Comparison of γδ T cell responses and farnesyl diphosphate synthase inhibition in tumor cells pretreated with zoledronic acid

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Summary

Exposing human tumor cells to nitrogen-containing bisphosphonates (N-BPs), such as zoledronic acid (Zol), greatly increases their susceptibility to killing by γδ T cells. Based on this finding and other studies, cancer immunotherapy using γδ T cells and N-BPs has been studied in pilot clinical trials and has shown benefits. Although Zol treatment can render a wide variety of human tumor cells susceptible to γδ T cell killing, there has not been a systematic investigation to determine which types of tumor cells are the most susceptible to γδ T cell-mediated cytotoxicity. In this study, we determined the Zol concentrations required to stimulate half maximal TNF-α production by γδ T cells cultured with various tumor cell lines pretreated with Zol and compared these concentrations with those required for half maximal inhibition of farnesyl diphosphate synthase (FPPS) in the same tumor cell lines. The inhibition of tumor cell growth by Zol was also assessed. We find that FPPS inhibition strongly correlates with γδ T cell activation, confirming that the mechanism underlying γδ T cell activation by Zol is isopentenyl diphosphate (IPP) accumulation due to FPPS blockade. In addition, we showed that γδ TCR-mediated signaling correlated with γδ T cell TNF-α production and cytotoxicity. Some lymphoma, myeloid leukemia, and

mammary carcinoma cell lines were relatively resistant to Zol treatment suggesting that assessing tumor sensitivity to Zol may help select those patients most likely to benefit from immunotherapy with γδ T cells.

Abbreviations

FPPS, farnesyl diphosphate synthase;

IPP, isopentenyl diphosphate;

N-BP, nitrogen-containing bisphosphonate;

NK, natural killer;

TCR, T cell receptor;

Zol, zoledronic acid.

Introduction

The majority of human peripheral blood γδ T cells express Vγ2 (also termed Vγ9) and Vδ2 T cell receptor genes[1-4] and display cytotoxicity against a wide spectrum of tumor cells.[5, 6] γδ T cells kill tumors cells through recognition by γδ T cell receptors (TCR)[7, 8] as well as by natural killer (NK) receptors.[9-12] Recent clinical trials demonstrated that zoledronic acid (Zol), a nitrogen-containing bisphosphonate (N-BP), provided clinical benefits when added to standard therapies for patients with mammary carcinoma and multiple myeloma.[13-17] Because N-BPs inhibit farnesyl diphosphate synthase (FPPS) in tumor cells and increase the intracellular level of isopentenyl pyrophosphate (IPP), leading to the activation of γδ T cells expressing Vγ2Vδ2 TCRs,[18-20] it has been suggested that γδ T cells might contribute to the therapeutic effect of Zol in cancer treatment.[21]

Although in vitro and in vivo studies have demonstrated that Zol renders many types of tumor cells susceptible to γδ TCR-mediated cytotoxicity,[5, 15, 22-29] there has not been a systematic examination to determine if it would be possible to predict which types of tumors would be most likely to respond to immunotherapy with γδ T cells and Zol. In this study, we have tested a variety of cancer cell lines to determine the Zol concentration required to inhibit FPPS by 50% (as assessed by rap1A prenylation) and compared these

concentrations to those required to stimulate half maximal TNF-α production by γδ T cells cultured with Zol-pretreated tumor cells. We find that the Zol concentrations required for FPPS inhibition closely correlates with those required for stimulation of TNF-α production by γδ T cells but not with the Zol concentrations required to inhibit tumor cell proliferation. Additionally, γδ TCR-mediated signaling correlated with FPPS inhibition.

Materials and Methods

Inhibition of FPPS

Zol was purchased from Novartis Pharmaceuticals Corp. (Basel, Switzerland) and converted to its sodium salt using a Na^+ form of Dowex 50W×8 (Muromachi Kogyo Kaisha Ltd., Chuo-ku, Tokyo, Japan). Zol inhibition of FPPS was determined by assessing the degree of rap1A prenylation (geranylgeranylation) on Western blotting with varying concentrations of Zol as described in Fig. S1.

Derivation of Vγ2Vδ2 T cell lines

Recombinant human IL-2 was kindly provided by Shionogi Pharmaceutical Co., Ltd. (Chuo-ku, Osaka, Japan). After institutional review board approval and with written informed consent, peripheral blood mononuclear cells (PBMC) were purified and stimulated with 5 μM Zol and 100 U/ml IL-2 for 10 days as described in Fig. S2 to derive Vγ2Vδ2 T cell lines.

Flow cytometry

Flow cytometric analyses were performed using a FACSCalibur system (Becton Dickinson, Franklin Lakes, NJ). The gating strategy is detailed in Fig. S2.

Cytokine production

Tumor cells listed in Table S1 were grown, harvested, and resuspended at 1×10^6 cells/0.5 ml in 10-fold serial dilutions of Zol in complete RPMI1640 media (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (Sigma), 10^{-5} M 2-mercaptoethanol (Nacalai Tesque Inc., Nakagyo-ku, Kyoto, Japan), 100 IU/ml penicillin (Meiji Seika Kaisha, Ltd., Chuo-ku, Tokyo, Japan), and 100 μg/ml streptomycin (Meiji Seika Kaisha). After incubation at 37^{o}C with 5% CO_2 for 4 h, the cells were washed three times with 5 ml of the medium and resuspended in 0.5 ml of the same medium. A total of 0.1 ml (2×10^5 cells/well) of the tumor cell suspension was placed on flat-bottomed 96-well plates and 0.1 ml of γδ T cells (2×10^5 cells/well) was added (Fig. S2). The plates were incubated at 37°C with 5% CO_2 for 16 h and the culture supernatants stored overnight at -80^{o}C. The samples were then thawed and TNF-α concentrations determined by ELISA (Peprotech, Rocky Hill, NJ) using an ARVO spectrophotometer (PerkinElmer, Foster City, CA). All experiments were performed in triplicate.

Tumor cell growth inhibition assay

Tumor cells listed in Table S1 were grown, harvested, and resuspended at 1×10^4 cells/ml in complete RPMI1640 medium. A total of 0.05 ml of the cell suspension was added to flat bottomed 96-well plates, followed by 0.05 ml of 3-fold serial dilutions of Zol. After incubation at 37°C with 5% CO_2 for 4 h, the supernatant was removed and Zol-free medium added. After an additional 16 h, 0.1 ml of the CellTiter-Glo reagent (Promega Corp.) was added and the luminescence due to released ATP was measured using an ARVO luminometer (PerkinElmer). All experiments were performed in triplicate.

γδ TCR Jurkat Transfectant and IL-2 assay

β^- Jurkat cells expressing Vγ2Vδ2 TCR were prepared and IL-2 release assayed as described previously.[8] Briefly, 2×10^5 TCR transfectant cells in 100 μl were mixed with 2×10^5 tumor cells in 100 μl. Tumor cells were pretreated with serial dilutions of Zol. After 16 h, the supernatants were collected and assayed for IL-2 by their ability to support the proliferation of the IL-2-dependent CTLL-2 cell line. CTLL-2 cell numbers were determined using the CellTiter-Glo reagent as described above. All experiments were

performed in triplicate.

γδ T cell Cytotoxicity assay

Tumor cells (1×10^6) were treated with serial dilutions of Zol for 4 h and then labeled with 100 μCi of $Na^{51}Cr$ for 1 h. γδ T cells were incubated with the labeled tumor cells (1×10^4 cells/well) at an effector to target ratio of 40:1. Specific ^{51}Cr release was determined as described previously.[5]

Results

High Zol concentrations were required for FPPS inhibition in many lymphoma and some myeloid leukemia cell lines in vitro

Zol inhibits FPPS rendering tumor cells susceptible to γδ TCR-mediated lysis.[5, 30] Zol inhibition of FPPS results in intracellular accumulation of upstream metabolites such as IPP.[18-20] Downstream metabolites, such as farnesyl diphosphate and geranylgeranyl diphosphate, are depleted leading to the accumulation of unprenylated Rap1A, a small G protein required for cellular adhesion.[31] The accumulation of unprenylated Rap1A was therefore used as a measure of FPPS inhibition. The Zol concentrations required for half maximal inhibition (IC_{50}) of Rap1A prenylation were determined by culturing tumor cell lines with Zol for 16 h and measuring the level of unprenylated Rap1A by Western blotting (Fig. S1). The proportion of tumor cell lines with Zol IC_{50} of 100 μM or greater was 85.7% for lymphoma, 57.1% for myeloid leukemia, and 28.6% for mammary carcinoma cell lines but only 5.8% for the other 52 tumor cell lines (Fig. 1). Of the 52 other tumor cell lines examined, 9 had IC_{50} values less than 10 μM, including the 786-0W and ACHN renal cell carcinoma, the EJ-1 and T24 bladder carcinoma, the MZChA2 bile duct carcinoma, the

TGBC1TKB gallbladder carcinoma, the HuO osteosarcoma, the PC-3 prostatic carcinoma, and the HT-1080 fibrosarcoma cell line.

High Zol concentrations are required for γδ T cell activation by Zol-pretreated lymphoma and myeloid leukemia cell lines in vitro

We next determined the Zol concentrations required to stimulate half maximal TNF-α secretion (EC_{50}) by γδ T cells (Fig. S2) in response to tumor cell lines incubated with Zol (Table S1). The EC_{50} values for most tumor cell lines were between 10 μM and 100 μM (Fig. 2). The proportion of tumor cell lines with EC_{50} values of 100 μM or greater was 85.7% for lymphoma, 42.9% for myeloid leukemia, and 42.9% for mammary carcinoma cell lines. In contrast, only 3.8% of the other 52 tumor cell lines had EC_{50} values greater than 100 μM. Although both the Daudi Burkitt's lymphoma and the RPMI 8226 plasmacytoma cell lines stimulate γδ T cells through their γδ TCRs, most other lymphoma and myeloid leukemia cell lines stimulated only poor γδ T cell responses in vitro even with exposure to Zol. Some mammary carcinoma cell lines also required high concentrations of Zol to elicit TNF-α responses by γδ T cells. The requirement for relatively high concentrations of Zol for γδ T cell activation exhibited by some lymphoma, myeloid

leukemia, and mammary carcinoma cell lines correlated with the greater Zol concentrations (EC_{50}) required for FPPS inhibition by these cell lines. In contrast, 13 out of 73 tumor cell lines had EC_{50} values less than 10 μM, including the ACHN and UOK111 renal cell carcinoma, the EJ-1 bladder carcinoma, the GCIY, KATO III, MKN28, and MKN74 gastric carcinoma, the Saos-2 osteosarcoma, the DLD-1 colorectal carcinoma, the C32TG and G-361 melanoma, the PC-3 prostatic carcinoma, and the HT-1080 fibrosarcoma cell lines.

Inhibition of FPPS was closely correlated with TNF-α production by γδ T cells

To assess the degree of correlation between FPPS inhibition and γδ T cell activation, we compared Zol concentrations for FPPS inhibition (IC_{50}) to those for γδ T cell activation (EC_{50}) for each of the tumor cell lines. As shown in Fig. 3, the Zol concentrations required for FPPS inhibition (prenylation inhibition) were well correlated with those required for γδ T cell production of TNF-α. For example, the MOLT-3 lymphoma required Zol concentrations of 500 μM for prenylation inhibition and 530 μM for TNF-α production (Fig. 3A). Similarly, BxPC-3 required 55 μM for prenylation inhibition and 58 μM for TNF-α production (Fig. 3G).

Direct cytotoxicity of Zol on tumor cell lines

Because some lymphoma, myeloid leukemia, and mammary carcinoma cell lines were relatively resistant to FPPS inhibition and γδ T cell activation by Zol, we next determined whether direct killing of certain tumor cell lines by Zol was inhibiting their ability to stimulate γδ T cell secretion of TNF-α. As shown in Fig. 4A for a representative sample of tumor cell lines, tumor cell growth inhibition curves were similar. The Zol concentrations required for half maximal tumor cell line growth inhibition (IC_{50}) were similar between the different types of tumors without high variability (Fig. 4B). These findings clearly demonstrate that the differences in Zol concentrations required for FPPS inhibition and γδ T cell activation were not due to the direct effects of Zol on tumor cell growth. In fact, much higher concentrations of Zol were required to inhibit tumor cell growth than those required to stimulate γδ T cells (Fig. S3). In addition, specific lysis of tumor cells by γδ T cells in the absence of Zol at an effector to target ratio of 1:1 was less than 6%, confirming further that tumor cell viability was not a critical factor determining the difference in IC_{50} and EC_{50} values between different tumor cell types.

γδ TCR-mediated recognition of Zol-treated tumor cells

We next examined the correlation between γδ TCR-mediated signaling and TNF-α production. Tumor cell lines were cultured with serial dilutions of Zol and used to stimulate IL-2 production by γδ TCR-expressing Jurkat cells. Because the production of IL-2 requires signaling through the γδ TCR, Zol concentrations required for γδ TCR-mediated signaling can be determined. The Zol concentrations that stimulated half maximal IL-2 production by the transfectants were well correlated with those stimulating half maximal TNF-α secretion by γδ T cells (Fig. 4C). These results demonstrate that γδ TCR-mediated signaling is a key factor determining cytokine production by γδ T cells in response to Zol.

γδ T cell cytotoxicity against Zol-treated tumor cells

Because activated γδ T cells express NK receptors such as NKG2D, γδ T cells exhibit cytotoxic activity against tumor cells expressing NK ligands, especially at higher effector to target ratios. Thus, γδ T cells lysed THP-1 myeloid leukemia cells and VMRC-RCW renal carcinoma cells, even in the absence of Zol-treatment, at an effector to target ratio of 40:1 (Fig. 4D). This is in stark contrast to cytokine secretion where γδ T cells did not produce TNF-α in the absence of Zol (Fig. 2). Treating tumor cells with Zol increased γδ T cell killing in a Zol concentration-dependent manner. For THP-1 and VMRC-RCW cell lines,

tumor cytotoxicity by γδ T cells was half maximally increased by Zol concentrations of 100-1000 μM and 5-20 μM, respectively. These values were similar to the Zol concentrations required to stimulate production of TNF-α by γδ T cells (100-1000 μM versus 440 μM for THP-1and 5-20 μM versus 13 μM for VMRC-RCW). Thus, γδ TCR-mediated recognition of Zol-treated tumor cells is critical not only for cytokine production but also for maximal cytotoxicity.

Discussion

Recent clinical trials have provided evidence that the addition of Zol to the treatment of patients with multiple myeloma and breast cancer provides benefits,[13, 14, 16, 17] although the mechanisms underlying this antitumor activity of Zol have not been determined.[32] One potential mechanism for Zol antitumor activity is the activation of γδ T cells expressing Vγ2Vδ2 TCRs. Zol inhibits the FPPS enzyme in isoprenoid synthesis. This results in the accumulation of the upstream metabolite, IPP, that stimulates γδ T cells.[18-20] To date, however, no comprehensive study has been reported comparing the Zol concentrations required for γδ T cell activation to those required for FPPS inhibition in different types of tumors. In this study, we have examined 73 human tumor cell lines originating from a variety of tissues to determine the Zol concentrations required for γδ T cell activation and FPPS inhibition. We find that the Zol concentrations required for γδ T cell activation strongly correlated with those for FPPS inhibition. Our findings clearly show that the accumulation of IPP by FPPS inhibition is closely related to the activation of γδ T cells in a variety of different types of tumor cell lines and is consistent with a study restricted to eight breast cancer cell lines.[33] Moreover, signaling through the γδ TCR was required for Zol to stimulate cytokine secretion and maximal cytotoxicity.

Why do different types of tumor cell lines vary in the Zol concentration required for FPPS inhibition and γδ T cell activation? In most tumor cell lines, Zol elicited half maximal γδ T cell responses at 10-100 μM. However, some but not all lymphoma, myeloid leukemia, and mammary carcinoma cell lines required much higher Zol concentrations of 100 μM or more. One possibility is that somatic mutation of FPPS in the cell lines alters their response to inhibition by Zol. There have been 14 mutations in FPPS reported out of 106 cancer samples analyzed (summarized on the Catalogue Of Somatic Mutations In Cancer website). However, none of the mutations were found in lymphomas, leukemias, or breast cancers making this explanation unlikely. As an alternative explanation, we speculate that certain types of tumors require higher concentrations of Zol for FPPS inhibition because Zol is not efficiently taken up through fluid-phase endocytosis[(34)] due to differences in their metabolism or rate of nutrient uptake. Supporting this hypothesis, the opposite is clearly the case. Lipophilic pyridiuium aminobisphosphonates (e.g. BPH-716), that are likely to enter cells more efficiently due to their much higher hydrophobicity, are up to ~12.5-fold more potent activators of γδ T cells than non-lipophilic aminobisphosphonates, such as Zol[(35)], despite being 631-fold less potent inhibitor of FPPS.[(36)]

The addition of Zol to standard treatments for breast cancer patients improved disease-free survival in the subset of patients that have estrogen receptor-positive cancers in a low estrogen environment (either through anti-estrogen treatment or menopause)[13-16]. Similarly, improved overall survival was noted with patients with newly diagnosed multiple myeloma.[17] Surprisingly, these improvements were independent of the prevention of skeletal-related events in myeloma[17] and, in the case of breast cancer in postmenopausal women, were related to a decrease in both skeletal and non-skeletal metastases.[15, 16] We have shown that the majority of patients with early-stage breast cancer will respond to Zol and many have elevated $V\delta2^+$ T cell frequencies.[37] However, we find in this study that there is heterogeneity in the ability of mammary carcinoma cell lines to stimulate γδ T cell responses with 42.9% requiring half maximal Zol concentration of >100 μM. If some of the survival benefits of Zol are due to γδ T cells as has been proposed,[21] heterogeneity in the tumor response to Zol may explain some of the variability of the patient response to Zol treatment. Moreover, in vitro examination of the ability of a patient's breast cancer cells to stimulate γδ T cell cultured with Zol or the cancer cell's sensitivity to FPPS inhibition by Zol might be useful for selecting patients that would be the most likely to benefit from Zol-based therapy.

As demonstrated in this study, renal cell carcinoma cell lines required relatively low concentrations of Zol to inhibit FPPS and to stimulate γδ T cell responses in vitro. Recent clinical trials have shown that γδ T cell/Zol-based therapies provide clinical benefits for patients with lung metastasis of renal cell carcinoma.[38-40] Like the breast cancer and myeloma studies, these observations suggest that the effect of Zol is not solely limited to preventing skeletal metastasis. Instead, Zol may serve to potentiate the effector functions of γδ T cells in patients with a variety of tumor types including those not metastatic to bone.

Clinical studies assessing γδ T cell therapy have been performed in patients with lymphoma,[41] mammary carcinoma,[27, 40] myeloma,[42] renal cell carcinoma,[24, 38, 43-45] melanoma,[45] prostate cancer,[26] , acute myeloid leukemia,[45] non-small cell lung cancer, [46] and assorted solid tumors[40] through either direct in vivo immunization with IL-2 or by adoptive immunotherapy after ex vivo expansion of γδ T cells. However, the correlation between Zol sensitivity of a tumor and the clinical outcome remains unclear. Assessing the in vitro sensitivity of tumor cells to Zol may help to predict which tumor types are most likely to respond to therapy and, if cancer cells from individual patients can be tested for Zol sensitivity, aid in deciding which patients to recruit for Zol-based clinical trials. Currently, many laboratories are attempting to develop N-BPs that have affinity for the

tumors themselves or that can be targeted to tumors. This medicinal chemistry approach may be help optimize N-BPs for γδ T cell-based cancer immunotherapy.

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Disclosure Statement

C. T. M. is a co-inventor of US Patent 8,012,466 on the development of live bacterial vaccines for activating γδ T cells. The other authors have no financial or commercial conflict of interest.

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Figure Legends

Fig. 1. Differential effects of Zol on FPPS inhibition in tumor cell lines. (A) Dose-dependent Zol inhibition of geranylgeranylation of Rap 1A for various types of tumor cell lines including (a) lymphomas, □ MOLT-3, Δ PEER, ○ C1R, ♦ J.RT3-T3.5, ■ Raji, ▲ RAMOS-RA1, ● MOLT-4; (b) myeloid leukemias, □ HL60, Δ U937, ○ THP-1, ♦ SCC-3, ■ P31/FUJ, ▲ K562, ● NOMO-1; (c) mammary carcinomas, □ YMB-1-E, Δ MRK-nu-1, ○ HMC-1-8, ♦ MCF-7, ■ MDA-MB-231, ▲ T-47D, ● SK-BR-3; (d) renal cell carcinomas, ▲ 786-0, Δ VMRC-RCZ, ● UOK121, ○ Caki-1, ■ A-704; (e) pancreatic carcinomas, ● BxPC-3, ▲ KP4-1, ○ KP4-2, □ KP4-3, Δ MIAPaCa-2; and (f) other tumor cells, ● TGBC24TKB, ▲ ACS, ○ MG-63, □ LK-2, Δ EJ-1. (B) Comparison of Zol concentrations (IC_{50}) required for half maximal inhibition of prenylation of Rap 1A in various types of tumor cells. Ly, lymphoma; My, myeloid leukemia; Ma, mammary carcinoma; Others, other tumor cell lines.

Fig. 2. Comparison of TNF-α secretion by γδ T cells stimulated with Zol-treated tumor cells. (A) TNF-α production by γδ T cells in response to tumor cells pretreated with various Zol concentrations: (a) lymphomas, □ MOLT-3, Δ PEER, ○ C1R, ♦ J.RT3-T3.5, ■ Raji, ▲

RAMOS-RA1, ● MOLT-4; (b) myeloid leukemias, □ HL60, Δ U937, ○ THP-1, ♦ SCC-3, ■ P31/FUJ, ▲ K562, ● NOMO-1; (c) mammary carcinomas, □ YMB-1-E, Δ MRK-nu-1, ○ HMC-1-8, ♦ MCF-7, ■ MDA-MB-231, ▲ T-47D, ● SK-BR-3; (d) renal cell carcinomas, ▲ 786-0, Δ VMRC-RCZ, ● UOK121, ○ Caki-1, ■ A-704; (e) pancreatic carcinomas, ● BxPC-3, ▲ KP4-1, ○ KP4-2, □ KP4-3, Δ MIAPaCa-2; (f) other tumor cells, ● TGBC24TKB, ▲ ACS, ○ MG-63, □ LK-2, Δ EJ-1. (B) Comparison of Zol concentrations (EC_{50}) required for half maximal TNF-α secretion by γδ T cells in response to stimulation with different tumor cell lines. Ly, lymphoma; My, myeloid leukemia; Ma, mammary carcinoma; Others, other tumor cell lines.

Fig. 3. Correlation between Zol concentrations required for FPPS inhibition and γδ T cell activation. Zol concentrations required for half maximal inhibition of prenylation of Rap 1A and half maximal stimulation of TNF-α secretion by γδ T cells. Each line connects IC_{50} (prenylation inhibition) and EC_{50} (TNF-α production) for the same tumor cell line: (a) lymphomas, ● C1R, ▲ Raji, ■ MOLT-3, ♦ PEER; (b) myeloid leukemias, ● THP-1, ▲ SCC-3; (c) mammary carcinomas, ● YMB-1-E, ▲ MCF-7, ■ MDA-MB-231; (d) renal cell carcinomas, ● 786-0, ▲ VMRC-RCZ, ■ A-704, ♦ UOK121, ▼ Caki-1; (e) cholangiocell

carcinoma, ● TGBC24TKB, ▲ TFK-1; (f) gastric carcinomas, ● ACS, ▲ AGS, ■ MKN1; (g) pancreatic carcinomas, ● BxPC-3, ▲ KP4-1, ■ KP4-2, ♦ KP4-3, ▼ MIAPaCa-2; (h) osteosarcomas, ● HOS, ▲ MG-63; (i) other tumors, ● LK-2, ▲ GCT-IZ, ■ CW-2, ♦ hu2, ▼ EJ-1.

Fig. 4. Correlation between TCR-mediated signaling and cytokine secretion and cytotoxicity by γδ T cells. (A) Dose-dependent inhibition of tumor cell growth by Zol. Tumor cell lines were treated with serial dilutions of Zol and cell growth inhibition examined for: ● J.RT3.T3.5, ▲ RAMOS-RAI, ■ Colo320, ♦ MG63. (B) Comparison of Zol concentrations required for half maximal growth inhibition of various tumor cell lines. Direct inhibition of tumor cell growth by Zol was determined for: Ly, lymphoma; My, myeloid leukemias; Ma, mammary carcinoma; Others, other tumor cell lines. (C) Correlation between TNF-α production by γδ T cells and γδ TCR-mediated signaling. Zol concentrations required for half maximal production of TNF-α by γδ T cells stimulated with Zol-treated tumor cells were compared with those required for half maximal production of IL-2 by γδ TCR-expressing J.RT3-T3.5 cells stimulated with Zol-treated tumor cell lines including: ● PK-9, ▲ KP4-3, ■ BxPC3, ○ MKN28, Δ AGS, □ G361. (D)

Cytotoxic activity of γδ T cells against tumor cells. THP-1 myeloid leukemia and VMRC-RCW renal carcinoma cells were treated with serial dilutions of Zol and used as target for cytotoxicity by γδ T cells.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of tumor cell lines used in this study.

Fig. S1. Zol inhibition of geranylgeranylation of Rap 1A.

Fig. S2. Flow cytometric analysis of $V\delta2^{+}$ γδ T cells before and after expansion from PBMC by Zol/IL-2 and the gating strategy.

Fig. S3. Comparison between Zol concentrations required for γδ T cell responses and tumor cell growth inhibition.

Fig. 1

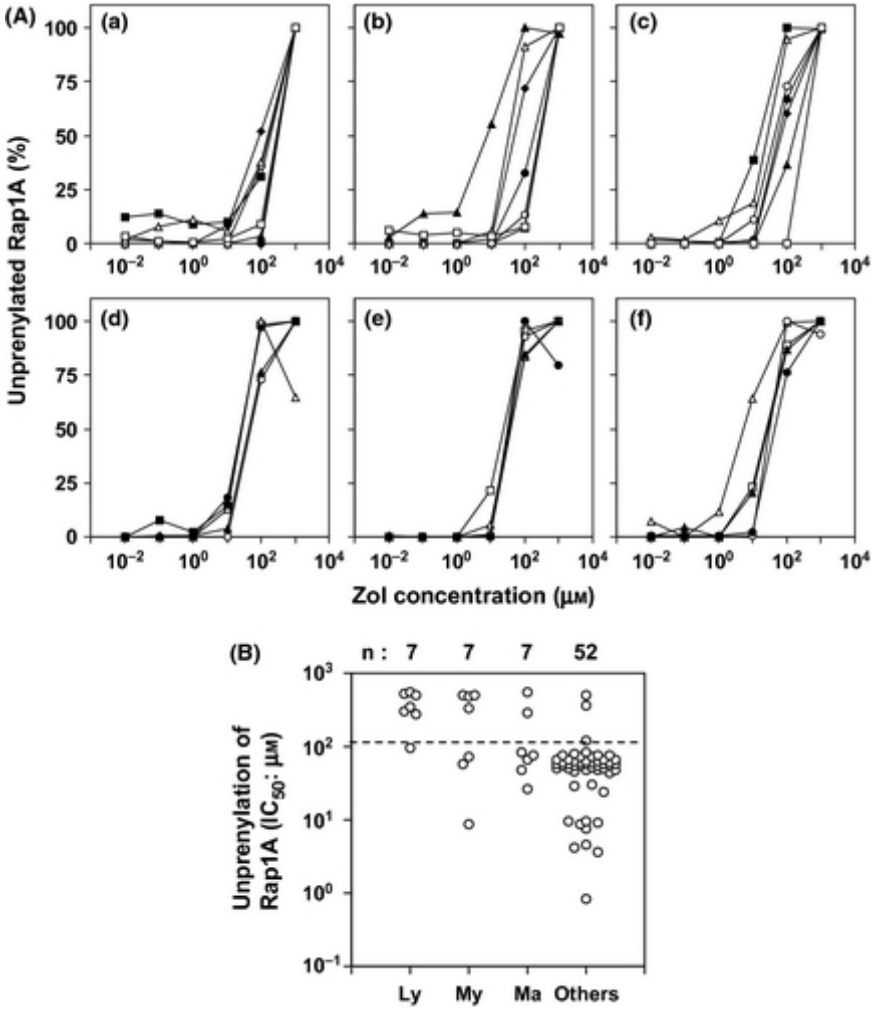
(A)
(a)
(b)
(c)
(d)
(e)
(f)
Unprenylated Rap1A (%)
100
75
50
25
0
10^{-2}
10^{0}
10^{2}
10^{4}
Zol concentration (μM)
(B)
n : 7 7 7 52
Unprenylation of Rap1A (IC_{50}: μM)
10^{3}
10^{2}
10^{1}
10^{0}
10^{-1}
Ly
My
Ma
Others

Fig. 2

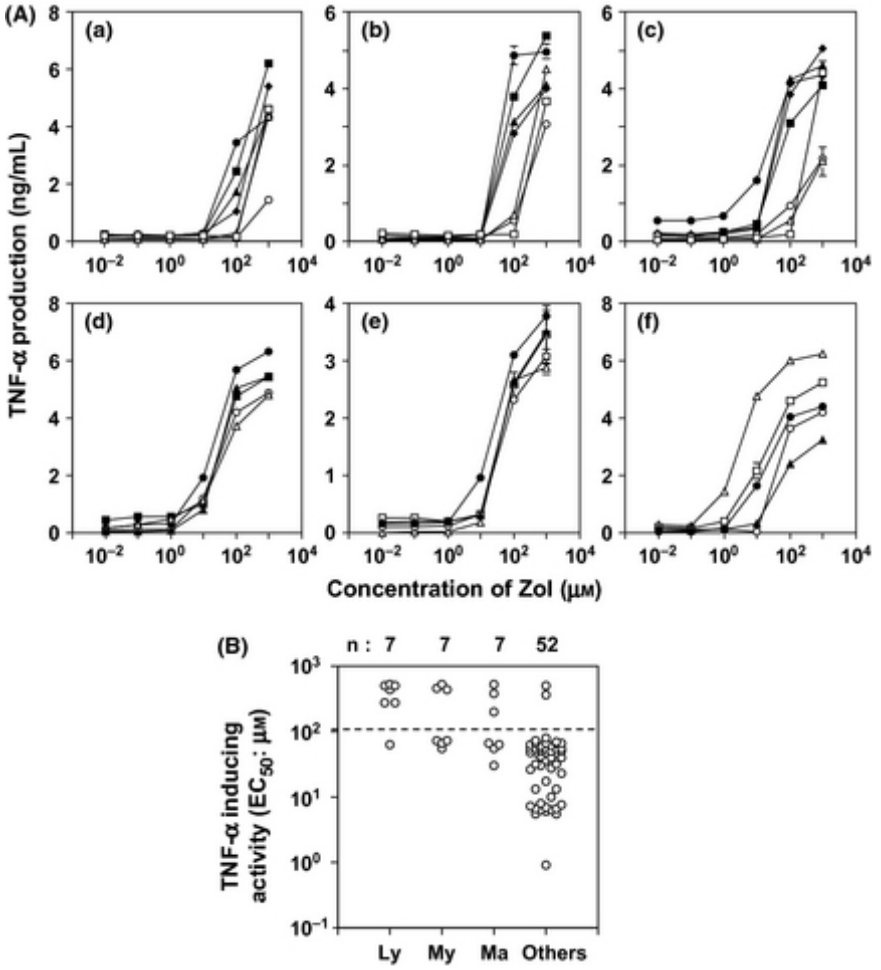
(A)
(a)
(b)
(c)
(d)
(e)
(f)
TNF-α production (ng/mL)
Concentration of Zol (μM)
(B)
n : 7 7 7 52
TNF-α inducing activity (EC50: μM)
Ly
My
Ma
Others

Fig. 3

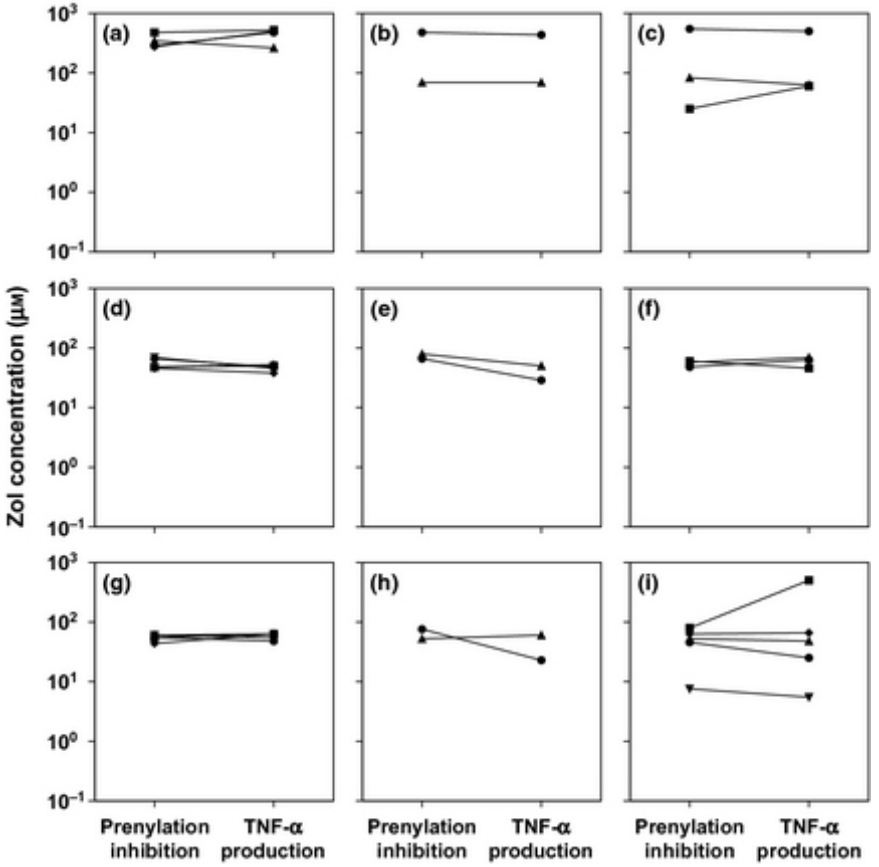
Zol concentration (μM)
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
Prenylation inhibition
TNF-α production

Fig. 4

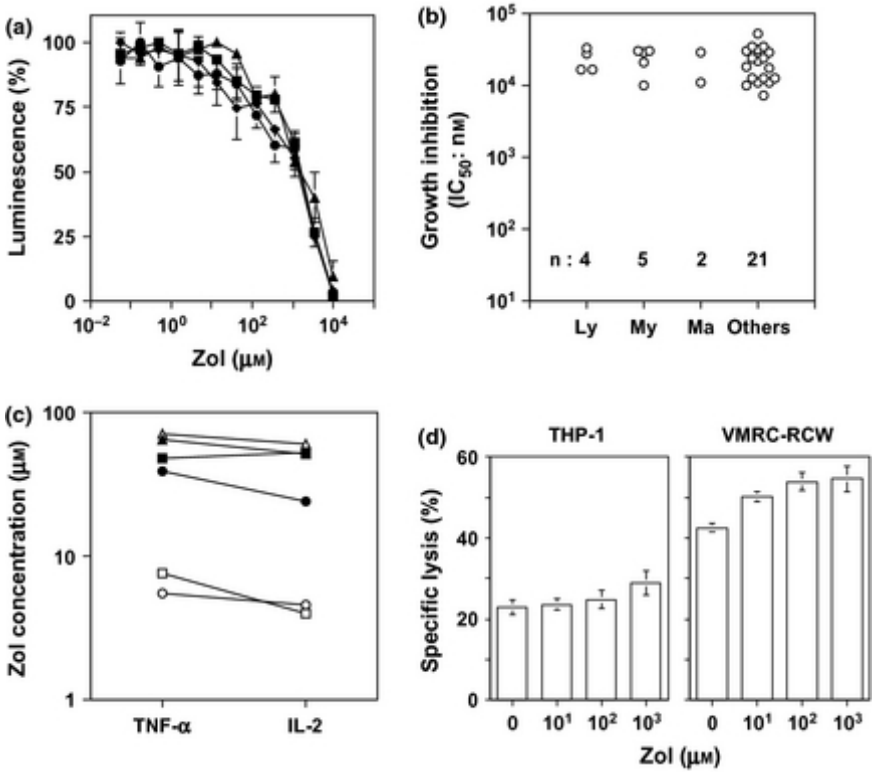
(a)
Luminescence (%)
100
75
50
25
0
10^{-2}
10^{0}
10^{2}
10^{4}
Zol (μM)
(b)
Growth inhibition (IC_{50}: nM)
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
n : 4
5
2
21
Ly
My
Ma
Others
(c)
Zol concentration (μM)
100
10
1
TNF-α
IL-2
(d)
THP-1
VMRC-RCW
Specific lysis (%)
60
40
20
0
0
10^{1}
10^{2}
10^{3}
Zol (μM)

Table S1. List of tumor cell lines used in this study.

Tumor	Description	Source	Tumor	Description	Source
C1R	B lymphoblast	1	TFK-1	Bile duct carcinoma	6
RAMOS-RA1	Burkitt's lymphoma	2	ACS	Gastric carcinoma	6
Raji	Burkitt's lymphoma	2	AGS	Gastric carcinoma	6
J.RT3-T3.5	T cell lymphoma	1	GCIY	Gastric carcinoma	3
MOLT-3	T cell lymphoma	2	KATOIII	Gastric carcinoma	2
MOLT-4	T cell lymphoma	2	MKN1	Gastric carcinoma	2
PEER	T cell lymphoma	2	MKN28	Gastric carcinoma	2
HL60	Monocyte-like cell line	2	MKN74	Gastric carcinoma	2
NOMO-1	Monocyte-like cell line	2	AsPC-1	Pancreatic carcinoma	1
SCC-3	Monocyte-like cell line	2	BxPC-3	Pancreatic carcinoma	1
THP-1	Monocyte-like cell line	2	KP4-1	Pancreatic carcinoma	3
U937	Monocyte-like cell line	2	KP4-2	Pancreatic carcinoma	3
P31/FUJ	Monocyte-like cell line	2	KP4-3	Pancreatic carcinoma	3
K562	Erythrocytoma	2	MIA PaCa-2	Pancreatic carcinoma	2
HMC-1-8	Mammary carcinoma	2	PANC-1	Pancreatic carcinoma	3
MCF-7	Mammary carcinoma	2	PK-1	Pancreatic carcinoma	3
MDA-MB-231	Mammary carcinoma	1	PK-8	Pancreatic carcinoma	3
MRK-nu-1	Mammary carcinoma	2	PK-9	Pancreatic carcinoma	6
SK-BR-3	Mammary carcinoma	1	T3M4	Pancreatic carcinoma	6
T-47-D	Mammary carcinoma	1	HOS	Osteosarcoma	2
YMB-1-E	Mammary carcinoma	2	HuO	Osteosarcoma	7
786-0	Renal cell carcinoma	1	MG-63	Osteosarcoma	2
786-0W	Renal cell carcinoma	4	OST	Osteosarcoma	7
A-704	Renal cell carcinoma	1	Saos-2	Osteosarcoma	3
ACHN	Renal cell carcinoma	1	TAKAO	Osteosarcoma	7
Caki-1	Renal cell carcinoma	2	Colo320	Colorectal carcinoma	2
UOK111	Renal cell carcinoma	5	CW2	Colorectal carcinoma	3
UOK121	Renal cell carcinoma	5	DLD-1	Colorectal carcinoma	2
VMRC-RCW	Renal cell carcinoma	2	C32TG	Melanoma	2
VMRC-RCZ	Renal cell carcinoma	2	G-361	Melanoma	2
EJ-1	Bladder carcinoma	2	LK-2	Lung carcinoma	2
T24	Bladder carcinoma	2	SBC-2	Lung carcinoma	2
TGBC1TKB	Gallbladder carcinoma	3	hu2	Hepatic carcinoma	8
TGBC2TKB	Gallbladder carcinoma	3	GCT-IZ	Osteoclast-like cell line	7
TGBC24TKB	Gallbladder carcinoma	3	PC-3	Prostatic carcinoma	2
HuCCT1	Bile duct carcinoma	2	HT-1080	Fibrosarcoma	2
MZChA2	Bile duct carcinoma	6			

Tumor cell lines indicated as 1 were purchased from American Type Culture Collection, Manassas, VA, U.S.A., 2 from Health Science Research Resources Bank, Sennan, Osaka, Japan, 3 from RIKEN BioResource Center, Tsukuba, Ibaraki, Japan, 4 kindly provided by Dr. Kazuhiro Iwai, Kyoto University, Sakyo, Kyoto, Japan, 5 by Dr. Hirohito Kobayashi, Tokyo Women's Medical University, Shinjuku, Tokyo, Japan, 6 by Dr. Hidenori Tanaka, Kyoto University, Sakyo, Kyoto, Japan, 7 by Dr. Junya Toguchida, Kyoto University, Sakyo, Kyoto, Japan, and 8 by Dr. Atsushi Aruga, Tokyo Women's Medical University, Shinjuku, Tokyo, Japan.

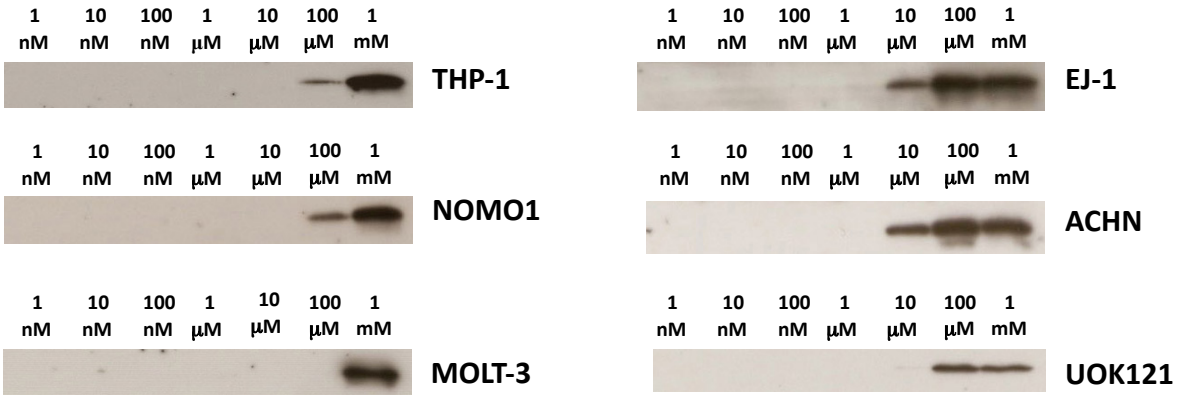

Fig. S1. Zol inhibition of geranylgeranylation of Rap 1A. Tumor cells were resuspended in 90 ml of complete RPMI1640 medium supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO), 10^{-5} M 2-mercaptoethanol (Invitrogen corp., Carlsbad, CA), 100 IU/ml of penicillin (Meiji Seika Kaisha, Ltd., Chuo-Ku, Tokyo, Japan), and 100 μg/ml of streptomycin (Meiji Seika Kaisha, Ltd., Chuo-Ku, Tokyo, Japan) and grown overnight at 37°C with 5% CO_2 in 225 cm^2 flasks. Zol was then added to the flasks to the concentrations indicated above. After incubation for 16 h, the cells were harvested and resuspended in 100 μl of lysis solution containing 1% NP-40 (Wako Pure Chemical Industries Ltd., Chuo-ku, Osaka, Japan), 0.1% sodium dodecyl sulfate (Tokyo Chemistry Industry Co., Ltd., Chuo-Ku, Tokyo, Japan), and 0.5% sodium deoxycholate (Wako) in microcentrifuge tubes. After centrifugation at 15,000 rpm for 10 min, the supernatants were transferred to new tubes and SDS-urea buffer containing 6.7 M urea (Wako), 5% sodium dodecylsulfate (Tokyo Chemistry Industry), 100 mM Tris–HCl buffer, pH 7.4 (Wako), 0.25% bromophenol blue (Wako), and 50 mM dithiothreitol (Wako) were added to give a protein concentration of 5 mg/ml. The samples were loaded on 15% polyacrylamide slab gels (Daiichi Pure Chemicals Co., Ltd., Chuo-ku, Tokyo, Japan) at 50 μg/lane, and electrophoresed at 120 mA/h. Then, the proteins were transferred onto Polyscreen (R) PVDF Transfer Membranes (PerkinElmer Inc., Waltham, MA) treated with goat anti-unprenylated Rap 1A mAb (×500, Santa Cruz Biotechnology Inc., Santa Cruz, CA), and horse radish peroxydase-conjugated anti-goat IgG mAb (×5,000, KPL Inc., Gaithersburg, MD), followed by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). Although not shown, controls using goat anti-Rap1A and anti-GAPDH mAbs (Santa Cruz Biotechnology) were included in this study. Chemiluminescence was detected on Amersham Hyperfilm™ MP (GE Healthcare Ltd., Little Chalfont, Buckinghamshire, UK) using a Fuji Medical Film Processor FPM100 (Fuji Film Co., Ltd., Ashigara, Kanagawa, Japan). Of the 73 tumor cell lines tested, images from six representative cells are shown above. The strength of signal for each protein band was determined by the brightness of the corresponding part of the image scanned using a LAS-4000 mini Luminescent Image Analyzer (Fuji Film Co., Ltd.). The dose-dependency curves in Figure 2 are based on digitalized data.

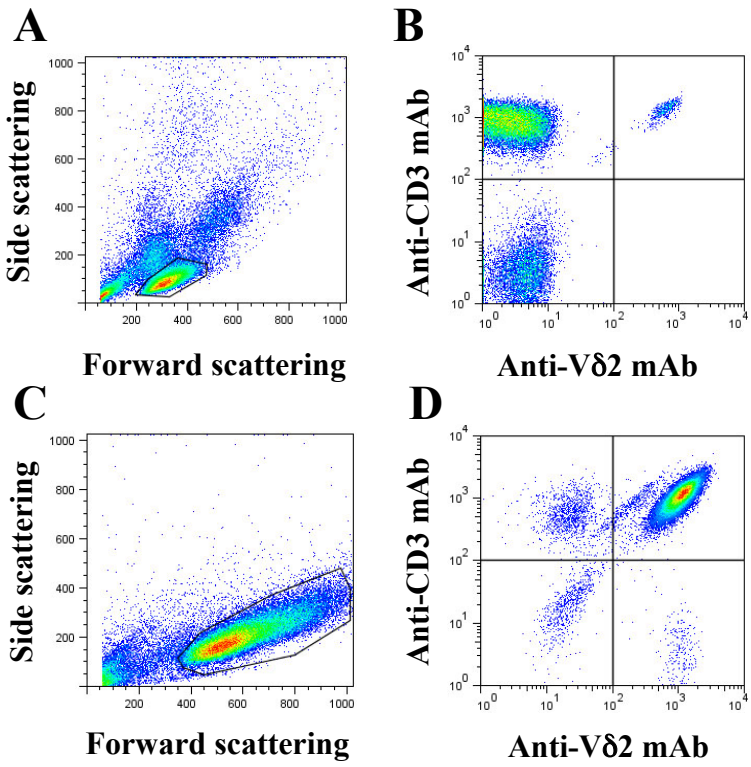

Fig. S2. Flow cytometric analysis of Vδ2$^+$ γδ T cells before and after expansion of PBMC by Zol/IL-2 and the gating strategy. PBMCs before and after stimulation with Zol/IL-2 were stained as described below and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ); Gating strategy (A) and two-color flow cytometric analysis (B) of PBMCs before stimulation with Zol and (C and D) after expansion over 10 days. The proportion of Vδ2$^+$ T cells relative to CD3$^+$ cells was 2.7% and 97.3% before and after expansion. γδ T cells on day 10 were harvested and used for the TNF-α production assay.

Preparation of PBMCs and cell culture. Peripheral blood samples were obtained from a patient with mammary carcinoma after institutional review board approval and with written informed consent. PBMCs were purified by Ficoll-Paque™ PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) gradient centrifugation. The cells were washed two times with PBS, then resuspended in modified Yssel's medium [1] supplemented with 10% human AB serum (Cosmobio., Co., Ltd., Koto-ku, Tokyo, Japan). They were cultured for 10 days at 2.5×10^6/1.5 ml in modified Yssel's medium with 5 μM Zol and 100 U/ml IL-2 (Shionogi Pharmaceutical Co., Ltd., Chuo-ku, Osaka, Japan) in a 24-well plate (Corning Incorp., Corning, NY). The culture medium was replaced everyday from day 2 with fresh medium containing IL-2.

Cell staining. PBMCs before and after expansion were plated out at 2×10^5 cells/50 μl in a 96-well plate (Corning Incorporated, Corning, NY). The cells were then treated with 3 μl of fluorescein isothiocyanate (FITC)-conjugated anti-TCR Vδ2 mAb (Beckman Coulter Inc., Flullerton, CA), and phycoerythrin (PE)-conjugated anti-CD3 mAb (BD Biosciences, San Diego, CA) on ice for 30 min. After being washed three times with PBS, the cells were resuspended in 200 μl of 1% paraformaldehyde in PBS and subjected to flow cytometry.

1.Yssel, H., De Vries, J.E. Koken, M., Van Blitterswijk, W. and Spits,H., Serum-free medium for generation and propagation of functional human cytotoxic and helper T cell clones.
J. Immunol. Method 1984. **72**:219-227.

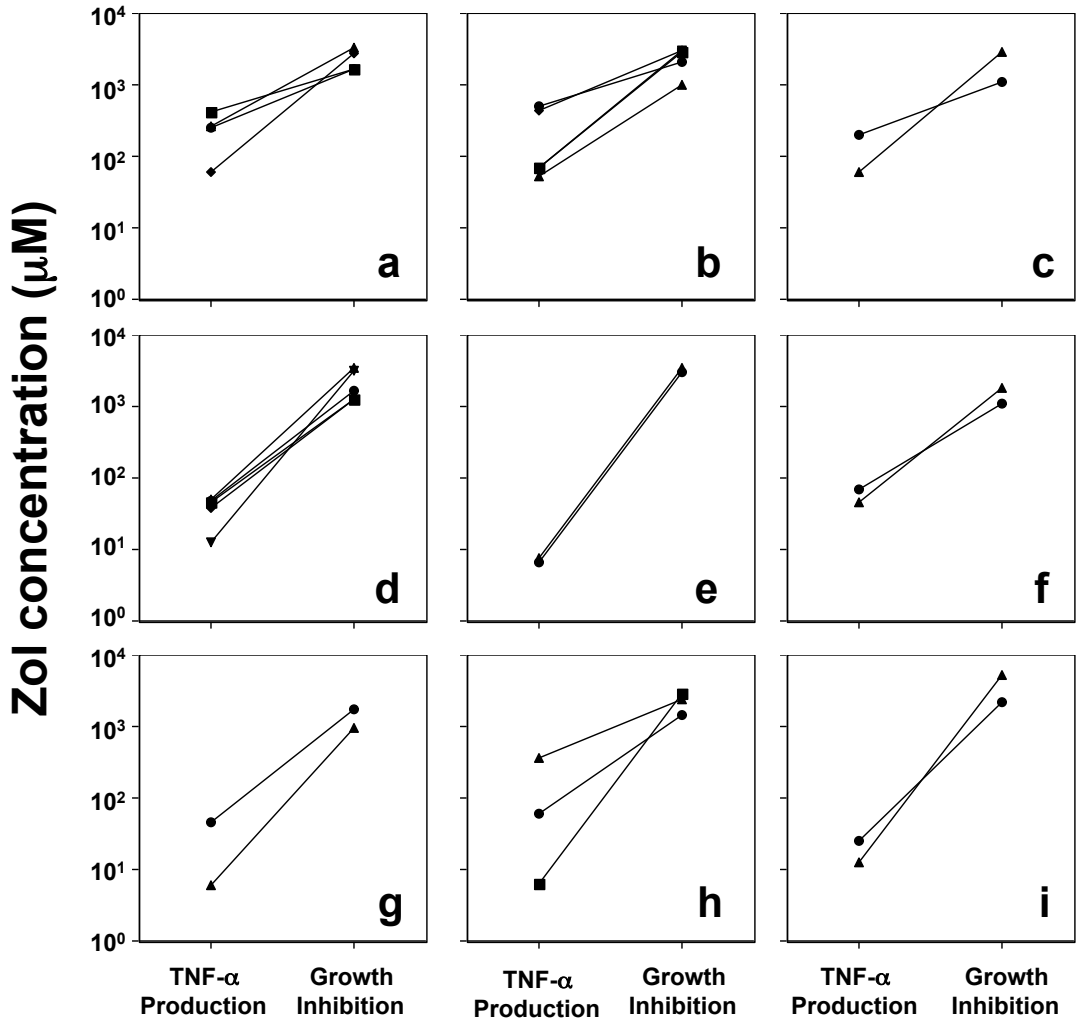

Fig. S3. Comparison between Zol concentrations required for γδ T cell responses and tumor cell growth inhibition. Zol concentrations required for half-maximal inhibition (IC_{50}) of tumor cell growth (Growth Inhibition) were examined as described in Material and Methods and compared with those required for half maximal production (EC_{50}) of TNF-α by γδ T cells stimulated with Zol-pretreated tumor cells (TNF-α Production). Each line connects EC_{50} (TNF-α production) and IC_{50} (Growth inhibition) for the same tumor cell line: lymphomas (a), ● RAMOS-RAI, ▲ Raji, ■ J.RT3-T3.5, ♦ MOLT-4; myeloid cells (b), ● HL60, ▲ NOMO-1, ■ SCC-3, ♦ THP-1, ▼ P31/FUJ; mammary carcinomas (c), ● HMC-1-8, ▲ MDA-MB-231; renal cell carcinoma (d), ● 786-0, ▲ A-704, ■ Caki-1, ♦ UOK121, ▼ VMRC-RCW; melanoma (e), ● C32TG, ▲ G361; gastric carcinoma (f), ● AGS, ▲ MKN1; colorectal carcinoma (g), ● Colo320, ▲ DLD-1; osteosarcoma (h), ● MG63, ▲ OST, ■ SAOS2; lung carcinoma (i), ● LK-2, ▲ SBC-2.