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**Tittle:**

Cytotoxic human peripheral blood-derived γδT cells kill glioblastoma cell lines: implications for cell-based immunotherapy for patients with glioblastoma

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**Abstract**

Glioblastoma (GBM) is a highly aggressive brain tumor for which novel therapeutic approaches, such as immunotherapy, are urgently needed. Zoledronate (ZOL), an inhibitor of osteoclastic activity, is known to stimulate peripheral blood-derived γδT cells and sensitize tumors to γδT cell-mediated killing. To investigate the feasibility of γδT cell-based immunotherapy for patients with GBM, we focused on the killing of GBM cell lines by γδT cells and the molecular mechanisms involved in these cell–cell interactions. Peripheral blood mononuclear cells were expanded in ZOL and interleukin (IL)-2 for 14 d, and γδT cells were enriched in the expanded cells by the immunomagnetic depletion of αβT cells. Gliomas are resistant to NK cells but susceptible to lymphokine-activated killer cells and some cytotoxic T lymphocytes. When the γδT cell-mediated killing of three GBM cell lines (U87MG, U138MG and A172 cells) and an NK-sensitive leukemia cell line (K562 cells) were tested, 32% U87MG, 15% U138MG, 1% A172, and 50% K562 cells were killed at an effector:target ratio of 5:1. The γδT cell-mediated killing of all three GBM cell lines was significantly enhanced by ZOL and this ZOL-enhanced killing was blocked by an anti-T cell receptor (TcR) antibody. These results indicated that TcR γδ is crucial for the recognition of ZOL-treated GBM cells by γδT cells. Since the low level killing of GBM cells by the γδT cells was enhanced by ZOL, γδT cell-targeting therapy in combination with ZOL treatment could be effective for patients with GBM.

## Introduction

Glioblastoma (GBM) is a highly aggressive brain tumor. Despite aggressive treatment combining surgery, radiation, and chemotherapy, the prognosis remains poor, with a median survival time of only 12–15 months after diagnosis. The failure of conventional chemotherapy reflects the resistance to alkylating agents, including temozolomide, which is frequently shown by GBM tumors due to the expression of the DNA-repair protein $O^6$-methylguanine methyltransferase [1, 2]. Thus, the development of novel therapeutic approaches for patients with GBM is crucial. Immunotherapy has shown promise in experimental models, but little success in the clinic, principally due to the brain's protected environment and the immunosuppressive effect of the tumor itself [3–5].

Human γδT cells account for about 10% of peripheral blood T cells, and have an important role in innate immune surveillance and defense against cellular stress. They can recognize conserved determinants upregulated during inflammation, infection, or cell transformation [6–8]. Most human peripheral blood γδT cells express a Vγ9Vδ2 T cell receptor (TcR), and recognize cellular stress in a major histocompatibility complex (MHC)-independent manner [9]. Vγ9Vδ2 TcRs recognize nonpeptidic prenyl pyrophosphate metabolites, generically known as phosphoantigens (PAgs) [10]. Natural PAgs include isopentenyl pyrophosphate (IPP), a metabolite of the mevalonate pathway in mammalian cells [10] and the deoxyxylulose phosphate pathway present in many microorganisms [11]. Bisphosphonate, a drug widely used to prevent bone resorption, promote the production of IPP in some tumor cells by

inhibiting farnesylpyrophosphate (FPP) synthase in the mevalonate pathway, and can enhance γδT cell-mediated cytotoxicity [10]. The γδT cells recognize IPP and IPP derivatives in the context of ecto-F1-ATPase [12] and/or CD277 [13] in tumor cells. Human peripheral blood derived-γδT cells have been reported to express some NK receptors and to be involved in the cytotoxic killing of human hepatocellular carcinoma cells [14]. NK receptor-ligand interactions are also crucial in γδT cell-mediated killing of malignant tumor cells.

Previous studies have indicated that γδT cells elicit potent antitumor activity against a broad range of malignant tumors [15–19]. In brain tumors, Fujimiya et al. first reported that peripheral blood γδT cells displayed significant tumoricidal activity [20]. Bryant et al. showed that γδT cells can kill GBM cells *in vitro*, and reduce tumor progression *in vivo* [21, 22]. However, these results did not progress beyond the pre-clinical stage, as there was no easy and safe procedure for expanding γδT cells to obtain sufficient quantities for clinical studies. Human peripheral blood γδT cells have now been shown to proliferate well *in vitro* in the presence of zoledronate (ZOL), a bisphosphonate, and IL-2 [23]. This procedure has been used in clinical trials of γδT cell-based immunotherapy for patients with several tumors [24-26]. This methodology was shown to be clinically beneficial and safe, providing a promising therapeutic strategy for patients with GBM.

To investigate the possibility of using γδT cell-based immunotherapy for patients with GBM, we focused on the cytotoxic effect of γδT cells on standard GBM cell lines, and the effect of ZOL on

sensitizing GBM cell lines as cytotoxic target cells. In addition, to elucidate the molecular mechanisms involved in these cell–cell interactions, the expression of ligands on GBM cell lines that could be recognized by γδT cell expressing receptors were analyzed.

**Materials and methods**

Preparation of human γδT cells, cytotoxic T lymphocytes and lymphokine-activated killer cells

Informed consent was obtained for the collection of peripheral blood from healthy volunteers, and peripheral blood mononuclear cells (PBMCs) were prepared by density gradient centrifugation (Lymphoprep; Axis-Shield PoC AS, Oslo, Norway). For the preparation of γδT cells, human PBMCs were placed in a T25 culture flask (Corning, NY, USA) containing AIM-V medium (Life Technologies Co., Carlsbad, CA, USA) supplemented with 5% autologous plasma, 1 μM zoledronate (Novartis Pharma K.K., Tokyo, Japan) and 200 IU/ml recombinant human (rh) IL-2 (Nipro Co., Osaka, Japan). The cultures were maintained and expanded in AIM-V medium supplemented with 200 IU/ml rhIL-2 for 14 d. The expanded cellular population was depleted of αβT cells by magnetic-activated cell separation (MACS; Miltenyi Biotech, Bergisch-Gladbach, Germany), which produced a TcR γδ-positive cell population that was usually over 95% pure.

To prepare polyclonal cytotoxic T lymphocytes (CTL), PBMCs were cultured with an immobilized anti-CD3 antibody (OKT3; Nipro Co., Osaka, Japan) in AIM-V medium containing 5% autologous

plasma and 200 IU/ml rhIL-2. After expansion in AIM-V supplemented with 200 IU/ml rhIL-2 for 14 d, the cell population contained 98% TcR αβ+, 39% CD4+, and 71% CD8+ cells. To prepare lymphokine-activated killer (LAK) cells, PBMCs were cultured in AIM-V containing 5% autologous plasma and 3,000 IU/ml rhIL-2 for 5 d. After expansion in AIM-V supplemented with 200 IU/ml rhIL-2 for 14 d, the cell population consisted of over 98% CD3- CD56+, characteristic of natural killer cells.

Cell lines

Human GBM cell lines U87MG, U138MG, and A172 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Human leukemia cell line K562 cells was provided by Professor Matsuda (Kyoto Prefectural University of Medicine, Japan). Cell lines were maintained in D-MEM (for GBM cells) or RPMI640 medium (for K562 cells; Life Technologies Co.) with 10% heat-inactivated FBS (Thermo Fisher Scientific Inc., Waltham, MA, USA), 100 μg/ml penicillin and streptomycin (Life Technologies Co.) at 37°C in a humidified atmosphere containing 5% $CO_2$.

Flow cytometry

Samples were stained with appropriate antibodies (see Supplemental materials and methods) and analyzed using a BD FACS Calibur flow cytometer. The data were analyzed using CellQuest software

(BD Biosciences, San Jose, CA, USA). The ratio of fluorescence intensity (RFI) was calculated as the mean fluorescence intensity obtained with the sample/mean fluorescence intensity obtained with the isotype-matched control.

Cytotoxicity assay

Cytotoxic killing was measured using a calcein-AM release assay, as described previously [27]. Briefly, cell lines used as targets were incubated with 1 μM calcein-AM (Life Technologies Co.) for 30 min. TcR γδ-positive T cells were added to 96-well round-bottomed plastic plates (Corning) containing $1\times10^4$ labeled target cells per well at various effector-to-target ratios (E:T). After incubation for 4 h at 37 °C in a humidified atmosphere, supernatants were collected, and calcein-AM release was measured using Fluoroskan Ascent (Thermo Fisher Scientific Inc.). GBM cell lines were treated with ZOL by culturing them in medium containing defined doses of this drug for 20 h prior to performing cytotoxicity assays.

For cold inhibition assays, calcein-AM-labeled (hot) and unlabeled (cold) cell lines were used as target cells. These were incubated at defined hot-to-cold target ratios and E:T ratios. In blocking experiments, γδT cells were treated with 10 μg/ml anti-TcRVγ9 (BD Pharmingen; B3, $IgG_1$) or an isotype-matched mAb for 1h at 4°C, and then used as effector cells.

Statistical methods

Results are given as mean ± standard error (SE). The statistical significance of differences was determined using a t-test or one-way analysis of variance (ANOVA) followed by Scheffe's test. Single and double asterisks indicate $P$ values $< 0.05$ and $< 0.01$, respectively.

**Results**

Phenotypic analysis of γδT cells expanded in culture

The expression of cell-surface antigens on γδT cells expanded in culture was analyzed by flow cytometry using the mAbs described in Supplemental materials and methods (Figure 1). The γδT cells expressed the TcR co-receptor CD3 and the lineage marker CD8, but also very low levels of CD4. The cells expressed the adhesion molecules CD2 (LFA-2) and CD11a (LFA-1α) at high levels. Of the NK cell markers, CD56 (N-CAM) and CD161 (NKR-P1A) were expressed. Of the markers associated with activating NK cell receptors, CD16 (FcγRIII), CD226 (DNAM-1), CD244 (2B4), and CD314 (NKG2D) were highly expressed. CD335 (NKp46) was almost absent, while CD336 (NKp44) and CD337 (NKp30) were slightly expressed. Of the markers associated with inhibitory NK cell receptors, CD94 and CD159a (NKG2A) were highly expressed. CD158a (KIR2DL1) was almost absent. CD158b (KIR2DL2/DL3) was highly expressed in a small percentage (3.7%) of γδT cells and expressed at low levels in a large percentage of the cells. CD178 (Fas-L) was almost absent.

Cytotoxic effect of γδT cells on GBM cell lines

We used a calcein-AM release cytotoxicity assay to test the effect of γδT cells on standard GBM cell lines and the cytotoxicity-sensitive leukemia cell line K562. At E:T ratios of 1:1 and 5:1, the γδT cells killed 28% and 50% of K562 cells, 18% and 32% of U87MG cells, 7% and 15% of U138MG cells, and 4% and 1% of A172 cells, respectively (Figure 2). The γδT cells had a significant cytotoxic effect on U87MG and U138MG cells, which was cell number-dependent, but had little cytotoxic effect on A172 cells. In addition, the cytotoxic effect of γδT cells on the GBM cell lines was significantly lower than on K562 cells.

Effect of ZOL on sensitivity of GBM cell lines to γδT cell-mediated cytotoxicity

We tested the ability of ZOL to sensitize GBM cell lines to γδT cell-mediated cytotoxicity by pre-incubating U87MG cells with 0–10 μM ZOL for 20 h. Untreated or ZOL-treated cells were co-cultured with γδT cells at an E:T ratio of 5:1. After pre-treatment with 1 μM ZOL, 86% U87MG cells were lysed, which was a 4.1-fold increase in lysis compared to untreated cells. This enhancement of lysis by ZOL was a dose-dependent effect (Figure 3a). After pre-treatment with 1 μM ZOL, the other glioma cell lines, U138MG and A172 cells, and the leukemia cell line, K562 cells, showed 43%, 67%, and 55% lysis, respectively, which were 3.6, 16.8, and 1.1-fold increases in lysis compared to untreated cells

(Figure 3b). Therefore, pre-treatment with 1 μM ZOL significantly enhanced the cytotoxic killing of all three GBM cell lines. As previously reported [23], ZOL pre-treatment did not enhance the cytotoxic effect on K562 cells. Interestingly, the killing of the resistant GBM cell line A172 cells by the γδT cells was strongly induced by ZOL pre-treatment.

Mechanisms of ZOL-induced sensitization to γδT cell-mediated killing

To elucidate the molecular mechanisms involved in the interactions between γδT cells and GBM cell lines, cold inhibition assays were performed (Figure 4a). The γδT cell-mediated lysis of calcein labeled, ZOL-treated U87MG cells was competitively blocked by unlabeled, ZOL-treated U87MG, while the lysis of unlabeled-U87MG cells was not enhanced by unlabeled, ZOL-treated U87MG cells.

Next, we analyzed the expression of ligands on GBM cell lines specific for receptors expressed by γδT cells. As shown in Table 1, the expression of HLA-ABC (the ligand for CD158b) was high on all GBM cell lines, but HLA-E (the ligand for CD94/NKG2A) was mostly absent. PVR was expressed by all cell lines and nectin-2 (ligands for DNAM-1) was variably expressed. The expression of the NKG2D-specific ligands MICA, MICB, ULBP1, ULBP2, and ULBP3 was low, but at least one ligand could be detected on every cell line. ICAM family adhesion molecules, which interact with CD11a, were variably expressed on U87MG and U138MG, but mostly absent on A172 cells. Unlike GBM cell lines, the leukemia cell line K562 cells lacked HLA-ABC and HLA-E, expressed the NKG2D-specific ligands

with the exception of MICA, and expressed ICAM-1 and ICAM-2, although ICAM-3 was absent. We also looked at the effect of ZOL on the expression of these ligands by incubating the cell lines with or without 1 μM ZOL before analyzing the ligands present (Figure 4b). After ZOL treatment, MICA was slightly up-regulated on A172 and K562 cells, and HLA-ABC was down-regulated on U87MG cells, but up-regulated on K562 cells. To further investigate the involvement of TcRγδ in these cell-cell interactions, antibody blocking experiments were performed. As shown in Figure 4c, the ZOL-enhanced killing of GBM cells was inhibited by anti-TcRVγ9 mAb in all three cell lines tested.

ZOL-enhanced killing of GBM cells is not specific to γδT cells

To gain further insights into the sensitizing effect of ZOL on GBM cells, we investigated whether this was specific to γδT cells by using LAK and polyclonal CTL as effector cells. After treatment with 1 μM ZOL, 43.5% U87MG cells, 34.8% U138 cells and 15.4% A172 cells were killed by LAK cells, and these were 1.1, 1.1, and 0.9-fold, respectively, of the lysis levels seen with untreated cells. In contrast, when CTLs were incubated with untreated GBM cells, they killed -1.6% U87MG cells, 2.6% U138 cells, and 6.3% A172 cells, but after treatment with 1 μM ZOL these lysis levels were increased -10.6, 3.8, and 0.4-fold, respectively. We therefore observed that ZOL not only enhanced γδT cell-mediated killing, but also CTL-mediated killing (Figure 4d). However ZOL had no effect on LAK-mediated killing.

**Discussion**

To our knowledge, this is the first report of γδT cells having different cytotoxic effects on different GBM cell lines. The cytotoxic effect seen on GBM cell lines was significantly lower than that on the leukemia cell line K562 cells, which is known to show cytotoxic sensitivity. Therefore, to achieve anti-tumor effects in patients with GBM, it will be important to enhance the cytotoxic effect on GBM cells.

To investigate whether bisphosphonate can sensitize GBM cell lines to cytotoxic killing, we selected ZOL, and found it to have a strong sensitizing effect. Cold inhibition assays showed that the γδT cells recognized ZOL-pretreated GBM cells using specific membrane receptors and that they killed the cells by a direct cytotoxic mechanism. In addition, ZOL-enhanced killing did not seem to involve the release of soluble mediators, which would mediate an indirect cytotoxic mechanism.

Cimini et al. reported that ZOL synergistically enhanced the γδT cell-mediated apoptosis of GBM cell lines [28]. The adhesion molecule ICAM-1 has a critical role in the cytotoxic killing of colon cancer cells. The cell lines that lacked ICAM-1 failed to be recognized by γδT cells, even after bisphosphonate treatment. In transfection experiments, the only molecule that was shown dramatically to enhance γδT cell-mediated cytotoxicity after bisphosphonate treatment was ICAM-1 [16]. However, we observed that ZOL could not only enhance the killing of U87MG and U138MG cells that expressed ICAM-1, but could also induce the killing of A172 cells that lacked ICAM-1. Our investigation has confirmed the expanded

γδT cells strongly expressed DNAM-1, and that its specific ligand, PVR, was one of the most highly expressed molecules on all three of the GBM cell lines tested. The expression of PVR, rather than ICAM-1, on GBM cells might mediate the enhanced cell-mediated killing by γδT cells. We also observed that ZOL did not appreciably change the expression of the other ligands tested on the GBM cell lines. Furthermore, mAb specific for TcRVγ9, which is a major component of the TcR repertoire in peripheral blood [10], inhibited ZOL-enhanced killing in all GBM cell lines tested. Although further investigations using mass spectrometry will be needed to measure the accumulation of IPP in target cells, a possible explanation for the effect of ZOL on target cell killing is that it strongly promotes the intracellular accumulation of IPP, thus favoring the recognition of GBM cell lines by γδT cells. Taken together, our results showed that TcR γδ has an important role in the ZOL-enhanced killing of GBM cells. However, polyclonal CTL-mediated killing was also enhanced by ZOL treatment in two GBM cell lines, although LAK-mediated killing was unaffected in all GBM cell lines tested. This suggested that ZOL-enhanced killing may not be entirely specific to γδT cells and other mechanisms remain to be elucidated in CTL-mediated killing.

It has been reported that ZOL reduced anti-apoptosis defense mechanisms, thus allowing ZOL-treated cells to induce apoptosis in tumor cells [29]. We saw no increase in spontaneous fluorescence release from any ZOL-treated cells, compared to untreated cells, in 4 h fluorescence-release cytotoxicity assays. We saw no evidence that ZOL induced direct cytotoxicity in these experiments. We

have confirmed that ZOL can enhance γδT cell-mediated killing of GBM cell lines recoginized by TcR γδ, and consider that PVR might be involved in enhancing γδT cell-mediated killing of GBM cells.

Our study showed that γδT cells killed low numbers of GBM cells, despite being allogeneic, and that ZOL could enhance allogeneic γδT cell-mediated killing. We observed that γδT cells killed virtually no allogeneic normal B lymphoblast cell lines (data not shown). These results indicated allogeneic γδT cell-based immunotherapy would be safe and that administration of allogeneic γδT cells in combination with ZOL may provide a novel strategy for allogeneic cell-based immunotherapy for human GBM.

Our results are encouraging for the development of γδT cell-based immunotherapy for patients with GBM; however, some problems have yet to be overcome for clinical application. It was reported γδT cells might be unable to infiltrate human GBM parenchyma [21]. Our observation that GBM cell lines express low levels of ligands for the receptors on γδT cells, which had a mild cytotoxic effect on GBM cell lines, suggests a possible mechanism for the failure of γδT cells to accumulate in GBM parenchyma. A subpopulation of clonally expanded Vδ2-γδT cells that respond to heat shock protein (HSP) was identified within demyelinated regions of the brain in a multiple sclerosis patient [30]. This implies that γδT cells might accumulate in a GBM tumor if the production of IPP derivatives could be enhanced by ZOL in GBM cells.

For ZOL to penetrate GBM tumors, it must cross the brain–blood barrier (BBB), which is composed of endothelial cells, associated astrocytic end-feet processes, perivascular neurons, and

pericytes [31]. An early study showed that brain tumors such as GBM might partially disrupt the BBB by inducing large gaps between endothelial cells [32], suggesting ZOL might be able to penetrate BBB-disrupting GBM tumors without modification. In other cases, ZOL could potentially be delivered directly into the region around GBM tumors during surgery.

In summary, ZOL strongly sensitizes GBM cell lines as targets for γδT cell-mediated cytotoxic killing. Our investigation has provided a novel mechanistic basis for combining ZOL and γδT cell-based immunotherapy for GBM. This therapy could be an effective strategy to treat GBM.

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**Ethical standards**

The study design was approved by the ethics committee at Clinic Grandsoul Nara, Uda, Japan, and conformed to the Declaration of Helsinki.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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**Figure legends**

**Fig. 1 Phenotypic analysis of human peripheral blood-derived γδT cells**

Cells were stained with specific mAbs (see Supplemental materials and methods) and analyzed by flow cytometry. The top left panel shows the gated TcR γδ-positive cell population for which the histograms on the right show specific antibody staining. Open histograms show controls stained only with secondary PE-conjugated goat anti-mouse IgG, and filled histograms show specific mAb staining. For each histogram, the percentage of positive cells is shown and the ratio of mean fluorescence intensity (RFI) is given in parentheses. The RFI was calculated as the mean fluorescence intensity (MFI) of the indivudial sample/MFI of the isotype-matched control. The results shown are representative of three independent experiments.

**Fig. 2 Cytotoxic killing of human GBM cell lines by γδT cells expanded *in vitro***

The ability of γδT cells to kill $1\times10^4$ GBM cells or K562 leukemia cells was tested at different effector-to-target (E:T) ratios, as shown, by 4h calcein released assay. Each data point is a mean ± standard error (SE) of values obtained from at least three independent experiments. *P* values compared with K562 cells were determined using one-way ANOVA followed by Scheffe's test. * $P < 0.05$ and ** $P < 0.01$

**Fig. 3 ZOL sensitizes GBM cell lines to the γδT cell-mediated killing**

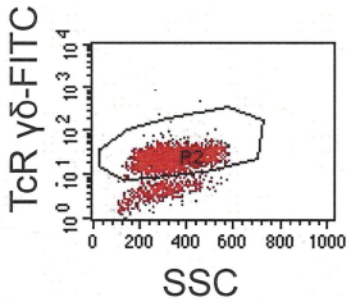
**a,** U87MG cells were pretreated with 0, 0.1, 0.5, 1, 5, and 10 μM ZOL for 20 h, and then washed extensively. Cells were labeled with calcein-AM and co-cultured with γδT cells at a 5:1 E:T ratio for 4 h. Supernatants from the co-cultures were measured by fluorometry. Each bar represents the mean ± SE of values obtained in three independent experiments. **b,** Three GBM cell lines and the leukemia cell line K562 were pretreated with 0 or 1 μM ZOL for 20 h, then labeled with calcein-AM and co-cultured with γδT cells for 4 h at an effector-to-target (E:T) ratio of 5:1. Supernatants from the co-cultures were measured by fluorometry. Each bar represents the mean ± SE of values obtained in three independent experiments. The open and filled bars show the cytotoxic killing of untreated and ZOL-treated cell lines, respectively. *P* values were determined using a t-test to compare untreated and ZOL-treated cell lines. ** $P < 0.01$.

**Fig. 4 Mechanisms of ZOL-enhanced killing of GBM cells by γδT cells**

**a,** Cold inhibition assay. Calcein-labeled, untreated or ZOL-pretreated U87MG cells were used as hot targets and were mixed with ZOL-pretreated U87MG (filled bars) or untreated U87MG cells (diagonal bars) as cold targets, at a hot-to-cold target ratio of 1:10. The γδT cell-mediated lysis of the mixture was determined as calcein release after 4 h at an effector-to-hot target ratio of 5:1. Each bar represents the mean ± SE of values obtained in triplicate experiments. *P* values were determined using a

t-test. ** $P < 0.01$. **b,** Effects of ZOL on expression of ligands on GBM cell lines specific for receptors expressed on γδT cells. Cell lines were pre-treated with 0 or 1 μM ZOL for 20 h, and the effect of ZOL on the expression of the molecules shown was then evaluated by flow cytometry. Open and filled bars show the results for untreated and ZOL-treated cells, respectively. Each bar shows the ratio of mean fluorescence intensity (RFI) ± SE obtained in three independent experiments. *P* values were determined using a t-test to compare untreated and ZOL-treated cells. *$P < 0.05$. **c,** Antibody blocking experiments. The γδT cells were treated with 10 μg/ml anti-TcRVγ9 mAb or an isotype-matched control antibody for 1h. ZOL-pretreated GBM cell lines were incubated with the antibody-treated γδT cells at an E:T ratio of 5:1 and the percent lysis was determined as calcein release after 4 h. Each bar represents the mean ± SE of values obtained in triplicate experiments. *P* values were determined using a t-test. ** $P < 0.01$. **d,** ZOL-enhanced killing is not specific to γδT cells. LAK and CTL were expanded from PBMCs cultures for 14 d. Effector LAK or CTL were then co-cultured with calcein labeled-GBM cell lines for 4 h at an E:T ratio of 5:1. Open and filled bars show the results for untreated or ZOL-pretreated target cells, respectively. Data are presented as the mean ± SE of triplicate determinations. *P* values were determined using a t-test comparing untreated and ZOL-pretreated cells. **$P < 0.01$.

Figure 1.


TcR γδ-FITC
SSC
P2
0
200
400
600
800
1000



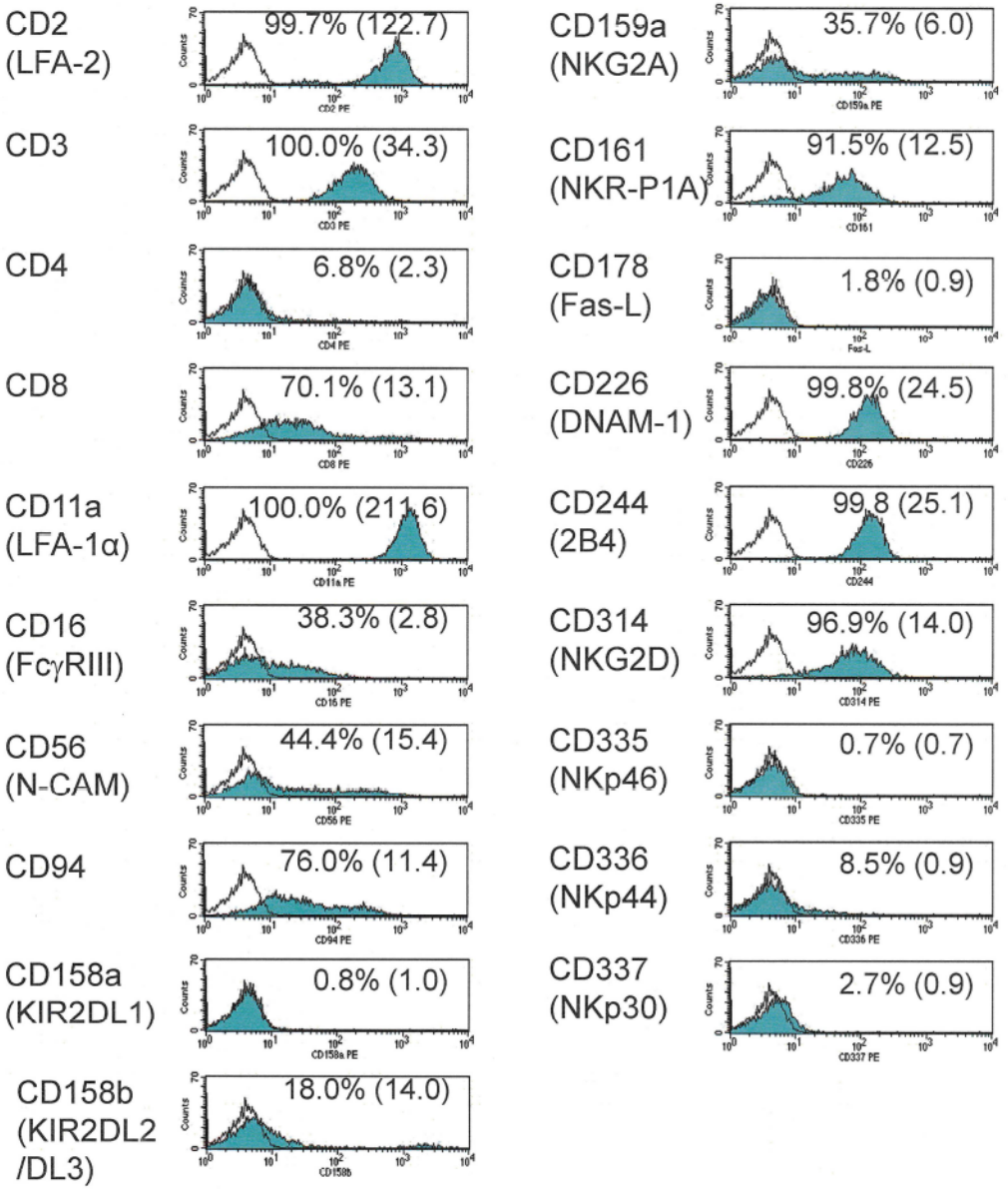

CD2 (LFA-2)
99.7% (122.7)
CD3
100.0% (34.3)
CD4
6.8% (2.3)
CD8
70.1% (13.1)
CD11a (LFA-1α)
100.0% (211.6)
CD16 (FcγRIII)
38.3% (2.8)
CD56 (N-CAM)
44.4% (15.4)
CD94
76.0% (11.4)
CD158a (KIR2DL1)
0.8% (1.0)
CD158b (KIR2DL2 /DL3)
18.0% (14.0)
CD159a (NKG2A)
35.7% (6.0)
CD161 (NKR-P1A)
91.5% (12.5)
CD178 (Fas-L)
1.8% (0.9)
CD226 (DNAM-1)
99.8% (24.5)
CD244 (2B4)
99.8 (25.1)
CD314 (NKG2D)
96.9% (14.0)
CD335 (NKp46)
0.7% (0.7)
CD336 (NKp44)
8.5% (0.9)
CD337 (NKp30)
2.7% (0.9)

Figure 2.

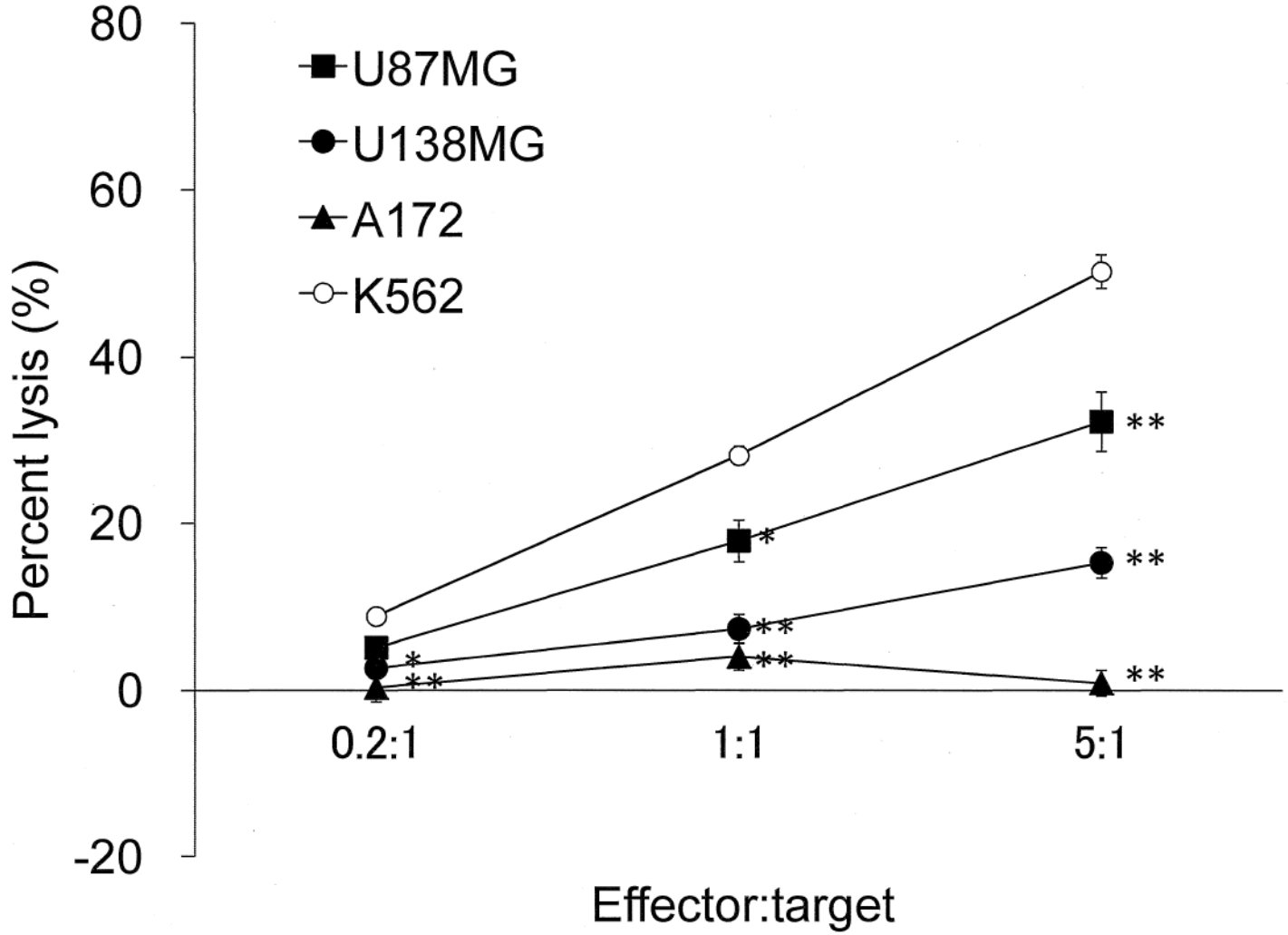

80
60
40
20
0
-20
Percent lysis (%)
U87MG
U138MG
A172
K562
*
**
**
**
**
**
0.2:1
1:1
5:1
Effector:target

Figure 3a.

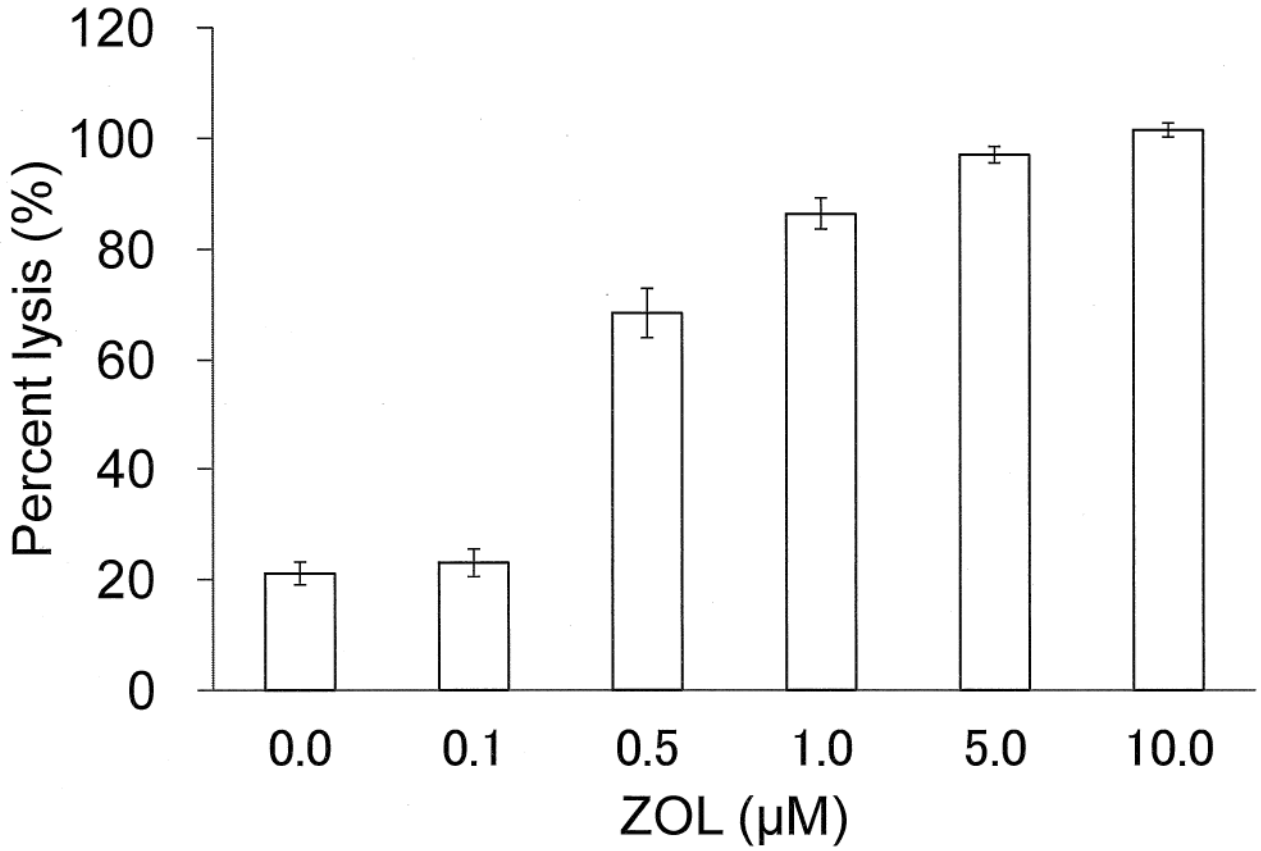


Figure 3b.

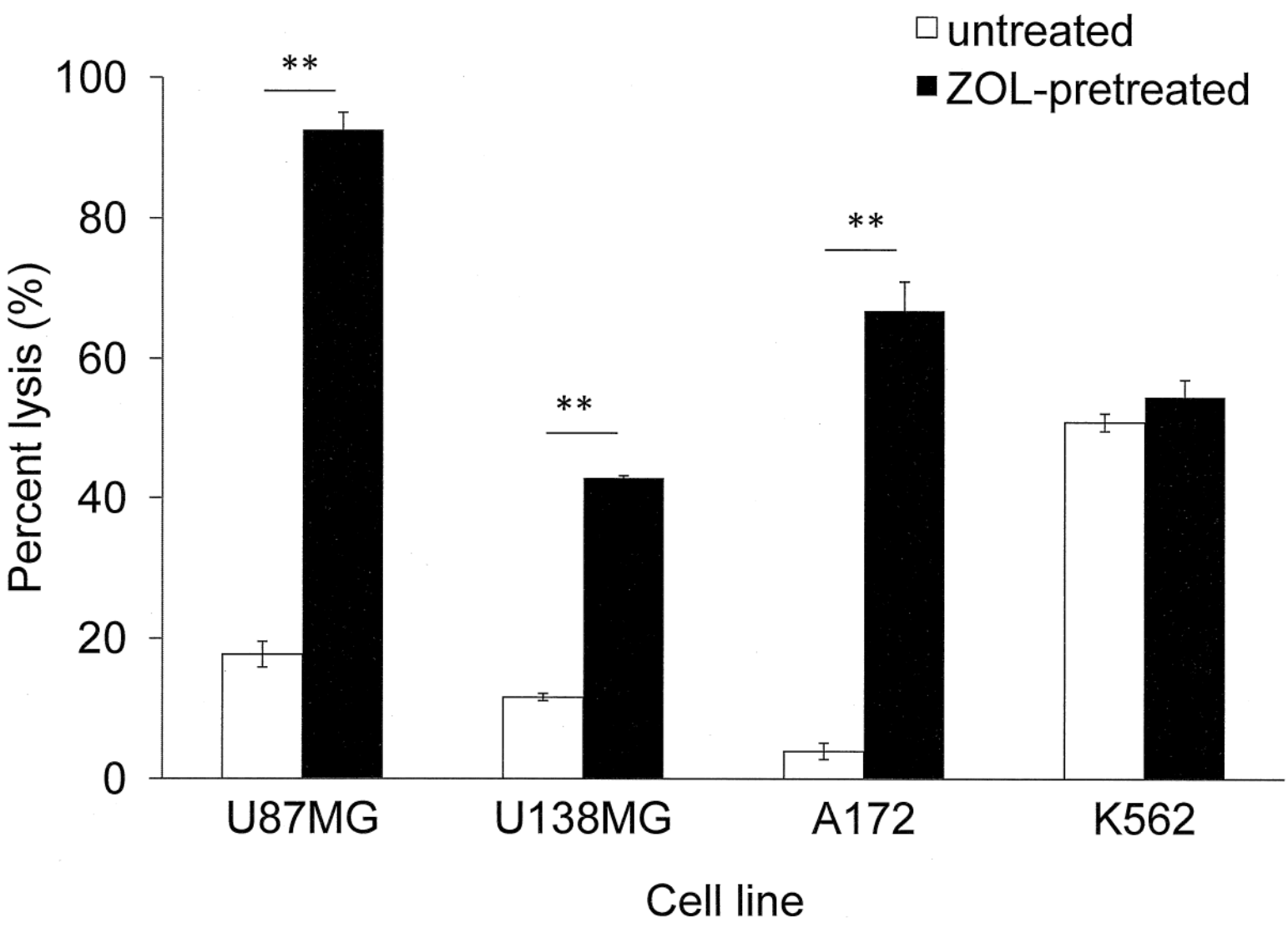

untreated
ZOL-pretreated
Percent lysis (%)
100
80
60
40
20
0
**
**
**
U87MG
U138MG
A172
K562
Cell line

Figure 4a.

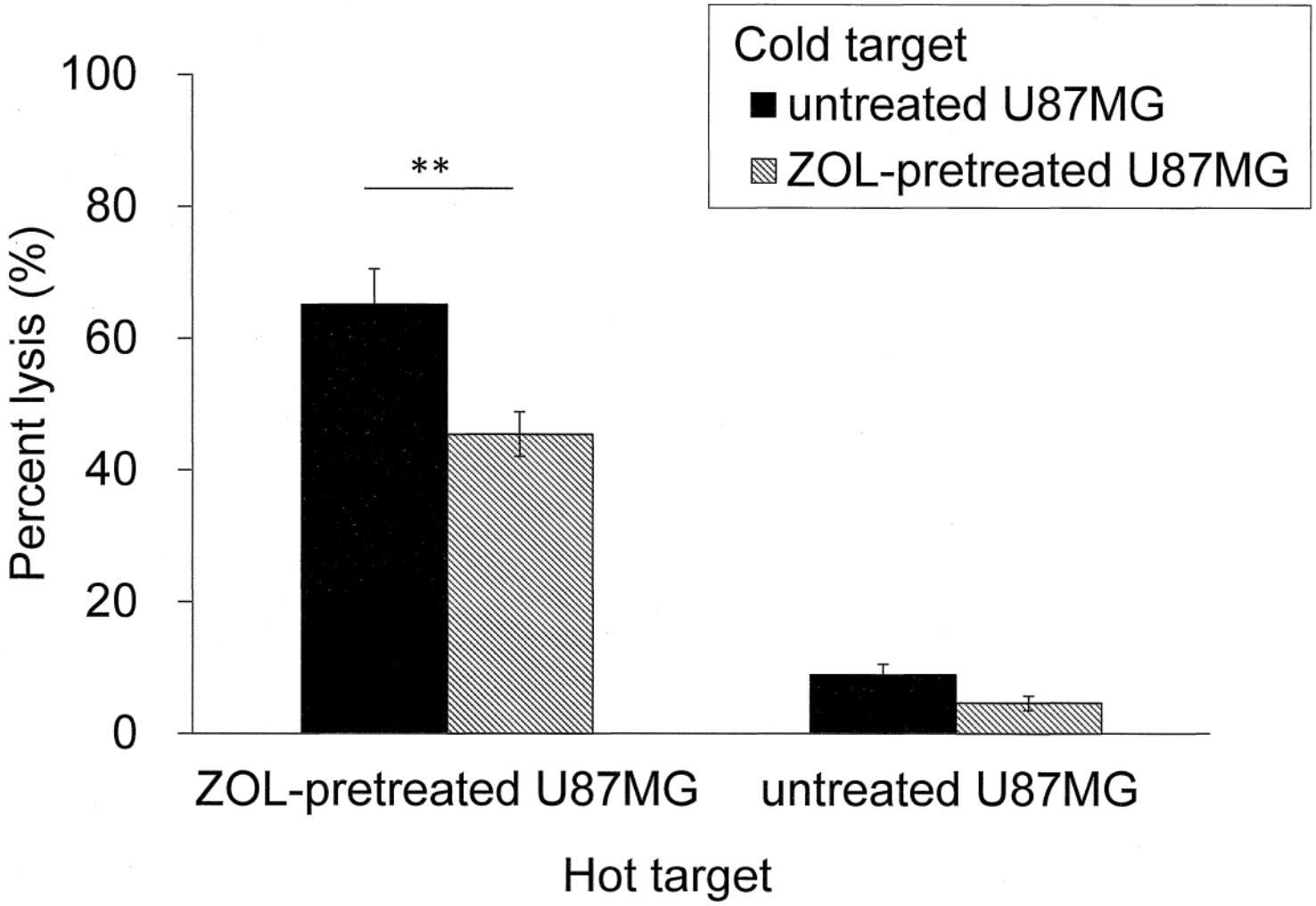

Cold target
untreated U87MG
ZOL-pretreated U87MG
Percent lysis (%)
100
80
60
40
20
0
**
ZOL-pretreated U87MG
untreated U87MG
Hot target

Figure 4b.

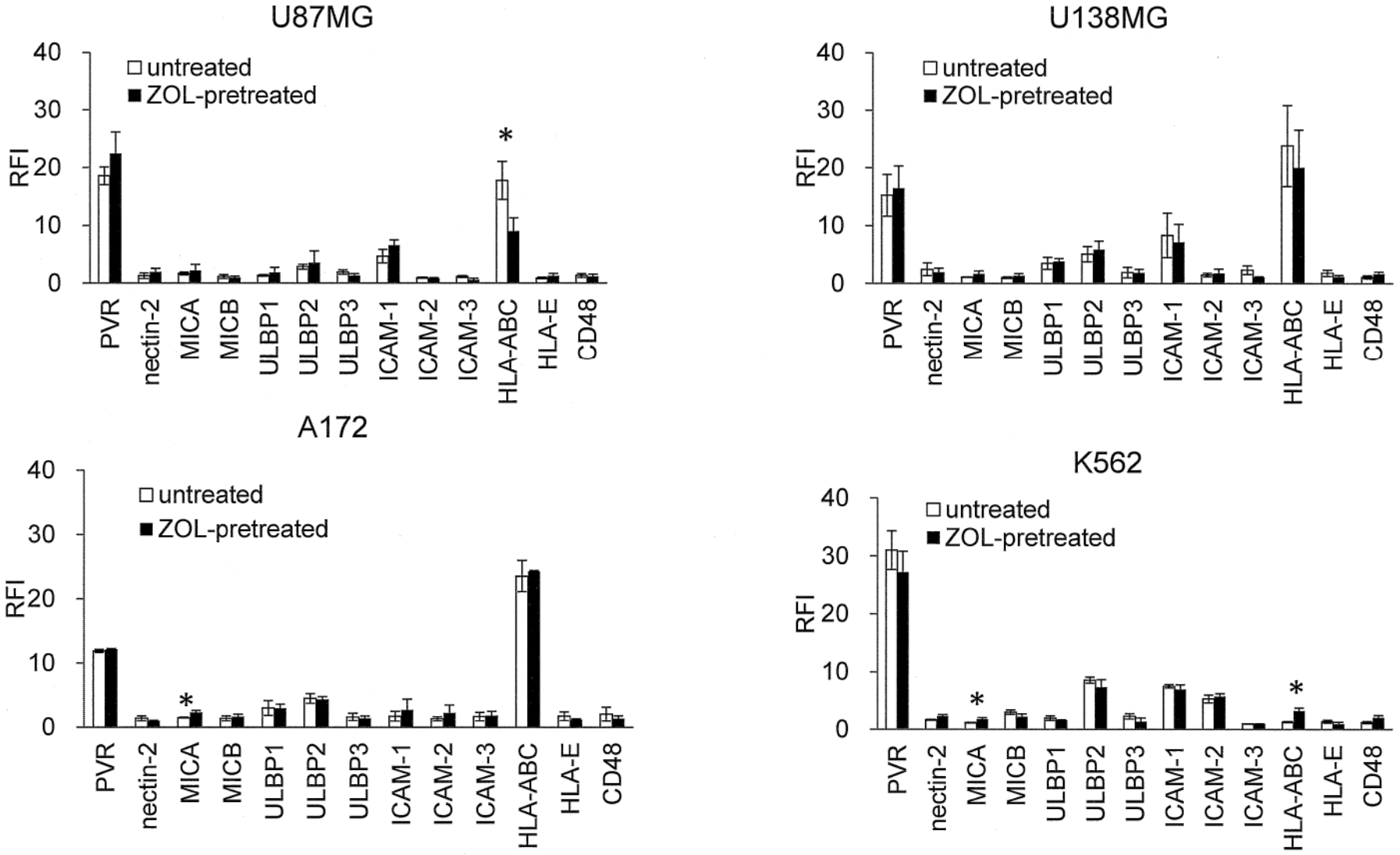

U87MG
U138MG
A172
K562
untreated
ZOL-pretreated
RFI
0
10
20
30
40
PVR
nectin-2
MICA
MICB
ULBP1
ULBP2
ULBP3
ICAM-1
ICAM-2
ICAM-3
HLA-ABC
HLA-E
CD48
*

Figure 4c.

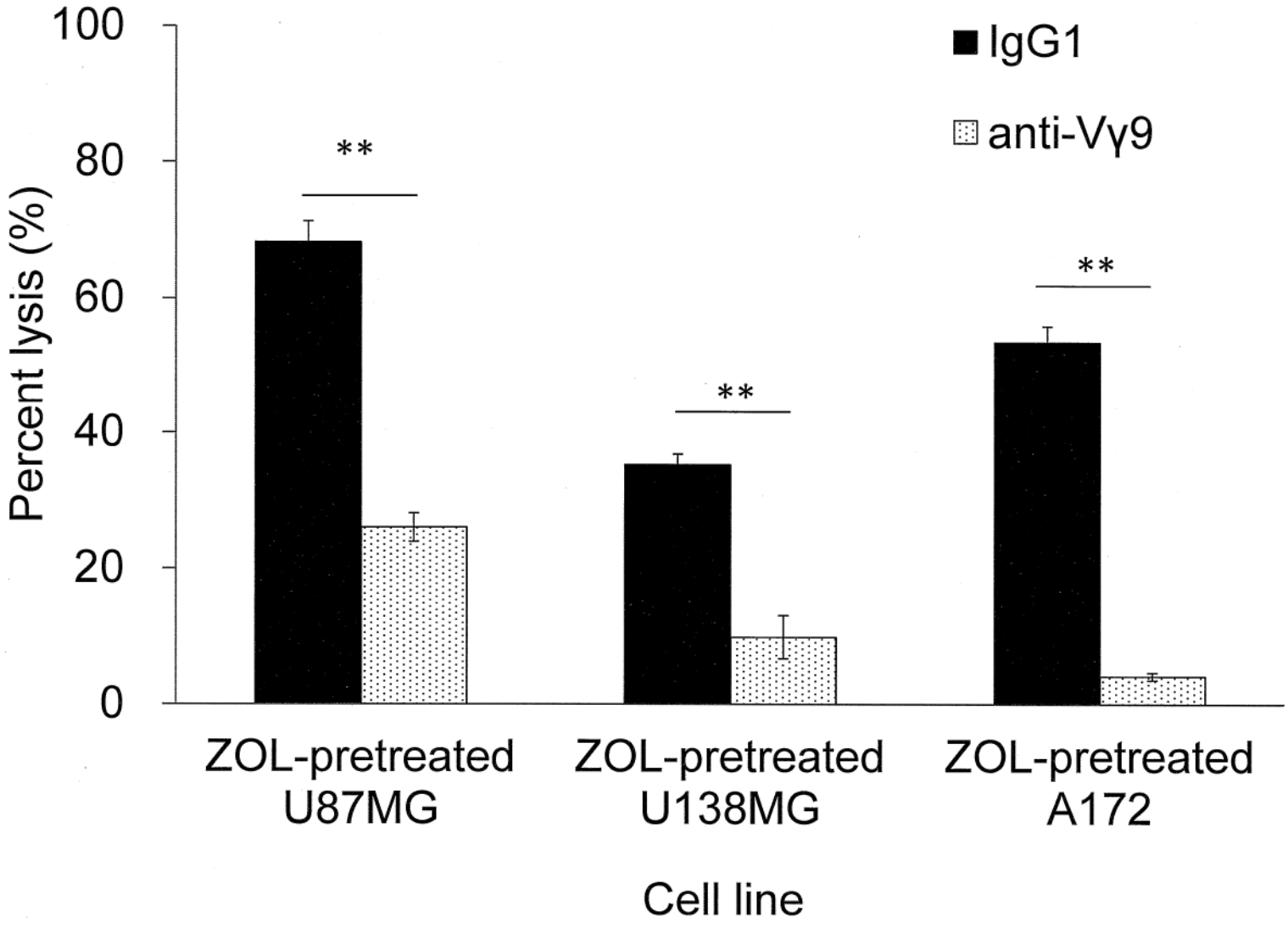

100
80
60
40
20
0
Percent lysis (%)
IgG1
anti-Vγ9
**
**
**
ZOL-pretreated U87MG
ZOL-pretreated U138MG
ZOL-pretreated A172
Cell line

Figure 4d.

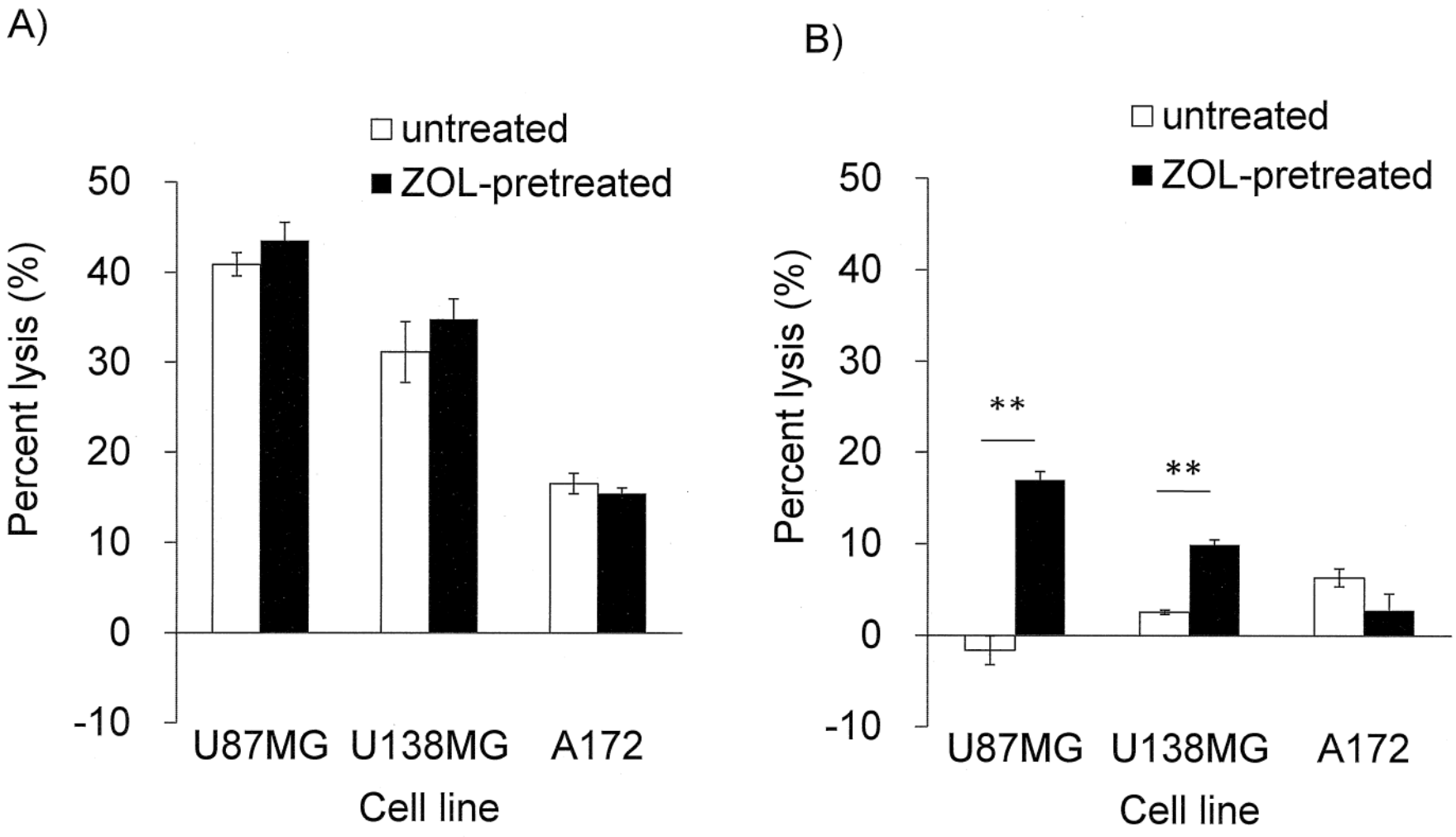

A)
untreated
ZOL-pretreated
Percent lysis (%)
50
40
30
20
10
0
-10
U87MG
U138MG
A172
Cell line
B)
untreated
ZOL-pretreated
Percent lysis (%)
50
40
30
20
10
0
-10
**
**
U87MG
U138MG
A172
Cell line

**Table 1 Expression of ligands on GBM cell lines specific for receptors expressed on γδT cells**

| | HLA-ABC | HLA-E | PVR | nectin-2 | CD48 | MICA | MICB | ULBP1 | ULBP2 | ULBP3 | ICAM-1 | ICAM-2 | ICAM-3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| U87MG | 17.7 | 0.9 | 18.6 | 1.2 | 1.3 | 1.7 | 1.1 | 1.3 | 2.9 | 1.9 | 4.7 | 0.9 | 1.1 |
| U138MG | 23.8 | 1.8 | 15.2 | 2.4 | 1.1 | 1.1 | 1.0 | 3.5 | 5.1 | 1.9 | 8.3 | 1.5 | 2.3 |
| A172 | 23.5 | 1.7 | 11.9 | 1.4 | 2.1 | 1.5 | 1.4 | 3.0 | 4.5 | 1.6 | 1.7 | 1.3 | 1.7 |
| K562 | 1.3 | 1.3 | 31.0 | 1.7 | 1.2 | 1.2 | 3.0 | 2.0 | 8.6 | 2.3 | 7.5 | 5.3 | 1.0 |

GBM cell lines were analyzed by flow cytometry for expression of the indicated molecules. The numbers given are the ratios of mean fluorescence intensity (RFI) of values obtained in three independent experiments. Since K562 cells were HLA-ABC negative, an RFI of $\leq 1.3$ was judged to be negative.

**Supplemental materials and methods**

Antibodies

PE-conjugated anti-TcR αβ (clone, T10B9.1A-31; isotype, IgM), FITC-conjugated anti-TcR γδ (11F2, $IgG_1$), anti-CD50 (TU41, $IgG_{2b}$), anti-CD94 (HP-3D9, $IgG_1$), anti-CD158a (HP-3E4, IgM), anti-CD158b (CH-L, $IgG_{2b}$), anti-CD161(DX12, $IgG_1$), PE-conjugated anti-CD226 (DX11, $IgG_1$) monoclonal antibodies (mAbs) were purchased from BD Biosciences (San Jose, CA, USA). Anti-CD16 (3G8, $IgG_1$), anti-CD56 (NKH-1, $IgG_1$), PE-conjugated anti-CD159a (Z199, $IgG_{2b}$), anti-CD178 (NOK-1, $IgG_1$), anti-CD244 (C1.7, $IgG_1$), anti-CD314 (ON72, $IgG_1$), PE-conjugated anti-CD335 (BAB281, $IgG_1$), PE-conjugated anti-CD336 (Z231, $IgG_1$), and PE-conjugated anti-CD337 (Z25, IgG1) mAbs were purchased from Beckman-Coulter (Fullerton, CA, USA). Anti-CD54 (HA58, $IgG_1$), anti-CD102 (CBRIC2/2, $IgG_{2a}$), anti-CD48 (156-4H9, $IgG_1$), PE-conjugated anti-PVR (2H7CD155, $IgG_1$), anti-HLA-E (3D12HLA-E, $IgG_1$), anti-nectin-2 (R.2.525, $IgG_1$), and anti-HLA-ABC (W6/32, $IgG_{2a}$) mAbs were purchased from eBioscience (San Diego, CA, USA). Anti-CD2 (NU-TER, $IgG_1$), anti-CD3 (NU-T3, $IgG_{2a}$), anti-CD4 (NU-TH/I, $IgG_1$), and anti-CD8 (NU-TS/C, $IgG_{2a}$) mAbs were purchased from Nichirei Co. (Tokyo, Japan). Anti-CD11a (TS1/22, $IgG_1$) was purchased from Thermo Fisher Scientific Inc. Anti-MICA (AMO1, $IgG_1$) and anti-MICB (BMO1, $IgG_1$) mAbs were purchased from MBL International Co. (Nagoya, Japan). Anti-ULBP1 (170818, $IgG_{2a}$), ULBP2 (165903, $IgG_{2a}$), and ULBP3 (166510, $IgG_{2a}$) mAbs were purchased from R&D systems (Minneapolis, MN, USA). Anti-mouse

PE-conjugated goat $F(ab)_2$ was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Isotype-specific control antibodies for mouse $IgG_1$, mouse $IgG_{2a}$, mouse $IgG_{2b}$, PE-conjugated mouse IgM, and FITC-conjugated mouse $IgG_1$ were purchased from eBioscience, and were used to measure background staining levels.