Original Research

Zoledronic acid modulates maturation of human monocyte-derived dendritic cells

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Abstract

Zoledronic acid (ZA) is a drug of the bisphosphonate class, which is widely used for the treatment of both osteoporosis and skeletal metastasis. Besides its main bone antiresorptive activity, ZA displays antitumor properties, by triggering the expansion and activation of $\gamma\delta$ T-cells, which exert an antitumor effect through dendritic cells (DCs). Several studies have reported the interaction between ZA and $\gamma\delta$ T-cells, but the potential immunoregulatory activity of this drug on DCs has scarcely been investigated. Therefore, in this paper, we evaluated the effects of a therapeutic dose of ZA on the *in vitro* generation and maturation of DCs derived from peripheral blood monocytes of healthy adult donors. We demonstrate that ZA treatment did not affect DC differentiation, but inhibited DC maturation on lipopolysaccharide activation, as shown by the impaired expression of maturation surface markers and reduced ability to induce allogeneic T-cell proliferation. Interestingly, IL-10 secretion by mature DCs was significantly lower in ZA-treated cells than in controls. We conclude that ZA exerts its immunological *in vitro* activity also by modulating the maturation of DCs.

Keywords: dendritic cells, zoledronic acid, immunomodulation

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Introduction

Bisphosphonates (BPs) are synthetic analogs of the pyrophosphate molecule, an endogenous inhibitor of bone mineralization. They are currently used for the prevention and treatment of osteoporosis in bone diseases and in postmenopause, as well as of skeletal metastases of patients with malignant diseases, such as breast cancer or multiple myeloma.[1] In the case of malignancies, where increased bone re-uptake is one of the central mechanisms in tumor-induced hypercalcemia, these compounds are effective inhibitors of osteoclast-mediated bone resorption,[2] inducing physico-chemical modifications of bone mineral composition.[3]

Zoledronic acid (ZA), one of the newer generation nitrogen-containing BPs, inhibits the enzyme farnesyl diphosphate synthase, which plays a role in the mevalonate pathway and in the subsequent prenylation of small GTPase proteins, such as Ras. These molecules are involved in the intracellular signaling cascades and are essential for osteoclast activity and survival.[4]

Recently, several *in vitro* and *in vivo* studies suggest that, besides their well-known antiosteoclastic activity, BPs have other direct and indirect antitumor effects, which could contribute to their efficacy in metastatic cancer treatments.[5] Among BPs, ZA stands out with much higher potencies than the other ones with regard to antitumor activity.[6]

An indirect mechanism of its antitumor activity seems to be exerted, at least in part, through the expansion of $\gamma\delta$ T-cells. Indeed, this compound and its metabolites share structural homologies with recently identified $\gamma\delta$ T-cells non-peptide ligands, thereby allowing their stimulatory effects.[7]

Recently, it has been demonstrated that $\gamma\delta$ T-cells activated by ZA play an adjuvant role, linking innate and acquired immunity, through interaction with dendritic cells (DCs), in a way that amplifies the proliferation of tumor antigen-specific CD8+ T cells.[8,9]

DCs are the most important antigen-presenting cells (APCs) belonging to the monocyte lineage, with a central role in innate and adaptive immunity. As immature cells,

DCs are ubiquitously distributed in all tissues, where they have a role as sentinels specialized in the uptake and processing of antigen. After capturing an antigen, DCs migrate to the lymph nodes, where they present the antigen to T lymphocytes, and activate the adaptive immune system by generating antigen-specific responses. During this migration, DCs undergo phenotypic and functional changes, acquiring mature state characteristics.[10]

Several studies have been performed focusing on the interaction between ZA and unconventional $\gamma\delta$ T-cells. However, the effects of this compound on DCs have not been widely investigated. Moreover, those studies which have looked at the effect of ZA on DCs have yielded inhomogenous results because of different experimental settings.[1,2,11–13]

In this paper, we investigated the effects of ZA on the generation and maturation of DCs derived from peripheral blood monocytes of healthy adult donors, in order to contribute to the comprehension of the immunological effects of this compound.

Materials and methods

Generation of DCs

Human heparinized peripheral blood samples were drawn from healthy adult donors with informed consent. The study was approved by the local ethical committee.

Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll–Paque density gradient centrifugation (Lympholyte-H, Cedarlane Laboratories Ltd, Burlington, Ontario, Canada). CD14-positive cells were purified from PBMCs by positive selection with microbeads coated with monoclonal mouse antihuman CD14, using the MIDIMACS Technique (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), according to the manufacturer's instructions and as previously described.[14] Monocytes were re-suspended at a concentration of 1×10^6 cells/mL and cultured at 37°C and 5% CO_2 for six days in a 24-well tissue culture plate in RPMI-1640 (Gibco Laboratories, Grand Island, NE, USA) supplemented with 10% of fetal bovine serum (FBS), 2 mmol/L L-glutamine (Euroclone SpA, Pavia, Italy), 100 μg/mL streptomycin (Sigma-Aldrich Co, St Louis, MO, USA) and 100 IU/mL penicillin (Sigma-Aldrich Co), in the presence of 50 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (PeproTech Inc, Rocky Hill, NJ, USA) and 20 ng/mL recombinant human interleukin-4 (IL-4; PeproTech Inc). Fresh cytokines were replaced every three days. For the induction of DC maturation, immature DCs (iDCs) received 100 ng/mL of bacterial lipopolysaccharide (LPS; Sigma-Aldrich Co) on day 6 for 24 h.

ZA (1 μmol/L; Novartis AG, Basel, Switzerland) was added on the first day of culture to evaluate its effect on differentiation and maturation of DCs. As ZA showed high toxicity on culture-derived cells at the concentrations of 5 and 10 μmol/L, we used the concentration of 1 μmol/L, being 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.[15]

Morphological evaluation

In order to evaluate the morphology of 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, Ramsey, MN, USA). Slides were air-dried and fixed with methanol (Carlo Erba Reagenti SpA, Milan, Italy), stained with May-Grünwald-Giemsa (Carlo Erba Reagenti SpA) and then examined by light microscopy (Olympus Corporation, Tokyo, Japan).

Phenotypic analysis

Cells harvested were labeled with the following mouse monoclonal antibodies for cell-surface staining: anti-CD14, antihuman leukocyte antigen D-related (HLA-DR) (Immunotools, Friesoythe, Germany), anti-CD40, anti-CD80, anti-CD83 (Immunotech SAS, Marseille, France), conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin. Washing was performed with a saline solution containing phosphate-buffered saline, 0.2% FBS and 0.05% sodium azide (NaN_3; Sigma-Aldrich Co). The negative control (cells without any monoclonal antibodies) was realized for each sample in order to exclude cell autofluorescence. Cell acquisition and data analysis were performed on Epics-XL (Beckman Coulter Inc, Miami, FL, USA) with Expo32 Software. Five thousand events were acquired for each sample. DCs were gated on the basis of their light-scattering properties, and dead cells were excluded from the analysis. Results were expressed as a percentage of positive cells or as mean fluorescence intensity (MFI) of positive cells. Apoptotic and necrotic DCs were evaluated by staining cells with annexin V and propidium iodide (PI) using the TACS® Annexin V Kit (Trevigen® Inc, Gaithersburg, MD, USA), in order to determine the direct cytotoxic effect of ZA on cultured cells.

Phagocytosis assay

Mannose receptor-mediated phagocytosis was measured as cellular uptake of FITC-dextran (Sigma-Aldrich Co) by cytofluorimetric analysis. Approximately 1×10^6 of immature ZA-treated or untreated cells were incubated in RPMI-1640 containing 10% FBS and 2 mg/mL FITC-dextran for 60 min at 37°C or on ice (negative control, in order to exclude unspecific external binding). After incubation, cells were washed twice with PBS to remove excess dextran, and fixed with cold 1% formaldehyde (Bio Optica Milano SpA, Milan, Italy). Five thousand events were acquired for each sample. DCs were gated on the basis of their light-scattering properties, and dead cells were excluded from the analysis. The quantification of phagocytosis was expressed as difference in MFI (ΔMFI) between the cell fractions of the same sample incubated at 37 and 0°C, respectively.

Allogeneic T-cell proliferation assay

For the primary mixed lymphocyte reaction assay, a T-cell population, isolated by the immunomagnetic method from healthy donors after Ficoll-separation of PBMCs, was used as responder cells to test the stimulatory capacity of DCs.

Graduated numbers of ZA-treated and untreated stimulator mature DCs were added to 2×10^5 allogeneic responder T-cells, and cultured in 96-well round-bottomed cell culture plates at 37°C and 5% CO_2. Control cultures were assessed with responder or stimulator cells only to evaluate the background proliferation. All experiments were performed in triplicate. After six days of culture, cells were collected, counted, diluted to 1×10^6 cells/mL, fixed with 70% ethanol and stored at −20°C for 24 h. Proliferation, as S phase of the cell cycle measure, was assessed by DNA staining with PI (20 μg/mL; eBioscence Inc, San Diego, CA, USA). Data acquisition and analysis were performed on an Epics-XL flow cytometer, using MultiCycle software.

Cytokine measurement

Supernatants of the ZA-treated and untreated cell cultures were harvested on day 7 to determine the following cytokines: IL-6, IL-12, IL-10 and TNF-α. Briefly, cell suspensions were collected and centrifuged; supernatants were filtered through 0.2-μm pore filters (International Pbi SpA, Milan, Italy) and stored at −80°C. Cytokine concentrations were measured with immunoenzymatic method (enzyme-linked immunosorbent assay), according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc, La Jolla, CA, USA). Results were expressed as mean ± standard error of the mean (SEM). Significant differences between ZA-treated and control samples were determined by two-tailed paired Student's *t*-test. The statistical analysis of cell viability was performed by one-way analysis of variance test. *P* values <0.05 were considered statistically significant.

Results

High ZA concentrations affected viability of DCs

In preliminary experiments, cells were treated with three different ZA concentrations (1, 5 and 10 μmol/L) to evaluate the effect of ZA on DC viability. Treatment with 5 and 10 μmol/L of ZA induced an enhanced rate of cell death compared with controls (iDCs: 58.55 ± 1.91% and 77.03 ± 2.07%, respectively, versus 10.69 ± 0.88%; $P < 0.01$ for ZA concentration of 10 μmol/L). Similar values were observed with both ZA concentrations in mature DCs (mDCs) (Figures 1a and 1b). At the highest ZA concentration of 10 μmol/L, the percentage of apoptotic and necrotic cells was about 35–38% and 40–42%, respectively.

As shown in Figure 1, ZA at the lowest concentration of 1 μmol/L did not show a cytotoxic effect on DCs. The percentage of annexin V positive–propidium iodide negative, and thus apoptotic cells, amounted to approximately 7% and 10% for control and ZA 1 μmol/L-treated DCs, respectively. Annexin V–propidium iodide double positive, and thus necrotic cells, were about 3–7% for the controls and 6–8% for ZA 1 μmol/L-treated DCs.

ZA treatment did not influence monocyte differentiation in immature DCs

The ZA treatment did not substantially modify DC immunophenotype on day 6 compared with the control. In fact, both untreated and ZA-treated iDCs did not express the CD14 molecule (6.54 ± 1.73% versus 4.52 ± 1.29%, respectively) and ZA-treated cells showed a slight, but not statistically significant, increase of class II molecule (HLA-DR) expression, compared with controls (81.07 ± 5.07% versus 77.02 ± 6.22%, respectively) (Figure 2a).

Phagocytic activity, a property of immature DCs, was evaluated by FITC-dextran uptake on day 6 on both treated and untreated DCs. Cells cultured in the presence of 1 μmol/L ZA showed a not-relevant increase in antigen capture capacity compared with control DCs (ΔMFI: 61.69 ± 10.06 versus 52.35 ± 7.37, respectively) (Figure 2c).

In addition, the morphological analysis showed that cells derived from ZA-treated cultures displayed typical features of mature myeloid DCs, including irregular membrane ruffling and laterally positioned nuclei (data not shown); however, ZA-treated cells harvested on day 7, after stimulation with LPS, showed a smaller size and shorter dendrites than control cells (Figures 3a and 3b).

ZA treatment affected DC maturation

On day 7, ZA treatment altered the immunophenotype of the mature cultured DCs, with regard to the CD40, CD80 and CD83 expression levels. In fact, the expression of these molecules on ZA-treated cells was significantly lower than in control DCs (CD40 MFI: 242.65 ± 16.89 versus 275.30 ± 17.87, respectively, $P < 0.01$; CD80 MFI: 115.44 ± 7.35 versus 135.08 ± 8.83, $P < 0.05$; CD83 MFI: 42.82 ± 2.02 versus 50.71 ± 3.49, $P < 0.05$). On the contrary, the HLA-DR expression showed a weak but not statistically significant increase, compared with control cells (MFI: 355.01 ± 46.07 versus 326.42 ± 39.61, respectively) (Figure 2b).

The ability of DCs to stimulate allogeneic T-cell proliferation is a function of their maturation status. Both treated and untreated DCs stimulated T-cell proliferation when compared with responder T-cells alone. However, the activity of ZA-treated stimulator DCs was significantly lower than that of untreated DCs derived from the same donor (S phase: 10.00 ± 1.64% versus 13.66 ± 2.34%, respectively; $P < 0.05$) (Figure 2d).

The evaluation of the levels of cytokines secreted in supernatants of cultures was performed on day 7. As shown in Figure 4, ZA significantly affected IL-10 secretion by mature DCs compared with controls (13.00 ± 5.22 versus 54.28 ± 13.50 pg/mL, respectively; $P < 0.05$), whereas it did not significantly inhibit TNF-α release. Both IL-6 and IL-12 secretion did not show any differences between ZA-treated and control cells.

Discussion

The capacity of BPs to reduce the bone loss by inhibiting osteoclastic activity is well known.[4] More recently, it has

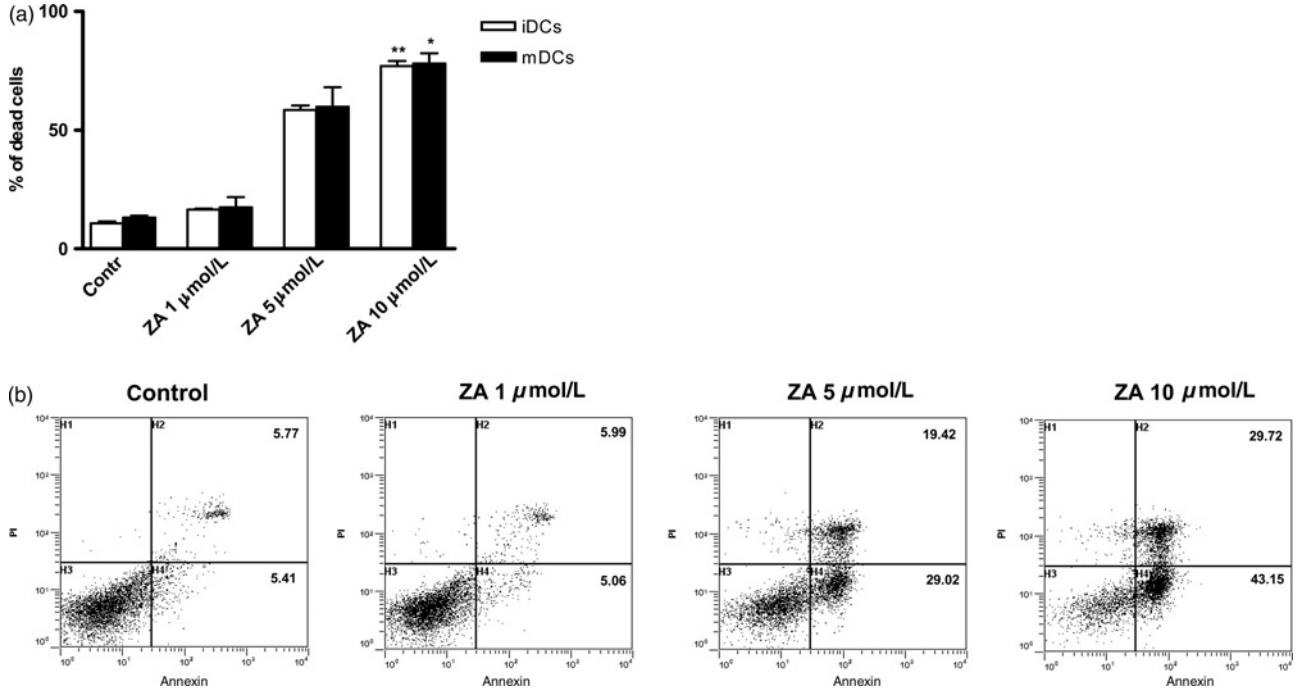

Figure 1 High ZA concentrations affect DC viability. The evaluation of the ZA cytotoxic effect on both immature and mature DCs was performed by annexin V and propidium iodide staining. (a) The percentage of total dead cells was calculated by summing of the apoptotic and necrotic rate. Data are shown as mean ± SEM of three independent experiments ($^{*}P \leq 0.05$; $^{**}P \leq 0.01$). (b) A representative staining performed on mature DCs is shown. DC, dendritic cell; ZA, zoledronic acid; iDC, immature DC; mDC, mature DC; PI, propidium iodide

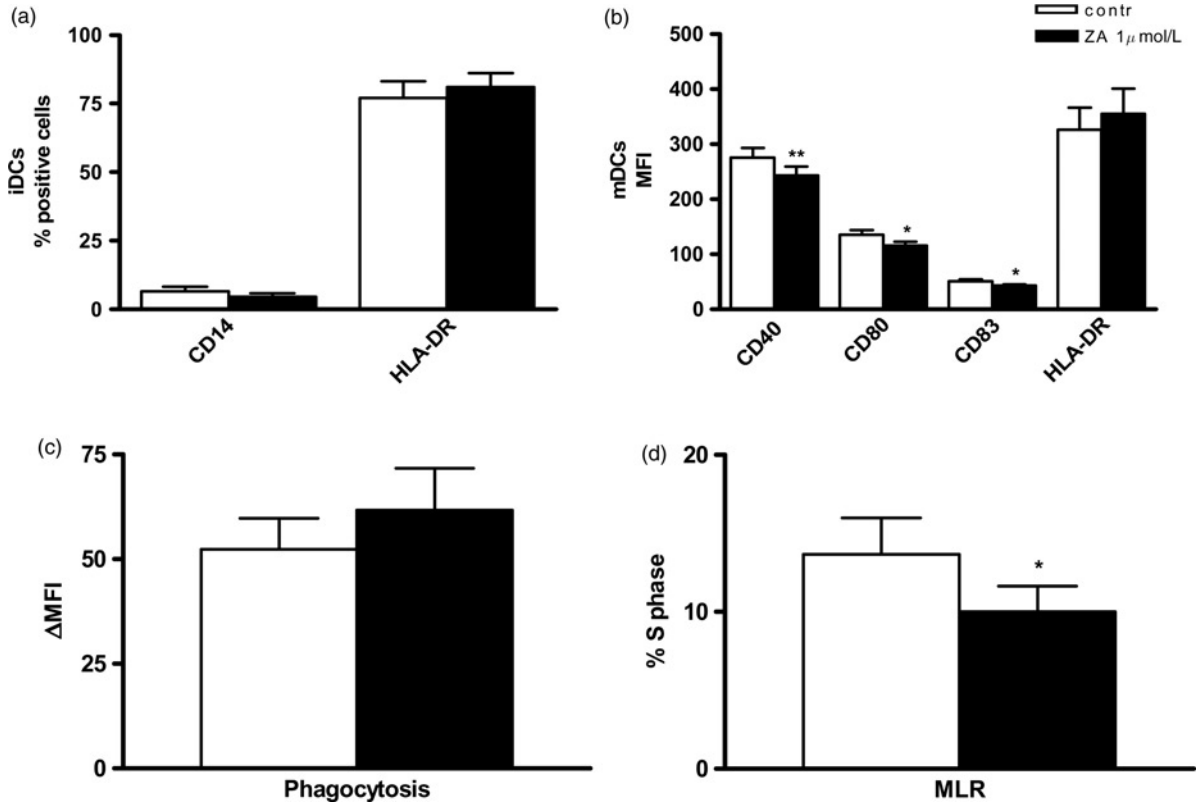

Figure 2 ZA impairs DC maturation. Phenotypic efunctional analysis were performed by flow cytometry. Percentage of positive cells was reported only when experimental groups included less than 90% of antigen-expressing cells. (a, b) The surface marker expression was evaluated on (a) iDCs and/or (b) mDCs. (c, d) Phagocytic and allo-stimulatory activites were evaluated on (c) iDCs and (d) mDCs, respectively. Data are shown as mean ± SEM of nine independent experiments ($^{*}P \leq 0.05$; $^{**}P \leq 0.01$). DC, dendritic cell; ZA, zoledronic acid; iDC, immature DC; mDC, mature DC; MFI, mean fluorescence intensity; MLR, mixed lymphocyte reaction

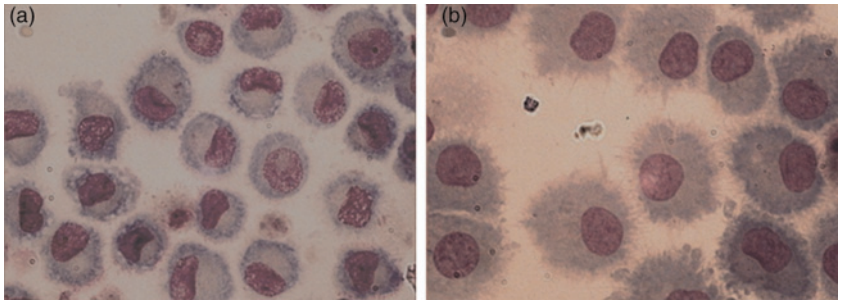

Figure 3 ZA modifies mature DC morpholgy. Morphological analysis was performed by light microscopy (magnification ×100) on (a) ZA-treated and (b) control mDCs previously stained with May–Grünwald–Giemsa. A representative experiment is shown. DC, dendritic cell; ZA, zoledronic acid; mDC, mature DC. (A color version of this figure is available in the online journal)

been widely reported that BPs inhibit tumor cell migration, proliferation and survival, through both apoptotic and cytostatic mechanisms.[16]

Interestingly, *in vitro* and *in vivo* models provided evidence that BPs exert indirect antitumor effects, by inducing antiangiogenic and immunological activities.[4,17,18] Concerning their immunological role, several studies focused on the effects of BPs on γδ T-cells.[7,19,20] Gammadelta T-cells play a pivotal role in innate and adaptive immune response, triggering DC maturation which in turn expands the numbers and function of both innate and adaptive lymphocytes.[21,22] ZA exerts its effects on γδ T-cells mainly by the selective activation and expansion of γδ T-cells via monocytes (MO).[7,11,23,24] The prevailing explanation of this effect is that BPs specifically target the mevalonate pathway of MO and induce the accumulation of phosphorylated metabolites naturally recognized by γδ T-cells.[11,25] The same mechanism is also observed in tumor cells which have been sensitized by ZA to the γδ T-cell cytotoxicity.[26,27]

The ZA γδ T-cell activating property as well as the antiosteolytic one has been shown to depend at least partially on its affinity for phagocytosing and/or APC types, such as osteoclasts and monocytes.[1,23]

As the monocytes are a major source of DCs, the aim of this study was to investigate if ZA exerts an immunoregulatory effect, by modulating DC generation and maturation.

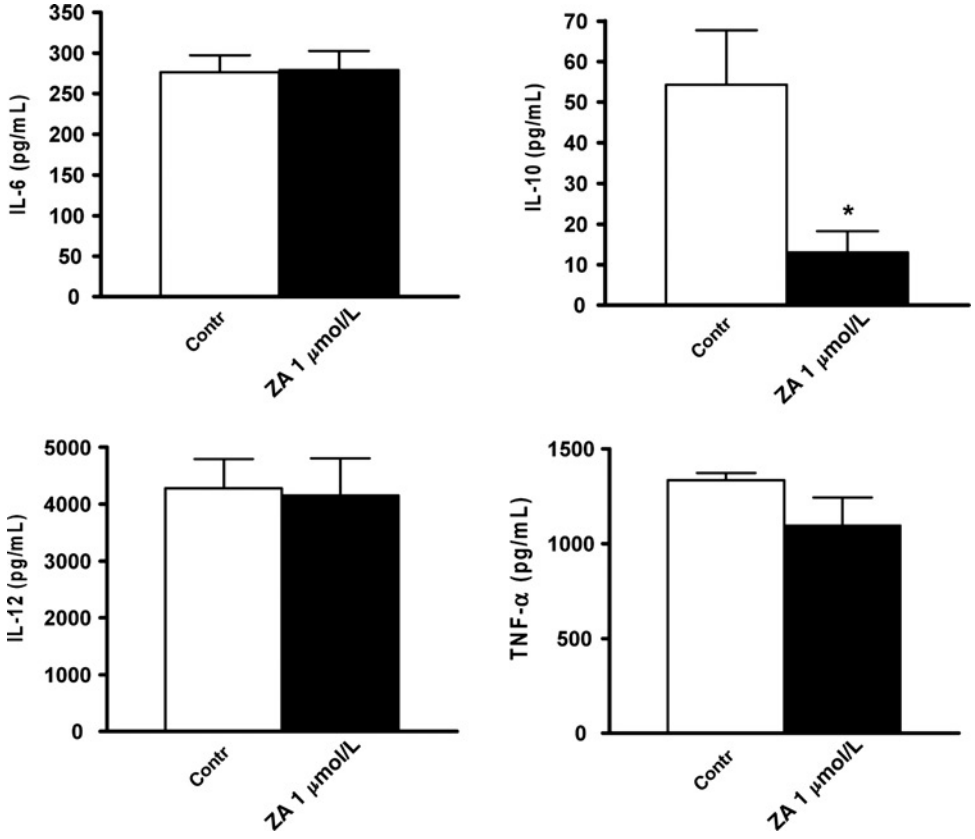

Figure 4 ZA reduces IL-10 secretion. Cytokine concentrations were measured in culture supernatants on day 7, using a commercial ELISA kit. Data are shown as mean ± SEM of nine independent experiments ($^{*}P \leq 0.05$). IL, interleukin; TNF-α, tumor necrosis factor-α; ELISA, enzyme-linked immunosorbent assay; ZA, zoledronic acid; mDCs, mature DCs

We demonstrated here that monocytes were not inhibited by ZA treatment, used at the clinical dose, in their capacity to generate DCs *in vitro* under conventional stimuli, as shown by the loss of CD14 antigen expression on the surface of ZA-treated immature DCs, according to previous reports.[2,11] Conversely, some authors observed an impairment of DC differentiation by employing experimental procedures different in drug concentration[12] or incubation times.[1]

Additionally, ZA treatment altered the maturation of iDCs, triggered by LPS; this stimulus physiologically induces up-regulation of co-stimulatory molecules, such as CD40 and CD80, as well as CD83 and HLA-DR, required for a correct cell contact between DC and T-cell and for an efficient T-cell response.[28] We observed a significant impairment of the expression of typical DC maturation markers in ZA-treated cells; accordingly, the capacity of DCs to stimulate the proliferation of T-cells in the allo-mixed lymphocyte reaction was impaired. Furthermore, the evaluation of the ZA-treated mDC morphological features, typical of DCs at a not fully mature stage, was in line with the above-described phenotypic analysis.

This resulting conclusion is in agreement with previous studies;[1,2,12] furthermore, Fiore *et al.*[11] reported a significant enhancement of the CD80 and HLA-DR expression by using a different culture protocol. The same author demonstrated the capacity of both mature and immature ZA-treated DCs to significantly expand the autologous $\gamma\delta$ T-cell population compared with untreated cells. The fact that the ZA-treated immature DC subset was higher than that of the ZA-treated mature DCs in inducing the proliferation of $\gamma\delta$ T-cells was in line with our observed capacity of ZA-monocytes to differentiate in immature DCs.

Interestingly, we showed that ZA treatment significantly impaired IL-10 release by mDCs, suggesting that it could play, through this mechanism, an immunogenic role in a tumor milieu.

In summary, our current report demonstrates that ZA displays inhibitory effects on both phenotypic and functional DC maturation patterns at the maximum plasma levels of the therapeutic dose.

Although the few studies about the effects of ZA on DC generation and maturation differ in terms of experimental design (drug concentration, time exposure, cell culture condition) and consequently in the observed phenotypic and functional DC aspects, they agree about the fact that this compound exerts its immunological action, at least in part, by modulating DC properties.

Taking an account of the crucial role of DCs in the immune response, our research contributes to the understanding of ZA mechanism of action and of its potential benefit in clinical practice.

Author contributions: Each author has reviewed the manuscript, believes it represent a valid work and approves it for submission. All authors contributed to the work presented in this paper. RC, AR and AL designed the study; GO and AF participated in the performance of the research; GO, AL and RC conducted data analysis and wrote the manuscript; and GO, AF, AL, BA, AR and RC discussed results and implications.

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