated mouse mAbs against anti-HLA-ABC-FITC (BD Biosciences Pharmingen), anti-Sox-2-APC (Becton Dickinson BD Biosciences Italy, Pharmingen) and CIK-target antigens [anti-MIC A/B (BD Biosciences Pharmingen), anti-ULBPs, anti CD112 and anti CD155 (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, Beckman Coulter s.r.l.) and analyzed using Summit Software.

Generation of hOct4.eGFP lentiviral vector

VSV-G pseudotyped third-generation lentiviral vectors were produced by transient 4-plasmid co-transfection into 293T cells as described by Follenzi et al.[53-55] The transfer vector pRRL.sin.PPT.hPGK.EGFP.Wpre (LV-PGK.EGFP) was kindly provided by Dr. Elisa Vigna and described elsewhere.

The phOct4.EGFP vector[56] 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-eGFP, cleaved from phOct4-eGFP vector, by insertion into SalI and XhoI restriction enzyme sites.[30,31] Physical titers for lentiviral vector stocks were determined on the basis of p24 antigen content (HIV-1 p24 ELISA kit; PerkinElmer).

Sarcoma cell cultures transduction

For each lentiviral vector transduction, OS and UPS primary cells were resuspended in fresh medium with 10% heat-inactivated Fetal Bovine Serum (FBS, Euroclone Spa). Virus-conditioned medium was added at dose of 400 ng P24/100,000 cells. After 16 hours, cells were washed twice and grown for at least 10 days before flow cytometric analysis to reach steady-state eGFP expression and to rule out pseudo transduction.

Tumorigenicity of putative sCSC

Fractions of eGFP$^+$ and eGFP$^-$ sarcoma cells were obtained by Flow Activated Cell Sorting (MoFlow, Beckman Coulter s.r.l.)

starting from 2 LV-Oct4.eGFP transduced UPS primary culture (S1 and S3). To assess the tumorigenic potential of putative eGFP$^+$sCSC, we subcutaneously transplanted six-week-old Non-Obese Diabetic/LtSz-scid/scid (NOD/SCID) (Charles River) female mice with eGFP$^+$ or eGFP$^-$ sorted UPS primary cultures (from 2.5×10^5 to 5×10^5 cells, 2 mice for each fraction), resuspended in sterile PBS 1X and BD Matrigel™ Basement Membrane Matrix (Becton Dickinson BD Biosciences, Pharmingen) 1:1.

Limiting dilution assay was performed with fractions of eGFP$^+$ (90%) and eGFP$^-$ (96%) sarcoma cells obtained by Flow Activated Cell Sorting (MoFlow, Beckman Coulter s.r.l.) starting from S3 LV-Oct4.eGFP transduced UPS primary cells. Six-week-old NOD/LtSz-scid/scid (NOD/SCID; Charles River Laboratories) female mice were subcutaneously implanted with progressively scalar doses (7×10^4 n = 6, 7×10^3 n = 6; 7×10^2 n = 6; 7×10^1 n = 6; 7 n = 6; 0.7 n = 6) of both eGFP$^+$ (right flank) and eGFP$^-$ (left flank) tumor cells resuspended in equal volume of sterile PBS 1X and BD Matrigel™ Basement Membrane Matrix (Becton Dickinson BD Biosciences, Pharmingen). Tumor growth was monitored weekly with calipers; volume was calculated using formula, $V = 4/3 \times \pi \times (l/2)^2 \times (L/2)$, where L is the length and l the width diameter of the tumor. Mice were sacrificed when tumor volume reaches a maximum of 2 cm (main diameter) and tumors were cut into 3-mm^3 pieces and processed for cell isolation. Tumor tissue was processed by mechanical and enzymatic dissociation (Collagenase Type I, Invitrogen, Life Technologies Italia) for 3 hours. Cells were then resuspended in KnockOut Dulbecco's Modified Eagle's: Nutrient Mixture F-12 Medium (KO DMEM:F12 medium, Gibco BRL, Life Technologies Italia) with the addition of penicillin (50 U/ml), streptomycin (50 µg/ml), Glutamax 100X (all from Gibco BRL, Life Technologies Italia); cells were seeded in 10% heat-inactivated Fetal Bovine Serum (FBS, Euroclone Spa) and plated at clonal density ($10^4 - 10^5$ cells per cm^2) in multi-well plates treated for anchorage-dependent cultures (Corning/Costar, VWR International PBI S.r.l.). Cell aliquots were analysed by flow-cytometry to evaluate the eGFP expression either after sorting and explantation.

In vitro assessment of sCSC sensitivity to chemotherapy or molecular targeted therapy

LV-Oct4.eGFP transduced sarcoma cells were treated with standard CHT and molecular targeted therapies. We used doxorubicin (from pharmacy leftover) as CHT for all 5 sarcomas, while we used pazopanib (Selleckchem Research Product) and sorafenib (Sequoia Research Product) as targeted therapy for UPS and OS, respectively. Each sarcoma culture was treated with scalar doses of drugs (doxorubicin: from 1 to 0.05 µM, pazopanib: from 30 to 5 µM, sorafenib: from 10 to 5 µM) and percentages of tumor lysis and the rate of residual sCSC were evaluated after 72 hours of each treatment. LV-Oct4.eGFP transduced sarcoma cells treated with equal volume of drug diluent have been utilized as control. At the end of each treatment, cells were harvested and counted. The cell viability was determined using Trypan Blue 0.1% exclusion dye (Sigma Aldrich). The IC50 dose corresponds to IC values ranging between 40% and 60%. While the IC75 dose corresponds to IC values ranging between 60% and 80%. Concentrations of drugs

that determined IC values ranging between 40% and 80% (IC50 and IC75 doses) are considered as therapeutic doses. Viable eGFP$^+$sCSC were determined by flow cytometry (Cyan ADP, Beckman Coulter s.r.l.). The eGFP positivity was calculated on viable cell fraction, detected by DAPI permeability exclusion assay (Thermo Fisher Scientific). The eGFP enrichment, expressed as fold increase (% of eGFP$^+$ cells post-drug exposure/% of eGFP$^+$ cells in untreated sample), was calculated for each experiment separately, comparing treated samples with their internal untreated control. After drugs treatment cell aliquots were stained with fluorescein isothiocyanate (FITC) or allophycocyanin (APC)-conjugated mouse mAbs against anti-HLA-ABC-PE (BD Biosciences Pharmingen) and CIK-target antigens [anti-MIC A/B (BD Biosciences Pharmingen), anti-ULBP3 and anti-ULBP2-5-6 and anti-CD112 (R&D System, Space Import Export)]. Labeled cells were read on FACS Cyan (Cyan ADP, Beckman Coulter s.r.l.) and analyzed using Summit Software.

CIK culture and ex vivo expansion

CIK were expanded from peripheral blood collected from 5 patients with histologic confirmed OS and UPS at the Candiolo Cancer Institute, Fondazione del Piemonte per l'Oncologia (FPO) – IRCCS. 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, Aurogene s.r.l.) and seeded in cell culture flasks at a concentration of 2×10^6 cells/mL in RPMI-1640 medium (Gibco BRL Life Technologies Italia) supplemented with 10% fetal bovine serum (Sigma Aldrich) 100 U/mL penicillin, and 100 U/mL streptomycin (Gibco BRL Life Technologies Italia) at 37°C and 5% CO$_2$. IFN-γ (Miltenyi Biotec S.r.l.; 1000 U/mL) was added on day 0; after 24 hours the recombinant human interleukin IL-2 (Miltenyi Biotec S.r.l.) and anti-CD3 antibody (Miltenyi Biotec S.r.l.) 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 $2 - 1.5 \times 10^6$ cells/mL. Phenotypic analyses of CIK were performed weekly, and cytotoxic activity was assessed at the end of 3–4 weeks of culture.

Immunophenotype

Phenotype of CIK was weekly analyzed by standard flow cytometric assays. The following monoclonal antibodies (mAb) were used: fluorescein isothiocyanate (FITC), phycoerythrin (PE), or allophycocyanin (APC)-conjugated mouse monoclonal antibodies (mAbs): CD3–FITC, CD8–PE, CD56–APC/PE and CD314–APC (aka anti-NKG2D) (mAbs) (CD3, CD8 and CD56 from BD Biosciences Italy, Pharmingen; anti-NKG2D from Miltenyi Biotec S.r.l.). Labeled cells were read on FACS Cyan (Cyan ADP, Beckman Coulter s.r.l.) and analyzed using Summit Software.

In vitro assessment of CIK cells activity against sCSC that survive chemotherapy or molecular targeted therapy.

CIK tumor-killing ability was assessed against 5 LV-Oct4. eGFP transduced sarcoma cultures previously treated for 72 hours with IC50 dose of doxorubicin (0.1 μM), or pazopanib (30 μM), or sorafenib (5 μM) and or with equal volume of diluent as control. The effector cells were assayed where possible against autologous tumor targets (4/5). In absence of autologous PBMC (S22) we utilized as allogeneic effectors, CIK generated from a third party metastatic OS patient. The CIK immune-mediated killing was analyzed by flow cytometry (Cyan ADP, Beckman Coulter s.r.l.) by DAPI permeability assay (Thermo Fisher Scientific) of target cells. CIK were co-cultured with either autologous or allogeneic sarcoma cells with a 40:1, 20:1, 10:1, 5:1, 2,5:1, 1:1, 1:2 and 1:4 effector/target ratio for 72 hours in 200 μL of medium with IL-2 at a concentration of 300 U/mL at 37°C 5% CO$_2$. Target cells were stained with PKH26 Red Fluorescent Cell Linker kit (Sigma Aldrich) or with the vital dye CFSE (5, 6-carboxy-fluorescein diacetate succinimidyl ester; Molecular Probes) in accordance with the manufacturer's protocol. As confirmation test, a method of determining the number of viable cells in culture based on quantitation of the ATP present, an indicator of metabolically active cells (CellTiter-Glo® Luminescent Cell Viability Assay, Promega Italia s.r.l) has been utilized (data not shown). Tumor cells plated in the absence of CIK were used as a control to assess spontaneous mortality. The percentage of tumor-specific lysis for each effector/target ratio was calculated according to the following formula: (experimental-spontaneous mortality/100 – spontaneous mortality) x 100. To evaluate the percentage of the residual eGFP$^+$ cells viable at each effector/target ratio of the killing curve, a duplicate of the cytotoxicity test was plated using tumor target not previously stained with PKH26 Red Fluorescent Cell Linker kit or with the vital dye CFSE. The percentage of viable eGFP$^+$ cells was determined by flow cytometry (Cyan ADP, Beckman Coulter s.r.l.) in any point of the immunotherapy CIK killing curve. The eGFP positivity was calculated on viable cell fraction, detected by DAPI permeability exclusion assay (Thermo Fisher Scientific). The enrichment (fold) of viable sCSC was calculated as the ratio between residual percentage of sCSC post and pre CIK immunotherapy for each point of the immunotherapy killing curve.

In vivo activity of chemotherapy and CIK-immunotherapy against sCSC

Six-week-old NOD/LtSz-scid/scid (NOD/SCID; Charles River Laboratories) female mice were subcutaneously injected with 3×10^6 LV. Oct4.eGFP–transduced patient-derived sarcoma cells (S1, n = 18) resuspended in sterile PBS 1X and BD Matrigel™ Basement Membrane Matrix (Becton Dickinson BD Biosciences, Pharmingen) 1:1. Treatments started when tumors became palpable. Mice from chemotherapy group (n = 11) received a single tail vein injection of doxorubicin (5 mg/kg, day 1). Mice from CIK immunotherapy group (n = 4) received 3 intravenous infusions (1 $\times$ 10^7/injection, days 1, 3, 5) of autologous mature CIK cells (resuspended in 200 mL of PBS 1X) while mice injected with

PBS 1X alone (n = 3) represented untreated controls. To explore the sequential treatment with chemotherapy and CIK immunotherapy, 6 mice from the CHT cohort (treated with doxorubicin on day 1) received an intravenous infusion with CIK cells (1×10^7/ mouse, day 5). Tumor growth was monitored every 2 days with calipers and volume calculated according to the formula: $V = 4/3 \times \pi \times (l/2)^2 \times (L/2)$, where L is the length and l the width diameter of the tumor. 72 hours from the last treatment (day 8), animals were euthanized and the recovered tumors were processed by mechanical and enzymatic dissociation using the Tumor Dissociation kit, human and the gentleMACS dissociator, according to the manufacturer's instructions (Miltenyi Biotec S.r.l.). Single cell suspensions thus obtained were enriched of human tumor cells with the mouse cell depletion kit (Macs Miltenyi Biotec.) and the percentage of eGFP$^+$ cells was determined by flow cytometry (CyAn ADP, Beckman Coulter s.r.l.) and analyzed using Summit Software.

The volume fold increase was calculated according to the formula:

Tumor volume at day of sacrifice/Tumor volume 72 hours before treatment started.

Statistical analysis

Descriptive analyses of CIK and sarcoma features were reported as median values and ranges, or mean ± SEM, as appropriate. The relative increase of eGFP$^+$ sCSC after treatments *in vitro* was reported as fold change compared to untreated controls. The statistical significance was calculated by one sample *t*-test, transforming each fold change into the corresponding log2 value. The mixed model analysis of variance (ANOVA) was employed to assess CIK cytotoxic activity curves *in vitro*. P value concerning the tumor onset frequency in limiting dilution assay was calculated with Fisher's exact test.

Results with p value < 0.05 were considered as statistically significant.

Data were analyzed by software GraphPad Prism 6.

Conflict of interest disclosure

The authors declare no competing financial interests.

ORCID

Lorenzo D'Ambrosio http://orcid.org/0000-0003-3294-8819

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