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Original Article

Zoledronate increases γδT cell proliferation through co-culturing peripheral blood mononuclear cells with autologous dendritic cells

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Abstract: Objective: Adoptive immunotherapy with γδT cells may be a promising cancer treatment because of its specificity. Zoledronate can be used in γδT cell expansion protocol in vitro. However, more effective amplification strategies should be explored. This study aims to investigate the effect of γδT cell proliferation following co-culturing peripheral blood mononuclear cells (PBMCs) with autologous dendritic cells (DCs) pretreated with zoledronate. Method: Peripheral blood derived DCs and co-culturing PBMCs were treated with 1 μmol/L zoledronate for 48 h at the fifth day of incubation at 37°C with 5% CO_2. Cell flow cytometry was performed for checking the cells ratio and content. Modulation by zoledronate of DC surface marker expression and changes in γδT cell proportions were measured by ELISA and Q-PCR. Total RNA was extracted by TRIZOL method. The isopentenylpyrophosphate (IPP) content in the supernatant of the specimen under different stimulating conditions was analyzed by high performance liquid chromatographytandem mass spectrometry (LC-MS/MS). Results: Approximately 1 μmol/L zoledronate did not induce maturation of immature DCs, as manifested by the reduced expressions of HLA-DR, CD80 and CD86, whereas specific marker CD11c remained unchanged. Following treatment of DCs and co-culturing PBMCs with zoledronate, the number of γδT cells significantly increased (from 4.99% to 65.7%, n = 3, $P < 0.05$). In comparison, γδT cells increased from 4.96% to 34.1% (n = 3, $P < 0.05$) with the treatment of PBMCs with zoledronate. Cytoplasmic granule-associated proteins perforin, granzyme B, and CD107a were enhanced correspondingly. Hence, increasing zoledronate concentration caused the increase of IPP, and the stimulating ability was abrogated by mevastatin. Conclusion: Following treatment of DCs and co-culturing PBMCs with zoledronate, γδT cell proliferation can be significantly induced. This method of generating γδT cells is eligible for γδT cell adoptive immunotherapy for lung cancer.

Keywords: Immunotherapy, zoledronate, dendritic cell, γδT cell, isopentenylpyrophosphate, lung cancer

Introduction

Lung cancer is one of the most common malignant tumors with poor prognosis and limited therapeutic options. Common treatment options for patients with advanced non-small cell lung cancer include chemotherapy, radiotherapy, targeted therapy, and immunotherapy. The use of immunotherapies designed to target tumor cells may be a promising approach in cancer treatement because of their specificity and mild side effects [1, 2]. Many previous studies have been conducted regarding this possible treatment [3, 4]. T cells bearing the T-cell receptor (TCR)-γδ represent a minor subset of human peripheral T cells (about 5%) [5], which display a non-MHC restricted lytic activity against a broad panel of tumors [6-8]. These γδT cells are active in immunosurveillance of tumors as components of innate immunity [9]. Hence, these could be one of the candidates for passive immunotherapy against tumors.

As a natural ligand of γδT cells, isopentenylpyrophosphate (IPP) produced in mammalian cells through the mevalonate pathway can stimulate γδT cell proliferation [9]. As the third generation of aminobisphosphonates (ABPs), such as zoledronate, used in the management of bone metastases, IPP inhibits the generation of intermediate products in the mevalonate synthesis pathway by suppressing the key enzyme, farnesyl pyrophosphate synthase, which leads to an accumulation of IPP [10]. Some therapeutic approaches consist of expanding γδT cells in vitro from the peripheral blood mononuclear

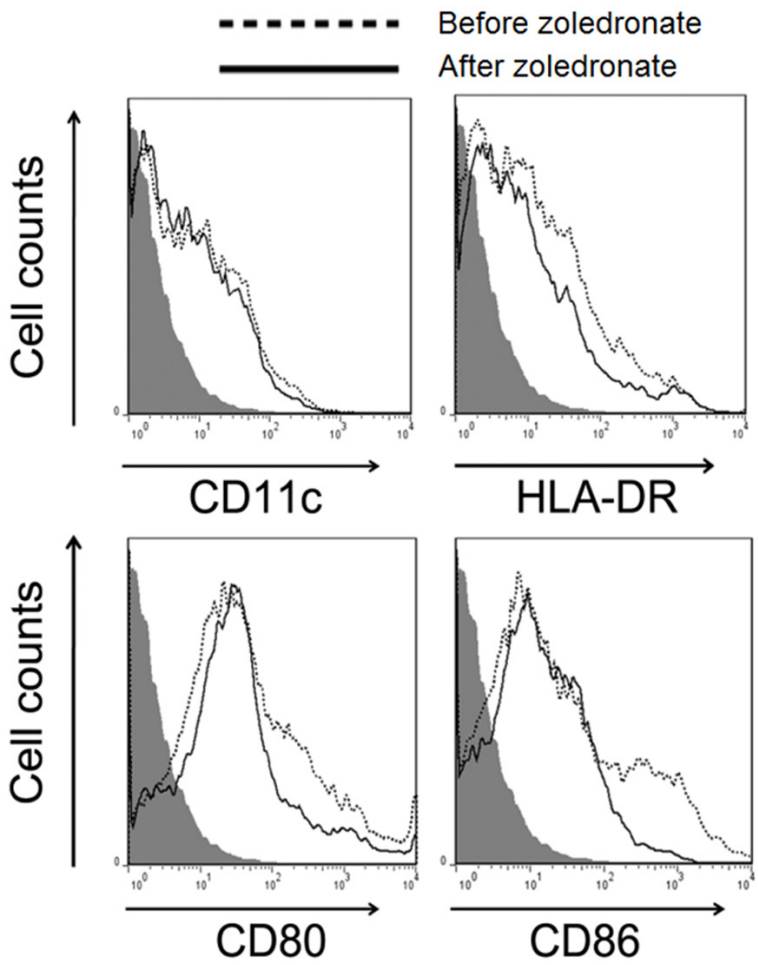

Figure 1. Phenotypic changes of DCs before and after zoledronate stimulation.

cells (PBMCs) of patients using ABP or a synthetic analog of IPP (i.e., bromohydrin pyrophosphate) to infuse a large number of highly cytolytic γδT cells into the patient. However, some of the trials revealed that this strategy was hindered by the unsuccessful γδT cell amplification that occurred in about 50% of cancer patients [11, 12]. Thus, a new design of expansion protocol is needed for the development of γδT cell amplification.

Dendritic cells (DCs) are the most capable antigen-presenting cells currently known in the body, which can recognize, capture, process and present tumor antigens, as well as initiate immune responses. They can stimulate initial T cell proliferation and activate the killing effect of toxic T cells. Reciprocal influences have been elaborated between DCs and γδT cells [13, 14]. Hence, mimicking this interaction in vitro in a co-culture model could be useful to improve γδT cell amplification.

In the present study, we observed the proliferation of γδT cells after stimulating DCs and co-culturing PBMCs with zoledronate. This study was approved by the Ethical Committee of Shanghai Chest Hospital, and written informed consent was obtained each volunteer.

Materials and methods

Preparation of DCs

PBMCs collected from 100 mL of venous blood samples of healthy volunteers from Shanghai Chest Hospital were enriched by density gradient centrifugation with Ficoll-Paque, resuspended in X-VIVO with 1% autologous heat-inactivated serum, plated at a concentration of 5×10^6 cells/ml, and allowed to adhere to 10 cm^2 dishes. After 4 hours, the adherent cells were cultured in X-VIVO supplemented with 1% heat-inactivated autologous serum in the presence of 1000 U/mL granulocyte macrophage colony-stimulating factor (Leukomax; Novartis International AG, Basel,Switzerland), as well as 500 units/mL IL-4 (Strathmann Biotec AG, Hannover, Germany) at 37°C for 7 days.

Examining the surface molecules of DCs and related cytokines

The supernatant was collected from the DCs on Day 5 and treated with 1 μmol/L of zoledronate (Novartis, Basel, Switzerland) for 48 hours to examine the expression of related cytokines and surface molecules. The antibody for human TCR-γδ was from Immutech France; antibodies for CD11c, CD80 and CD86 were supported by the USA company Fitzgerald; HLA-DR antibody came from Abcam England.

γδT cell culture, phenotype identification and A549 cell activity

The cultured DCs on Day 5 were collected after being treated with 1 μmol/L zoledronate for 48

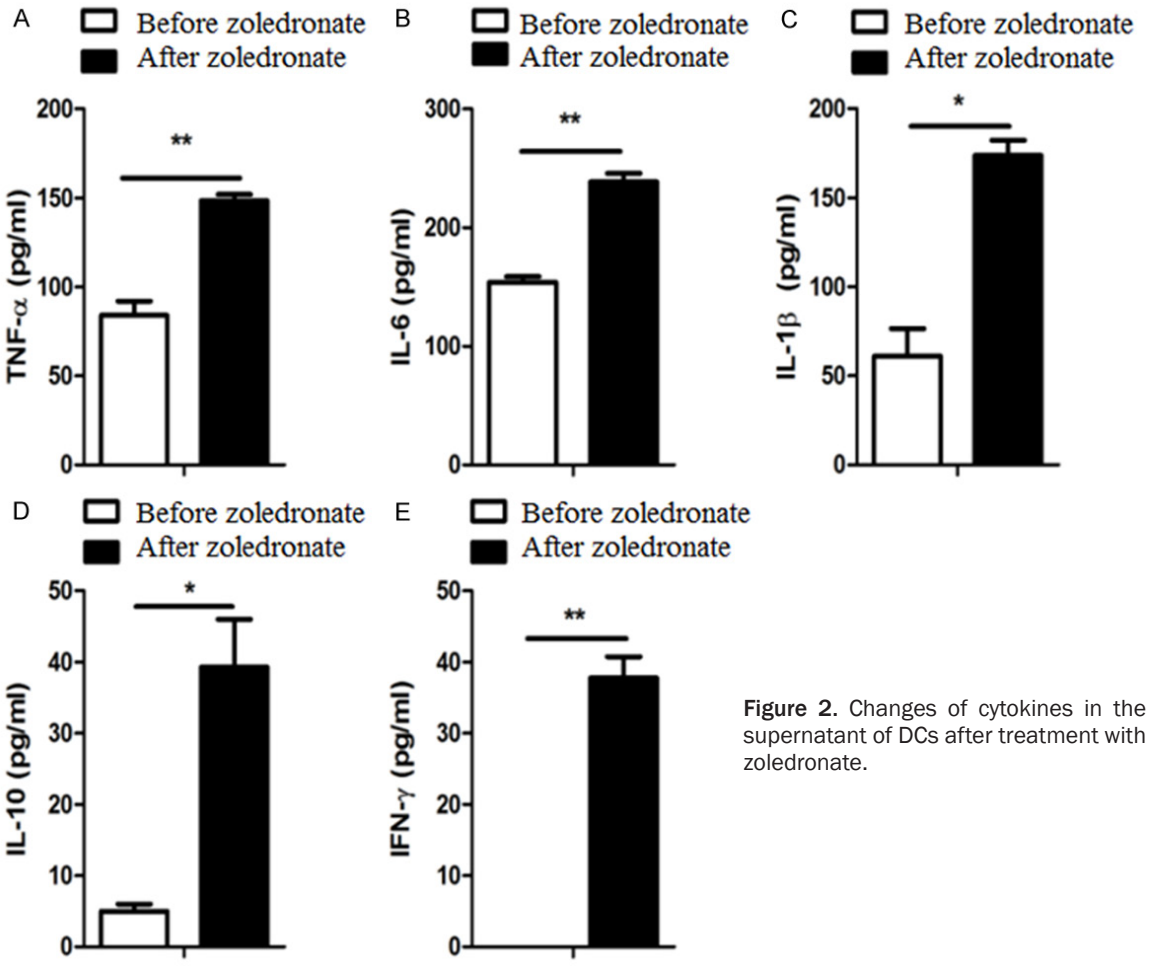

Figure 2. Changes of cytokines in the supernatant of DCs after treatment with zoledronate.

hours and rinsed with PBS once. Pretreated DCs were incubated with autologous PBMCs in a 1:20 ratio and then coated on cell culture dishes containing RPMI1640 (Gibco) culture medium (IL-2 400 U/ mL, 10% bovine serum). The dishes were cultured in 5% CO_2 cell incubator at 37°C; half culture medium was changed every 2 days, and the cells were harvested on Day 8 to examine γδT cell proliferation.

Next, γδT cells were then rinsed with staining PBS once and stained with FITC-Anti-human gamma delta TCR antibody (Affymetrix) and PE-anti-human CD3 antibody (Affymetrix). After being kept away from light, the cells were examined on Calibur flow cytometry as the control. A batch of γδT cells was cultured with the same method and treated with 1 μmol/L zoledronate for 48 hours as the experimental group.

Finally, γδT cells and A549 cells (bnon-small cell lung cancer cell lines) were blended (E/T 10:1 and 20:1, respectively), and about 10 uL of CCK8 solution was added after 48 hours of culture. A549 Cell activity was examined at 0.5, 1, and 2 hour time points to investigate the tumor cell killing potency of γδT cells.

Isopentenylpyrophosphate examination

IPP contents in supernatant treated with different concentrations of zoledronate were examined with LC-MS/MS. Welch Ultimate® XB-C18 was used as matrix and sample was performed at 0.2 mL/min.

Results

The expression of surface markers on immature DCs after zoledronate treatment

Approximately 1 μmol/L zoledronate stimulation had no effect on DC specific marker CD11c (P = 1.541), suggesting that zoledronate cannot promote its maturation (**Figure 1**). Compared with the control group, the propor-

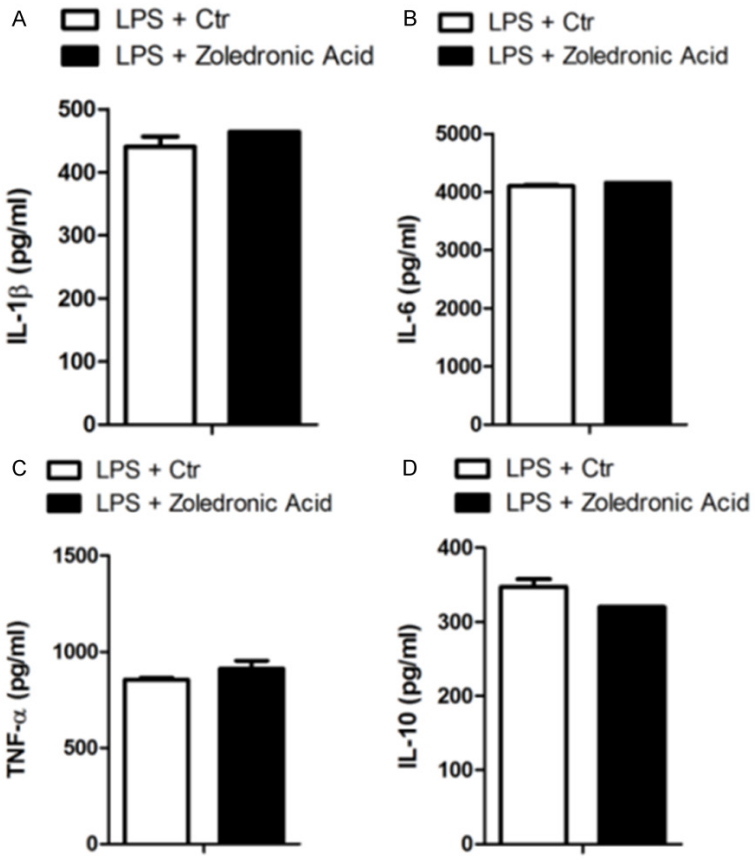

Figure 3. Cytokine levels of mature DCs after treatment with zoledronate for 48 hours (from immature DCs).

tions of the HLA-DR, CD80, and CD86 groups slightly reduced with no statistical differences ($P > 0.05$ in all).

Changes of cytokines in supernatant of immature DCs after being treated with zoledronate

After the 1 μmol/L zoledronate stimulation, the expression levels of various inflammatory factors, such as TNF-α, IL-6, IL-1β and IL-10, increased (**Figure 2**). Moreover, IFN-γ expression also increased. Significant difference ($P < 0.05$) was observed in comparison with each control.

Changes of cytokine levels of mature DCs after being treated with zoledronate

After stimulating with LPS (Sigma, Saint Louis, USA) for 6 hours, DCs were treated with zoledronate for 48 hours to examine whether zoledronate can affect the expressions of cytokines of mature DCs. The lack of effect on either various inflammatory factors by zoledronate pretreatment suggested that zoledronate did not affect the cytokine expression of mature DCs (**Figure 3**). No statistical differences were observed between IL-1β, IL-6, TNF-α, and IL-10 compared with the control group ($P = 1.276$, 0.926, 0.476 and 0.728, respectively).

γδT cells and related killing gene detection

Higher proportions of γδT cells were induced after treating immature DCs with zoledronate and co-culturing PBMCs. γδT cell proportion was 4.99% for the non-stimulated group, which increased to 65.7% after stimulation ($n = 3$, $P < 0.05$) (**Figure 4**).

When treating PBMCs with zoledronate, the stimulated group can also induce higher proportion of γδT cells. The proportion of the non-stimulated group of γδT cells was 4.96%, which increased to 34.1% after stimulation ($n = 3$, $P < 0.05$) (**Figure 5**).

Enhanced corresponding killing activities in γδT cells

After treating DCs and PBMCs with zoledronate, the corresponding killing activities of γδT cells also increased significantly ($n = 3$, $P < 0.01$) (**Figure 6A**). In addition, γδT cells induced by stimulating PBMCs with zoledronate also showed significant increase in killing activities compared with the control ($n = 3$, $P < 0.01$) (**Figure 6B**).

Enhanced tumor cell-killing capability of γδT cells

As seen in **Figure 7**, γδT cells induced by stimulating imDCs/PBMCs with zoledronate had significant killing activities related to A549 cells compared with the control group. The effector-target ratio 20:1 (below) had better killing effect than that of 10:1 (above), and the results

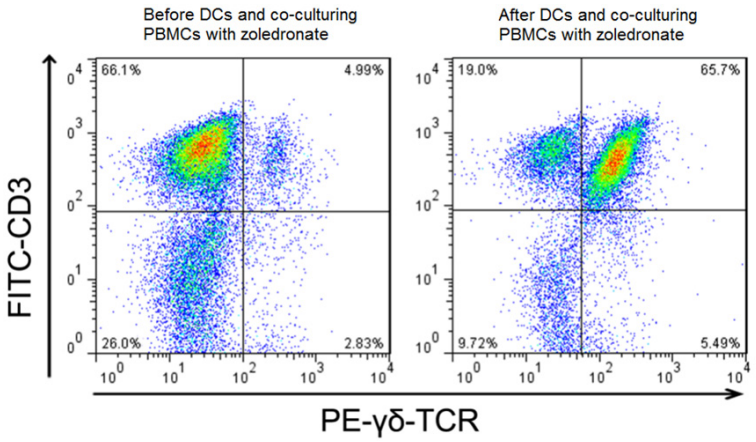

Figure 4. Higher proportion of γδT cells after treatment of DCs and co-culturing PBMCs with zoledronate.

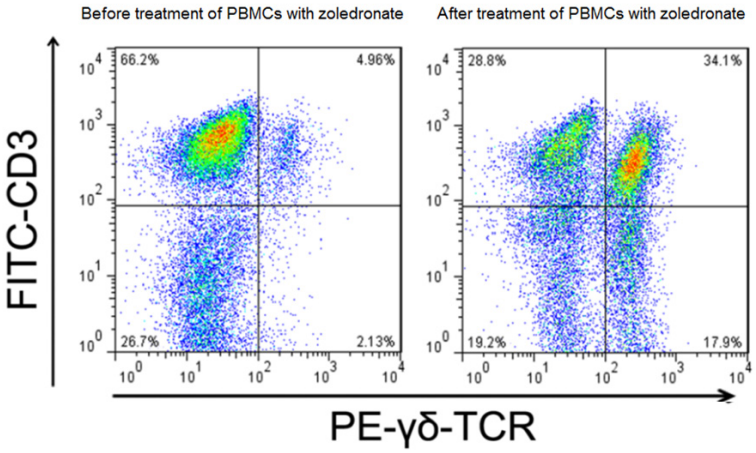

Figure 5. Increased γδT cells after treatment of PBMCs with zoledronate.

remained stable 2 hours later. Significant difference ($n = 3$, $P < 0.05$) was observed in both groups.

Increase in isopentenylpyrophosphate content through the stimulation of zoledronate

The experiment examined the IPP concentrations in the supernatant of specimens stimulated by different methods with LC-MS/MS as shown in **Table 1**. The results revealed that IPP was undetectable in the hemocyte supernatant or in the supernatant of imDCs without the stimulation of zoledronate. The IPP contents determined in the supernatant of peripheral blood cells stimulated with 1, 5, and 30 μmol/L zoledronate for 48 hours were 0.634, 0.385 and 1.728 μg/mL, respectively, whereas those for imDCs were 0.667, 0.578, and 1.740 μg/mL, respectively. Of note, zoledronate-pretreated imDCs displayed high IPP level, indicating that a mevalonate pathway metabolite is likely involved in the activation. Hence, the IPP content induced by stimulating imDCs with different concentrations of zoledronate decreased significantly ($n = 3$, $P < 0.05$) upon the addition of mevastatin, an inhibitor of the mevalonate pathway.

Discussion

Our results revealed that zoledronate-an effective aminobisphosphonate against pathological bone loss-modulated the maturation of human monocyte-derived dendritic cells and induced imDCs to generate antineoplastic effect. Zoledronate induced distinct γδT cell expansion in primary PBMC cultures of healthy donors at clinically relevant concentrations with co-culturing imDCs. Zoledronate-pretreated imDCs displayed high IPP levels, indicating that mevalonate pathway metabolite was likely involved in the activation. Aminobisphosphonates revealed a lower stimulatory capacity of γδT cells than the already described potent natural antigen (IPP).

γδT cells belong to a distinct T cell lineage; thus, they can recognize self ligands, such as MICA/MICB molecules or complexes comprising ATP-synthase subunits, which are induced or upregulated on the surface of some tumor cells; γδT cells also represent a non-MHC restricted lytic activity and play a pivotal role in innate immune responses against tumor cells [15-17]. These abilities are the bases of the putative role of γδT cells in tumor immunosurveillance.

A considerable number studies have found that ABPs could induce the amplification of γδT cells via PBMCs [18-20]. However, based on a num-

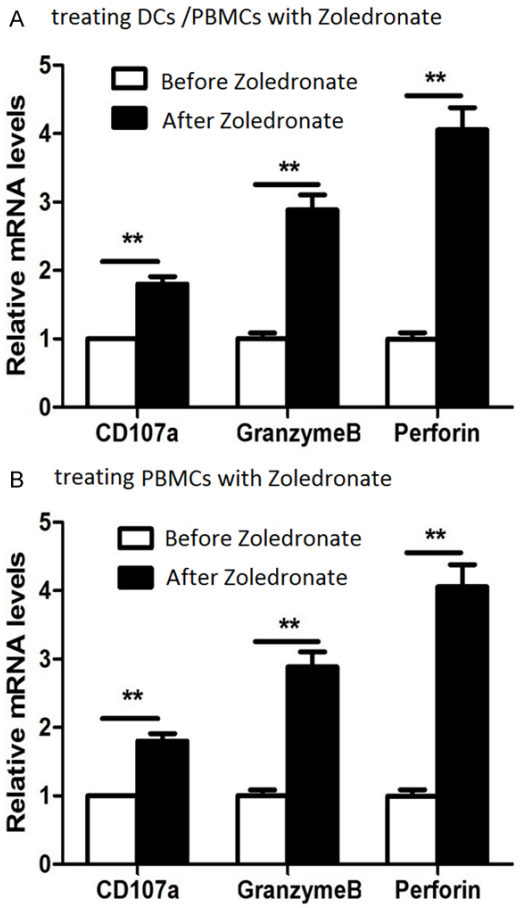

Figure 6. Expression of γδT cell's CD107a, cellular granzyme B, and perforin after treating imDCs/PBMCs (A) and PBMCs (B) with Zoledronate, respectively.

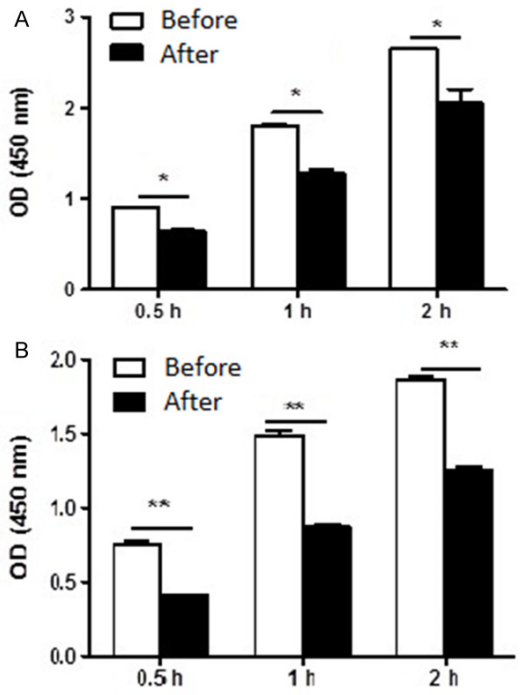

Figure 7. Decrease of A549 cells related to γδT cells induced by stimulating imDCs/PBMCs with zoledronate (E:T 10:1, A) and zoledronate (E:T 20:1, B).

ber of studies that reported a reciprocal stimulation between γδT cells and DCs [21, 22] most of the experiments were performed with PBMCs treated with ABPs during the amplification, not with freshly generated imDCs as in the current study. Among ABPs, zoledronate is approximately 100-fold more potent than pamidronate in blocking the farnesyl pyrophosphate synthase [23]. Hence, we performed the study to observe the proliferation of γδT cells after stimulating imDCs and co-culturing PBMCs with zoledronate. The maximal rate of γδT purity is expected.

The current experiment revealed that zoledronate cannot induce imDC maturation, as shown by the very similar phenotypic characterization of imDCs among mDCs. The expression of various inflammatory factors, such as TNF-α, IL-6, IL-1β, IL-10 and IFN-γ, generated in imDCs after zoledronate stimulation all increased significantly ($P < 0.05$), suggesting that zoledronate can induce imDCs to generate the antineoplastic effect, and the stronger immunostimulatory ability is associated with a successful expansion of γδT cells. However, we also observed that zoledronate cannot stimulate mDCs to generate the antineoplastic effect.

Our findings revealed that co-culture of PBMCs with zoledronate-pretreated imDCs induced a strong expansion of γδT cells than stimulating PBMCs only with zoledronate, suggesting that zoledronate mainly induced the organism to increase γδT cells specific to the treatment of imDCs, associated with high IPP level. Meanwhile, the mRNA expression levels of the γδT cell characterization killing activity gene perforin, granzyme, and CD107a all significantly increased ($n = 3$, $P < 0.01$), with killing effect to lung cancer cell line also increased to an extent ($n = 3$, $P < 0.05$). However, whether this will benefit cancer immunotherapy is not clear. The improvement of the new therapy need to be checked in the future study *in vivo.*

Furthermore, the inhibitive effect of zoledronate on farnesyl pyrophosphate synthase gradually intensified with the increase of zoledro-

Table 1. Determination results of samples

Sample	IPP concentration (μg/mL)
Culture medium	UD
Blood 0	UD
Blood 1	0.634
Blood 5	0.785
Blood 30	1.728
DC 0	UD
DC 1	0.667
DC 5	0.858
DC 30	1.740
DC 1-M1	0.348
DC 5-M1	0.493
DC 30-M1	0.416
DC 1-M5	0.308
DC 5-M5	0.441
DC 30-M5	0.393

Blood X: Peripheral blood treated with x μmol/L zoledronate, DC X: Dendritic cells treated with x μmol/L zoledronate, DC x1-M x2: Dendritic cells treated with x1 μmol/L zoledronate and x2 μmol/L mevastatin.

nate concentration, which led to high IPP levels. Moreover, the IPP concentration induced by zoledronate-treated imDCs is abrogated by mevastatin, indicating that the DC-mevalonate pathway is involved in γδT cell activation. The addition of HMG-CoA reductase in the form of mevastatin inhibited the formation of mevalonate. This phenomenon affected the formation of IPP in the second stage of cholesterol synthesis and had a significant inhibitive effect on the generation of IPP.

ImDCs pretreated with zoledronate induced the proliferative expansion of γδT cells efficiently. Here, zoledronate played the main role because following the stimulation of imDCs with zoledronate, the number of γδT cells significantly increased, and the immunostimulatory ability of zolodronate-pretreated imDCs showed as IPP was abrogated by mevalonate. Unlike the zoledronate/IL-2 stimulation, our study indicates that the imDC is the main source of IPP and is responsible for the recruit of the γδT cells via IPP.

One limitation of this study is that the venous blood samples were gathered from healthy volunteers. And some important factors, which may involve in zoledronate γδT cells stimulation, are not checked in this study. For example, H250 Vitamin D and Butyrophilin, which according to the recent studies, play important roles in zoledronate stimulation of γδT cells proliferation. In our future study we will check these factores and test this treatment with sample from lung cancer patients. What's more, many studies showed that zoledronate may cause metastatic bone disease and increased bone loss (cite R. Rizzoli, J.-J. Body, M.-L. Brandi et al., "Cancer-associated bone disease," Osteoporosis International; Lee CY1, Suzuki JB. Medication-related osteonecrosis of the jaws from once per year intravenous zoledronic acid (Reclast): report of 4 cases.), which should be concerned in the futhre study and practical usage.

In conclusion, a combination of imDCs and co-culturing PBMCs pretreated with zoledronate induced γδT cell proliferation significantly. This expansion protocol is an eligible approach for γδT cell adoptive immunotherapy in lung cancer patients.

Acknowledgements

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Disclosure of conflict of interest

None.

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References

[1] Harshman LC, Drake CG, Wargo JA, Sharma P, Bhardwaj N. Cancer Immunotherapy Highlights from the 2014 ASCO Meeting. Cancer Immunol Res 2014; 2: 714-9.

[2] Kakimi K, Nakajima J, Wada H. Active specific immunotherapy and cell-transfer therapy for the treatment of non-small cell lung cancer. Lung cancer 2009; 65: 1-8.

[3] Reck M. What future opportunities may immuno-oncology provide for improving the treat-

ment of patients with lung cancer? Ann Oncol 2012; 23 Suppl 8: viii28-34.
[4] Brahmer JR. Harnessing the immune system for the treatment of non-small-cell lung cancer. J Clin Oncol 2013; 31: 1021-8.
[5] Allison TJ, Winter CC, Fournie JJ, Bonneville M, Garboczi DN. Structure of a human gammadelta T-cell antigen receptor. Nature 2001; 411: 820-4.
[6] Bouet-Toussaint F, Cabillic F, Toutirais O, Le Gallo M, Thomas de la Pintiere C, Daniel P, Genetet N, Meunier B, Dupont-Bierre E, Boudjema K, Catros V. Vgamma9Vdelta2 T cell-mediated recognition of human solid tumors. Potential for immunotherapy of hepatocellular and colorectal carcinomas. Cancer Immunol Immunother 2008; 57: 531-9.
[7] Corvaisier M, Moreau-Aubry A, Diez E, Bennouna J, Mosnier JF, Scotet E, Bonneville M, Jotereau F. V gamma 9V delta 2 T cell response to colon carcinoma cells. J Immunol 2005; 175: 5481-8.
[8] Viey E, Fromont G, Escudier B, Morel Y, Da Rocha S, Chouaib S, Caignard A. Phosphostim-activated gamma delta T cells kill autologous metastatic renal cell carcinoma. J Immunol 2005; 174: 1338-47.
[9] Bonneville M, Scotet E. Human Vgamma9-Vdelta2 T cells: promising new leads for immunotherapy of infections and tumors. Curr Opin Immunol 2006; 18: 539-46.
[10] Gober HJ, Kistowska M, Angman L, Jeno P, Mori L, De Libero G. Human T cell receptor gammadelta cells recognize endogenous mevalonate metabolites in tumor cells. J Exp Med 2003; 197: 163-8.
[11] Bennouna J, Bompas E, Neidhardt EM, Rolland F, Philip I, Galea C, Salot S, Saiagh S, Audrain M, Rimbert M, Lafaye-de Micheaux S, Tiollier J, Négrier S. Phase-I study of Innacell gammadelta, an autologous cell-therapy product highly enriched in gamma9delta2 T lymphocytes, in combination with IL-2, in patients with metastatic renal cell carcinoma. Cancer Immunol Immunother 2008; 57:1599-609.
[12] Kobayashi H, Tanaka Y, Yagi J, Osaka Y, Nakazawa H, Uchiyama T, Minato N, Toma H. Safety profile and anti-tumor effects of adoptive immunotherapy using gamma-delta T cells against advanced renal cell carcinoma: a pilot study. Cancer Immunol Immunother 2007; 56: 469-76.
[13] Marcu-Malina V, Heijhuurs S, van Buuren M, Hartkamp L, Strand S, Sebestyen Z, Scholten K, Martens A, Kuball J. Redirecting alphabeta T cells against cancer cells by transfer of a broadly tumor-reactive gammadeltaT-cell receptor. Blood 2011; 118: 50-9.
[14] Rincon-Orozco B, Kunzmann V, Wrobel P, Kabelitz D, Steinle A, Herrmann T. Activation of V gamma 9V delta 2 T cells by NKG2D. J Immunol 2005; 175: 2144-51.
[15] Kabelitz D, Wesch D, Pitters E, Zoller M. Potential of human gammadelta T lymphocytes for immunotherapy of cancer. Int J Cancer 2004; 112: 727-32.
[16] Smyth MJ, Godfrey DI, Trapani JA. A fresh look at tumor immunosurveillance and immunotherapy. Nat Immunol 2001; 2: 293-9.
[17] Groh V, Rhinehart R, Secrist H, Bauer S, Grabstein KH, Spies T. Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. Proc Natl Acad Sci U S A 1999; 96: 6879-84.
[18] Abe Y, Muto M, Nieda M, Nakagawa Y, Nicol A, Kaneko T, Goto S, Yokokawa K, Suzuki K. Clinical and immunological evaluation of zoledronate-activated Vgamma9gammadelta T-cell-based immunotherapy for patients with multiple myeloma. Exp Hematol 2009; 37: 956-68.
[19] Noguchi A, Kaneko T, Kamigaki T, Fujimoto K, Ozawa M, Saito M, Ariyoshi N, Goto S. Zoledronate-activated Vgamma9gammadelta T cell-based immunotherapy is feasible and restores the impairment of gammadelta T cells in patients with solid tumors. Cytotherapy 2011; 13: 92-7.
[20] Roelofs AJ, Jauhiainen M, Monkkonen H, Rogers MJ, Monkkonen J, Thompson K. Peripheral blood monocytes are responsible for gammadelta T cell activation induced by zoledronic acid through accumulation of IPP/DMAPP. Br J Haematol 2009; 144: 245-50.
[21] 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.
[22] Devilder MC, Maillet S, Bouyge-Moreau I, Donnadieu E, Bonneville M, Scotet E. Potentiation of antigen-stimulated V gamma 9V delta 2 T cell cytokine production by immature dendritic cells (DC) and reciprocal effect on DC maturation. J Immunol 2006; 176: 1386-93.
[23] Dunford JE, Thompson K, Coxon FP, Luckman SP, Hahn FM, Poulter CD, Ebetino FH, Rogers MJ. Structure-activity relationships for inhibition of farnesyl diphosphate synthase in vitro and inhibition of bone resorption in vivo by nitrogen-containing bisphosphonates. J Pharmacol Exp Ther 2001; 296: 235-42.