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Osteosarcoma cell line growth inhibition by zoledronate-stimulated effector cells

M. Muraro *, O.M. Mereuta, F. Carraro, E. Madon, F. Fagioli

Stem Cell Transplantation and Cellular Therapy Unit, Regina Margherita Children's Hospital, Piazza Polonia 94, 10126 Turin, Italy

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Abstract

Bisphosphonates have a profound effect on bone resorption and are widely used in the treatment of osteoclast-mediated bone diseases. Zoledronic acid (ZA), a third-generation biphosphonate, has a potent antitumor activity and expands gammadelta (γδ) T cells endowed of major histocompatibility complex-unrestricted lytic activity. Many solid tumors express tumor-specific antigens on their surface, representing targets for immune effector T cells. Nevertheless, the immune surveillance against clinically manifested tumors is relatively inefficient. Therefore, we investigated the hitherto unknown effects of ZA activated γδ T cells of normal donors on osteosarcoma cell lines. γδ T cells were stimulated with ZA and low doses of interleukin-2, and then analyzed for proliferation and generation of effector activity against osteosarcoma cell lines. Our results show the potent anti-tumor activity of ZA-stimulated γδ T cells and the enhanced immunosensitivity of osteosarcoma cell lines to γδ T cells suggesting that osteosarcoma is another γδ T cell susceptible tumor type.

Keywords: Gammadelta T cells; Osteosarcoma; Cytotoxicity

1. Introduction

Bone tissue sarcomas are aggressive malignant tumors of mesenchymal origin. The treatment of osteosarcoma patients has dramatically improved over the last decade. This progress has been brought about through advances in diagnosis, surgical techniques, conformal radiotherapy and combination chemotherapy [1,2]. For patients with high grade osteosarcoma the overall survival rate raised from 15% to 70%; nevertheless, for patients with relapsed disease, prognosis is still very poor [3].

In recently reported studies, early relapsed patients 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 [4]. Therefore, additional maintenance treatments need to be explored, such as the induction of an immune reaction against the tumor. New therapeutic options other than conventional surgery and chemotherapy are needed.

The identification of tumor associated antigens (Ags) in osteosarcoma allows a selective cell-mediated immunotherapy by the *ex-vivo* production of a large number of specific T lymphocytes, even though the variability of the subtypes leads to difficulty in identifying tumor-specific targets for immunotherapy and/or other therapeutic modalities [5–7].

Nevertheless, the immune surveillance against clinically manifested tumors is relatively inefficient. As evidenced for bone metastases, there might be a complex interaction between osteoclasts, bone stromal cells/osteoblasts, and cancer cells during the progression of primary bone tumors [8].

Several investigators have enhanced the potential effect of osteoclast-regulating drugs on tumor growth. Among these drugs, aminobisphosphonates (NBPs) are an important class of molecules with different molecular mechanisms of action used in the treatment of bone diseases.

* Corresponding author. Fax: +390113135487.
E-mail address: michela.muraro@unito.it (M. Muraro).

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NBPs act by inhibiting the recruitment, proliferation, and differentiation of preosteoclasts, or by impeding the resorptive activity of mature osteoclasts [9–12]. They also shorten the life span of osteoclasts by inducing their apoptosis [13].

Previous studies revealed that NBPs can reduce the osteolytic bone resorption associated with multiple myeloma and breast cancer [14,15]. Recent pre-clinical *in vitro* and *in vivo* models provided evidence that high doses of bisphosphonates may not only reduce bone loss by inhibition of osteoclast activity, but may also exert direct anti-tumor and anti-angiogenic effects [16–18].

Newer generation bisphosphonates, such as zoledronic acid (ZA) are 10,000- to 100,000-fold more potent than the older generation bisphosphonates [19]. ZA, the most potent NBPs clinically available, has shown efficacy in bone cancer metastases due to prostate cancer and other solid tumors, demonstrating that NBPs can reduce skeletal morbidity in both osteolytic and osteoblastic diseases [20,21]. Moreover, preclinical data show that NBPs can act on tumor cells by inhibiting tumor cell adhesion to mineralized bone as well as tumor cell invasion and proliferation [22,23].

The role of innate effector cells such as γδ T cells in natural tumor immunity and tumor immunotherapy has also been the subject of further research [24,25]. Circulating γδ T cells constitute about 1–5% of peripheral blood T cells and express the CD3+ CD4− CD8− phenotype [26]. Their T cell receptor (TCR) enables them to recognize families of unprocessed non-peptide compounds of low molecular weight (100–600 Da) with conserved patterns including microbial metabolites such as pyrophosphomonoesters and alkylamines [27,28].

NBPs share the chemical features of non-peptide compounds naturally recognized by γδ T cells, since they contain both a short hydrocarbon chain attached to a pyrophosphomonoster and an alkyl amine moiety [29].

Certain hemopoietic tumor cell lines, such as the Daudi Burkitt's lymphoma and the RPMI 8226 myeloma line are also recognized and lysed by γδ T cells *in vitro* [29,30].

Since no similar study has been performed on osteosarcoma cell lines, our purpose was to investigate the *in vitro* immunomodulatory effects of ZA-stimulated γδ T cells of healthy donors on this tumor type.

2. Materials and methods

2.1. Samples

Peripheral blood mononuclear cells (PBMCs) were prepared from Buffy coats from healthy donors, after written informed consent was obtained from each individual. PBMCs were separated by density gradient centrifugation over Lymphocytes separation medium (Eurobio, Analitica De Mori, Milano, Italy). Aliquots of the isolated PBMCS were frozen and stored at −180 °C until further use.

2.2. Culture conditions

PBMCs were cultured at 1×10^6/ml in round-bottom microtiter plates at 37 °C and 5% CO_2. The standard culture medium was Roswell Park Memorial Institute Medium (RPMI 1640; Sigma, Saint Louis, MI, USA) supplemented with 10% foetal bovine serum (FBS; Sigma, Saint Louis, MI, USA), L-glutamine 2 mM (Biochrom KG, Berlin, Germany), penicillin 100 U/ml (Sigma, Saint Louis, MI, USA) and streptomycin 100 μg/ml (Sigma, Saint Louis, MI, USA). Different culture conditions were tested in order to determinate the optimal T cell stimulation: medium alone, IL-2 (Chiron, Emeryville, CA) 10 U/ml, ZA (Novartis Pharma, Origgio, Italy) at different concentrations (0.25, 0.5, 1, 5, 10, and 20 μM) alone or with IL-2 (10 U/ml). The appropriate culture conditions selected were: ZA (1 μM) + IL-2 (10 U/ml) and IL-2 (10 U/ml) as control.

2.3. Flow cytometry

Immunophenotyping was performed with the following monoclonal antibodies (mAbs): anti CD3 (Beckman Coulter, Fullerton, CA); anti CD16 (Becton Dickinson, San Jose, CA, USA); anti CD 19 (Immunotech, Marseille Cedex, France); anti CD56 (Immunotech, Marseille Cedex, France); anti CD14 (Becton Dickinson, San Jose, CA, USA); anti TCR αβ (Immunotech, Marseille Cedex, France); anti TCR pan-γδ (Immunotech, Marseille Cedex, France), anti TCRVγ9 (Immunotech, Marseille Cedex, France). Appropriate combinations of fluorescein isithiocyanate (FITC)-, phycoerythrin (PE)-, 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, CA, USA) with the XL2 software program. The lymphocytes were gated using forward/sideward scatter analysis.

The increase of γδ T cells was calculated by counting the number of viable cells per well and by cytofluorimetric identification of γδ T cells using Beckman Coulter analysis on day 7.

Total counts of specific cell subsets per well were calculated by multiplying total counts of viable cell per well by the percentage of cells, as identified by two colour flow cytometry. At least 100,000 events were acquired for each sample, specifically gating on characteristic size and granularity of lymphocytes (FSC vs SSC) and propidium iodide (PI).

2.4. Osteosarcoma cell lines

The human osteosarcoma cell lines, SJSA-1, MG-63, HOS, U2OS and SAOS2, were obtained from the Ameri-

can Type Culture Collection. The SJSA-1, U2OS and SAOS2 cell lines were maintained in RPMI 1640 (Sigma, Saint Louis, MI, USA), while MG-63 and HOS cell lines were maintained in Dulbecco's Modified Eagle Medium (D-MEM; Invitrogen, Carlsbad, CA, USA), both supplemented with 10% FBS and 0.5 mg/ml penicillin/streptomycin. Once a week, cells were detached with trypsin/EDTA (Cambrex, Verviers, Belgium), counted and re-seeded at 1×10^6 cell/flask.

2.5. Antitumoral activity of ZA-activated γδ T cells

In the first set of experiments, culture was initiated at day 0 with PBMCs in the presence of the appropriate stimuli as described above. At day 6 of PBMCs culture, osteosarcoma cell lines were added to PBMCs for a further 24 h. Different effector-target (E:T) ratios (40:1, 20:1, 10:1, and 5:1) were tested in order to determinate the lysis efficiency of expanded γδ T cells.

To confirm the antitumoral activity of ZA-activated γδ T cells, we also used a flow cytometric assay based on CFSE staining of target cells and identification of dead cells by PI staining of CFSE-labeled target cells.

Osteosarcoma cell lines growth in presence of IL-2 was considerate as control. The results were expressed as percentage of growth inhibition, according to the formula:

$$1 - \frac{\text{mean number of viable osteosarcoma cells/experimental well}}{\text{mean number of viable osteosarcoma cell/control well}} \times 100$$

Total number of viable osteosarcoma cells after stimulation with IL-2 or ZA + IL-2 was determined at day 7 of culture.

To determinate whether the blockade of γδ TCR was sufficient to prevent antitumoral activity and was specific, in three experiments, PBMCs were left untreated, or incubated with anti-γδ TCR mAb, immediately before the addition of osteosarcoma cell lines.

In the second set of experiments, on day 5 of PBMCs culture, the osteosarcoma cell lines were pretreated for 18 h with ZA 1 μM and then mixed to PBMCs at different E:T ratios for a further 24 h in presence of IL-2 alone.

In both sets of experiments, the osteosarcoma cell lines were cultured alone in presence of ZA 1 μM to determine any direct effect on their growth rate and/or viability. PBMCs stimulated with IL-2 alone were used as control.

2.6. Cytolytic assay

The osteosarcoma cell lines were used as targets in a standard ^{51}Cr-release assay with PBMCs stimulated with IL-2 or ZA + IL-2 as effectors. Briefly, ^{51}Cr-labeled target cells (5×10^3 cells in 0.1 ml) were mixed in triplicate wells at the indicated effector to target cell ratios in round-bottomed microtiter plates. After 4 h of incubation at 37 °C, 50 μl of supernatants were collected and ^{51}Cr release was determined with a TopCount NXT^{TM} counter (Packard Instrument Company, CT, US). The percentage of specific ^{51}Cr release was calculated as reported in the legend of Fig. 5.

2.7. RT-PCR

Cells were pelletted and RNA was extracted using NucleoSpin RNA II (Macherey-Nagel GmbH & Co. KG, DE) according the manufacturer's protocol. Reverse transcription (RT) was performed using 1μg of total RNA and high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA).

For quantitative real-time RT-PCR (Q-RT-PCR), an Applied Biosystems 7900 HT termocycler with SYBR green (SYBR® Green PCR Master Mix Applied Biosystems, Foster City, CA) labelling was used and each sample was run in triplicate. Threshold cycle values for each gene in Q-RT-PCR were normalized against a housekeeping gene (PGK), relative quantization was done by using an excel manual method and results were expressed in log2 ratio. Primers used for PCR amplifications are listed below:

2.7.1. Human granzyme A

FW: 5′-CACACGCGAAGGTGACCTTA-3′
RE: 5′-CACATCGTCCCCCTTTTTAGG-3′

2.7.2. Human granzyme B

FW: 5′-GAGCCGACCCAGCAGTTTATC-3′
RE: 5′-TCTCCAGCTGCAGTAGCATGA-3′

2.7.3. Human perforin

FW: 5′-CGAGGCCCAGGTCAACATAG-3′
RE: 5′-GGCCGTCATCTTGTGCTTCT-3′

2.7.4. Human PGK

FW: 5′-AGCTGCTGGGTCTGTCATCCT -3′
RE: 5′-TGGCTCGGCTTTAACCTTGT -3′

Values derived from PBMCs cultured for 7 days in IL-2 alone or ZA + IL-2. Melting curves from Q-RT-PCR products were used as quality control for those analyses throughout.

2.8. Statistical analysis

Results were expressed as means of three different and independent experiments for each culture condition. Data were analyzed for statistical significance using the two-sided Student's t test. Statistical significance was set at $p < 0.05$.

2.9. Experimental design

	Day 0	Day 5	Day 6	Day 7
Ist set of experiments:	PBMCs + ZA 1μM + IL-2 10U/ml PBMCs + IL-2 10 U/ml (CTRL)	Co-culture of PBMCs with osteosarcoma cell lines	—	Cytofluorimetric analysis: - γδ T cells expansion - % PI positive osteosarcoma cell lines ^{51}Cr Assay RT-PCR
2nd set of experiments:	PBMCs + ZA 1μM + IL-2 10U/ml PBMCs + IL-2 10 U/ml (CTRL)	18 h ZA pre-treatment of osteosarcoma cell lines	Co-culture of ZA pre-treated osteosaroma cell lines with PBMCs	Cytofluorimetric analysis: - γδ T cells expansion - % PI positive osteosarcoma cell lines ^{51}Cr Assay RT-PCR

3. Results

3.1. Selective response to ZA stimulation

Based on preliminary experiments, ZA 1 μM + IL-2 10 U/ml were selected as the standard culture condition. This combination of stimuli induced the largest expansion of γδ T cells in all the healthy donors, compared to the expansion obtained after stimulation only with medium or IL-2 or ZA (at different concentrations).

Results are expressed as a percentage per well of viable γδ T cells: 10% in presence of IL-2 vs 50% in presence of ZA 1 μM + IL-2 ($p = 0.0021$) (Fig. 1). Flow cytometric analysis also revealed no significant expansion of other PBMCs subpopulations: CD14+, αβ T cells, CD8 + T cells and CD16 + cells were unaffected after 7 day culture in presence of IL-2 or ZA 1 μM + IL-2 ($p > 0.05$) (Fig. 2).

On the contrary, CD56+ cells were slightly increased upon stimulation with IL-2 or ZA + IL-2 ($p < 0.05$) compared to medium alone; CD4+ T cells, instead, were moderately decreased upon stimulation with ZA + IL-2 ($p < 0.05$). This variation in the NK and CD4+ T cell numbers do not compromise the selective and specific response of γδ T cells to ZA stimulation, as demonstrated by the anti-γδ TCR mAb treatment (Fig. 4).

The relative increase of γδ T lymphocytes in response to ZA 1 μM + IL-2 also reflects an increase in absolute cell numbers, as determined by counting the number of γδ T cells per well on day 7: 32,000 ± 1700 in presence of IL-2 vs 213,000 ± 6083 in presence of ZA + IL-2

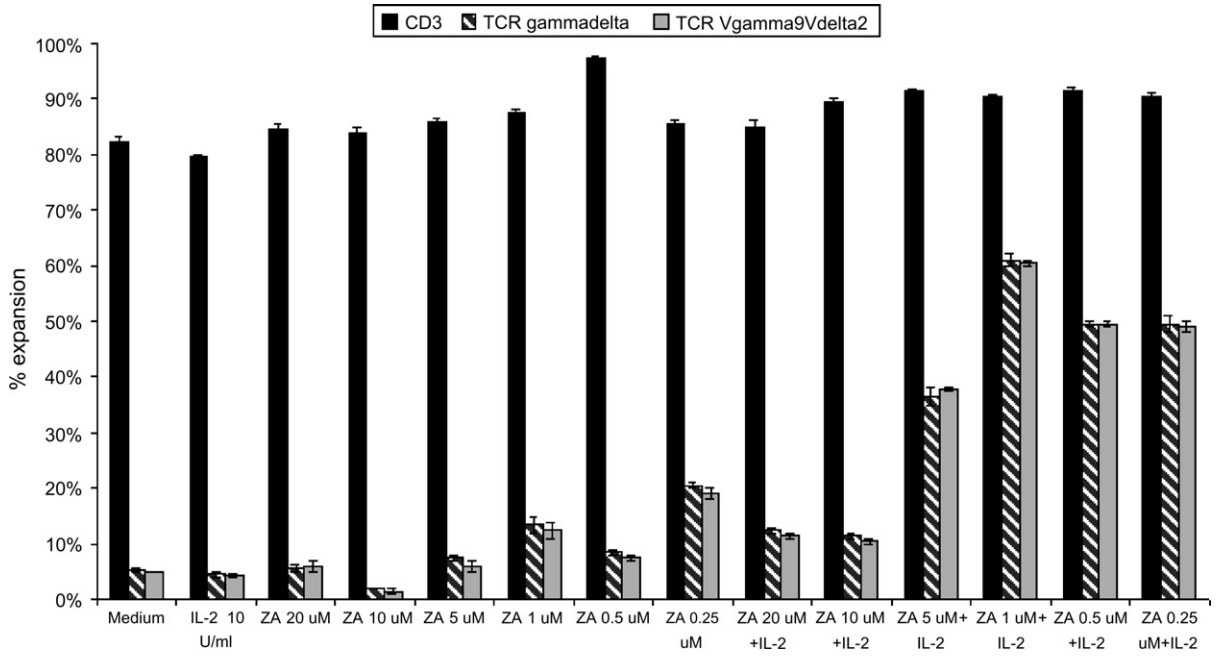

Fig. 1. Percentage of PBMC expansion after 7 days of culture with different concentrations of stimuli. PBMCs of healthy donors were cultured for 7 days in the following culture conditions: medium alone, IL-2 (10 U/mL) alone, ZA (0.25, 0.5, 1, 5, 10, and 20 μM) alone and ZA + IL-2, respectively. Results are expressed as a percentage per well of viable γδ T cells and represent the mean value of three independent experiments. ZA 1 μM + IL-2 (10 U/mL) culture condition induced the largest expansion of γδ T cells ($p = 0.0012$) compared to the IL-2 (10 U/mL) culture condition.

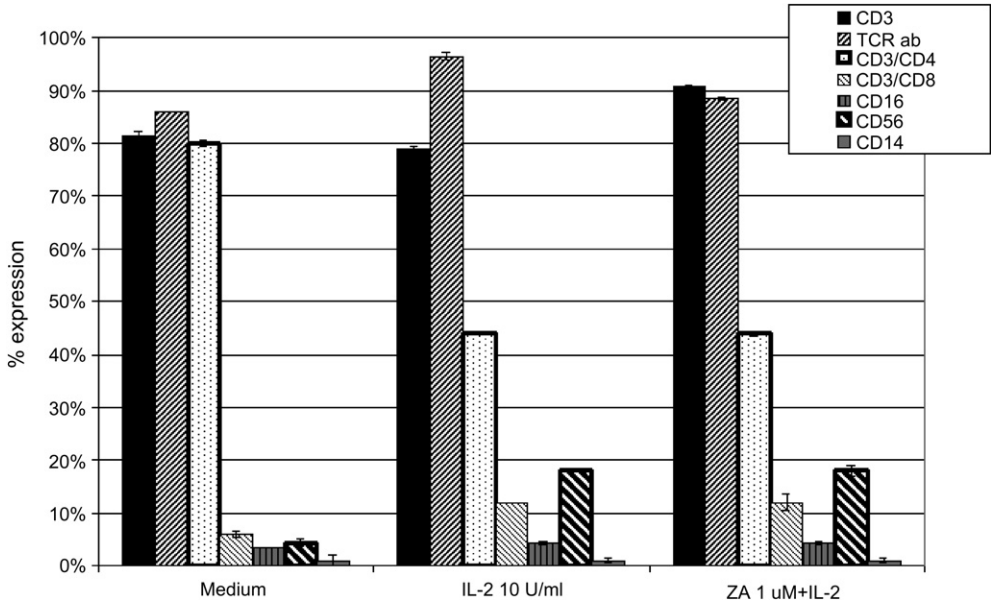

Fig. 2. Expansion of different PBMCs subpopulations after 7 days of culture. No significant expansion of monocytes, B cells, NK cells, αβ T cells, CD4 and CD8 T cells was observed in presence of medium or IL-2 10 U/ml or ZA 1 μM + IL-2. Results represent the mean value of three independent experiments.

($p = 0.00007$). The counts refer to the mean ± standard deviation (SD) of three experiments for each culture condition. This selective γδ T-cell outgrowth required the presence of low doses of IL-2 (10 U/mL).

3.2. Cytotoxic effect of ZA-activated γδ T cells

In the first set of experiments, on day 6 of PBMCs culture, the osteosarcoma cell lines were added at different ratio for a further 24 h. On day 7, we revealed a significant cytotoxic activity of ZA + IL-2 stimulated γδ T cells compared to IL-2 stimulated γδ T cells.

The average percentage of osteosarcoma cell lines killed at different E:T ratios by γδ T cells stimulated with IL-2 was strongly reduced compared to the average percentage of osteosarcoma cell lines killed by γδ T cell stimulated with ZA + IL-2: 9% ± 3% and 30% ± 3%, respectively ($p = 0.00004$) (Fig. 3A and B). The significant increase of γδ T cells percentage after ZA + IL-2 stimulation underlies an enhanced cytotoxic activity of these cells against all the osteosarcoma cell lines.

In three experiments, PBMCs were left untreated, or incubated with anti-γδ TCR mAb, immediately before the addition of osteosarcoma cell lines.

When PBMCs were treated with this mAb, the antitumoral activity was completely abrogated (−7% ± 0.8% and −11% ± 1% cell growth inhibitions in presence of IL-2 and ZA + IL-2, respectively), thus indicating an increased proliferation of all osteosarcoma cell lines (Fig. 4). Flow cytometry of residual γδ T cells showed that the TCR was still engaged by the mAb used.

These results show that ZA specifically engaged the effector γδ T cells, which were able to exert an anti-tumor activity at different effector-target ratio, only if they were stimulated with ZA and their receptor acted on target cells.

3.3. ^{51}Cr release assay

Evidence of ZA superiority over IL-2 alone was clearly detected when γδ T cells were tested in the standard ^{51}Cr release assay. The osteosarcoma cell lines were used as targets and PBMCs stimulated with IL-2 or ZA + IL-2 as effectors.

The ^{51}Cr assay revealed that γδ ? cells stimulated with ZA + IL-2 showed cytolytic activity versus all osteosarcoma cell lines used. In fact, when effector cells were stimulated with ZA + IL-2, the ^{51}Cr release was revealed for all lines tested and the more susceptible cell lines to γδ T cells activity were U2Os and MG-63 cell lines (Fig. 5).

This assay detects the added value of ZA + IL-2 ($p < 0.05$ in all E:T and osteosarcoma cell lines tested) over the baseline cytotoxicity induced by IL-2 alone (the so-called LAK activity), underlining a significantly higher γδ T cells activity after stimulation with ZA + IL-2 compared to IL-2.

3.4. Increased sensitivity of osteosarcoma cell lines to the γδ activity after ZA pre-treatment

As tumor cell lines become more susceptible to γδ T-cell mediated cytotoxicity after brief pulsing with NBPs, we therefore studied whether a direct effect on osteosarcoma cells contributed to the antitumor activity induced by ZA. On day 5 of PBMC culture, $SJSA_{-1}$, $SAOS_2$, U_2OS, HOS and MG-63 osteosarcoma cell lines were pretreated with ZA 1 μM for 18 h and then mixed with PBMCs on

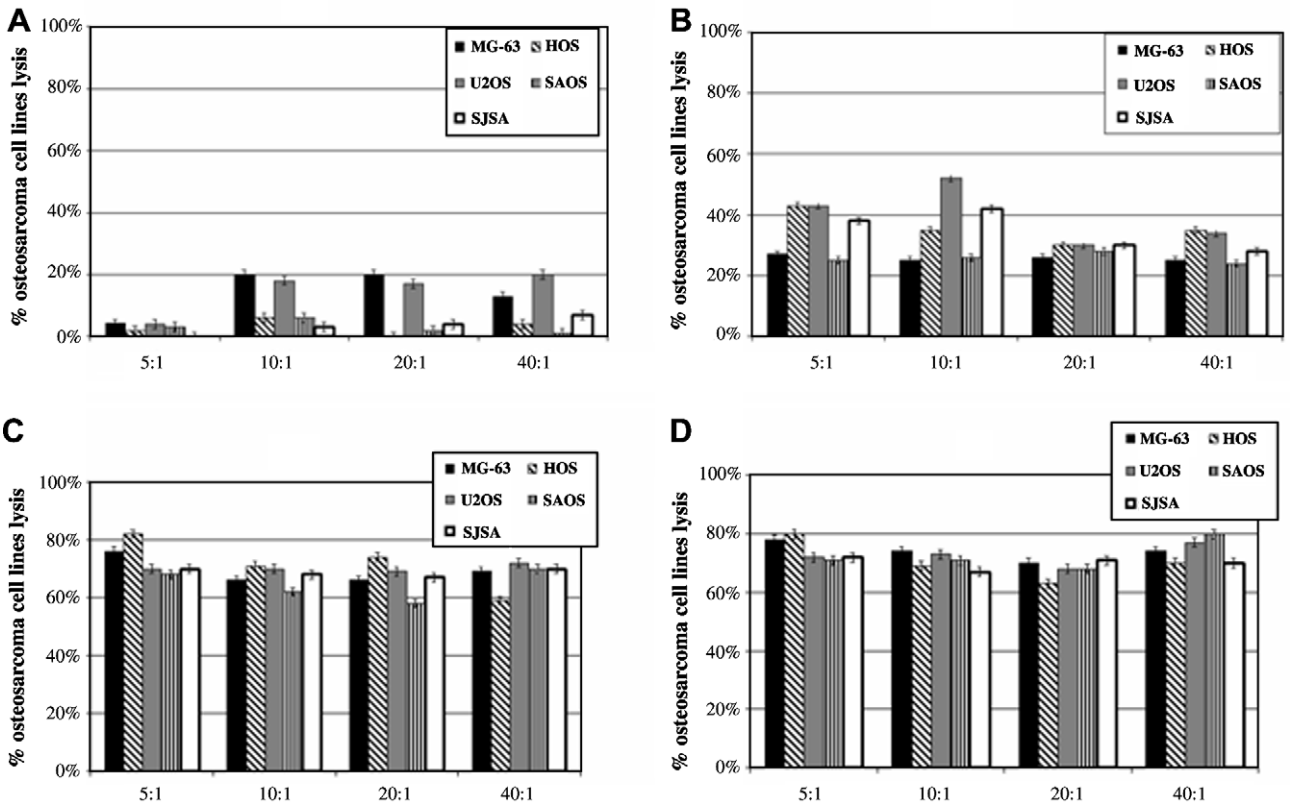

Fig. 3. Inhibition of osteosarcoma cell lines growth by expanded γδ T cells with different stimuli. The percentage of osteosarcoma cell lines killed by γδ T cells stimulated with IL-2 (10 U/ml) (A) was strongly reduced compared to the percentage of osteosarcoma cell lines killed by γδ T cell stimulated with ZA 1 μM + IL-2 (B) ($p < 0.05$). After a ZA 1 μM pre-treatment of 18 h all the osteosarcoma cell lines become more susceptible to the γδ mediated cytotoxic activity (C,D) ($p < 0.005$). Results did not depend on the E:T ratio tested and represent the mean value of three independent experiments for each culture condition.

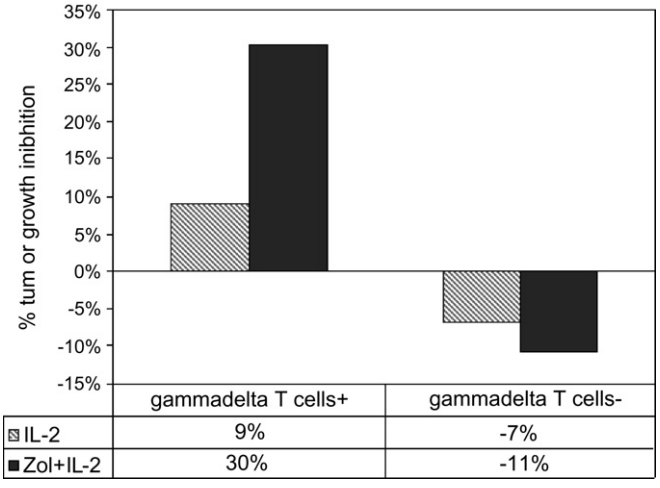

Fig. 4. ZA-induced antitumoral activity is dependent on γδ T cells. PBMCs were incubated with IL-2 or ZA + IL-2 for 5 days. On day 6, PBMCs were left untreated or treated with anti TCR γδ Ab and co-cultured for further 24 h with fixed numbers of osteosarcoma cell lines. On day 7, total counts of viable osteosarcoma cells and γδ T cells were calculated. The figure shows the mean percentage of osteosarcoma cell growth inhibition in PBMCs left untreated (open bars) or treated (solid bars). Inhibition was calculated according to the formula: 1 − (mean number of viable osteosarcoma cell lines in ZA + IL-2 wells/mean number of viable osteosarcoma cell lines in IL-2 wells) × 100. Negative values indicate increased proliferation as compared with IL-2 alone that was set as control.

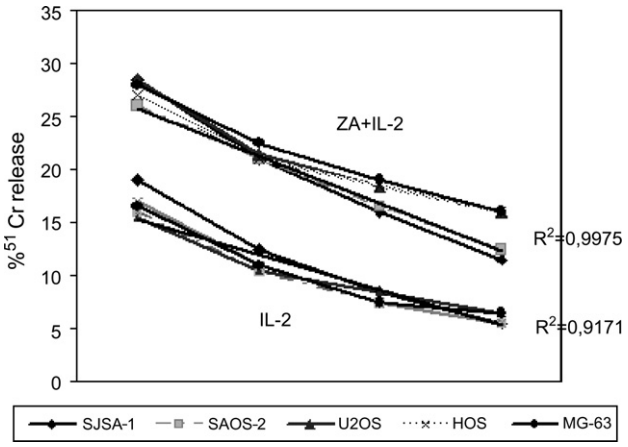

Fig. 5. Cytolytic activity of PBMCs after stimulation with ZA + IL-2. ^{51}Cr-labeled osteosarcoma cell lines were used as target cells at effector to target cell ratios of 12:1, 6:1, 4:1, 2:1. PBMCs effectors were stimulated for 7 days with ZA + IL-2 (*closed symbols*) or IL-2 (*open symbols*), respectively. The percentage of ^{51}Cr release was calculated from the expression: (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100, where the spontaneous release represents the amount of ^{51}Cr released by target cells incubated without effector cells, and the maximum release is that given by target cells upon treatment with 1% Triton-X in normal saline.

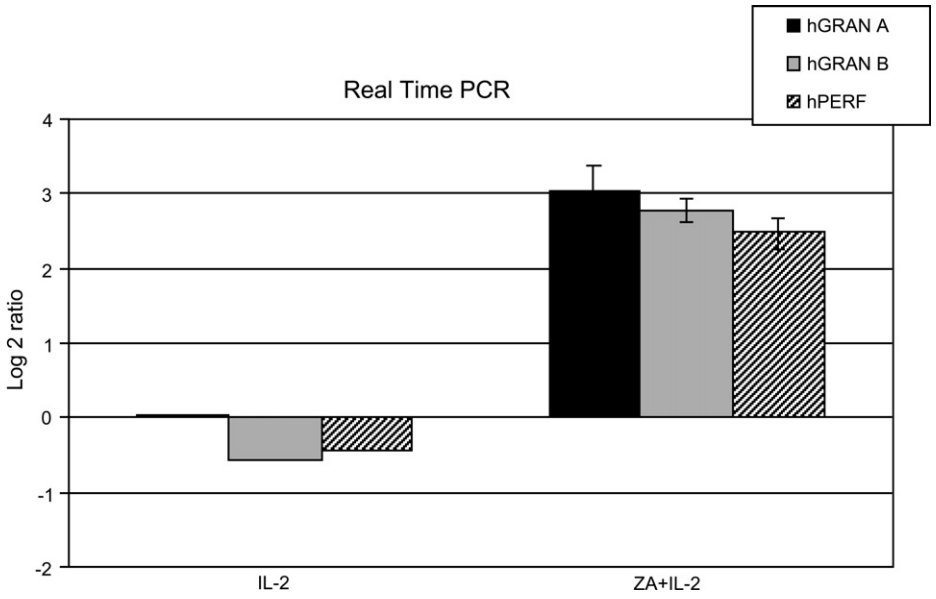

Fig. 6. Activation-induced changes in transcript levels for various effector molecules in γδ T cells. Gene expression levels were evaluated by Q-RT-PCR for ZA + IL-2 or IL-2 stimulated PBMCs. Results represent mean values of three independent experiments. Significantly increased levels of transcripts for perforin, granzyme A and B in γδ T cells, but not in αβ T cells, were obtained ($p < 0.01$).

day 6 and co-cultured for a further 24 h only in presence of IL-2. PBMCs incubated with IL-2 alone and untreated osteosarcoma cell lines were used as controls. The growth of ZA-treated tumor cell lines was strongly inhibited (Fig. 3C and D). These results are due to the increased ZA-induced sensitivity of tumor cells to γδ T cells activity. In fact, after the ZA-pre-treatment all the osteosarcoma cell lines become more susceptible to the γδ mediated cytotoxic activity, showing an average value of $69\% \pm 3\%$ ($p < 0.00005$) of osteosarcoma cell lines lysis in presence of IL-2 and $72\% \pm 3\%$ in presence of ZA + IL-2 ($p < 0.00005$), respectively.

Under these conditions, the antitumoral activity of ZA is optimized via the combined effects on γδ T cells mediated by monocytes and osteosarcoma cell lines.

These results indicate that a short-term incubation with ZA confers the following properties on osteosarcoma cell lines: (1) the ability to induce the proliferative expansion of γδ T cells; (2) a higher susceptibility to the effector mechanisms of γδ T cells.

In both sets of experiments, the different osteosarcoma cell lines were cultured alone with ZA 1 μM as negative control for its cytotoxicity. Indeed, no direct effect on the osteosarcoma cell lines growth rate and/or viability was revealed (data not shown).

3.5. Effector molecule expression

Transcripts for several effector molecules were compared using *in vitro* PBMCs stimulated for 7 days with IL-2 alone or ZA + IL-2 (Fig. 6).

We revealed high levels of perforin released by ZA + IL-2 expanded γδ T cells, while no perforin release was observed after IL-2 stimulated αb T cells ($p < 0.01$). A significantly increased level of transcript for granzyme A and B in γδ T cells, but not in αβ T cells, was obtained ($p < 0.01$).

4. Discussion

Most current immunotherapeutic approaches aim at inducing antitumor response via the stimulation of the adaptive immune system, which is dependent on major histocompatibility complex (MHC)-restricted αβ T cells. However, loss of MHC molecules is often observed in cancer cells, rendering tumor cells resistant to αβ T cell-mediated cytotoxicity [31,32]. The γδ T cells exhibit potent MHC-unresticted lytic activity against different tumor cell lines *in vitro*, such as lymphoma or myeloma cells, suggesting their potential utility in anticancer therapy [33–35].

In particular, human Vγ9/Vδ2 γδ T cells represent the majority of circulating γδ T cells in healthy adults and are polyclonally activated by low molecular-mass non-peptide Ags without prior priming. The non-peptide Ags include microbial metabolites such as pyrophosphomonoesters and alkylamines, as well as synthetic aminobisphosphonates such as pamidronate [36].

Unlike αβ T cells, activation of γδ T cells by the former non-peptide Ags does not seem to require the specialized APCs. However, it has been shown that the intimate cellular contact was a prerequisite for the γδ T cell clones to be activated by these Ags, which might be presented on γδ T cells themselves.

Several NBPs, such as pamidronate, ibandronate, alendronate, risendronate as well as zoledronate, have been reported to induce significant dose-dependent expansion of γδ T cells both *in vitro* and *in vivo*, mainly to the Vγ9Vδ2

subset [33,38,39]. NBPs analogues lacking one of the two phosphate groups lose their stimulatory activity, suggesting that both, the nitrogen atom and the P–C–P moieties are essential for the activation of γδ T cells by NBPs. The proliferative expansion of these cell subsets depends on monocytes, as previously reported for pamidronate [36]. Hence, it is tempting to speculate that ZA exerts immuno-regulatory properties by modulating monocytic cell function and differentiation.

Human Vγ2Vδ2 T cells recognize non-peptide antigens derived from pathogenic microbes in a TCR-dependent manner, such as pyrophoshomonoester compounds from mycobacteria and malaria parasite and alkyl amines from *Proteus*, suggesting that this subset of γδ T cells is involved in infectious immunity. Although activated γδ T cells exhibit a cytolytic activity against most of tumor cells, only a small fraction of tumor cells, like Burkitt lymphoma cells and multiple myeloid cells, is recognized by human γδ T cells in a TCR-dependent manner [27]. Indeed, Vγ9Vδ2 T-cells exert potent anti-tumor activity *in vitro* by killing myeloma cell lines and primary myeloma cells isolated from patients with multiple myeloma [29]. NBPs may manipulate the immune system to target and eliminate cancer cells [37].

As a response to NBP-mediated T cell activation and proliferation, γδ T cells secrete cytokines, such as TNFa and IFN-γ, enhancing antitumor activity by inhibiting tumor cell growth and angiogenesis. Vγ9Vδ2 T cells may also stimulate the activity of NK and NKT cells, macrophages and αβ T cells through the secretion of IFN-γ. Another mechanism through which γδ T cells may mediate cytotoxicity after target cell recognition is the perforin/granzyme pathway. Vγ9Vδ2 T lymphocytes also express the activating NK cell receptor NKG2D. This cell surface receptor is important for tumor cell recognition by bisphosphonate-activated γδ T cells; it recognizes cancer cells (carcinomas, myelomas, lymphomas) that express the stress-inducible MHC-I-related MICA/ MICB proteins. Vγ9Vδ2 T cells also recognize a complex formed between apolipoprotein (Apo) A–I and ATP synthase (AS), a mitochondrial enzyme which is translocated to the surface of some tumor cell lines, in a TCR-dependent fashion. The biological relevance of AS recognition by Vγ9Vδ2 T cells is still unclear, however, it may involve AS in NBPs presentation to γδ T cells [40].

Interestingly, it has been proposed that some tumor cells may have an innate susceptibility to γδ T-cell recognition because they accumulate phosphorylated mevalonate metabolites that are similar to the phosphorylated non-peptidic ligands of γδ T cells. This accumulation is due to the increased expression of hydroxy-methylglutaryl-CoA reductase (HMGR), the rate-limiting enzyme in the mevalonate pathway. ZA is a very potent inhibitor of farnesyl-pyrophosphate synthase (FPP), an enzyme acting downstream from HMGR in the mevalonate pathway.

One can predict that ZA-treated tumor cells are more susceptible to γδ T cells because they accumulate the phosphorylated mevalonate metabolites by means of two mechanisms: inhibition of FPP and putative increased expression of HMGR. Based on this model, one can also predict that inhibition of HMGR will abrogate the susceptibility to γδ T cells because the metabolites are not accumulated and FPP inhibition has no effect [29].

A recent study was performed using Daudi cells, but also a breast carcinoma line (YMB-1) activates the Vγ9Vδ2 population by mevalonate pathway dysregulation. Thus, accumulation of mevalonate metabolites in tumor cells is a powerful danger signal that activates the immune response [41].

Therefore, the anticancer effects of γδ T cells may be achieved through three main mechanisms: (1) inhibition of tumor growth by IFN-γ secreted from γδ T cells, (2) direct killing of cancer cells by perforin (secreted by cytotoxic T lymphocytes) following interaction of γδ T cells with cancer cells, and (3) IPP-dependent immunomodulatory mechanism, which has yet to be clarified *in vivo*. Vγ9Vδ2 T cells may therefore play a major role in cancer and the stimulation of these cells using NBPs could be an effective way to combat cancer.

These effects may represent a potential novel anti-tumor mechanism induced by aminobisphosphonates, which has been linked to the selective expansion of Vγ9Vδ2 T-cells. In support of this concept, it was found that the combination of pamidronate and low-dose IL-2 induced the *in vivo* expansion of Vγ9Vδ2 T-cells in a small cohort of patients with non-Hodgkin's lymphoma [42]. A recent study further demonstrated that ZA induces γδ T-cell expansion and function in patients suffering from epithelial malignancies [39]. T cells expressing the Vγ9Vδ2 T cell receptor (TCR) play an important role in immune system surveillance and defence [43–45].

As the aim of this study was to investigate the effects of ZA-stimulated γδ T cells of healthy donors on osteosarcoma cell lines, we used low doses of ZA (1 μM) and analyzed its *in vitro* immuno-modulatory action.

Our results demonstrate that ZA has a potent immunomodulatory effect *in vitro* on osteosarcoma cell lines, even at micromolar concentrations. It induces a rapid expansion of γδ T cells in healthy donors in the presence of very low doses of IL-2. These data are also supported by *in vitro* findings which show that the action of ZA is highly specific and, except for pan-γδ and Vγ9 T cells, it does not activate other immune effector cells.

On the other hand, it has been shown that tumor cell lines become more susceptible to γδ T-cell mediated cytotoxicity after brief pulsing with NBPs [38,46].

Highly activated γδ T cells efficiently killed a variety of tumor cells, while less activated γδ T cells showed only weak tumoricidal activity. When TCR usage was examined, activated γδ T cells were found to exert their cytotoxic activity on most of the tumor cells in a TCR-independent manner, except for a small fraction of tumor cells like RPMI8226 and Daudi. Cytotoxic activity for most of the tumor cells was significantly reduced as the activation state

of γδ T cells declined. After pretreatement of tumor cells with non-peptide antigens, however, γδ T cells exhibited a TCR-dependent cytotoxicity for most human tumor cells in a species-specific manner. Although the pretreatment efficiency of pyrophosphomonoester antigens and nitrogen-containing bisphosphonate compounds were almost the same in *in vitro* assay systems, pyrophosphomonoesters may be less active *in vivo* due to their susceptibility to alkaline phosphatase in plasma [47].

In fact, our experiments demonstrated that the pretreatment with ZA 1 μM for 18 h induced an enhanced antitumor activity of ZA-stimulated γδ T cells leading to a strong inhibition of osteosarcoma cell lines growth.

Thus, the enhanced immunosensitivity of tumor cells to γδ T cells can be another mechanism involved in the antitumoral activity induced by ZA.

Two non-mutually exclusive interpretations have been proposed to explain the enhanced immunosensitivity of tumor cells after exposure to NBPs: (1) NBPs bind to the tumor cell surface and directly target the effector functions of γδ T cells [39]; (2) NBPs are internalized by tumor cells and indirectly enhance their immunosensitivity to γδ T cells by modulating the mevalonate pathway [41].

Besides, we extended our analysis of stimulated T cells by directly comparing gene expression profiles of γδ T cells stimulated with ZA + IL-2 or IL-2.

The ZA-activated γδ T cell subsets, when compared with unstimulated PBMCs (cultured in medium alone) or IL-2 stimulated PBMCs, exhibited increased levels of transcript for several molecules involved in cytotoxic functions including those in the perforin/granzyme pathways.

Therefore, our study identifies the osteosarcoma as yet another γδ T cell susceptible tumor type. This could be effectively used as a starting point for further studies regarding an osteosarcoma-specific immunotherapy.

Thus, immunomodulation should be added to the list of ZA activities. Notably, immunomodulation is observed at significantly lower concentrations than those required for its anti-angiogenic, anti-metalloproteinase or pro-apoptotic activity.

To conclude, γδ T cells can control osteosarcoma cell lines by different mechanisms, including direct cytotoxicity and perforin/granzyme A and B release. ZA has immunomodulatory properties at concentrations significantly lower than the other aminobisphosphonates. This and its well-proven track record of safety should encourage its investigational use as an immunomodulatory drug.

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