

## Dendritic cells based immunotherapy of patient with chondrosarcoma - case report

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**Abstract:** We present a case report of patient with intracranial chondrosarcoma and attempt to use vaccination of dendritic cells as the salvage therapy. To our knowledge, this is the first case report of DCs vaccination in the head and neck chondrosarcoma. Immunotherapy with allogeneic DCs stimulated with tumor cell lysates in this case was demonstrated to be feasible, safe and well tolerated. Unfortunately we did not observe any clinical or immune response during vaccination. CD4+ and CD8+ regulatory cells could be responsible for ineffectiveness of immunotherapy.

**Keywords:** Chondrosarcoma of the head and neck - Dendritic cells - Immunotherapy

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### Introduction

Chondrosarcoma constitute nearly 8% of primary bone tumors overall and 10% of them are diagnosed in the head and neck region [1]. This type of neoplasm may arise from cartilage, mesenchymal cells or embryonal rests. Chondrosarcoma of the head and neck demonstrate variable growth and their aggressiveness is dependent on histologic grade. These tumors grow locally with involvement of adjacent visceral and neurovascular structures [2]. Regional and remote metastases are uncommon. The clinical symptoms depend upon whether neighbouring structures such as orbit, paranasal sinuses, eustachian tube, or cranial compartments are involved. Complete resection is an initial treatment, however local recurrence after surgery alone is greater than 65% [3]. Radiotherapy is used as adjuvant therapy for residual cancer, as salvage therapy for relapses, and prophylactically for large tumors with negative resection margins [4], but nearly 80% of

patients die of uncontrolled local disease [3]. Some investigators underline ineffectiveness of radiation and chemotherapy [3,5] and on the other hand suggest that immunotherapy against chondrosarcoma could be possible [5].

Since conventional therapies, such as surgery, chemotherapy, and irradiation are limited, there is an urgent need for new therapeutic approaches. Immunotherapy with the use of DCs is becoming of interest for different solid tumors including sarcoma. Dendritic cells (DCs) are the most effective professional antigen presenting cells with an ability to prime both a primary and secondary immune response to tumor antigens. Large amounts of DCs can be generated from peripheral blood monocytes with the use of GM-CSF and IL-4 [6] and different stimuli are used to induce their maturation [7]. Numerous strategies are present for loading tumor antigens into DCs. Pulsing with synthetic peptides and genetic modification of DCs with RNA or DNA vectors are used for well known tumor antigens. There are also methods with the use of whole tumor cells as a source of antigen: tumor lysates, tumor-DC fusion techniques, acid eluted peptides or apoptotic tumor cells. Several clinical trials with the use of tumor antigens loaded dendritic

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cells were conducted in the therapy of neoplasms [7-11]. We present a case report of patient with intracranial chondrosarcoma and attempt to use vaccination of dendritic cells as the salvage therapy.

## Materials and methods

**Patient.** A 55-year-old woman was admitted to Department of Otolaryngology and Laryngological Oncology for the diagnosis of nasal septum tumor. The tumor filling patient's left nasal cavity was diagnosed in 2003. The surgical resection of the tumor was performed in March 2003. Postoperative pathomorphologic diagnosis was: chondroma and chondromatous infiltration of the bones. Because of the local recurrence another surgical procedure involving: resection of the tumor with ethmoid sinuses, fundus of sphenoid sinus, posterior part of the palatal bone, nasal septum, left-sided rhinotomy was performed in 2004. Postoperative pathomorphologic diagnosis was: Chondroma in statu malignisationis/Chondrosarcoma - G1. After the surgical treatment was finished, the patient was qualified for radiotherapy with  $Co^{60}$ , with the total dose of 6600cGy/g. The postradiation reaction I - II° was observed. Another surgery of paranasal sinuses (ethmoid and maxillary) with Denker's method was done in February 2005. In postoperative histopathologic result the neoplastic infiltration of the skull was described. The resection of the ethmoid sinuses and frontal cranial fossa tumor was performed in February 2006. In June 2006 the patient was disqualified from radiotherapy. The patient was admitted to the Department of Otolaryngology with a suspicion of a recurrence in ethmoid bones and the medial wall of the orbit in November 2006. The partial resection of the tumor was done and the material for immunotherapy was taken. The immunotherapy protocol was approved by the Ethics Committee of the Medical University of Lublin and patient gave a written informed consent to the therapy. After the procedure swelling of the left eye conjunctiva arised and the limitation of left eyeball mobility concerning its abduction and adduction was observed. The patient didn't complain about diplopia, both pupils were round, equal, with proper both side eyelids closing. The patient was admitted to the Department of Otolaryngology on 25<sup>th</sup> of February 2007, unconscious, with spastic paresis of upper and lower limbs. Very high fever up to 41.3°C occurred. The patient's general state was getting constantly worse. Because of breathing problems the patient was ventilated by a respirator. Fatal arrhythmia was observed. The patient died on 2<sup>nd</sup> of March 2007.

**Dendritic cells generation.** Peripheral blood mononuclear cells (PBMC) were isolated from 150 ml of peripheral blood, taken by venipuncture, by gradient centrifugation on GradiSol L (Aqua-Medica, Poland) and washed twice in PBS without  $Ca^{2+}$  and  $Mg^{2+}$  (Biochrome, Germany). CD14<sup>+</sup> monocytes were enriched by positive selection of CD14<sup>+</sup> cells in magnetic field according to the manufacturer's protocols (anti-CD14 MicroBeads and VarioMACS, Miltenyi-Biotec, Germany). After the isolation, CD14<sup>+</sup> cells were washed twice in PBS and seeded into three level cell culture flasks (Cell Factory<sup>TM</sup>, Nunc, Denmark) in RPMI 1640 medium (Biochrome, Germany) supplemented with 2% human albumin (ZLB Bioplasma AG, Switzerland), 100 IU/ml penicillin, 50 mg/ml streptomycin and 100 mg/ml neomycin for 7 days. On days 1, 3 and 5 of the culture 1,000 IU/ml rhGM-CSF (Leukine, Berlex, USA) and 500 IU/ml rhGM-CSF rhIL-4 (Strathmann, Germany) were added. Human tumor necrosis factor  $\alpha$  (rhTNF $\alpha$ , [Strathmann, Germany]) at a concentration of 50 ng/ml and the tumor cells lysates were added during the last 48 hours of culture for the maturation and antigen loading of the DCs. On day 8, generated dendritic cells were harvested using Trypsin/EDTA solution (Biochrome, Germany), washed twice in

0.9% NaCl. Viability was checked by Trypan Blue staining in light microscopy. Flow cytometry analysis was performed using mouse monoclonal antibodies (mAb) against CD80, CD86, CD83, CD1a and HLA-DR, all purchased from BDPharmingen, USA. The vaccine product was divided to single doses and stored in liquid nitrogen.

**Preparation of the neoplastic cells lysates.** Neoplastic cells were obtained during palliative operation. Tumor samples (without necrotic areas) were digested with 1 mg/ml of type I collagenase (Biochrome AG, Germany), 1 mg/ml of type I deoxyribonucleic (Sigma, Germany) and 0.1 mg/ml of hyaluronidase (Sigma, Germany) and washed twice in RPMI 1640 (Biochrome, Germany). Tumor cells were homogenized by five repeated cycles of quick freezing and thawing. Large particles were removed by centrifugation and after that supernatants were passed through a 0.2  $\mu$ m pore filter, the protein content was determined by OD measurement (DU<sup>®</sup>530 spectrophotometer, BeckmanCoulter, USA) and aliquots were stored at -80°C until use. 100  $\mu$ g/ml protein was used for stimulation of DCs.

**Proliferation assay.** CD3<sup>+</sup> cells were separated from the patient peripheral blood. Magnetic labeled anti-CD3 antibodies and separation column (Miltenyi Biotec) were used according to the manufacturer's instructions. Next  $5 \times 10^4$  cells/well of CD3<sup>+</sup> cells were seeded in 96-well flat-bottom plates (Nunc). CD3<sup>+</sup> lymphocytes cultured with autologous DCs loaded with lysates (at a 20:1 ratio) or with lysates only for 72 hours. CD3<sup>+</sup> cells cultured separately were used as a negative control. CD3<sup>+</sup> cells stimulated with PMA (Sigma-Aldrich, Germany) were used as a positive control. <sup>3</sup>H-thymidine (1  $\mu$ Ci/well, Lecom, Czech Republic) was added for the last 16 hours of culture. Cells were harvested onto filter papers with a Skatron (Lier, Norway) cell harvester. Thymidine incorporation was measured in mean counts per minute (cpm) from triplicate experiments using a beta scintillation counter (Wallac 1409, Perkin Elmer, USA).

**Peripheral blood lymphocytes immunophenotyping.** The percentages of the cells positive for the following surface antigens: CD14/CD45, CD3/CD19/CD69, CD3/CD19/CD25, CD3/CD19/CD95, CD4/CD8/CD69, CD4/CD8/CD25, CD4/CD8/CD95 were examined (BD Pharmingen, USA). The samples were collected and measured using the FACS Calibur cytometer (Becton Dickinson, USA) and the data were analyzed by the CellQuest<sup>TM</sup> software (Becton Dickinson, USA).

**T regulatory cells estimation.** Treg lymphocytes were estimated by Human Treg Flow<sup>TM</sup> Kit (FOXP3 Alexa Fluor<sup>®</sup> 488/CD4 PE-Cy5/CD25 PE, Biolegend, USA) according to the manufacturer's protocol. Results are calculated as mean CD4<sup>+</sup>/CD25<sup>+</sup>/FOXP3<sup>+</sup> cells percentage in CD4<sup>+</sup> gate from triplicate counting for each sample.

**Vaccination protocol.** A single dose of vaccine product was thawed directly before vaccination. DCs vaccines were injected intradermally in the close vicinity of cervical and nuchal lymph nodes. Patient received 3 doses of DCs vaccines biweekly. Due to the progression of diseases the vaccination protocol was stopped.

**Evaluation of the clinical response and the toxicity of the DC vaccination.** Patient was checked before each vaccination by physical examination, differential blood count and serum chemistry. Patients was monitored for local and systemic toxicity according to the grading system of the National Cancer Institute (NCI-CTC).

**Cutaneous delayed-type hypersensitivity (DTH).** Simultaneously to vaccination tumor cell lysates (300  $\mu$ g/ of total protein),

**Table 1.** Immunophenotype of dendritic cells generated from patient's peripheral blood CD14<sup>+</sup> monocytes.

Antigen	Percentage
CD83 <sup>+</sup>	99.91%
CD1a <sup>+</sup>	91.54%
CD80 <sup>+</sup>	99.36%
CD86 <sup>+</sup>	98.90%
CD206 <sup>+</sup>	90.75%
CD209 <sup>+</sup>	99.96%
B7-H2 <sup>+</sup>	38.91%
HLA-DR <sup>+</sup>	99.74%

25×10<sup>4</sup> of loaded dendritic cells and 100 µl 0.9% NaCl were administered intradermally into the forearm. DTH reactivity (induration) was measured at 72 h.

**Enzyme-linked immunosorbent spot (ELISPOT) assay for the detection of interferon gamma (IFN-γ) secretion.** IFN-γ ELISPOT assays were performed according to the manufacturer's protocol (Mabtech, Germany) and as described earlier in detail [13]. Briefly, 96-well nitrocellulose plates (Millipore, Germany) were coated with INF-γ mAbs (Mabtech) and incubated overnight at 4°C. After washing with PBS (Invitrogen Gibco, USA), plates were blocked with 10% human AB serum (German Red Cross Blood Bank, Ulm) for 2 h at 37°C. 5×10<sup>4</sup> CD3<sup>+</sup> T-lymphocytes and 1×10<sup>4</sup> target cells (tumor cells) were added to each well. As a positive lectin control 1 µg/ml Pokeweed Mitogen (PWM) (Sigma-Aldrich) was used and CD3<sup>+</sup> T lymphocytes served as negative control. After the incubation in RPMI 1640 medium (Biochrom AG) overnight, plates were washed with PBS. 0.2 µg/ml IFN- mAbs (Mabtech) were added to each well, plates were incubated for 2 h at room temperature. Cells were washed with 1 µg/ml streptavidin-alkaline phosphatase (Mabtech) for 2 h. Plates were washed and BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate p-toluidine / nitroblue tetrazolium, Sigma-Aldrich) was used for colorization according to the manufacturer's instructions.

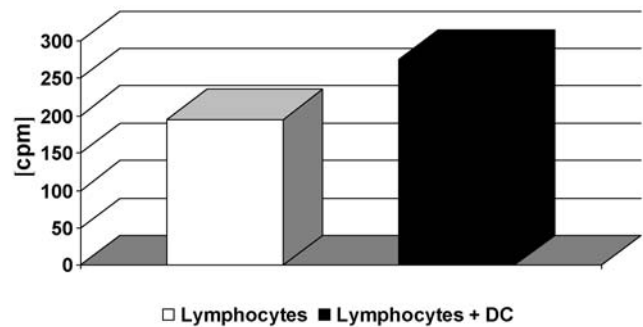
Plates were dried up at room temperature and thereafter evaluated by the use of an ELISPOT reader consisting of a video camera and a computer system with pattern recognition software (CTL, Germany).

**Granzyme B ELISPOT assay.** The granzyme B ELISPOT assay was performed as described elsewhere [14] to determine specific lysis of target cells according to the manufacturer's instructions (Becton Dickinson, USA).

## Results

### Generation of dendritic cells

From 150 ml of peripheral blood 70×10<sup>6</sup> of CD14<sup>+</sup> monocytes were isolated and after 7-days culture 20×10<sup>6</sup> of moDC was harvested. Cells were divided into 3 doses (1st - boost dose 10×10<sup>6</sup>, 2nd and 3rd both 5×10<sup>6</sup>) and stored in liquid nitrogen. The harvested cells express typical moDC morphology and immunophenotype (Table 1). Generated dendritic cells



**Fig. 1.** Lymphocytes proliferation assay. DCs used in all vaccines were checked for ability to prime proliferation of autologous CD3<sup>+</sup> cells.

were able to stimulate proliferation of CD3<sup>+</sup> lymphocytes (Fig. 1).

### Cutaneous delayed-type hypersensitivity (DTH)

There was no positive cutaneous DTH reaction to autologous tumor cells lysates or moDC loaded with lysates after any of vaccinations.

### Evaluation of the clinical response and the toxicity of the DC vaccination

All 3 vaccinations were well tolerated and no severe side effects were associated with the intradermal injections of pulsed DCs. Immunotherapy with allogeneic DCs stimulated with tumor cell lysates in this case was demonstrated to be feasible, safe and well tolerated.

During the first month of therapy patient reported the improvement in the quality of life and the local symptoms of tumor growth were not present. During the monthly break between 3<sup>rd</sup> and 4<sup>th</sup> vaccination the patient's general state was getting constantly worse, that made 4<sup>th</sup> vaccination impossible due to neurological state. Because of breathing problems the patient was ventilated by a respirator and fatal arrhythmia was observed. CT examination revealed massive local progression of tumor (Fig. 2). The patient died on 74<sup>th</sup> day from the first vaccination.

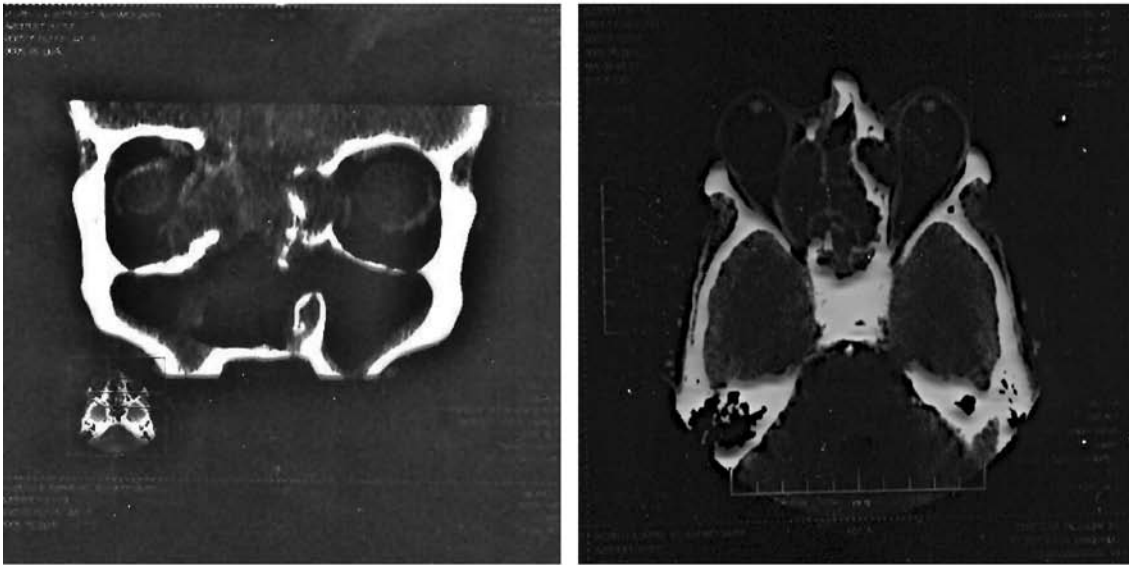
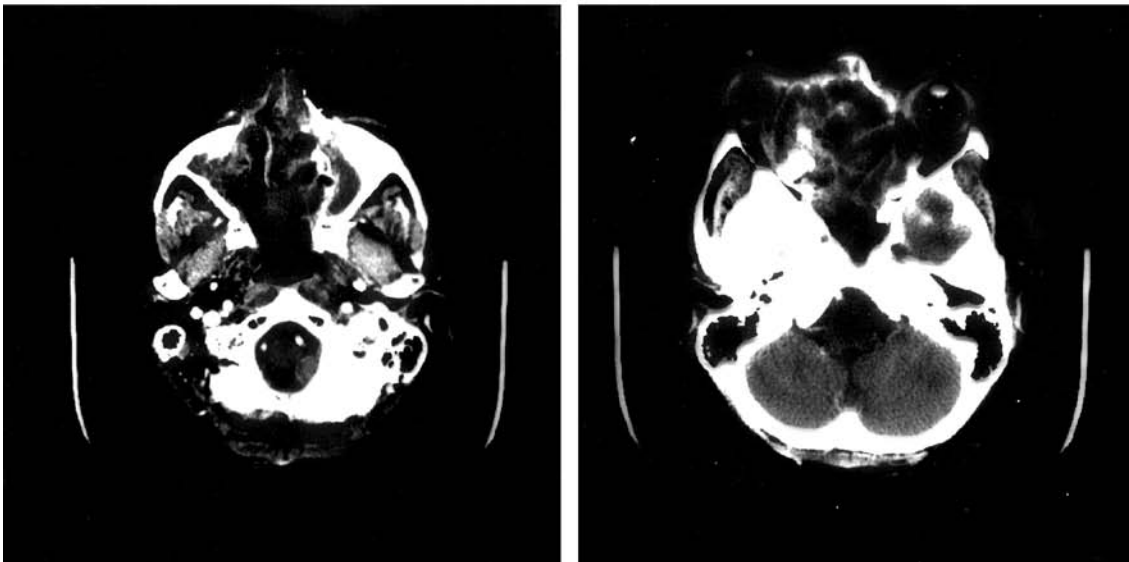
### Peripheral blood lymphocytes

We did not observe any particular changes except continuous increase in CD8<sup>+</sup>/CD25<sup>+</sup> lymphocytes during vaccination (Fig. 3).

### T regulatory lymphocytes estimation

We noticed that percentage of CD4<sup>+</sup>/CD25<sup>+</sup>/FOXP3<sup>+</sup> was strongly reduced after 1st vaccination, but increased during therapy (Fig. 4).



**Before vaccination****Progression of tumor, the last CT examination before death of the patient.**

**Fig. 2.** CT examinations of patients before vaccination and 7 days before death of patients. Large progression of tumor mass is present.

### ***Specific immune response to autologous tumor cells***

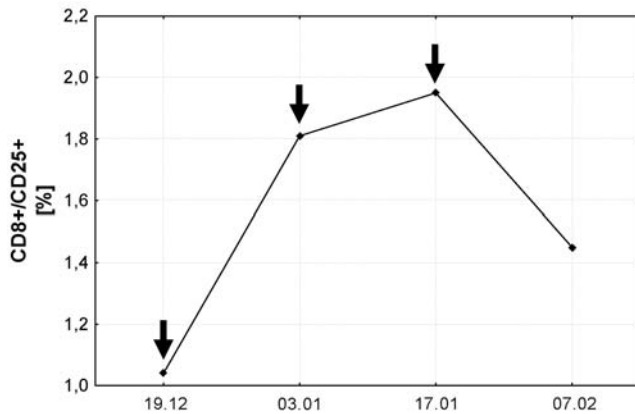
In ELISPOT experiments we did not notice any specific INF- $\gamma$  and granzyme B release against autologous tumor cells by CD3<sup>+</sup> lymphocytes.

### **Discussion**

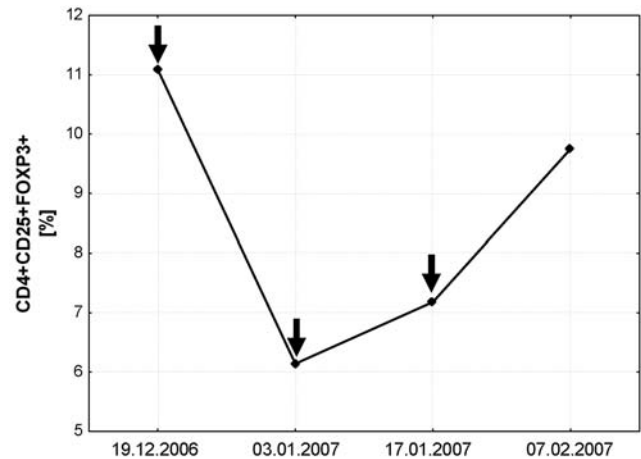
In the present work we use vaccination with tumor lysate loaded DCs as a salvage therapy for the patient with intracranial chondrosarcoma. Chondrosarcoma comprise less than 15% of intracranial chondromatous neoplasm and 0.15% of all primary intracranial tumors

and typically arise at the age between 40 and 50 without any gender preference (2,14). The radical surgical resection is the preferred treatment of choice, but unfortunately skull based chondrosarcoma has a strong tendency for local recurrence, mainly due to the complicatedness of complete resection. The effectiveness of adjuvant therapy as irradiation and chemotherapy is still under discussion [1,5]. *In vitro* experiments showed that chondrosarcoma cells can be destroyed by antigen specific lymphocytes and these results suggest that immunotherapy directed against chondrosarcoma cells is possible [5,15].

In our study we generated fully mature monocyte derived DCs from peripheral blood of patient with



**Fig. 3.** Changes of the CD8+/CD25+ cells percentage during immunotherapy. Arrows symbolize injections of DCs.



**Fig. 4.** Changes of the CD4+/CD25+/FOXP3+ cells percentage during immunotherapy. Arrows symbolize injections of DCs.

advanced intracranial chondrosarcoma. Generated dendritic cells were able to stimulate proliferation of autologous CD3+ lymphocyte. Three doses of vaccine were injected and after that treatment was stopped due to the progression of disease. Immunotherapy with allogeneic DCs stimulated with tumor cell lysates in this case was demonstrated to be feasible, safe and well tolerated. During vaccination we did not observe any clinical or immune response. DTH tests against autologous tumor lysates were negative and we also did not find any specific cytotoxic response with the use of ELISPOT experiments.

In many clinical trials a fraction of vaccinated patients developed immune response against vaccinating antigens, on the other hand in some experiments even half of the patients did not show any signs of specific immune response [7,16]. Despite encouraging clinical response in some trials [7,9,17] we still do not know whether the modest clinical response was caused by immunotherapy or they reflect patients with better prognosis capable of eliciting efficient immune response. Investigators involved in DC vaccinations suffered a serious defeat when a phase III clinical trial in patients with stage IV melanoma did not succeed to show that DC vaccination provided an increased benefit compared with standard chemotherapy [18].

After the first vaccination we observed strong reduction in circulating T regulatory lymphocytes, however their percentage was continuously increasing during therapy. CD4+/CD25+/FOXP3+ Treg cells are well known as immunosuppressive agents hampering anti-tumor response and elimination of these cells enhances immunity and effectiveness of DCs vaccinations [19,20]. Failure in the maintenance of CD4+/CD25+/FOXP3+ Treg lymphocytes on the low level could be one of the reason of ineffectiveness of vaccination and lack of both clinical and immune response.

Interestingly we noticed continuous increase in circulating CD8+/CD25+ cells, which also could represent population of regulatory cells. It is well documented that CD8+/CD25+ cells, especially with simultaneous expression of FOXP3, participate in immunosuppression [21-23]. CD8+/CD3+/FOXP3+ cells and produce transforming growth factor beta, which is also well known as cytokine participating in tumor immune escape. On the other hand CD8+/CD25+ are strong inducers of Graft versus Host Disease and Graft versus Tumor effect [24].

The effectiveness of DCs vaccination in treatment of the head and neck chondrosarcoma require further investigation and clinical trials. CD4+ and CD8+ T regulatory cells seem to play as the agents which influence cancer immunotherapy and inhibit priming of effective immune response. Perhaps elimination of regulatory T lymphocytes during DCs vaccinations is needed to enhance clinical and immune response.

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