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Tumor-associated-antigens or osteosarcoma cell line lysates: Two efficient methods for *in vitro* generation of CTLs with special regard to MHC-I restriction

Michela Muraro *, Oana Madalina Mereuta, Francesco Saglio, Francesca Carraro, Cristina Cravero, Franca Fagioli

Stem Cell Transplantation and Cellular Therapy Unit, Pediatric Onco-Hematology Division, Regina Margherita Children's Hospital, Turin, Italy

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ABSTRACT

The expression of tumor associated antigens (TAA) in osteosarcoma cell lines allowed us to design an *in vitro* model for the generation of TAA-specific CTLs. Since the MHC-I-restriction of these peptides represents the major obstacle to clinical applications, we studied a second method for the generation of CTLs starting from osteosarcoma cell line lysates and PBMCs of HLA-I compatible healthy donors. TAA-specific CTLs showed high and homogeneous cytotoxic response against each peptide; high levels of IFN-γ were released by osteosarcoma cell line lysate specific-CTLs in response to the osteosarcoma cell line they were activated for. The MHC-I dependent osteosarcoma cell line lysate-specific CTLs activity was proved by the indifference against the HLA-I-negative erytroleukaemia cell line K562 and by the absence of IFN-γ production with the addition of HLA-class I blocking antibodies. These two methods may be considered the model for the autologous setting in the context of immunotherapeutic approaches for osteosarcoma patients.

1. Introduction

Bone and soft tissue sarcomas are aggressive malignant tumors whose treatment has dramatically improved over the last decade thanks to advances in diagnosis, surgical techniques, conformal radiotherapy, and combination chemotherapy. For patients with high-grade osteosarcoma the overall survival rate raised from 15% to 70%. Nevertheless, for patients with relapsed disease, the prognosis is still poor. In recently reported series, patients with early relapse, treated with high dose chemotherapy with stem cell rescue and surgery, had a high remission rate, but the disease-free survival remained low (12%) at 3 year follow-up [1–8]. Reports of tumor regression after allogeneic non myeloablative stem cell transplantation (SCT) indicate the potential of an immunotherapeutic approach [9,10]. However, as few patients maintained complete remission (CR), additional maintenance treatments need to be explored [11].

The identification of tumor antigens and their recognition by tumor-specific cytotoxic T lymphocytes (CTLs) has fuelled the development of immunotherapeutic strategies in solid malignancies [12]. Therefore, immunotherapy may represent an important option for these patients [13].

Many solid tumors, such as sarcomas and carcinomas, express tumor-specific associated antigens (TAA) on their surface, which can serve as targets for immune effector T cells. Nevertheless, the immune-surveillance against clinically manifested tumors is relatively inefficient and the number and function of dendritic cells (DCs) are dramatically reduced in patients with cancer, implying a defective presentation of TAA to the effector cells [14,15]. A large number of TAA, like the universal antigen Survivin (i.e. antigen expressed on practically all human tumors but not or very weakly expressed in normal tissues) and the germinal antigens of the MAGE family, were identified. The ability of patients' T cells to recognize these TAA in the context of HLA, makes adoptive immunotherapy with T cells directed to such antigens an appealing approach for patients with residual disease after standard treatment. The expression of HLA-A24 restricted MAGE, SSX and SART-3 family antigens has been identified in osteosarcoma cell lines and fresh osteosarcoma tissues [16–21], allowing the development of immunotherapeutic strategies in high-grade osteosarcoma patients.

The purpose of our study was to design an *in vitro* model for the generation and expansion of cytotoxic lymphocytes with special regard to HLA compatibility in the prospect of clinical application.

In the first step, we generated TAA-specific CTLs through activation by a mix of HLA-A24+ restricted peptides. That's why HLA24+ healthy donors were chosen for the generation of CTLs.

However, in spite of their specificity and well known characterization, the employment of peptides is unfavourable especially for

* Corresponding author. Address: Stem Cells Transplantation and Cellular Therapy Unit, Regina Margherita Children's Hospital, P.za Polonia 94, 10126 Turin, Italy. Fax: +39 0113135596.
E-mail address: michela.muraro@unito.it (M. Muraro).

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their MHC-I-restriction, which represents an obstacle to clinical applications. Moreover, these peptides are limited by their short permanence on the surface of antigen presenting cells (APCs) with consequent weak cytotoxic response.

For this reason, in the second step we designed a different method for the generation of CTLs, starting from osteosarcoma cell line lysates. In this case CTLs were generated from donors specifically selected for their HLA-I compatibility corresponding to every single cell line.

2. Materials and methods

2.1. Samples

In the first series of experiments we generated TAA-specific CTLs with a mix of peptides of MAGE, SSX and SART-3 family antigens starting from peripheral blood mononuclear cells (PBMCs) of five HLA-A24+ healthy donors. In the second series of experiments we generated CTLs starting from four osteosarcoma cell line lysates and PBMCs of four HLA-I compatible healthy donors. PBMCs were then separated by density gradient centrifugation over Lymphocytes separation medium (Eurobio, Les Ulis Cedex B, France) and used immediately or cryopreserved in fetal bovine serum (FBS) supplemented with 10% dimethylsulfoxide (DMSO) and stored in liquid nitrogen.

2.2. HLA typing

HLA-class I typing was performed by the Blood Bank Laboratory of the Regina Margherita-S.Anna Hospital with the molecular method.

2.3. HLA-class I-restricted peptides

The following HLA-A24+ restricted peptides were used (Laboratory INBIOS, Napoli, Italia):

peptide 1: MAGE-A1 – NYKHCFPEI
peptide 2: MAGE A2 – EYLQLVFGI
peptide 3: MAGE A3a – TFPDLESEF
peptide 4: MAGE A3b – IMPKAGLLI
peptide 5: SART-1 – EYRGFTQDF
peptide 6: SART-3a – VYDYNCHVDL
peptide 7: SART-3b – YIDFEMKI
peptide 8: SSX-2 – AWTHRLRER
The purity of all peptides was >95%.

2.4. Osteosarcoma cell line lysates

SJSA-1, U2-OS, HOS and MG-63 cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). SJSA-1 and U-2OS osteosarcoma cell lines were maintained in RPMI 1640 (Sigma, Saint Louis, MI, USA), containing 10% FBS (Sigma), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. HOS and MG-63 cell lines were maintained in Dulbecco's Modified Eagle Medium (D-MEM; Invitrogen, Carlsbad, CA, USA), containing 10% FBS (Sigma), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Once a week, cells were detached with trypsin/EDTA (Cambrex), counted and re-seeded at 1×10^6 cell/flask.

The osteosarcoma cell lines were harvested without serum for 24 h, washed twice with PBS and collected by scraping. Cells were then lysed through 5 cycles of freezing in liquid nitrogen and thawed at 37 °C. Lysis was monitored at optical microscope. After centrifugation, supernatants were filtered (0.2 μm) and frozen at −80 °C. Protein concentration of supernatants was then determined by Bio Rad Protein Assay, according to the product instructions.

2.5. Generation and loading of dendritic cells (DCs)

In the first series of experiments for the generation of TAA-specific CTLs, DCs were generated from PBMCs of HLA-A24+ healthy donors. In the second series of experiments DCs were isolated from PBMCs of healthy donors HLA-I compatible to each osteosarcoma cell line.

DCs were selected using a magnetic isolation method with CD14 microbeads and Mini-MACS columns (Milteny Biotec, Auburn, CA, USA), according to the manufacturer's instructions (Miltenyi Biotech). To evaluate purity, the CD14+ population was stained with the PE-anti-CD14 and the ECD-anti-CD3 mouse anti-human antibodies (mAb) (Beckman Coulter, Fullerton, CA, USA). The CD14 positive cells were cultured in CellGro DC medium (CellGenix, Freiburg, Germany) containing 10% FBS (Cambrex, Verviers, Belgium) and incubated with 800 U/ml recombinant human GM-CSF (rhGM-CSF, CellGenix) and 500 U/ml recombinant human interleukin-4 (rhIL-4, CellGenix). Fresh cytokines were added on day 2.

Different experiments were performed to determinate the optimal timing of DCs uptake with peptides and cell line lysates, respectively.

In the first series of experiments, DCs were collected on day five and loaded with the mix of HLA-I-restricted peptides (1 μM/each peptide) by three-hour incubation at 37 °C. In the second series of experiments, DCs were loaded with the lysate of the corresponding HLA-I compatible osteosarcoma cell line (50 μg lysate proteins/2×10^6 DCs) by two-hour incubation at 37 °C. Then, rhGM-CSF (800 U/ml) and rhIL-4 (500 U/ml) were added to the cell culture after the three-hour and two-hour incubation period, respectively.

The following day, DCs were maturated with a cytokine cocktail: rhGM-CSF (800 U/ml), rhIL-4 (500 U/ml), recombinant human interleukin-6 (rhIL-6 100 ng/ml, CellGenix), recombinant human TNF-α (rhTNF-α 10 ng/ml, CellGenix,), recombinant human interleukin-1β IL-1β (rhIL-1β 10 ng/ml, CellGenix) and recombinant human PG-E2 (rhPG-E2 1 μg/ml, Cayman chemical, Ann Arbor, MI, USA).

2.6. Generation of antigen-specific cytotoxic T lymphocytes (CTLs)

CD8 enriched cells were obtained by immunomagnetic separation (Miltenyi Biotec) from the samples of PBMCs. Briefly, PBMCs were incubated with CD8 microbeads at 20 μl/10×10^6 PBMC in 80 μl/10×10^6 PBMC MACS Buffer (PBS 1× + 0.5 BSA + EDTA 2 mM) for 15 min at 4 °C. Cells were passed through Mini-MACS columns and the positive fraction was collected.

Different culture conditions were tested in order to determinate the optimal number of stimulations and the effector-to-stimulator (E:S) ratio for the CTLs stimulations. Three specific antigen stimulations were necessary to generate specific CTLs endowed of cytotoxic activity. The appropriate E:S ratios selected were 40:1 for the first stimulation and 4:1 for the second and third stimulation.

In the first stimulation CD8 enriched cells were resuspended at 2×10^6/ml and co-cultured with autologous, irradiated (30 Gy) DCs pulsed with the mix of peptides (1 μM/each peptide) or osteosarcoma lysates (50 μg lysate proteins/2×10^6 DCs) at an E:S ratio 40:1 in presence of rhIL-12 (5 ng/ml) (R&D Systems, Minneapolis, USA) and rhIL-7 (5 ng/ml) (R&D Systems). After 7 days, for the second stimulation the cultures were restimulated with autologous pulsed and irradiated DCs at the E:S ratio of 4:1 in presence of rhIL-12 (5 ng/ml), rhIL-15 (1 ng/ml) (R&D Systems), rhIL-2 (100 U/ml) and rhIL-7 (5 ng/ml) (R&D Systems). Finally, after further 7 days the third stimulation was performed with autologous pulsed and irradiated DCs at the E:S ratio of 4:1 in presence of rhIL-2 (100 U/ml), rhIL-12 (5 ng/ml), rhIL-15 (1 ng/ml) (R&D Systems), rhIL-2 and rhIL-7.

2.7. Immunophenotyping

Mature DCs staining was performed with the PE-anti-CD86, PE-anti-CD83, PE-anti-CD80, PE-anti-CD40, PE-anti-CD14, and FITC-anti-HLA-DR monoclonal antibodies (mAbs) (Beckman Coulter). Mature DCs were also tested for negativity for the lineage markers PE-anti-CD3, PE-anti-CD19, PE-anti-CD20, PE-anti-CD16 and PE-anti-CD56 mAbs (Beckman Coulter). Control samples labelled with appropriate isotype-matched antibodies (Beckman Coulter) were used in each experiment.

CTLs immunophenotyping was performed with the following mAbs: anti-CD3 (Beckman Coulter); anti-CD4 (Becton Dickinson, San Jose, CA, USA); anti-CD8 (Becton Dickinson); anti-CD16 (Becton Dickinson); anti-CD19 (Immunotech, Marseille Cedex, France); anti-CD56 (Immunotech); anti-CD4 (Becton Dickinson). Appropriate combinations of fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- and R phycoerythrin-Texas Red-X (ECD)-conjugated mAbs were used. About 10×10^4 cells from each sample were analyzed on an Epics XL Cytometer (Beckman Coulter) with the XL2 software program. The lymphocytes were gated using forward/sideward scatter analysis. At least 100,000 events were acquired for each sample, specifically gating on characteristic size and granularity of lymphocytes (FSC vs SSC).

2.8. ELISPOT assay

Ninety-six-well polyvinylidene difluoride-backed plates (MAIPS, Millipore, Bedford, MA) were precoated overnight with a 10 μg/ml concentration of an anti-IFN-γ monoclonal antibody (1-DIK, Mabtech, Nacka, Sweden).

CTLs were added to triplicate wells in the number of 0.1×10^6/well in the presence of 2.5 μM peptides in the first series of experiments and in presence of 0.1×10^6/well cells of each osteosarcoma cell line in the second series of experiments (see below). The plates were incubated overnight at 37 °C in 5% CO_2. The cells were discarded the following day and a biotinylated anti-IFN-γ mAb (7-B6–1, Mabtech) was added at 1 μg/ml and left for 2–4 h at room temperature. Plates were then processed according to standard procedure.

In the first series of experiments, CTLs functional activity and specifity were tested against the following targets:

CTLs against peptide 1 (MAGE-A1),
CTLs against peptide 2 (MAGE A2)
CTLs against peptide 3 (MAGE A3a)
CTLs against peptide 4 (MAGE A3b),
CTLs against peptide 5 (SART-1),
CTLs against peptide 6 (SART-3a),
CTLs against peptide 7 (SART-3b),
CTLs against peptide 8 (SSX-2),
CTLs against indifferent peptide (negative CTRL),
Single peptides (negative CTRL),
CTLs alone (negative CTRL).

In the second series of experiments, CTLs functional activity and specifity against each osteosarcoma cell line were tested in the follwing conditions:

SJSA-1-activated CTLs against each osteosarcoma cell line,
U2-OS-activated CTLs against each osteosarcoma cell line,
HOS-activated CTLs against each osteosarcoma cell line,
MG-63-activated CTLs against each osteosarcoma cell line,
CTLs against PHA autologous blasts (negative CTRL),
Each osteosarcoma cell line alone (negative CTRL),
CTLs alone (negative CTRL),
CTLs against K562 cell line (negative CTRL).

In order to assure the specificity of osteosarcoma-lysate-activated CTLs in ELISPOT assay we performed two further negative control conditions using CTLs against autologous PHA blasts and CTLs against K562 cell line.

From the samples of PBMCs, autologous PHA blasts were obtained resuspending 5×10^6 cells in 10 ml of RPMI with 5 μg/ml of Phytohemagglutinin (PHA) (Invitrogen, Carlsbad, CA, USA).On day 2 and then every other day, 1 ml of fresh culture medium with 200 U/ml of IL-2 was added. The HLA-I missing erytroleukaemia cell line K562 was used to study the MHC-I dependent CTLs functional activity. K562 cell line grew in suspension in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Furthermore, blocking with anti-human MHC class-I antibody was performed to confirm MHC-I-restricted activity of CTLs in both sets of experiments. In fact, when HLA-class I blocking antibody was added in the well containing TAA-specific CTLs or lysate-specific CTLs, cytotoxicity was blocked and no IFN-γ production was revealed, confirming the MHC-I-restricted activity.

The spots were counted using an AID ELISPOT Reader System ELR03 (Nanogen Corporate Headquarters, San Diego, CA). The number of spots per well was calculated after subtracting assay background, defined as an average of the number of spots in 24 wells containing only complete medium, and specific background, defined as the number of spots in wells with responder CTLs alone or plated with DMSO solvent control, as appropriate.

2.9. ELISA

Human IFN-γ concentration in CTLs culture supernatants were measured by ELISA test (enzyme linked immunosorbent assay) (R&D Systems kit).

This kit is based on a sandwich ELISA. Human interleukine present in the test sample is captured by anti-human monoclonal antibody that has been pre-adsorbed on the surface of microtiter wells. After sample binding, unbound proteins and molecules are washed off, and a biotinylated detection antibody is added to the wells to bind to the captured interleukine. A strepavidin-conjugated horseradish peroxidase (SA-HRP) is then added to catalyze a colorimetric reaction with the chromogenic substrate TMB (3,3′,5,5′-tetramethylbenzidine). The colorimetric reaction produces a blue product, which turns yellow when the reaction is terminated by addition of dilute sulfuric acid. The absorbance of the yellow product at 450 nm is proportional to the amount of analyte present in the sample and a four-parameter standard curve can be generated. The interleukine concentrations in the test samples can then be quantified by interpolating their absorbance from the standard curve generated in parallel with the samples. After factoring sample dilutions, the interleukine concentrations in the original sample can finally be calculated.

We used the following procedure: we added 100 μl of diluted coating antibody (Coating Buffer, 0.05 M Carbonate-Bicarbonate, pH 9.6) to each well and incubated at room temperature (RT) (20–25 °C) for 1 h. Then we washed (Wash Solution, 50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) the plate five times, we added 200 μl of blocking solution (Blocking Solution, 50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) to each well and we incubated at RT for 30 min. After further five washing, 100 μl of standard or sample (Sample/Conjugate Diluent, 50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20) was added to well and incubated at RT for 1 h. The plate was washed five times and 100 μl of diluted HRP detection antibody was added to each well. The plate was incubated at RT for 1 h and then washed five times.

We added 100 μl of TMB (3,3′,5,5′-Tetramethylbenzidine) substrate solution to each well and developed the plate in the dark

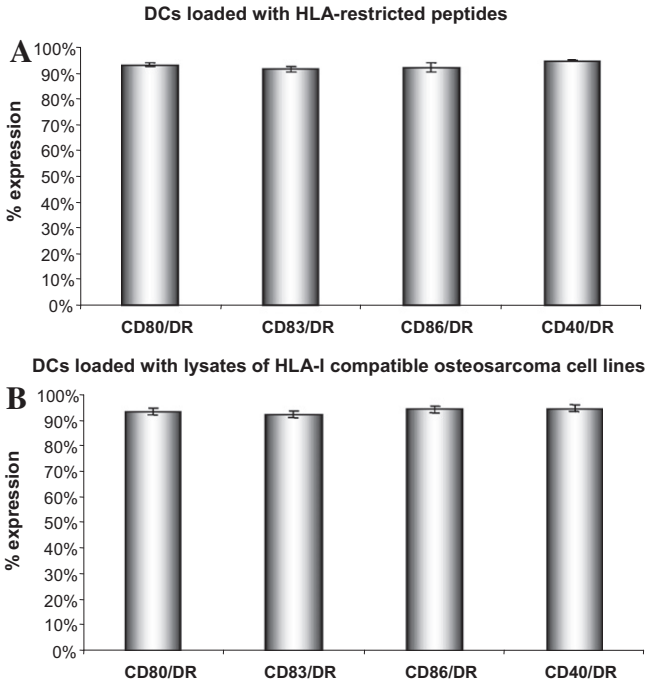

Fig. 1. (A, B) DCs immunophenotype: the expression of CD80, CD83, CD86, and CD40 on DCs that are MHC-II type DR was evaluated. Cytofluorimetric analysis shows a high percentage of all the co-stimulatory molecules expression in both experimental conditions. Data concerning DCs loaded with peptides and DCs loaded with osteosarcoma cell line lysates are mean of five and four experiments, respectively. Bars are standard errors.

at RT for 15 min. We stopped the reaction by adding 100 μl of 0.18 M H_2SO_4 to each well. We measured the absorbance on a plate reader at 450 nm.

2.10. Statistical analysis

For the generation of TAA-specific CTLs five independent experiments were performed, while for each osteosarcoma cell line lysate-specific CTLs three independent experiments was conducted. Data were given as mean ± standard error. Data were analyzed for statistical significance using the two-sided Student's *t* test. $p < 0.05$ was considered significant.

3. Results

3.1. Immunophenotyping

Before each stimulation, the maturation of DCs, pulsed with the mix of immunogenic peptides or with the lysate of the corresponding HLA-I compatible osteosarcoma cell line, was confirmed by cytofluorimetric analysis.

Thus, the expression of CD80, CD83, CD86 and CD40 on DCs that are MHC-II type DR was evaluated.

Cytofluorimetric analysis shows a high percentage of all the co-stimulatory molecules expression in both experimental conditions (Fig. 1 A, B). Data concerning DCs loaded with peptides and DCs loaded with osteosarcoma cell line lysates are mean of five and four experiments, respectively.

After three stimulations of CD8+ with DCs loaded with peptides or lysates we obtained the following cell population: in the first series of experiments 78.6% of CD3+ CD8+, 14% of CD3+ CD4+ cells, 2.5% of CD3–CD16+ cells and 3.9% of CD3–CD56+ cells; in the second series 81% of CD3+ CD8+, 9% of CD3+ CD4+ cells, 4% of CD3-CD16+ cells and 6% of CD3–CD56+ cells (Fig. 2 A, B).

3.2. CTLs specificity

ELISPOT assay was performed in order to test the faculty of CTLs to secrete IFN-γ upon specific recognition of antigens after the two types of stimulations in the first and second series of experiments respectively. In the first set of experiments, at the end of the third stimulation, TAA-specific CTLs showed a high and homogeneous cytotoxic response in terms of SFC (spot forming cell for 1×10^6 CTLs) against each peptide they were activated for.

No IFN-γ release was detected in the negative control conditions (CTLs against indifferent peptide, CTLs against each peptide alone and CTLs alone). Results are expressed as mean ± SE (Fig. 3).

In the second set of experiments, at the end of the third stimulation, high levels of IFN-γ in terms of SFC (spot forming cell for 1×10^6 CTLs) were released by osteosarcoma cell line lysate specific-CTLs in response to the osteosarcoma cell line they were activated for (Fig. 4). A minimal production of IFN-γ was observed against the other osteosarcoma cell lines without statistical significance. No IFN-γ release was detected against autologous PHA blasts; osteosarcoma cell lines alone and CTLs alone didn't produce IFN-γ Moreover, the MHC-I dependent CTLs functional activity was proved by the indifference against the HLA-I-negative erytroleukaemia cell line K562 (Fig. 4).

HLA-I restricted CTLs cytotoxicity was confirmed by means of HLA-class I blocking antibodies with no IFN-γ production (data not shown). Results are expressed as mean ± SE.

3.3. IFN-γ release in ELISA assay

Cytotoxicity was studied in terms of IFN-γ release in the supernatants of CTLs collected 24 h after each stimulation. TAA-specific CTLs produced improving amounts of IFN-γ up to a concentration of 3606 ± 256.85 pg/ml (Fig. 5A). Results are expressed as mean ± SE of five independent experiments and represent a pool of CTLs stimulated by all the different peptides.

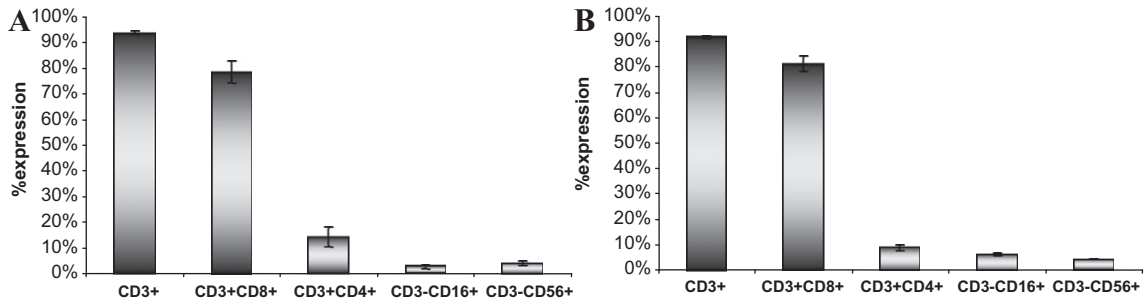

Fig. 2. (A, B) Cytofluorimetric analysis: the histograms show the different cellular populations obtained at the end of the third stimulation of CD8 enriched cells with DCs loaded with peptides (A) or lysates (B), respectively. Bars are standard errors.

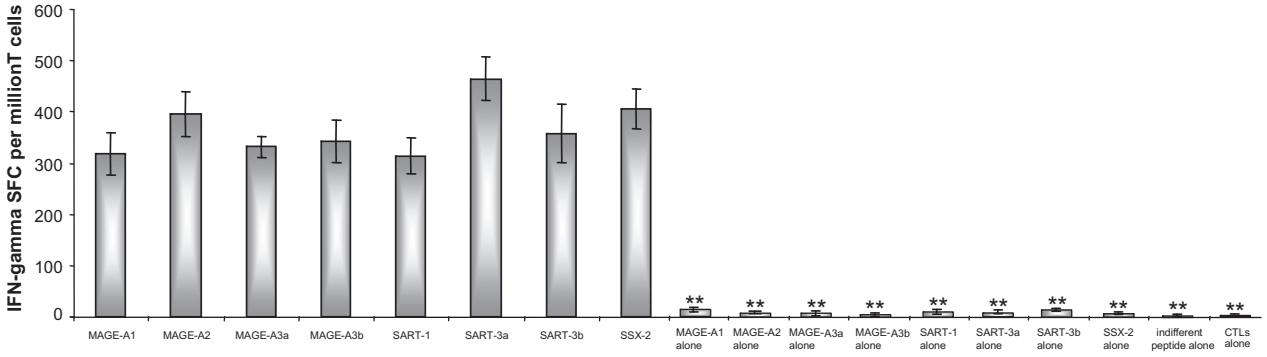

Fig. 3. IFN-γ release in response to peptides: the histograms show the IFN-γ release of TAA-specific CTLs at the end of the third stimulation in response to each peptide they were activated for (in terms of spot forming cell, SFC, for 1×10^6 CTLs) comparing this value with peptide alone controls. The statistical analysis was performed between each experimental condition and its own control. Bars are standard errors. (** $p < 0.01$).

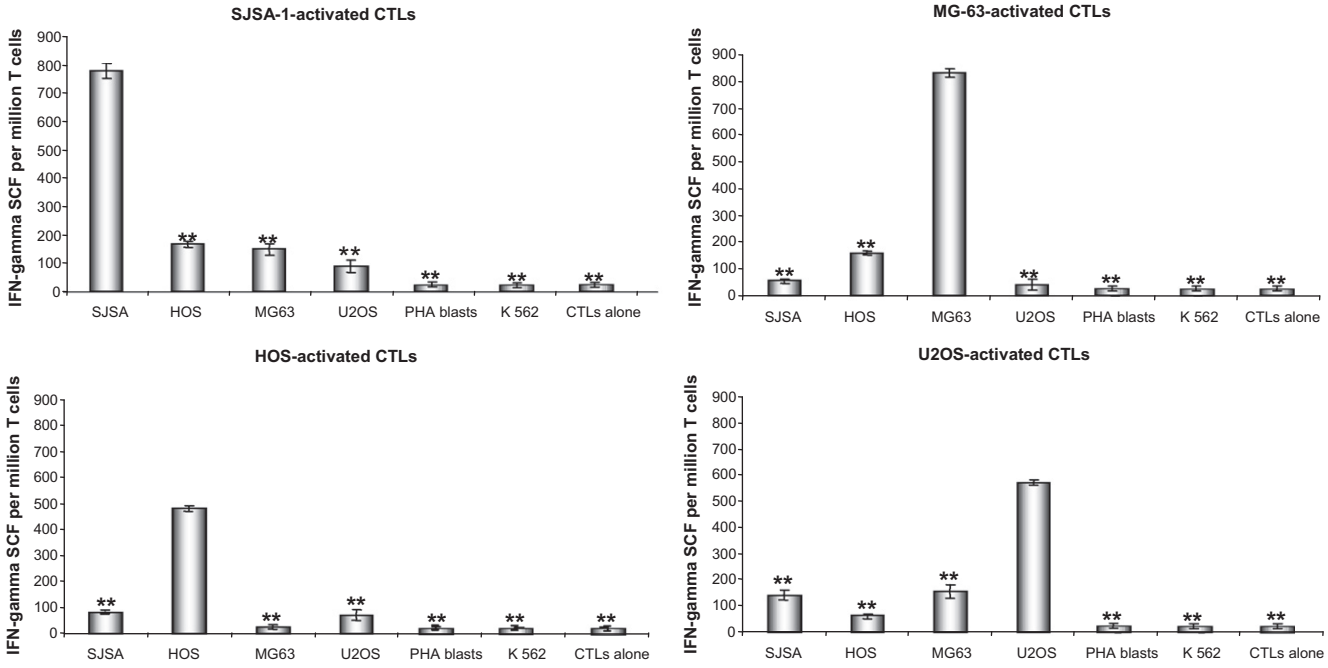

Fig. 4. IFN-γ release in response to osteosarcoma cell lines: High levels of IFN-γ in terms of SFC(spot forming cell for 1×10^6 CTLs) were released by osteosarcoma cell line lysate specific-CTLs in response to the osteosarcoma cell line they were activated for compared to the controls. A minimal production was observed against the other osteosarcoma cell lines. K562 cell line and autologous PHA blasts and CTLs alone were used as negative controls. The statistical analysis was performed between each experimental condition and its own control. Bars are standard errors. (** $p < 0.01$).

Similar results were observed in the supernatants of SJSA-1, U2OS, HOS- activated CTLs. Higher levels of IFN-γ were detected in the supernatant of MG-63-activated CTLs up to a concentration of 5831 ± 79 pg/ml (Fig. 5B). Results are expressed as mean ± SE of three independent experiments.

4. Discussion

T-cell therapy has attracted a great deal of interest in the treatment of viral infection [22], EBV-associated-tumors [23] and relapses of haematological diseases [24], while the identification of tumor antigens and their recognition by tumor-specific CTL has fuelled the development of immunotherapeutic strategies in cancer [25]. Moreover, the persistence of adoptively transferred T-cells and reconstitution of virus specific immunity has been demonstrated in the absence of noticeable side effects [12].

Nonetheless, tumor tolerance is relative and not absolute, leaving open a potential window on immunotherapeutic opportunities. The ability to grow tumor-specific T-cells from patients does not mean that *in vivo* tolerance has been broken. Many of the T-cell clones grown from these patients recognise peptides that bind poorly to their presenting Major Histocompatibility Complex (MHC) alleles or possess T-cell receptors with relatively low affinity for their cognate peptide-MHC complex. Thus, these T-cells weakly recognise tumor antigens compared with typical virus-specific T-cells and presumably fail to become activated *in vivo*, thereby ignoring tumor cells [13].

Experiments *in vitro* or in mouse models support the notion that tumor-specific T-cells can be activated and expanded *in vitro* and that they can inhibit tumor growth [26–31]. Recently, the expression of antigens of MAGE, SSX and SART-3 families has been identified in osteosarcoma cell lines and fresh osteosarcoma tissues

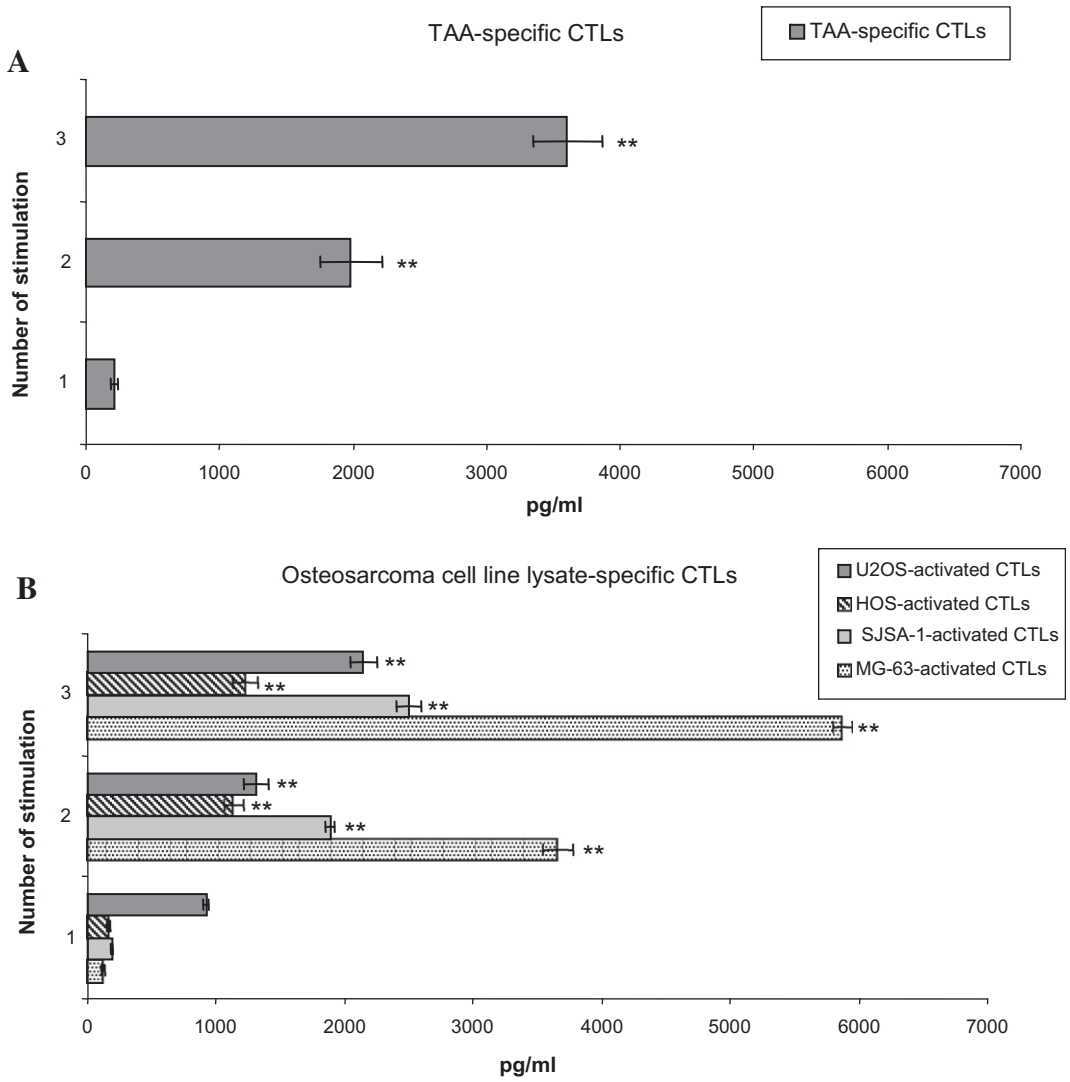

Fig. 5. IFN-γ secretion in ELISA assay: (A) Cytotoxicity was studied in terms of IFN-γ release in the supernatants of CTLs collected 24 h after each stimulation with the pull of all different peptides. TAA-specific CTLs produced IFN-γ up to a concentration of 3606 ± 256.85 pg/ml after the third stimulation. The statistical analysis was performed between each experimental condition and its own control. Results are expressed as mean ± SE of five independent experiments (** $p < 0.01$). (B) Similar results were observed in the supernatants of SJSA-1, U2OS, HOS- activated CTLs. MG-63-activated CTLs produced up to a concentration of 5831 ± 79 pg/ml after the third stimulation. The statistical analysis was performed between each experimental condition and its own control. Results are expressed as mean ± SE of three independent experiments. Bars are standard errors. (** $p < 0.01$).

[16–21], giving the opportunity to develop new immunotherapeutic approaches also for osteosarcoma patients.

We designed an *in vitro* model for the generation and expansion of cytotoxic lymphocytes with special regard to HLA compatibility in the prospect of clinical application.

In a first step starting from a mix of well known peptides of MAGE, SSX and SART-3 family antigens we demonstrated that it is possible to generate MHC-class I-restricted TAA-specific CTLs. At the end of the expansion procedure we obtained 78.6% of CD3+ CD8+ cells, 14% of CD3+ CD4+ cells, 2.5% of CD3-CD16+ cells and 3.9% of CD3-CD56+ cells.

In order to determinate the *in vitro* specific tumor reactivity of CTLs after three stimulations, CD8+ enriched cells were tested for IFN-γ production in ELISPOT assay, showing a homogeneous cytotoxic response against each peptide used for their stimulation.

Despite the advantage of their specificity, the employment of peptides for the generation of CTLs is unfavourable for their MHC-I-restriction, which represents the major obstacle to clinical applications. For this reason, in a second step we designed a different method for the generation of CTLs starting from osteosarcoma cell line lysates and PBMCs of HLA-I compatible healthy donors. Despite their antigenic variability, the use of cell line lysates with regard to HLA-compatibility may be considered the model for the autologous setting. In fact the major advantage of cellular lysates is represented by their easy reproducibility.

With this second method of CTLs generation we obtained 81% CD3+ CD8+ cells, 9% of CD3+ CD4+ cells, 4% of CD3–CD16+ cells and 6% of CD3–CD56+ cells.

The functionality and specificity of osteosarcoma cell line lysate-specific CTLs was analyzed in ELISPOT assay for IFN-γ production. High levels of IFN-γ were released by osteosarcoma cell line lysate specific-CTLs in response to the osteosarcoma cell line they were specifically activated for. A minimal production of IFN-γ was observed against the other osteosarcoma cell lines, due to a minimal alloreactivity without statistical significance. Moreover, in our model CTLs cytotoxic activity was proved to be MHC-I dependent by the indifference against the HLA-I-negative erytroleukaemia cell line K562. This result was further confirmed by the absence of IFN-γ production with the addition of HLA-class I blocking antibodies (data not shown). The functional activity of

CTLs generated in the two sets of experiments was also studied in terms of IFN-γ release in ELISA assay. Similar results were observed in the supernatants of TAA-specific CTLs and osteosarcoma cell line lysate-specific CTLs, suggesting a similar performance between the two methods.

Therefore, our *in vitro* study resulted in the effective generation and expansion of TAA-specific CTLs and osteosarcoma cell line lysate-specific CTLs. The good performance of both methods allows us to assume that the generation of CTLs starting from osteosarcoma cell line lysates and PBMCs of HLA-I compatible healthy donors may be considered the model for autologous setting in the context of immunotherapeutic approaches for osteosarcoma patients.

The potential clinical applicability of the future approach rests on the availability of a large number of tumor cells, which is to be employed as source of tumor antigens in the generation of anti-tumor CTLs.

We strongly believe that the manipulation of patients' biological materials in the preclinical setting may be transferred into an innovative treatment protocol, concerning the development of a direct immune response against specific MHC-restricted cancer targets.

We expect our project to have a potential impact at both scientific and clinical levels, providing the bases for effective immunotherapies that can be transferred and adopted as models in other solid incurable tumors.

Conflict of interest

The authors declare that they have no competing interests.

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