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Interactions between osteosarcoma cell lines and dendritic cells immune function: An *in vitro* study

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ABSTRACT

Dendritic cells (DCs) might be partly responsible for the defective immune response in tumor bearing hosts, but no study in osteosarcoma patients is still available. Therefore, we investigated *in vitro* whether human osteosarcoma cell lines have an inhibitor effect on different types of DCs: CD14+DCs, DC1 and DC2. DCs derived from healthy donors were cultured with osteosarcoma cell lines and appropriate cytokine cocktails and analysed for the expression of co-stimulatory molecules (CD40, CD80, CD83, CD86, HLA-DR). Each interaction resulted in a lower phenotypic expression of the DCs maturation markers, especially on DC2. Moreover, the addition of various cytokines and compounds (rhIL-12, CD40L, Indometacin) induced the DC1 and DC2 subsets towards the Th1 pattern as shown by ELISA. Osteosarcoma highly interferes with an *in vitro* DCs immune function as antigen presenting cells. The understanding of tumor biology underlines the need for a specific osteosarcoma immunotherapy able to reverse this immune-surveillance inhibition.

1. Introduction

Osteosarcoma is the most frequent bone tumor and is most common between the ages of 15–25. A high number of patients have a high-grade tumor, which prognosis has dramatically improved from 10–15% to 65–70% in the last 25 years, thanks to the use of adjuvant and neo-adjuvant chemotherapy [1,2]. Nevertheless, despite continuing scientific research into the treatment, the prognosis of patients with osteosarcoma in metastatic relapse, especially in bones [3], is still very poor. Even in the last concluded Italian and Scandinavian phase II protocol including high-dose chemotherapy, the 3 years overall survival rate and the 3 years disease free survival rate were only 20% and 12%, respectively [4].

Many solid tumors, such as sarcomas and carcinomas, express tumor-specific antigens on their surface, which can serve as targets for immune effector T cells. Nevertheless, the immune-surveillance against clinically manifested tumors is relatively inefficient.

The growth and metastatic spread of tumors, to a large extent, depend on their capacity to overcome host defenses. Tumor escape from immune effectors is most often caused by weak immunogenicity of tumor antigens, antigen masking or overall immunosuppression, a characteristic of advanced cancer. Failure of antigen processing or binding to major histocompatibility antigen (MHC) molecules, inadequate or low-affinity binding of MHC complexes to T-cell receptors, or inadequate expression of co-stimulatory adhesion molecules in conjunction with the antigen-presenting MHC complex may all lead to poor immunogenicity of tumor-associated peptides and impaired antitumor response [5].

Because host professional Antigen Presenting Cells (APCs) are one of the most important elements in the induction of specific anti-tumor immune response, dendritic cells (DCs) might be partly responsible for the defective immune response in tumor bearing hosts. To date, two peripheral blood DC subsets have been described, which are distinguishable by their ability to express CD11c [6,7]: the CD11c+ subset represents myeloid-derived DCs (type 1 DCs or DC1), whereas the CD11c-negative (CD11c−) DCs, which express high levels of CD123, also known as IL-3 receptor, are designated as lymphoid-derived DCs (type 2 DCs or DC2) [8]. Although both subsets express high levels of HLA-DR and lack the lineage markers CD3, CD14, CD19, CD20, CD16 and CD56, functional differences between DC1 and DC2 have been described [9]. The DC1s have greater T cell-stimulatory activity [7,10], produce pro-inflammatory cytokines, up-regulate co-stimulatory molecules [11,12] and prime a Th1 response [13]. DC2 can produce IFN-α [14–16] and seem to support the generation of a Th2 response.

It has been shown that the number and the function of DCs are dramatically reduced in patients with head and neck squamous cell carcinoma, breast, renal, colon and prostate cancer [17,18]. Most of the DCs' inhibition effect by tumors has been attributed to soluble factors [19]. Till now, no study has been made in osteosarcoma patients. Therefore, with this work, we determinate whether osteosarcoma cells generate a similar immunosuppressive effect *in vitro*. Since the presence of co-stimulatory molecules on the

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DCs' surface is crucial in determining whether engaged T lymphocytes become anergic or develop productive immunity [20], we evaluated the DCs maturation, quantified as CD80, CD86, CD40 and HLA-DR expression and the cytokines release after co-culture with different concentrations of osteosarcoma cells. Given that another important aspect of DCs function is the maturation state, we also evaluated the expression of the CD83 molecule, a widely used DCs maturation marker thought to be involved in antigen presentation and cellular interactions [6]. We also studied possible differences between the DCs subpopulations: DCs derived from the CD14 positive cells (CD14+DCs), DC1 and DC2. Furthermore, we also tested whether the addition of some cytokines or compounds could change the immunosuppressive effect of the osteosarcoma cell lines on the maturation of different DCs types.

2. Materials and methods

2.1. Samples

Peripheral blood mononuclear cells (PBMCs) were prepared from buffy coats obtained from healthy donors (kindly provided by the local blood bank after a written informed consent was obtained from each individual). PBMCs were separated by density gradient centrifugation over lymphocytes separation medium (Eurobio, Les Ulis Cedex B, France).

2.2. Generation of DCs

CD14+DCs: DCs were selected from PBMCs using a magnetic isolation method, with CD14+ microbeads and Mini-MACS columns, according to the manufacturer's instructions (Miltenyi Biotech, Auburn, CA, USA).

The standard culture medium was the CellGro DC medium (CellGenix, Freiburg, Germany) containing 10% fetal bovine serum (FBS; Cambrex, Verviers, Belgium). Resulted cells were cultured at 1×10^6/ml in standard culture medium supplemented with 800 U/ml recombinant human GM-CSF (rhGM-CSF; CellGenix, Freiburg, Germany) and 500 U/ml recombinant human IL-4 (rhIL-4; CellGenix, Freiburg, Germany) in 24 flat-bottomed plates (Greiner Bio-One, Longwood, FL, USA) for 2 days at 37 °C in 5% CO_2; fresh cytokines were added on day 2. On day 4, cells were maturated with a cytokine cocktail: 500 U/ml of rhIL-4, 800 U/ml of rhGM-CSF, 100 ng/ml of recombinant human IL-6 (rhIL-6; CellGenix, Freiburg, Germany), 10 mg/ml of recombinant human IL-1β (rhIL-1β; CellGenix, Freiburg, Germany), 10 ng/ml of recombinant human TNF-α (rhTNF-α; CellGenix, Freiburg, Germany) and 1 μg/ml of recombinant human PG-E_2 (rhPG-E_2; Cayman Chemical, Ann Arbor, MI, USA). On day 5, mature DCs were immunophenotyped.

DC1: DC1 was selected from PBMCs using a two-step magnetic isolation method, with the BDCA-1 Dendritic Cell Isolation Kit, according to the manufacturer's instructions (Miltenyi Biotech, Auburn, CA, USA). First, the CD1c expressing B cells were depleted using CD19-labeled magnetic beads. Secondly, the B-cell depleted flow-through fraction was labeled with biotin-labeled CD1c antibody and selected by the anti-biotin antibody labeled micro-magnetic beads. These labeled cells were separated from the unlabeled fraction utilizing Mini-MACS magnetic columns (Miltenyi Biotech, Auburn, CA, USA). Resulted cells were cultured at 1×10^6/ml in CellGro DC medium supplemented with 10% FBS and 800 U/ml rhGM-CSF and 500 U/ml rhIL-4 in 24 flat-bottomed plates for 2 days at 37 °C in 5% CO2; fresh cytokines were added on day 2. On day 4, cells were maturated with the same cytokine cocktail of DC14+. On day 5, mature DCs were immunophenotyped.

DC2: DC2 was isolated directly from PBMC by immunomagnetic sorting using BDCA-4 Dendritic Cell Isolation Kit and Mini-MACS columns, according to the manufacturer's instructions (Miltenyi Biotech, Auburn, CA, USA). Resulted cells were cultured at 1×10^6/ml in CellGro DC medium supplemented with 10% FBS and 10 ng/ml of recombinant human IL-3 (rhIL-3; Stemcell Technologies, Vancouver, Canada) in 24 flat-bottomed plates for 2 days at 37 °C in 5% CO_2. Fresh cytokines were added on day 2 and, on day 4, cells were maturated with 100 ng/ml of lipopolysaccharide (LPS; Sigma, Saint Louis, MI, USA). On day 5, mature DCs were immunophenotyped.

2.3. Osteosarcoma cell Lines

The human osteosarcoma cell lines $SJSA_1$, MG-63, HOS, U_2OS and $SAOS_2$, were obtained from the ATCC (American Type Culture Collection). The SJSA-$_1$, U_2OS and $SAOS_2$ cell lines were maintained

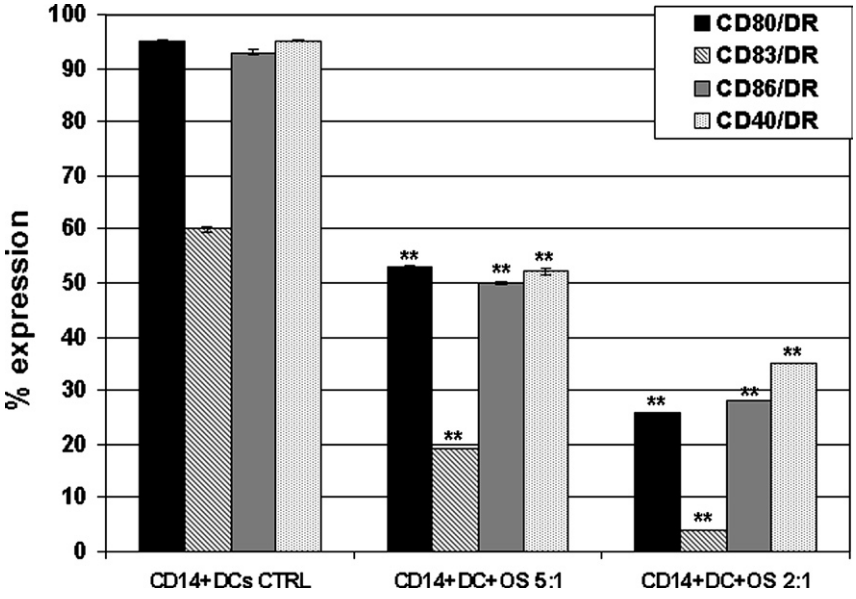

Fig. 1. CD14+DCs surface markers after 5 days of co-culture with osteosarcoma (OS) cell lines at 2:1 and 5:1 ratios. Cytofluorimetric analysis shows a significant decrease of all the co-stimulatory molecules expression for both E:T ratios. CD14+DCs were co-cultured with all OS cell lines and a mean percentage of the CD14+DCs surface markers expression was reported in the graphic. Three independent experiments were performed for each condition. Bars are standard errors. $^{**}p < 0.01$.

in Roswell Park Memorial Institute Medium (RPMI; 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 reseeded at 1×10^6 cell/flask.

2.4. Experimental culture conditions

Cell to cell contact co-culture experiments were performed. Different DC: tumor cell ratios were tested and the appropriate culture conditions were selected (2:1 and 5:1 ratios).

In the first set of experiments, the osteosarcoma cell lines were used separately and co-cultured with different DCs types in order to evaluate their effects on DCs maturation. Since no differences were revealed, we decided to use a mix of osteosarcoma cell lines for each co-culture experiment.

In the second set of experiments, DCs populations were cultured on day 0 with all osteosarcoma cell lines (2:1 and 5:1 ratios) in presence of the appropriate cytokine cocktails mentioned above. At the end of the culture, on day 5, cytofluorimetric analysis was performed to evaluate HLA-DR, CD80, CD83, CD86, CD40, CD14, CD16 and CD11b expression levels. For different culture conditions, a mean value for each DCs surface marker was considered. Cell viability of either DC type was also assessed in each co-culture experiment.

In the third set of experiments, on day 0, we added also 5 ng/ml of recombinant human IL-12 (rhIL-12), 2,5 μl/ml of CD40 Ligand (CD40L), 5 μM of Indometacin or 1000 U/ml of Interferon-γ (IFN-γ) to the DCs cultured with different osteosarcoma cell lines (2:1 and 5:1 ratios), in order to evaluate their potential to change the immunosuppressive effect of the osteosarcoma lines on maturation of different DCs types. Preliminary experiments were performed in order to decide the optimal concentration of these cytokines and compounds. On day 5, we tested the maturation markers expression.

For all the experiments, combined treatment (CD14+DCs, DC1, DC2 plus osteosarcoma cell lines) was compared with controls (CD14+DCs, DC1 or DC2 alone).

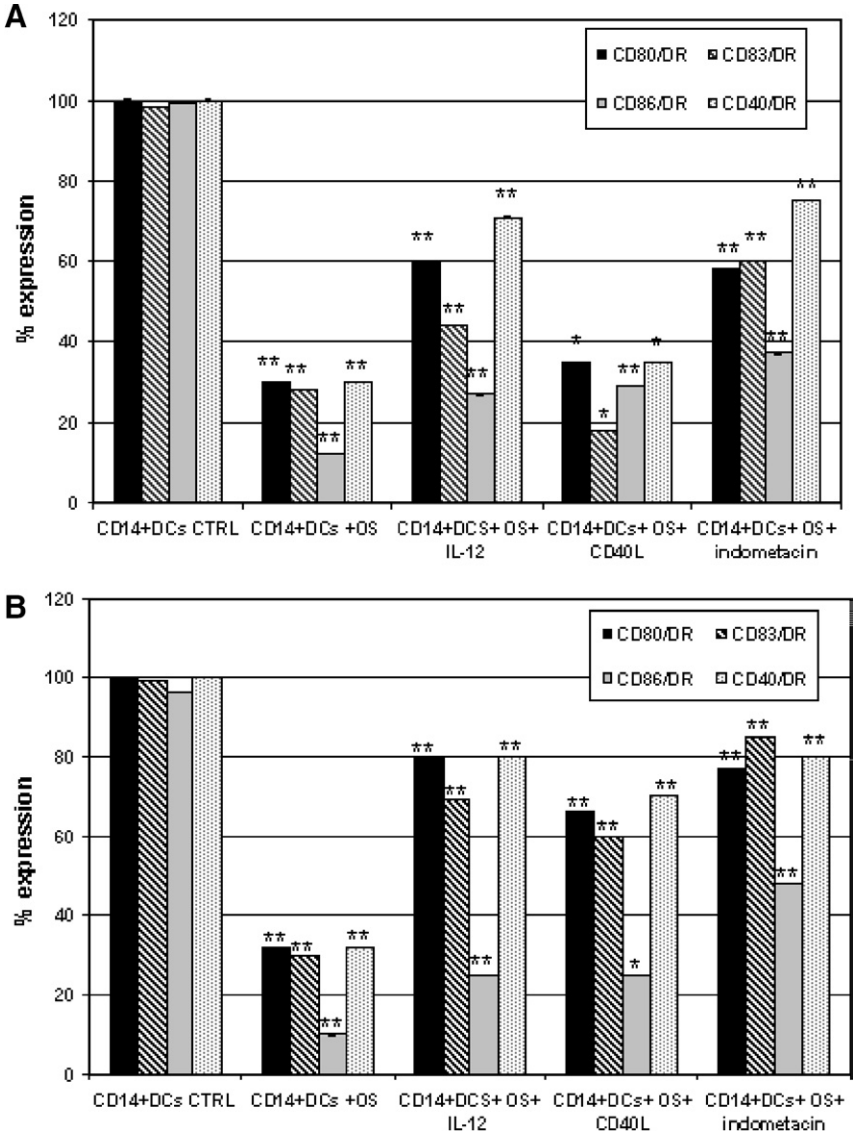

Fig. 2. CD14+DCs surface markers after the addition of different cytokines and compounds. CD14+DCs co-cultured with osteosarcoma (OS) cell lines at 2:1 (A) and 5:1 ratios (B): the immunosuppressive effect of OS cell lines on CD14+DCs maturation is significantly blocked in all culture conditions tested. Different co-culture conditions were tested and the mean percentage of the CD14+DCs surface markers expression was reported in the graphic. Three independent experiments were performed for each condition. Bars are standard errors. $^{*}p < 0.05$, $^{**}p < 0.01$.

2.5. Immunophenotyping

To evaluate purity, the CD14+DCs population was stained with the PE-anti-CD14 and the ECD-anti-CD3 mouse anti-human antibodies (mAb) (Coulter, Fullerton, CA, USA), the DC1 population with the PE-anti-BDCA-1 mAb (Miltenyi Biotech, Auburn, CA, USA) and the DC2 population with the PE-anti-BDCA-2 mAb (Miltenyi Biotech, Auburn, CA, USA). On days 0 and 5, DCs staining was performed with the PE-anti-CD86, PE-anti-CD83, PE-anti-CD80, PE-anti-CD40, PE-anti-CD14, PE-anti-CD16, PE-anti-CD11b and FITC-anti-HLA-DR mAbs (Coulter, Fullerton, CA, USA). Mature DCs were also tested for negativity at the lineage markers PE-anti-CD3, PE-anti-CD19, PE-anti-CD20 and PE-anti-CD56 mAbs (Coulter, Fullerton, CA, USA). Control samples labeled with appropriate isotype-matched antibodies (Coulter, Fullerton, CA, USA) were used in each experiment. In particular, cells were incubated with the mAbs for 30 min on ice and washed twice in phosphate buffer saline (PBS; Cambrex, Verviers, Belgium), containing 0.1% human serum albumin (HAS; Kedrion, Lucca, Italy) and 0.1% NaN3. After staining, the cells were analysed or fixed with 1% paraformaldehyde in PBS at room temperature before flow cytometry. At least 100,000 events were acquired for each sample, specifically gating on characteristic DCs size and granularity (FSC vs. SSC). To exclude background fluorescence, the percentage of positive cells was determined by subtracting the isotype-matched control antibody from the specific mAb histogram after normalization.

2.6. ELISA

Human IL-10 and IL-12p70 protein levels in the DCs culture supernatants were measured by sandwich ELISA (R&D Systems).

2.7. Statistical analysis

Three separate experiments were performed for each condition. Data were given as means ± standard deviation or standard error of mean. Data were analysed for statistical significance using the two-sided Student's *t*-test. $p < 0.05$ was considered significant.

3. Results

3.1. CD14+DCs surface markers

On day 0, CD14+DCs expressed HLA-DR, CD14 and CD11b but not CD80, CD83, CD86, CD40 and CD16. Moreover, on day 5, control monocyte-derived DCs differentiated along a pathway leading to the loss of CD14 and, following a maturation step, the up-regulation of co-stimulatory molecules CD80, CD83, CD86 and CD40, as well as CD11b expression. No CD16 expression by the control CD14+DCs was detected.

After 5 days of co-culture, CD14+DCs plus osteosarcoma cells at 2:1 and 5:1 ratios were compared with control (CD14+DCs alone) by cytofluorimetric analysis for CD80, CD86, CD40, HLA-DR and CD83 expression. We found a significantly decreased percentage of the co-stimulatory molecules expression in both experimental conditions ($^{**}p < 0.01$) (Fig. 1). No significant changes of CD14 and CD16 expression were found after the co-culture of CD14+DCs with osteosarcoma cell lines, whereas the percentage of CD11b expression was partially reduced compared to the control CD14+DCs (CD14+DCs alone). Histograms for different mAbs and isotype-matched controls are shown in Fig. 3.

The addition of rhIL-12 or CD40L or Indometacin to the CD14+DCs co-culture with osteosarcoma cell lines (Fig. 2) blocked the immunosuppressive effect of the osteosarcoma cell lines on CD14+DCs maturation enhancing the percentage of CD80, CD83, CD86 and CD40 surface markers expression, especially for the 5:1 ratio co-culture ($^{*}p < 0.05$, $^{**}p < 0.01$). Histograms for different mAbs and isotype-matched controls are shown in Fig. 4.

3.2. DC1 surface markers

On day 0, DC1 expressed HLA-DR and low levels of CD14 and CD11b. DC1 was also negative for CD80, CD83, CD86, CD40 and CD16. On the contrary, mature DC1 expressed high levels of HLA-DR, CD80, CD83, CD86 and CD40. The expression of CD14 was down-regulated, whereas the expression of CD11b was partially increased.

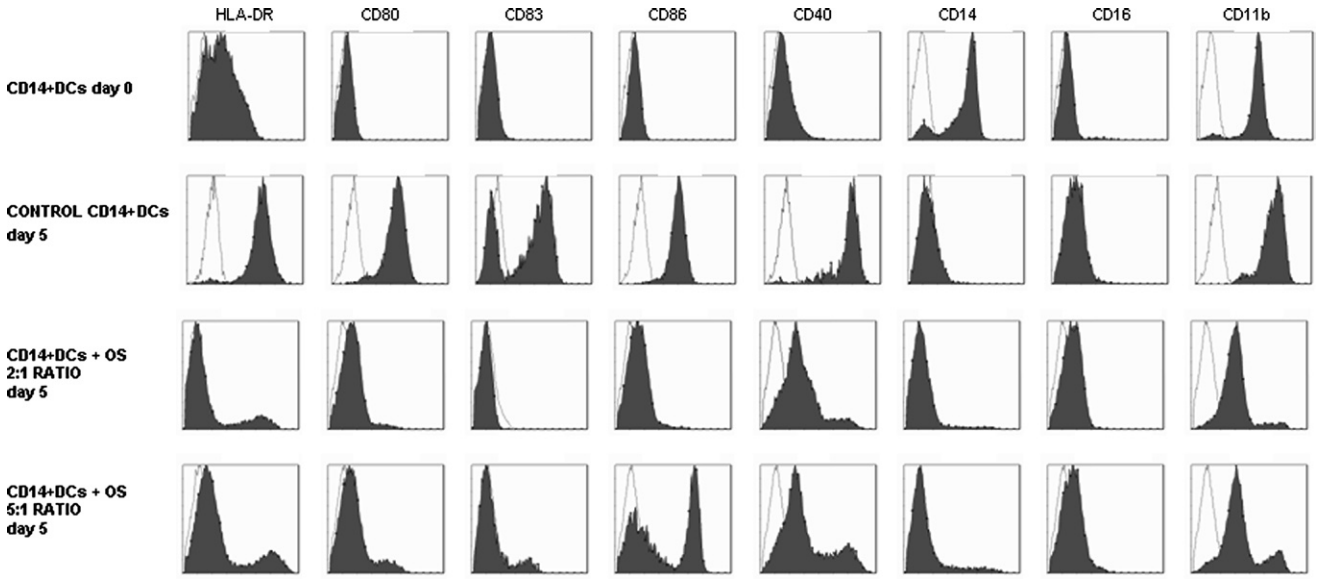

Fig. 3. CD14+DCs surface markers histograms before and after maturation and after the co-culture with OS cell lines at 2:1 and 5:1 ratios. On days 0 and 5, CD14+DCs were analysed by flow cytometry after labeling with isotype-matched controls and the indicated mAbs. On day 5, CD14+DCs co-cultured with OS cell lines (2:1 and 5:1 ratios) were compared to the control DCs (CD14+DCs alone). Histograms show profiles of isotype controls (dotted lines) and mAbs (solid lines). At least 100,000 gated events were acquired for each sample. Data are representative of three similar experiments.

After 5 days of co-culture, DC1 plus osteosarcoma cells were compared with control (DC1 alone). In both co-culture conditions tested (2:1 and 5:1 ratios), the percentage of all the maturation markers significantly decreased ($^{*}p < 0.05$, $^{**}p < 0.01$) (Fig. 5). Furthermore, DC1 was negative for CD14, CD16 and CD11b surface markers. Histograms for different mAbs and isotype-matched controls are shown in Fig. 6.

The addition of rhIL-12, CD40L or Indometacin to the DC1 co-culture did not change the immunosuppressive effect of the osteosarcoma cell lines on DC1 maturation (data not shown).

3.3. DC2 surface markers

On day 0, DC2 was negative for all the surface markers tested, except for the HLA-DR marker. On day 5, DC2 showed a significantly increased percentage of HLA-DR, CD80, CD83, CD86 and CD40 expression, but remained negative for CD14, CD16 and CD11b.

After 5 days of co-culture, DC2 plus osteosarcoma cells, were compared with the control (DC2 alone) by cytofluorimetric analysis for CD80, CD86, CD40, HLA-DR and CD83. DC2 population showed similar features for both ratios used: 2:1 and 5:1 (Fig. 7). In particular, the percentage of CD80, CD83, CD86 and CD40 surface markers expression significantly decreased after co-culture of DC2 with osteosarcoma cell lines ($^{**}p < 0.01$). The negativity for CD14, CD16 and CD11b was maintained after the co-culture. Histograms for different mAbs and isotype-matched controls are shown in Fig. 9.

Indometacin and rhIL-12 added to the co-culture partially reversed the immunosuppressive effect of osteosarcoma cell lines promoting a significant DC2 maturation for both ratio used ($^{**}p < 0.01$) as shown by the increased percentage of the surface

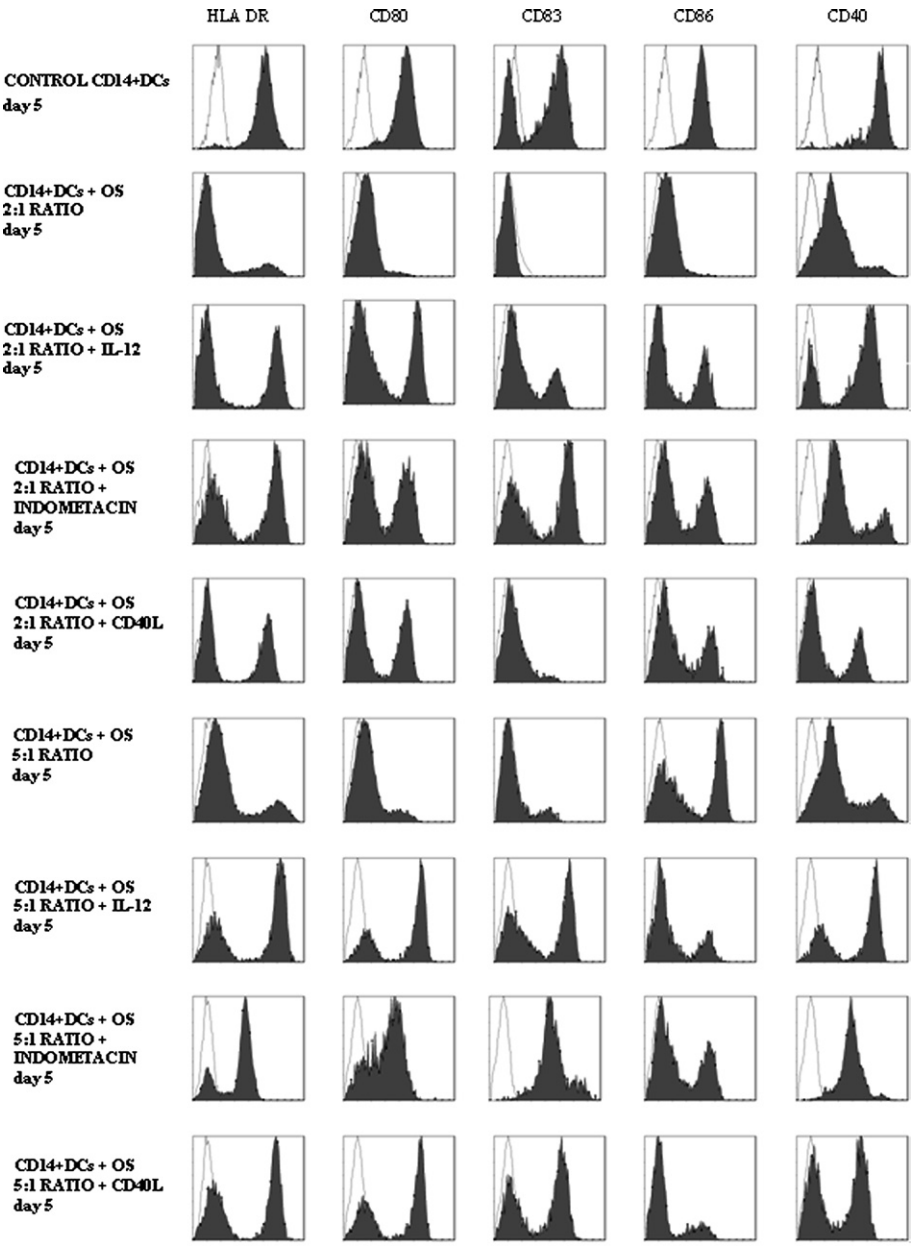

Fig. 4. CD14+DCs co-stimulatory molecules histograms after the addition of different cytokines and compounds. In some experiments, 5 ng/ml of recombinant human IL-12 (rhIL-12), 2,5 μl/ml of CD40 Ligand (CD40L) or 5 μM of Indometacin were added to the co-cultures on day 0 and the maturation surface markers were analysed on day 5. Histograms show profiles of isotype controls (dotted lines) and mAbs (solid lines). At least 100,000 gated events were acquired for each sample. Data are representative of three similar experiments.

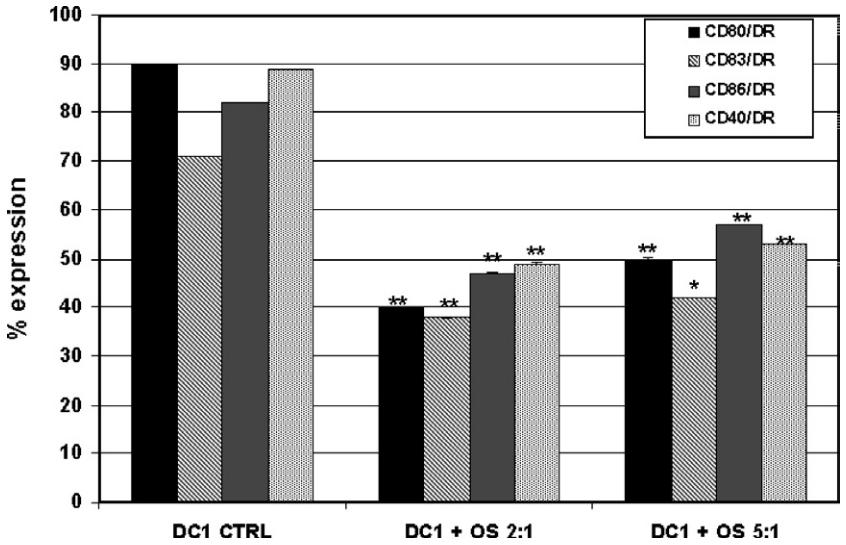

Fig. 5. DC1 surface markers after 5 days of co-culture with osteosarcoma (OS) cell lines at 2:1 and 5:1 ratios. A significant decrease of all the co-stimulatory molecules expression for both E:T ratios was revealed. DC1 was co-cultured with all OS cell lines and a mean percentage of the DC1 surface markers expression was reported in the graphic. Three independent experiments were performed for each condition. Bars are standard errors. $^{*}p < 0.05$, $^{**}p < 0.01$.

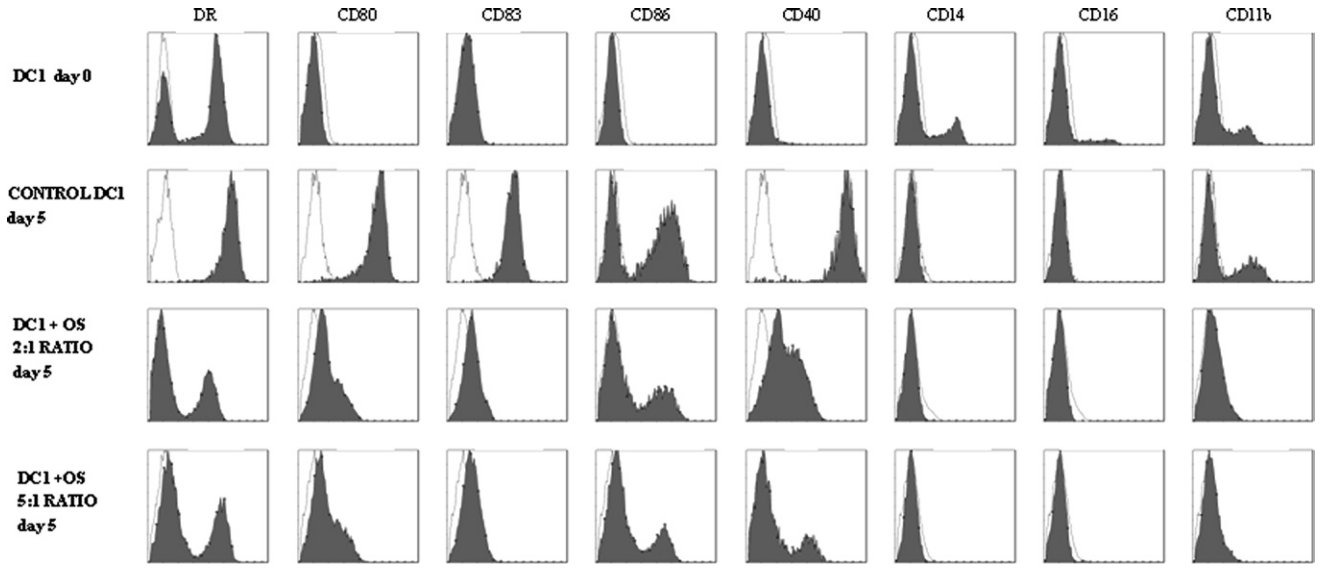

Fig. 6. DC1 surface markers histograms before and after maturation and after the co-culture with OS cell lines at 2:1 and 5:1 ratios. On days 0 and 5, DC1 was analysed by flow cytometry after labeling with isotype-matched controls and the indicated mAbs. On day 5, DC1 co-cultured with OS cell lines (2:1 and 5:1 ratios) was compared to the control DC1 (DC1 alone). Histograms show profiles of isotype controls (dotted lines) and mAbs (solid lines). At least 100,000 gated events were acquired for each sample. Data are representative of three similar experiments.

markers expression (Fig. 8). Histograms for different mAbs and isotype-matched controls are shown in Fig. 10.

3.4. ELISA cytokines quantification

To investigate the effect of osteosarcoma cell lines on the secretion of the immunomodulatory cytokines, such as IL-10, and pro-inflammatory cytokines, such as IL-12, supernatants were collected at day 5 of DC1 and DC2 co-cultures. The results obtained are shown in Fig. 11.

DC1 subset: Large amounts of IL-12, but no IL-10 production were revealed for the control DC1 (DC1 alone), whereas large amounts of IL-10 were produced by DC1 cultured with osteosarcoma cell lines ($^{**}p < 0.01$).

DC2 subset: The DC2 control (DC2 alone) produced the immunomodulatory cytokine IL-10; in presence of osteosarcoma cell lines, the IL-10 secretion significantly enhanced ($^{**}p < 0.01$).

The addition of rhIL-12, CD40L, Indometacin to the DC1 and DC2 co-cultures diminished the IL-10 production and induced the IL-12 secretion, thus reducing the immunosuppressive action of osteosarcoma cell lines and polarizing both DCs subsets towards the Th1 phenotype ($^{**}p < 0.01$).

4. Discussion

Tumors employ two strategies to avoid recognition: they either hide themselves from immune cells, down-regulating surface molecules, or they proceed to disable or eliminate immune system cells, which are thought to be the major mechanism.

The functional characteristics of DCs evolve with their stage of maturation. Immature DCs are found at sites of antigen capture and excel at antigen processing, but lack significant stimulatory capacity. After antigen uptake, DCs undergo further differentiation characterized by the loss of phagocytic capacity and by the

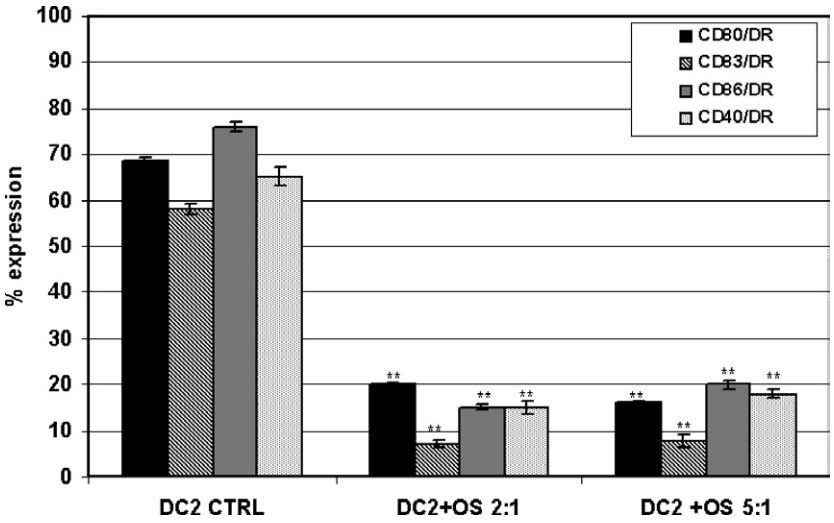

Fig. 7. DC2 surface markers after 5 days of co-culture with osteosarcoma (OS) cell lines at 2:1 and 5:1 ratios. All the maturation markers are strongly inhibited in both E:T ratios. Different co-culture conditions were tested and the mean percentage of the DC2 surface markers expression was reported in the graphic. Three independent experiments were performed for each condition. Bars are standard errors. $^{**}p < 0.01$.

increased expression of co-stimulatory molecules necessary for T cell activation (CD80, CD83, CD86, CD40, DR). Mature DCs have the ability to produce very high levels of IL-12, a pro-inflammatory cytokine that primes T-helper 1 (Th1) antitumor responses [21].

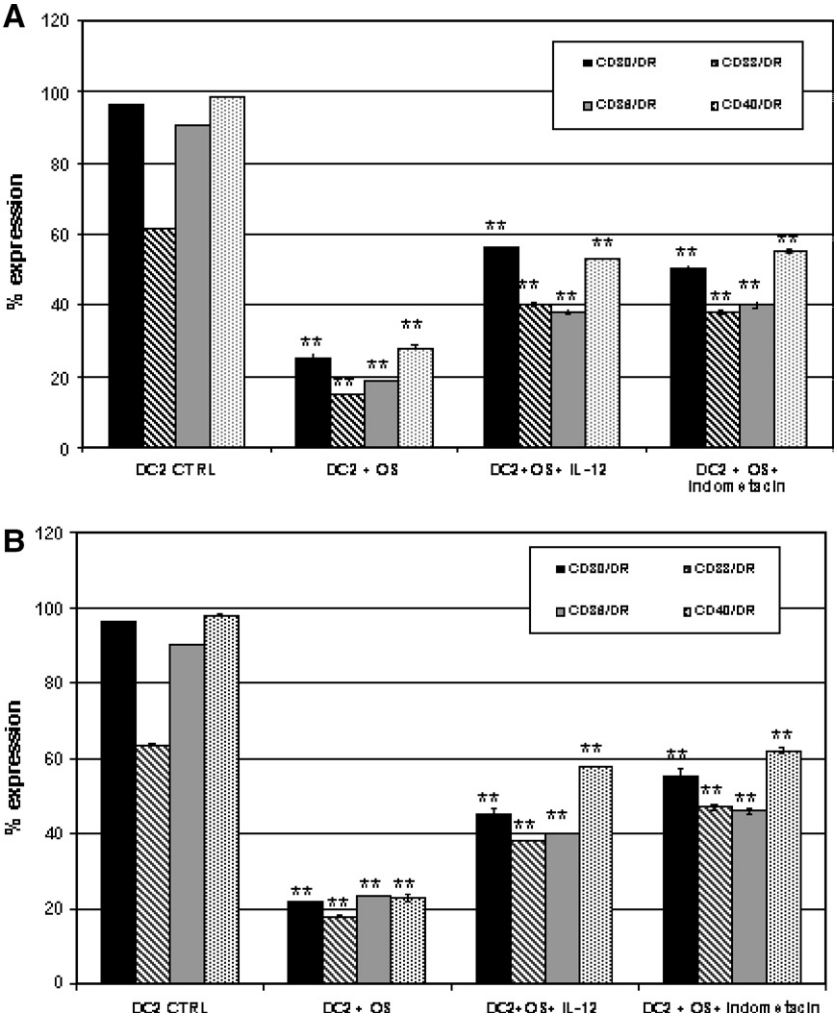

Fig. 8. DC2 surface markers after the addition of different cytokines and compounds. DC2 was co-cultured with all osteosarcoma (OS) cell lines at 2:1 (A) and 5:1 (B) and a mean percentage of the DC2 surface markers expression was reported in the graphic. The adding of rhIL-12 and Indometacin to the co-culture reversed the immunosuppressive effect of OS cell lines allowing a slight DC2 maturation. Three independent experiments were performed for each condition. Bars are standard errors. $^{**}p < 0.01$.

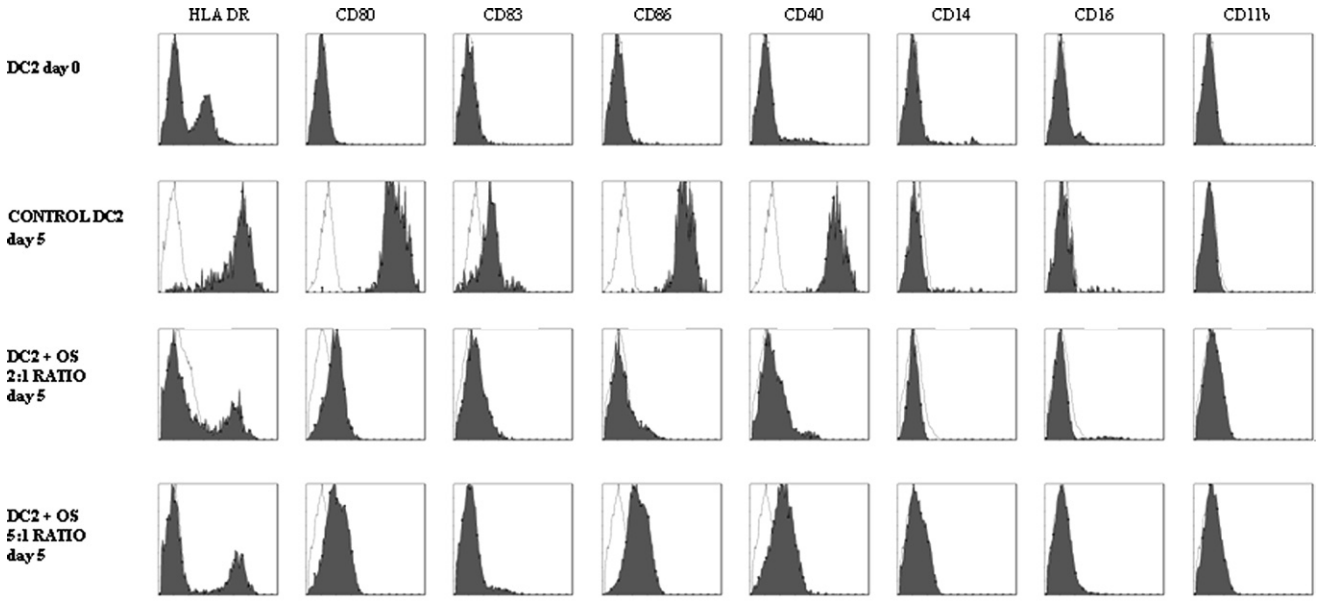

Fig. 9. DC2 surface markers histograms before and after maturation and after the co-culture with OS cell lines at 2:1 and 5:1 ratios. On days 0 and 5, DC2 was analysed by flow cytometry after labeling with isotype-matched controls and the indicated mAbs. On day 5, DC2 co-cultured with OS cell lines (2:1 and 5:1 ratios) was compared to the control DC2 (DC2 alone). Histograms show profiles of isotype controls (dotted lines) and mAbs (solid lines). At least 100,000 gated events were acquired for each sample. Data are representative of three similar experiments.

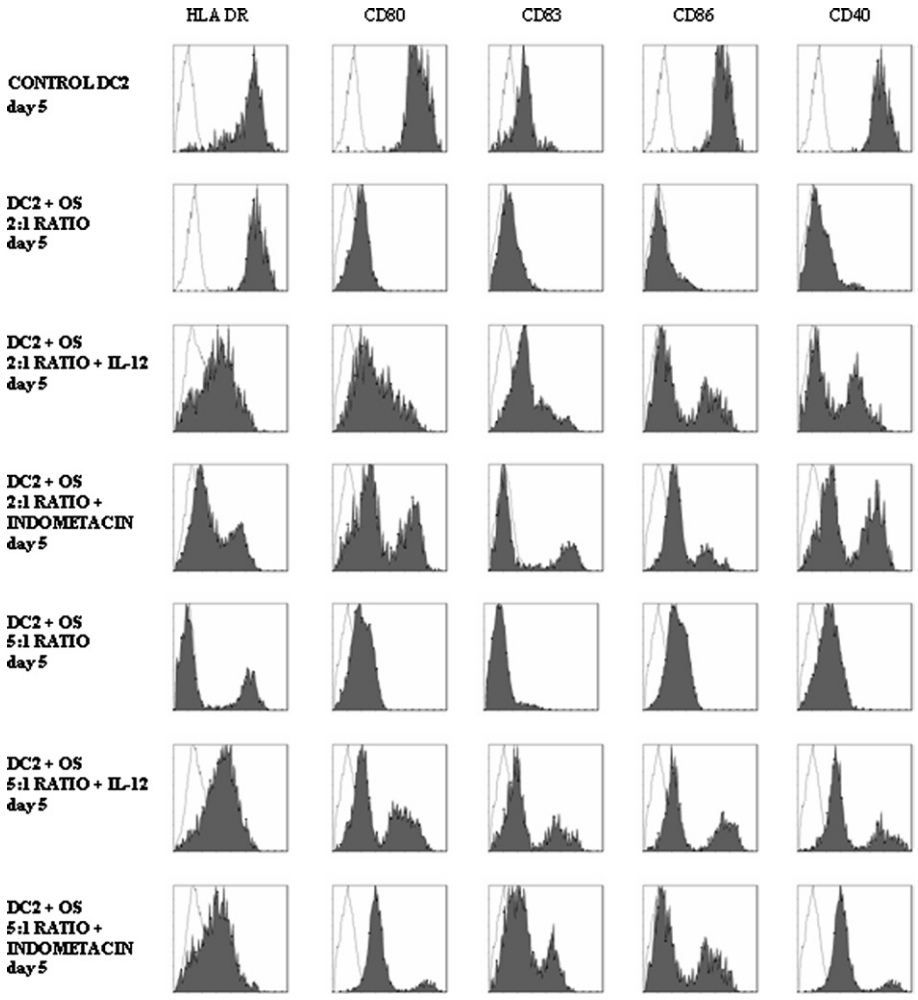

Fig. 10. DC2 co-stimulatory molecules histograms after the addition of different cytokines and compounds. In some experiments, 5 ng/ml of recombinant human IL-12 (rhIL-12), 2.5 μl/ml of CD40 Ligand (CD40L) or 5 μM of Indometacin were added to DC2 co-cultured with OS cell lines on day 0 and the maturation surface markers were analysed on day 5. Histograms show profiles of isotype controls (dotted lines) and mAbs (solid lines). At least 100,000 gated events were acquired for each sample. Data are representative of three similar experiments.

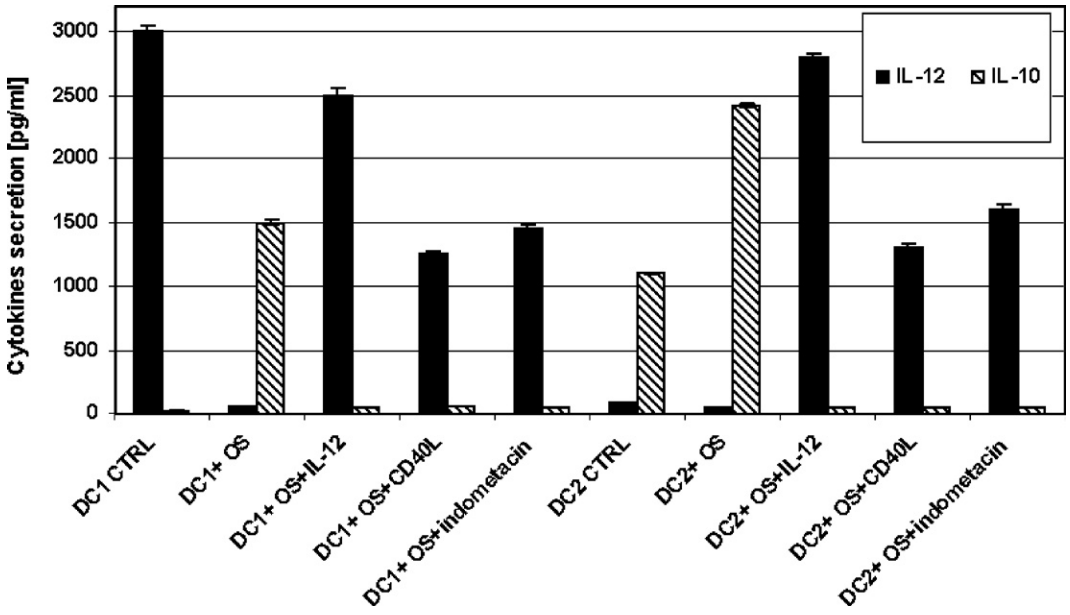

Fig. 11. DC1 and DC2 cytokines secretion in different culture conditions. DC1 and DC2 co-cultures supernatants were collected at day 5. Large amounts of IL-12, but no IL-10 production were revealed for the control DC1 (DC1 alone), whereas large amounts of IL-10 were produced by DC1 cultured with osteosarcoma (OS) cell lines (**$p < 0.01$). The DC2 control (DC2 alone) produced the immunomodulatory cytokine IL-10; in presence of OS cell lines, the IL-10 secretion significantly enhanced (**$p < 0.01$). The addition of rhIL-12, CD40L, Indometacin to the DC1 and DC2 co-cultures diminished the IL-10 production and induced the IL-12 secretion.

Down-regulated IL-12 secretion by DC results in an inadequate or absent immune response, and may allow a state of tolerance or anergy to emerge.

It has been shown that the number and the functions of DCs are dramatically reduced in cancer patients. In fact, DCs isolated from cancer patients exhibit quantitative and functional deficiencies [17,22–24].

DCs subsets analysis in patients with breast cancer and head and neck squamous cell carcinoma has revealed that decreases in cell numbers are confined to DCs of the myeloid lineage in comparison with those of the lymphoid lineage. The number of DCs was halved in early-stage patients, with a further decrease in patients with more advanced disease. This effect was closely associated with an accumulation of immature myeloid cells in peripheral blood [25,22]. Surgical debulking of tumor mass results in a corresponding increase in myeloid-derived DCs [23,26].

Della Bella et al. also found that DCs constitutive production of IL-10 was higher in patients with invasive cancer. The T-helper lymphocytes of cancer patients were also polarized towards the Th2 phenotype [26]. In patients with renal, colon and prostate cancer, DCs have low levels of co-stimulatory molecules and a decreased capacity to stimulate allogeneic T cell proliferation [24,27].

Since no study has been made in osteosarcoma patients, we evaluated the effects of osteosarcoma cell lines on the maturation and cytokines secretion of different types of DCs, in order to improve the knowledge of this tumor biology.

In our experiments, we obtained results which perfectly fit with those described in the literature, like the presence of an *in vitro* inhibition of the DCs function by the osteosarcoma cells. Three major points have been evaluated in this study.

The first was aimed at the study of the effect of the osteosarcoma cell lines on the CD14+DCs. All the surface markers were reduced, especially in the 2:1 co-culture condition where they were halved.

The second point concentrated on the effect of the osteosarcoma cell lines on the DC1.

Importantly, the inhibition of co-stimulatory molecules expression on the DC1 surface was similar for both ratios tested (2:1 and 5:1), but less evident in comparison with CD14+DCs and DC2 subsets suggesting that DC1 subset is relatively resistant to tumor-induced co-stimulatory molecule modulation.

The third point evaluated was the study of the effect of the osteosarcoma cell lines on DC2. The level of all the surface markers fell in both co-culture conditions carried out. To confirm the immunosuppressive effect of osteosarcoma cell lines, the IL-10 and IL-12p70 production was assayed by ELISA. Indeed, control DC1 did not produce IL-10, whereas high amounts of IL-10 were produced by control DC2 and by DC1 co-cultured with osteosarcoma cell lines.

Therefore, the IL-10 production in presence of osteosarcoma cell lines primes a T-helper 2 (Th2) response which accounts for an inadequate immune response and may allow a state of tolerance or anergy. On the contrary, the addition of rhIL-12, CD40L and Indometacin diminished the IL-10 production and enhanced the IL-12 secretion by DC1 and DC2, thus inducing the Th1 pattern.

One of the mechanisms for the inhibition of immune-cells function seems to be the production of soluble factors by tumor cells, such as IL-4, IL-5, IL-6, IL-10, TNF-α, TGF-β, prostaglandin-E_2 (PG-E_2) and vascular endothelial growth factor (VEGF) [19,28,29]. On the contrary, the literature states that the DCs generated from monocytes in presence of tumor culture supernatant appear mature, expressing the cell surface molecules [30].

Another important feature, such as the possibility of a reversible effect adding different compounds to the co-culture, was also evaluated.

Indometacin, a prostaglandin-E_2 (PG-E_2) inhibitor, IL-12 and CD40L have been described as stimulators of the DCs maturation and function [31–34]. Therefore, we added these compounds to all co-culture conditions, in order to evaluate their capacity to change the immunosuppressive effect of osteosarcoma cell lines promoting the DCs differentiation and maturation.

We observed an enhanced expression of CD14+DCs maturation markers. Importantly, the level of CD80, CD83, CD86 and CD40 surface markers significantly increased after the addition of rhIL-12, CD40L and Indometacin, especially in the 5:1 ratio condition.

Similar results were also obtained by adding rhIL-12 and Indometacin to the DC2 co-cultures, which promoted a significant DC2 differentiation and maturation. No effect was revealed by the addition of the same compounds to the DC1 co-cultures. As shown by ELISA, the addition of rhIL-12, CD40L and Indometacin to the DCs co-cultures reduced the IL-10 production and enhanced the IL-12 secretion by DC1 and DC2.

Collectively, these results suggest that osteosarcoma cell lines have a strong impact on DCs subsets *in vitro* and, therefore, on the immune system. Thus, it is possible to understand how this tumor can grow in patients avoiding the immune-surveillance.

As shown by the addition of rhIL-12, CD40L and Indometacin to the co-cultures, the immunosuppressive action of osteosarcoma cell lines could be reversed and both DCs subsets polarized towards the Th1 phenotype.

The knowledge of the immune-surveillance process is important for the future treatment of high risk osteosarcoma patients, especially for a specific immunotherapy able to overcome the state of anergy induced by the tumor. Indeed, if DCs are inhibited by the tumor cells, the efficacy of an infusion of osteosarcoma specific T-lymphocytes might be reduced by the lack of tumor-antigen presentation by the APCs.

Therefore, this aspect underlines the need for finding new therapeutic approaches in order to reverse the immune-surveillance inhibition in osteosarcoma.

Disclosures

The authors declare that they have no competing interests.

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