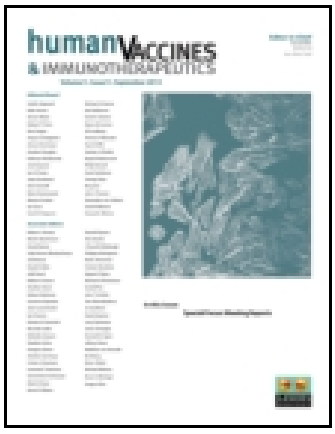

Human Vaccines & Immunotherapeutics
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/khvi20

The effects of zoledronate on monocyte-derived dendritic cells from melanoma patients differ depending on the clinical stage of the disease

Alessandra Failli[a], Annalisa Legitimo[a], Giulia Orsini[a], Antonella Romanini[b] & Rita Consolini[a]
[a] Department of Clinical and Experimental Medicine; University of Pisa; Pisa, Italy
[b] Medical Oncology Unit; S. Chiara Hospital; Pisa, Italy

Accepted author version posted online: 01 Nov 2014.Published online: 27 Jan 2015.

RESEARCH PAPER

Human Vaccines & Immunotherapeutics 10:11, 3375–3382; November 2014; Published with license by Taylor & Francis Group, LLC

The effects of zoledronate on monocyte-derived dendritic cells from melanoma patients differ depending on the clinical stage of the disease

Alessandra Failli[1,†], Annalisa Legitimo[1,†], Giulia Orsini[1], Antonella Romanini[2], and Rita Consolini[1,*]

[1]Department of Clinical and Experimental Medicine; University of Pisa; Pisa, Italy; [2]Medical Oncology Unit; S. Chiara Hospital; Pisa, Italy

[†]These authors contributed equally to this work.

Keywords: melanoma, dendritic cells, zoledronic acid

Zoledronic acid has shown indirect anticancer effects on angiogenesis, the tumor microenvironment and immune responses. Its immunological action is exerted, at least in part, via its modulating properties. The aim of this study was to investigate the in vitro effects of zoledronic acid on the dendritic cells of melanoma patients. Peripheral blood samples were collected from 26 patients with melanoma and 11 healthy donors. Dendritic cells were derived from purified monocytes, and zoledronic acid (ZA) was added on the first day of culture. The phenotype and function of the generated cells were evaluated by flow cytometry. The ZA-treated monocytes from patients with early-stage disease generated DCs characterized by reduced endocytic activity and increased allostimulatory capacity compared with the untreated samples, allowing restoration of the DC function observed in normal subjects. In contrast, the ZA-treated monocytes from patients at stage III generated cells with higher CD14 antigen expression and endocytosis than the untreated samples. Therefore, in melanoma patients, the in vitro ZA effects differ according to the progression of the disease. In addition, our preliminary results appear to suggest that ZA effects are also influenced by the expression of CD14 antigen, indicating that the DC phenotype together with clinical characteristics must be considered in the choice of patients to be treated with ZA. Our work focus on the effect of ZA on monocyte-derived DCs from melanoma patients, showing that the effects of therapeutic doses of this drug might be mediated at least in part by modulation of myeloid cell function.

Introduction

Cutaneous melanoma is an aggressive tumor with a high metastatic potential and marked resistance to the currently available antitumor therapy strategies.[1] The high mortality rate of malignant melanoma, the poor efficacy of chemotherapy in advanced stages of the disease, and the high toxicity of the classical regimens have stimulated intensive research for new alternatives to the therapy.[1]

It has been previously demonstrated that the nitrogen-containing bisphosphonate pamidronate is able to induce apoptosis and to inhibit proliferation in melanoma cells in vitro.[1,2]

Aminobisphosphonates (ABPs) are synthetic compounds that have shown direct and indirect anticancer actions.[3] A pronounced effect on the immune system, which might contribute to their in vivo anti-tumor activity, has been described.[4]

The enhancement of immune-mediated antitumor activity appears to be exerted, at least in part, through the expansion of γδ T-cells.[3]

Zoledronic acid (ZA), one of the newer generation nitrogen-containing BPs, has been particularly well investigated, both preclinically and in clinical practice.[4] ZA inhibits the enzyme farnesyl diphosphate synthase (FPPS), which plays a role in the mevalonate pathway and in the subsequent prenylation of small GTPases proteins such as Ras. These molecules are involved in the intracellular signaling cascades and are essential for osteoclast activity and survival.[4,5]

Recently, it has been demonstrated that γδ T-cells activated by ZA play an adjuvant role, linking innate and acquired immunity, through interactions with dendritic cells (DCs) in a way that amplifies the proliferation of tumor antigen-specific CD8+ T cells.[6-8]

DCs are unique specialized antigen-presenting cells that are capable of stimulating naive T cells during primary immune responses and of inducing antigen-specific cytotoxic T lymphocytes (CTLs) and helper T cells.[9]

The interaction between tumor and dendritic cells is an emerging theme. Most tumors are infiltrated by DCs, which may

*Correspondence to: Rita Consolini; Email: rita.consolini@med.unipi.it
Submitted: 04/02/2014; Revised: 05/23/2014; Accepted: 05/31/2014
http://dx.doi.org/10.4161/hv.29416

determine the balance of several (often opposing) molecular interactions that regulate the recognition, uptake, processing and ultimately presentation of cellular antigens to the immune system. DCs also interact with living tumor cells and have non-immunologic effects on tumor cells and their microenvironment.[10]

Many ongoing clinical trials make use of monocyte-derived DCs as vaccines in cancer patients; therefore the aim of this study was to analyze the effects of ZA on the properties of DCs derived from the peripheral blood monocytes of melanoma patients to investigate its potential use in reversing the immunosuppressive environment of the tumor.

Results

ZA effects on cultured cell morphology

Using cells from 10 melanoma patients, we investigated the morphological profile of cells harvested on day 7 of culture after stimulation with LPS. The cells derived from cultures displayed features of mature myeloid DCs, including irregular membrane ruffling, dendrites and a laterally positioned nucleus.

ZA treatment did not induce substantial changes in the morphological appearance of the cells (**Fig. 1**).

ZA effects on dendritic cell phenotype and function

It is known that the generation of DCs from the monocyte population is characterized by the loss of CD14 antigen. Therefore, we used this marker to assess DC generation on the sixth day of culture using cells from 26 melanoma patients.

Collected data were examined using two different analytical approaches.

First analytical approach

We evaluated the entire patient population and patients divided on the basis of clinical stage (stage I-II, stage III, and stage IV) in comparison with the healthy control group. The effects of ZA treatment were thus investigated (**Fig. 2**).

As previously reported,[11] melanoma patients exhibit a higher percentage of CD14-expressing cells than healthy controls (15.5± 4.4% vs. 5.1 ± 1.4%, respectively; $P > 0.05$). CD14 expression was highly variable among the patients (range: 0.6%–94.7%), with high values especially observed for patients in a more advanced stage of the disease (CD14+ cells: 32.6± 10.3%; $P = 0.009$, compared with HCs).

The expression levels of HLA-DR antigen in immature DCs and those of CD40, CD80, CD83, and HLA-DR in the mature DCs from the patients were found to be similar to those of the controls.

However, the immature DCs obtained from melanoma patients at all stages of the disease showed a significantly higher endocytic activity than those from the healthy donors. The ability of the mature DCs obtained from patients to induce allogeneic T cell proliferation was reduced, particularly in the group of patients in disease stage IV, without significant differences from the control DCs.

ZA treatment did not substantially modify the percentage of CD14-expressing cells compared with the untreated samples. In fact, the ZA treated cells did not show a significant decrease in CD14 expression, except for those from the stage III patients, which exhibited a significant increase in expression compared with the baseline sample (10.14 ± 5.1% vs. 7.7 ± 4.7%; $P < 0.05$). In addition, the ZA treated immature cells did not show a significant increase in HLA-DR antigen expression compared with the untreated cells (data not shown).

Endocytic activity was modulated to different extents by ZA treatment. In fact, it induced a significant reduction in endocytic activity in the cells from patients with early disease (ΔMFI: 60.1± 17.6 vs. 88.4± 15.0; $P < 0.05$), whereas a significant increase was found in the cells from patients at stage III (ΔMFI: 243.4± 59.2 vs. 119.8± 33.1; $P < 0.05$) compared with the untreated samples.

ZA treatment did not significantly affect the phenotype of mature DCs, with similar values of HLA-DR, CD40, CD80, and CD83 expression observed between the treated and untreated groups.

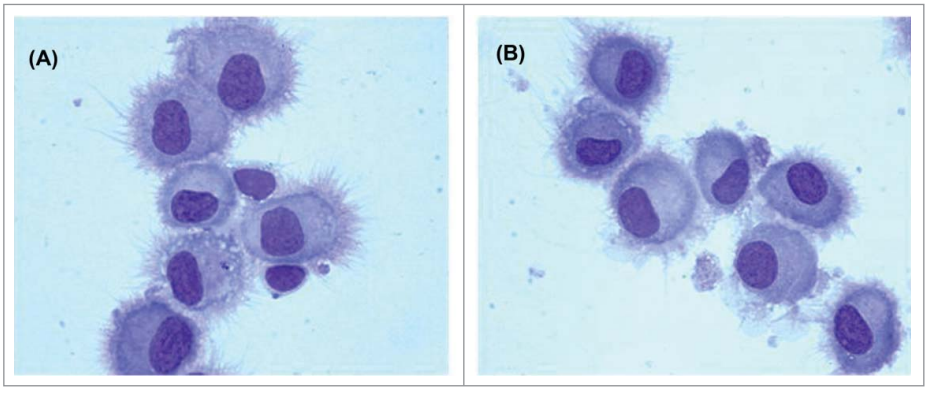

Figure 1. Zoledronic acid treatment does not modify the morphology of monocyte-derived DCs. Mature DCs were differentiated from untreated (**A**) and ZA-treated (**B**) CD14+ cells from melanoma patients. DC preparation was examined by light microscopy after May–Grunwald-Giemsa staining. A representative experiment using the cells from a patient in stage III of the disease is shown. DCs, dendritic cells; ZA, zoledronic acid.

However, ZA treatment did induce a significant increase in allostimulatory capacity in the cells from both the combined patient population and early-stage patients compared with the untreated samples (15.2 ± 1.7 vs. 13.2 ± 1.4 and 5.5 ± 2.2 vs. 13.8 ± 2.0, respectively; $P < 0.05$).

Second analytical approach

As we observed a variable trend in CD14 antigen expression in the cells from melanoma patients, we performed an additional analysis by dividing the population into two groups on the basis of CD14 expression: one with levels comparable to normal controls (group A: 4.05± 0.95%) and the other one with 1

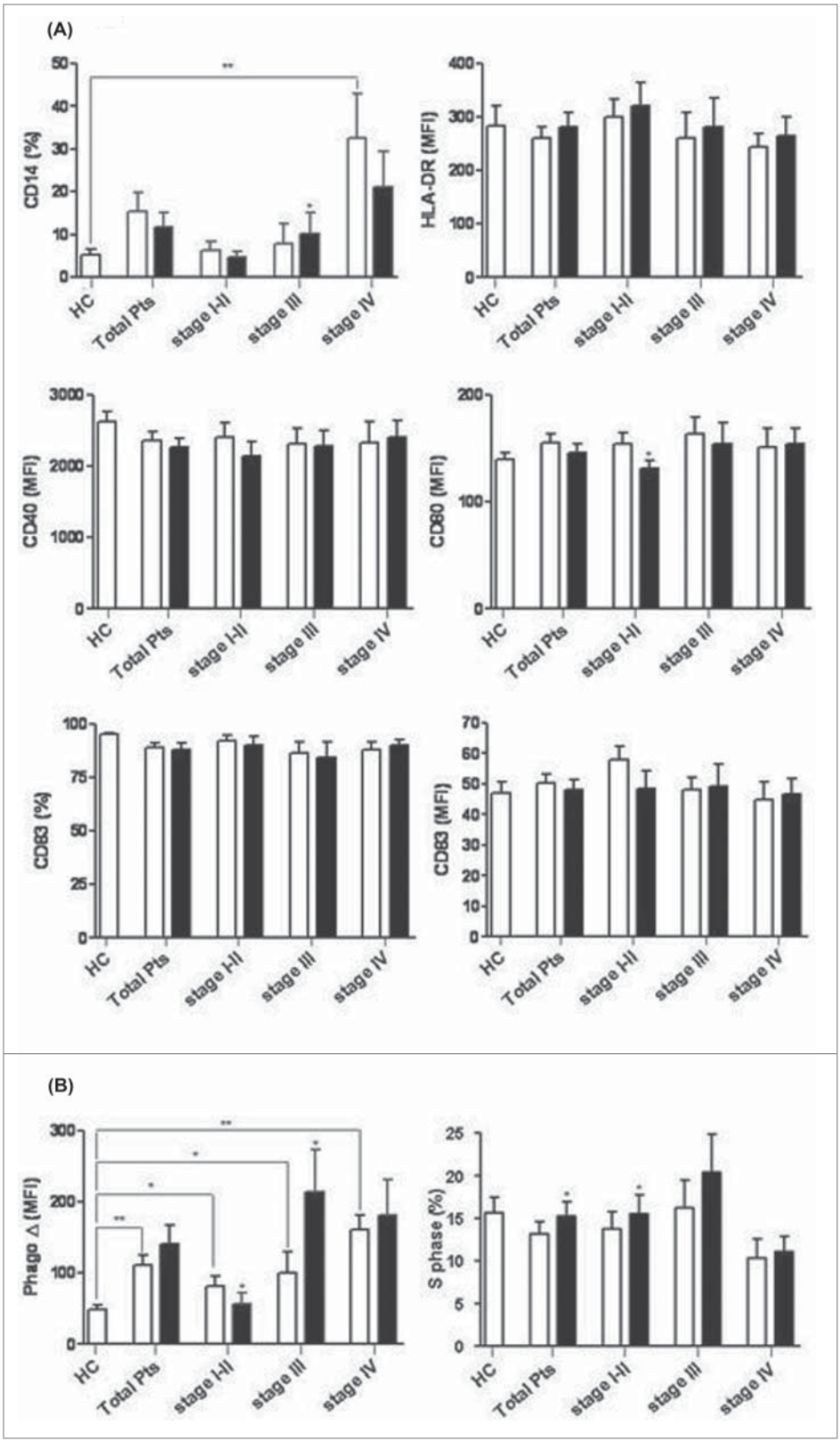

Figure 2. Effects of zoledronic acid treatment on the phenotype (**A**) and function (**B**) of monocyte-derived DCs. CD14 expression and phagocytic activity (evaluated by FITC-dextran uptake) were measured using immature DCs. HLA-DR, CD40, CD80, and CD83 expression and allostimulatory activity (assessed by DNA staining for S phase of the cell cycle) were measured using mature DCs. Bars represent the mean ± SEM of percent positive cells or the mean fluorescence intensity (MFI) of each observation. Black bars indicate the ZA-treated samples. Significant differences are indicated: $*P < 0.05$, $**P < 0.01$. HC, healthy controls; ΔMFI, difference in MFI between the cell fractions of the same sample incubated at 37°C and 0°C, respectively

SD more than the mean of the controls (group B: 40.13± 8.4%).

Groups A and B were composed of 17 patients (7 at stages I-II, 6 at stage III, and 4 at stage IV) and 9 patients (3 at stages I-II, 1 at stage III, and 5 at stage IV), respectively. The effects of zoledronic acid in the cells from these groups are displayed in **Figure 3**.

In group A, ZA treatment induced a significant increase in the percentage of CD14-expressing cells in comparison with the untreated samples (6.4± 1.3% vs. 4.05 ± 0.95%; $P = 0.0024$); this increase was observed in cells from patients in all disease stages, reaching a significant value only in the patients with early disease (4.1 ± 1.3% vs. 2.3± 0.7%; $P = 0.04$). Although ZA treatment increased the percentage of CD14-expressing cells, the obtained values were still comparable to those ones of the healthy subjects. Conversely, in group B, ZA treatment induced a substantial reduction in CD14-expressing cells (24.0 ± 8.1% vs. 40.1 ± 8.4%; $P = 0.0056$), with significant values in stages I-II and IV (6.3 ± 2.8% vs. 15.3 ± 2.0% and 28.1 ± 14.6% vs. 51.8± 13.2; $P = 0.03$ and $P = 0.023$, respectively).

The cells from the patients in both groups A and B showed an endocytic activity that was significantly higher than that for the cells from the healthy controls (130.1 ± 19.4 and 106.1 ± 15.8 vs. 49.0 ± 6.2; $P = 0.003$ and $P = 0.002$, respectively). In group A, the ZA-treated cells showed an increase in this activity compared with the untreated samples (183.3 ± 40.4 vs. 130.1 ± 19.4), particularly notable for stage III of the disease (250.8 ± 67.8 vs. 133.8 ± 34.6; $P = 0.046$); in group B cells, ZA treatment induced low effects.

The expression of costimulatory molecules (CD40 and CD80), CD83, and HLA-DR did not differ between groups A and B and the controls. However, in group A (both combined population and early-stage patients), the ZA-treated samples compared with untreated samples showed a significant reduction in both CD40 (2206 ± 151.8 vs. 2389 ± 148.8 and 2057 ± 322.7 vs. 2414 ± 317.6; $P = 0.02$ and $P = 0.001$, respectively) and CD80 (145.9 ± 11.6 vs. 163.7 ± 10.1 and 135.4 ± 11.4 vs. 160.1 ± 13.0; $P = 0.04$ and $P = 0.02$, respectively). Following ZA treatment, the cells from patients at stages I-II of this group showed a significant increase in HLA-DR antigen (364.8 ± 50.0 vs. 320.6 ± 40.2; $P = 0.03$). In addition, ZA treatment induced a low reduction in the percentage of CD83-expressing cells and a weak increase in allostimulatory activity.

In contrast, in group B cells, ZA treatment did not induce any significant effect.

Discussion

In melanoma, due to its high immunogenicity, the role of DCs is crucial for understanding its pathogenesis and developing efficacious immunotherapy.[11] In the present work, we found that monocyte-derived immature DCs from melanoma patients in advances stages show phenotypical/functional changes, as previously observed.[11]

Zoledronic acid is used for the prevention of osteoporosis and the treatment of bone metastasis in multiple myeloma and other cancers.[12-14]

In vitro and in vivo studies indicate that, in addition to their well-known antiosteoclastic activity, bisphosphonates exert other direct and indirect antitumor effects, which could contribute to their efficacy in metastatic cancer treatments. Among the several bisphosphonates, zoledronic acid is considered to be more potent than the others with regard to antitumor activity.[4,6] One of the mechanisms implicated in the antitumor effect of ZA is the inhibition of the enzyme farnesyl diphosphate synthase, which results in the altered synthesis of enzymes such as Rho, Rac, and Rab, which are thought to be involved in cell proliferation, cell motility, angiogenesis, and cell migration.[15-22] Several in vitro and in vivo models provide evidence that ZA also exerts antitumor effects by inducing immunological activities.

Recently, it has reported that ZA blocks the interaction between mesenchymal stem cells and breast cancer cells, which is thought to reduce the effect of mesenchymal stem cells on the progression of breast cancer.[22,23]

However, the most investigated and recognized effect of ZA is the induction of inflammatory responses, which are accompanied by the expansion of γδ T cells.[24]

These γδ T cells activated by ZA play an adjuvant role, linking innate and acquired immunity, through interactions with DCs in a way that amplifies the proliferation of tumor antigen-specific CD8+ T cells.[6-8] In fact, it has been reported[25-27] that a rapid and robust proliferative expansion of γδ T cells with antitumor activity can be induced by ZA treatment of monocytes or dendritic cells. Therefore, γδ T cell activation by ZA is highly dependent on the presence of APCs, including DCs.[7,28-30]

There are few studies about the effects of ZA on DC generation and maturation that differ in terms of experimental design and in the observed phenotypic and functional DC aspects.[6] However, these studies agree that this compound exerts its immunological action, at least in part, by modulating DC properties.

Fiore et al.[25] have shown that ZA improves the immunostimulatory ability of immature and mature DCs toward unconventional γδ and conventional αβ T cells.

Bringmann et al.[24] reported that the application of high-dose ZA has inhibitory effects on DC differentiation and activation, suggesting that the immunosuppressive effects may have a negative impact on tumor surveillance or control of minimal residual disease or promote infectious complications.

Other studies,[31,32] demonstrated that ZA modulates the differentiation, maturation, and functions of DCs, indicating that ZA may act at the differentiation stage of DCs to suppress the generation and maturation phenotype of DCs.

Recently, we demonstrated in normal subjects that ZA treatment did not affect DC differentiation from monocytes but modulated their maturation process, as shown by its inhibitory effects on both phenotypic and functional features.[6]

Here, we show that in melanoma patients, the in vitro ZA effects differ according to the progression of the disease. Indeed, ZA-treated monocytes from patients with early-stage disease generated DCs characterized by reduced endocytic activity and increased allostimulatory capacity compared with untreated samples, allowing restoration of the DC function observed in normal subjects. In contrast, ZA-treated monocytes from patients at stage III generated cells with a higher CD14 antigen expression and higher endocytosis than untreated samples.

Therefore, these results are similar to those observed in a recent work,[33] showing that the progressively reduced cytotoxicity against the melanoma cells of purified γδ T cells from melanoma patients, after IL-2/zoledronate expansion, depended on the stage of the disease.

However, the results obtained by a further analysis evaluating the expression of CD14 antigen appear to suggest that the DC phenotype together with clinical characteristics must be considered in the choice of patients to be treated with ZA. Furthermore, this observation derived from a low number of patients deserves further investigation in a larger sample.

Tumor cells use several mechanisms to escape the immune system. DCs, which are important cells for the initiation of a specific antitumor T-cell response, are also possible target cells susceptible to tumor-mediated immunosuppression.

Takahara et al.[7] suggest that the physical size of the immune response is crucial for successful antitumor therapy and that new methods to increase the immune response are needed for further therapeutic progress.

Studies to revert or overcome γδ lymphocyte paralysis have to be encouraged to evaluate possible therapeutic strategies aimed at controlling melanoma.[33]

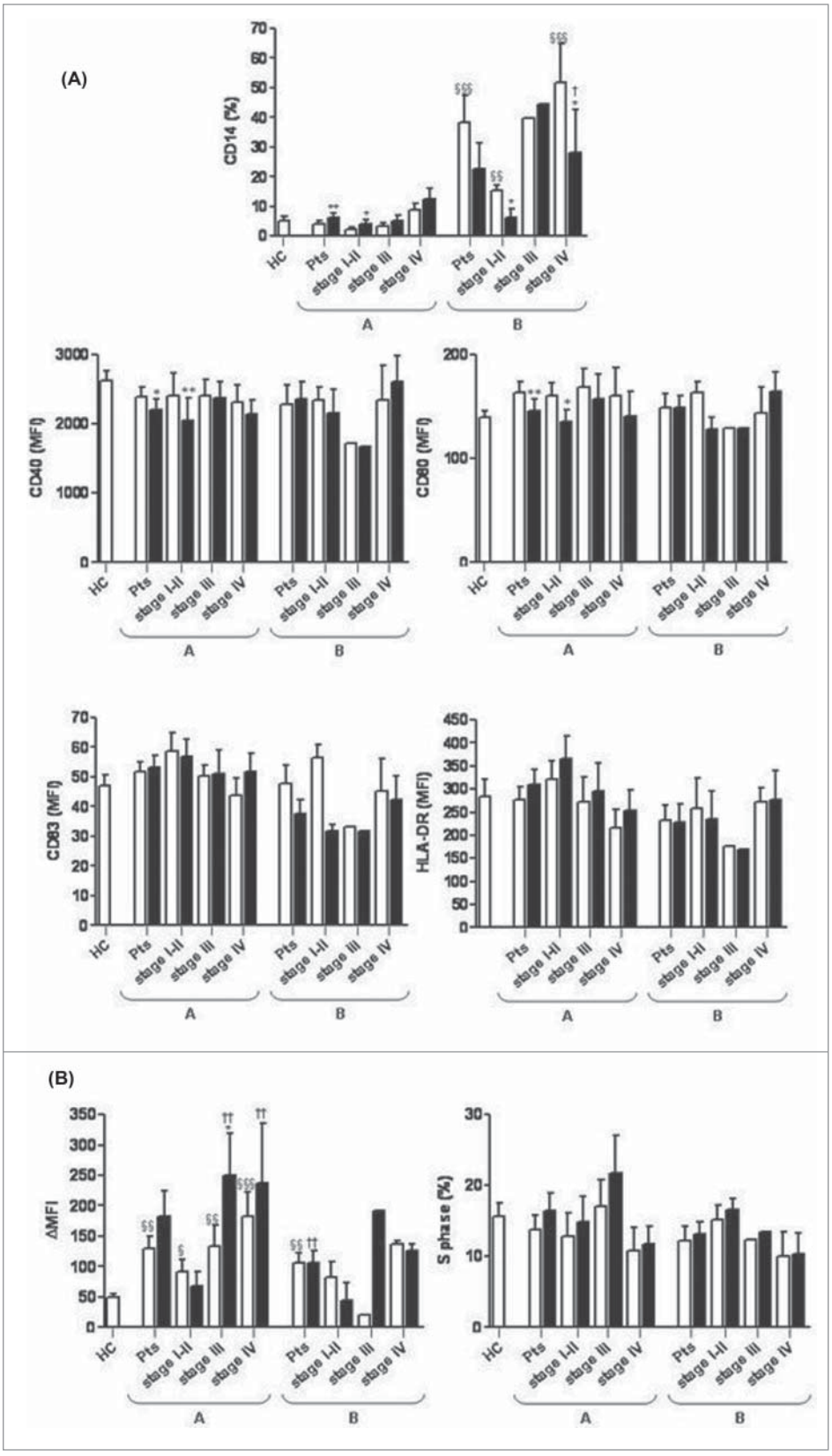

Figure 3. Effects of zoledronic acid treatment on the phenotype (A) and function (B) of monocyte-derived DCs. The patient population was divided into two groups based on CD14 expression: one with levels comparable to normal controls (group A) and the other one with 1 SD more than the mean of the controls (group B). Bars represent the mean ± SEM of percent positive cells or the mean fluorescence intensity (MFI) of each observation. Black bars indicate the ZA-treated samples. Statistical significance is in comparison to untreated samples, unless otherwise indicated: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$, $^{\S\S\S}P < 0.001$, untreated samples vs. healthy controls; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, ZA-treated samples vs. healthy controls. HC, healthy controls; Pts, overall patient population; ΔMFI, difference in MFI between the cell fractions of the same sample incubated at 37°C and 0°C, respectively.

Interestingly, it has been shown that copulsing immature DCs with zoledronate and melanoma-derived peptides increased antigen-specific CD8+ T cells in healthy individuals, confirming that the adjuvant effect of ZA can enhance responses to cross-presented tumor antigens.[7]

In conclusion, our work focused on the effect of ZA on monocyte-derived DCs from melanoma patients, showing that the effects of therapeutic doses of this drug might be mediated at least in part by modulation of myeloid cell function.

Material and Methods

Study population

The study protocol was approved by the local Ethics Committee review board (protocol 2806/09), and informed donor consent was obtained in accordance with the Declaration of Helsinki. In this study, we included 26 patients (n = 10 females and n = 16 males) with a new, histologically confirmed diagnosis of cutaneous melanoma. This population was divided into three groups based on the clinical stage: no evidence of regional lymph node or distant metastasis (stages I-II; n = 10), lymph node involvement without distant metastasis (stage III; n = 7), the presence of distance metastasis (stage IV; n = 9). None of these patients presented with a history of neoplastic diseases (except cutaneous melanoma), infectious diseases or others disorders, therapies or conditions known to interfere with DC expression. Blood samples were drawn from the melanoma patients prior to

chemotherapeutic and/or radiation treatment. None of these patients was previously treated for melanoma.

Drug

Zoledronic acid (ZA), kindly provided by Novartis Pharma AG, was added on the first day of culture to evaluate its immuno-modulatory effects on DCs. The drug concentration was selected on the basis of a previous study,[6] in which we demonstrated the high toxic effect of ZA on culture-derived cells at concentrations of 5 and 10 μmol/L; therefore, we used the concentration of 1 μmol/L, the maximum plasma level reached after the standard clinical administration of 4 mg of the drug in 15 min for the treatment of bone metastasis.[6] The same effect was observed in further experiments performed on a small group of melanoma patients (data not shown).

Generation of dendritic cells

Peripheral blood was collected from 26 patients with melanoma and from 11 healthy donors with a similar gender and age distribution.

Following venous blood collection in heparinised tubes, peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation using Histopaque 1077 (Sigma, St Louis, MO, USA). CD14+ monocytes were isolated from PBMCs by magnetic bead separation using anti-CD14 microbeads (Miltenyi Biotech) to a purity that was generally >90%, as determined by FACS analysis.[34]

Briefly, PBMCs were incubated with microbeads conjugated with monoclonal mouse anti-human CD14 antibodies for 15 min on ice and washed in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin and 2 mM ethylenediamine tetraacetic acid (EDTA). The labeled and positively enriched cells were eluted from magnetic columns by the removal of the columns from the magnetic device. The purified CD14+ cells were resuspended at a concentration of 1×10^6 cells/mL and cultured for 6 d in multi-well tissue culture plates in RPMI-1640 (Gibco Laboratories) supplemented with 10% fetal calf serum (FCS; Euroclone SpA), 2 mM L-glutamine (Euroclone SpA), 100 mg/mL streptomycin and 100 IU/mL penicillin (Sigma) in the presence of 50 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; PeproTech EC Ltd) and 50 ng/mL recombinant human interleukin 4 (IL-4; PeproTech Inc.).

For maturation, DCs were cultured with 100 ng/mL of bacterial lipopolysaccharide (LPS; Sigma-Aldrich) for 24 h.

DCs were harvested after 6 or 7 d of culture and used to analyze cell morphology and cell-surface markers.

Morphological evaluation

To evaluate the morphology of the cultured cells, cytospin slides were prepared by cytocentrifuging 5×10^4 cells/mL at 800 × g for 10 min (Shandon Cytospin-2 Centrifuge, GMI Inc.). The slides were air-dried and fixed with methanol (Carlo Erba Reagenti SpA), stained with May–Grunwald-Giemsa (Carlo Erba Reagenti SpA) and examined by light microscopy (Olympus Corporation).

Flow cytometric analysis

The antibodies used for cell surface staining included anti-CD14 and anti-human leukocyte antigen D-related (HLA-DR) (Immunotools) and anti-CD40, anti-CD80, and anti-CD83 (Immunotech SAS) conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Data acquisition and analysis were performed with Epics-XL (Beckman Coulter Inc.) with Expo32 Software. Five thousand events were acquired for each sample.

DCs were gated according to their light-scattering properties, and dead cells were excluded from the analysis.

The results were expressed as a percentage of positive cells or as the mean fluorescence intensity (MFI) of positive cells.

Uptake of FITC–dextran

Mannose receptor-mediated endocytosis was measured as the cellular uptake of FITC-dextran (Sigma-Aldrich Co) using cytofluorimetric analysis. Approximately 1×10^6 immature ZA-treated or untreated cells were incubated in media containing FITC-dextran (2 mg/mL; Sigma) for 60 min at 37°C or on ice (the latter for the assessment of background staining due to non-specific external binding).

After incubation, the cells were washed twice with PBS to remove the excess dextran and fixed in cold 1% formaldehyde (Bio Optica Milano SpA). The cells were then analyzed using flow cytometry. At least 5000 gated events were evaluated for each condition. DCs were gated based on their light-scattering properties, and dead cells were excluded from the analysis. The level of antigen uptake by DCs was expressed as the difference in MFI (ΔMFI) between the cell fractions of the same sample incubated at 37°C and 0°C, respectively.

Allogeneic T cell proliferation assay

The primary T cell stimulatory capacity of DCs was tested in an allo-mixed lymphocyte reaction (MLR).

Allogeneic purified T cells, derived from the same donor, were used as the responder cells in 96-well round-bottomed tissue culture plates in a culture volume of 200 mL. All experiments were performed in triplicate. Graded numbers of stimulator cells were added to 2×10^5 responder cells; control cultures, used to evaluate the background proliferation, only contained responder or stimulator cells.

After 6 d of culture in 5% CO_2 at 37°C, the cells were harvested, counted and diluted to approximately 1×10^6 cells/mL and fixed in 70% ethanol. Proliferation was assessed using flow cytometry for the S phase of the cell cycle with propidium iodide (PI) staining for DNA (20 mg/mL; eBioscence Inc.). Data acquisition and analysis were performed using an Epics-XL (Beckman Coulter) with MultiCycle software.

Statistical analyses

A statistical analysis was performed either on the entire population or by dividing the patients based on clinical stage using GraphPad Prism software (GraphPad Software Inc.).

The data were expressed as the mean ± standard error of the mean (SEM). The statistical significance of the differences between the ZA-treated and untreated samples was determined

by a paired two-tailed Student's t test; an association was considered statistically significant when $P < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We express our sincere appreciation to the study participants.

Funding

This work was supported by Novartis Farma (Origgio, Italy) and University of Pisa. A.F. was supported by a fellowship of the Associazione Contro il Melanoma Onlus (Pisa, Italy).

Author Contributions

All listed authors have reviewed and approved the final version of the manuscript. All authors contributed to the work presented in this paper. Conceived and designed the experiments: A.L., A. R., R.C. Performed the experiments: A.F., G.O. Analyzed the data: A.F., A.L. Recruited the patients: A.R. Wrote the paper: A. F., A.L., R.C. Discussed the results and implications: A.F., A.L., A.R., R.C.

References

1. Forsea AM, Müller C, Riebeling C, Orfanos CE, Geilen CC. Nitrogen-containing bisphosphonates inhibit cell cycle progression in human melanoma cells. Br J Cancer 2004; 91:803-10; PMID:15280922
2. Riebeling C, Forsea AM, Raisova M, Orfanos CE, Geilen CC. The bisphosphonate pamidronate induces apoptosis in human melanoma cells in vitro. Br J Cancer 2002; 87:366-71; PMID:12177810; http://dx.doi.org/10.1038/sj.bjc.6600476
3. Neville-Webbe HL, Gnant M, Coleman RE. Potential anticancer properties of bisphosphonates. Semin Oncol 2010; 37(Suppl 1):S53-65; PMID:20682373; http://dx.doi.org/10.1053/j.seminoncol.2010.06.008
4. Clézardin P, Ebetino FH, Fournier PG. Bisphosphonates and cancer-induced bone disease: beyond their antiresorptive activity. Cancer Res 2005; 65:4971-4; PMID:15958534; http://dx.doi.org/10.1158/0008-5472.CAN-05-0264
5. Rogers MJ. New insights into the molecular mechanisms of action of bisphosphonates. Curr Pharm Des 2003; 9:2643-58; PMID:14529538; http://dx.doi.org/10.2174/1381612033453640
6. Orsini G, Failli A, Legitimo A, Adinolfi B, Romanini A, Consolini R. Zoledronic acid modulates maturation of human monocyte-derived dendritic cells. Exp Biol Med (Maywood) 2011; 236:1420-6; PMID:22075551; http://dx.doi.org/10.1258/ebm.2011.011168
7. Takahara M, Miyai M, Tomiyama M, Mutou M, Nicol AJ, Nieda M. Copulsing tumor antigen-pulsed dendritic cells with zoledronate efficiently enhance the expansion of tumor antigen-specific CD8+ T cells via Vgamma9gammadelta T cell activation. J Leukoc Biol 2008; 83:742-54; PMID:18156189; http://dx.doi.org/10.1189/jlb.0307185
8. Saito A, Narita M, Yokoyama A, Watanabe N, Tochiki N, Satoh N, Takizawa J, Furukawa T, Toba K, Fuse I, et al. Enhancement of anti-tumor cytotoxicity of expanded gammadelta T Cells by stimulation with monocyte-derived dendritic cells. J Clin Exp Hematop 2007; 47:61-72; PMID:18040145; http://dx.doi.org/10.3960/jslrt.47.61
9. Nakai N, Hartmann G, Kishimoto S, Katoh N. Dendritic cell vaccination in human melanoma: relationships between clinical effects and vaccine parameters. Pigment Cell Melanoma Res 2010; 23:607-19; PMID:20579307; http://dx.doi.org/10.1111/j.1755-148X.2010.00736.x
10. Dhodapkar MV, Dhodapkar KM, Palucka AK. Interactions of tumor cells with dendritic cells: balancing immunity and tolerance. Cell Death Differ 2008; 15:39-50; PMID:17948027; http://dx.doi.org/10.1038/sj.cdd.4402247
11. Failli A, Legitimo A, Orsini G, Romanini A, Consolini R. Numerical defect of circulating dendritic cell subsets and defective dendritic cell generation from monocytes of patients with advanced melanoma. Cancer Lett 2013; 337:184-92; PMID:23684927; http://dx.doi.org/10.1016/j.canlet.2013.05.013
12. Cohen MH, Dagher R, Griebel DJ, Ibrahim A, Martin A, Scher NS, Sokol GH, Williams GA, Pazdur R. U.S. Food and Drug Administration drug approval summaries: imatinib mesylate, mesna tablets, and zoledronic acid. Oncologist 2002; 7:393-400; PMID:12401901; http://dx.doi.org/10.1634/theoncologist.7-5-393
13. Yu J. Zoledronate activates NK cells. Blood 2011; 118:2642-3; PMID:21903898; http://dx.doi.org/10.1182/blood-2011-06-362707
14. Oster G, Lamerato L, Glass AG, Richert-Boe KE, Lopez A, Chung K, Richhariya A, Dodge T, Wolff GG, Balakumaran A, et al. Use of intravenous bisphosphonates in patients with breast, lung, or prostate cancer and metastases to bone: a 15-year study in two large US health systems. Support Care Cancer 2014; 22:1363-73; PMID:24389827; http://dx.doi.org/10.1007/s00520-013-2094-y
15. Santini D, Vincenzi B, Galluzzo S, Battistoni F, Rocci L, Venditti O, Schiavon G, Angeletti S, Uzzalli F, Caraglia M, et al. Repeated intermittent low-dose therapy with zoledronic acid induces an early, sustained, and long-lasting decrease of peripheral vascular endothelial growth factor levels in cancer patients. Clin Cancer Res 2007; 13:4482-6; PMID:17671133; http://dx.doi.org/10.1158/1078-0432.CCR-07-0551
16. Santini D, Zoccoli A, Gregorj C, Di Cerbo M, Iuliani M, Pantano F, Zamarchi R, Sergi F, Flammia G, Buscarini M, et al. Zoledronic acid induces a significant decrease of circulating endothelial cells and circulating endothelial precursor cells in the early prostate cancer neoadjuvant setting. Oncology 2013; 85:342-7; PMID:24335337; http://dx.doi.org/10.1159/000347226
17. Mönkkönen H, Auriola S, Lehenkari P, Kellinsalmi M, Hassinen IE, Vepsäläinen J, Mönkkönen J. A new endogenous ATP analog (ApppI) inhibits the mitochondrial adenine nucleotide translocase (ANT) and is responsible for the apoptosis induced by nitrogen-containing bisphosphonates. Br J Pharmacol 2006; 147:437-45; PMID:16402039; http://dx.doi.org/10.1038/sj.bjp.0706628
18. Oades GM, Senaratne SG, Clarke IA, Kirby RS, Colston KW. Nitrogen containing bisphosphonates induce apoptosis and inhibit the mevalonate pathway, impairing Ras membrane localization in prostate cancer cells. J Urol 2003; 170:246-52; PMID:12796698; http://dx.doi.org/10.1097/01.ju.0000070685.34760.5f
19. Denoyelle C, Hong L, Vannier JP, Soria J, Soria C. New insights into the actions of bisphosphonate zoledronic acid in breast cancer cells by dual RhoA-dependent and -independent effects. Br J Cancer 2003; 88:1631-40; PMID:12771933; http://dx.doi.org/10.1038/sj.bjc.6600925
20. Paepke S, Blohmer JU, Warm M, Ohlinger R, Kiechle M. Zoledronic Acid in Adjuvant Therapy of Receptor Negative Early Breast Cancer - Rationale and First Experience in Individual Therapy Strategy. Geburtshilfe Frauenheilkd 2011; 71:135-9;; http://dx.doi.org/10.1055/s-0030-1250685
21. Insalaco L, Di Gaudio F, Terrasi M, Amodeo V, Caruso S, Corsini LR, Fanale D, Margarese N, Santini D, Bazan V, et al. Analysis of molecular mechanisms and anti-tumoural effects of zoledronic acid in breast cancer cells. J Cell Mol Med 2012; 16:2186-95; PMID:22260151; http://dx.doi.org/10.1111/j.1582-4934.2012.01527.x
22. Fasching PA, Jud SM, Hauschild M, Kümmel S, Schütte M, Warm M, Hanf V, Grab D, Krocker J, Stickeler E, et al. FemZone trial: a randomized phase II trial comparing neoadjuvant letrozole and zoledronic acid with letrozole in primary breast cancer patients. BMC Cancer 2014; 14:66; PMID:24499441; http://dx.doi.org/10.1186/1471-2407-14-66
23. Gallo M, De Luca A, Lamura L, Normanno N. Zoledronic acid blocks the interaction between mesenchymal stem cells and breast cancer cells: implications for adjuvant therapy of breast cancer. Ann Oncol 2012; 23:597-604; PMID:21551002; http://dx.doi.org/10.1093/annonc/mdr159
24. Bringmann A, Schmidt SM, Weck MM, Brauer KM, von Schwarzenberg K, Werth D, Grünebach F, Brossart P. Zoledronic acid inhibits the function of Toll-like receptor 4 ligand activated monocyte-derived dendritic cells. Leukemia 2007; 21:732-8; PMID:17301819
25. Fiore F, Castella B, Nuschak B, Bertieri R, Mariani S, Bruno B, Pantaleoni F, Foglietta M, Boccadoro M, Massaia M. Enhanced ability of dendritic cells to stimulate innate and adaptive immunity on short-term incubation with zoledronic acid. Blood 2007; 110:921-7; PMID:17403919; http://dx.doi.org/10.1182/blood-2006-09-044321
26. Cabillic F, Toutirais O, Lavoué V, de La Pintière CT, Daniel P, Rioux-Leclerc N, Turlin B, Mönkkönen H, Mönkkönen J, Boudjema K, et al. Aminobisphosphonate-pretreated dendritic cells trigger successful Vgamma9Vdelta2 T cell amplification for immunotherapy in advanced cancer patients. Cancer Immunol Immunother 2010; 59:1611-9; PMID:20582413; http://dx.doi.org/10.1007/s00262-010-0887-0
27. Miyagawa F, Tanaka Y, Yamashita S, Minato N. Essential requirement of antigen presentation by monocyte lineage cells for the activation of primary human gamma delta T cells by aminobisphosphonate antigen. J Immunol 2001; 166:5508-14; PMID:11313389; http://dx.doi.org/10.4049/jimmunol.166.9.5508
28. Miyagawa F, Tanaka Y, Yamashita S, Mikami B, Danno K, Uehara M, Minato N. Essential contribution of germline-encoded lysine residues in Jgamma1.2 segment to the recognition of nonpeptide antigens by human gammadelta T cells. J Immunol 2001; 167:6773-9; PMID:11739492; http://dx.doi.org/10.4049/jimmunol.167.12.6773

29. Kato Y, Tanaka Y, Tanaka H, Yamashita S, Minato N. Requirement of species-specific interactions for the activation of human γδ T cells by pamidronate. J Immunol 2003; 170:3608-13; PMID:12646624; http://dx.doi.org/10.4049/jimmunol.170.7.3608
30. Conti L, Casetti R, Cardone M, Varano B, Martino A, Belardelli F, Poccia F, Gessani S. Reciprocal activating interaction between dendritic cells and pamidronate-stimulated gammadelta T cells: role of CD86 and inflammatory cytokines. J Immunol 2005; 174:252-60; PMID:15611247; http://dx.doi.org/10.4049/jimmunol.174.1.252
31. Chen YJ, Chao KS, Yang YC, Hsu ML, Lin CP, Chen YY. Zoledronic acid, an aminobisphosphonate, modulates differentiation and maturation of human dendritic cells. Immunopharmacol Immunotoxicol 2009; 31:499-508; PMID:19555208; http://dx.doi.org/10.1080/08923970902814103
32. Wolf AM, Rumpold H, Tilg H, Gastl G, Gunsilius E, Wolf D. The effect of zoledronic acid on the function and differentiation of myeloid cells. Haematologica 2006; 91:1165-71; PMID:16956814
33. Petrini I, Pacini S, Galimberti S, Taddei MR, Romanini A, Petrini M. Impaired function of gamma-delta lymphocytes in melanoma patients. Eur J Clin Invest 2011; 41:1186-94; PMID:22775565; http://dx.doi.org/10.1111/j.1365-2362.2011.02524.x
34. Mucci I, Legitimo A, Compagnino M, Consolini R, Migliaccio P, Metelli MR, Scatena F. The methodological approach for the generation of human dendritic cells from monocytes affects the maturation state of the resultant dendritic cells. Biologicals 2009; 37:288-96; PMID:19665905; http://dx.doi.org/10.1016/j.biologicals.2009.05.004