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Zoledronic acid sensitizes rhabdomyosarcoma cells to cytolysis mediated by human γδ T cells

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Abstract. Rhabdomyosarcoma (RMS) is the most common type of soft-tissue sarcoma in children. Immunotherapy has been proposed as a treatment for this deadly tumor. In the present study, the cytotoxicity of *ex vivo* expanded γδ T cells on RMS cell lines was evaluated and the molecular interactions involved were investigated. γδ T cells were expanded *in vitro* using peripheral blood mononuclear cells from 5 healthy donors and were stimulated with zoledronic acid (Zol) and interleukin 2. RMS cell lines RD and A-673 were used as target cells. The cytotoxicity of the γδ T cells against RMS was assessed *in vitro* and *in vivo*. γδ T cells were cytotoxic to RMS cells. Importantly, Zol markedly increased their cytotoxic potential. RMS cells treated with Zol-stimulated γδ T cells to produce interferon γ. γδ T cell-mediated cytotoxicity was primarily through the T cell receptor-dependent signaling pathway in blocking studies. Transfer of γδ T cells together with Zol into nude mice induced the regression of RD tumor xenotransplants. The results of the present study provide the rationale for the clinical evaluation of γδ T cells in RMS.

Introduction

Rhabdomyosarcoma (RMS) is the most common type of soft tissue sarcoma in children. However, overall survival times for patients with RMS have remained unchanged since the 1970s (1). Despite the use of systemic chemotherapy, metastatic or unresectable RMS remains an unmet clinical challenge. The cure rate of this disease is particularly poor for those patients, with a 3-year event-free survival rate of only 27% (2).

Immunotherapy has been proposed as a treatment for this RMS (3,4). Adoptive T-cell therapy utilizing cytotoxic T lymphocytes (CTLs) directed against tumor-associated antigens represents a promising immunotherapy approach. A major limitation, however, is that CTLs recognize and kill tumor cells in a major histocompatibility complex (MHC)-restricted manner, but almost half of the bone and soft tissue sarcoma cases have developed to evade immune recognition by decreasing MHC expression (5). To this end, alternative approaches using MHC-independent immune effectors may circumvent this problem and allow for more universal application.

γδ T cells express T cell receptors (TCRs) composed of γ and δ chains (6). Unlike tumor antigen-specific αβ T cells, identification of tumor target antigens is not required for γδ T cells. A previous study focused primarily on peripheral Vδ2-positive γδ T cells (Vδ2 T cells) with potential antitumor reactivity (7). This subset typically accounts for between 50 and 95% of the total γδ T cells in peripheral blood, and contributes to the cytotoxic response against a broad range of tumors. Vδ2 T cells recognize isopentenyl pyrophosphate (IPP) as phosphoantigens without MHC-restriction (8,9). In tumor cells, the mevalonate signaling pathway is frequently dysregulated, leading to the upregulation of an intermediate IPP. Cumulative evidence indicates that γδ T cells are capable of lysis of a broad range of tumor cells, including ovarian, breast, renal cell cancer, glioblastoma and other solid tumors (6). Most noteworthy, nitrogen-containing bisphosphonates (N-BPs), including zoledronic acid (Zol), sensitize tumor cells to the Vδ2 T cell cytotoxicity (10,11). Recently, we also characterized the cytotoxicity of γδ T cells against osteosarcoma and chondrosarcoma cells in a preclinical setting (12,13).

The known potential of γδ T cells in anticancer surveillance suggests their possible role in cellular immunotherapy. Although the effectiveness of γδ T cells is increasingly well-described, further studies are required in the area of sarcoma in particular. In the present study, the antitumor activity of γδ T cells against RMS cells was demonstrated for the first time, to the best of our knowledge. Furthermore, it was demonstrated that Zol enhances this cytolytic effect mediated by human γδ T cells. The potential underlying molecular mechanism of

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Abbreviations: Zol, zoledronic acid; mAb, monoclonal antibody; IPP, isopentenyl pyrophosphate; E:T ratio, effector/target ratio; N-BP, nitrogen-containing bisphosphonate

Key words: γδ T cells, zoledronic acid, rhabdomyosarcoma, cytotoxicity, immunotherapy

the interaction between γδ T cells and Zol-treated RMS cells is also discussed.

Materials and methods

Cell lines. The RMS cell lines RD and A-673 were purchased from the Cell Collection of Chinese Academy of Science (Shanghai, China). The firefly luciferase-expressing RD cell line RD-LUC was purchased from Invabio Biotechnology, Ltd. (Shanghai, China). RMS cells were cultured in complete Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin and 1% L-glutamine (all from Invitrogen; Thermo Fisher Scientific, Inc.).

Ex vivo expansion and phenotype of γδ T cells. γδ T cells were expanded from peripheral blood collected from healthy donors (n=5). Informed written consent was obtained from all donors, and the research was approved by the Human Research Ethics Committees of the Second Affiliated Hospital, College of Medicine, Zhejiang University (Hangzhou, China). Peripheral blood mononuclear cells (PBMCs) were separated from peripheral blood by density gradient centrifugation (Cedarlane Laboratories, Burlington, ON, Canada) and seeded on 24-well culture plates at a concentration of $1.5x10^6$ cells/ml in RPMI-1640 medium (Gibco), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin and 1% L-glutamine. Zol (Zometa®; Novartis International AG, Basel, Switzerland) at 1 μM and 400 IU/ml interleukin 2 (IL-2; PeproTech, Inc., Rocky Hill, NJ, USA) were added on day 0. After 3 days, half of the culture medium was replaced with fresh medium containing 400 IU/ml IL-2. Fresh medium and IL-2 (400 U/ml) were added every 3 days during culture. At day 14, γδ T cells were purified by negative magnetic-activated cell sorting isolation (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The phenotype of γδ T cells was analyzed using standard flow cytometric assays. Briefly, the cells were stained with the indicated fluorescently labeled antibodies for 30 min at 4˚C, washed, and analyzed by flow cytometry according to the manufacturer's instructions. The following monoclonal antibodies (mAbs) were obtained from BioLegend (San Diego, CA, USA): anti-Vδ2-fluorescein isothiocyanate (FITC) (cat. no. 331406; clone B6), anti-cluster of differentiation (CD) 3-FITC (cat. no. 344804; clone SK7), anti-interferon γ (IFN-γ)-FITC (cat. no. 506504; clone B27), anti-CD69-FITC (cat. no. 310904; clone FN50) and anti-TCR-γ/δ-phycoerythrin (cat. no. 331210; clone B1). Flow cytometry was performed using a FACS CantoII instrument (BD Biosciences, San Jose, CA, USA) and the data were analyzed using FlowJo software (version 9.3.2; Tree Star, Inc., Ashland, OR, USA).

Intracellular staining of IFN-γ. γδ T cells were co-cultured with tumor cells for 4 h at 37˚C in the presence of 20 μg/ml brefeldin A (BD Biosciences). γδ T cells stimulated with phorbol-12-myristate-13-acetate (PMA; 2.5 mg/ml; Sigma-Aldrich; Merck KGaA) and ionomycin (0.5 mg/ml; Sigma-Aldrich; Merck KGaA) for 2 h was used as positive control. Cells were re-suspended in PBS with 1% FBS (Gibco; Thermo Fisher Scientific, Inc.) and stained with specific monoclonal antibody TCR-γ/δ for 30 min in the dark at 4˚C. Following surface staining of TCR-γ/δ, cells were fixed and permeabilized using Cytofix/Cytoperm buffer (BD Biosciences). γδ T cells were washed with Perm/Wash buffer (BD Biosciences) and stained with FITC-labeled anti-human IFN-γ mAb for 30 min in the dark at 4˚C.

Cytotoxicity assays. The cytotoxicity γδ T cells was determined using a CellTiter 96 cytotoxicity MTS assay (Promega Corporation, Madison, WI, USA) as described previously (12). Briefly, $5x10^3$ tumor cells were seeded in 96-well flat-bottomed plates in triplicate. In certain experiments, Zol was used to sensitize tumor cells for 24 h after cell attachment. Subsequently, γδ T cells were added at the indicated effector/target (E:T) ratio and co-cultured with tumor cells for 4 h at 37˚C. The plates were washed gently three times, and the residual viable tumor cells were quantified using the MTS assay according to the manufacturer's protocol. In blocking studies, γδ T cells were incubated with 10 μg/ml (saturating concentrations) anti-human natural killer group 2, member D (NKG2D; clone 149810; R&D Systems, Inc., Minneapolis, MN, USA), anti-pan-γδ TCR (clone B1; BD Biosciences), anti-tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (clone RIK-2; BD Biosciences) or anti-human Fas ligand (FasL; clone NOK-2; BD Biosciences) for 30 min before co-culture to block the relevant cytotoxic pathways. All experiments were performed in triplicate. Cytotoxicity at each E:T ratio was calculated according to the following formula: Cytotoxicity (%) = 100 - 100x (optical density at 490 nm for co-culture well/optical density at 490 nm for target cell well).

ELISA. RMS cells ($1x10^4$) were co-cultured with $2x10^5$ γδ T cells in triplicate in 96-well flat-bottomed plates for 4 h. Supernatants were harvested and assayed for IFN-γ using a Human IFN-γ ELISA kit (Dakewe Biotech Co., Ltd., Shenzhen, China), according to the manufacturer's protocol.

Adoptive immunotherapy with human γδ T cells. Female 4-week-old athymic nude mice weighing ~20 g were purchased from the Experimental Animal Center of the Zhejiang Traditional Medical University (Hangzhou, China), and were housed under specific pathogen-free conditions at 25˚C in an atmosphere with 50% humidity and at 12/12 h light/dark cycle with free access to food and water. RD-LUC tumor cells ($5x10^6$) were implanted subcutaneously (s.c.) into the upper right flank of mice under anesthesia. Mice were randomized into four groups (6 mice/group) 7 days after tumor implantation: i) Control mice, treated with PBS; ii) mice treated with intraperitoneal (i.p.) injections of Zol (50 μg/kg); iii) mice treated with intravenous (i.v.) injections of γδ T cells through the tail vein ($5x10^6$ cells/mouse in 100 μl serum-free culture medium); and iv) mice treated with Zol followed by γδ T cells 1 day later. All treatments were performed once a week for 4 weeks. The survival and general status of mice was monitored daily. Tumor bioluminescence was observed using an IVIS Lumina Series III Imaging platform (PerkinElmer, Inc., Waltham, MA, USA) as described previously (13). Tumor size was measured and calculated according to the formula: Volume = 1/2 x length x $width^2$. All animal procedures and protocols followed the guidelines of the Institutional Authority

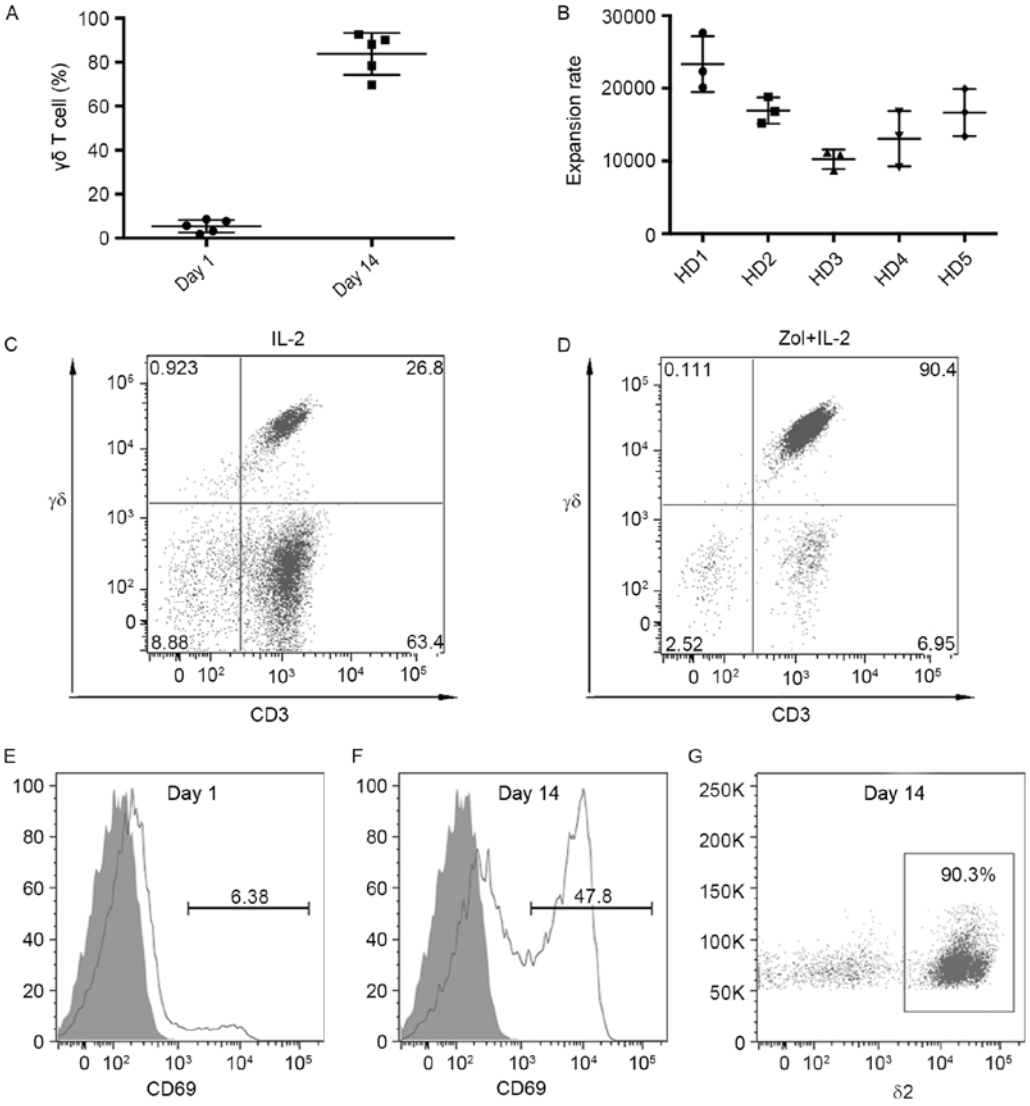

Figure 1. *Ex vivo* expansion and activation of γδ T cells. Peripheral blood mononuclear cells were isolated and cultured in the presence of Zol (1 μM) and IL-2 (400 IU/ml). (A) Proportion of γδ T cells expanded from 5 HDs at days 1 and 14. Results are presented as the mean ± SD. (B) Expansion rate of γδ T cells following 14-day culture from 5 HDs. Results are presented as the mean ± SD. The expansion rate was calculated according to the formula: (Percentage of γδ T cells at day 14 x total cell number at day 14)/(percentage of γδ T cells at day 1 x total cell number at day 1). (C) Representative flow cytometry of γδ T cells expanded without Zol at day 14. (D) Representative flow cytometry of γδ T cells expanded with Zol at day 14. Immunophenotype analysis of CD69 expression at (E) day 1 and (F) day 14. Unfilled histograms represent isotype controls and filled histograms indicate the specific staining. (G) Representative flow cytometry of δ2-positive γδ T cells at day 14. Zol, zoledronic acid; SD, standard deviation; HD, healthy donor; CD, cluster of differentiation; IL-2, interleukin 2.

for Laboratory Animal Care of the Zhejiang University and were approved by the Ethics Committee of the Second Affiliated Hospital, School of Medicine, Zhejiang University (Hangzhou, China).

Statistical analysis. Comparison of quantitative data between two groups was performed using Student's t-test. Analysis of variance was used to determine the difference among three or more groups. Differences between paired samples were tested by Wilcoxon's tests. All data were analyzed using SPSS software (version 11.0; SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Zol and IL-2 induced the ex vivo expansion and activation of γδ T cells. According to flow cytometric analysis, the proportion of γδ T cells in the T-cell population from healthy donors was low, ranging between 1.9 and 8.5% (Fig. 1A). However, Zol and IL-2 induced the robust expansion of γδ T cells in peripheral blood. At the end of the 14-day culture, γδ T cells were successfully expanded. Although the expansion varied between donors, stimulation of PBMCs with Zol and IL-2 resulted in >10^4-fold higher levels of the numbers of γδ T cells for all the donors tested (Fig. 1B). Notably, the preferential expansion of γδ T cells was dependent on Zol stimulation, because in culture with addition of IL-2 alone, the percentage of γδ T cells averaged only 25.7% on day 14 (range, 12.5-34.2%; Fig. 1C), whereas the median percentages of γδ T cells on day 14 was 88.1% (range, 69.7-92.5%; Fig. 1D). With the expansion of γδ T cells, the expression of activation marker CD69 was also upregulated. At the onset of culture, γδ T cells expressed little CD69 (Fig. 1E). By contrast, on day 14, ~45% of the γδ T cells were observed to express CD69 (Fig. 1F). In accordance with

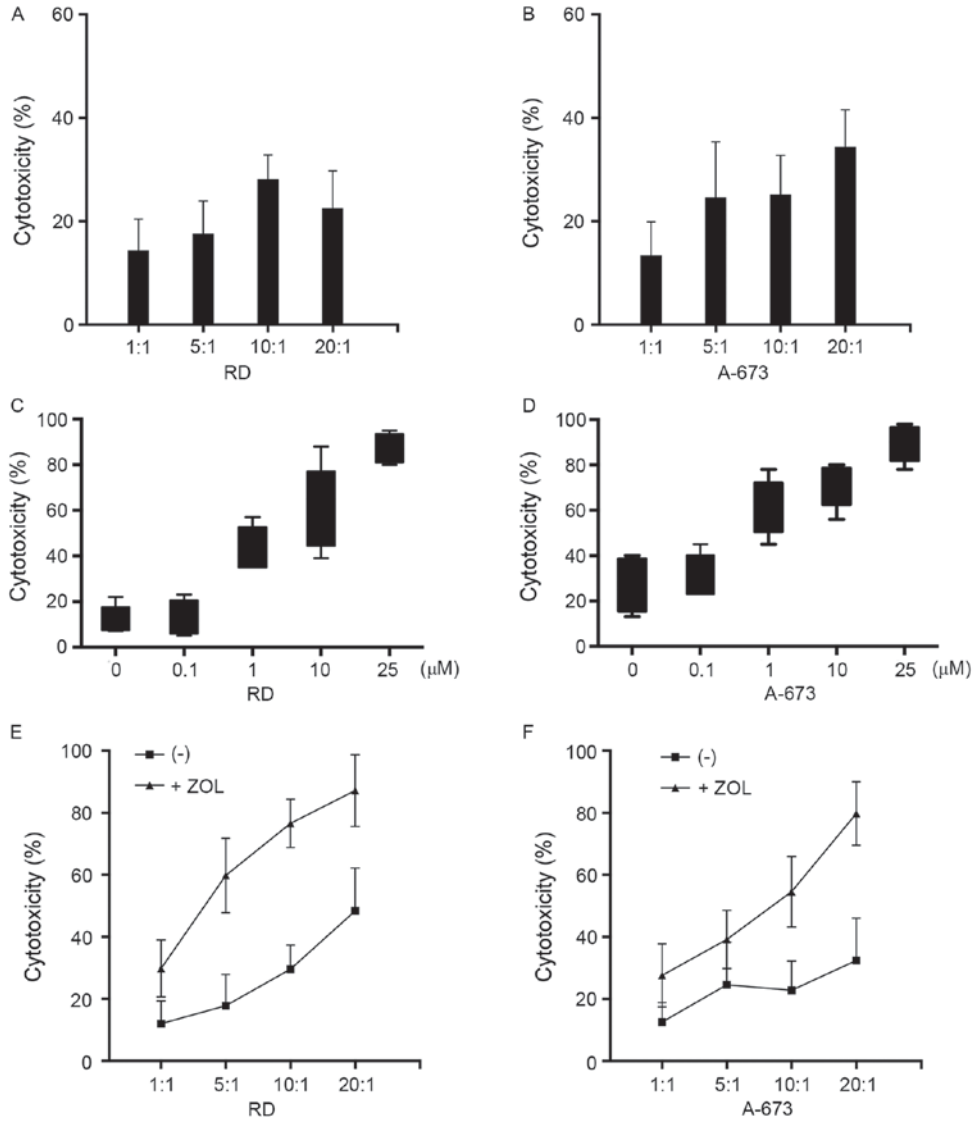

Figure 2. Zol pretreatment enhances the *in vitro* tumor-killing activity of γδ T cells against rhabdomyosarcoma cells. (A) Cytotoxic activity of γδ T cells from different HDs against untreated RD cells at the indicated E:T ratios (mean ± SD; n=5). (B) Cytotoxic activity of γδ T cells from distinct HDs against untreated A-673 cells at the indicated E:T ratios (mean ± SD; n=5). (C) Cytotoxic activity of γδ T cells from different HDs against RD cells treated with various concentrations (0. 1, 1, 10 and 25 μM) of Zol for 24 h at an E:T ratio of 10:1 (mean ± SD; n=5). (D) Cytotoxic activity of γδ T cells from distinct HDs against A-673 cells treated with different concentrations (0. 1, 1, 10 and 25 μM) of Zol for 24 h at an E:T ratio of 10:1 (mean ± SD; n=5). (E) Cytotoxic activity of γδ T cells against untreated (squares) and Zol-treated (triangles; 1 μM Zol for 24 h) RD cells at the indicated E:T ratios. Results are presented as the mean ± SD from five independent experiments using 1 HD. (F) Cytotoxic activity of γδ T cells against untreated (squares) and Zol-treated A-673 (triangles; 1 μM Zol for 24 h) cells at the indicated E:T ratios. Results are presented as the mean ± SD from five independent experiments using 1 HD. Zol, zoledronic acid; E:T ratio, effector/target ratio; SD, standard deviation.

previous reports, a subset of δ2-positive γδ T cells was preferentially expanded. The majority of the expanded γδ T cells were δ2-positive T cells (Fig. 1G).

Zol pretreatment enhances the in vitro tumor-killing activity of γδ T cells against RMS cells. The sensitivity of RMS cell lines RD and A-673 to lysis by γδ T cells was determined using an MTS assay. Results presented in Fig. 2A and B indicated that γδ T cells exhibited only moderate cytotoxicity towards RMS cells, with 28.2 and 25.2% lysis for RD and A-673, respectively, at an E:T ratio of 10:1. The effect of Zol pretreatment on the susceptibility of the RMS cells to γδ T cell-mediated cytotoxicity was determined. Target cells were cultured in medium supplemented with a graded concentration of Zol for 24 h before a 4 h MTS assay at an E:T ratio 10:1. When Zol was used at 0.1 μM, no appreciable increase in cytotoxicity against the RD cell line was observed (P>0.05; Fig. 2C). γδ T cells began to exhibit enhanced levels of cytotoxicity with 1 μM Zol. Increased cytotoxicity was detected with an increase in Zol concentration, and peaked at a concentration of 25 μM. This experiment revealed that the sensitization effect of Zol was dose-dependent. Similarly, γδ T cells demonstrated comparable cytotoxic activity with that towards A-673 cells (Fig. 2D). A detectable increase was already observed when target cells were treated with 1 μM Zol, therefore a concentration of 1 μM was used in the subsequent experiments. The increase in cytotoxicity towards Zol-treated tumor cells was consistently observed at all E:T ratios used (Fig. 2E and F). Not unexpectedly, a ratio-dependent increase in cytotoxicity was observed, and almost complete killing could be achieved

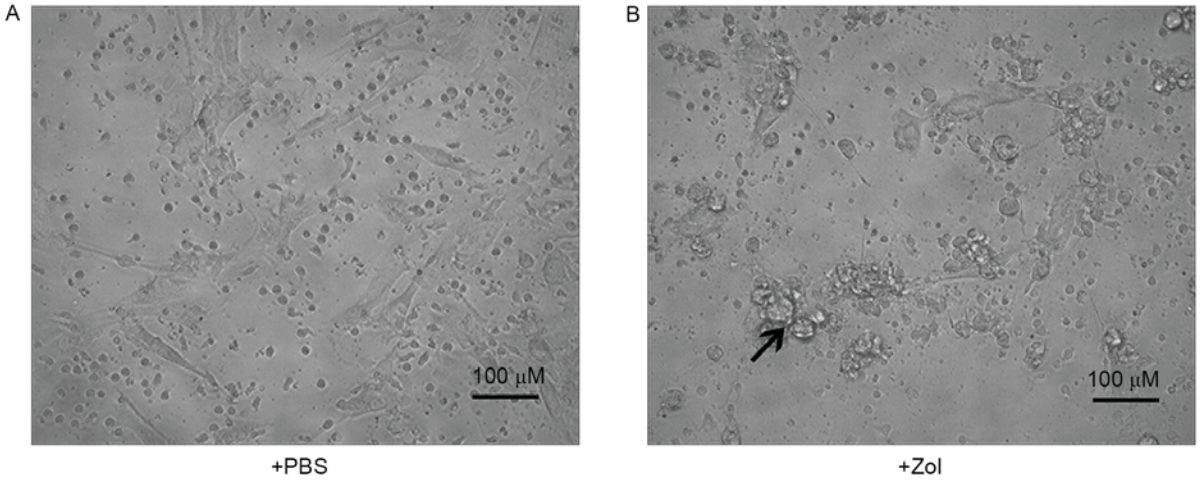

Figure 3. Zol increases the lysis of rhabdomyosarcoma cells by γδ T cells *in vitro*. Representative light microscopic fields of γδ T cells co-cultured with (A) Zol-treated (1 μM for 24 h) or (B) untreated RD cells. The arrow indicates that Zol-treated RD cells were surrounded by γδ T cells, leading to cell death induced by γδ T cells. By contrast, fewer T cells were bound to untreated RD cells, many of which remained intact throughout the 4-h co-culture. Zol, zoledronic acid.

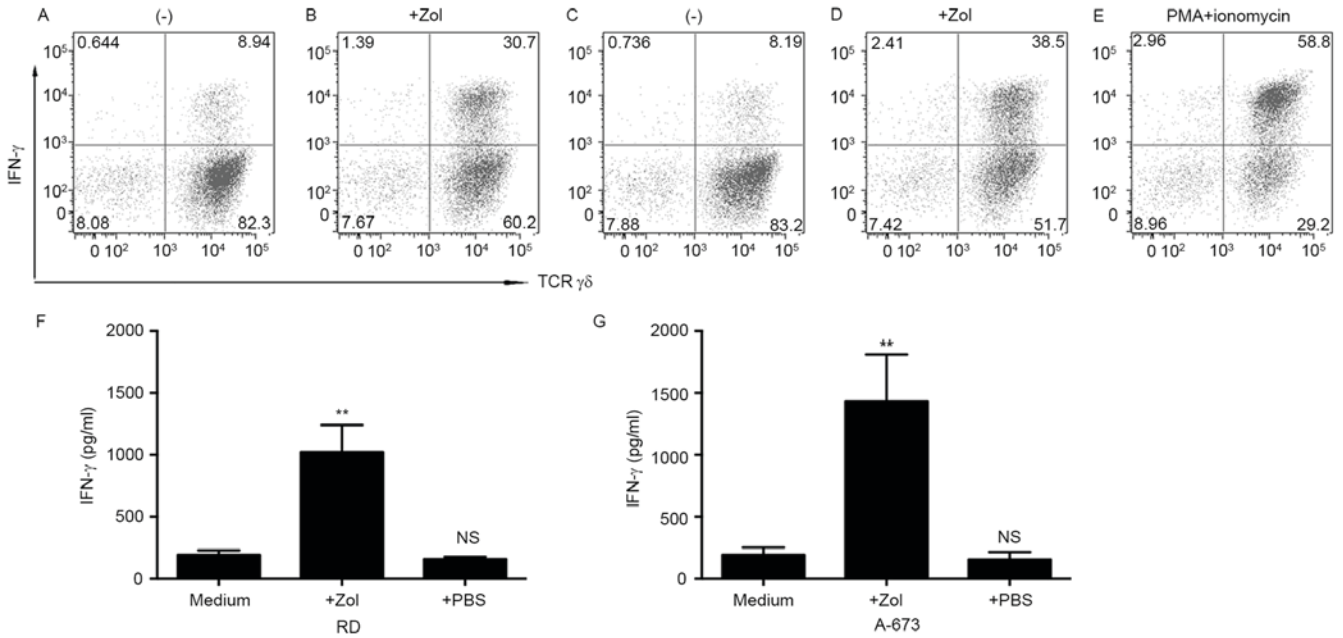

Figure 4. Zol potentiates IFN-γ secretion in γδ T-lymphocytes stimulated by RMS cells. Flow cytometric plots of intracellular IFN-γ expression in γδ T cells of 1 healthy donor after 4 h co-culture with RMS cells at an effector/target ratio of 20:1. RD cells were pretreated with (A) PBS or (D) 1 μM Zol for 24 h before co-culture. A-673 cells were pretreated with (B) PBS or with (E) 1 μM Zol for 24 h before co-culture. PMA and ionomycin were used as positive controls (C). Culture supernatants of (F) RD or (G) A-673 cells were harvested and analyzed for IFN-γ content by ELISA. Results are presented as the mean ± standard deviation from five independent experiments using 1 HD. **P<0.01 vs. culture medium. RMS, rhabdomyosarcoma; Zol, zoledronic acid; PMA, phorbol myristate acetate; IFN-γ, interferon γ; TCR, T cell receptor; NS, not significant.

at an E:T ratio of 20:1, suggesting that optimal cytotoxicity requires sufficient effector cells. Notably, no apparent tumor cell death was observed using the MTS assay when cultured for 24 h in medium supplemented with the indicated concentration of Zol, indicating that Zol alone did not induce direct tumor cell lysis (data not shown). To further investigate the effect of Zol on the lysis of RMS cells by γδ T cells, target cells were treated with or without Zol, the cell lines were co-cultured and visualized microscopically. As presented in Fig. 3A, Zol-treated RMS cells were surrounded by γδ T cells, leading to cell death induced by γδ T cells. By contrast, fewer T cells were bound to untreated RMS cells, many of which remained intact throughout the 4-h co-culture period (Fig. 3B). Overall, these data suggest that Zol pre-treatment sensitized the γδ T cell-mediated cytotoxicity to RMS cells.

RMS cells treated with Zol induce γδ T cells to produce IFN-γ. IFN-γ production in γδ T cells was examined in response to RMS cells. Flow cytometry of the intracellular staining of IFN-γ was performed. Culture of γδ T cells with untreated tumor cells resulted in relatively low levels of IFN-γ (Fig. 4A and B). The poor response towards human RMS cells was not an intrinsic property of the γδ T cells, because a markedly increased level of IFN-γ was observed in γδ T cells stimulated

with phorbol myristate acetate and ionomycin (Fig. 4C). The Zol-sensitized immune response of γδ T cells was evaluated. Pretreatment of RD cells with Zol led to marked levels of intracellular IFN-γ within γδ T cells (Fig. 4D). Likewise, γδ T cells displayed increased intracellular IFN-γ levels in response to Zol-treated A-673 cells (Fig. 4E).

To confirm the ability of γδ T cells to secrete IFN-γ, the supernatants of co-culture was determined by ELISA. γδ T cells were cultured with tumor cells as aforementioned. After 4 h of co-culture, supernatants were harvested and analyzed for IFN-γ content. In line with the flow cytometric data, γδ T cells produced moderate levels of IFN-γ when co-cultured with either untreated RD or A-673 cells. However, Zol pretreatment increased IFN-γ protein production. The results presented in Fig. 4F and G demonstrated that γδ T cells secreted significantly increased amounts of IFN-γ in the Zol-sensitized RMS cell lines (P<0.01). These results indicate that Zol enhanced γδ T cell responsiveness compared with that in untreated target cells.

Zol sensitizes RMS cells susceptible to the γδ T cell-mediated cytotoxicity in a TCR-dependent manner. To study the molecular mechanisms involved in the interaction between γδ T cells and Zol-treated RMS cells, a blocking assay was used to test the effect of surface molecules on γδ T cell cytotoxicity. γδ T cells were incubated with anti-pan-γδ TCR, anti-NKG2D and anti-human TRAIL mAbs for 30 min before co-culture. Pre-incubation of γδ T cells with anti-pan-γδ TCR antibody significantly inhibited the cytotoxicity against the RMS cell lines (P<0.01; Fig. 5), whereas anti-TRAIL antibody did not result in an appreciable decrease in γδ T cell cytotoxicity. Previous study has indicated the role of NKG2D pathway in the lysis of distinct tumors (14). However, as presented in Fig. 5, anti-NKG2D mAb blockade had no discernible effect on the cytolysis of Zol-treated RD or A-673 cell line by γδ T cells. These results suggest that γδ T cell-mediated cytolysis of RMS cells was dependent on TCR pathways.

In vivo antitumor effect of infused γδ T cells against RD xenograft tumors. To examine the *in vivo* immunotherapeutic effects of γδ T cells, a RMS xenograft nude mouse model was established by subcutaneous injection into mice with established firefly luciferase-expressing RD cell line RD-LUC cells (Fig. 6A). At 1 week after tumor inoculation, mice were treated weekly with γδ T cells ($5x10^6$ cells/mouse, i.v.), or Zol (50 μg/kg/mouse, i.p.), or a combination of γδ T cells and Zol (injection of Zol and then γδ T cells 1 day later) for 4 weeks. PBS treatment was set as control. As presented in Fig. 6B, all untreated control mice demonstrated progressive tumor growth. The volume of single treatment alone was not significantly different from that of control mice, whereas a significant decrease in the tumor volume in mice injected with combined treatment was observed (P<0.01). These results demonstrated that a combination of γδ T cells and Zol significantly inhibited the growth of RMS cells *in vivo*.

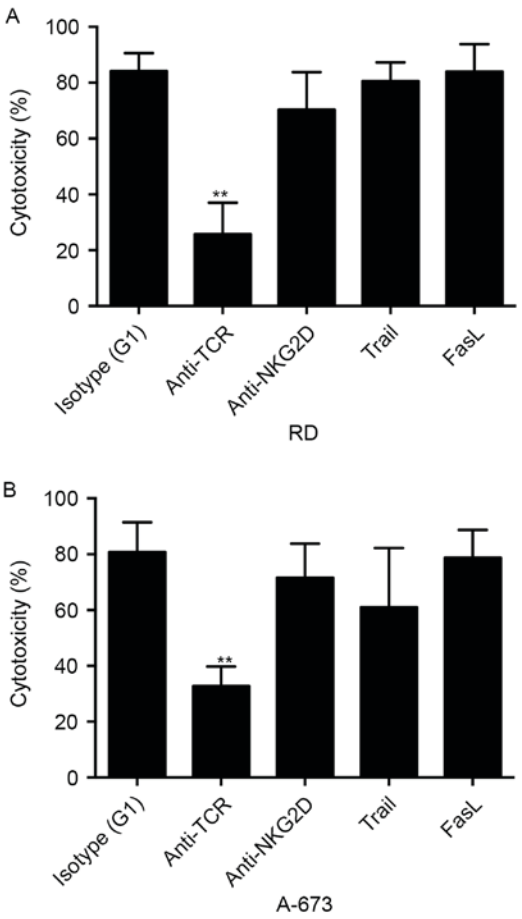

Figure 5. γδ T cells kill Zol-sensitized RMS cells through the TCR-dependent signaling pathway. Expanded γδ T cells were co-cultured for 4 h with Zol-incubated (A) RD cells or (B) A-673 cells for 4 h at an effector/target ratio of 20:1. Cytotoxicity of γδ T cells was determined using an MTS assay. γδ T cells were pre-incubated for 30 min with 10 μg/ml anti-human NKG2D, anti-pan-γδ TCR or anti-TRAIL mAbs. Wilcoxon's paired test was used to compare differences between immunoglobulin control treatment and mAb treatment. Results are presented as the mean percentage of cytotoxicity ± SD from five independent experiments using 1 healthy donor. **P<0.01 vs. isotype control. Zol, zoledronic acid; mAb, monoclonal antibody; NKG2D, natural killer group 2D, member 2; TCR, T cell receptor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; FasL, Fas ligand.

Discussion

Current immunotherapeutic approaches that target RMS cells mainly focus on natural killer (NK) cells or CTLs. It remains challenging to expand NK cells or antigen-specific αβ T cells *ex vivo* to the amount required for efficacious adaptive immunotherapy. Conversely, the results of the present study confirmed that γδ T cells could be sufficiently obtained from PBMCs of healthy donors in short-term culture. Additionally, the cytotoxic activity of γδ T cells against RMS cells is, to the best of our knowledge, reported for the first time. The results of the present study revealed that γδ T cells had direct cytotoxic activity towards RMS cell lines. Importantly, Zol sensitization markedly increased the susceptibility of RMS cells to γδ T cells.

Zol is an N-BP and exerts pharmacological effects by specifically inhibiting farnesyl pyrophosphate synthase, a key enzyme in the mevalonate signaling pathway (15). This process leads to the accumulation of the upstream metabolite of the mevalonate signaling pathway, including IPP, which is sensed by γδ T cells as stimulating antigens. The use of Zol may represent a double strategy for adoptive γδ T cell-based immunotherapy (16). On one hand, when Zol is internalized

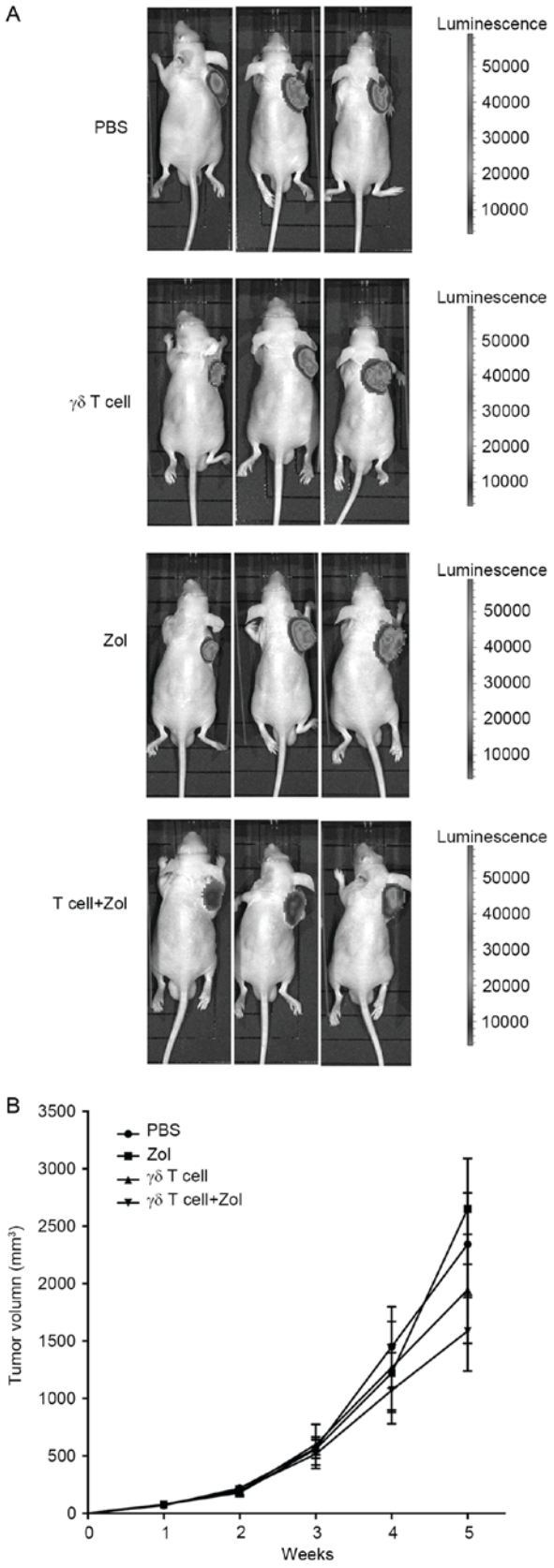

Figure 6. Treatment of nude mice with γδ T cells suppresses the development of RMS *in vivo*. Female athymic nude mice (4-week-old) were subcutaneously xenografted into the right upper flank of mice with human firefly luciferase-expressing RD-LUC cells. After 1 week, mice were randomized into 4 groups. (A) Whole-body images of tumor bioluminescence of RMS tumor-bearing mice were captured 4 weeks after tumor inoculation. (B) Mean ± standard deviation tumor volume of 6 mice. The tumor volume of mice receiving human γδ T cells and Zol was significantly less compared with the other groups ($P<0.01$).

by monocytes and dendritic cells, γδ T cells in PBMCs are expanded and activated to an effector phenotype (17). On the other hand, tumor cells pretreated by Zol are sensitized to the cytolysis mediated by human γδ T cells (10). Zol is a clinically approved drug widely used in the treatment of bone resorption-associated disease for patients with cancer (18). It has been demonstrated that Zol may also exert direct antitumor effects *in vitro* and in animal models (16). Therefore, this therapeutic strategy is of practical value in a clinical scenario, particularly in settings in which there are limited options for treating metastatic RMS.

The second major result is that the γδ T cell response to Zol-treated RMS cells was primarily through the TCR-mediated signaling pathway. NKG2D was originally described as a stimulatory receptor for NK cells. Several lines of evidence indicate that tumor cell lysis by γδ T cell may be modulated by TCR and NKG2D ligation (14,19). However, the results of the antibody blockade assay in the present study indicated that the NKG2D signaling pathway may serve a lesser role in the recognition of Zol-treated RMS cells, because blocking NKG2D on γδ T cells was largely ineffective. This result is not unexpected. As reported previously, NKG2D activates γδ T cells in an antigen-independent manner (19), whereas Zol-produced IPP on the tumor surface is mainly recognized by γδ TCR. The underlying molecular mechanisms mediating the cytotoxic effect of γδ T cells to Zol-treated RMS cells were investigated in the present study. Zol treatment caused γδ T cells to secrete increased levels of IFN-γ. It coincides with the results by Rincon-Orozco *et al* (19) that IFN-γ production may not be induced by NKG2D ligation. Mattarollo *et al* (20) also observed that NKG2D interactions did not significantly contribute to the cytotoxicity of Zol-sensitized tumor cells, but did not develop this issue further in their study.

The present study has some limitations. Only PBMCs from healthy donors were used to expand γδ T cells. Whether the expansion efficiency of γδ T cells from patients with RMS is comparable with that of healthy donors remains to be determined. In previous studies, γδ T cells have been successfully expanded from patients with lung cancer (21), neuroblastoma (22) and follicular lymphoma (23). Therefore, the proliferative responses of γδ T cells from certain patients with RMS are presumably not impaired. Owing to the lack of alloreactivity of γδ T cells, for patients with impaired autologous γδ T cell expansion capacities, it is possible to transfer sufficient allogeneic γδ T lymphocytes expand from normal donors. As only the cytotoxic activity of γδ T cells against RD and A-673 cells was examined, other RMS cell lines and autologous tumor cells are required to confirm the results of the present study. Finally, the *in vivo* results indicate that a combination of Zol and γδ T cells yielded marked antitumor responses compared with other single treatment, and it was consistent with *in vitro* studies. Considering the promising results of the present study, further studies including immunohistochemical analysis of localization and kinetics of infused γδ T cells are warranted to explore the mechanism of the synergistic antitumor activity of human γδ T cells in combination with Zol.

The results of the present study confirmed that Zol is able to sensitize RMS cells to γδ T cell cytotoxicity. Adoptive γδ T cell therapy combined with Zol may serve as a novel

approach for the treatment of RMS and therefore warrants further scientific investigation.

Acknowledgements

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