Activated and expanded natural killer cells target osteosarcoma tumor initiating cells in an NKG2D–NKG2DL dependent manner

L. Fernández a, J. Valentín b, M. Zalacain c, W. Leung d, A. Patiño-García c, A. Pérez-Martínez e,*

a Clinical Research Department, Spanish National Cancer Research Centre CNIO, C/Melchor Fernández Almagro, 3, 28029 Madrid, Spain
b Tumor Immunology Lab, IDiPZ, Paseo de la Castellana, 261, 28046 Madrid, Spain
c Pediatrics Lab, Universidad de Navarra, C/Irunlarrea s/n, 31008 Pamplona, Spain
d Bone Marrow and Cell Therapy, St Jude Children’s Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, USA
e Pediatric Hemato-Oncology, Hospital Universitario La Paz, Paseo de la Castellana, 261, 28046 Madrid, Spain

ABSTRACT

Current therapies fail to cure most metastatic or recurrent bone cancer. We explored the efficacy and the pathways involved in natural killer (NK) cells’ elimination of osteosarcoma (OS) cells, including tumor initiating cells (TICs), which are responsible for chemotherapy resistance, recurrence, and metastasis. The expression of ligands for NK cell receptors was studied in primary OS cell lines by flow cytometry. In vitro cytotoxicity of activated and expanded NK (NKAE) cells against OS was tested, and the pathways involved explored by using specific antibody blockade. NKAE cells’ ability to target OS TICs was analyzed by flow cytometry and sphere formation assays. Spironolactone (SPIR) was tested for its ability to increase OS cells’ susceptibility to NK cell lysis in vitro and in vivo. We found OS cells were susceptible to NKAE cells’ lysis both in vivo and in vitro, and this cytolytic activity relied on interaction between NKG2D receptor and NKG2D ligands (NKG2DL). SPIR increased OS cells’ susceptibility to lysis by NKAE cells, and could shrink the OS TICs. Our results show NKAE cells target OS cells including the TICs compartment, supporting the use of NK-cell based immunotherapies for OS.

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Original Articles

Introduction

Osteosarcoma (OS) is the most common type of solid bone cancer, mainly affecting children and young adults. OS usually affects long bones, in particular the distal femur and proximal tibia. For the last three decades, mainstream treatments consisting of radical surgery and chemotherapy have yielded a 5-year survival rate of 67% [1]. About 20% of patients present metastases at diagnosis primarily in the lungs. Once the metastases have occurred, the prognosis is very poor, and the current therapeutic strategies have shown limited efficacy. Furthermore, prognosis of patients with recurrent disease is also dismal. OS remains the second leading cause of cancer-related death in children and young adults [2], therefore, there is a need for new therapeutic approaches. Current treatment failure could be related to the inability to target OS TICs [3]. TICs, also called cancer stem cells, are a subset of tumor cells characterized by self-renewal capacity, increased tumorigenicity, and resistance to chemotherapeutic agents [4]. TICs from OS have been described by their capacity to grow as spheres (sarcospheres) under serum-starvation conditions [5], by their expression of different surface markers such as c-kit, CD133, or Stro-1 [6,7], or as a “side population” through the exclusion of the vital dye Hoechst 33342 [8]. OS TICs also express CXCR4, a metastasis-associated stem cell marker. CXCR4 expressing cells metastasize to organs such as lung that produce high levels of SDF-1 (or CXCL12). Clinically, greater expression of CXCR4 correlates with disease severity [9–11].

NK cells are lymphocytes of the innate immune system that can eliminate virally infected or transformed cells without prior sensitization [12]. The interactions between inhibitory and activating NK cell receptors and their ligands are determinants of NK cell activation and anti-tumor activity [13]. The main NK cell activating receptors are NKG2D, DNAM-1, and the natural cytotoxicity receptors (NCRs). NKG2D receptor recognizes human MICA/B and ULBP1-6 [14–16] ligands, whereas DNAM-1 recognizes CD112 (Nectin-2) and...
CD155 (PVR) [17], NKGD2 ligands (NKGD2Ls) are expressed selectively or at low levels by normal cells but are upregulated during stress and cellular transformation. NKGD2 stimulation of NK cells leads to strong activation and tumor cell rejection. Although NKGD2Ls are up-regulated in most tumor cells, sarcomas are the tumor type most sensitive to NK cell cytotoxicity [18]. However, during tumor progression, tumors evade immunosurveillance by different mechanisms such as shedding or downregulating NKGD2L. Different therapeutic agents such as spironolactone (SPIR) [19], irradiation [18], and gemcitabine [20, 21] enhance NKGD2L expression in different tumor cell lines, which then become more sensitive to NK cell-mediated lysis.

In this report, we demonstrate that activated and expanded NK cells (NKAE) can efficiently eliminate primary and metastatic OS cells, including OS TICs. The elimination of OS by NK cells relies in NKGD2–NKGD2L interaction. We also found OS cells being highly responsive to SPIR, triggering up-regulation of surface NKGD2L and increasing their susceptibility to NK cell lysis both in vitro and in vivo. Finally, we found NKAEs and SPIR could reduce OS TIC ability to form sarcomospheres and to express c-kit and CXCR4. Together our data support the use of the NKAE cells in combination with SPIR as a new treatment for OS.

Materials and methods

Osteosarcoma cells

A total of 22 human OS primary cell lines (10 from primary OS: 531B, 588B, 595B, 491B, 524B, 473B, 475B, 486B, 738B, 6998; 11 from lung metastasis: 588M, 595M, 491M, 491MI, 658MI, 728M, 722MI, 654M, 709M, 319M; and 1 from a metastasis in the rib: 531MI) were used for this study. All the primary cell lines were provided by Dr. Patiño (University of Navarra, Spain) and cultured in MEM (GIBCO, 10270-086) and penicillin-streptomycin (P/S) (GIBCO, 15140-122). MG-63 cells were cultured in ATCC, MG-63 cell line was purchased from ATCC. MG-63 cells were cultured in EMEM (ATCC, 30-2003), supplemented with 10% FBS and P/S. We tested if SPIR, low dose irradiation or gemcitabine treatments could increase the expression of ligands for NK cell receptors in primary OS cell lines. SPIR was obtained from Sigma Aldrich (S3378) and or gemcitabine treatments could increase the expression of ligands for NK cell receptors in primary OS cell lines. SPIR was obtained from Sigma Aldrich (S3378) and NKAE cells were incubated with anti-NKG2D or anti-DNAM-1 antibody at a 10:1 E:T ratio. Cells were then sorted, plated, and analyzed as explained above.

CD155 (PVR) [17], NKGD2 ligands (NKGD2Ls) are expressed selectively or at low levels by normal cells but are upregulated during stress and cellular transformation. Flowjo VX.0.7 software (TreeStar, San Carlos, USA) was used for data analysis. The mouse IgG2a anti-human HLA class I mAb was used in blocking experiments (clone W6/32) [20] and anti-DNAM-1 (clone 102511) [21] was used in blocking experiments (clone 102511). The mouse IgG2b anti-human Fas Ligand mAb (clone 100419) (MAB126) and anti-DNAM-1 (clone 102511) [21] were used in blocking experiments (clone 102511). The mouse anti-NKGD2 (clone 1D11) [22] was used in blocking experiments (clone 1D11). IgG1 mAb was used as an isotype control. All mAbs were used at a concentration of 10 μg/mL except for anti-HLA class I, and anti-DNAM-1 that we used 20 μg/mL. All mAbs were added to cultures for 1 hour at 37 °C, and then washed once with complete medium.

Cytotoxicity assays

The cytotoxicity of NKAE cells against OS cells was evaluated at 8:1, 4:1, 2:1, and 1:1 effector:target (E:T) ratios by performing conventional 4-hour euromix-TPA release assays (Perkin Elmer) [20] as described previously [22]. In brief, target cells were labeled with a fluorescent-enhancing ligand (BATDA); this hydrophobic ligand quickly penetrates the cell membrane. Within the cell, hydrolysis of ester bonds results in the ligand becoming hydrophilic and, therefore, unable to pass through the cell membrane. Cytolysis, however, results in the release of the ligand and, ultimately, a reaction of the ligand with the europium to form a stable, fluoro- rescing chelate, which was evaluated fluorometrically [1240 VICTOR Perkin Elmer, Finland]. The number of NK cells was calculated by multiplying the lymphocyte counts by the percentage of CD3/CD56− NK cells. The following formulæ were used to calculate spontaneous and specific cytotoxicity: 5 specific release (excess spontaneous release-background)/maximum release (spontaneous release-background)+maximum release-background) × 100.

Sphere formation assay

The ability of TICs to grow as spheres was tested by culturing 1×105 cells/mL in DMEM F12 medium (GIBCO, 12331-020) supplemented with B27 (17054-044), FGF2 (12356-029) (both from GIBCO), ITS+ and 1% L-Glu (25030-081) from Gibco, in ultra-low attachment plates (Corning) (3747 and 3814). The presence of TIC markers (c-kit, CD133, and CXCR4) was assayed by flow cytometry. To assay the ability of NKAE cells to shrink the OS TIC compartment, we co-cultured 531MI, 654MI, MG-63, and U2-OS spheres with NKAE cells in a 1:1 E:T ratio for 4 or 24 h and analyzed the percentage of c-kit and CXCR4 positive OS cells by performing flow cytometry. To determine whether NKAE cells also impaired OS cells’ ability to grow as spheres, MG-63 cells were transduced with a lentiviral vector encoding GFP to allow fluorescent-activated cell sorting (FACS). We then co-cultured MG-63 GFP+ cells with NKAE cells at a 10:1 E:T ratio for 4 or 24 h. After the culture, living GFP+ cells were sorted by using a FACSaria II flow cytometer (BD Biosciences, San Jose, CA) and re-plated in spheres medium at a concentration of 1×105 cells/mL. At 7 days, images were taken and spheres were counted by using an inverted Nikon Ti microscope and NIS Elements imaging software (v 3.1) (Tokyo, Japan). In a different experiment, we co-cultured MG-63 GFP+ cells for 24 hours with NKAE cells that were untreated or treated with an NKGD2-blocking antibody at a 10:1 E:T ratio. Cells were then sorted, plated, and analyzed as explained above.

In vivo studies

All procedures were approved by the ethics committee of the Instituto de Salud Carlos III or by the St. Judge Animal Care and Use Committee. For the orthotopic intratibial model, 531MI OS cells (5×106) were injected by injection through the tibial plateau in the primary spongosia of both tibias of 10- to 12-week-old NOD/ scid IL2rgnull (NSG) mice, with or without effector cells (5×105 NKAE cells/tibia). Mice were divided in four different treatment groups: Control: receiving 531MI osteosarcoma cells alone, SPIR: 531MI cells previously treated with SPIR (56 μM) for 3 days, NKAE: 531MI and NKAE cells, and NKAEs:SPIR receiving 531MI cells previously treated with SPIR for 3 days plus NKAE cells. Those groups receiving NKAE cells also received IL-2 (10,000 U/mouse) injected intraorbitally for 5 days after the cells infusion. A total of 48 NSG mice were used in two independent experiments. NKAE cells from a 531MI haploidentical donor were used in the first experiments, and NKAEs from a healthy unrelated donor were used in the second. At day 56, tumor volumes were measured and bone damage was studied by plain X-rays. In vivo radiography images were acquired by using a Faxitron specimen radiograph system (Faxitron LLC, Tucson, Arizona). Animals were anesthetized with isoflurane for the data acquisition. Data were acquired at 30 μm2 in-plane resolution with 26 kV energy and ~12-s exposure time. Prone position data were acquired with 2 (beam coverage diameter, 27.7 cm; source to object distance, 28.6 cm). MicroDicom software version 0.1.5 (2007) was used for image analysis. At shelf position 2 (beam coverage diameter, 27.7 cm; source to object distance, 28.6 cm).
lesions were evaluated and quantified by a veterinary pathologist. A total of six lung sections at 100 μm intervals were collected and the total number of pulmonary nodules was counted. The presence of NK cells in the tibia was studied by immune staining using anti-human CD45 mAbs purchased from Abcam (ab6728). ChromoMap DAB (Roche) (760-2208) was used as chromogen. Sections were counterstained using Hematoxylin II (Roche) (790-2208).

Results

**OS cells express ligands for NK cell receptors**

Cell surface expression of ligands for NK cell receptors on tumor cells was assessed by flow cytometry. The MFI ratio was determined by calculating the fold-increase over isotype control using mouse anti-human mAbs. We found moderate to high levels of expression (5- to more-than-10-fold increase over isotype) of at least one NKG2DL (MICA, MICAB, or ULBP1, 2 or 3) in all of them. We also found high expression of Fas (14-fold increase over isotype), which binds FasL, and of CD155 (5-fold increase over isotype) and CXCL6.

**Statistical analysis**

Results are shown as means ± SD. Non-parametric Wilcoxon tests were used to compare NKG2DL MFI expression and cytotoxicity. In the mouse model, survival was estimated by using the univariate Kaplan–Meier method and compared by using the log-rank test. Statistical significance was defined as P ≤ 0.05.

**NKA cells target OS cells using NKG2D–NKG2DL interactions**

All 14 primary OS cell lines were sensitive to lysis by NKA cells, albeit at different levels (Fig. 2A). Although bone and metastatic primary OS cell lines differed in NKG2DL expression, their susceptibility to cell lysis by NKA cells was similar (Fig. 2B): higher expression of NKG2DL was not correlated with higher sensitivity to NKA cell elimination. For the 531MII cell line, we used NKA cells from a haploidentical donor or from other unrelated donors. Haploidentical NKA cells with a KIR receptor-ligand mismatch showed higher cytotoxicity than those from other donors and was enhanced when HLA class I molecules were blocked in the tumor cells (Fig. S1).

Because most OS primary cell lines had high expression of NKG2DLs (MICA, MICAB, and ULBP1, 2, and 3), Fas receptor, and ligands for DNAM-1 receptor, we explored whether blocking any of these receptors with specific antibodies affected NK cells’ cytotoxicity against OS. NKG2D receptor blockade (but not that of DNAM-1 nor FasL) significantly decreased NK cells’ cytotoxicity against OS (P = 0.03 for ratio 8:1 and P = 0.05 for ratios 4:1, 2:1 and 1:1; Fig. 2C), suggesting the direct involvement of NKG2D–NKG2DL interactions in the elimination of OS by NK cells.

![Fig. 1](image-url) Expression of inhibitory and activating NK ligands by osteosarcoma cells. A total of 22 human osteosarcoma primary cell lines (10 bone: 531B, 588B, 595B, 491B, 524B, 473B, 475B, 486B, 738B, and 699B; and 12 metastatic: 531MII, 588M, 595M, 491M, 491MII, 491MIII, 524B, 473B, 475B, 486B, 738B, 685MII, 722M, 722MII, 654M, 709M, 728M, 319M, and 699B) were evaluated for the expression of NK cell ligands by flow cytometry. (−) MFI ratio of specific staining versus staining of isotype control between 1 and 5. (++) MFI ratio between 10 and 15. (++) MFI ratio > 15. (B) MICA expression is significantly enhanced in metastatic tumors. **P ≤ 0.05.** (C) For those tumors from the same patient, the expression of MICA, MICAB, ULBP1, CD112, and CD69 is significantly higher in metastatic tumors than in their corresponding bone tumors. **P ≤ 0.05, ***P ≤ 0.01. 
SPIR up-regulates expression of NK cell ligands in OS cells and enhances their sensitivity to NK cell mediated cytolysis

Because of the importance of NKG2D–NKG2DL interactions for the NK cell-mediated elimination of OS, we tried to strengthen these interactions by up-regulating NKG2DL expression in OS cells. Recently it was reported that spironolactone (SPIR), an FDA-approved diuretic drug with a long-term safety profile, can up-regulate NKG2DL expression in multiple colon cancer cell lines by activating the ATM–Chk2-mediated checkpoint pathway, which in turn enhances tumor elimination by natural killer cells [19]. We found that SPIR treatment increased the expression of MICA, MICA/B, ULBP2 and ULBP3, in addition to CD155, and CD112 expression in 531MII, 491M, 654M, and 473B primary cell lines (Fig. 3A). To determine whether the increased expression of NKG2DL and CD112/CD112 induced by SPIR enhanced tumor cell killing by NKAE cells, we used BATDA assays to evaluate NK cells’ cytotoxicity against exposed and non-exposed (DMSO) 531MII cells. SPIR treatment significantly increased the susceptibility of 531MII cell to NKAE cell mediated lysis, particularly at low E:T ratio. (P = 0.03 and P = 0.009 for ratios 4:1 and 2:1 respectively, Fig. 3B). To determine whether the increased susceptibility to NKAE cell-mediated elimination induced by SPIR treatment was directly correlated with NKG2DL up-regulation, we performed cytotoxicity assays in the presence of NKG2D or DNAM-1
blocking antibodies. NKG2D blockade completely abolished the enhancement of NKAE cell cytotoxicity (P ≤ 0.001 for all ratios), but DNAM-1 blockade had no effect, confirming the direct involvement of NKG2D–NKG2DL interactions in NK cell-mediated elimination of SPIR treated OS cells (Fig. 3C).

**NKAE cells reduce the TIC compartment using NKG2D–NKG2DL interactions**

To evaluate whether NKAE cells were capable of decreasing the number of OS cells with TIC characteristics, we first analyzed the presence of TIC markers in the pool of 531MII and MG-63 cell lines and found that 531MII and MG-63 cells could grow as sphere-like structures under serum-starvation conditions. Both also had a high percentage of c-kit+ and CXCR4+ cells. We then co-cultured 531MII or MG-63 cells with NKAE cells and performed flow cytometry to analyze the percentage of c-kit+ and CXCR4+ cells. After 4 hours of co-culture, percentages of c-kit and CXCR4-positive cells in the 531MII and MG-63 pool were lower, and this decrease was even more noticeable after 24 hours of co-culture (Fig. 4A). NKAEs showed the ability to disrupt spheres that were already formed, but they were unable to completely eliminate them. However, when NKAE

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**Fig. 3.** SPIR treatment increases NKG2DL expression and sensitivity to NKAE-mediated cell lysis in primary bone and metastatic osteosarcoma cells. (A) Cell-surface expression of MICA, MICA/B, ULBP 1-3, CD155, and CD112 was analyzed in different primary and metastatic osteosarcoma cell lines treated with DMSO (solvent control) or SPIR (56 μM) for 3 days. (B) NK cells’ cytotoxicity on 531MII cells treated with DMSO or SPIR (56 μM) for 3 days was determined by performing BATDA release assays. Results shown are the average of 3 different experiments. (C) SPIR-treated 531MII cells were used for BATDA release assays performed with NKAE cells in the presence or absence of anti-NKG2D or anti-DNAM-1 blocking antibodies (10 or 20 μg/mL, respectively). *P < 0.05, **P ≤ 0.01, *** P ≤ 0.001.
Fig. 4. NKAE cells reduce the OS TICs compartment using NKG2D–NKG2DL interactions. (A) OS spheres were co-cultured with NKAE cells at an E:T ratio of 10:1 for 4 and 24 h, and then analyzed by FCM. Co-culture with NKAE cells reduces CXCR4 and c-kit positive subsets. (B) MG-63 GFP+ cells were cultured alone or with NKAE cells at an E:T ratio of 10:1 in SFA conditions. 5 days later no spheres are present when co-cultured with NKAE cells. (C) NKAE cells eliminate the OS TICs compartment by NKG2D–NKG2DL interactions. MG-63 GFP+ cells were co-cultured for 4 or 24 h with NKAE cells at an E:T ratio of 10:1, sorted and cultured in spheres medium. After 24 h remaining alive MG-63 GFP+ cells are unable to grow as spheres. However, when NKAE cells are previously treated with an NKG2D blocking antibody, they grow as spheres. (D) Number of spheres recovered after co-culture with NKAEs untreated or NKG2D blocked.
cells and MG-63 GFP+ cells were co-cultured at the same time in SFA conditions, no spheres formed, even after 8 days of co-culture (Fig. 4B). To further prove the ability of NKAEC cells to eliminate the TIC compartment, we co-cultured NKAEC cells with GFP-expressing MG-63 cells, sorted the living GFP-positive cells, and re-plated them in sarcosphere medium. Although 4 hours of co-culture was insufficient to eliminate the TIC compartment, 24 hours of co-culture totally abolished OS TICs’ ability to grow as spheres (Fig. 4C). Additionally, blocking NKAEC cells’ NKG2D receptor with an NKG2D blocking antibody enabled OS cells to grow as spheres (Fig. 4C and D), indicating that NK cells’ elimination of OS TICs occurs via NKG2D–NKG2DL interactions.

**NKAEC cells and SPIR synergistically shrink the TIC compartment**

To determine whether SPIR affected the ability of TICS to grow as spheres, we cultured 531MII cells in sarcosphere medium alone or in sarcosphere medium containing either DMSO (control solvent) or SPIR (56 μM). Five days later, untreated cells and those treated with DMSO had similar numbers of spheres. However, those cells that received SPIR treatment had an impaired capacity to grow as sarcospheres (Fig. 5A). Total cell numbers recovered were also lower when SPIR treatment was added (Fig. 5B). Flow cytometry analysis of the recovered cells revealed lower percentages of CXCR4 and c-kit positive cells, indicating that SPIR was affecting the OS TIC compartment (Fig. 5C).

We then evaluated whether NKAEC cells and SPIR could have a synergistic effect that impairs OS TICs’ ability to grow as spheres. The number of spheres was significantly reduced after the addition of NKAEC cells, particularly if the medium contained SPIR (Fig. 5D).

**NKAEC cells reduce the tumorigenicity of OS in an orthotopic mouse model**

Having shown NKAEC cells’ ability to kill OS cells and shrink the TIC compartment in vitro, we next tested the in vivo effect of NKAEC cells alone or in combination with SPIR in a xenograft orthotopic model.

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**Fig. 5.** SPIR treatment affects osteosarcoma TICs’ ability to form spheres. 531MII cells were grown in spheres under serum-starvation conditions in medium alone or in medium, with DMSO or SPIR (56 μM) for 12 days. (A) Pictures of 531MII (10x). (B) Total number of cells recovered. (C) Expression of CXCR4 and c-kit markers analyzed by flow cytometry. (D) MG-63 GFP+ cells were cultured as spheres with no treatment, DMSO or SPIR for 5 days. Then, NKAEC cells were added at an E:T ratio of 10:1. 72 hours later, sphere numbers were reduced.
OS model (Fig. 6A). Mice receiving NKAE cells, also received IL-2 IP injections (10000 IU) for 5 days. Compared with the control group and mice treated with SPIR alone, those groups treated with NKAE cells had less bone damage (Fig. 6B), smaller tumor volumes (Fig. 6C), absence of lung metastases (Fig. 6D), and significantly extended survival times (Fig. 6E). SPIR treatment alone seemed to have no effect on survival or tumor growth; however, in combination with NKAE cell treatment, it increased survival and reduced tumor volume (Fig. 6C and E). No differences were found in the in vivo model when haploidentical NKAEs were compared to unrelated NKAEs.

**Discussion**

Over the past 30 years the outcome of OS has not improved, with metastatic diseases with an overall survival of only 20%. OS TICs have increased tumorigenicity and ability to metastasize; therefore, there is a critical need for new therapeutic approaches targeting this compartment. Clinical data showed that NK cells may play an important role in OS prevention and treatment response. In patients with OS, a lower number of circulating NK cells was observed in peripheral blood compared to normal controls suggesting that NK cells play a preventive role in OS tumor development [23]. Furthermore, patients undergoing treatment for OS demonstrate better survival outcome with faster absolute lymphocyte recovery compared to patient with slow lymphocyte recovery denoting the antitumor role of the immune system in treatment response [24]. Finally, patients with OS treated with IL-2 in addition to polychemotherapy and surgery showed augmentation in the number and activity of NK cells, the magnitude of which correlated with an improved clinical outcome [25]. In the present study, we took advantage of NK cells’ ability to eliminate tumor cells without prior sensitization to develop a new cellular therapy to treat OS. Here, we show that a combination of activated and expanded NK cells and spironolactone efficiently eliminate OS cells, including those in the TIC compartment through NKG2D receptor/ligands interactions.

Fig. 6. Mice receiving NKAE cells show significantly reduced tumor growth, no lung metastases and longer survival. (A) Experimental groups and treatment. (B) X-rays from representative animals of each group taken at day 56. Mice from groups treated with NKAE cells + IL-2 or NKAE cells + IL-2 + SPIR show less bone damage than those mice untreated (CONTROL) or treated with SPIR alone (white arrows). (C) Tumor volumes are significantly smaller in groups treated with NKAE cells compared with CONTROL and SPIR alone. (D) Groups treated with NK cells show no lung metastases, while CONTROL and SPIR groups do (black arrows). (E) Survival is significantly longer in groups that received NKAE cells + SPIR.
and/or local ionizing irradiation might sensitize tumor cells to immune recognition, leading to synergistic antitumor effects. These observations are in agreement with one previous report [27] but are in contrast with one that showed that DNAM-1 receptor also had a main role in NK cells’ cytotoxicity against OS [28]. In vivo, OS primary and metastatic tumors have been shown to lose or downregulate HLA class I expression, thus becoming more susceptible to NK cell killing [29].

In agreement with a previous report in which NK cell-mediated killing was predicted by the degree of KIR receptor–ligand incompatibility [30], we observed an enhanced in vitro cytotoxicity of haploidentical NKA cells bearing a KIR receptor–ligand mismatch in the 531MII cell line. These data could indicate a beneficial effect of the use of haploidentical NKA cells as effector cells.

Induced expression of NKG2D ligands on tumors is a promising therapeutic strategy in chemo-resistant metastatic solid tumors, multiple myeloma, and myeloid leukemia [19,21,31,32]. Hence, we explored different mechanisms of NKG2D-dependent up-regulation in OS cells to enhance their sensitivity to NKA cell-mediated lysis. Although NKG2D up-regulation was not as specific as for colorectal carcinoma cell lines [19], SPIR treatment also enhanced OS cells’ susceptibility to NKA cells in an NKG2D–NKG2D dependent manner. Similarly, new generation cancer drugs such as the proteasome inhibitors and the histone deacetylase inhibitors can upregulate the death receptor DR5, sensitizing tumor cells to TRAIL-mediated killing by NK cells [33,34].

Tumor-initiating cells (TICs) are a subpopulation of chemoresistant tumor cells that have been shown to cause tumor recurrence and metastasis. Targeting and eliminating of TICs are therefore priorities for the development of new therapeutic paradigms. Several studies reported how immunotherapies as cytokine-induced killer (CIK) cells were capable of clearing cancer cells with stemness features in lymphoma, melanoma, hepatocellular carcinoma, bone and soft-tissue sarcomas [35]. Recently it was reported how innate immunity (natural killer cells and gamma delta T cells) and also adaptive immunity (cytotoxic T lymphocyte-based cellular immunity and antibody-based humoral immunity) can recognize TICs in vitro efficiently [36]. In the present study, we defined OS CSC/TICs according the ability of growing as spheres, because the presence of TIC markers (c-kit and CXCR4) and the in vivo ability to develop metastasis. Our study demonstrates that SPIR treatment can phenotypically and functionally reduce the subpopulation of OS TICs in vitro, as treated OS cells showed lower expression of c-kit and CXCR4, and a reduced ability to form sarcomas.

Previous studies have shown NK cells preferentially target oral squamous, glioblastoma, colon cancer stem cells [37–39], and clonogenic multiple myeloma cells with stem cell characteristics [40]. Castriconi et al. reported how NK cells efficiently killed glioma stem cells, probably through cytotoxicity receptor NKP46 and DNAM-1 [39]. Gamma-delta T cells can also target colon cancer CSCs/TICs [41]. Our study also agrees with these previous data regarding the ability of NK cells to eliminate TICs, although our focus is on OS cells, and the main role of NKG2D receptor.

Re-directed T cells have also been shown to target OS TICs [42]. Here, we demonstrate for the first time that NKA cells are able to eliminate the OS TICs in an NKG2D–NKG2D dependent manner. Exposing OS cells to NKA cells decreased both their capacity to form sarcomeres and their TIC fraction, and this effect was enhanced with the upregulation of NKG2D by SPIR treatment, showed to increase NK cell ability to reduce OS TICs compartment. An in vivo study using a xenograft model showed that mice receiving OS cells in conjunction with NKA cells had better survival rates, lower tumor volumes and less lung metastases than did mice receiving OS cells alone. These differences in tumor volume, lung metastases and survival rates are difficult to explain if NKA cells are not targeting the OS TIC compartment.

In summary, we provide strong evidence that NKA cells can kill OS cells, including the TIC compartment, in an NKG2D–NKG2D dependent manner. Furthermore, we demonstrate that NKA cells’ killing capacity can be further enhanced by increasing NKG2D expression on OS cells by using SPIR. Altogether, our data suggest that patients with OS could benefit from a combination of NK cells and SPIR as a new therapy for this disease.

Funding
This work was supported by National Health Service of Spain grant FIS PI12/01622 to Antonio Pérez-Martínez and a CRIS Cancer Foundation (http://www.criscancer.org/en/index.php) grant to Lucía Fernández.

Authors’ contributions

Acknowledgements
We thank Francisco Borrego for valuable discussions, Laura Janke for help with histopathology results and interpretation, and Jieun Kim for help with X-rays studies. We also thank St. Jude’s Scientific Editing Department for language correction.

Conflict of interest
None.

Appendix: Supplementary material
Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.07.042.

References
[8] None.