Hepatocellular carcinoma cell sensitivity to Vγ9Vδ2 T lymphocyte-mediated killing is increased by zoledronate

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Abstract. The limited efficacy of vaccines in hepatocellular carcinoma (HCC), due to the low frequency of tumor-infiltrating cytotoxic T lymphocytes (CTLs), indicates the importance of innate immune surveillance, which assists acquired immunity by directly recognizing and eliminating HCC. Innate Vγ9Vδ2 T cells have major histocompatibility complex-unrestricted antitumor activity and are activated by phosphoantigens, which are upregulated in cancer cells by the nitrogen-containing bisphosphonate, zoledronate (Zol). A better understanding of HCC susceptibility to Zol and downstream γδ T cell-mediated killing is essential to optimize γδ T cell-mediated immunotherapy. This study systematically examined the interactions between γδ T cells and Zol-treated HCC cell lines (HepG2, HLE, HLF, HuH-1, JHH5, JHH7, and Li-7) *in vitro*. All HCC cell lines expressed the DNAX accessory molecule-1 ligands, poliovirus receptor, and Nectin-2, and γδ T cell-mediated killing of these cells was significantly enhanced by Zol. Small interfering RNA-mediated knockdown of these ligands did not affect the susceptibility to γδ T cell lysis. This killing activity was partly inhibited by mevastatin, an inhibitor of the mevalonate pathway, and markedly reduced by a monoclonal antibody to γ- and δ-chain T cell receptor, indicating that this is crucial for Zol-induced HCC killing. In addition, Zol-treated HCC cell lines triggered γδ T cell proliferation and induced production of Th1 and Th2, but not Th17, cytokines. The Zol concentration that enhanced HCC cell susceptibility to γδ T cell killing was lower than that required to directly inhibit HCC proliferation. Thus, γδ T cells may be important effector cells in the presence of Zol, especially where there are insufficient number of cancer antigen-specific CTLs to eliminate HCC. Our *in vitro* data support the proposal that Zol-treatment, combined with adaptive γδ T cell immunotherapy, may provide a feasible and effective approach for treatment of HCC.

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Abbreviations: CTL, cytotoxic T lymphocyte; DNAM-1, DNAX accessory molecule-1; FPP, farnesyl pyrophosphate; GM-CSF, granulocyte macrophage colony-stimulating factor; GPC3, glypican-3; HCC, hepatocellular carcinoma; IL, interleukin; Ig, immunoglobulin; IFN-γ, interferon-γ; IPP, isopentenyl pyrophosphate; MHC, major histocompatibility complex; MICA/B, MHC class I chain-related A/B; mAb, monoclonal antibody; MTT, 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide; NKG2D, natural killer group 2D; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; PD-1, programmed cell death-1; PVR, poliovirus receptor; TCR, T cell receptor; TIGIT, T cell immunoreceptor with Ig and ITIM domains; TNF-α, tumor necrosis factor-α; ULBP, UL 16-binding protein; Zol, zoledronate

Key words: cancer immunotherapy, hepatocellular carcinoma, γδ T cell, zoledronate, isopentenyl pyrophosphate

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and is the leading cause of cancer death (1). It is characterized by a poor prognosis due to a high potential for metastasis and recurrence within a short time. The tyrosine kinase inhibitor, sorafenib, was recently shown to prolong overall survival in patients with advanced HCC and has become the standard drug for first-line systemic treatment (1-3). However, only a minority of patients show objective evidence of a clinical response and their median overall survival remains less than one year. These findings, and the incidence of adverse reactions, suggest that novel treatment approaches are required.

Immunotherapy has now been clinically validated as an effective treatment for cancer (4-6). Tumor antigen-specific immune targeting by cytotoxic T lymphocytes (CTLs) may provide an additional treatment modality to eliminate residual

disease and prevent relapse in HCC patients. We have reported that glypican-3 (GPC3), an oncofetal antigen overexpressed in HCC, is a useful target for CTL-mediated cancer immunotherapy and we performed clinical trials of a GPC3 peptide vaccine (7,8). GPC3 peptide-specific CTLs were detected in the peripheral blood of some vaccinated patients and a very small number of the CTLs were also present in the HCC tissue, indicating an ongoing local immune response (8). However, this tumor response was rare and many HCC patients are unlikely to benefit from a vaccination strategy targeting a single CTL epitope. The rarity of tumor antigen-specific CTLs in the T cell repertoire in the thymus suggests a requirement for a type of therapy that coordinates the interplay between the innate and acquired immune systems in order to eradicate HCC (9).

γδ T cells are a small subset of T lymphocytes expressing γ- and δ-chain T cell receptor (γδ TCR); these occur at a frequency of 0.5-10% of total T lymphocytes in peripheral blood and play an important role in innate immune surveillance (10,11). The most abundant circulating γδ T cells, Vγ9Vδ2 T cells, can be activated by small non-peptide phosphorylated antigens synthesized in the mevalonate pathway, major histocompatibility complex (MHC) class I-related molecules A/B (MICA/B), various members of the UL16-binding protein (ULBP) family, or poliovirus receptor (PVR, nectin-like molecule 5, CD155) in an MHC-independent manner (12-15).

A nitrogen-containing bisphosphonate, zoledronate (Zol), which is widely used to prevent bone metastasis, increases the isopentenyl pyrophosphate (IPP) mevalonate metabolite in some tumor cells by inhibiting farnesyl pyrophosphate (FPP) synthase, leading to γδ T cell-mediated killing of malignant tumor cells (16-18). This process has been used in clinical trials of γδ T cell-based immunotherapy for patients with several cancers and was shown to be beneficial and safe, providing a promising therapeutic strategy (19). Although *in vivo* and *in vitro* studies indicated that Zol rendered many types of tumor cells susceptible to γδ T cell-mediated killing, there has not been a systematic examination of whether HCC would respond to immunotherapy using γδ T cells and Zol. The present study comprehensively examined the expression of γδ T cell ligands on a variety of HCC cell lines and the effects of Zol treatment on the responses of γδ T cells. We demonstrated that the γδ T cell-mediated killing of all examined HCC cell lines was significantly enhanced by Zol treatment, indicating that the recognition of Zol-treated HCC cell lines by γδ T cells was likely γδ T cell receptor-dependent. In addition, Zol-treated HCC cell lines triggered γδ T cell proliferation and cytokine productions. Our findings could contribute to the development of an immunotherapeutic approach combining Zol with γδ T cells for the treatment of HCC.

Materials and methods

Cytokines and chemicals. Recombinant human interleukin (IL)-2 and IL-15 were purchased from Nipro (Osaka, Japan) and PeproTech Inc., (Rocky Hill, NJ, USA). Zol (Zometa) was purchased from Novartis (Basel, Switzerland). Mevastatin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Antibodies. Anti-ULBP1 (170818), anti-ULBP2 (165903), anti-ULBP3 (166510), anti-natural killer group 2D (NKG2D) (140810), and mouse immunoglobulin (Ig) G2a (20102) were purchased from R&D Systems (Minneapolis, MN, USA). Anti-MICA/B (6D4), anti-CD3 (UCTH1), anti-Nectin-2 (TX31), anti-PVR (SKII.4), anti-DNAX accessory molecule-1 (DNAM-1) (11A8), anti-NKG2D (1D11), anti-CD27 (O323), anti-CD45RA (H100), mouse IgG2b, κ (MPC-11) and mouse IgG1, κ (MOPC-21) were purchased from BioLegend (San Diego, CA, USA). Anti-TCRVγ9 (IMMU360) and anti-TCR-pan-γδ (IMMU510) were purchased from Beckman Coulter (Fullerton, CA, USA). Anti-DNAM-1 (DX11) was from Abcam (Cambridge, UK).

Cells. Human HCC cell lines (HLE, HLF, HuH-1, JHH5, and JHH7) were purchased from the Health Science Research Resources Bank (Osaka, Japan). The HepG2 and Li-7 HCC cell lines, the T2 lymphoblastoid cell line, and the K562 erythroleukemia cell line were purchased from the RIKEN BioResource Center (Ibaraki, Japan). The EJ1 bladder cancer cell line was provided by the Cell Resource Center for Biomedical Research (Miyagi, Japan). The pancreatic cancer cell line, MIAPaCa-2, was purchased from the American Type Culture Collection (Rockville, MD, USA). All HCC cell lines, EJ1, and MIAPaCa-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 100 μg/ml L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA). T2 cells and K562 cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640; Sigma-Aldrich) supplemented with 100 μg/ml L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. Phytohemagglutinin (PHA) blasts were obtained by stimulating peripheral blood mononuclear cells (PBMCs) with PHA (Sigma-Aldrich; 1 μg/ml) in AIM-V medium (Gibco, Grand Island, NY, USA) supplemented with 10% human AB serum and IL-2 (100 IU/ml). Peripheral blood mononuclear cells from healthy donors were purchased from Cellular Technology Ltd. (Cleveland, OH, USA).

γδ T cells. $CD3^+V\gamma9^+$ cells were isolated using an automated cell sorter (FACS Aria II; BD Biosciences, San Jose, CA, USA), seeded in a 96-well plate, and stimulated by PHA (1 μg/ml) in the presence of irradiated (100 Gy) allogeneic PBMCs ($8.0x10^4$ cells/well) as feeder cells in AIM-V medium supplemented with 10% human AB serum, IL-2 (100 IU/ml), and IL-15 (10 ng/ml).

Flow cytometry. Cell samples were treated with human γ-globulin (Sigma-Aldrich) for 10 min in order to block Fc-receptors, stained with the relevant fluorochrome-conjugated monoclonal antibody (mAb) for 20 min, and washed with phosphate-buffered saline containing 2% FBS. The stained cell samples were analyzed on a flow cytometer (FACSCanto II; BD Bioscience) and the data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Cell proliferation. Cell proliferation was evaluated by a standard MTT assay and by [^{3}H]-thymidine incorporation assay. For the MTT assay, cells were seeded in 96-well culture plates

($4.0x10^3$ cells/well) and incubated with Zol. After 72 h, MTT reagent was added directly to the medium and incubated for 4 h. The supernatant was removed, and 200 μl of dimethyl sulfoxide was added to each well and thoroughly mixed for 3 min. The absorbance of the converted dye at 595 nm was measured on a Multiskan FC microplate reader (Thermo Scientific, Vantaa, Finland). For [^{3}H]-thymidine incorporation assays, the cells were cultured in 96-well culture plates ($5.0x10^3$ cells/well) for 72 h and 37 kBq/well [^{3}H]-thymidine was added to the culture for the last 16 h. The incorporated radioactivity was then measured by scintillation counting (MicroBeta2 LumiJET; PerkinElmer, Waltham, MA, USA).

Cytotoxicity assay. Target cells were labeled with Calcein-AM (Dojindo, Kumamoto, Japan) for 30 min at 37˚C and washed three times. The labeled cells were then incubated with effector cells at various effector: target (E:T) ratios. Cytotoxic activity against target cells was measured using the Terascan VPC system (Minerva Tech, Tokyo, Japan). Fluorescent intensity was measured before and after the 4-h incubation, and specific cytotoxic activity was calculated using the following formula: % cytotoxicity = {1- [(average fluorescence of the sample wells - average fluorescence of the maximal release control wells) / (average fluorescence of the minimal release control wells - average fluorescence of the maximal release control wells)]} x 100%.

Cytokine measurements. The cytokine levels in the culture supernatants were evaluated using Cytometric Bead Array Flex Sets (BD Bioscience), according to the manufacturer's protocol. The resulting data were analyzed using FCAP Array Software 3.0.

Small interfering RNA. Predesigned small interfering RNA (siRNA) targeting human Nectin-2, PVR, or a negative control siRNA (Stealth pre-designed RNAi siRNA; Invitrogen, CA, USA) were transfected into HCC cell lines in a 12-well plate using Lipofectamine RNAiMAX transfection reagent (Invitrogen), according to the manufacturer's protocol.

Statistical analysis. The statistical significance of differences was determined using one-way analysis of variance (ANOVA) with the Bonferroni post-hoc test. P-values <0.05 were considered to denote statistical significance.

Results

Expression of NKG2D and DNAM-1 ligands by HCC cells. NKG2D and DNAM-1 are known to positively modulate the cytotoxicity of γδ T cells (12,20). To investigate the effect of Zol on γδ T cell-mediated cytotoxicity towards HCC, we first examined the expression of surface NKG2D ligands (MICA/B and ULBP1-3) and DNAM-1 ligands (Nectin-2 and PVR) on a variety of HCC cell lines, as compared to γδ T cell-sensitive or non-sensitive cell lines (Fig. 1). The EJ1 bladder carcinoma cell line is known to be sensitive to γδ T cell-mediated lysis and expressed NKG2D ligands (MICA/B and ULBP1-3) and DNAM-1 ligands (Nectin-2 and PVR). In contrast, T2 cells show very little sensitivity to γδ T cell-mediated lysis and only expressed MICA/B. Although they showed different expression patterns and levels of MICA/B and ULBP1-3 (ranging from minimal to undetectable), all HCC cell lines consistently expressed both Nectin-2 and PVR. These results suggested that some ligands that may activate γδ T cells were expressed on all HCC cell lines. It also indicated that they may be susceptible to γδ T cell-mediated lysis.

Enhanced γδ T cell reactivity against Zol-treated HCC cells. Zol is a potent inhibitor of FPP synthase and this modifies the mevalonate pathway, leading to the accumulation of IPP mevalonate metabolites. IPP acts as a phosphoantigen and activates the expansion of human γδ T cells, which then exhibit cytotoxicity against Zol-sensitized malignant tumor cells (21-23). We isolated γδ T cells from PBMCs and stimulated with PHA in the presence of IL-2 and IL-15. In contrast to the γδ T cells in freshly isolated PBMCs, which showed $CD27^+CD45RA^+$ naïve or $CD27^+CD45RA^-$ central memory phenotypes, γδ T cells that had been expanded for 14 days showed a $CD27^-CD45RA^-$ effector memory phenotype (Fig. 2A). We examined the cytotoxic activity of these expanded γδ T cells against HCC cell lines that had been preincubated with or without Zol (Fig. 2B). In the absence of Zol pretreatment, γδ T cells showed weak cytotoxicity against HCC cell lines, but not against T2 cells. These weak cytotoxic activities toward HCC cell lines were possibly mediated by DNAM-1/PVR interactions (24). Irrespective of Zol treatment, T2 cells do not express PVR or Nectin-2. Accordingly, these molecules are not essential for the killing of T2 cells by γδ T cells. It is conceivable that Zol treatment may not induce the accumulation of IPP in T2 cells. The cytotoxicity of γδ T cells against all HCC cell lines was remarkably enhanced following pretreatment with 5 μM Zol. Since γδ T cells are multifunctional inflammatory cells that produce various cytokines and cytotoxic molecules, we next examined the production of cytokines [IL-2, IL-4, IL-5, IL-10, IL-13, IL-17A, interferon-γ (IFN-γ), granulocyte macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α)] and granzyme B in response to HCC cell lines that had been preincubated with or without Zol (Fig. 2C). Most Zol-treated HCC cell lines triggered the production of IL-4, IL-5, IL-13, IFN-γ, GM-CSF, TNF-α, and granzyme B by γδ T cells, but not that of IL-2, IL-10, and IL-17A. However, Zol treatment of T2 cells, which showed very little sensitivity to γδ T cell-mediated lysis, did not stimulate production of these cytokines. In addition, γδ T cells showed enhanced proliferative responses in the presence of Zol-treated HCC cell lines, as compared to the untreated cells (Fig. 2D). These results suggested that Zol treatment of HCC cell lines considerably enhanced the cytotoxicity, cytokine production, and proliferation of γδ T cells. The cytokine production and proliferation of γδ T cells in the presence of Zol-treated HCC cell lines tended to correlate with the γδ T cell-mediated cytotoxicity.

Enhanced sensitivity of Zol-treated HCC cell lines to γδ TCRs. γδ T cells express TCR Vγ9, NKG2D, and DNAM-1, and can recognize target cells via interactions with individual receptors or with a combination of receptors (Fig. 3A) (12,25,26). To determine the contributions of TCR, NKG2D, and DNAM-1 to the recognition of Zol-treated HCC cell lines, we performed cytotoxicity assays in the presence of blocking mAbs for these receptors. Zol-pretreated HCC cell lines were lysed

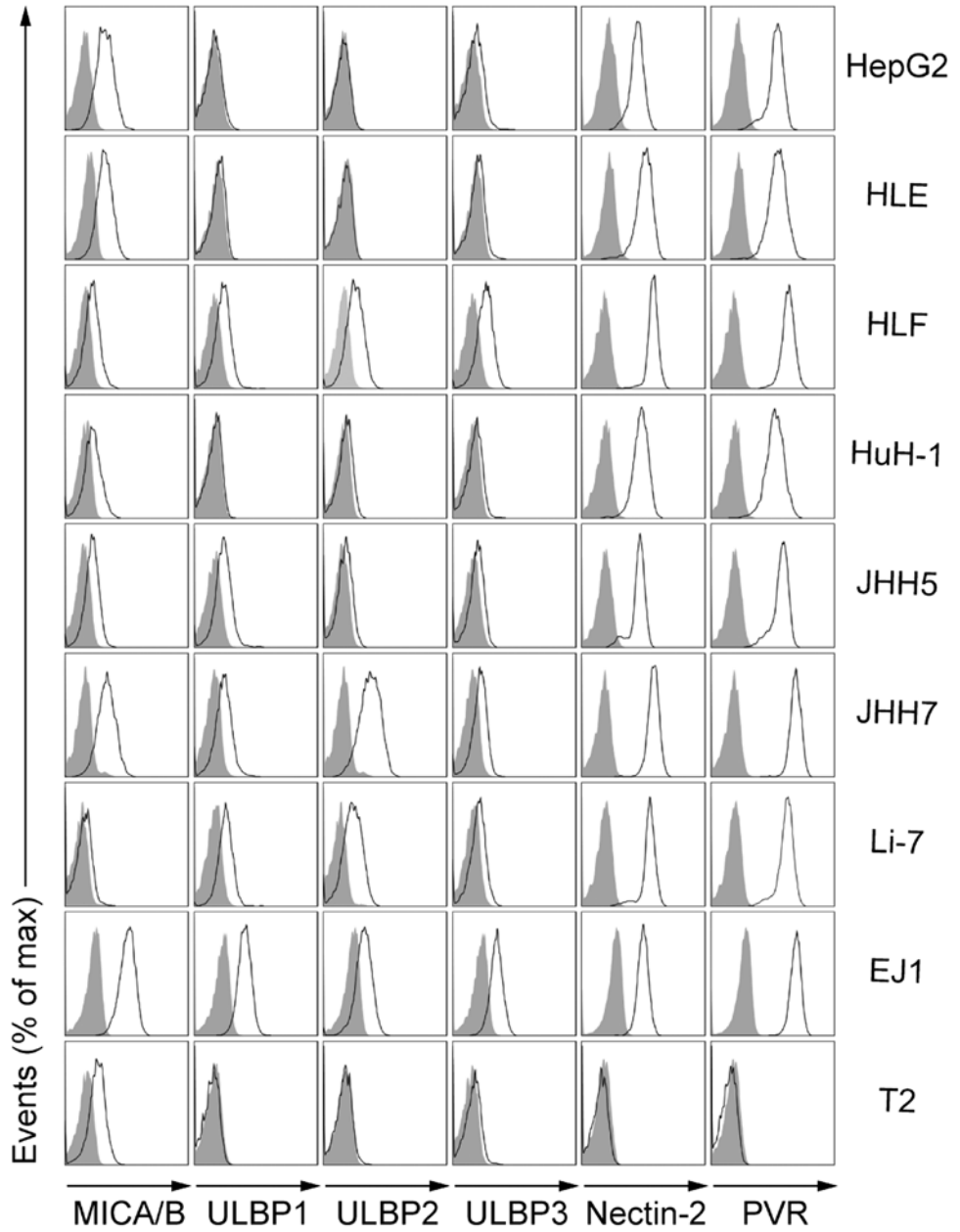

Figure 1. Expression of NKG2D and DNAM-1 ligands by HCC cell lines. Representative flow cytometry profiles showing surface-expressed NKG2D ligands (MICA/B, ULBP1, ULBP2, and ULBP3) and DNAM-1 ligands (Nectin-2 and PVR) on the indicated HCC cell lines (HepG2, HLE, HLF, HuH-1, JHH5, JHH7, and Li-7). EJ1 and T2 cell lines served as references. Open histograms represent specific staining for the indicated molecules, while gray histograms represent isotype control staining.

by γδ T cells at an E:T ratio of 30:1. The addition of anti-γδ TCR mAb markedly inhibited the cell lysis of all HCC cell lines tested (Fig. 3B). However, the addition of anti-NKG2D or anti-DNAM-1 mAbs only produced marginal inhibition of cell lysis. Zol treatment was not associated with any significant change in the expression of NKG2D or DNAM-1 ligands in any of the HCC cell lines tested (Fig. 3C). In addition, siRNA-mediated Nectin-2 and PVR knockdown had no effect on their susceptibility to γδ T cell-mediated lysis (Fig. 3D and E). These results indicated that γδ TCRs were important for the recognition of Zol-treated HCC cells.

Inhibition of γδ T cell recognition of Zol-treated HCC cell lines by mevastatin. The cytotoxicity of γδ T cells towards Zol-treated HCC cells decreased in the presence of mevastatin, which blocked IPP synthesis (Fig. 4). This finding suggests that increased levels of mevalonate pathway metabolites, such as IPP, are likely to be the ligands responsible for the enhanced susceptibility of HCC cells to γδ T cell-mediated killing.

γδ T cells exert specific killing activity against Zol-treated HCC cell lines without damaging Zol-untreated cells. To investigate the non-specific killing of target cells by activated γδ T cells, γδ T cells were co-cultured with Zol-treated HepG2 cells and with an equal number of Calcein-AM-labeled, Zol-untreated cells, and the killing activity against Zol-untreated cells was determined (Fig. 5). Activation of

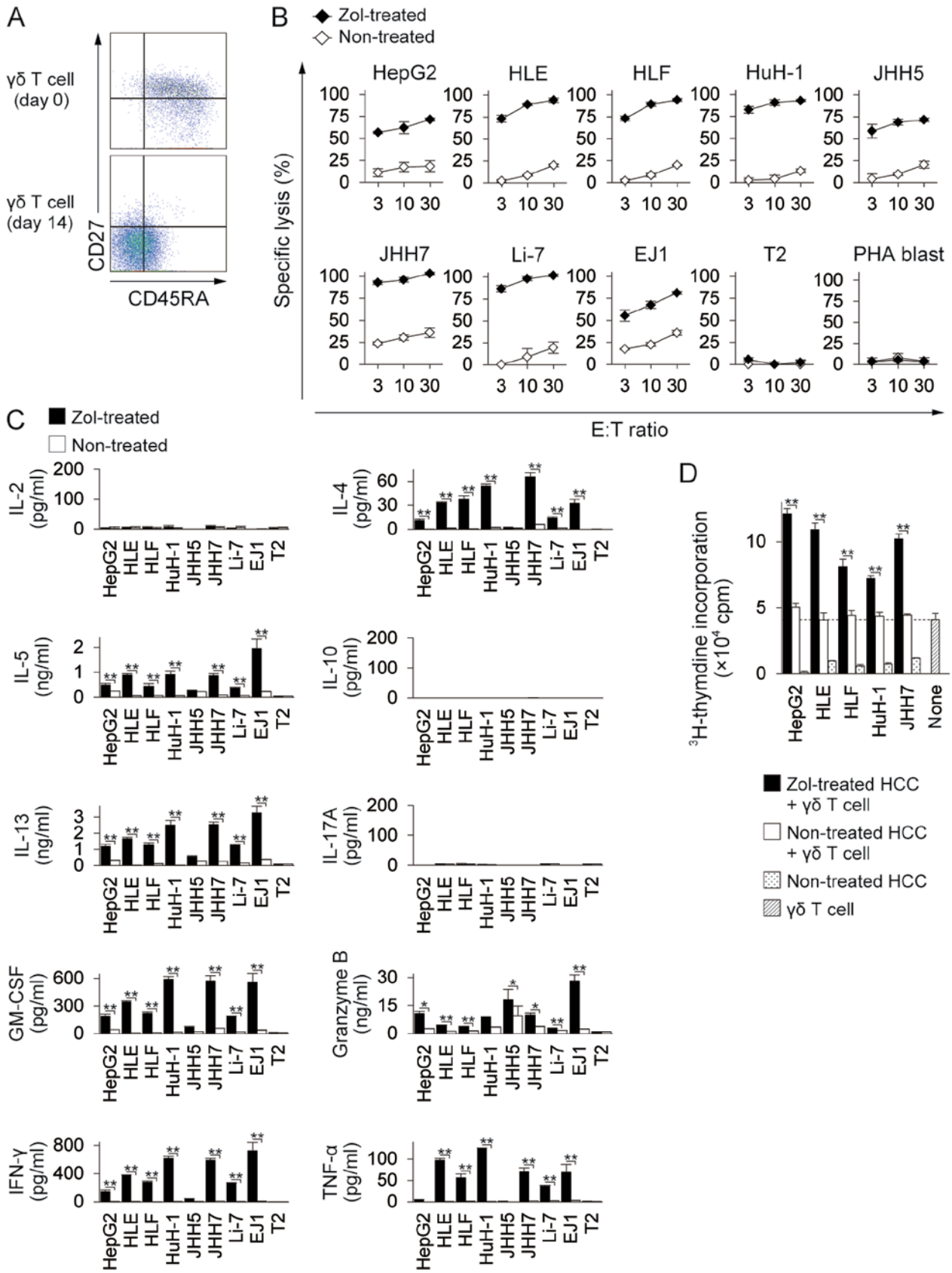

Figure 2. γδ T cell effects on Zol-treated HCC cell lines. (A) Maturation stage of γδ T cells, indicated by CD27 and CD45RA expression. Upper, representative plot of CD3$^+$TCRVγ9$^+$ γδ T cells gated from healthy donor PBMCs. Lower, maturation of CD3$^+$TCRVγ9$^+$ γδ T cells in 14-day cultures stimulated with PHA in the presence of IL-2 and IL-15. (B) Cytotoxic activity of γδ T cells towards target cells preincubated for 16 h in the presence or absence of 5 μM Zol. γδ T effector (E) cells were co-cultured for 4 h with indicated target (T) cells (1.0x10^4 cells/well) at the indicated E:T ratios. EJ1 cells, T2 cells, and PHA blast served as references. Data shown are representative of three independent experiments. (C) Cytokine production by γδ T cells (1.0x10^5 cells/well) after 24-h co-culture with the indicated target cells (5.0x10^4 cells/well). Data represent mean ± SD of triplicate cultures. (D) Proliferative response of γδ T cells (5.0x10^4 cells/well) co-cultured with irradiated (90 Gy) HCC cell lines (5.0x10^3 cells/well) for 72 h, as determined by [^{3}H]-thymidine incorporation assay. Data represent mean ± SD of triplicate cultures. Significance was determined by a one-way ANOVA with the Bonferroni post-hoc test; *P<0.05; **P<0.01.

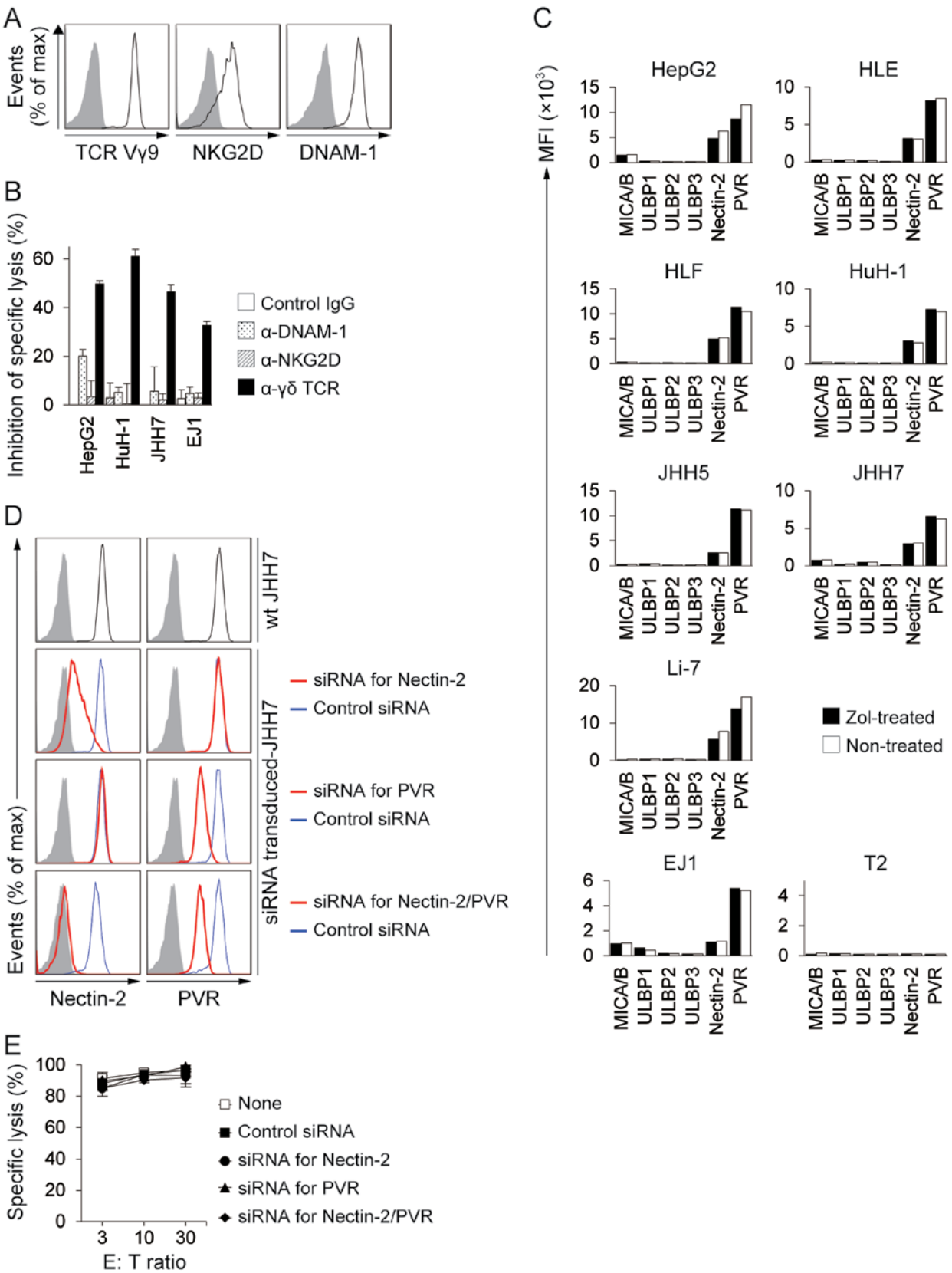

Figure 3. γδ T cells predominantly recognize Zol-treated HCC cell lines via γδ TCRs. (A) Representative flow cytometry profiles of surface expression of TCRVγ9, NKG2D, and DNAM-1 in γδ T cell lines. Open histograms represent specific staining for the indicated molecules, while gray histograms show isotype control staining. (B) Inhibition of γδ T cell-mediated cytotoxicity by mAbs raised against the indicated proteins. γδ T cells were preincubated for 60 min with a saturating mAb concentration and subsequently washed and co-cultured for 4 h with the indicated target cells ($1.0x10^4$ cells/well) at an E:T ratio of 30:1. Data are the mean ± SD of triplicate cultures and represent at least three independent culture experiments. (C) Mean fluorescence intensity (MFI) of the indicated ligands on HCC cell lines preincubated for 16 h with or without 5 μM Zol. (D) Representative flow cytometry profiles of the surface expression of Nectin-2 or PVR in JHH7 wild-type (wt) cells or those transfected with the indicated siRNA(s). Open histograms represent specific staining for the indicated molecules (blue, control siRNA; red, siRNA for indicated molecules), while gray histograms represent isotype control staining. (E) Knockdown of DNAM-1 ligands did not affect HCC susceptibility to γδ T cell-mediated lysis. γδ T cells were co-cultured for 4 h with HCC cell lines transfected with the indicated siRNA at the indicated E:T ratios. Data represent mean ± SD of triplicate cultures.

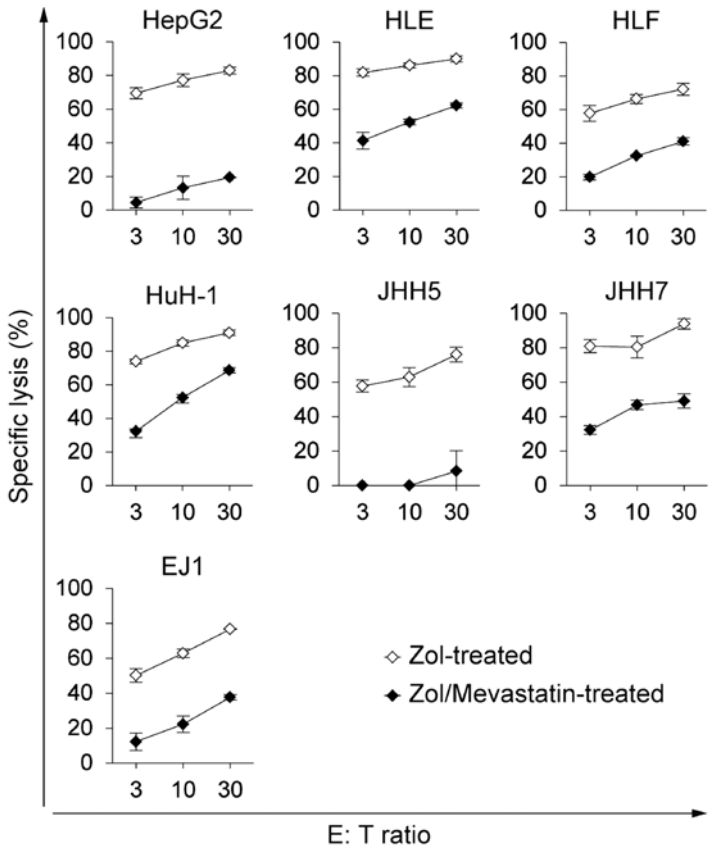

Figure 4. Mevastatin pretreatment of HCC cell lines reduced their susceptibility to Zol-induced γδ T cell lysis. The indicated target HCC cell lines ($1.0x10^4$ cells/well) were pretreated with 5 μM Zol for 16 h in the presence or absence of 100 μM mevastatin prior to 4-h co-culture with γδ T cells at the indicated E:T ratios. Data represent mean ± SD of triplicate cultures. Representative results of three independent experiments are shown.

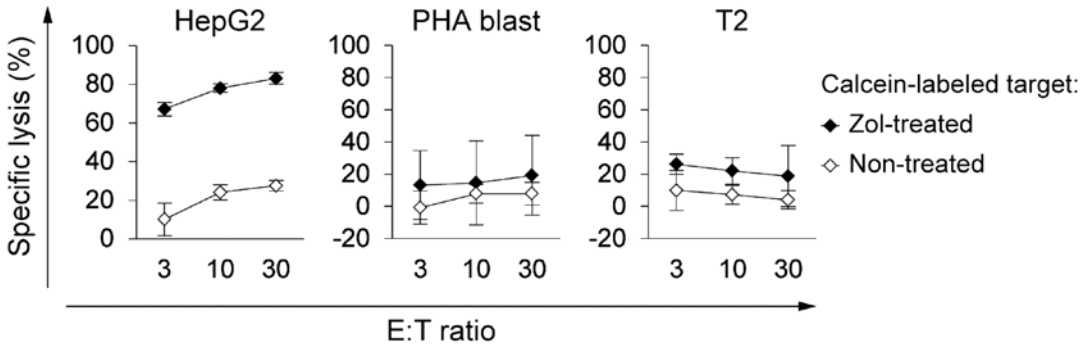

Figure 5. γδ T cells specifically kill Zol-treated HCC cell lines without killing Zol-untreated cells. Bystander cytotoxic activity of γδ T cells was determined in Zol-untreated target cells (HepG2, T2, or PHA blasts). γδ T effector (E) cells ($3.0x10^5$ cells/well) were co-cultured for 4 h with 5 μM Zol-pretreated (16 h) HepG2 cells ($1.0x10^4$ cells/well), along with Calcein-AM-labeled, Zol-untreated target (T) cells ($1.0x10^4$ cells). Zol-pretreated (5 μM, 16 h) HepG2, T2, or PHA blasts served as references. Data represent the mean ± SD of triplicate cultures. Representative results of three independent experiments are shown.

γδ T cells by Zol-treated HepG2 cells did not produce any cytotoxic effects on Zol-untreated HepG2, T2, or PHA blasts. In contrast, Zol-treated HepG2 cells were effectively killed by activated γδ T cells. Cells that were not susceptible to Zol, such as T2 and PHA blasts, were not killed, regardless of Zol treatment. These results suggested that activated γδ T cells specifically killed Zol-treated HCC cells, without affecting the Zol-untreated cells.

Lower Zol concentration enhances HCC susceptibility to γδ T cell killing than that required to inhibit HCC proliferation. Zol exerts direct antitumor effects on HCC cells, inhibiting cell proliferation, migration, and adhesion (27). We found striking differences between the concentration of Zol required to sensitize HCC cells to γδ T cell-mediated killing and that required to directly inhibit HCC proliferation. Zol consistently inhibited the proliferation of all HCC cell lines at concentrations >10-15 μM (Fig. 6A). In contrast, Zol consistently enhanced HCC susceptibility to γδ T cell killing at concentrations above 1.0-1.5 μM (Fig. 6B). In addition, the concentration of Zol required for these effects in HCCs was one or two orders of magnitude lower than those required in K562, MIAPaCa-2,

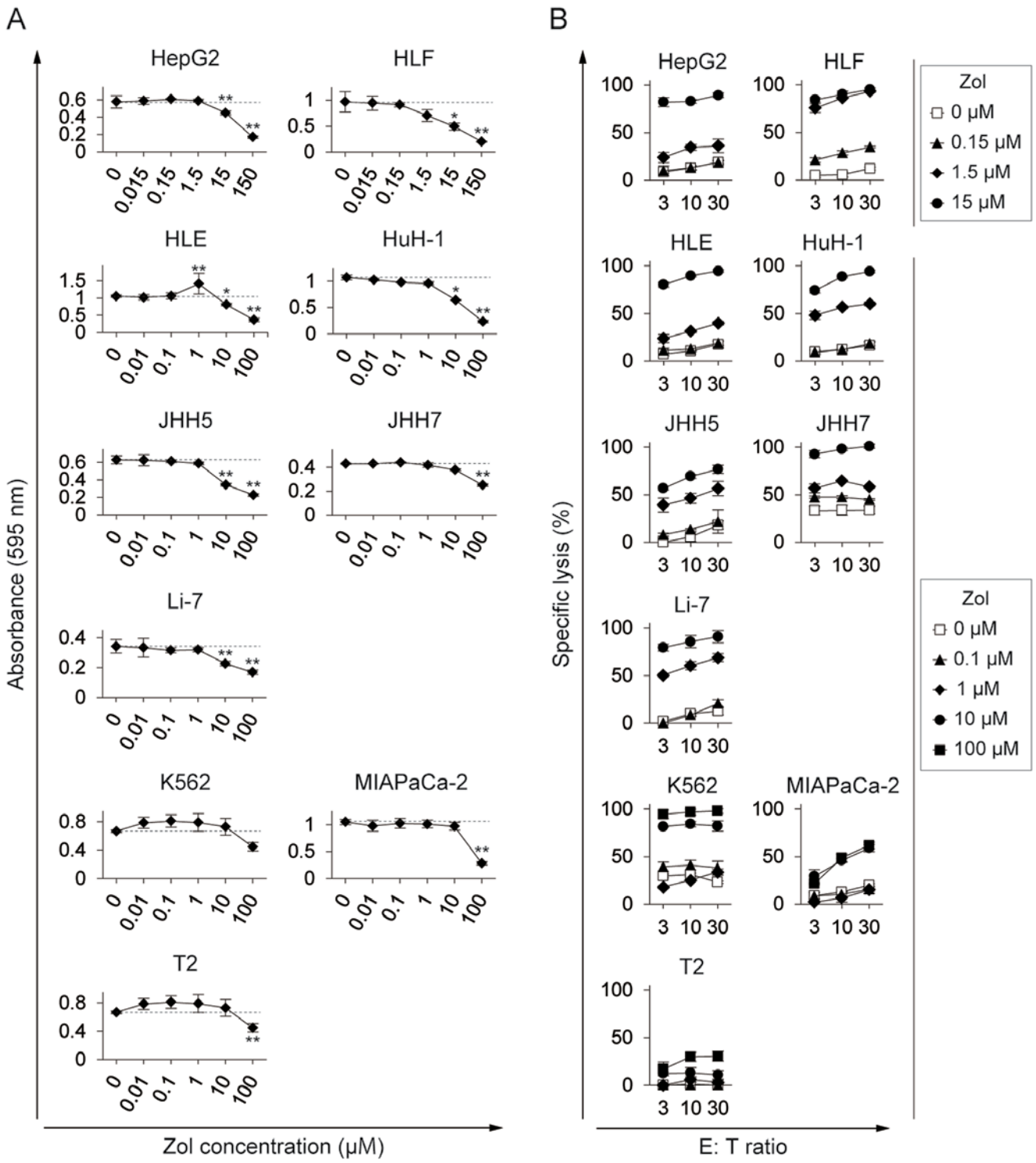

Figure 6. Low levels of Zol sensitized HCC cell lines to γδ T cell-mediated lysis. (A) High concentrations of Zol directly inhibited HCC cell proliferation. HCC cell lines were incubated with the indicated concentrations of Zol for three days in 96-well culture plates ($4.0x10^3$ cells/well) and proliferation was determined by MTT assay. Data represent the mean ± SD of triplicate cultures. (B) Target HCC cell lines ($1.0x10^4$ cells/well) were incubated with the indicated concentrations of Zol for 16 h prior to co-culture with γδ T cells for 4 h at the indicated E:T ratios. K562, MIAPaCa-2 and T2 cell lines served as references. Data represent the mean ± SD of triplicate cultures. The representative results of three independent experiments are shown. Significance was determined by a one-way ANOVA with the Bonferroni post-hoc test; $^{*}P<0.05$; $^{**}P<0.01$).

and T2 cells. This indicated that Zol treatment may serve to potentiate the effector functions of γδ T cells in many patients with HCC at a dose below that required to directly inhibit tumor proliferation.

Discussion

The anti-HCC potential of γδ T cells and the mechanisms involved in their cytotoxic activity have been well character-

ized previously (24,27-30). However, most of these studies have investigated primary HCC cells or only investigated a limited number of HCC cell lines, such as HepG2 and HuH-7. In the present study, we used a variety of HCC cell lines and found *in vitro* evidence supporting the roles of γδ T cells and Zol in immune surveillance against HCCs.

Some HCC cell lines are known to be targets for γδ T cells, either spontaneously or after Zol treatment (28,29). HCC cells may show impaired regulation of the mevalonate pathway and the endogenous mevalonate metabolite, IPP, can be recognized by γδ TCRs (31). The interaction between the costimulatory receptor, NKG2D, and its ligands is considered to play a role in triggering γδ T cell cytotoxicity (14,20,31). DNAM-1 has also been implicated in the cytotoxicity of these cells via a specific interaction with PVR, expressed by HCC cell lines (12,24). Following Zol treatment, all HCC cell lines showed enhanced susceptibility to γδ T cell-mediated killing (Fig. 2B), in the absence of any significant change in the expression of NKG2D and DNAM-1 ligands (Fig. 3C). It is conceivable that although NKG2D and DNAM-1 may exert additive effects on γδ T cell responses, treatment of HCC cells with Zol further increases intracellular accumulation of IPP, which generally enhances their susceptibility to γδ T cell-mediated killing. This indicated that treatment of HCC patients with Zol could improve the *in vivo* antitumor efficacy.

Zol was previously reported to directly inhibit HCC proliferation and migration at concentrations >10 μM *in vitro* (27). This was consistent with the present analysis of a wide variety of HCC cell lines, where Zol consistently inhibited their proliferation at concentrations >10-15 μM (Fig. 6A). The Zol concentration required to enhance HCC cell susceptibility to γδ T cell-mediated killing was on average one order of magnitude lower than this (Fig. 6B), indicating that γδ T cell-mediated HCC clearance may occur *in vivo* at safer doses than those required to directly inhibit HCC proliferation.

It is noteworthy that the Zol concentration required for γδ T cell killing in HCC cells was lower than those required in other cancer cell lines. It is conceivable that Zol-resistant cancer cells may have a high frequency of FPP synthase somatic mutations, leading to the reduction of upstream IPP accumulation. In contrast, Zol-susceptible cancer cells such as HCCs may have fewer FPP synthase mutations, thereby promoting the accumulation of IPP. It is also possible that there is a difference in the rate of Zol metabolism in these cancer cell types. Zol may be rapidly taken up through fluid-phase endocytosis, particularly in HCCs, resulting in intracellular Zol levels that are capable of activating γδ T cells (32). However, further investigation is required to define the molecular mechanism that determines susceptibility to γδ T cell-mediated killing.

Our established γδ T cell lines, which were expanded in the presence of IL-2 and IL-15, produced Th1 and Th2 cytokines, but not Th17 cytokines (Fig. 2C). This finding is in agreement with a previous report showing that IL-2 and IL-15 stimulation of naïve γδ T cells resulted in the differentiation of producers of IFN-γ, but not IL-17 (33). Recent studies indicated that IL-17-producing γδ T cells induce tumor-promoting effects by facilitating angiogenesis in patients with colorectal cancer (18,34). Although our established γδ T cells hardly produced any IL-17 *in vitro*, they may produce IL-17 and behave differently in the presence of high inflammatory cytokine levels, such as those found in the tumor microenvironment (35). Therefore, targeting the inflammatory cytokines responsible for IL-17 production (such as IL-1β, IL-6, transforming growth factor-β, and IL-23) *in situ* may potentiate γδ T cell-based immunotherapy.

γδ T cells showed proliferative responses in the presence of Zol-sensitized HCC cell lines *in vitro* (Fig. 2D), implying that they may also proliferate on encountering Zol-sensitized HCC cells *in vivo*. This may be beneficial as it could potentially increase the number of effector cells in the tumor microenvironment.

There is evidence that Vδ1- or Vδ2-TCR-expressing T cells in HCC tissues represent a functionally suppressed phenotype ($CD27^+CD45^-$) (30). However, Vδ2-TCR expressing T cells are present in HCC tissues more predominantly than Vδ1-TCR-expressing T cells, which implies that Zol treatment of HCC may activate the Vδ2-TCR-expressing T cells and change them to a $CD27^-CD45^-$ effector memory phenotype that play an important role in the eradication of HCCs.

All of the HCC cell lines used in this study expressed the DNAM-1 ligands, PVR and Nectin-2, indicating that many HCC cell lines were susceptible to DNAM-1-mediated killing (24). T cell immunoreceptor with Ig and ITIM domains (TIGIT) is an inhibitory molecule that was recently identified and found to be expressed on natural killer cells and CTLs; this inhibits DNAM-1 homodimerization and thus reduces DNAM-1-mediated killing activity (36). A recent clinical trial using anti-TIGIT and anti-programmed cell death-1 (PD-1) indicated a good clinical efficacy in melanoma patients (37). We have already identified increased expression of TIGIT and PD-1 in activated γδ T cells (data not shown) and co-administration of mAbs targeting these proteins may enhance the efficacy of γδ T cell-based immunotherapy. Novel regimens combining these mAbs and Zol are currently under investigation.

Recent studies have indicated that many immunologically relevant tumor antigens are neoantigens derived from point mutation of normal genes, which trigger potent antitumor immune responses (38). It is conceivable that the initial killing process triggered by γδ T cells may disseminate neoantigens that are subsequently taken up and cross-presented by DCs, inducing further CTL-mediated immune responses. Moreover, activated γδ T cells acquire a professional antigen-presenting cell function to express high levels of costimulatory molecules such as CD80 and CD86, as well as molecules associated with lymph node homing (39,40). This function may further boost the generation of a potent and long-lasting immune response.

In conclusion, many human HCC cells are highly sensitive to Zol and are most likely to produce phosphoantigens, such as IPP, which trigger γδ T cell-mediated antitumor responses. The *in vitro* data resulting from this study may foster the development of therapies targeting γδ T cells for the treatment of HCC patients, which can be optimized by Zol. It will be important to gather additional therapeutic evidence *in vivo* using appropriate animal models.

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